

Chronic fibroblasts cultured in 3D collagen  
matrices as an in vitro model for isolated  
healing processes in a chronic wound

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Title (English)	<b>Chronic fibroblasts cultured in 3D collagen matrices as an <i>in vitro</i> model for isolated healing processes in a chronic wound</b>	
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Abstract	<p>Wound healing is a complex process which involves numerous cell types, extracellular matrix components and cytokines. Chronic wounds fail to proceed through the normal phases of healing and are instead locked in a state of pathologic inflammation. In order to understand the underlying mechanisms causing the chronicity and to develop new and more effective treatments there is an immense need for improved models for chronic wounds.</p> <p>In the present project, a three dimensional <i>in vitro</i> model of dermal like tissue in chronic wounds has been developed. The model was utilized for the investigation of the differences in cell behavior between chronic dermal fibroblasts and normal dermal fibroblasts on cellular processes important for dermal repair and regeneration. Furthermore, the effect of a wound treatment substance, enamel matrix derivative (EMD), on these cellular processes has been examined.</p>	
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# **Chronic fibroblasts cultured in 3D collagen matrices as an *in vitro* model for isolated healing processes in a chronic wound**

Emma Stegberg

## ***Sammanfattning***

Sårläkning är en komplicerad process som involverar åtskilliga celltyper, vävnads-komponenter och signalmolekyler. Kroniska sår karakteriseras av en oförmåga att fortskrida genom läkningsprocessen, beroende av flera olika faktorer där infektion, syrebrist och försämrad blodförsörjning bara är några. För att kunna undersöka mekanismerna som orsakar kroniska sår och för att kunna utveckla nya och mer effektiva behandlingsmetoder så krävs förbättrade modeller av dessa sår.

I detta projekt utvecklades en tredimensionell modell av dermis i kroniska sår. Modellen användes för att analysera skillnaderna i beteende mellan kroniska och normala hudceller vad gäller de processer som är viktiga vid sårläkningen i huden. Dessutom undersöktes effekten av en sårläkningsprodukt, Xelma®, på dessa processer.

Resultaten visar att kroniska hudceller har nedsatt funktionalitet i flera av de undersökta processerna i jämförelse med normala hudceller. Vid behandling med Xelma® stimuleras de kroniska cellerna till ett mer normalt beteende, vilket visar att Xelma® är en produkt med bra potential att läka kroniska sår.

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## ABBREVIATIONS

BSA	Bovine serum albumin
CRL	Chronic human dermal fibroblasts
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EMD	Enamel matrix derivative
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FPCM	Fibroblast-populated collagen matrix
HAc	Acetic acid
HBSS	Hanks' balanced salt solution
IL	Interleukin
MCP-1	Monocyte chemoattractant protein 1
MMP	Matrix metalloproteinase
NaOH	Sodium hydroxide
NHDF	Normal human dermal fibroblasts
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
RPE	R-Phycoerythrin
TGF- $\beta$	Transforming growth factor $\beta$
TNF- $\alpha$	Tumor necrosis factor $\alpha$
VEGF	Vascular endothelial growth factor

# 1. INTRODUCTION

Wound healing is a complex process which involves cell proliferation and migration, matrix synthesis and contraction and the regulation of these events by the synthesis and release of numerous growth factors and cytokines.<sup>1</sup>

Chronic wounds, contrary to acute wounds, fail to proceed through the normal phases of healing and are instead locked in a state of pathologic inflammation. The healing process is consequently delayed and incomplete. Impaired healing may be evoked by numerous of different reasons; diabetes, venous stasis and pressure being the most common.<sup>2</sup>

To be able to analyze the different aspects of the wound healing process and the effect of wound healing treatments, improved models are needed. Numerous models have been developed, both in vitro and in vivo. However, cells cultured in vitro have demonstrated a different behavior compared to cells in vivo, especially cells cultured in monolayers. When modeling chronic wound healing in vitro it is therefore essential to create an environment with conditions similar to the tissues naturally surrounding the cells.<sup>3</sup> Fibroblasts, the most abundant cell type in human dermis, are of utmost importance in cutaneous wound healing.<sup>4</sup> The fibroblast-populated collagen matrix has therefore been utilized as an in vitro model for wound healing. It provides a reasonable representation of the dermal granulation tissue in a healing wound.<sup>5</sup>

Xelma® is a new wound healing treatment for hard to heal wounds (Mölnlycke Health Care AB, Gothenburg, Sweden). The active component in this product is enamel matrix derivative (EMD), a mixture of hydrophobic proteins extracted from developing porcine teeth. The principal component of EMD is amelogenins.<sup>6</sup> Under physiological conditions, EMD resembles a stable extra cellular matrix and is believed to serve as a temporary matrix for cell attachment and migration. Consequently, in chronic wounds where the extracellular matrix (ECM) is deficient, these proteins have a potential to promote cutaneous wound healing processes.<sup>7,8</sup> EMD has been shown to be advantageous in the treatment of chronic venous leg ulcers<sup>8,9</sup> and to accelerate wound closure in rabbits.<sup>6</sup> These proteins have demonstrated an ability to increase cell proliferation and contraction of three dimensional matrices and are also shown to stimulate the production of certain growth factors important for dermal repair and regeneration.<sup>6,10</sup>

## **2. AIM**

The objectives of this thesis project were to:

1. Improve a three dimensional in vitro model of dermal like tissue in chronic wounds and investigate the differences in cell behavior between chronic human dermal fibroblasts and normal human dermal fibroblasts on cellular processes important for dermal repair and regeneration.
2. Analyze the effect of a wound treatment substance, EMD, on these cellular processes.

## 3. BACKGROUND

### 3.1. The skin

The skin is the largest organ of the body, covering an area of about two square meters in adults.<sup>11</sup> The primary function of the skin is to serve as a barrier, protecting the body against noxious external conditions and preserving the balanced internal environment. It enables prevention of liquid loss, regulation of body temperature, transduction of cutaneous sensations and vitamin D synthesis.<sup>11,12</sup>

The skin consists of two different tissue layers: the outermost thin epidermis and the inner thicker dermis. The epidermis is a stratified epithelium, hosting four different cell types. The keratinocytes which are the most abundant, produce the fibrous protein keratin and differentiate from basal cells into dead corneocytes while migrating upwards. The epidermis can be subdivided into four layers based on the maturation of the keratinocytes. Aggregation of the keratin fibrils inside the cells gives the epidermis its strength to withstand damage.<sup>11,12</sup> Langerhans cells are immunologically active cells with the ability to catch, process and present exogenous antigens and are an important part of the immune system. Melanocytes synthesize the pigment melanin which protects the skin from harmful ultraviolet radiation. Merkel cells are located near neurons and act as transducers for the sense of touch.<sup>11,12</sup>

The epidermis and the dermis are separated by the basal membrane which functions as a semipermeable filter for transfer of nutrients and cells from dermis to epidermis. Furthermore, it provides mechanical support and cell adhesion sites.<sup>11,13</sup>

The dermis is a connective tissue mainly composed of collagen and elastin in a matrix of glucosaminoglycans. The proteins provide tensile strength and elasticity while the ground substance is responsible for the viscosity and hydration. The different components of the extracellular matrix are synthesized by fibroblasts which are the most abundant cell type in the dermis. Other cell types are lymphocytes, macrophages and mast cells. Contrary to epidermis where cells are closely adherent, the dermis shelters only few cells sparsely located in the extracellular matrix. In addition, the dermis contains blood vessels, nerve fibers, lymphatics, muscles, glands and hair follicles. Beneath, the dermis is attached to the hypodermis, a subcutaneous layer which mainly consists of connective tissue and fat.<sup>11,12</sup>

The structure of the skin is shown in figure 1.

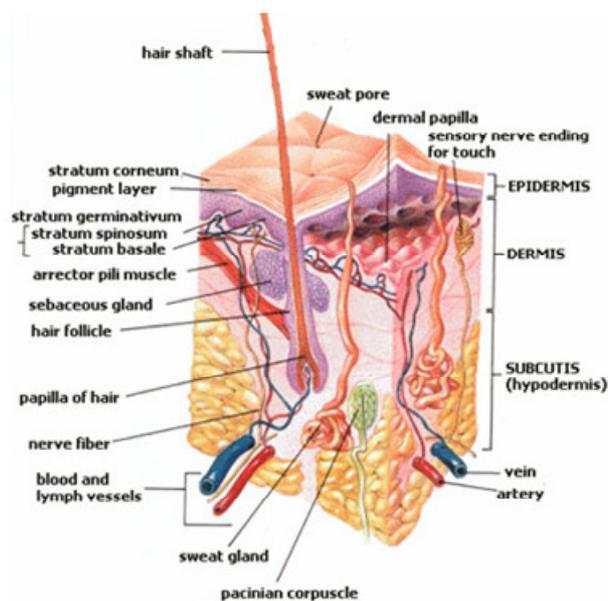


Figure 1. Structure of the skin. Modified from wikipedia.<sup>14</sup>

## 3.2. Cutaneous wound healing

Wound healing is a complex process which involves cell proliferation and migration, matrix synthesis and contraction and the regulation of these events by the synthesis and release of numerous growth factors and cytokines. The process is traditionally divided into three overlapping phases: Inflammation, proliferation and remodeling.<sup>1,15</sup> The main outlines of these phases will be described here.

### 3.2.1. Inflammation

Tissue injury usually causes the disruption of blood vessels and leakage of blood constituents. The immediate response to reestablish homostasis is the formation of a clot. Capillary permeability increases at first, to allow neutrophils, lymphocytes, monocytes and platelets to migrate into the wound and release chemokines. This results in vasoconstriction and prevention of further blood loss.<sup>1,16</sup>

Coagulation occurs as platelets aggregate with fibrin fibers, derived from the cleavage of fibrinogen by thrombin. The network formed serves as a shelter for temporary protection of the open wound and as a matrix for cell migration.<sup>16</sup>

Platelets release several factors, including transforming growth factor  $\beta$  (TGF-  $\beta$ ) and platelet derived growth factor (PDGF) which attract neutrophils and monocytes to the wound site. These cells cleanse the injured area from contaminating bacteria and foreign particles.<sup>1,17</sup> When the monocytes enter the wound site they become activated macrophages which phagocytose remaining pathogens, other cells and matrix debris. Furthermore, they secrete growth factors such as PDGF and vascular endothelial growth factor (VEGF), thus stimulating fibroblast recruitment and angiogenesis.<sup>1,16,17</sup>

### **3.2.2. Proliferation**

During the process of reepithelialization, i.e. formation of a new epidermal layer, the keratinocytes migrate over the provisional wound matrix, leaving a stratified layer of proliferating cells and an underlying basal lamina behind. To be able to move through the dense provisional matrix, the keratinocytes utilize specific enzymes, including matrix metalloproteinases (MMP), which degrade matrix proteins. Once a monolayer of keratinocytes has been produced, the epidermal migration ceases.<sup>16,18</sup>

Fibroblast migration is stimulated by a vast number of cytokines and growth factors. The cells navigate along the provisional matrix fibers and commence to synthesize components of a permanent extracellular matrix, including collagen, glucosaminoglycans and proteoglycans, but also growth factors.<sup>18</sup> The new granulation tissue replaces the provisional matrix and numerous new capillaries are created.<sup>17</sup>

Furthermore, the healing process comprises contraction of the wound. This involves myofibroblasts, a specific type of differentiated fibroblasts which possess contractile forces and therefore are able to reapproximate wound edges. The contraction process is a complex interaction of cells, the extracellular matrix and cytokines.<sup>17,18</sup>

### **3.2.3. Remodeling**

The remodeling phase of repair implies a tightly coordinated equilibrium between collagen synthesis and catabolism.<sup>1</sup> The degradation of collagen is performed by matrix metalloproteinases (MMP), produced by macrophages, epidermal cells, endothelial cells and fibroblasts.<sup>17</sup>

Over time, the proportion of collagen type I increases with the corresponding decrease in collagen type III, proteoglycans and water. Collagen fibrils become more organized and the number of intermolecular cross-links augments.<sup>18</sup> This remodeling persists for several months after wound closure but nevertheless, tensile strength of the remodeled tissue only reaches a maximum of about 70 – 80 % of that of unwounded tissue.<sup>1,18</sup>

At the end of the wound healing process, most of the cells in the wound tissue undergo apoptosis and a relatively acellular scar is formed. The molecular mechanisms of this regulation are still not fully elucidated.<sup>17</sup>

A summary of the wound healing process is shown in figure 2.

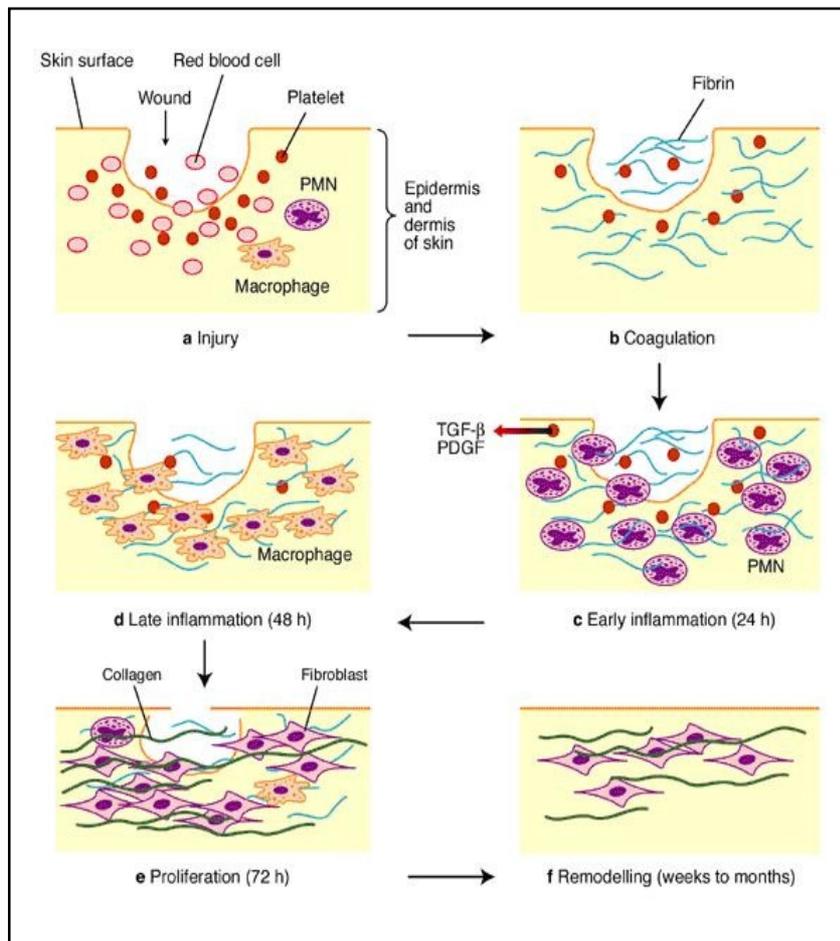


Figure 2. Summary of the wound healing process. (a) Immediately after cutaneous damage neutrophils, monocytes and platelets migrate into the wound from disrupted blood vessels. (b) Blood coagulation occurs as platelets aggregate with fibrin, a protein derived from the cleavage of fibrinogen by thrombin. (c) Platelets release several factors, including transforming growth factor  $\beta$  (TGF-  $\beta$ ) and platelet-derived growth factor (PDGF) which attract neutrophils and (d) monocytes to the wound site. These cells cleanse the injured area from debris and secrete growth factors. (e) Fibroblasts are recruited and commence to synthesize collagen. (f) The tissue is remodeled through collagen crosslinking and reorganization for months after injury. Modified from Beanes *et al.* (2003).

### **3.3. Chronic wounds**

Chronic wounds, contrary to acute wounds, fail to proceed through the normal phases of healing and are instead locked in a state of pathologic inflammation. The healing process is delayed, incomplete and consequently, the outcome is poorly functional.<sup>2</sup> Impaired healing may be caused by numerous different reasons; diabetes, venous stasis and pressure being the most common. Ischemia, the shortage of blood supply, is generally the underlying cause although several mechanisms have been proposed to explain the chronicity, including microbial contamination and the cellular and systemic effects of aging.<sup>2,19</sup>

The principal pathological characteristics of a chronic wound are prolonged inflammation, defective extracellular matrix and failed reepithelialization.<sup>20</sup> An excessive number of inflammatory cells are present in the tissue and the level of chemokines and pro-inflammatory cytokines, such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and interleukin 1 (IL-1) are persistently elevated.<sup>2,21</sup> The proteolytic activity is intensified due to an increased concentration of matrix metalloproteinases (MMP), leading to extracellular matrix degradation.<sup>21</sup> Moreover, a reduced concentration of certain growth factors, for example transforming growth factor  $\beta$  (TGF- $\beta$ )<sup>22,23</sup> decreases the level of mitogenic activity in the wound.<sup>2</sup>

The result of these conditions is an uncontrolled inflammatory response with additional tissue damage and deterioration of the chronic wound, which further promotes inflammation and prevents wound healing.<sup>2</sup>

#### **3.3.1. Chronic fibroblasts**

Fibroblasts are of utmost importance in cutaneous wound healing. Apart from synthesizing and remodeling the principal components of the extracellular matrix, fibroblasts also produce the mitogens for keratinocytes and endothelial cells.<sup>4</sup>

Fibroblasts obtained from chronic wounds show decreased proliferation and reduced mitogenic response to exogenous application of growth factors such as platelet derived growth factor-BB (PDGF-BB) and transforming growth factor  $\beta$  (TGF- $\beta$ ).<sup>4,21</sup> Besides, migration capacity of these cells is impaired and some studies have demonstrated that the cellular morphology is altered, making them resemble fibroblasts which have become senescent *in vitro*.<sup>2,21</sup> Furthermore, these features are dependent on the duration of the ulcer.<sup>4</sup>

It remains unclear whether the deterioration of the wound healing activities, such as decreased proliferation and mobility, demonstrate permanent modifications of the cellular phenotype or reversible behaviors caused by the chronic environment.<sup>24</sup>

### **3.4. Models for wound healing**

In order to analyze the different aspects of the wound healing process and the effect of wound healing treatments, improved models are needed. Numerous models have been developed, both in vitro and in vivo. Which model to choose depends upon a variety of factors, such as laboratory facilities, financial resources but also ethical and legal aspects.<sup>3</sup>

The main advantage of an in vivo model in wound healing research is the similarity to wounds in clinical practice. In the case of cutaneous wounds, studies can even be performed in humans. The disadvantages are the difficulties to examine single tissue components without influencing others, a consequence of the inherent heterogeneity of in vivo models.<sup>3</sup>

In vitro models are generally rapid, less costly and involve minimal ethical and legal considerations in comparison with in vivo models. Another advantage is the simplicity which provides the possibility to directly evaluate the effect of a single substance in the tissue. The major drawback is the difficulty of extrapolating the in vitro results to the in vivo situation.<sup>3</sup>

In vitro models can be divided according to the difference in complexity, ranging from single cell systems in two dimensions to multicellular systems in three dimensions and organ cultures.<sup>3</sup> Culturing cells in monolayers is the simplest technique of modeling wound tissue and has until recently been the primary approach applied.<sup>3</sup> A significant and inherent disadvantage with this model is the absence of dorsal cell adhesion points. These conditions are therefore an unfair representation of the tissue environment, particularly improper for cells that naturally are completely surrounded by a matrix that can be remodeled, e.g. fibroblasts.<sup>25,26</sup>

Cells cultured in three dimensional matrices behave very differently when compared to cells in two dimensional environments. Remarkable changes in cell morphology, matrix adhesion, migration strategies, gene and protein expression are reported, among others.<sup>26</sup> Although the underlying mechanisms of these differences remain unclear, dimensionality and compliance of the matrix are the plausible key factors.<sup>26</sup>

#### ***3.4.1. The fibroblast-populated collagen matrix (FPCM)***

The fibroblast-populated collagen matrix (FPCM) has earlier been utilized as an in vitro model for wound healing. It provides a reasonable representation of the dermal granulation tissue in a healing wound.<sup>5</sup>

The major disadvantage as well as the advantage of this model is its simplicity. Granulation tissue is a complex composition of several cell types and numerous proteins including fibrin, fibronectin and vitronectin as well as collagen, hyaluronan and a variety of growth factors and release products. There is a continuous evolution of the matrix and as the concentration of the components change, the granulation tissue matures.<sup>5</sup> The FPCM on the other hand, consists of only one cell type, fibroblasts, and initially there is only one protein, collagen.

However, incubation of this matrix in the presence of serum provides a source of some extracellular matrix molecules. Besides, fibroblasts cultured in this milieu produce a variety of proteins which also are incorporated into the matrix, creating a model with the ability to mature in a similar way as granulation tissue. The most significant matrix constituent absent in the FPCM is the fibrin network, which initiates the wound healing process *in vivo*.<sup>5</sup> Consequently, the simplicity of the fibroblast-populated collagen matrix in comparison with granulation tissue in a healing wound is advantageous because it enables experimental precision. Nevertheless, it is difficult to extrapolate the *in vitro* results to the much more complex *in vivo* situation.<sup>5</sup>

### **3.5. Extracellular matrix proteins**

The extracellular matrix is a complex, heterogeneous, dynamic and tissue specific composition of different biopolymers and water, including proteins and glucosaminoglycans.<sup>22,26</sup> The two key extracellular matrix proteins of the present project are described below.

#### **3.5.1. Collagen**

Collagen is the most abundant protein in the body and exists in at least ten different forms in tissues of vertebrates.<sup>26,27</sup> The collagen in skin, bone and tendon is predominantly type I collagen. Type II collagen is the major constituent of cartilage whereas type III collagen is predominant in blood vessel walls.<sup>27</sup>

The tropocollagen is the essential unit of a collagen fiber and consists of three polypeptide strands twisted into a triple helix. The subunits are synthesized and processed intracellularly, subsequently exported to the extracellular matrix where they self-assemble into the triple helical collagen molecule. Several molecules then aggregate into microfibrils and several microfibrils aggregate to form large collagen fibers.<sup>18,27</sup>

Collagen possesses cell-adhesion domain sequences which facilitate cell attachment and allow specific cellular interactions. This feature may be important in preserving the phenotypes and activities of many cell types.<sup>26</sup> Collagen is easily purified from animal tissues by acid digestion and forms a gel when returned to neutral pH. By altering the collagen concentration, pH or ionic strength during gelation (polymerization), pore size and fiber diameter can be moderately adjusted. As the gel forms, the microfibrils aggregate laterally into fibers which in turn entangle into a matrix. Reconstituted collagen matrices are mechanically weaker and more highly hydrated than natural tissues.<sup>26</sup>

### **3.5.2. Enamel matrix derivative (EMD)**

Enamel matrix derivative (EMD) is the active component in Xelma®, a wound healing product for hard to heal wounds (Mölnlycke Health Care AB, Gothenburg, Sweden). In clinical studies, Vowden *et al.* (2006, 2007) have demonstrated the advantages of EMD in the treatment of chronic venous leg ulcers; the wound area was reduced and the exudate production decreased.

EMD are extracted from the enamel matrix of developing porcine teeth.<sup>6</sup> It is a mixture of hydrophobic proteins where the principal component is amelogenins. The unique amino acid sequences seem to facilitate aggregation of the proteins into large hydrophobic complexes instead of promoting the formation of the usual secondary structures. Under physiological conditions, these aggregates resemble a stable extra cellular matrix, thus serving as a temporary matrix for cell attachment and migration.<sup>7</sup>

Topical application of EMD has earlier been utilized in periodontal surgery and in acute periodontal injuries since it demonstrated positive effects on periodontal wound healing and tissue regeneration.<sup>28</sup> The molecular mechanisms underlying these results are still not fully elucidated but the presence of amelogenin seems to regulate or induce several essential cell responses, including proliferation, migration and differentiation.<sup>7,8</sup>

EMD have been shown to enhance cell adhesion of numerous cell types.<sup>29</sup> The broad cell-type specificity of these proteins proposed the appliance of EMD on injured skin in order to promote the wound healing processes. Mirastschijski *et al.* (2003) showed that topical treatment with EMD in full-thickness wounds in rabbits increased formation of granulation tissue and accelerated wound closure. Furthermore, it is demonstrated that incubation of human dermal fibroblasts *in vitro* with EMD generated a strong biological response including increased cell proliferation and contraction of three-dimensional matrices as well as augmented levels of vascular endothelial growth factor (VEGF) and transforming growth factor  $\beta$ 1 (TGF- $\beta$ 1).<sup>6,10</sup>

### 3.6. Cell mediators

Numerous cell mediators are involved in the wound healing processes. Besides several growth factors such as vascular endothelial growth factor (VEGF) and platelet derived growth factor (PDGF), three important cytokines synthesized by fibroblasts are interleukin 6 (IL-6), interleukin 8 (IL-8) and monocyte chemoattractant protein 1 (MCP-1).<sup>30</sup>

IL-6 is a proinflammatory cytokine shown to be strongly upregulated during the inflammatory phase of wound healing.<sup>30</sup> This protein has demonstrated a mitogenic effect for keratinocytes and a chemoattractive effect on neutrophils and is therefore believed to be essential for initiating the healing process. Complete lack of IL-6 seems to impair wound healing whereas extreme levels of the cytokine are related to scar formation. Thus, a coordinated expression of IL-6 seems to be crucial for normal tissue repair.<sup>30</sup>

IL-8 is a key mediator of the inflammatory response. This chemotactic cytokine was reported to recruit polymorphonuclear leukocytes to the wound site<sup>30</sup>, stimulate keratinocyte proliferation<sup>31</sup> but exhibit an inhibitory effect on wound contraction.<sup>30</sup> IL-8 has furthermore demonstrated angiogenic properties.<sup>32</sup> Human chronic wound fluid is shown to contain high levels of this cytokine.<sup>30</sup>

MCP-1 is another chemotactic cytokine, a major chemoattractant for monocytes and macrophages. Moreover, it is demonstrated to recruit T-cells and mast cells<sup>30</sup>, influence endothelial cell migration<sup>33</sup> and stimulate collagen production by fibroblasts.<sup>34</sup> Total absence of MCP-1 was suggested to result in reduced collagen synthesis and delayed reepithelialization and angiogenesis.<sup>30</sup>

## 4. MATERIALS AND METHODS

### 4.1. Materials

Normal human dermal fibroblasts (NHDF) were derived from a healthy volunteer and purchased from Karocell (Stockholm, Sweden). Near-senescent human dermal fibroblasts (cell line CRL-7815) were derived from a chronic dermatitis patient and purchased from the American Type Culture Collection (Rockville, MD). The latter cells are here referred to as “chronic” fibroblasts.

Dulbecco’s modified Eagles’s medium (DMEM) with phenol red and 1 g/L GlutaMAX™, Dulbecco’s modified Eagle medium 2x (2x DMEM) without phenol red, Hanks’ balanced salt solution (HBSS) without Ca and Mg, penicillin (100 units/mL), streptomycin (100 µg/mL), trypsin (0.25 %) / EDTA (1 mM), fetal bovine serum (FBS), Trypan Blue Stain and Fluorescein isothiocyanate (FITC)-conjugated phalloidin were purchased from Invitrogen (Paisley, UK). Phosphate buffered saline (PBS) was purchased from Bakteriologiska laboratoriet (Göteborg, Sweden). Rat tail collagen type I, T-25 tissue culture flasks, 24-well plates and Cultureslides were purchased from BD Biosciences (Erembodegem, Belgium). Collagenase type I clostridium histolyticum and Bovine serum albumin (BSA) were purchased from EMD Biosciences (San Diego, US). Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) was purchased from Vector Laboratories (Peterborough, UK). Enamel matrix derivative (EMD) was purchased from Biora AB (Malmö, Sweden) and Triton X-100 was purchased from Sigma-Aldrich (Basel, Switzerland).

### 4.2. Cell culture

Normal human dermal fibroblasts (NHDF) and chronic human dermal fibroblasts (CRL) were maintained and expanded in T-25 tissue culture flasks in Dulbecco’s modified Eagle medium (DMEM) with Glutamax 1 g/L, supplemented with 10 % of fetal bovine serum (FBS) and 1 % of penicillin/streptomycin. Cultures were incubated at 37°C with 5 % CO<sub>2</sub> and 95 % humidity. Culture medium was changed three times weekly and confluent cultures were washed with Hanks’ balanced salt solution (HBSS, without Ca and Mg) and passaged using trypsin/EDTA (0.25 % trypsin in 1 mM EDTA). Cell viability was determined by means of trypan blue exclusion and cell counts were performed in a counting chamber (Bürker hemocytometer). All experiments were performed on cells between passage 4 and 12.

### 4.3. Fabrication of collagen matrices

Collagen matrices were fabricated according to the manufacturer's instructions and Grayson *et al.* (2006) with some modifications. In the optimized method, 10 units of 2x DMEM were mixed with 2 units of FBS, 2 units of 0.1 M NaOH, 4 units of ice cold rat-tail type I collagen solution (stock 10 mg/mL) and 2 units of cells suspended in supplemented DMEM. Acellular matrices were formed by replacing the cell suspension with 2 units of supplemented DMEM.

Matrices were cast with 400  $\mu$ L per well in 24-well plates and with a cell density of  $6 \times 10^4$  cells per mL matrix. The plates were incubated for 1 hour at 37°C with 5 % CO<sub>2</sub> and 95 % humidity to allow collagen polymerization. Thereafter, 1 mL of supplemented DMEM was added to each well and the plates were incubated for 1, 3 and 7 days at 37°C with 5 % CO<sub>2</sub> and 95 % humidity without the change of medium.

EMD treatment of matrices was performed by topical addition. Instead of adding 1 mL of supplemented DMEM to each well after polymerization, 1 mL of EMD (stock 20 mg/mL diluted in supplemented DMEM to 0.1 mg/mL and 1 mg/mL) was added to each well. The plates were then incubated for 1, 3 and 7 days as previously described.

Moreover, to assure that the mechanical conditions in all matrices would remain the same, wells were pre-coated with BSA (100  $\mu$ g/mL diluted in PBS) in some of the experiments in order to prevent collagen adsorption to the walls of the wells.

### 4.4. Proliferation analysis

After 1, 3 and 7 days the supernatants from each well were collected in Eppendorf tubes and centrifuged for 5 min at 1000 rpm. The supernatants were collected in two new tubes and stored in -70°C for future cytokine analysis. The pellets in the original tubes were suspended in 20  $\mu$ L of supplemented DMEM.

To each matrix, 400  $\mu$ L of pre-warmed (37 °C) collagenase solution (1 mg/mL diluted in PBS) were added and the plates were incubated for 120 min at 37°C with 5 % CO<sub>2</sub> and 95 % humidity to allow collagen degradation. The solutions from each well were transferred into the Eppendorf tubes from above. Then, 150  $\mu$ L of trypsin/EDTA were added to each well and incubated for 5 min at 37°C with 5 % CO<sub>2</sub> and 95 % humidity to release the cells still attached to the bottom of the wells. The trypsin was inactivated using 150  $\mu$ L of supplemented DMEM. These solutions were also added to the Eppendorf tubes from above and centrifuged for 5 min at 1000 rpm. The supernatants were discarded and the pellets were dissolved in 20  $\mu$ L of supplemented DMEM.

The cell numbers were then measured using the NucleoCounter™ automatic cell counting system (ChemoMetec, Allerød, Denmark). According to the manufacturer's instructions, the cell sample was mixed with an equal volume of reagent A-100 (lysis / disaggregation buffer) and reagent B (stabilizing buffer). Nucleo-Cassettes™ were loaded with 50 µL of sample mixture and placed in the NucleoCounter™ instrument for measurement.

#### **4.5. Histology analysis**

Collagen matrices were fabricated according to the procedure described above with a cell density of 60 000 cells per mL matrix. When EMD treatment of matrices was performed, the substance was added topically after polymerization.

After 1, 3 and 7 days the supernatants from each well were discarded and the matrices were fixed with 1 mL of 4 % formaldehyde in PBS for 2 hours at room temperature. The formaldehyde was removed and the matrices were washed twice with PBS and once with 70 % ethanol. The samples were then kept in 70 % ethanol in room temperature until preparation. Paraffin-embedding, microscope sectioning and van Gieson's staining were performed by HistoCenter – Skandinaviskt Centrum för Histoteknik AB (Västra Frölunda, Sweden).

#### **4.6. Morphology analysis in 2D and 3D**

This experiment was performed according to Jiang *et al.* (2005) with some modifications. Two dimensional collagen surfaces were obtained by coating cultureslides with collagen solution (100 µg/mL in 2x DMEM) following incubating at 4°C over night. Three dimensional collagen surfaces were obtained by incubating cultureslides with 200 µL of collagen solution (2 mg/mL) for 1 hour at 37°C with 5 % CO<sub>2</sub> and 95 % humidity to allow collagen polymerization.

The collagen solution for the three dimensional surfaces was prepared as previously described with some modifications. Briefly, 11 units of 2x DMEM were mixed with 2 units of FBS, 2 units of 0.1 M NaOH, 4 units of ice cold rat-tail type I collagen solution (stock 10 mg/mL) and 1 unit of 17 mM HAc.

Thereafter, 100 µL of fibroblasts suspended in 2x DMEM with 5 mg/mL BSA and 10 % FBS were seeded on top of the coated surfaces and polymerized matrices (cell concentration 50 cells/mm<sup>2</sup>). The cultureslides were incubated for 30, 60 and 120 min at 37°C with 5 % CO<sub>2</sub> and 95 % humidity.

The supernatants were removed and the cells were fixed with 200  $\mu$ L of 4 % formaldehyde in PBS for 10 min at room temperature. The cells were washed twice with PBS, permeabilized with 100  $\mu$ L of 0.5 % Triton X-100 in PBS for 5 min and washed twice with PBS again. Cells were then incubated with 200  $\mu$ L of 1 % BSA in PBS for 30 min and washed twice with PBS. The cells were treated with 100  $\mu$ L of FITC-conjugated phalloidin (2 U/mL in PBS containing 1 % BSA) for 30 min in room temperature and washed twice with PBS to remove surplus fluorescence. Samples were mounted with one droplet of DAPI medium and kept in darkness to avoid fading of the fluorophores. The cells were examined under a fluorescence microscope (Zeiss) and images were collected using a Canon Powershot G6 camera.

EMD treatment of matrices was performed by adding EMD in the collagen solution prior to polymerization. Two different concentrations of EMD were used, namely 0.1 mg/mL and 1 mg/mL, corresponding to collagen concentrations 1.9 mg/mL and 1 mg/mL in order to keep the total protein concentrations of the matrices at 2 mg/mL.

Briefly, 11 units of 2x DMEM were mixed with 2 units of FBS, 2 units of 0.1 M NaOH and 4 units of ice cold rat-tail type I collagen solution (stock 10 mg/mL diluted in 17 mM HAc to 9.5 mg/mL and 5 mg/mL for the matrices with lower collagen concentrations). 1 unit of EMD (stock 20 mg/mL diluted in 17 mM HAc to 2 mg/mL for the matrices with low EMD concentration) or 1 unit of 17 mM HAc (control) was added. Thereafter, the matrices were treated as described above.

## 4.7. Cytokine analysis

Cell culture supernatants were collected from the first four proliferation experiments and stored at -70 °C prior to analysis. Cytokine concentrations in these samples were then analyzed using the Human Cytokine Twenty-Five-Plex Antibody Bead Kit from BioSource™ (Invitrogen, UK). This kit enables the measurement of human IL-1 $\beta$ , IL-1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40/p70, IL-13, IL-15, IL-17, TNF- $\alpha$ , IFN- $\alpha$ , IFN- $\gamma$ , GM-CSF, MIP-1 $\alpha$ , MIP-1 $\beta$ , IP-10, MIG, Eotaxin, RANTES and MCP-1.

The experiment was performed according to the manufacturer's instructions. Samples were clarified by centrifugation at 14,000 rpm at 4°C for 10 min. Each protein standard was diluted in 0.5 mL (0.25 mL Assay Diluent and 0.25 mL supplemented DMEM) and after complete reconstitution, further serially diluted according to the protocol. The wells of the filter bottom microplate were washed by adding 0.2 mL of Wash Solution and aspirated using a vacuum manifold. The beads of defined spectral properties conjugated to analyte specific antibodies were vortexed for 30 seconds followed by sonication in a sonicating water bath for 30 seconds. Into each well 25  $\mu$ L of the antibody beads were added.

The plate was washed twice with Wash solution and 50  $\mu$ L of Incubation Buffer were added to each well. To the wells selected for the standard curve 100  $\mu$ L of appropriate standard dilution were added. To the wells selected for the samples 50  $\mu$ L of Assay Diluent followed by 50  $\mu$ L of sample were added. The microplate was incubated for two hours at room temperature on an orbital shaker in order to bind the analytes to the antibodies on the beads.

The plate was washed twice and 100  $\mu$ L of analyte-specific Biotinylated Detector Antibody were added to each well. The plate was incubated for one hour at room temperature on an orbital shaker in order to bind these antibodies to the immobilized analytes. Excess biotinylated detector antibodies were removed by washing the plate twice with Wash Solution.

After the addition of 100  $\mu$ L of Streptavidin conjugated to the fluorescent protein R-Phycoerythrin (Streptavidin-RPE) to each well, the plate was incubated for 30 min at room temperature on an orbital shaker in order to bind the streptavidin-RPE to the biotinylated detector antibodies. Unbound streptavidin-RPE were removed by washing the plate three times with Wash Solution.

Finally, 100  $\mu$ L of Wash solution were added to each well and the plate was incubated for 2-3 min at room temperature on an orbital shaker in order to resuspend the beads. The plate was then analyzed using a Luminex™ 100 Cytometer (Applied Cytometry, Sheffield, UK). The concentrations of the analytes were determined by monitoring the spectral properties of the beads and the amount of associated RPE fluorescence.

#### **4.8. Contraction analysis**

Cellular matrices cast in BSA coated wells were all contracted upon incubation. Contraction measurements were performed on all matrices incubated for more than 24 hours. After 1, 3 and 7 days images of the matrices were collected using a Canon Digital IXUS 750 camera. The reduction in matrix diameter was utilized as a measure of contraction.

## 5. RESULTS

Cellular processes important for dermal repair and regeneration have been analyzed in the three dimensional in vitro model using different methods. The results are described below.

### 5.1. Proliferation analysis

The proliferation assay was performed by culturing cells in collagen matrices for 1, 3 and 7 days. The cell concentrations were then measured using the NucleoCounter™ automatic cell counting system. This technique is based on the quantification of cell nuclei stained with propidium iodide. The results from the proliferation experiment are shown in figure 3 and 4.

The collagen matrices were cast in wells either uncoated or coated with BSA. This coating prevents collagen adsorption to the walls of the wells, thus reducing the mechanical tension in the matrices and facilitating matrix contraction.

Figure 3 shows the proliferation of normal fibroblasts. Both for the matrices with and without topical addition of EMD, the proliferation of cells were increased in uncoated, attached matrices in comparison with BSA-coated, released matrices. An exception was the measurement taken on day 1 of the matrices without EMD which showed a higher proliferation in released matrices. The standard deviation was however rather high here which necessitates careful interpretation of these results. Between day 3 and day 7 fibroblasts in the released matrices did not seem to proliferate at all.

For chronic fibroblasts, the effect of coating the wells was not evident, as seen in figure 4. Only occasionally, the proliferation of these cells seemed to be higher in attached matrices than in released matrices. The reason for this variation is not clear; still the relatively large standard deviations of some measurements need to be taken into account before conclusions can be drawn.

The number of fibroblasts increased in all groups of normal fibroblasts, with the exception of the matrices cast in BSA-coated wells without EMD. As mentioned above, the large differences between the replicates need to be considered here. For the chronic fibroblasts, this development was not apparent; for the matrices without EMD the number of cells seemed to be almost constant, or even decreasing. For the matrices with EMD, cell number first increased but after seven days a reduction was observed.

## RESULTS

The results further demonstrate that the general ability of chronic fibroblasts to proliferate is reduced compared to normal fibroblasts. The effect of topical addition of EMD in a concentration of 1 mg/mL was however obvious for both cell types, increasing the cell concentration in the matrices approximately threefold.

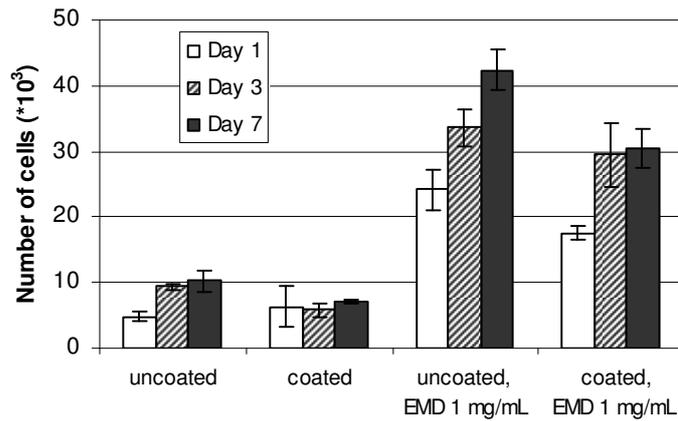


Figure 3. Number of normal fibroblasts cultured in collagen matrices for 1, 3 and 7 days with or without topical addition of EMD (1 mg/mL) and cast in wells either uncoated or coated with BSA. (n=4)

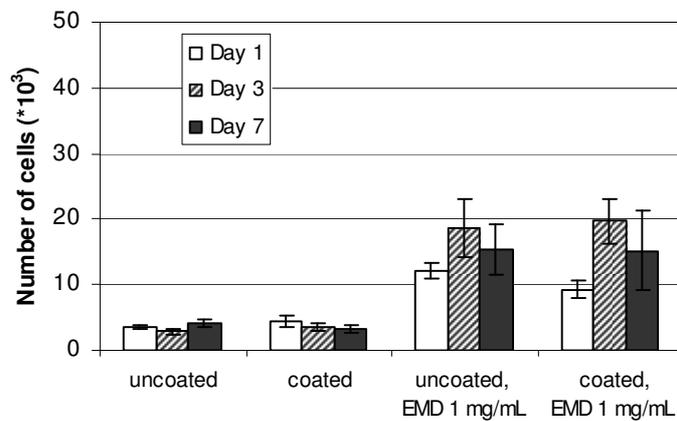


Figure 4. Number of chronic fibroblasts cultured in collagen matrices for 1, 3 and 7 days with or without topical addition of EMD (1 mg/mL) and cast in wells either uncoated or coated with BSA. (n=4)

## 5.2. Cellular morphology analysis

To investigate the cellular morphology of fibroblasts in three dimensions in comparison with two dimensions using fluorescence microscopy, cells were incubated for 30, 60 and 120 minutes on surfaces or on collagen matrices and stained for F-actin. Images of cells with representative morphologies were collected.

After one hour of incubation the differences between chronic fibroblasts seeded on collagen coated surfaces (2D) and on collagen matrices (3D) were obvious. Figure 5A shows a chronic fibroblast seeded on a collagen coated surface. It is broadly spread and has a typical flattened unnatural morphology. Fibroblasts seeded on collagen matrices on the other hand, show a spindle shape and a reduced cell size, as seen in figure 5B (chronic fibroblast) and 5C (normal fibroblast).

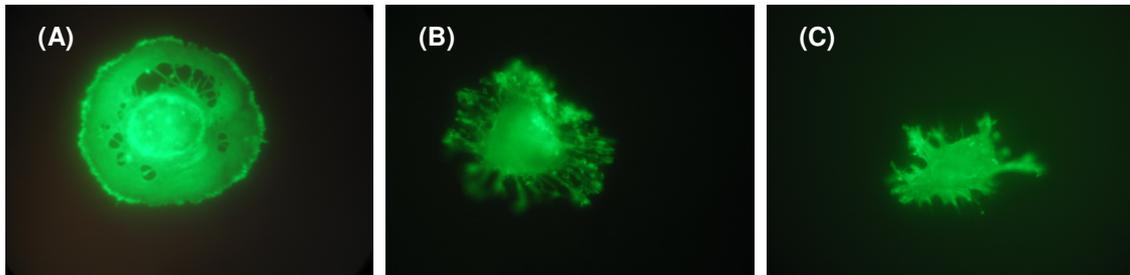


Figure 5. Chronic fibroblasts seeded for 1 hour on (A) a collagen coated surface and (B) a collagen matrix. (C) Normal fibroblast seeded for 1 hour on a collagen matrix. Original magnification x1000.

Increasing the incubation time from 30 to 60 and 120 minutes led to elongated extensions of the cells, as seen in figure 6. However, with the longer incubation the cells commenced to penetrate the protein matrix, complicating the visualizing of the cells in the microscope.

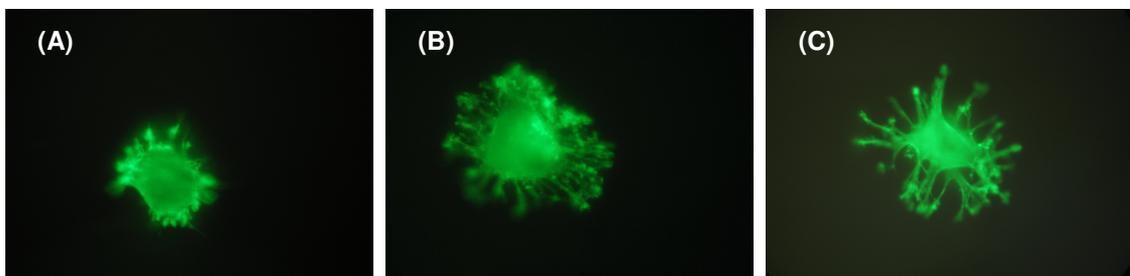


Figure 6. Chronic fibroblasts seeded on collagen matrices for (A) 30 minutes, (B) 60 minutes and (C) 120 minutes. Original magnification x1000.

No obvious difference in cellular morphology was seen between normal fibroblasts and chronic fibroblasts neither in 2D nor in 3D, implying that the chronicity of chronic fibroblasts do not affect their ability to attach to and spread on surfaces or matrices (Figure 5B and 5C).

The effect of EMD on cell morphology was also analyzed but these results are difficult to interpret. The appearance of the matrices fabricated with a collagen concentration of 1 mg/mL and an EMD concentration of 1 mg/mL aroused suspicions of whether high EMD concentrations impair collagen polymerization. If these suspicions were justified, studying cells on these matrices for the evaluation of the EMD effect on fibroblast morphology would not generate a fair result. In addition, these matrices were difficult to handle which decreased the possibility to get representative images of the cells. No evident effect of EMD on cellular morphology could be observed, as seen in figure 7.

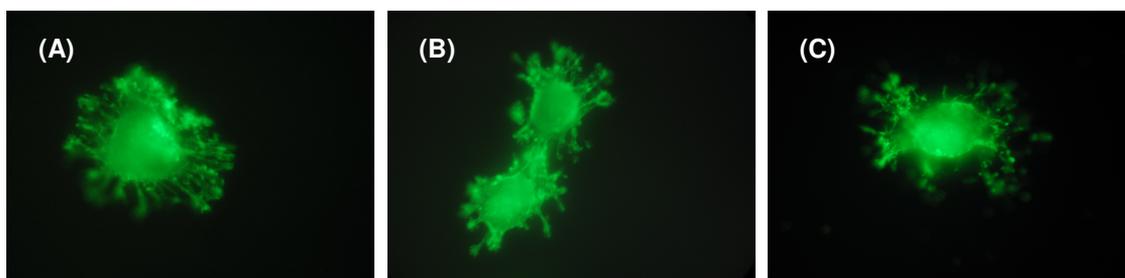


Figure 7. Chronic fibroblasts seeded on collagen matrices (A) without EMD, (B) with an EMD concentration of 0.1 mg/mL and (C) with an EMD concentration of 1 mg/mL. Original magnification x1000.

### 5.3. Matrix contraction analysis

Contraction measurements were performed on all matrices incubated for more than 24 hours. The collagen matrices were cast in wells either uncoated or coated with BSA. This coating prevents the matrices from attaching to the walls of the wells, thus reducing the mechanical tension in the matrix and facilitating matrix contraction.

Figure 8 shows collagen matrices incubated for 1, 3 and 7 days. During all experiments collagen matrices cast in wells coated with BSA were detached from the well walls whereas matrices cast in uncoated wells remained attached during the entire time of the study, i.e. 7 days. Contraction of detached matrices containing fibroblasts commenced directly after polymerization while contraction of detached acellular matrices never occurred. Moreover, a faster contraction was observed for the matrices with normal fibroblasts in comparison with the matrices containing chronic fibroblasts.

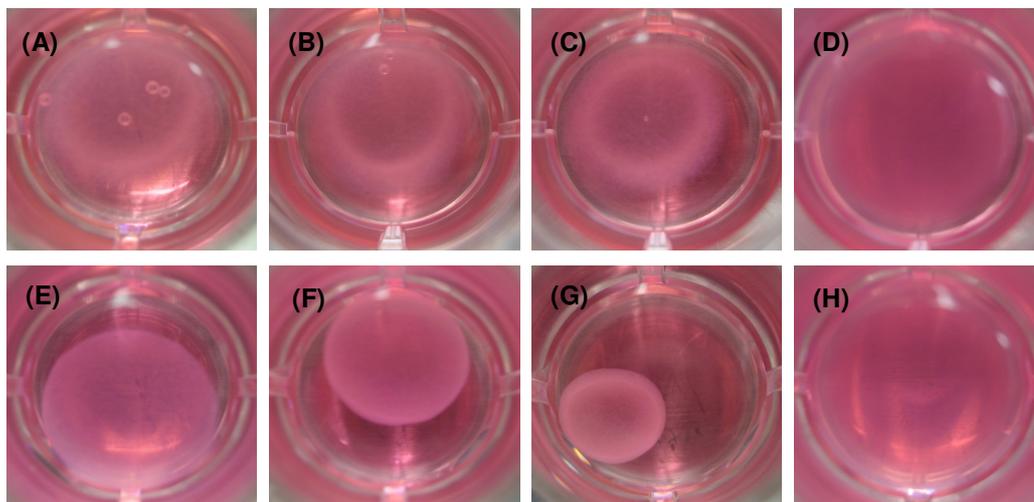


Figure 8. Collagen matrices containing chronic fibroblasts cast in wells coated with BSA and incubated for (A) 1 day, (B) 3 days and (C) 7 days. (D) Acellular collagen matrix cast in a well coated with BSA and incubated for 7 days. Collagen matrices containing normal fibroblasts cast in wells coated with BSA and incubated for (E) 1 day, (F) 3 days and (G) 7 days. (H) Collagen matrix containing normal fibroblasts cast in an uncoated well and incubated for 7 days. (Well diameter 15.5 mm)

The effect of topical addition of EMD (1 mg/mL) to the collagen matrices is shown in figure 9. An increased contraction was observed after EMD incubation, both for matrices containing chronic fibroblasts and for matrices containing normal fibroblasts. This is seen by the slightly reduced diameters of the matrices in figure 9 B and D compared to A and C. Topical addition of EMD did not initiate contraction of cellular matrices cast in uncoated wells, as seen in figure 9 E-H. Nor did addition of EMD initiate contraction of acellular matrices. (data not shown)

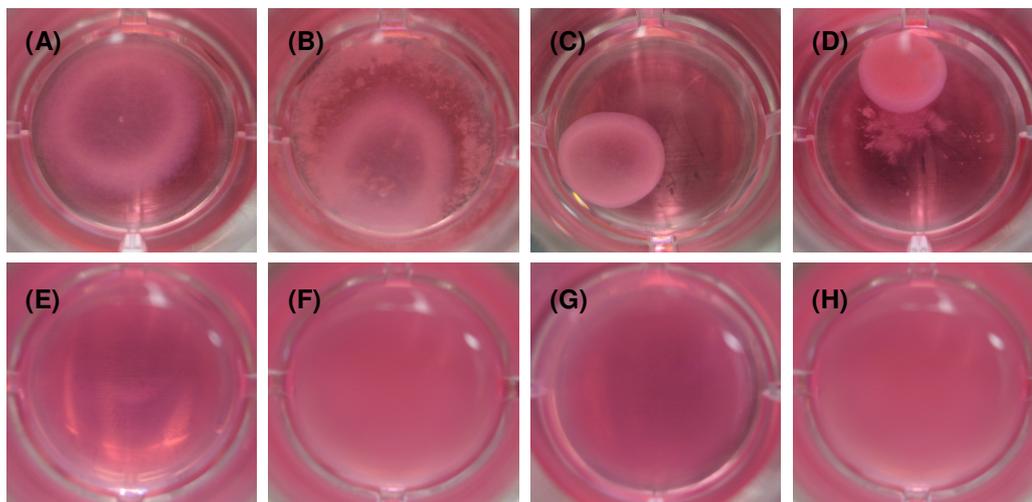


Figure 9. Collagen matrices containing chronic fibroblasts cast in wells coated with BSA and incubated for 7 days (A) without EMD and (B) with 1 mg/mL EMD. Collagen matrices with normal fibroblasts cast in wells coated with BSA and incubated for 7 days (C) without EMD and (D) with 1 mg/mL EMD. Collagen matrices with chronic fibroblasts cast in uncoated wells and incubated for 7 days (E) without EMD and (F) with 1 mg/mL EMD. Collagen matrices with normal fibroblasts cast in uncoated wells and incubated for 7 days (G) without EMD and (H) with 1 mg/mL EMD. (Well diameter 15.5 mm)

#### 5.4. Histology analysis

Histology analysis was performed by culturing cells in collagen matrices for 1, 3 and 7 days. Cross-sections were then stained with van Gieson's staining for tissue analysis.

Figure 10 shows the van Gieson's staining of the collagen matrices. Collagen fibers are stained red and cell nuclei are stained dark purple. After 1 day of incubation the matrices containing chronic fibroblasts and normal fibroblasts were comparable; the collagen fibers were straight and oriented in the same direction. After 3 and 7 days of incubation the matrices with chronic fibroblasts still exhibited a rather similar structure although a tendency of less oriented fibers could be observed after 7 days.

The structure of the matrices containing normal fibroblasts changed more noticeably with increased incubation time. After 3 days of incubation a darker staining appeared in the close vicinity of the cells, implying an increasing concentration of collagen in these areas. As the incubation time increased further an intensification of the staining could be visualized in the entire matrix and simultaneously the alignment of the collagen fibers became indistinct.

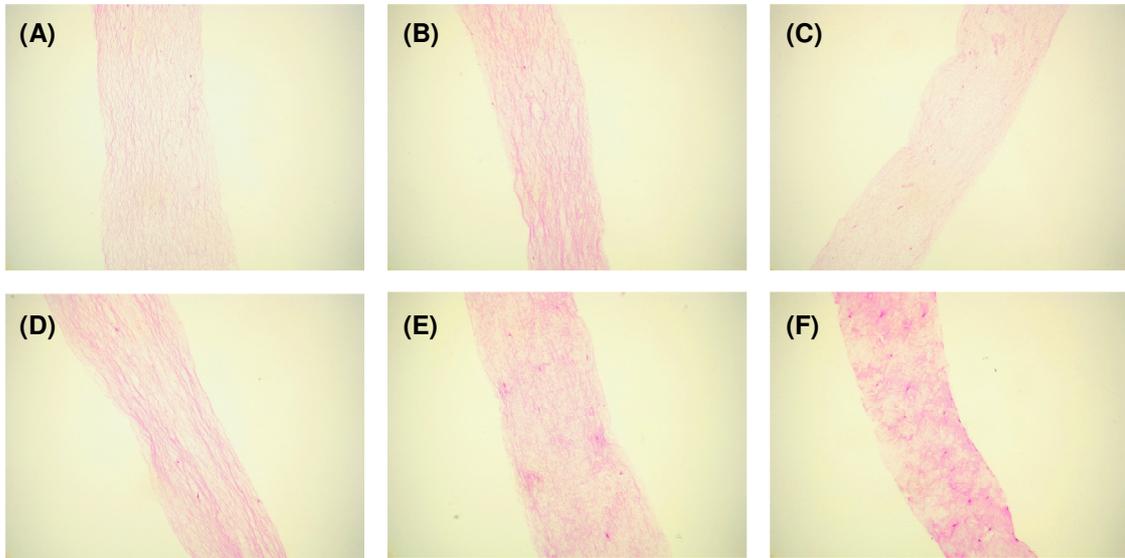


Figure 10. van Gieson's staining of collagen matrices containing chronic fibroblasts, incubated for (A) 1 day, (B) 3 days and (C) 7 days. van Gieson's staining of collagen matrices containing normal fibroblasts, incubated for (D) 1 day, (E) 3 days and (F) 7 days. Original magnification x 100.

The effect on the histological appearance of the collagen matrices upon topical addition of EMD is shown in figure 11. For matrices containing chronic fibroblasts, addition of EMD seemed to increase the remodeling rate. After 7 days, the collagen fibers are still oriented in almost the same direction in the matrices incubated without EMD whereas the matrices incubated with an EMD concentration of 1 mg/mL exhibited many collagen fibers without a particular orientation. The effect is less pronounced in the matrices incubated with an EMD concentration of 0.1 mg/mL.

For matrices containing normal fibroblasts the effect of EMD was even more apparent; After 7 days, the staining of the matrices incubated with an EMD concentration of 1 mg/mL was much more intense than the staining of the matrices incubated without EMD. The color intensity and hence the high collagen concentration even complicated the visualization of the collagen fibers. The low EMD concentration elicited intermediate effects.

EMD can be visualized as light orange areas on the left side of the matrix containing normal fibroblasts and incubated for 7 days. However, no proteins are detectable inside the matrix according to these images.

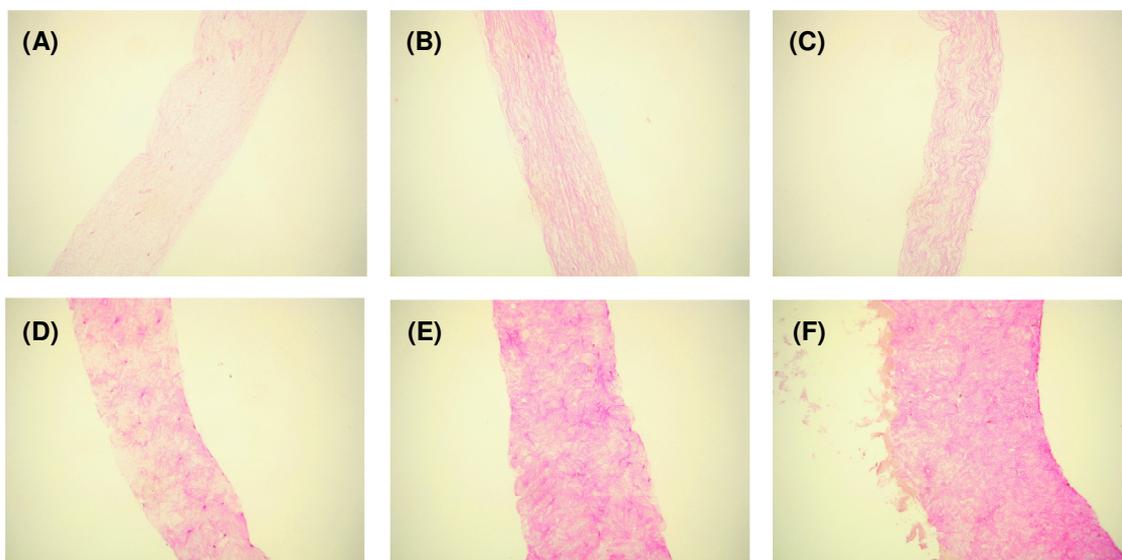


Figure 11. van Gieson's staining of collagen matrices containing chronic fibroblasts, incubated for 7 days (A) without EMD, (B) with an EMD concentration of 0.1 mg/mL and (C) with an EMD concentration of 1 mg/mL. van Gieson's staining of collagen matrices containing normal fibroblasts, incubated for 7 days (D) without EMD, (E) with an EMD concentration of 0.1 mg/mL and (F) with an EMD concentration of 1 mg/mL. Original magnification x 100.

Whether the cell concentration in the matrices increased during the longer incubation time or with addition of EMD is difficult to see on these images, partly because the cell concentration is very low and partly because the red color is intensified, complicating the discrimination of the cells.

The thicknesses of the matrices seen on these images should not be analyzed in depth. It seems as if these are more a reflection of the angle with which the cross-sections are cut than the actual thicknesses of the matrices.

## 5.5. Cytokine analysis

Cell culture supernatants were collected from matrices incubated for 1, 3 and 7 days and cytokines were analyzed using the Human Cytokine Twenty-Five-Plex Antibody Bead Kit, originally designed for inflammatory cells.

The results from the analysis show that the fibroblasts used here produce at least three of the analyzed cytokines, namely IL-6, IL-8 and MCP-1. The chronic fibroblasts seemed to synthesize larger amounts of IL-6 and MCP-1 than normal fibroblasts while the relationship was the opposite for the synthesis of IL-8. The other cytokines were not produced in amounts above the detection limit.

## RESULTS

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The standard deviations of the measurements were rather high which complicates the interpretation of the results but in general it appears as if the presence of a high EMD concentration (1 mg/mL) reduced the production of all three cytokines, IL-6, IL-8 and MCP-1.

## 6. DISCUSSION

### 6.1. Proliferation analysis

It has been demonstrated that proliferation of cells requires a combination of signals, where mechanical tension within the cells seems to be involved.<sup>36</sup> Thus, fibroblasts cultured in attached, mechanically loaded matrices proliferate whereas cells cultured in released or floating matrices tend to become quiescent and apoptotic.<sup>36</sup> This phenomenon can clearly be seen for normal fibroblasts, where proliferation was increased in uncoated, attached matrices in comparison with BSA-coated, released matrices. Between day 3 and day 7 fibroblasts in the released matrices did not seem to proliferate at all, indicating the presence of many quiescent cells.

For chronic fibroblasts, the effect of coating the wells was not evident. Only occasionally, the proliferation of these cells seemed to be higher in attached matrices than in released matrices. The reason for this variation is not clear; still the relatively large standard deviations of some measurements need to be taken into account before conclusions can be drawn. The reduction in cell number observed after 7 days in EMD treated matrices implies an increased amount of apoptotic cells.

The effect of topical addition of EMD in a concentration of 1 mg/mL was obvious for both cell types, increasing the cell concentration in the matrices approximately threefold. Grayson *et al.* (2006) have earlier demonstrated increased proliferation of normal fibroblasts cultured within collagen matrices with EMD incorporated inside, although these effects were less pronounced. Furthermore, it is shown that EMD increased proliferation of the CRL chronic cell line when cultured in monolayer.<sup>37</sup>

As described previously by several groups<sup>4,37</sup> these results also demonstrate that the general ability of chronic fibroblasts to proliferate is reduced compared to normal fibroblasts. The fascinating observation was however that topical treatment of chronic fibroblasts with EMD enabled the cells to proliferate even better than untreated normal fibroblasts. This result is of great importance when striving to change the behavior of chronic fibroblasts into a behavior resembling the one of normal fibroblasts.

Initially proliferation experiments in the present study were performed on collagen matrices with different EMD concentrations incorporated inside the matrices, a technique earlier used by Grayson *et al.* (2006). The results from the present experiments were however not consistent and the standard deviations were high (data not shown). Together with the appearance of the matrices, this aroused

suspensions of whether a high EMD concentration impairs collagen polymerization. In order to make the conditions in all matrices as similar as possible, EMD was instead added on top of the matrix after polymerization. Furthermore, this new approach closer mimics the treatment practice of topical appliance of EMD on a wound. The results are presented in this report.

Apparently, EMD does not need to be incorporated in the matrix in order to affect the proliferation of cells. Possibly, small aggregates or monomers released from the aggregated protein enter the collagen matrix in a concentration high enough to influence the fibroblasts in the matrix. The mechanism underlying this stimulatory effect of EMD is not fully elucidated.

To avoid large variations in the measurements and assure more statistically reliable results for chronic cells, a suggestion would be to utilize more replicates in this study. This experiment has however been performed several times and the outcome is most often the same; chronic fibroblasts seem to be more sensitive and have more varying ability to proliferate than normal fibroblasts, making these analyses complicated to interpret.

## 6.2. Cellular morphology analysis

Fibroblasts seeded on collagen coated surfaces demonstrated a typical flattened unnatural morphology. The extreme spreading observed was probably caused by the absence of dorsal adhesion points. The balance between spreading and retraction is altered which stimulates excessive spread out.<sup>38</sup> Fibroblasts seeded on collagen matrices on the other hand, show a spindle shape and a reduced cell size. This natural morphology is seen when fibroblasts are cultured in a three dimensional environment with adhesion points in all directions.<sup>26</sup>

No obvious difference in cellular morphology was seen between normal fibroblasts and chronic fibroblasts neither in 2D nor in 3D, implying that the chronic fibroblasts are not affected in their ability to attach to and spread on surfaces or matrices. These results are consistent with the findings of Cook *et al.* (2000) who showed that chronic fibroblasts exhibited no difference in morphology compared to patient-matched normal fibroblasts. In contrast, other groups, Ågren *et al.* (1999) being one example, demonstrate a morphology of chronic fibroblasts that resemble in vitro senescent fibroblasts.

Since amelogenin, the principal component of EMD, is an adhesion protein which has been shown to enhance cell adhesion of numerous cell types,<sup>29</sup> the expected effect of EMD on cell morphology would be an increase in cell adhesion points. However, these observations could not be made in this experiment.

The present morphology analysis illustrates the importance of a careful selection of wound model when examining the wound healing process. Apparently, cells express different adhesion patterns and assume different morphologies depending on the dimensionality of the substrate. Culturing cells in two dimensions instead of their natural three dimensional environment therefore increases the risk to misinterpret significant biological behaviors of cells.

To extend the morphology study in order to investigate morphology differences over a longer time scale would obviously be of interest. Analyzing morphology of cells cultured within the three dimensional matrices would provide results which more adequately reflect the morphology of cells in tissues. However, these studies require other equipment than an ordinary fluorescence microscope for visualizing cells, confocal laser scanning microscope being one example. Another approach for extension of the morphology study would be to stain cells for other components than F-actin, myosin being a component of interest.

### **6.3. Matrix contraction and histology analyses**

The histology and contraction analysis demonstrated that the remodeling rate and contraction ability is lower in the matrices containing chronic fibroblasts in comparison with the matrices containing normal fibroblasts.

It is earlier shown that fibroblast remodeling of collagen matrices by their migratory activity occurs at two levels. Locally, the density of collagen fibrils increases in the close vicinity of the cells, typically reaching the collagen concentrations of mature tissues. Globally, the total increase in matrix density leads to a decrease in matrix volume, i.e. contraction occurs.<sup>25</sup> These remodeling processes can clearly be observed here in the matrices containing normal fibroblasts.

Why this phenomenon seems to be deficient in the matrices containing chronic fibroblasts is not clear. However, earlier studies have demonstrated that the ability of cells to reorganize the extracellular matrix *in vitro* is strongly associated with the wound healing capacity of the corresponding tissue *in vivo*. Fibroblasts derived from chronic wounds would therefore exhibit an impaired ability to remodel collagen matrices which corresponds to the defective remodeling processes *in vivo*.<sup>20</sup>

The exact mechanisms underlying the extracellular matrix remodeling in collagen matrices remain unclear. By comparing cell numbers of the matrices with their ability to contract, it is however demonstrated in the present study that the differences in reorganization of the collagen matrices between chronic and normal fibroblasts was not related only to the higher cell number of the normal fibroblasts in comparison with the chronic cells.

Since contraction seems to depend on other factors than the ability of cells to proliferate, other explanations have been examined. Cook *et al.* (2000) for example, have demonstrated that the impaired reorganization by chronic fibroblasts may be associated with alterations in the activity of matrix metalloproteinases, specific enzymes which degrade matrix proteins.

According to the present histology results, it appears as if the ability of chronic fibroblasts to remodel the matrices is almost absent whereas the contraction analysis demonstrate that these cells do contract the matrices, although less than normal fibroblasts. (Figure 8 and 10) The reason for this discrepancy could be that the matrices containing chronic fibroblasts fabricated for the histology analysis did not contract as much as the corresponding matrices utilized during the contraction measurements. Probably, the former matrices were attached to the well bottom at some points, which decreased the contraction rate further in comparison with the matrices containing normal fibroblasts. This could explain the larger differences in remodeling ability between the cell types seen on the histology images than on the contraction measurements.

It is furthermore important to remember that these contraction estimates only have embraced the horizontal contractions observed, i.e. reduction in diameter have been used as a measure of contraction. Optimally these measurements should also include the vertical contraction and thus be performed using the reduction in matrix volume as a measure of contraction. However, these analyses require other more complicated methods, analysis of three dimensional images using confocal microscopy being one example.

Remodeling and contraction of collagen matrices by fibroblasts seems to be enhanced with topical addition of EMD. This effect is observed for both chronic and normal fibroblasts. Grayson *et al.* (2006) have earlier described similar effects for normal dermal fibroblasts in three dimensional collagen matrices. The mechanisms of EMD and its effects on extracellular reorganization are still not fully elucidated. However, the present results also demonstrate the possibility of adding EMD on top of the matrices and still affect activities of fibroblasts residing inside the matrices. From a clinical point of view this is of utmost importance in the treatment of chronic wounds.

At first, the cell concentrations of the collagen matrices seen on the histology images seem to be very low, but in comparison with natural human dermal tissues this is an adequate concentration of fibroblasts. (data not shown) The cross-sections are moreover very thin and calculations of the measured proliferation results confirmed the visualized cell concentrations.

An interesting continuation of this remodeling analysis would be to use immunohistochemistry in order to investigate whether EMD accelerates the differentiation

of fibroblasts into myofibroblasts, the type of fibroblasts that possess contractile forces. Moreover, it would be useful to examine how EMD is distributed in the matrix after topical appliance.

#### **6.4. Cytokine analysis**

The results from the cytokine analysis showed that the presence of an EMD concentration of 1 mg/mL reduced the fibroblast production of all three cytokines that were produced in detectable amounts, i.e. IL-6, IL-8 and MCP-1. Since a chronic wound is a complex composition of different cell types and extracellular proteins, including numerous growth factors and cytokines it is however unclear whether a similar effect of EMD would be seen in clinical practice.

To analyze the effect of single tissue components in order to assign each of them functions in this complicated system is not an easy task. Hence, although IL-6 has demonstrated a mitogenic effect for keratinocytes<sup>30</sup> it is not certain that EMD, through its demonstrated inhibitory effect on IL-6 production, would decrease proliferation of keratinocytes.

Contrary to the present results, EMD increased the IL-8 secretion by chronic fibroblasts cultured in monolayer in a previous study.<sup>37</sup> MCP-1 production was however inhibited by EMD addition<sup>37</sup>, which is supported by present data. The variance of these findings could be due to differences in dimensionality.

There are probably several reasons for the relatively sparsely amount of cytokines found in these samples. Firstly, the Human Cytokine 25-plex kit utilized in this experiment is developed for the analysis of cytokines primarily involved in inflammation; many of the cytokines included in this assay have never been shown to be produced by fibroblasts. Other cytokines have been shown to be produced by fibroblasts in an inflammatory environment but not during the prevalent conditions of this assay. Therefore, neither of these cytokines should be expected to be found in this analysis. Finally, even if some of the cytokines have been demonstrated to be synthesized by fibroblasts in three dimensional collagen matrices in other studies, small differences in experimental design can lead to remarkable differences in cytokine production.

It would be of great interest to investigate the synthesis and release of other cytokines and growth factors important in wound healing processes. Especially analyses of chronic fibroblast expression of vascular endothelial growth factor (VEGF) and transforming growth factor  $\beta$ 1 (TGF-  $\beta$ 1) would provide important information. Grayson *et al.* (2006) have earlier demonstrated increased levels of TGF-  $\beta$ 1 production by normal fibroblasts cultured in collagen matrices with EMD.

## 7. CONCLUSION

Our knowledge of the wound healing process has progressed significantly during the last years and numerous models have been developed for the analysis of the cellular mechanisms regulating these processes. During times when the population ages and the incidence of diabetes increases the prevalence of chronic wounds will inevitably rise as well. In order to understand the underlying mechanisms causing the chronicity and to develop new and more effective treatments there is an immense need of improved models for chronic wounds.<sup>3,39</sup>

In the present project, a three dimensional model which provides a reasonable representation of the dermal granulation tissue in a chronic wound has been developed. The model was utilized for the investigation of the differences in cell behavior between chronic human dermal fibroblasts and normal human dermal fibroblasts on cellular processes important for dermal repair and regeneration, i.e. proliferation, cellular morphology, cytokine production, matrix contraction and remodeling.

My results demonstrate that the ability of chronic fibroblasts to proliferate and to contract and remodel the matrix is reduced in comparison with normal fibroblasts. No obvious difference in cellular morphology was seen between normal fibroblasts and chronic fibroblasts neither in 2D nor in 3D, implying that the chronicity of chronic fibroblasts do not affect their ability to attach to and spread on surfaces or matrices.

Furthermore, the effect of a wound treatment substance, EMD, on these cellular processes has been examined. My results demonstrate that topical addition of EMD affected both cell types by increasing the ability to proliferate and to contract and remodel the matrix. These results are of great importance, especially for chronic fibroblasts as EMD improved their deficient capacities. When combined with basic medical care, EMD and Xelma® therefore have an excellent potential to provide an effective treatment of chronic wounds.

In summary, my results show that it is possible to measure the differences in cell behavior between chronic and normal fibroblasts on cellular processes important for dermal repair and regeneration in this three dimensional in vitro model. It was furthermore possible to analyze the effect of a wound treatment substance, EMD, on these cellular processes when topically applied. Moreover, the results are reproducible which gives this model high potential when studying wound healing processes in chronic wounds.

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