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In Vitro Wound Healing Characteristics of Amelogenins

by

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Göteborg 2011
Cover picture:
Micrograph displaying a cell extension from a human dermal fibroblast adhering to spherical micrometre-sized amelogenin aggregates.

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To my sunshine Emil
ABSTRACT

Wound healing involves the co-ordinated actions of several cell types, soluble cell mediators and extracellular matrix (ECM). This research project intended to investigate the role of certain ECM proteins in different processes during tissue repair by studying the interaction between dermal cells and ECM. The focus has been on amelogenins, ECM proteins that under physiological conditions aggregate into spherical structures. As a resorbable biomaterial, amelogenins enhance periodontal tissue regeneration and have been introduced in the treatment of hard-to-heal ulcers. However, the mechanisms of action need to be delineated. The aim of this project was to increase the knowledge on the effects of amelogenins on cell behaviour, to further understand the role of this specific ECM protein in tissue repair and regeneration.

To study the in vitro effects of amelogenins on wound healing, three human cell types; macrophages, fibroblasts, and endothelial cells, all essential for successful tissue repair, were utilised. The study designs included cell cultures, in monolayer, 3D-culture and an ex vivo model (chick aortic arch assay) for the angiogenesis studies. The evaluation methods included cell quantification, mitogenesis and apoptosis studies by BrdU incorporation and TUNEL measurements respectively, cytokine analysis by ELISA and multiplex bead array, cell surface integrin adhesion assay, gene microarray analysis, phase contrast and fluorescence microscopy for morphology and viability, and ultrastructural studies by electron microscopy.

The results demonstrate that amelogenins influence the in vitro cell behaviour of all three cell types investigated. The interaction and uptake of amelogenin aggregates was demonstrated for both macrophages and fibroblasts. In addition, the possible involvement of integrin-dependent adhesion was demonstrated for fibroblasts and endothelial cells, with increased cell binding by multiple integrins subunits and αvβ3, αvβ5 and α5β1. Amelogenin treatment of cultured macrophages displayed anti-inflammatory properties, directing the release of several pro- and anti-inflammatory cytokines. In particular, induced secretion of the specific marker of alternative macrophage activation AMAC-1, along with vascular endothelial growth factor was seen, most probably resulting from a switch of macrophage phenotype to an alternatively activated cell, with tissue repair characteristics. Also, amelogenins increased cell proliferation and induced the expression of genes involved in cellular growth, migration and differentiation in normal dermal fibroblasts. Moreover, amelogenins had the capacity to restore an acute-like phenotype in senescent fibroblasts. Finally, amelogenins displayed pro-angiogenic properties in vitro and ex vivo.

In conclusion, the effects of amelogenins on wound healing are plausibly, at least partly, conducted by providing macrophages, fibroblasts, and endothelial cells with tissue repair characteristics. These effects are most probably conducted through cell adhesion via integrin interaction.

Keywords: Amelogenins, extracellular matrix, cell culture, macrophage, fibroblast, endothelial cell, integrin, cytokine, inflammation, wound healing/tissue repair
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V. Effects of Amelogenins on Angiogenesis-Associated Processes of Endothelial Cells
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ABBREVIATIONS

AMAC-1     Alternative macrophage activation-associated CC chemokine-1 (CCL-18)
ANOVA      Analysis of variance
bFGF       Basic fibroblast growth factor
BMP        Bone morphogenetic proteins
BrdU       5-bromo-2’-deoxyuridine
BSP        Bone sialoprotein
cAMP       Cyclic adenosine monophosphate
CICP       C-terminal propeptide of type I collagen
DAPI       4’,6-diamidino-2-phenylindole
DMEM       Dulbecco’s modified Eagle’s medium
DNA        Deoxyribonucleic acid
ECGS       Endothelial cell growth supplement
ECM        Extracellular matrix
EGF        Epidermal growth factor
ELISA      Enzyme-linked immunosorbent assay
EMD        Enamel matrix derivate
ERK        Extracellular signal-regulated kinase
E-SFM      Endothelial serum-free medium
FCS        Foetal calf serum
GAG        Glucosaminoglycan
GM-CSF     Granulocyte-macrophage colony-stimulating factor
HDMEC      Human dermal microvascular endothelial cells
HUVEC      Human umbilical vascular endothelial cells
ICAM       Inter-cellular adhesion molecule
IFN-γ      Interferon-gamma
IGF-1      Insulin-like growth factor-1
IL         Interleukin
IL-1Ra      Interleukin-1 receptor antagonist
IP-10       Immune protein-10 (CXCL-10)
LDH         Lactate dehydrogenase
LPS         Lipopolysaccharide
LRAP        Leucine-rich amelogenin peptide
MAPK       Mitogen-activated protein kinase
MCP-1       Monocyte chemoattractant protein-1 (CCL-2)
M-CSF       Macrophage colony-stimulating factor
MIG         Monokine induced by gamma-interferon
MIP-1α      Macrophage inflammatory protein-1 alpha (CCL-3)
MIP-1β      Macrophage inflammatory protein-1 beta (CCL-4)
MMP         Matrix metalloproteinase
mRNA        Messenger ribonucleic acid
NHDF        Normal human dermal fibroblasts
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<td>PDL</td>
<td>Periodontal ligament</td>
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<tr>
<td>RANTES</td>
<td>Regulated upon activation, normal T-cell expressed and secreted (CCL-5)</td>
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<tr>
<td>RGD</td>
<td>Arginine - Glycine - Aspartic acid</td>
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<tr>
<td>RPE</td>
<td>R-Phycoerythrin</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
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<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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<tr>
<td>TGF-β</td>
<td>Transforming growth factor-beta</td>
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<tr>
<td>TIMP</td>
<td>Tissue inhibitor metalloproteinase</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-alpha</td>
</tr>
<tr>
<td>TRAP</td>
<td>Tyrosine-rich amelogenin peptide</td>
</tr>
<tr>
<td>TUNEL</td>
<td>TdT-mediated dUTP nick end labelling</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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1. BACKGROUND

*Healing is a matter of time, but it is sometimes also a matter of opportunity.*

(Hippocrates 400 BC)
When skin integrity is disrupted and a wound results, healing begins. The intricate healing process is a progression of multiple interacting processes, involving the coordinate action of multiple cell types, soluble cell mediators and extracellular matrix (ECM), which finally leads to repair of the injury. However, some wounds fail to follow this complex healing progression, due to different underlying aetiologies, e.g. ischemia or infection. As a result of impaired healing, these wounds are often described as being stuck in a chronic inflammatory phase. Over time, chronic inflammation leads to degradation of the ECM vital for cell attachment and signalling and key cells become senescent. Understanding and correcting the hindrance of tissue repair may be an effective strategy to stimulate the formation of granulation tissue and progression of healing. One possible treatment strategy is to introduce a temporary matrix for cell adhesion and activation, triggering the healing process to resolve inflammation and restore a normal, acute-like wound environment.

The following background section will discuss the structure and function of human skin and three essential cell types in tissue repair; macrophages, fibroblasts and endothelial cells. Next, a description of the ECM followed by cell-ECM and cell-cell interactions is given. Further, the wound healing process both in acute and hard-to-heal ulcers is then described. Finally, the chapter ends with an introduction to amelogenins, enamel matrix proteins, a novel treatment of chronically inflamed tissues, with a mode of action that needs to be delineated.

### 1.1 Structure and function of the human skin

The skin is the largest organ, covering the entire outer surface of the human body. It has a surface area of around two square meters for an adult, weighing approximately 15% of the total body weight. The skin is the principal site of interaction with our surroundings, performing several vital functions. It is a protective barrier, preventing entrance of pathogens and hazardous substances, protects from ultraviolet radiation and shields internal organs from trauma. The skin also controls evaporation, thermoregulation, sensory perception, synthesis of vitamin D and immunologic surveillance. This dynamic organ undergoes constant changes throughout life as the outer layers are shed and continuously replaced. It varies in thickness depending on age, sex and anatomic location. Children have relatively thin skin, which gradually thickens until middle age, when the tissue begins to thin due to loss of connective tissue.

The skin is composed of essentially three different layers: the keratinised and stratified epidermis, the thicker collagen-rich dermis and the hypodermis also referred to as the subcutaneous tissue, consisting of loose connective tissue with adipocytes (Fig. 1).

#### 1.1.1 The epidermis

The epidermis is the outermost layer of the skin. It consists of cellular layers that form a diffusion limiting barrier and, when undamaged, prevents most pathogens and foreign substances from entering the body. The epidermis contains no blood vessels and is
entirely dependent on the underlying dermis for nutrient delivery and waste disposal via
diffusion through the basement membrane, connecting the epidermis to the underlying
dermis. The epidermis consists primarily of keratinocytes, forming multiple layers (strata)
of closely adherent cells, originating from the deepest layer of the epidermis called
stratum basale or basal layer. This single cell layer of keratinocytes is attached to the
basement membrane via hemidesmosomes, specialised anchoring structures. As
keratinocytes divide and slowly migrate up the strata, they change shape and composition,
becoming keratinised as they differentiate and finally die due to the isolation from blood
supply and lysosomal enzyme release. Once the keratinocytes reach the skin surface
called stratum corneum, they are gradually shed in the process of epidermal turnover
entitled desquamation.

In addition to keratinocytes, melanocytes are scattered throughout the basal layer
and are also found in hair follicles. Their primary function is production of the pigment
melanin. Melanin is transferred in melanosomes to adjacent keratinocytes, where they
remain as granula and function to absorb ultraviolet radiation from sunlight. The
epidermis also contains Langerhans cells, serving as antigen-presenting cells for activation
of the immune system, as well as Merkel cells, which are specialised in perception of light
touch sensation.

1.1.2 The dermis
Underneath the epidermis, the dermis consists primarily of connective tissue, giving
flexibility and strength to the skin and cushions the body from external trauma. The
dermis is a more complex structure than the epidermis, with markedly less cellularity.

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**Fig. 1.** The structure of skin. The skin is composed of three layers: the epidermis, dermis and subcutis.
Instead the majority of the composition is ECM. The dermis is composed of two layers, the superficial papillary region and the deeper reticular region. The papillary region is thinner, consisting of loose connective tissue containing capillaries, elastin and collagen fibres. The name originates from the finger-like projections called papillae extending toward the epidermis. These structures provide the dermis with an enlarged interface to the epidermis, strengthening the connection between the two layers of skin in the basement membrane.¹

The reticular region consists of a thicker layer of dense connective tissue containing bundles of collagen fibres and closely interlaced elastic, elastin fibres embedded in ground substance, composed of glucosaminoglycans, proteoglycans, metabolites, ions and water.¹ The reticular dermis also contains larger blood vessels, fibroblasts, mast cells, nerve endings, hair follicles, sweat glands, sebaceous glands, apocrine glands and lymphatic vessels.¹ The fibroblast is the major cell type of the dermis. These cells produce and secrete extracellular matrix proteins e.g. pro-collagen and elastic fibres.⁵ Pro-collagen is proteolytically cleaved into collagen that aggregates and becomes cross-linked. These tightly cross-linked collagen fibres provide tensile strength to the skin. Collagen type I is the major protein component of the dermis followed by type III collagen.⁶ Elastin constitute less than 1% of the weight of the dermis, but play an enormous functional role by resisting deformational forces and returning the skin to its resting shape.⁷

1.1.3 The subcutis
The dermis rests on a fatty subcutaneous layer that offers insulation, protective padding, and also serves as an energy storage area.⁴ The fat layer varies in thickness, from a few micrometres on the eyelids to several centimetres on the abdomen and buttocks. The subcutaneous layer also attaches the skin to underlying bone and muscles as well as supplying it with blood vessels and nerves.² The fat is contained in adipocytes, held together by loose connective tissue and elastin. Besides adipocytes, the main cell types found in subcutis are fibroblasts and tissue macrophages.⁴

1.2 Cell types in skin and tissue repair
The skin is an intricate structure comprising numerous cell types with specific roles essential for tissue structure and function. The complexity enhances when skin integrity is compromised due to e.g. tissue injury. The following section describes three different cell types all essential cellular components of our body both under normal, homeostatic situations, as well as during tissue repair. These involve the macrophages; an important cell in inflammation and tissue repair, the fibroblasts; the major cell type in the dermis producing vital growth factors and ECM molecules, and the endothelial cells; constituting the inner lining of blood vessels and a key component during angiogenesis, the formation of new blood vessels.
1.2.1 Macrophages

Leukocytes or white blood cells are found in blood and lymph and they participate in both innate and adaptive immune functions. They can be divided in three different groups; the granulocytes, the lymphocytes and the monocytes. Monocytes are developed in the bone marrow and circulate in a dormant state with low phagocytic ability and relatively inert cell surface receptors and ligand complements until activation. When activated e.g. upon injury, they migrate to surrounding tissue through the vascular endothelium and differentiate into macrophages. The differentiation is triggered by secreted chemokines and other cytokines, immune complexes, bacterial products and degraded or newly synthesised ECM molecules. In addition, macrophages are further activated by the process of phagocytosis. Macrophages can also become resident in certain tissues, especially the lungs, spleen and liver. Here, the macrophages are positioned to rinse the blood from bacteria and cellular debris.

Macrophages play key roles in the regulation of inflammation and wound healing, both by clearing the wound of microbes and debris and by orchestrating the repair process through the production of growth factors and other mediators. However, the precise role of leukocytes remains controversial with studies on macrophage knockout mice suggesting either that macrophages promote wound healing or that healing may be enhanced without one or more of the inflammatory cell types. One classical way to activate macrophages is by bacterial products such as endotoxins e.g. lipopolysaccharides (LPS). LPS is a component of the cell walls of Gram-negative bacteria and is a major activator of innate immune responses, and a potent inducer of the expression of cytokines and inflammatory mediators by macrophages.

Macrophages are exquisitely sensitive to their microenvironment and can be phenotypically polarised, developing into different subsets broadly known as classically or alternatively activated macrophages. Depending on specific exogenous or endogenous stimuli, they modulate their expression of an array of cytokines and growth factors. Classically activated cells, also referred to as M1 cells in the literature, are activated by interferon-gamma (IFN-γ) and other pro-inflammatory cytokines e.g. tumour necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β) and IL-12, bacterial DNA and endotoxins, e.g. LPS, opsonised particles, binding to T lymphocytes, and hypoxia. Classical activation mediates a Th1 response with high levels of pro-inflammatory cytokines, ECM degradation by direct or indirect production of matrix metalloproteinases (MMPs), strong antimicrobial activity by the production of nitric oxide and the induction of apoptosis in cells at the inflamed site.

Alternatively activated macrophages, or M2, are instead activated by IL-4 and IL-13. Other stimuli implicated for the alternative pathway described in the literature are IL-10, transforming growth factor-beta (TGF-β), macrophage colony-stimulating factor (M-CSF) and glucocorticosteroids. However, the inclusion of all these modulators remain controversial and is presumably an oversimplification, instead there are plausibly many subgroups (M2a, b, c) to the alternatively activated macrophage, with distinct but overlapping phenotypes. The alternatively activated phenotype promotes Th2-associated activities, resolving inflammation and promoting tissue repair. The alternatively activated macrophages express a specific marker known as alternative...
macrophage activation-associated CC-chemokine-1 (AMAC-1) and anti-inflammatory cytokines, e.g. IL-10, IL-1 receptor antagonist (IL-1Ra). Further, these cells stimulate ECM protein deposition by fibroblasts and produce fibronectin and the matrix protein β1G-H3 themselves. Also, they express tissue transglutamase that cross-links matrix proteins. In addition, alternatively activated macrophages have been shown to promote angiogenesis through the secretion of pro-angiogenic factors e.g. vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF-1) and TNF-α, along with induction of endothelial cell proliferation.

Both the classical and alternative phenotypes are necessary to resolve inflammation and the right balance of the respective population is vital during the inflammatory progression.

### 1.2.2 Fibroblasts

Fibroblasts are the major cellular component of the dermis and are not only responsible for the deposition of ECM macromolecules, e.g. collagen, they also communicate with each other and other cell types, thereby playing a crucial role in regulating skin physiology. Skin fibroblasts are normally quiescent cells, but after tissue injury they become activated by the secretion of soluble cell mediators and degraded ECM. These activated fibroblasts play a central role in tissue repair by producing and remodelling ECM components and also by providing paracrine stimulation of wound healing processes including granulation tissue formation and angiogenesis. Vascular endothelial growth factor (VEGF) is one of the important growth factors produced by fibroblasts and it is a major mitogen in angiogenesis. Fibroblasts are also important sources of immunomodulators. Examples of fibroblast-derived mediators are interleukin-8 (IL-8) and monocyte chemotactic protein-1 (MCP-1), which are potent chemokines for neutrophils and monocytes, respectively.

Fibroblasts can differentiate into contractile myofibroblasts during the remodelling phase of wound healing. This is an essential feature that is triggered by the growth factor TGF-β, splice variant of fibronectin ED-A and mechanical cues within the wound matrix. Myofibroblasts are specialised cells sharing phenotypic characteristics with both fibroblasts and smooth muscle cells. They express α-smooth muscle actin and resemble muscle cells by their ability to generate strong contractile forces. Myofibroblasts can contract and remodel the granulation tissue by pulling fibrous proteins, primarily collagen. This reduces the wound surface area, enabling re-epithelialisation, thus essential for rapid wound healing. However, myofibroblasts are also responsible for hypertrophic scarring and can persist to form pathological scars.

### 1.2.3 Endothelial cells

Endothelial cells are a specialised type of epithelial cell which forms the inner layer of blood vessels called the endothelium. The endothelium functions as an interface between circulating blood in the lumen and the rest of the vessel wall. Endothelial cells are involved in many aspects of vascular biology. The structure and integrity of the cells are important in the maintenance of the vessel wall and circulatory function. Under basal,
homeostatic conditions the endothelium is involved in maintaining a non-thrombogenic interface due to a variety of distinct mechanisms. For example, heparin is sequestered on the endothelial lumenal surface, and acts as a co-factor for activating anti-thrombin, a protease that cleaves several factors in the coagulation cascade.\textsuperscript{41} Also, the endothelium is responsible for reducing the turbulence of blood flow.\textsuperscript{40} If an injury occurs, the endothelium is instead involved in striving for a haemostasis by controlling the formation of a blood clot.\textsuperscript{3} The semi-permeable barrier controls the transit of leukocytes from the vessel lumen, e.g. during inflammation, as well as the transport of cell mediators and molecules.\textsuperscript{40} Endothelial cells also participate in both metabolic and synthetic functions, e.g. exerting paracrine and endocrine signalling.\textsuperscript{39} Endothelial cells play a key role in angiogenesis, the development of new blood vessels from pre-existing vessels. Angiogenesis is a multi-step process important for both physiological and pathological development. During angiogenesis, endothelial cells are activated in response to environmental cues, especially angiogenic growth factors such as VEGF, and start secreting MMPs, which degrade the vascular basement membrane.\textsuperscript{42} A specific cell, called the tip cell, then starts to migrate through the basement membrane into the surrounding tissue followed by proliferating stalk cells, together forming a sprout that grows in length.\textsuperscript{43,44} This angiogenic process requires changes in cell adhesion, which are mediated by specific integrins.\textsuperscript{43} When the tip cell encounters another vessel outgrowth, the sprouts are coupled and a loop is formed with a lumen that allows blood flow. Finally, interactions of newly formed capillary networks with pericytes or vascular smooth muscle cells are needed to stabilise the vascular structure.\textsuperscript{45}

1.3 The extracellular matrix (ECM)

The extracellular matrix (ECM) is the acellular component present within all tissues and organs. The ECM has traditionally been thought of as a physical scaffolding material, providing support and protection for cells and tissues. However, research during the last decades has changed the perspective and established the importance of the ECM. The ECM is now considered to be a complex and ordered composition of different macromolecules whose structural integrity and functional composition are important to maintain normal tissue structure, development, and tissue specific functions e.g. wound healing.\textsuperscript{46}

Basically, the ECM is composed of water, proteins and polysaccharides. However, the ECM is functionally diverse. Some components are rigid, others elastic; some hydrated, others sticky. All have modular structures that impart diverse roles, yet allow for highly specialised functions.\textsuperscript{47} Thus, each tissue has an ECM with a unique composition created through a dynamic and reciprocal interaction of the various cell types present in the microenvironment, which synthesise and structurally remodel the ECM proteins. Hence, the ECM is a flexible structure that is constantly altered, and its molecular components are subject to numerous post-translational modifications. Through these physical and biochemical characteristics the ECM generates the biochemical and mechanical properties of each organ, such as its tensile and compressive strength and elasticity, and also mediates protection by a buffering action that maintains extracellular homeostasis and water retention.\textsuperscript{47}
The ECM consists of four main classes of macromolecules: structural proteins, cell-adhesive glycoproteins, glucosaminoglycans/proteoglycans and matricellular proteins. The main structural ECM proteins are collagen and elastin. Collagens are the most abundant fibrous proteins found in the body and there are at least 19 different types, constituting the main element of the ECM. Collagen in the skin is primarily type I and type III and provide structure, strength, and integrity. Type IV collagen is a component of epidermal and endothelial basement membranes. Collagen associates with elastin, another major ECM fibre. Elastin provides elasticity to tissues that undergo repeated stretch, e.g. arteries, lungs and skin, by assuming an elongated, linear organisation when stretched and returning to a more coiled structure when released.

Cell-adhesive glycoproteins include e.g. fibronectin, laminin, vitronectin, thrombospondin, tenascin and entactin. These macromolecules generally contain precise peptide domains specialised for binding to specific cell surface receptors, as well as to multiple components of the ECM, thus mediating binding between cells and the ECM or between ECM molecules. As examples, fibronectin can mediate cell adhesion and cell migration during embryonic development and wound healing, while laminin can promote diverse processes like axonal outgrowth, metastasis and maintain the polarised and differentiated phenotype of epithelial cells on a basement membrane. Glycoproteins can also serve as modulators for e.g. growth factor activity.

Glucosaminoglycans (GAGs) and proteoglycans fill the majority of the extracellular interstitial space within the tissue in the form of a hydrated gel. They have a wide variety of functions that reflect their unique buffering, hydration, binding and force-resistance properties. GAGs, e.g. hyaluronic acid, can bind large amounts of water into their structures, allowing them to resist compression forces. They are also fairly rigid. This combination allows for cell migration and transport of nutrients and other substances through their structures. Most GAGs are bound to a protein core to form proteoglycans. An exception is hyaluronic acid, which remains as a GAG without a protein core. GAGs interact with other ECM proteins, e.g. the adhesive protein fibronectin. Through this interaction, glucosaminoglycans anchor proteins at specific locations and affect their biological activity. Proteoglycans also serve as co-receptors for growth factors, participate in cell signalling, and help regulate the activity of many other molecules.

There are also proteins within the ECM without any structural role, the matricellular proteins. Examples of members of this group include the galectins, tenascins, thrombospondins, osteonectin and osteopontin. These proteins bind to several cell surface receptors and ECM proteins, thereby stimulating cell activities such as motility, proliferation, apoptosis and differentiation. The expression of these proteins is high during the development of tissues while in normal adult tissue there is only an up-regulation during tissue repair.

1.3.1 ECM-cell interactions

Cell adhesion to the ECM plays a key role in the assembly of cells into functional multicellular organisms (Fig. 2). The composition, three-dimensional organisation and remodelling of the ECM are the major signal determinants from the microenvironment,
which initiates crucial biochemical and biomechanical cues that influence the behaviour of cells, by controlling cellular shape, growth, migration, differentiation and survival.\textsuperscript{54} Through cooperative mechanisms, the ECM together with growth factors and other cytokines, hormones and cell-to-cell contact, influence the phenotype of the affected cell.\textsuperscript{54,55} The importance of ECM signalling has been emphasised by the evidence that impairment of cell–ECM interactions can contribute to many diseases, including developmental, immune, haemostasis, degenerative and malignant disorders.\textsuperscript{56} Cell-ECM binding is also important because essential signalling of growth factors occurs optimal if the cells are attached to the appropriate ECM.\textsuperscript{54}

Cell adhesion to the ECM is mediated by ECM receptors, primarily integrins, but also syndecans and discoidin domain receptors.\textsuperscript{57-62} Integrin-mediated adhesion couples the cytoskeleton to the surrounding ECM, a receptor binding involved in e.g. cell migration.\textsuperscript{63} In addition, the ECM directs essential morphological organisation and physiological function by binding growth factors and interacting with cell-surface receptors to elicit signal transduction and regulate gene transcription.\textsuperscript{54}

\subsection*{1.3.2 Integrins}
Integrins are transmembrane cell adhesion proteins that transfer information from the surrounding ECM to the intracellular signalling system and some integrins also mediate important cell-cell adhesion events. These receptors are heterodimeric structures consisting of non-covalently associated alpha and beta subunits. So far, 18 $\alpha$ and 8 $\beta$ subunits have been identified, which can form at least 24 different receptors that bind

\begin{figure}[h]
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\caption{Schematic picture of the interaction of the cell surface of two cells with the surrounding ECM.}
\end{figure}
ECM ligands.\textsuperscript{50,64} Some integrins are ubiquitously expressed, while others are cell, tissue or event specific. Furthermore, some integrins bind only to a single ECM protein, while others mediate binding to several different ligands.\textsuperscript{58} No cell expresses all integrins, cells rather tend to express integrins that match the ECM ligands present within their local microenvironment.\textsuperscript{65}

Integrins are normally expressed on the cell surface in an inactive form but binding to a cognate ligand activates the integrin which increases the binding affinity.\textsuperscript{66} This binding affinity is relatively low compared with, e.g. antibody-antigen binding, however the effect of integrin clustering on the cell surface into so-called focal adhesions, increases the overall affinity.\textsuperscript{64} Integrin binding induces structural and signalling changes within the cell that ultimately leads to changes in gene expression.\textsuperscript{66}

The extracellular domains of the integrins bind to ECM ligands and cations, but they also interact with other proteins on the cell surface such as growth factor receptors, proteases, and matricellular proteins. The intracellular domains form complexes with proteins involved in cell signalling and adaptor proteins that connect to the cytoskeleton.\textsuperscript{66} Cell adhesion to the ECM transmits information that regulates intracellular signalling, called outside-in signalling, influencing e.g. cell spreading and migration. Conversely, intracellular signalling can influence the conformation and activation of integrins that alter the ligand binding activity, named inside-out signalling.\textsuperscript{67}

Integrins recognise specific peptide sequences of ECM proteins. An important cell-binding domain; arginine (R) - glycine (G) - aspartic acid (D), is recognised by specific integrins, and several ECM proteins, e.g. fibronectin, fibrinogen, osteopontin, tenascin, vitronectin, contain this RGD-site. Often, the RGD-sequence is cryptic and only exposed after proteolysis, adsorption to a surface, when the protein is multimerised, or denatured, thus allowing the site to be exposed only under specific conditions. Molecular structural alterations of ligands like these, together with soluble mediators thus constitute a mean for cell signalling in e.g. wound healing.\textsuperscript{68}

### 1.4 Cell communication

In addition to the importance of cell-cell and cell-ECM interactions, all phases of wound healing are dependent on the secretion of multiple cell mediators. As an example, tissue repair is initiated immediately after injury by the release of numerous cytokines and low molecular-weight compounds from both degranulating platelets and serum from injured blood vessels.\textsuperscript{69}

#### 1.4.1 Cytokines

A major mechanism by which cells inter-communicate is through the secretion of, and response to cytokines. These are a diverse class of soluble, secreted signalling proteins which act through cognate trans-membrane receptors. Cytokines are small proteins, often 8-25 kDa, which are able to regulate cellular functions, e.g. protein synthesis and cell proliferation.\textsuperscript{70} As expected of mediators with regulatory effects, cytokines are rarely produced at a constant rate but rather induced or suppressed by specific stimuli to which
the body needs to respond. Also, the lifetime in the bloodstream or other extracellular fluids is usually short to ensure activity only during limited time periods.\textsuperscript{70}

There are numerous known cytokines and most of them are produced by T helper cells or macrophages, but also by e.g. fibroblasts, endothelial cells and keratinocytes.\textsuperscript{70} In general, cytokines are secreted from one cell and bind to specific receptors on a target cell. Some cytokines act in an autocrine manner, affecting the cell that releases the cytokine, while others have paracrine, adjacent cell stimulation or endocrine effect, influencing behaviour of distant cells.\textsuperscript{30}

As an exceptional large family of mediators, the cytokines are difficult to classify, however, setting growth factors aside, there are three major structural families of cytokines. Firstly, there is the haematopoietic family, which includes growth hormones and many interleukins. The name interleukins comes from the originate task, to help leukocytes to communicate with each other, the name meaning between leukocytes.\textsuperscript{71} Next, the tumour necrosis factor (TNF) family, which functions in both innate and adaptive immunity. Members of the TNF family are often membrane-bound and are quite distinct in their properties from other cytokines. They are synthesised upon antigen recognition by T lymphocytes, and affect the behaviour of the target cell often into a pro-inflammatory response.\textsuperscript{72}

Thirdly, the chemokines, which are members of a family with chemoattractant properties, inducing cells to migrate toward the source of the chemokine through chemotaxis. These are among the first cytokines to be released in the earliest phases of inflammation and infection. Chemokines were initially named as interleukins, with IL-8 as the first chemokine to be cloned and characterised, with characteristics typical of this family. Other family members include e.g. MCP-1, macrophage inflammatory protein-1 alpha (MIP-1\textalpha) and MIP-1\textbeta.\textsuperscript{72} Many different types of cells can release chemokines and they are involved in the innate and adaptive immunity, organogenesis and angiogenesis, e.g. in lymphocyte development and cell migration.\textsuperscript{72,73}

In addition to cytokines, there is also a wide group of cell mediators called growth factors. Growth factors are generally known to be mitogenic, causing cellular growth by stimulating cell proliferation and differentiation.\textsuperscript{74} Some growth factors affect only a few cell types, while most of them stimulate growth and differentiation in a wide range of cells. The nomenclature of cytokines and growth factors are used inconsistently in literature. Some growth factors are classified as part of one of the cytokine family, e.g. TGF-\beta, while many constitute separate families, usually based on cell receptor specificity.\textsuperscript{70} Examples of families are the vascular endothelial growth factor family, platelet-derived growth factor family, fibroblast growth factor family, insulin-like growth factor family and epidermal growth factor family.\textsuperscript{69}

1.5 \textbf{Wound healing}

Maintaining skin integrity is a complex and continuous process often taken for granted until an injury occurs. Most cutaneous wounds resulting in minimal tissue damage heal quickly and efficiently. However, large injuries can result in life threatening situations due to fluid loss through evaporation and risk of infection.
The wound healing process involves the coordinate actions of several cell types, soluble cell mediators and ECM. This intricate process can be subdivided into three consecutive and overlapping phases following after haemostasis, referred to as inflammation, proliferation and maturation (Fig. 3).

1.5.1 Haemostasis
The wound healing process is initiated by clot formation at the site of an injury. Injury to damaged blood vessels stimulates the clotting cascade resulting in proteolytic cleavage of fibrinogen by the enzyme thrombin and formation of an insoluble fibrin clot that restore haemostasis. The clot also consists of platelets and other blood cells embedded in the cross-linked fibrin fibres, along with smaller amounts of plasma fibronectin, vitronectin and thrombospondin. As the barrier function of the skin is compromised at the wound site, the clot holds damaged tissues together and functions as a temporary shield. Structurally, it provides a provisional matrix for cell migration. The large number of platelets is essential as a source for release of cell mediators that promotes the wound healing cascade, by recruiting inflammatory cells, initialising re-epithelialisation, stimulating angiogenesis and tissue contraction.

1.5.2 The inflammatory phase
In addition to the cytokines released from the degranulating platelets in the fibrin clot, a wide range of elements also serve as chemotactic signals to recruit leukocytes to the

![Fig. 3. Schematic picture of the timeline of the overlapping phases of the normal wound healing process. (Inspired by Diegelmann et al. 2004)](image-url)
wound bed, including cleaved ECM components, microbial signals and cellular debris. Neutrophils are the first inflammatory cells to respond to the soluble mediators released by platelets and the coagulation cascade. They extravasate from the vasculature into the injured tissue. Their primary role is to protect against infection by phagocytising and killing microorganisms, and by breaking down foreign materials and damaged tissue. Neutrophils also produce and release inflammatory mediators, which further recruit and activate macrophages, fibroblasts and epithelial cells.

Neutrophils are usually depleted in the wound after two to three days, gradually replaced by macrophages. Macrophages begin as circulating monocytes and are attracted to the wound site about one day after injury, both by cell mediators and degraded components of the ECM. Monocytes then differentiate into macrophages initiated by the released cell mediators, serum factors, microbial signals and by binding to ECM proteins. Macrophages have a dual role in the healing process. They are efficient phagocytes, ingesting bacteria, damaged tissue and neutrophils. Macrophages also mediate the transition from the inflammatory to the proliferative phase of healing. They release a wide variety of growth factors and other cytokines. Some of these soluble mediators recruit and activate fibroblasts, which will synthesise, deposit and organise the new tissue matrix, while others promote angiogenesis and epithelialisation.

1.5.3 The proliferative phase

During the proliferative phase, the provisional wound matrix is remodelled and replaced with granulation tissue, consisting of new collagen fibres, proteoglycans and elastin fibres, which partially restore the structure and function of the tissue. This is accomplished by the migration, proliferation and differentiation of epithelial cells, dermal fibroblasts and vascular endothelial cells from adjacent tissue and stem cells that originate in the bone marrow and circulate to the wound site.

After one or two days, epithelial cells at the wound margin start to proliferate behind actively migrating cells. This onset of reepithelialisation is probably stimulated by the absence of neighbouring cells at the wound margin, called the "free edge effect", in addition to the secreted growth factors in the wound bed. Hence, the epithelial cells start to climb over the newly formed granulation tissue under the clot and will with time reepithelialise the injury.

About three to four days after injury fibroblasts in the adjacent intact dermis begin to express new integrin receptors and migrate into the provisional wound matrix. The migration is driven by chemokines and growth factors released from platelets and macrophages in the wound bed, as well as part of angiogenesis. The fibroblasts follow a concentration gradient of chemotactic factors and migrate along the alignment of the fibrils into the provisional matrix. Fibroblasts in native skin are typically quiescent and sparsely distributed. In contrast, fibroblasts found in the provisional wound matrix and granulation tissue are numerous and active. Growth factors and proteins secreted and then contained within the provisional matrix help to stimulate the infiltrating fibroblasts to begin proliferating and synthesising new ECM components, e.g. collagen, elastin and proteoglycans. Thus, the provisional wound matrix functions as much more than an inert
scaffold. It also acts as a reservoir to help entrap growth factors that stimulate fibroblasts, epithelial and endothelial cells, also providing adhesion via their adhesion receptors, e.g. integrins, to differentiate the cells into activated wound cells that will repair the injury.78

The process of angiogenesis, the formation of new blood vessels, is stimulated by local factors in the wound microenvironment, including hypoxia, low pH and high lactate levels.79 Several growth factors, including VEGF, TGF-β and basic fibroblast growth factor (bFGF) are also potent angiogenic signals for endothelial cells.30 Angiogenic factors cause endothelial cells on nearby blood vessels to begin secreting proteases that degrade the surrounding ECM to allow for migration into the provisional matrix, along with cell proliferation, which forms new buds and sprouts. As the tip of a newly formed endothelial sprout comes into contact with another, a loop is formed that grows into a new capillary. Angiogenesis continues until the capillary system is sufficiently repaired, so that the oxygen level and metabolic needs are met. It is these new capillary loops embedded in a loosely deposited collagen matrix that give granulation tissue its characteristic rough or granular appearance.3

At this stage the newly formed granulation tissue is comprised of a dense population of macrophages, fibroblasts and newly formed blood vessels embedded in a loose matrix of fibrin, fibronectin, collagen and other ECM proteins.78

1.5.4 Maturation

Maturation is the final phase of wound healing that overlaps with the proliferation phase. It starts at the wound edges while the granulation tissue is still forming in the centre of wound in all except for the smallest wounds. Once the granulation tissue is formed and the wound is covered by a neoeipidermis, some fibroblasts transform into myofibroblasts that contract the wound, and epithelial cells starts to differentiate to re-establish the permeability barrier.7 The maturation of granulation tissue into scar tissue involves reduction in the number of capillaries as smaller vessels are aggregated into larger ones, and a decrease in the cellular density and metabolic activity of other involved cells, e.g. fibroblasts and macrophages.3 Changes also occur in the type, amount and organisation of collagen, leading to increased tensile strength of the tissues. Initially during wound healing, type III collagen is synthesised at high levels, but this is at a later stage replaced by type I collagen, the dominant collagen type in native skin.3 Healed tissue never has the same breaking strength as uninjured skin. The tensile strength of a recently reepithelialised skin wound is only about 20% of normal tissue after about three weeks.3 The tensile strength is enhanced primarily by the remodelling of collagen fibres, which are deposited rapidly and at random during the proliferation phase and have been compacted during the contraction of the wound,3 along with increased covalent cross-linking of collagen molecules by the enzyme lysyl oxidase, which is secreted into the ECM by fibroblasts.78 The collagen remodelling occurs through the actions of several classes of proteases produced by cells in the scar tissue. Two of the most important families are MMPs and serine proteases. MMPs are a family of zinc-dependent endopeptidases that degrade components of the ECM, as well as other protein substrates, including cytokines, chemokines, and their receptors.40 The destructive actions of
proteolytic enzymes are carefully regulated by specific enzyme inhibitors, which are also produced by cells in the scar tissue, e.g. tissue inhibitor metalloproteinases (TIMPs) that inhibit MMPs. Over several months or more, changes in collagen organisation in the repaired tissue will slowly increase the tensile strength to a maximum of about 80% of normal tissue.\textsuperscript{78}

\subsection*{1.6 Hard-to-heal wounds}

All wounds begin as acute wounds with the formation of a fibrin clot, but instead of progressing through the normal phases of healing, chronic or hard-to-heal ulcers are often locked in a self-amplifying detrimental cascade with persistent chronic inflammation, defective extracellular matrix and failed reepithelialisation.\textsuperscript{91,92} Impaired healing can be due to many underlying aetiologies, as ischemia, diabetes mellitus, venous stasis, and pressure.\textsuperscript{83,84} Several other mechanisms include infection, tissue maceration, the presence of foreign bodies, as well as malnutrition and cellular and systemic effects of aging.\textsuperscript{83,84}

The prolonged inflammatory phase found in hard-to-heal ulcers differs markedly from acute wound healing. Acute inflammation is a self-limiting process, whereas chronic inflammation instead is self-amplifying. Macrophages are present in high amounts.\textsuperscript{85,86} An increased number of inflammatory cells present in the tissue persistently elevate the level of chemokines and pro-inflammatory cytokines.\textsuperscript{83,87,88} Prolonged inflammation may cause augmented levels of oxygen free radicals and proteases, e.g. MMPs, leading to ECM degradation and alteration of growth factors and possibly their respective cell receptors, resulting in reduced levels of active cell mediators to promote cell proliferation and matrix deposition.\textsuperscript{78,89-93} Growth factors may also become trapped by ECM molecules.\textsuperscript{84,94} In an acute wound, MMPs can be inhibited by protease inhibitors, but in some non-healing ulcers, this balance is shifted resulting in excessive tissue degradation.\textsuperscript{95,96} As a result, fibroblasts are unable to make progress in ECM deposition due to rapid degradation of matrix molecules.

Dermal fibroblasts have an age-related decrease in proliferation potential, called senescence.\textsuperscript{97} The accumulation of senescent fibroblasts is a sign of chronic inflammation.\textsuperscript{97,98} The quantity of senescent fibroblasts increases with ulcer duration and it has been estimated that ulcers populated by more than 15\% senescent fibroblasts become hard-to-heal.\textsuperscript{99} Fibroblasts cultured from non-healing wounds exhibit diminished proliferative ability\textsuperscript{97,99} and impaired ECM remodelling capacity,\textsuperscript{100} plausibly reflecting an undermined migratory activity.\textsuperscript{101,102} In addition, wound fibroblasts have a reduced responsiveness to growth factors and hormones.\textsuperscript{97,99,103} It appears that the decreased reaction to growth factors is not due to fewer cell surface receptors on the senescent cells, but possibly to dysfunctional intracellular signalling pathways.\textsuperscript{97,104,105} One explanation might be an incongruous ECM composition that fails to activate adhesion receptors on the fibroblasts, a prerequisite for maximal growth factor stimulation.\textsuperscript{84}

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.\textsuperscript{83}
1.6.1 ECM in acute vs. hard-to-heal wounds

Injury to the skin resulting in a wound is known to trigger a repair process that is characterised by major alterations in both the composition and structure of ECM. Cell adhesion to ECM proteins that provide new binding sites triggers the surrounding cells and regulates cellular attachment, migration, growth and differentiation during the process of healing.\textsuperscript{75}

There is limited information about the difference in ECM composition regarding many proteins in chronic wounds. Histology of wound biopsies has not shown any clear differences in presence of laminin, tenascin or collagen type I and III in venous leg ulcers or diabetic foot ulcers.\textsuperscript{106-108} However, the proteoglycans syndecan-1 and syndecan-4 have shown abnormal immunostaining patterns in venous leg ulcers\textsuperscript{109} and fibronectin has been reported absent in the base of non-healing venous leg ulcers, while prominent immunostaining reaction to fibronectin was seen in healing wounds.\textsuperscript{108} Fibronectin deficiency may impair both cellular migration and proliferation.\textsuperscript{110-113} In contrast, increased fibronectin mRNA levels have been found in venous leg ulcers compared to acute wounds.\textsuperscript{114} Further, cultured fibroblasts derived from venous leg ulcers have been proven capable of producing the full range of ECM proteins synthesised in fibroblasts from healing wounds.\textsuperscript{115} This implicates that the low amount of fibronectin detected in chronic ulcers is probably due to degradation rather than decreased synthesis.\textsuperscript{115} This is supported by detection of fibronectin degradation product as well as vitronectin and tenasin in chronic wound fluid.\textsuperscript{116,117} Also, increased levels of MMPs in chronic ulcers compared to acute wounds has been reported,\textsuperscript{118-120} and the unfavourable distribution and persistence of MMP-9, a gelatinase, in non-healing wounds has been shown.\textsuperscript{121}

1.7 Amelogenins

Enamel matrix proteins, in this work referred to as amelogenins, are ECM proteins applied in the treatment of chronically inflamed tissues both in the periodontal field and recently also in cutaneous wound healing. Amelogenins are found in the enamel matrix during tooth development, regulating the orientation and growth of enamel mineral crystals.\textsuperscript{71,122} The amelogenin sequence is highly preserved throughout evolution, suggesting a critical role of the molecule for tissue formation.\textsuperscript{123,124}

1.7.1 Amelogenin origin

Enamel is the hardest bioceramic composite in the vertebrate body and covers the outer layer of the teeth. The biomineralisation occurs at the inner enamel epithelium consisting of ameloblasts positioned along the dentin-enamel junction. The ameloblasts secrete extracellular matrix proteins, where the principal component is amelogenins constituting over 90% of the protein content.\textsuperscript{125} The enamel matrix also contains enamelines, ameloblastins, tuftelin, serum proteins like albumin, and proteinases.\textsuperscript{126} The amelogenins demonstrate a high overall level of sequence homology across species examined (>80%), where the tyrosine-rich amino terminal (N-terminal) and the carboxy-terminal (C-terminal) are almost identical between species.\textsuperscript{123} The high level of sequence conservation
suggests that the entire amelogenin molecule is crucial in enamel formation and biomineralisation.\textsuperscript{123,127,128}

The amelogenin “core” is rich in hydrophobic amino acids, and the majority of the charged residues are located in the C-terminal.\textsuperscript{136} This distribution of amino acids provides amelogenin with a hydrophobic–hydrophilic polarity that plays an important role in the self-assembly of the protein. In particular, the hydrophobic proline residues in the core of the protein are thought to inhibit the formation of classic secondary structures such as α-helices, β-sheets, and random coil, producing an intrinsically disordered protein.\textsuperscript{129} However, this disorder also allows amelogenin molecules to self-assemble into hydrophobic supramolecular monodisperse assemblies, named nanospheres (Fig. 4).\textsuperscript{122} This assembly is presumably composed of core particles sized 5 nm containing six amelogenin molecules,\textsuperscript{130,131} which through further aggregation forms spherical structures measuring about 20 nm,\textsuperscript{122,132} composed of roughly 100 monomers.\textsuperscript{133} The size corresponds to the gap between developing enamel crystallites, where amelogenins function as spacers between the tiny crystals to inhibit lateral fusion.\textsuperscript{134} Thus, in the mineralising enamel matrix these amelogenin nanospheres bind to hydroxyl apatite crystallites to control crystal orientation and modulate the crystal elongation and morphology.\textsuperscript{71,122,135} The amelogenin nanospheres are gradually removed by proteolytic processing leaving room for the deposit of the enamel crystallites that grow in width to occlude the space. During this maturation, the matrix converts from containing around 30% mineral and the remainder organic material, to a highly organised structure >99% inorganic.\textsuperscript{136}

The intact amelogenin molecule is a 25 kDa protein when newly secreted by the ameloblasts. This parent protein is rapidly processed into a 23 kDa intermediate with the loss of a telopeptide in the C-terminal (Fig. 5).\textsuperscript{137,138} This protein is then further degraded into a 20 kDa molecule, constituting residues 1-148 on the N-terminal, which accumulates in the enamel matrix and thus represents the principal element of the amelogenins.\textsuperscript{137,138}

Fig 4. A schematic model of amelogenin self-assembly. The amelogenin molecule can fold into a globular shape preserving a bipolar nature, with the hydrophilic C-terminal (black thread) exposed on the surface. Nanospheres are formed through self-assembly of monomers and oligomers. (Based on Du \textit{et al.} 2005)\textsuperscript{136}
This protein can then be further enzymatically cleaved by two pathways (Fig. 5). The major pathway results in the 5 kDa tyrosine-rich amelogenin peptide (TRAP), comprising residue 1-45 on the N-terminal, and a 13 kDa product.\textsuperscript{125,137,139} While TRAP is relatively insoluble, the 13 kDa peptide is highly soluble and diffuses out of the enamel matrix into the enamel fluid.\textsuperscript{140} The minor pathway for the 20 kDa amelogenin cleavage results in a 11 kDa soluble peptide that also diffuses into the enamel fluid\textsuperscript{140} and a 7 kDa intermediate that is further cleaved into the 5 kDa TRAP peptide.\textsuperscript{141} TRAP can also be further degraded into smaller peptides, presumably lost into the enamel fluid.\textsuperscript{141} There are also other alternative gene splicing products and peptides, e.g. leucine-rich amelogenin peptide (LRAP).\textsuperscript{141}

Amelogenins are associated with the mineralising enamel in developing teeth and were previously thought of as tissue specific and exclusively expressed by ameloblasts. However, amelogenins are also implicated in both dentinogenesis and cementogenesis,\textsuperscript{142} the formation of the structural elements dentin and cementum in teeth. Also, amelogenin expression has been identified in bone and cartilage; from chondrocytes, osteoblasts, osteoclasts, bone marrow stromal cells and epiphyseal bone cells.\textsuperscript{150} Further, reports indicate that amelogenin is also expressed in soft tissues like the brain, salivary gland, blood vessels and in macrophages, megakaryocytes and hematopoietic stem cells.\textsuperscript{151} The findings that amelogenins are also expressed in soft tissues suggest that amelogenins are indeed multifunctional.

![Diagram of amelogenin processing](image-url)

*Fig 5. Extracellular processing of amelogenin, (Based on Brookes et al. 1994 and Robinson et al. 1998, with data derived primarily from porcine origin)\textsuperscript{125,137,139} 29*
1.7.2 Amelogenins in tissue repair

As a resorbable biomaterial, the amelogenin mixture for clinical use (Xelma®, Emdogain®) consists of enamel matrix proteins that are extracted from the enamel matrix of developing porcine teeth. The principal component of the enamel matrix proteins is amelogenins, where the 20 kDa protein is the dominant fraction. Unlike the enamel matrix in developing teeth, the mixture has been purified during preparation, resulting in a product with non-detectable levels of some non-amelogenin proteins like enamelin, ameloblastins and albumin, instead enriched in amelogenins.

Amelogenins are insoluble at physiological pH, but can be dissolved at either low or high pH. The solubility is also influenced by temperature due to hydrophobic interactions, with the best solubility obtained at low temperature. For clinical use, the amelogenin mixture is dissolved in an aqueous, acidic viscous solution of propylene glycol alginate (PGA) in a gel formulation. When applied to a patient, the temperature increases and the acidity of the gel is slowly neutralised, releasing amelogenins that undergo self-assembly and precipitate on the exposed tissue surface. The self-assembly of amelogenins under physiological conditions results in a further aggregation of the nanospheres found in the enamel matrix into micrometre sized structures. The size difference compared to the aggregates found during enamel formation is most probably due to the supersaturated, high mineral condition present in the enamel forming compartment, which is completely different from other physiological microenvironments.

Amelogenin mixture has been utilised in periodontal treatment since 1997 to enhance the regeneration of connective tissue after inflammatory destruction in tooth-supporting tissues in conjunction with surgical treatment. The mechanisms of the reparative properties of amelogenins have not been fully elucidated, although regarding the periodontal area, there are numerous experimental studies to support the clinical findings. Amelogenins have been shown to promote adhesion, migration and proliferation of cultured periodontal ligament (PDL) and gingival fibroblasts. Also, the increased synthesis of ECM molecules, e.g. osteopontin, bone sialoprotein, osteocalcin, proteoglycans, hyaluronic acid and collagen type I, TGF-β1, IGF-1, VEGF, IL-6, PDGF-AB and bone sialoproteins and up-regulation of second messengers as cyclic adenosine monophosphate (cAMP) and alkaline phosphatase have been reported in vitro. Further, similar effects by amelogenins have been seen on cultured osteoblasts. By contrast, the reported findings on the influence of amelogenins on epithelial cell proliferation are opposite to the findings on cells of mesenchymal origin. Here, amelogenins seem to have a growth-inhibiting effect, by hindering cell proliferation, although without the induction of apoptosis, instead possibly promoting cellular attachment, although the published results are indecisive.

In recent years, amelogenin mixture has also been applied in wound care to enhance the healing process of hard-to-heal cutaneous wounds. Randomised control trials involving patients with hard-to-heal venous leg ulcers, in particular with ulcer size larger than 10 cm² and duration of more than six months treated with amelogenin mixture in conjunction with standard compression therapy, demonstrated a significant reduction in ulcer size, improvement of ulcer state, generally low levels of exudate and reduced pain in
comparison to compression alone.\textsuperscript{199-202} Also, similar results have been shown in case reports of hard-to-heal venous leg ulcers, rheumatoid ulcers, neuropathic ulcers and diabetic foot ulcers.\textsuperscript{202-206} In contrast to the periodontal research field the number of studies in relation to cell types involved in cutaneous wound healing are still limited and the mode of action is still under investigation.

In experimental studies, topical application of amelogenin mixture accelerated granulation tissue formation and wound closure in a rabbit model.\textsuperscript{207} Also, amelogenins promote cell proliferation,\textsuperscript{169,207,208} secretion of the growth factors TGF-β and VEGF\textsuperscript{207,208} and MMP-2\textsuperscript{207} in cultured normal human dermal fibroblasts, whereas senescent-like fibroblasts have not yet been explored. Amelogenins increased the number of blood vessels in two murine subcutaneous models of angiogenesis,\textsuperscript{209,210} chick chorioallantoic membrane assay\textsuperscript{211} and sprouting in canine dental pulp\textsuperscript{212}. These findings strongly suggest that amelogenin mixture enhances angiogenesis during tissue repair. However, the mechanism of amelogenin-promoted angiogenesis has not been elucidated. VEGF secretion from human fibroblasts\textsuperscript{207,208,213} and angiopoietin-2 expression in human umbilical vein endothelial cells (HUVEC) were increased \textit{in vitro} in the presence of amelogenins.\textsuperscript{214} Also, amelogenin mixture promoted migration of endothelial cells in Boyden chemotaxis chambers\textsuperscript{210,213} and denuded endothelial monolayers\textsuperscript{214} indicating the involvement of integrin-dependent mechanisms.\textsuperscript{43} Furthermore, the effect of amelogenins on proliferation of endothelial cells is inconclusive in contrast to studies on other cell types of mesenchymal origin.\textsuperscript{198,207,213-215}

A possible influence by amelogenins on inflammation was first addressed when the attenuation of two pro-inflammatory cytokines, TNF-α and IL-8, induced by LPS and peptidoglycan, along with increased levels of cAMP was demonstrated in cultured human blood cells.\textsuperscript{216} Also, cultured LPS-treated rat monocytes, reduced the secreted TNF-α levels and increased prostaglandin E2 release after amelogenin stimulation.\textsuperscript{217} A possible systemic anti-inflammatory treatment of amelogenins has been investigated without effect,\textsuperscript{218} although, the mixture is intended for topical application.

There are still great limitations to the current understanding of the effects on amelogenins on dermal cells, especially cell types essential for cutaneous wound healing. Amelogenins have been reported to be taken up by osteoblasts.\textsuperscript{196} Similar studies on the interaction of amelogenins and dermal cells are absent. The involvement of integrin-dependent adhesion to amelogenins is limited to studies on PDL and gingival fibroblasts and these are limited to a few subunits.\textsuperscript{166,180,219} The interactions between amelogenins and macrophages, dermal fibroblasts or endothelial cells, crucial cells for cutaneous tissue repair and the possible involvement of integrin adhesion have, to the author’s knowledge, not been explored.
2. AIMS

*If we knew what we were doing, it wouldn't be called research, would it?*

(Albert Einstein 1879-1955)
The aim of this research project was to contribute to the knowledge of the effects of amelogenins on cell behaviour related to wound healing, thereby increasing the understanding of the role of this specific ECM protein in tissue repair and regeneration.

In particular, the aims were:

- To study the *in vitro* effect of amelogenins on the inflammatory cytokine response by non-stimulated and LPS-activated human monocyte-derived macrophages.
- To examine the cell interactions of human monocyte-derived macrophages and normal human dermal fibroblasts and amelogenin aggregates by scanning and transmission electron microscopy.
- To partially characterise and compare a near-senescent human dermal cell line and normal human dermal fibroblasts.
- To study the *in vitro* effects of amelogenins on normal and near-senescent human dermal fibroblasts regarding integrin cell adhesion, cytokine secretion, proliferation and collagen matrix remodelling.
- To study the *in vitro* and *ex vivo* effects of amelogenins on human dermal microvascular endothelial cells with respect to angiogenic processes involving integrin cell adhesion, mitogenesis, apoptosis and sprouting in an *ex vivo* model.
3. MATERIALS AND METHODS

*If you're not part of the solution, you're part of the precipitate*

Steven Wright (1955-)

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3.1 Proteins

3.1.1 Amelogenins (Paper I, II, III, IV, V)
Amelogenins are extracted from the enamel matrix of developing porcine teeth. Lyophilised amelogenin mixture (enamel matrix derivative, EMD) was produced by Biora-Straumann (Malmö, Sweden) and dissolved in 17 mM acetic acid to provide a 10 mg/ml stock solution.

3.1.2 Tyrosine-rich amelogenin peptide (Paper I)
The 5 kDa tyrosine-rich amelogenin peptide (TRAP), constituting residues 1-45 on the N-terminal of the full-length amelogenin, constitutes about 9% of the total protein content according to the manufacturer’s QC certificate (Biora-Straumann). The TRAP peptide was isolated and purified by S.Petter Lyngstadaas (see paper I for further description). The purified TRAP was dissolved in 17 mM acetic acid to a final concentration of 1 mg/ml, prior to use in the cell cultures.

3.2 Cell culture

3.2.1 Isolation and culture of human monocytes (Paper I, II)
Human monocytes were obtained from healthy blood donors by a 2-step isolation procedure using the separation gradient Percoll™ (Pharmacia, Sweden). Buffy coat was layered over 1.076 g/ml Percoll in conical centrifugation tubes and centrifuged at 800 g for 30 min in room temperature. The layer containing mononuclear cells was then aspirated, washed twice, layered on top of 1.064 g/ml Percoll and centrifuged at 800 g for 60 min. The layer of monocytes was harvested and washed three times in HBSS. The cell concentration was estimated by the NucleoCounter® (ChemoMetec, Allerød, Denmark; see 3.3.1). The isolated monocytes were then re-suspended to a concentration of 5x10⁵ cells/ml in Roswell Park Memorial Institute (RPMI) 1640 culture medium supplemented with 5% foetal calf serum (FCS). The viability of the cells was checked by trypan blue dye exclusion. After monocyte isolation, cells were seeded in 24-well plates and cultured for 24 h at 37°C with 5% CO₂ and 95% humidity. Thereafter, supernatants, including non-adherent cells, were removed resulting in increased purity of the monocyte population.

3.2.2 Fibroblast culture (Paper III, IV)
Normal human dermal fibroblasts (NHDF; Karocell Tissue Engineering AB, Stockholm, Sweden) and near-senescent CRL-7815™ cell line (ATCC, Rockville, MD, USA) were cultured at 37°C in humidified 5% CO₂ in complete medium composed of Dulbecco’s modified Eagle’s medium with GlutaMAX™ supplemented with 5% FCS. Culture medium was changed three times weekly. At confluence, cells were washed and split at a ratio of 1:3. NHDF and CRL-7815 fibroblasts were used for the experiments between passages 5 and 12.
3.2.3 Endothelial cell culture (Paper V)

Human dermal microvascular endothelial cells (HDMEC) purchased from Promocell (Heidelberg, Germany) were cultured in complete growth medium recommended by the manufacturer. Medium was changed three times weekly. At 75-80% confluence, cells were washed, detached and seeded at 10,000 cells/cm² according to manufacturer’s instructions. Cells were cultured at 37°C in a humidified 5% CO₂. Endothelial serum-free growth medium (E-SFM) used in studies was purchased from Invitrogen (Paisley, UK). HDMEC between passage 3 and 7 were used for the studies.

3.3 Evaluation methods

3.3.1 Cell quantification (Paper I, II, III, IV, V)

Cell quantification was utilised to determine the correct starting number of cells in each experiment as well as for the proliferation studies in paper III and IV. Cells were quantified using the NucleoCounter® system (ChemoMetec, Allerød, Denmark). This included the cells first being lysed and lysates stabilized with disaggregation buffer. The cell nuclei were stained with propidium iodide in the NucleoCassette™ and the nuclei counted by the NucleoCounter® system according to the manufacturer’s protocol.

3.3.2 Enzyme-linked immunosorbent assay (Paper I, II, IV)

Enzyme-linked immunosorbent assay (ELISA) was used to determine the concentration of cytokines, growth factors and collagen propeptide in the supernatants from the cultured cells after incubation. Conditioned media were collected, centrifuged at 400 g for 5 min at 4°C and supernatants stored at -80°C until analysis. Commercial human ELISA kits for AMAC-1, IGF-1, IL-8, MCP-1, TNF-α, VEGF, and the C-terminal propeptide of type I collagen (CICP) were utilised according to manufacturers’ instructions. The optical density was measured at 450 nm with a plate reader (SPECTRAMax, Molecular devices, UK) and translated to cytokine levels using SoftPro software (Molecular devices, UK). The content of respective cytokine/growth factor and CICP in the complete medium was subtracted from the test samples.

3.3.3 Multiplex bead array (Paper I, II)

Conditioned medium from the monocyte studies was analysed for cytokine concentrations using the human cytokine 25-plex bead immunoassay (BioSource™, Invitrogen). This kit enables the detection of human eotaxin, granulocyte-macrophage colony-stimulating factor (GM-CSF), IFN-α, IFN-γ, IL-1β, 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, immune protein-10 (IP-10), MCP-1, monokine induced by gamma-interferon (MIG), MIP-1α, MIP-1β, regulated upon activation, normal T-cell expressed and secreted (RANTES) and TNF-α. The measurements were performed according to the manufacturer’s instructions. Shortly, samples were clarified by centrifugation and added to a filter bottom microplate containing beads conjugated to
cytokine-specific antibodies. The microplate was incubated for 2 h at room temperature on an orbital shaker. Thereafter, analyte-specific biotinylated detector antibodies were added to each well followed by 1 h incubation. Streptavidin conjugated to R-Phycoerythrin (RPE) was pipetted to each well and the microplate was incubated for 30 min. The beads were re-suspended in wash solution and the microplate was analysed using a Luminex 100™ Cytometer (Applied Cytometry System, Sheffield, UK). The concentrations of the cytokines were determined by monitoring the spectral properties of the beads while simultaneously quantifying associated RPE fluorescence.

3.3.4 Electron microscopy studies (Paper I, III)
The interactions between human peripheral blood monocyte-derived macrophages and normal human dermal fibroblasts (NHDF) and amelogenin aggregates were visualised with transmission electron microscopy (TEM), along with scanning electron microscopy (SEM) for the fibroblasts. The cells were seeded on Thermofax™ coverslips in 24-well plates and cultured for 24 h at 37°C with 5% CO2 and 95% humidity. The supernatants were discarded, including non-adherent cells and fresh medium alone (control) or medium containing amelogenin was added. After 24 h incubation, the wells were washed with PBS and cells were fixated in 2% paraformaldehyde, 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer for 2 h at room temperature.

3.3.4.1 SEM preparation
The cells were washed with 0.15 M sodium cacodylate buffer and post-fixated with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 h at 4°C. Contrast enhancement was performed with 1% thiocarbohydrazide for 10 min at room temperature followed by incubation in 1% osmium tetroxide for 1 h at 4°C according to a modified version of the OTOTO post-fixation method. Dehydration was performed with a series of ethanol solutions ranging from 70-95.5% and critical point-drying by hexamethyldisilizane evaporation. The specimens were mounted on stubs and sputtered with palladium. The samples were analysed using SEM (LEO 982 Gemini; Zeiss, Oberkochen, Germany) operated at 3 kV.

3.3.4.2 TEM preparation
The cells were washed with 0.15 M sodium cacodylate buffer and post-fixated in 1% osmium tetroxide with 1% potassium ferrocyanide for 2 h at 4°C. Contrast enhancement was performed with 1% uranyl acetate in distilled water for 1 h at room temperature in the dark. The cells were dehydrated with a series of ethanol solutions ranging from 70-95.5% followed by transition to epoxy resin (Agar 100; Agar Scientific Ltd., Stanstead, UK) by dilution in ethanol in two steps before embedding in fresh epoxy resin by polymerisation at 40°C for 16 h and then at 60°C for 48 h. Ultra-thin (60-70 nm) sections were cut using a Reichert Ultracut E ultramicrotome (Reichert, Vienna, Austria) and collected on Formvar-coated copper grids. The sections were counterstained with uranyl acetate and lead citrate before analysis in TEM (LEO 912 Omega; Zeiss) operated at 120 kV. Digital images were taken with a Megaview III camera (SIS, Münster, Germany).
3.3.5 Integrin-mediated adhesion assay (Paper III, V)

The cell surface subunit (α1, α2, α3, α4, α5, αv, β1, β2, β3, β4, β6) or heterodimer (αvβ3, αvβ5 and α5β1) integrins in the cell plasma membrane were quantified using an integrin-mediated cell adhesion array (Millipore, Billerica, MA, USA). The kit utilises 96-well plates with immobilised monoclonal antibodies directed against specific human subunit or heterodimer integrins. Briefly, the cells were seeded at 20,000 cells/cm² in T-75 culture flasks in complete culture medium alone or supplemented with amelogenin. Fibronectin served as a positive adhesion protein control with concentration based on required amount for monolayer coverage. The cells were incubated for 24 h and harvested non-enzymatically with 5 mM EDTA/PBS. The cells were re-suspended to a final concentration of 10⁶ cells/ml, aliquoted and allowed to adhere for 2 h at 37°C. Thereafter, non-adherent cells were washed off and the remaining adherent cells were stained with crystal violet. The stain was then extracted for 5 min at room temperature and the optical density of extracts was measured at 540 nm on a SpectraMax® microplate reader (Molecular Devices, Crawley, UK). The optical density of controls for unspecific adhesion was subtracted from all values.

3.3.6 Gene microarray (Paper III)

The effects of amelogenin on gene expression in normal human dermal fibroblasts (NHDF) were studied by gene microarray. NHDF were seeded at 10,000 cells/cm² in T-75 culture flasks with or without the addition of 1,000 μg/ml amelogenin mixture and cultured at 37°C with 5% CO₂ and 95% humidity. RNA was isolated and purified from triplicates after 48 h and 72 h incubation, using DNase I kit, Qiashredder columns and RNeasy® mini kit (Qiagen, Solna, Sweden) according to manufacturer’s instructions. The RNA analysis was then performed at Sgewe (Lund, Sweden) using the GeneChip® Human Genome 1.0 ST Arrays (Affymetrix Inc., Santa Clara, CA, USA). Pathway analysis and bio-function analysis were performed by S.Petter Lyngstadaas using the Ingenuity Pathway Analysis (IPA) 7.4 software (Ingenuity Systems, Redwood City, CA, USA) using gene expression values, given by the Affymetrix MAS 5.0 software, with an 8-fold change or more between test and solvent control as significance level. Results were sorted according to the number of genes involved in each pathway.

3.3.7 Fibroblast-populated 3D collagen matrices (Paper IV)

The relaxed 3D collagen matrix model was used to study normal human dermal fibroblasts (NHDF) and near-senescent CRL-7815 fibroblasts collagen remodelling ability. Fibroblast-populated collagen matrices were fabricated on ice with rat tail type I collagen (354249; BD Biosciences) containing fibroblasts with or without the addition of amelogenin. In parallel, acellular matrices were made to serve as negative controls. Matrices containing 28,000 fibroblasts with 2 mg/ml collagen were cast in 24-well plates, pre-coated with 100 μg/ml bovine serum albumin. The matrices were allowed to polymerise for 60 min at 37°C. Thereafter, 1 ml complete medium was added to each well, the matrices gently
detached with a spatula and the plates incubated for 24 h. The areas of the dermal matrices were measured with an image analysis software (Easy Image 2000, TeknoOptik, Skårholmen, Sweden) from digital photographs acquired using Nikon COOLPIX 4500 at baseline (0 hour), 4, 8 and 24 h. Cell morphology was examined and documented by an inverted microscope equipped with Nikon DS-5M-L1 digital camera. At the end of the 24-hour incubation period, the matrices were weighed and then treated with 400 μL of 1 mg/ml type I collagenase (234153; Calbiochem) for 2 h at 37°C. The number of fibroblasts was counted by the NucleoCounter® System (see 3.3.1).

3.3.8 Mitogenesis assay (Paper IV, V)

The effect of amelogenins on mitogenesis was measured by BrdU incorporation, a measure of DNA synthesis in replicating cells. DNA synthesis was assessed by the incorporation of 5-bromo-2′-deoxyuridine (BrdU) in fibroblasts and endothelial cells during culture. BrdU was added at a final concentration of 10 μM to the wells during the final 24 h of the incubation periods. The cells were then fixed and the amount of incorporated BrdU was determined with an enzyme-linked immunoassay system according to the manufacturer’s protocol (GE Healthcare, Uppsala, Sweden). The fixed cells were reacted with a peroxidase-labelled anti-BrdU antibody for 90 min at room temperature and the immune complexes were detected by a subsequent substrate reaction. The optical density was read at 450 nm using a SpectraMax® microplate reader (Molecular Devices, Crawley, UK).

3.3.9 Apoptosis assay (Paper V)

Apoptosis was measured by the detection of DNA strand breaks with fluorescein staining by TdT-mediated dUTP nick end labelling (TUNEL) technology according to the manufacturer’s protocol (Roche Diagnostics, Mannheim, Germany). After incubation, the cells were fixed in 4% paraformaldehyde for 1 h at room temperature and permeabilised with 0.1% (v/v) Triton X-100 in 0.1% sodium nitrate for 2 min on ice. Positive controls were created by inducing DNA strand breaks with 10 U/ml recombinant DNase I in 50 mM Tris-HCl, 1 mg/ml bovine serum albumin for 10 min at room temperature. Thereafter, TUNEL solution conjugated with fluorescein was added and the cells were incubated for 1 h at 37°C. The slides were mounted with Vectashield (Vector Laboratories, Burlingame, USA) containing 4’,6-diamidino-2-phenylindole (DAPI) to visualise cell nuclei. Analyses were performed by fluorescence microscopy (Nikon Eclipse E600, Tokyo, Japan) and quantified with the image analysis software Easy Image 2000 (TeknoOptik AB, Göteborg, Sweden). Each treatment consisted of six wells with triplicate images taken from centre, 6 and 12 o’clock in each well. The number of apoptotic cells in percentage was calculated by comparing DAPI and fluorescein staining.

3.3.10 Chick aortic arch assay (Paper V)

This ex vivo model was utilised to study the effect of amelogenins on the early events in the formation of blood vessels by considering the combined outcome of cell proliferation,
migration and tubule formation. Sprouting was assessed using 1 mm thick transverse sections of aortic arches of 13-day-old chick embryos with the lumen visible, attached in 48-well plates with 30 µl of Matrigel (BD Biosciences, Bedford, MA, USA). An additional 25 µl Matrigel sealed the tissue sections in place. Amelogenin or the unspecific protein control porcine serum albumin (A2764, Sigma-Aldrich) were dissolved in 17 mM acetic acid and added to the wells in endothelial serum-free medium (E-SFM). Controls comprised of E-SFM medium alone or with endothelial cell growth supplement. The plates were incubated for up to 48 h. Sprouting was scored from 0 to 6 in arbitrary units (a.u.) by a blinded observer (Hynda K. Kleinman) based on the length and density of the outgrowth, with 0 being no outgrowth and 6 being the most extensive sprouting. This study was performed in the USA and according to US government regulations, approval from ethics committee or alike is not mandatory for experiments conducted on chicken embryos before hatching (day 21).

3.4 Statistics
Statistical analyses to compare cytokine levels (paper I, II, IV), integrin expression (paper III, V), DNA synthesis (IV, V), apoptosis and sprouting (paper V) was performed using two-way ANOVA test followed by Fisher’s protected least significant difference (Statview 5.0 software), where $p < 0.05$ was considered statistically significant.

In the proliferation studies (paper III, IV), the number of fibroblasts at given days was plotted against time of incubation as abscissa. In the fibroblast-populated 3D collagen matrices experiment (paper IV), the matrix areas were plotted against time. Area under curves (AUC) was calculated using the trapezoid method between day 1 and 7, day 1 and 9, and between 0 and 24 h, respectively, using the FigSys 2.4.3 software (Biosoft, Cambridge, U.K.). ANOVA-test followed by the Tukey test (paper III) or the student’s t-test for unpaired observations (paper IV) was then used to compare the AUC values between amelogenin treatment and control. Differences were considered statistically significant at $p < 0.05$. 
4. RESULTS

_Nothing shocks me. I'm a scientist._

Harrison Ford (1942 - ) _as Indiana Jones_
4.1 Electron microscopy studies (Paper I, III)

The interaction between amelogenin aggregates and both human macrophages and normal dermal fibroblasts (NHDF) after 24 h incubation was visualised with transmission electron microscopy (TEM), as well as scanning electron microscopy (SEM) for the fibroblasts. Amelogenins formed aggregates typically sized 0.5-1 μm (Fig. 6). Ultrastructural examination revealed interaction between the cells and amelogenin aggregates. Thin extensions from the cell membrane were observed in SEM, adhering to amelogenin aggregates found on the culture plastic or bound to the fibroblast cell surface (Fig. 7A, B). TEM analysis also displayed extensions adhering to

Fig. 6. SEM micrograph displaying the micrometer-sized amelogenin aggregates at 5,000 times magnification.

Fig. 7. SEM micrographs displaying the interaction of NHDF with amelogenins, showing extensions adhering to the protein at A) 3,000 and B) 30,000 times magnification after 24 h and also possible internalisation of amelogenin of NHDF, showing C) protrusions (arrows) under the cell membrane in similar size and shape as amelogenin spheres, and D) possible uptake of amelogenin after 24 h, showing a larger sphere that is partially enveloped by the cell membrane and a relatively smaller sphere.
amelogenin spheres in the microenvironment of both the fibroblasts (Fig. 8A) and the macrophages (Fig. 9B). Moreover, TEM sections of both cell types displayed a condensation of the cell membrane, visualised by a relative increase in electron-dense staining of the plasma membrane in areas of close proximity to amelogenins (Figs. 8B and 4C). A thin membrane was detected around internalised amelogenins (Figs. 9D and 4D).

Further, cellular uptake of amelogenin was visible for NHDF in the SEM micrographs, shown both with spherical protrusions of the cell surface (Fig. 7C), most likely representing internalized protein spheres, and membrane-enclosed spheres (Fig. 7D). The uptake of amelogenin was verified in TEM, occurring in high degree for many fibroblasts in microscopic sections (Fig. 8C). The uptake of amelogenins was also detected in the macrophages (Fig. 9C and D), although to a much lesser extent.

4.2 Cytokine measurements (Paper I, II, IV)

4.2.1 Macrophages (Paper I, II)

Isolated peripheral blood monocyte-derived macrophages were cultured for 6, 24 and 72 h with amelogenins (0, 10, 100 and 1,000 µg/ml) and with or without the inflammatory stimulus LPS. The supernatants were analysed for cytokine concentrations with a 25-plex
Fig. 9. TEM micrographs of macrophages after 24 h of cultivation in the presence of amelogenins (A, scale bar = 2 μm). Cell extension adhering to the protein is shown in B (scale bar: 0.5 μm) and condensations of the membrane in close proximity to amelogenins are marked with arrows (C, scale bar = 1 μm). Note the internalised amelogenins (C, D) with a thin membrane (arrows) surrounding the densely packed spheres (D, scale bar = 1 μm).

bead immunoassay and ELISA. All the kinetics study results are visualised in paper I and II. In general, amelogenin treatment of non-stimulated cells resulted in increased concentrations of the cytokines IL-1β, IL-1RA, IL-2R, IL-6, IL-8, IL-10, IL-12, MCP-1, MIP-1α and MIP-1β, all at significant levels after 24 h and everyone except IL-12 after 72 h. Amelogenin treatment of LPS-stimulated macrophages resulted in that IL-1β, IL-1RA, IL-2R, IL-10, IL-6, MIP-1α and RANTES were all significantly increased in the cell culture supernatant. Further, amelogenins attenuated the levels of the four classically pro-inflammatory cytokines studied, TNF-α, MCP-1, IL-8 and IL-12. MIP-1α was slightly decreased after 6 and 24 h and increased after 72 h for the highest amelogenin concentration.

The expression of AMAC-1, a marker for alternatively activated macrophages, was low in non-stimulated macrophages. The levels of AMAC-1 after treatment with 100 and 1,000 μg/ml amelogenins were significantly elevated and increased over time by a maximal 570% (p < 0.001) and 560% (p < 0.001), respectively, in comparison to medium control after 72 h (Fig. 10A). In LPS-stimulated cells, the amelogenin-induced AMAC-1 secretion was comparable to the levels obtained by the positive control IL-4. 50% after 24 h and 75% after 72 h with 100 μg/ml (Fig. 10B). Moreover, the levels of the two analysed
growth factors, VEGF (Fig. 11) and IGF-1, were increased in the presence of amelogenins in the cell culture medium (measured after 24 h).

**Influence of phagocytosis (Paper I)**

To investigate the involvement of phagocytosis in cytokine secretion after amelogenin treatment, human macrophages were treated with cytochalasin B prior to stimuli. The levels of all the analysed cytokines were independent of the opportunity for the cells to internalise amelogenin aggregates.

**Comparison of amelogenins and TRAP (Paper I)**

The secretion of cytokines induced by amelogenins was compared with that of the peptide TRAP. Macrophages were cultured for 24 h with amelogenins (10, 100 and 1,000 µg/ml) or TRAP (10 and 100 µg/ml) and the supernatants were analysed for 27 different cytokines. The expression after amelogenin treatment corresponded to the pattern in the cytokine kinetics study after 24 h, with a significant augmentation in the matching cytokines. The growth factor VEGF was also elevated after 24 h of incubation with all three concentrations of amelogenins (Figure 11B). The peptide TRAP slightly affected the VEGF levels at 10 µg/ml (Fig. 11B), but had no effect on the other 26 cytokines analysed.
**4.2.2 Fibroblasts (Paper IV)**

The levels of cytokines and collagen pro-peptide (CICCP) in conditioned media from cultured normal human dermal fibroblasts (NHDF) and near-senescent CRL-7815 fibroblasts after 1, 2 and 3 days were analysed with commercial ELISA kits. IL-8 levels in CRL-7815 fibroblast controls were reduced by 90% ($p < 0.001$), 88% ($p < 0.01$) and 85% ($p < 0.01$) compared with NHDF after 1, 2, 3 days of incubation. Amelogenins at 1,000 µg/ml elicited a significant increase in the amount of IL-8 in CRL-7815 fibroblasts at 1 ($p < 0.001$), 2 ($p < 0.01$) and 3 days ($p < 0.001$) (Fig. 12A). The production of MCP-1 was almost non-detectable in normal fibroblasts throughout the 3-day incubation period while the

![Graph A](image1.png)

*Fig. 11.* Levels of growth factor VEGF in conditioned medium from human monocyte-derived macrophages measured by ELISA cultured in the presence of (A) amelogenins 0, 10, 100 or 1,000 µg/ml or (B) 10 and 100 µg/ml TRAP (grey bars) for 24 h. White amelogenin mixture bars, without LPS stimulation; black bars, with simultaneous LPS stimulation (10 ng/ml). The cytokine concentration is presented as the mean value ± SEM, n = 6, * $p < 0.05$, **$p < 0.001$ vs. control (0 µg/ml).

![Graph B](image2.png)

*Fig. 12.* IL-8 (A) and MCP-1 (B) per cell from control medium normal (NHDF, open bars) and CRL-7815 (filled bars) fibroblasts, treated with 100 µg/ml amelogenins (hatched) or with 1,000 µg/ml amelogenins (cross-hatched) for 1, 2 and 3 days. Mean ± SEM (n = 3). * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$ compared with NHDF control. § $p < 0.01$, § § $p < 0.001$ compared with control-treated CRL-7815 fibroblasts.
constitutive MCP-1 secretion was significantly elevated in CRL-7815 fibroblasts and increased with incubation time. Amelogenin treatment at 100 and 1,000 µg/ml reduced the MCP-1 levels in the near-senescent CRL-7815 fibroblasts at 2 days ($p < 0.001$) and 3 days ($p < 0.001$) (Fig. 12B).

Further, VEGF levels and collagen biosynthesis, measured by CICP concentration in the conditioned medium, was elevated in CRL-7815 fibroblasts compared to NHDF. Amelogenins increased the concentration of VEGF in CRL-7815 fibroblasts throughout the experiment. The CICP was attenuated after 72 h by 100 µg/ml amelogenin. IGF-1 and TNF-α levels were below detection limits.

4.3 Cell proliferation (Paper III, IV, V)

4.3.1 Fibroblasts (Paper III, IV)

The effect of amelogenins on fibroblast proliferation was studied by culture of normal human dermal fibroblasts (NHDF) and near-senescent fibroblasts (CRL-7815) for up to 9 or 7 days, respectively. Cells were counted in separate wells every 24 h. Overall cell growth was assessed by area under the curves (AUC). The general proliferative ability, assessed by the area under the growth curves from day 1 to day 9, of near-senescent fibroblasts (CRL-7815) was significantly ($p = 0.0013$) lower, by 59%, compared with NHDF (Fig. 13A). Amelogenins at 1,000 µg/ml significantly increased the proliferation ($p = 0.0017$) of the CRL-7815 fibroblasts and this proliferation level did not differ significantly ($p = 0.11$) from the untreated normal fibroblasts (Fig. 13A).

Fig 13. Growth of normal (A, B, NHDF) and near-senescent human dermal fibroblasts (A, CRL-7815). Control medium (NHDF, open circles) and CRL-7815 (filled circles) fibroblasts were treated with 100 µg/ml (triangles) or 1,000 µg/ml amelogenins (squares) for up to 9 or 7 days. Overall cell growth, estimated by AUC from day 1 to day 9 or day 7. Mean ± SEM (n = 3)
amelogenins increased the proliferation of NHDF significantly with 42% at 100 µg/ml ($p = 0.022$) and 112% with 1,000 µg/ml ($p < 0.001$) compared to medium control (Fig. 13B).

The kinetic experiment was, in part, repeated by studying cell counts at days 1, 3 and 6. The control CRL-7815 fibroblasts again ceased replicating after reaching a saturation density of maximally 3500 fibroblasts compared with > 8000 CRL-7815 fibroblasts treated with 1 mg/ml amelogenin and > 8000 NHDF at day 6 (Fig. 14).

4.3.2 Endothelial cells (Paper V)

The effect of amelogenins on human dermal microvascular endothelial cells (HDMEC)

![Graph](image)

**Fig. 14.** Growth of normal (NHDF) and near-senescent human dermal fibroblasts (CRL-7815). Control medium (NHDF, open bars) and CRL-7815 (filled bars) fibroblasts were treated with 100 µg/ml (hatched) or 1,000 µg/ml amelogenins (cross-hatched) for 1, 3, and 6 days. Mean ± SEM ($n = 6$), $^*p < 0.05$, $^{***}p < 0.001$ compared with NHDF control.

![Graph](image)

**Fig. 15.** Effects of amelogenin mixture (A, $n = 6$) and VEGF165 (B, $n = 8$) on BrdU incorporation in HDMEC cultured on Matrigel in E-SFM after 24 h (○) and 48 h (●) of treatment. Mean ± SEM $^*p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ compared to control-treated cells.
proliferation was measured by BrdU incorporation, a measure of DNA synthesis in replicating cells. DNA synthesis increased in all the groups from 24 to 48 h (n = 8).

Compared with the control, BrdU levels in the 1,000 µg/ml amelogenin mixture increased significantly by 134% (p < 0.001) after 48 h (Fig 15A). No significant effects were found with the 10 µg/ml and 100 µg/ml amelogenin mixtures.

In a separate and expanded experiment (n = 8), amelogenin mixture was compared with human recombinant VEGF165 and the complete medium containing 5% FCS and growth factor supplements. The effect of VEGF on BrdU incorporation increased significantly at 5-100 ng/ml, corresponding to a 165% (p < 0.001) maximal increase at 24h and an 86% (p < 0.01) at 48h, compared with medium control (Fig 15B). Amelogenins (1,000 µg/ml) increased DNA synthesis by 65% after 24 h (p < 0.001) and by 148% after 48 h (p < 0.001) compared with control also in this second experiment.

4.4 Integrin-mediated adhesion (Paper III, V)

The integrin expression of normal human dermal fibroblasts (NHDF) and human dermal microvascular endothelial cells (HDMEC) upon ECM protein interaction was analysed after 24 h incubation. The binding capacity of the subunits α1, α2, α3, α5, αv, β1, β2 and heterodimers αvβ3, αvβ5 and α5β1 of NHDF (Fig. 16A) were significantly (p < 0.01, n = 5)

![Integrin-specific adhesion](image)

**Fig. 16.** Integrin-specific adhesion of heterodimers on (A) fibroblasts (NHDF) and (B) endothelial cells (HDMEC) treated for 24 h with amelogenin mixture at 100 µg/ml (open bars) or 1,000 µg/ml (filled bars), and fibronectin at 20 µg/ml (hatched) in comparison to complete medium alone expressed in percentage. Results are pooled from two separate experiments. Mean ± SEM (n=5 except for medium control n=7 where n=number of wells). ** p < 0.01 and *** p < 0.001 compared to medium control.
increased with 1,000 µg/ml amelogenin mixture compared to control. Amelogenins at 100 µg/ml showed no statistically significant effect on integrin expression of NHDF.

However, treatment with 100 µg/ml amelogenin mixture of HDMEC significantly ($p < 0.01, n = 5$) increased the adhesion of the integrin subunits α1, α2, α3, α5, αv, β1, β2 and heterodimers αvβ3, αvβ5 and α5β1 compared to medium control. By contrast, the higher amelogenin mixture concentration (1,000 µg/ml) significantly ($p < 0.01, n = 5$) decreased the adhesion by the same integrins (Fig. 16B).

The subunits β3, β4 and β6 on NHDF and α4, β3, β4 and β6 on HDMEC were barely detectable under these culture conditions. The results for both cell types were comparable to fibronectin treatment, an ECM protein involved in several phases of tissue repair, influencing cell growth.50

4.5 Gene microarray analysis (Paper III)

The gene microarray studies showed that amelogenin treatment of normal dermal fibroblasts caused more than three-fold up or down-regulation of 856 genes out of the 28,869 genes represented in the Affymetrix GeneChip® Human Gene 1.0 ST Array.

An eight-fold regulation was observed for 550 genes of which most are involved in cellular growth, migration, survival and differentiation (Table 1). Several of these genes are also involved in canonical pathways, especially in the hepatic fibrosis pathway and IL-6 and FGF signalling. The microarray studies displayed no changes in integrin gene expression above the three-fold cut off level after 48 or 72 h of amelogenin exposure. However, a significant (three-fold) increase in gene expression for some ECM proteins, e.g. several collagens and fibronectin was observed at both time points.

Table 1. Number of genes, sorted by function, regulated by amelogenins more than 8-fold in Affymetrix experiments.

<table>
<thead>
<tr>
<th>Molecular and cellular functions</th>
<th>Total no. of genes regulated</th>
<th>No. of regulated genes after 48 h</th>
<th>No. of regulated genes after 72 h</th>
<th>No. of regulated genes both at 48 and 72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth and proliferation</td>
<td>166</td>
<td>76 (36)</td>
<td>130 (90)</td>
<td>40</td>
</tr>
<tr>
<td>Cell migration</td>
<td>99</td>
<td>47 (19)</td>
<td>80 (52)</td>
<td>28</td>
</tr>
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<td>Cell survival</td>
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<td>39 (24)</td>
<td>97 (82)</td>
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<tr>
<td>Cell cycle</td>
<td>61</td>
<td>11 (3)</td>
<td>58 (50)</td>
<td>8</td>
</tr>
<tr>
<td>Cell differentiation</td>
<td>103</td>
<td>53 (23)</td>
<td>80 (50)</td>
<td>30</td>
</tr>
<tr>
<td>Signalling &amp; interaction</td>
<td>64</td>
<td>26 (0)</td>
<td>64 (38)</td>
<td>26</td>
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<tr>
<td>Gene expression</td>
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<td>35</td>
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<td>0</td>
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<td>0 (0)</td>
<td>0</td>
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<tr>
<td>Total number of genes*</td>
<td>550</td>
<td>218 (98)</td>
<td>424 (336)</td>
<td>132</td>
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</tbody>
</table>

Genes per functions are listed according to Ingenuity analysis. Figures in parenthesis indicate number of genes unique to that particular time point. * Some genes participate in several functions.
4.6 Collagen matrix remodelling (Paper IV)

The relaxed 3D collagen matrix model was used to study normal human dermal fibroblasts (NHDF) and near-senescent CRL-7815 fibroblasts remodelling ability by measuring the matrix area over a 24-h period. NHDF shrinkage of the matrix started after 4 h, whereas the overall activity of CRL-7815 fibroblasts was attenuated \((p < 0.001)\) compared with NHDF and size reduction was evident first at 24 h (Fig. 17). No area changes occurred for acellular collagen matrices. Amelogenins incorporated at 1,000 \(\mu\)g/ml in the CRL-7815-populated collagen matrix increased \((p = 0.0011)\) matrix contraction compared with control (Fig. 17C). There were no significant differences in the number of fibroblasts at 24 h among the four groups. Morphologically, prominent cell extensions were observed from most of the NHDF at 4 h while even at 24 h cell extensions from CRL-7815 fibroblasts were rare and poorly developed (Fig. 17D,E).

![Figure 17](image_url)

**Fig 17.** The effect of the fibroblast phenotype and amelogenins on collagen matrix remodelling. Images of collagen matrices with NHDF (A) and CRL-7815 (B) fibroblasts after 24 h of incubation. (C) The measured collagen matrix areas were plotted against time. The overall effect, estimated by AUC from 0 to 24 h, was 3,460 ± 60 mm\(^2\) · hour for NHDF, 4,410 ± 20 mm\(^2\) · hour for CRL-7815 (control), 4,370 ± 20 mm\(^2\) · hour for CRL-7815 (100 \(\mu\)g/ml amelogenins), and 4,260 ± 30 mm\(^2\) · hour for CRL-7815 (1,000 \(\mu\)g/ml amelogenins). The dotted line represents the acellular collagen matrices. Mean ± SEM \((n = 6)\). Phase-contrast images at 24 h of NHDF (D) and CRL-7815 (E) populated matrices. Note prominent cell extensions from NHDF compared with CRL-7815 fibroblasts. Scale bar 100 \(\mu\)m.
4.7 Chick aortic arch assay (Paper V)

The ex vivo chick aortic arch assay measures the combined effects of cell proliferation, migration and tubule formation. In the first of two experiments (n = 6), the positive control, endothelial cell growth supplement, significantly increased sprouting after both 24 h and 48 h of incubation, compared with medium alone. No bioactivity was observed with the negative control protein porcine serum albumin. A precipitate was observed with 1,000 µg/ml amelogenin mixture, as has previously been noted. At this concentration, the amelogenin mixture displayed its strongest activity following 24 h of incubation. At 48 h the promoting effect had ceased and tubules regressed rather than formed.

Amelogenins at 100 µg/ml were found to increase microvessel outgrowth, both in this experiment and in a subsequent enlarged experiment that assessed 12 replicates per treatment (Fig 18). In the second, enlarged experiment (n = 12), the outgrowth from explants treated with 100 µg/ml amelogenin mixture increased by 76% (p < 0.01) compared with the medium control, and by 42% (p < 0.01) compared with the solvent control after 48 h (Fig 18C).

4.8 Apoptosis assay (Paper V)

DNA strand break is a feature of the apoptotic process and was measured using a TUNEL method (n = 6). The positive control DNase I induced DNA cleavage in 97 ± 0.7% and 96 ± 1.4% of the cells after 24 h and 48 h, respectively, of incubation. By contrast, DNA cleavage was unexpectedly rare in the treatment groups since fewer than 2% of the cells went into apoptosis, irrespective of treatment.
5. DISCUSSION

Aerodynamically the bumblebee shouldn’t be able to fly, but the bumblebee doesn’t know that so it goes on flying anyway

Mary Kay Ash (1918-2001)
Interactions between the cellular component and the ECM are prerequisites for tissue repair. The present thesis was defined based on the hypothesis that amelogenins function as a temporary extracellular matrix for cell adhesion, thereby stimulating wound healing. This research demonstrates that amelogenins influence the in vitro cell behaviour of the three human cell types studied: macrophages, fibroblasts and endothelial cells, all essential in the progression of wound healing, where the effects are plausibly conducted by the interaction of amelogenins with cells via integrins.

5.1 Methodological considerations
In hard-to-heal wounds, the normal wound healing response is impaired, which may involve underlying aetiologies in addition to overwhelming local wound responses. To add further to the complex situation, all wounds are heterogeneous, both between patients, but also in the fact that different sites within the wound area may be located in different phases of the wound healing process. Thus, pre-clinical studies of a treatment implicated for non-healing wound is not easy to design. It is important to emphasise that the studies in the present thesis were performed in vitro and ex vivo, in controlled and simplistic models. The in vivo situation is an intricate environment with multiple cell types, soluble mediators and ECM proteins creating numerous influential interactions of cell-ECM and cell-cell contacts. This taken together with systemic factors affecting the cellular response to different treatment strategies precludes mimicking this with an experimental in vitro model. Moreover, the complexity also makes results harder to interpret, and thus the need for both in vitro and in vivo models are necessary in the study of cell-ECM interactions and their effect on tissue repair. In the present studies of human macrophages and dermal fibroblasts, the use of cell culture under serum-enriched conditions has been used to mimic the wound environment. Also, a 3D matrix of type I collagen as a model for matrix remodelling was utilised. In the endothelial cell studies the physiological substrate Matrigel was added where the studies were performed under serum-free conditions. Endothelial cells cultured on Matrigel differentiated into capillary-like structures as reported earlier. A non-specific protein control of BSA was used to clarify that the effects measured were specific effects of amelogenins and not the stimulation of random nature due to serum deprivation. Further, the positive control fibronectin was used in the integrin studies to compare the results in relation to an important tissue repair protein involved in several healing processes. Finally, the angiogenic response of amelogenins was demonstrated ex vivo in a chick aortic arch assay, also placed in Matrigel to resemble the basement membrane surrounding blood vessels.

5.2 Interactions between cells and amelogenins
Amelogenin is described as a protein with cell adhesive activities. The present thesis, has demonstrated an increased integrin-dependent adhesion of normal human dermal fibroblasts and microvascular endothelial cells after amelogenin treatment. The relation of integrin expression on dermal cells and amelogenin exposure has not been studied
previously, however the involvement of integrin binding through αv, β1, and αvβ3 to amelogenin coated surfaces has been suggested for PDL and gingival fibroblasts, supporting the present findings.\textsuperscript{166,180,219} Further, in contrast to earlier publications, the studies in paper III and V included numerous subunits and heterodimers and also demonstrated an involvement of several of these, including integrins αvβ3, αvβ5 and α5β1. These results were comparable to fibronectin treatment, an ECM protein involved in several phases of tissue repair, influencing cell growth.\textsuperscript{50} The mechanism through which the amelogenin mixture influenced integrin-mediated adhesion is most likely multifactorial. Amelogenins may influence the synthesis of integrins but they may also redistribute and activate existent integrins in the plasma membrane.

However, there was a discrepancy in the findings between the human dermal endothelial cells and fibroblasts. While the fibroblast surface integrin expression was further up-regulated at the highest amelogenin concentration, the endothelial cell adhesion was instead attenuated. Importantly, these cells differ in origin. Fibroblasts being mesenchymal cells, generally spread out scarcely in tissue, interacting predominantly with surrounding ECM in a three-dimensional manner, not to neighbouring cells.\textsuperscript{226} In contrast, the endothelial cells are epithelial cells, forming tight junctions with adjacent cells, creating cellular sheets in e.g. the endothelium and the epidermis (keratinocytes).\textsuperscript{227} Endothelial cells within the endothelium have a cellular polarity that is maintained by lateral positional cues from cell–cell contacts to adjacent cells and by integrin adhesion to the basement membrane on the basal side, along with soluble mediators.\textsuperscript{228,229} The complexity of this is not elucidated, however endothelial cell polarisation is required for vessel stability and lumen formation during angiogenesis.\textsuperscript{228}

The geometry and density of the ECM is an important determinant of whether capillary endothelial cells will enter S-phase, differentiate or undergo apoptosis.\textsuperscript{230} Ingber et al. has demonstrated how the fate of cultured capillary endothelial cells, whether to grow, differentiate or die, is dictated by the microenvironment by local binding interactions between cells and ECM molecules.\textsuperscript{230-232} These in vitro studies using coatings of fibronectin show a density-dependent switch between differentiation and cell growth in the presence of soluble mitogens. Very low densities induce cell apoptosis, while increasing ECM density promotes differentiation, and at a high density the endothelial cells start to proliferate. The switches are suggested to be dependent on cell geometry or cell spreading which in turn is dependent on integrin activation.\textsuperscript{230-232} Thus, the attenuated cell adhesion at high amelogenin mixture concentrations demonstrated here could therefore be due to an ECM density-dependent switch from differentiation to cell division. All these factors influencing the polarised endothelial cell, may explain the difference between the responses on integrin-dependent adhesion of dermal fibroblasts with endothelial cells after treatment with amelogenins.

Interestingly, the amino acid sequence of amelogenin does not contain the cell binding RGD sequence,\textsuperscript{137,138} although cell adhesion to amelogenin coated surfaces was hindered by addition of RGD-peptides.\textsuperscript{210} The RGD sequence is recognised by several integrins, e.g. αvβ3, αvβ5 and α5β1,\textsuperscript{233} an essential domain often cryptic within ECM proteins, e.g. in fibronectin and collagen during tissue repair.\textsuperscript{68} Possible explanations for the integrin-dependent adhesion could be that proteins from the serum-enriched medium
might interact with the amelogenins. However, previous published studies demonstrated integrin dependent adhesion under serum-free conditions, argues against this possibility.\textsuperscript{180,219} Another explanation could consist of the interaction between amelogenins and ECM proteins synthesised by the cultured cells themselves. Indeed, cells cultured for 24 h allows for the deposition of ECM proteins. Narani \textit{et al.} have demonstrated that amelogenins can bind both fibronectin and type I collagen, however this resulted in decreased cellular attachment by both PDL and epithelial cells compared to single proteins alone.\textsuperscript{180} Further, the binding of PDL fibroblasts to amelogenins did not require \textit{de novo} protein synthesis.\textsuperscript{180} Although, the amelogenin mixture may contain other peptides in small concentrations that could contribute to the influence of amelogenins on cell behaviour and be the source of the RGD-sequence, there are reports showing similar effects by recombinant amelogenin in comparison to the amelogenin mixture.\textsuperscript{182,208,234,235} In relation to other ECM proteins, the multimeric ECM matrix form of fibronectin has adhesive properties distinct from soluble, protomeric fibronectin.\textsuperscript{113} Similar to this, the self-assembled amelogenin aggregates may expose binding sites for several different integrins. Thus, the integrin-dependent adhesion may be conveyed by the protein aggregates themselves, where hypothetically, the self-assembly of amelogenins may create RGD-like motifs.

The surface interactions and cellular uptake of amelogenin aggregates were visualised by scanning and transmission electron microscopy for human peripheral blood monocyte-derived macrophages in paper I and normal human dermal fibroblasts in paper III. Micrographs displayed thin cell extensions adhering to amelogenin aggregates and membrane-enclosed spheres. Also, the ultrastructural studies displayed a high degree of amelogenin uptake by the cultured fibroblasts. Amelogenin treatment resulting in abundant internalisation of protein aggregates had no effect on cell viability compared to control cells, but instead promoted both cell proliferation and cytokine release. Fibroblasts are not thought of as typical phagocytes. However, fibroblasts remodel the surrounding ECM partly by phagocytosis and have been shown to ingest both collagen and fibronectin-coated beads in the size-range of amelogenin spheres in vitro.\textsuperscript{226-228} Thus, amelogenin aggregates could also be subject to internalisation for the intent of matrix remodelling. Also, recent studies suggest that several integrins have a natural ability to mediate phagocytosis of ECM components as an extension of their adhesive capacity.\textsuperscript{239} This is true for several integrins mediating binding to ECM components such as fibronectin.\textsuperscript{239} Thus, the observed internalisation of amelogenin aggregates could be part of the remodelling activity of fibroblasts, probably involving integrin interactions.

Amelogenin aggregates were only sporadically taken up by the macrophages, in contrast to the extensive internalisation of amelogenins by the dermal fibroblasts. The phagocytic activity of macrophages are usually linked to inflammation with the clearance of invading microorganisms, cellular debris and other foreign particles, to keep tissues free from possible harmful components.\textsuperscript{240} However, phagocytosis can also occur without an inflammatory response, e.g. when apoptotic cells are phagocytised as an important part of homeostasis.\textsuperscript{241} The micrometre-sized spheres of the amelogenin aggregates would appear to be an optimal size for cellular uptake. Although, amelogenins are known to be found in the developing enamel matrix, Deutsch \textit{et al.} have shown expression of amelogenins in
soft tissues and specific cell types, including macrophages. Thus, speculatively, the sporadic phagocytosis by macrophages, may indicate that amelogenins are not considered as phagocytic prey, but may instead be perceived as an ECM matrix, supported by the upregulation of cell surface integrins found in paper III and V. Further, the low uptake was in accordance to the phagocytosis blockage study where the addition of cytochalasin B did not result in significant changes in the secretion of cell mediators, indicating that mechanisms other than phagocytosis were responsible for the effects induced by amelogenins.

The present studies on the interactions between amelogenins and macrophages, dermal fibroblasts and endothelial cells, reveal amelogenin aggregates in the size range of 0.5–1 μm which interacted and become internalised by the cells. This is most probably through integrin adhesion and/or resulting in the upregulation of multiple integrins on the cell surface. Gestrelius et al. have also displayed protein aggregates in the same size range under physiological conditions, much larger than the nanospheres found in the developing enamel matrix. An important factor in cellular uptake is the size of the protein aggregates, where the amelogenin spheres are precisely between endocytosis and phagocytosis. Endocytosis is the process of receptor-mediated internalisation, the specific process through which macromolecules, viruses, and small particles enter cells. Endocytosis involves a clathrin-based mechanism and usually occurs independently of actin polymerization. By contrast, phagocytosis, the cellular uptake of particles larger than approximately 0.5 μm, occurs by an actin-dependent mechanism and is usually independent of clathrin. Cellular uptake of amelogenin aggregates has previously been detected in osteoblasts, possibly through clathrin-coated pits, implying endocytosis. Also, interactions between amelogenin and the transmembrane proteins LAMP1 and CD63, involved in endocytosis have been reported, where CD63 is known to form complexes with integrins. The precise route and function for the internalisation of amelogenin aggregates still needs further attention.

The primary amelogenin protein can be enzymatically cleaved to a variety of smaller proteins with molecular masses down to a fifth of the primary protein as discussed in further detail in the introduction. One of these proteins, 5 kDa TRAP, has been reported to affect gene expression in cementoblasts in vitro. The studies in paper I therefore attempted to investigate whether TRAP accounted for the elevated levels of cytokines released from the cultured macrophages. However, TRAP only increased VEGF levels, while the other 26 cytokines that were analysed were unaffected. The possible involvement of other amelogenin peptides present in low concentrations in the mixture, e.g. LRAP cannot be precluded. LRAP has been shown to have cell signalling activities.

There are several reports demonstrating upregulation of TGF-β1, bone morphogenetic proteins (BMPs) and bone sialoprotein (BSP) in different cell types by amelogenins. The possible presence of BSP-like or TGF-β-like molecules, including BMPs, which are part of the TGF superfamily, in the amelogenin mixture has been reported. However, several cytokines including TGF-β, could not be detected in the amelogenin mixture in an earlier study by Gestrelius et al. Hence, the results are elusive and need to be resolved.
The interaction and cellular uptake of amelogenin by human macrophages and dermal fibroblasts and the involvement of integrin-dependent adhesion of dermal fibroblasts and endothelial cells demonstrated in the present thesis imply that cellular attachment to the protein seems to be a crucial factor. Importantly, this indicates that amelogenins do function as a temporary matrix for cell adhesion.

After discussing plausible routes for cellular interactions with amelogenins, the next interesting question is how this affects the in vitro cell behaviour in regards to tissue repair. Macrophages, fibroblasts and endothelial cells are all essential in both promoting and regulating wound healing. Albeit, there are differences in cellular response to the amelogenin treatment, the overall results implicate that amelogenins stimulate a cell phenotype with tissue repair characteristics in all three cell types studied here.

5.3 Amelogenins and macrophages

In chronic inflammatory lesions, the rigidly controlled repair process is locked in the late inflammatory stage and healing only proceeds if the inflammation is attenuated.\textsuperscript{35-37} Treatment strategies to reduce inflammation, combined with the careful control of infection, are thus regarded as beneficial to healing.

In the present in vitro studies, firstly the effect of amelogenins on the cytokine release was studied in non-stimulated macrophages to recognise the response of macrophages when in contact with the amelogenins (paper I). The study resulted in elevated levels of multiple pro- and anti-inflammatory cytokines over 72 h. Also increased VEGF levels in the macrophage culture medium were demonstrated, in accordance to previous findings.\textsuperscript{207,208,213} In addition, amelogenins markedly augmented the production of AMAC-1. AMAC-1 is a specific marker for alternatively activated macrophages.\textsuperscript{251} The large increase in AMAC-1 and VEGF levels indicates macrophage activation towards a reparative phenotype. The release of cytokines seemed to be independent on phagocytosis of the amelogenin aggregates as discussed earlier. These results demonstrate the wide-ranging activation of previously non-stimulated macrophages when in contact with the amelogenin proteins.

The next step was to study the effects of amelogenins on LPS-stimulated macrophages (paper II) over time to evaluate the possible immunomodulatory effect of amelogenins in vitro. As with the non-stimulated macrophages, amelogenin treatment resulted in elevated expression of AMAC-1, indicating a re-orientation of the cultured macrophages from classical activation to an alternatively activated cell. Differentiation from a classically activated macrophage with a Th1 phenotype to an alternatively Th2-immuno-responsive macrophage has been well described (see introduction).\textsuperscript{20,251-253} Consistent with alternative activation,\textsuperscript{9,24,25,252,254,255} the study showed the increased release of IL-1RA, IL-6 and IL-10 and the growth factors IGF-1 and VEGF, along with reduced expression of the classical pro-inflammatory cytokines TNF-α, IL-8, IL-12 and MCP-1, confirming a switch in macrophage phenotype upon amelogenin treatment. This is supported by previous findings on decreased TNF-α and IL-8 levels after addition of
amelogenin mixture to human whole blood and rat macrophages.216,217 Thus, amelogenins may have the capacity to mediate a re-polarisation of the macrophage phenotype. A switch in macrophage phenotype may explain the findings after amelogenin treatment (Emdogain®) in a murine model of periodontitis where the possible involvement of macrophages has been discussed.296

Importantly, when comparing paper I and II, the cytokine levels in non-stimulated macrophages after amelogenin treatment were generally relatively low when compared to the response by macrophages to the inflammatory stimulus of LPS. In contrast, the levels of AMAC-1 were independent on the LPS stimuli, implicating that the secretion of this marker was due to the amelogenins and not e.g. to autocrine secretion after LPS-stimulation.

It has been suggested that re-polarisation of the macrophage activation state might be a fruitful path to modulate inflammation during wound healing.29 Thus, the observed changes in macrophage cytokine expression upon amelogenin treatment reveal anti-inflammatory properties of the protein and a plausible switch of the macrophage phenotype from a classically to an alternatively activated cell, with tissue repair characteristics is a possible therapeutic mechanism.

5.4 Amelogenins and fibroblasts

Fibroblasts are central in wound healing by secreting and remodelling ECM components and expressing important cell mediators to regulate cellular processes in e.g. inflammation, epithelialisation and angiogenesis.3,79,81 The chronically inflamed environment in hard-to-heal wounds is detrimental to fibroblast function and with time, cellular functions as cell proliferation and response to growth factors, diminish.88,97,99,257,258 Further, fibroblasts isolated from hard-to-heal ulcers display impaired ECM remodelling, presumably due to incapacitated migratory activity.102 This results in cellular senescence, a hallmark of hard-to-heal ulcers.97,99,259 The examined human CRL-7815 fibroblast cell line exhibited characteristics of fibroblasts cultured from non-healing chronic venous leg ulcers,97,98,100-102,260 such as an enlarged flattened morphology, cytokine and collagen secretion, matrix remodelling, as well as the gradual loss of proliferation resulting in reduced saturation density during culture.

A major finding in paper IV was the pronounced effect of amelogenin mixture on the increased proliferation of this near-senescent cell line to the level of normal fibroblasts. Amelogenins also increased the growth of the normal dermal fibroblasts studied in paper III, supported by previous findings.207,208 There are several possible mechanisms behind the ceased proliferation of CRL-7815 fibroblasts and the restoration of cell growth with amelogenins. Firstly, the CRL-7815 cells are reported to have impaired mitogenic response to EGF, due to reduced numbers of EGFR receptors (EGFR).261 Interestingly, Matsuda et al. showed that the proliferative effect of both EGF and amelogenins in serum-starved PDL cells involved phosphorylation of extracellular signal-regulated kinases 1/2 (ERK 1/2).164 The induced mitogenesis by amelogenins activated the same intracellular signalling pathway as EGF, but without the involvement of EGFR.164 Thus, amelogenins may have compensated for deficient EGFR in the CRL-7815 cells, by
activating the ERK 1/2 pathway. In addition, amelogenins stimulate proliferation of gingival fibroblasts via ERK.\textsuperscript{179} Secondly, as indicated previously,\textsuperscript{262,263} amelogenins possibly inhibited the increased apoptosis in fibroblasts found with cellular senescence.\textsuperscript{264} Where elevated TNF-α levels has been reported as a factor in accelerated cellular senescence.\textsuperscript{287} Interestingly, protection against TNF-α-induced apoptosis in gingival fibroblasts has been suggested.\textsuperscript{263} In the present thesis, TNF-α was not detected in the conditioned media in agreement with Fahey \textit{et al.}\textsuperscript{32} Finally, a synergistic effect of amelogenins and PDGF-BB, an important fibroblast mitogen in serum,\textsuperscript{265} was observed in PDL fibroblasts.\textsuperscript{178} An appropriate ECM composition stimulating cell adhesion is a prerequisite for maximal growth factor stimulation.\textsuperscript{44,266} The delayed proliferative response after addition of amelogenins in the present studies could be due to autocrine growth factor induction.\textsuperscript{165}

Fibroblasts are important sources of modulators of the innate immunity.\textsuperscript{31,32} The augmented secretion of MCP-1 by the CRL-7815 fibroblasts in comparison to the normal fibroblasts was considerably inhibited by amelogenins. Further, the low IL-8 secretion in near-senescent fibroblasts was augmented to the levels of normal fibroblasts after treatment of amelogenin mixture. IL-8 preferentially attracts neutrophils and is thought to be a regulatory molecule of acute inflammatory reactions.\textsuperscript{287} In contrast, MCP-1 is primarily chemotactic to monocytes and macrophages and contributes to chronic inflammatory cell infiltration and possibly to delayed ulcer healing.\textsuperscript{33,268,269} Whether this mechanism is operative in vivo is unknown. Nonetheless, these findings imply, hypothetically, that amelogenin could indirectly via cytokine modulation switch the chronic mononuclear cell infiltrate into a more acute-like cell composition in hard-to-heal wounds, although the exact role of IL-8 in wound healing remains elusive.\textsuperscript{267,270}

The constitutive expression of the pro-angiogenic growth factor VEGF by the CRL-7815 cells was more than four-fold greater than by normal dermal fibroblasts, in accordance with a previous study on senescent lung fibroblasts.\textsuperscript{271} The VEGF secretion was further increased in the presence of amelogenins in accordance to normal human fibroblasts.\textsuperscript{287} Because of augmented VEGF levels in tissue and wound fluids from non-healing compared with healing venous leg ulcers\textsuperscript{272} the clinical consequence of elevating already high VEGF secretion is unclear. However, the inhibitory soluble VEGFR-1 receptor, has also been seen to be markedly increased in wound fluids in venous leg ulcers, compared to acute excisional wounds.\textsuperscript{273}

As compared to the VEGF results, the constitutive type I collagen production was twice as high in the CRL-7815 compared to the normal dermal fibroblasts. Brem \textit{et al.} reported increased type I collagen synthesis by ulcer-edge fibroblasts contributing to dermal fibrosis.\textsuperscript{101} Amelogenins only decreased collagen synthesis at 100 μg/ml after 72 h cultivation. Related studies on normal fibroblasts also resulted in a slight decrease in collagen production (unpublished data), but in contrast, amelogenins induced a significant increase in gene expression for some ECM proteins, e.g. several collagens and fibronectin (paper III). Studies in PDL cells have shown an increased radiolabelled proline incorporation after 24 h of amelogenin mixture treatment.\textsuperscript{179} Thus, the influence of amelogenins on collagen production could possibly be dependent on cell type and activity.

The impaired remodelling of type I collagen matrix found with the near-senescent
compared with normal fibroblasts was improved with addition of amelogenin mixture. The matrix remodelling impairment in the CRL-7815 was not dependent on cell proliferation. Instead, the normal fibroblasts developed prominent cell extensions interacting with the collagen fibrils resembling a pro-migratory phenotype274,275 as opposed to more rounded CRL-7815 fibroblasts with retracted protrusions. These findings indicate a decreased migratory ability of the CRL-7815 fibroblasts; also found previously for fibroblasts isolated from venous leg ulcers.100-102

The findings on the near-senescent cells were supported by an up-regulation of numerous genes involved to a large extent in cellular growth, migration and differentiation in normal dermal fibroblasts after treatment with amelogenins. These results are in accordance with observations on human periodontal ligament fibroblasts165,276-278 and on primary human osteoblasts.196 Although increased integrin adhesion was shown in paper III as discussed earlier, no significant changes in integrin gene expression in relation to amelogenin mixture addition were detected, This suggests that these factors are mainly regulated at a higher cellular level and/or is part of a secondary response not detectable in the experimental set up.

In conclusion, amelogenins seem to influence key pathways in connective tissue repair and regeneration. Further, the characteristics of the near-senescent fibroblast cell line may be a useful model cell for in vitro studies on cellular senescence in relation to hard-to-heal wounds. Amelogenins demonstrated a capacity to switch chronic fibroblasts into an acute-like phenotype.

5.5 Amelogenins and human dermal microvascular endothelial cells

Angiogenesis is crucial in the wound healing cascade and is often impaired in hard-to-heal ulcers due to e.g. excessive degradation of essential ECM components.199,200 The present investigation has shown that the amelogenin mixture enhances several cellular processes involved in angiogenesis.

The angiogenic effect of amelogenins was recognized in an ex vivo chick aortic arch assay, showing increased vessel outgrowth. This was in accordance with earlier studies on vessel formation in vitro and in vivo.209-212 Angiogenesis involves basement membrane disruption, cell migration, proliferation, and tubule formation.281 This angiogenic process requires changes in cell adhesion, which are mediated by specific integrins.43,64 In agreement with promoted sprouting, amelogenins increased integrin expression on cultured human dermal microvascular endothelial cells. Amelogenin mixture induced cell adhesion via multiple angiogenesis-associated subunits and integrins, including αvβ3, αvβ5 and α5β1. This result was comparable to the positive control, fibronectin. Fibronectin is absent in mature blood vessels, but when introduced, e.g. after injury, it triggers a broad range of integrins, like αvβ3, αvβ5 and α5β1, that regulate endothelial cell growth, survival, and migration.50,282

The ex vivo model of vessel outgrowth involves both cell proliferation and migration of endothelial cells. Earlier studies showed that amelogenins stimulate chemotaxis and migration of human umbilical vascular endothelial cells (HUVEC).210,214 implicating integrin-dependent mechanisms. The integrin αvβ3 has been localized abundantly on the
tips of newly formed vessels in granulation tissue, while being minimally expressed on mature blood vessels.\textsuperscript{282-284} While the subunits αv, α5 and β1, have proven vital for angiogenesis in transgenic mice models, the β3 subunit has not.\textsuperscript{285-288} These results implicate the importance of the αv subunit alone, which thus may explain the lack of adherent cells through the β3 subunit, considering the found up-regulation of αv and αvβ3 on the cell surface in the present integrin-adhesion study.

Degradation and remodelling of the ECM microenvironment is decisive for integrin-mediated cell adhesion and migration and a balanced expression of MMPs enables cells to move.\textsuperscript{289,290} The interaction between endothelial cells and the ECM changes by cleavage of existing ECM proteins, e.g. MMP-2 cleavage of collagen I leads to exposure of an RGD-site for αvβ3.\textsuperscript{291} It has previously been shown that amelogenins increase MMP-2 secretion from human dermal microvascular endothelial cells.\textsuperscript{297} Thus, endothelial cell migration is possibly facilitated by elevating MMP-2, which associates with αvβ3.\textsuperscript{292} Interestingly, specific blockage of the MMP-2-αvβ3 interaction profoundly reduced the angiogenic response in a chorio-allantoic membrane assay.\textsuperscript{292} Further, Bertl et al. reported increased mRNA levels of angiopoietin-2 after treatment with amelogenins,\textsuperscript{214} a factor up-regulated during angiogenesis and suggested to destabilise existing vessels, leading to a more plastic state during angiogenesis and inflammation.\textsuperscript{293}

In contrast, the amelogenin mixture at 1,000 μg/ml decreased microvessel formation despite being mitogenic at this concentration, in concurrence with reduced integrin-mediated adhesion, suggesting impeded migration. Decreased migration at higher concentrations of amelogenin has also been observed by other investigators,\textsuperscript{214} which could be attributed to reduced adhesion via integrins by multiple mechanisms. The amelogenin mixture possibly increased integrin internalisation, either directly or indirectly via up-regulation of e.g. angiopoietin-2.\textsuperscript{214,294} As discussed earlier, endothelial cells are sensitive to different ECM densities,\textsuperscript{230-232} thus the high concentration of amelogenins may induce cell proliferation. Previous findings on endothelial cell growth in response to amelogenin are indecisive and dependent on the experimental design.\textsuperscript{198,207,210,213-215} Further, the highest amelogenin mixture concentration previously studied on cultured endothelial cells is 250 μg/ml corresponding to 62.5 μg/cm\textsuperscript{2},\textsuperscript{207,210,214,215} while the concentration applied in clinical practise is equivalent to 1,500 μg/cm\textsuperscript{2}.\textsuperscript{199,200} Thus, the study in paper V also included a higher concentration in closer relation to the clinical situation and the mitogenesis study was performed on the physiological substrate Matrigel. Here, the proliferation assay in serum-free medium resulted in increased proliferation by 1,000 μg/ml amelogenin mixture, while the lower concentrations resulted in no effect or decreased cell proliferation after 48 h. This is in accordance with previous studies at low serum levels, where unaffected or decreased proliferation has been reported after longer incubation than three days.\textsuperscript{207,210} Augmented cell proliferation was only found with low amelogenin mixture concentrations in serum-enriched culture medium.\textsuperscript{213} Bertl et al. even observed cytotoxic effects on human umbilical vein endothelial cells when exposed to an amelogenin mixture exceeding concentrations of 50 μg/ml.\textsuperscript{214} Moreover, Schlueter et al. speculated that amelogenins at high concentrations induce apoptosis.\textsuperscript{213} No influence on the apoptotic rate in the cultured HDMEC after treatment with amelogenins was detected here that could explain the concentration-dependent effect seen both in
*vitro* and *ex vivo*. The Matrigel coating utilized has adhesion properties that seem to prevent apoptosis. This may explain the in general low level of apoptosis compared with that for cells cultured on uncoated tissue culture wells.

It is important to emphasise that only the very early events in the formation of new blood vessels have been studied in this thesis. Maturation into distinct, functional blood vessels requires the participation of surrounding mesenchymal cells. Previous studies on fibroblasts show that amelogenin mixture increases the expression of VEGF and TGF-β1, factors important for endothelial cell proliferation and ECM stabilisation. Also, topical application of recombinant amelogenin has been reported to promote the recruitment of mesenchymal progenitor cells associated with neovascularisation *in vivo*. Thus, the present *in vitro* observations suggest that the angiogenic effects of the amelogenin mixture are, at least partly, due to increased integrin-mediated adhesion and migration by endothelial cells.
6. SUMMARY AND CONCLUSIONS

There is one thing stronger than all the armies in the world; and that is an idea whose time has come.

Victor Hugo (1802-1885)
The present thesis was based on the hypothesis that amelogenins stimulate wound healing by providing cells with a temporary extracellular matrix for cell adhesion, a prerequisite for tissue repair. This research project showed that amelogenins influence the in vitro cell behaviour of all three human cell types studied, macrophages, fibroblasts and endothelial cells. Ultrastructural studies revealed the interaction and uptake of amelogenin aggregates for both macrophages and fibroblasts. In addition, the possible involvement of integrin-dependent adhesion was demonstrated for fibroblasts and endothelial cells, with increased cell binding via multiple subunits and integrins, including αvβ3, αvβ5 and α5β1.

Amelogenin treatment of cultured human peripheral blood monocyte-derived macrophages resulted in anti-inflammatory effects, measured by cytokine secretion. In particular, an induced secretion of VEGF and the specific marker of alternative macrophage activation AMAC-1 was demonstrated. These results suggest an amelogenin-promoted switch of the macrophage phenotype to an alternatively activated cell, with tissue repair characteristics.

Observations of a near-senescent human cell line suggested that the cell line shares functional anomalies with fibroblasts isolated from non-healing cutaneous wounds. Amelogenins had the capacity to restore an acute-like phenotype in near-senescent fibroblasts, with regards to cell proliferation, collagen matrix remodelling and secretion of IL-8 and MCP-1. Furthermore, amelogenin mixture increased cell proliferation and induced expression of genes involved in cellular growth, migration and differentiation in normal dermal fibroblasts. Finally, amelogenins displayed pro-angiogenic properties both in vitro and ex vivo.

In conclusion, the effects of amelogenin on wound healing are, at least partly, conducted by providing macrophages, fibroblasts, and endothelial cells with tissue repair characteristics. These effects are probably mediated via cell-integrin interactions.
7. TOPICS FOR FUTURE STUDIES

*If you can dream it, you can do it*

Walt Disney (1901-1966)
The present study presents initial support for an induction of cellular response with tissue repair characteristics by a self-assembling biomaterial, plausibly conducted by virtue of its cell adhesive capabilities. However, there are still many questions as to the mechanisms of action of amelogenins. During the interactions between amelogenin aggregates and the cell membrane, the role of aggregate size and shape for the cell response is an intriguing question. Therefore, an interesting approach for future studies would be to address the question of the protein versus the aggregate involvement in modulation of cell behaviour, as well as the size of amelogenin aggregates. Also, the cellular uptake of the protein aggregates would be interesting to study in more detail, e.g. the intracellular fate of the internalised aggregates. It would also be interesting to extend the integrin studies with the near-senescent cells, e.g. to explore if there are differences in the basal cell surface expression of integrins in these cells and normal fibroblasts and if amelogenins would increase the integrin-mediated adhesion as indicated in the thesis. Also, comparative studies of amelogenin mixture and recombinant amelogenin would help to further clarify the mechanisms of action.

It would also be interesting to use co-cultures of cells both in monolayers and in 3D matrices or skin equivalents to further understand both the direct and indirect effects of the amelogenin aggregates. The integrin-dependent binding to the amelogenin aggregates and the secretion of cytokines and growth factors would be topics for further studies using such models. As a next step, although the use of in vitro models offers controlled conditions to study cell-protein interactions, it would be interesting to investigate several of the findings in vivo, e.g. to study the repolarisation of macrophage phenotype and the restoration of an acute-like phenotype in near-senescent fibroblasts. The use of more complex model systems would help to further understand the influence of amelogenins on inflammation in vivo. However, there are difficulties in finding appropriate in vivo models for the studies of chronic, non-healing wounds and the heterogeneous environment within a wound would make it hard to delineate the actual effects on single cell basis. Thus, there is a need for reproducible model systems to study the events of hard-to-heal wounds, both in vitro and in vivo. Here, we have suggested the use of a near-senescent fibroblast cell line both in monolayer and 3D collagen matrix, an approach that could be extended to more complex model systems.
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*If you have knowledge, let others light their candles in it.*

Margaret Fuller (1810-1850)
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