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Amelogenin is phagocytized and induces changes in integrin configuration, gene expression and proliferation of cultured normal human dermal fibroblasts

Sofia Almqvist · Maria Werthén · Anna Johansson · Magnus S. Ågren · Peter Thomsen · S. Petter Lyngstadaas

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Abstract Fibroblasts are central in wound healing by expressing important mediators and producing and remodelling extracellular matrix (ECM) components. This study aimed at elucidating possible mechanisms of action of the ECM protein amelogenin on normal human dermal fibroblasts (NHDF). Amelogenin at 100 and 1000 μg/ml increased binding of NHDF via several integrins, including $\alpha v\beta 3$, $\alpha v\beta 5$ and $\alpha 5\beta 1$. Further, both surface interaction and cellular uptake of amelogenin by NHDF was observed using scanning and transmission electron microscopy. Gene microarray studies showed >8-fold up or down-regulation of genes, of which most are involved in cellular growth, migration and differentiation. The effect of amelogenin was exemplified by increased proliferation over 7 days. In conclusion, the beneficial effects of amelogenin on wound healing are possibly conducted by stimulating fibroblast signalling, proliferation and migration via integrin interactions. It is hypothesized that amelogenin stimulates wound healing by providing connective tissue cells with a temporary extracellular matrix.

S. Almqvist (✉) · M. Werthén · A. Johansson · P. Thomsen
Department of Biomaterials, Sahlgrenska Academy at University of Gothenburg, Box 412, 405 30 Göteborg, Sweden
e-mail: sofia.almqvist@biomaterials.gu.se

S. Almqvist · M. Werthén · A. Johansson · P. Thomsen
Institute of Biomaterials and Cell Therapy, Göteborg, Sweden

S. Almqvist · M. Werthén
Mölnlycke Health Care AB, Göteborg, Sweden

A. Johansson · P. Thomsen
BIOMATCELL VINN Excellence Center of Biomaterials and Cell Therapy, Göteborg, Sweden

M. S. Ågren
Department of Surgery K and Copenhagen Wound Healing Center, Bispebjerg Hospital, Copenhagen, Denmark

S. P. Lyngstadaas
Department of Biomaterials, Institute for Clinical Dentistry, University of Oslo, Oslo, Norway

1 Introduction

Tissue repair involves the coordinate actions of different cells, cytokines, growth factors and extracellular matrix (ECM). Fibroblasts are central in wound healing by secreting and remodelling ECM components and by providing paracrine stimulation of cellular processes in e.g. inflammation, epithelialization and angiogenesis [1–3].

Amelogenin is the principal component of enamel matrix proteins [4]. The 20 kDa ECM protein has an amino acid sequence thought to inhibit formation of ordinary secondary structures, instead enabling the proteins to self-assemble into nanospheres which under physiological conditions aggregate into micrometer sized structures [5, 6]. As a resorbable biomaterial for induction of tissue regeneration, amelogenin enhances regrowth of tooth supporting tissues in conjunction with surgical treatment [7–11] and is beneficial in the treatment of chronic venous leg ulcers of long duration [12, 13], although the mechanism is not yet elucidated. Preclinically, amelogenin converted near-senescent fibroblasts into an acute-like phenotype [14], stimulated angiogenesis [15, 16], and accelerated granulation tissue formation and wound closure in a rabbit model [17]. Further, the promotion of adhesion, migration and proliferation of periodontal ligament (PDL), gingival and dermal fibroblasts as well as their synthesis of ECM molecules, cytokine expression and up-regulation of

second messengers as cAMP and alkaline phosphatase has been demonstrated in vitro [14, 17–26].

The mechanisms of action of amelogenin remain to be delineated. Amelogenin has been reported to be taken up by osteoblasts by clathrin-coated pits [25] and the involvement of integrin dependent adhesion between amelogenin and both PDL and gingival fibroblasts has been reported [22, 27, 28]. However, the interactions between dermal fibroblasts and amelogenin have yet to be investigated.

In the present study the effect of amelogenin on cell surface integrin expression, gene expression (microarray analysis) and cell proliferation in normal human dermal fibroblasts was analyzed. Furthermore, ultrastructural studies on cellular attachment to and uptake of amelogenin aggregates were carried out.

2 Materials and methods

2.1 Amelogenin

Amelogenin was extracted and purified from the enamel matrix of developing porcine teeth [19]. Lyophilized amelogenin was produced by Biora-Straumann (Malmö, Sweden) and dissolved in 17 mM acetic acid to provide a 10 mg/ml stock solution.

2.2 Cell culture conditions

Normal human dermal fibroblasts (NHDF; Karocell Tissue Engineering AB, Stockholm, Sweden) were cultured at 37°C in humidified 5% CO_2/air in Dulbecco's Modified Eagle's Medium (Invitrogen, Paisley, UK) containing 10% heat-inactivated EU-approved foetal bovine serum (Invitrogen), 100 units/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml Fungizone®. Culture medium was changed thrice weekly. At 90–95% confluence, cells were washed with PBS (Invitrogen), detached using 0.25% trypsin in 1 mM EDTA (Invitrogen) and split at a ratio of 1:3. NHDF between passages 5 and 11 were used for the studies.

2.3 Integrin assay

The cell surface subunit (α1, α2, α3, α4, α5, αv, β1, β2, β3, β4, β6) or heterodimer (αvβ3, αvβ5 and α5β1) integrins were quantified using an integrin-mediated cell adhesion array (Millipore, Billerica, MA, USA). Briefly, NHDF were trypsinized and seeded at 20000 cells/cm^2 in T-75 culture flasks with medium alone or supplemented with amelogenin (100 or 1000 µg/ml). Fibronectin derived from human plasma (F2006, Sigma–Aldrich, St. Louis, MO, USA) at 20 µg/ml served as a positive adhesion protein control with concentration based on required amount for monolayer coverage [29, 30]. 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 10^6 cells/ml and aliquoted (100 µl/well) to 96-well plates and allowed to adhere for 2 h at 37°C. Thereafter, unbound cells were washed off and the remaining adherent cells stained with crystal violet for 5 min at room temperature. The stain was then extracted for 5 min at room temperature and the optical density of extracts 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.

2.4 Electron microscopy studies

The NHDF cells were seeded at 20000 cells/cm^2 on Thermanox™ coverslips (Nunc A/S, Roskilde, Denmark) in 24-well plates and cultured for 24 h at 37°C with 5% CO_2 and 95% humidity. The supernatants were discarded, including non-adherent cells and fresh medium alone (control) or medium containing amelogenin (100 and 1000 µg/ml) 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 (pH 7.2) for 2 h at room temperature.

2.4.1 SEM preparation

The cells were washed with 0.15 M sodium cacodylate buffer (pH 7.2) and post-fixated with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (pH 7.2) 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 [31]. Dehydration was performed with a series of ethanol solutions ranging from 70–99.5% and critical point-drying by hexamethyldisilizane evaporation. The specimens were mounted on stubs and sputtered with palladium. The samples were analyzed using SEM (LEO 982 Gemini; Zeiss, Oberkochen, Germany) operated at 3 kV.

2.4.2 TEM preparation

The cells were washed with 0.15 M sodium cacodylate buffer (pH 7.2) 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 70–99.5% 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 polymerization 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 cupper 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).

2.5 Gene microarray

For these studies NHDF were seeded at lower density (10000 cells/cm^2) to avoid confluence in T-75 culture flasks and cultured for 24 h at 37°C with 5% CO_2 and 95% humidity. The supernatants were discarded and fresh medium containing 1000 µg/ml amelogenin was added to triplicate flasks. Control comprised of 1.7 mM acetic acid (solvent control). The effects of 1000 µg/ml amelogenin on gene expression in NHDF were studied after 48 h and 72 h. RNA was isolated and purified 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 Swegene (Lund, Sweden). Briefly, RNA integrity and amount were analyzed by Agilent 2100 bioanalyzer RNA LabChip® kit. Total RNA was processed by the GeneChip® Expression 3′-Amplification Reagents One-cycle cDNA synthesis (Affymetrix Inc. Santa Clara, CA, USA). Biotin labelled cDNA was fragmented and hybridized onto 12 GeneChip® Human Genome 1.0 ST Arrays (Affymetrix Inc.) overnight, with three chips for both amelogenin treatment and control at each time point. The arrays were washed and stained. Scanning was carried out with the GeneChip® Scanner 3000 and image analysis was performed using GeneChip® Operating Software. The Affymetrix MAS 5.0 software processed the data sets, and signal values representing the expression level of each transcript were generated. Genes were only considered up or down-regulated if a more than threefold change (log change >1.6) between test and control was observed; if the false discovery rates (Q-values) for these genes were below 17%; and finally, only if less than 3/4 of the approximately 26 probes for each particular gene were marked "absent" by the Affymetrix MAS 5.0 software.

Pathway analysis and bio-function analysis were performed using the Ingenuity Pathway Analysis (IPA) 7.4 software (Ingenuity Systems, Redwood City, CA, USA) using Affymetrix gene expression values with an eightfold change (log change >3.0) or more between test and solvent control as significance level. Results were sorted according to the number of genes involved in each pathway.

2.6 Proliferation studies

NHDF at 1000 cells/well were allowed to attach for 24 h in 96-well tissue culture plates. The medium was then exchanged with fresh medium alone (control) or medium containing amelogenin (100 and 1000 µg/ml). Cells were cultured for up to 7 days. At indicated time points, cells were permeabilized and stabilized before being stained with propidium iodide in the NucleoCassette™ and counted by the NucleoCounter® (ChemoMetec, Allerød, Denmark) according to the manufacturer's protocol.

2.7 Statistics

The ANOVA-test followed by the Tukey test was applied to compare integrin expression between different treatments, using the StatView 5.0 software. Differences were considered statistically significant at P values <0.05. In the proliferation experiment, the number of fibroblasts days 1, 2, 3, 4, 5, 6 and 7 was plotted against time of incubation as abscissa. Area under the curve (AUC) was calculated using the trapezoid method between day 1 and day 7 using the FigSys 2.4.3 software (Biosoft, Cambridge, UK). ANOVA followed by the Tukey test was then used to compare the AUC values between the amelogenin treatment and control.

3 Results and discussion

3.1 Integrin assay

Previous studies have suggested integrin-mediated adhesion through $\beta 1$ and $\alpha v \beta 3$ of PDL and gingival fibroblasts on amelogenin coated surfaces [22, 27, 28]. In the present study, the regulation of integrin activity on NHDF upon ECM protein interaction was analyzed after 24 h incubation with amelogenin (100 and 1000 µg/ml) and fibronectin (20 µg/ml) and the results were compared to medium control. The binding capacity of the subunits $\alpha 1$, $\alpha 2$, $\alpha 3$, $\alpha 5$, αv, $\beta 1$, $\beta 2$ (Table 1) and heterodimers $\alpha v \beta 3$, $\alpha v \beta 5$ and $\alpha 5 \beta 1$ (Fig. 1) were significantly increased with 1000 µg/ml amelogenin compared to control. Amelogenin at 100 µg/ml showed no statistically significant effect on integrin expression. The subunits $\beta 3$, $\beta 4$ and $\beta 6$ were barely detectable on the NHDF in culture, irrespective of treatment. These results were comparable to fibronectin treatment, an ECM protein involved in several phases of tissue repair, influencing cell growth [32]. Recent studies suggest that several integrin heterodimers have a natural ability to mediate phagocytosis as an extension of their adhesive capacity. This is true for several integrins mediating binding to ECM components such as fibronectin [33].

Table 1 Quantification of alpha (α) and beta (β) integrin subunits on NHDF after 24 h treatment with amelogenin and fibronectin expressed in percent of medium control

Treatment	α1	α2	α3	α4	α5	αv	β1	β2
Amelogenin (100 μg/ml)	103 ± 8	126 ± 7	103 ± 6	104 ± 8	117 ± 8	124 ± 11	104 ± 11	108 ± 12
Amelogenin (1000 μg/ml)	188 ± 10***	231 ± 25***	174 ± 9***	119 ± 7	199 ± 8***	214 ± 14***	183 ± 9***	164 ± 16**
Fibronectin (20 μg/ml)	153 ± 4***	172 ± 6***	167 ± 8***	193 ± 7***	180 ± 6***	187 ± 15***	133 ± 6*	136 ± 8

Mean ± SEM ($n = 6$)

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

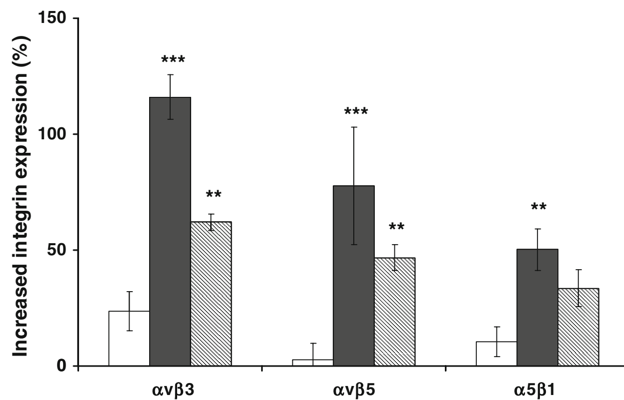

Fig. 1 The increased expression of integrin heterodimers on NHDF after 24 h cultivation with amelogenin at 100 μg/ml (*open*), 1000 μg/ml (*filled*) and 20 μg/ml fibronectin (*hatched*) expressed in percent compared to medium control. Mean ± SEM ($n = 6$), ** $P < 0.01$, *** $P < 0.001$

3.2 Electron microscopy studies

The interaction between NHDF and amelogenin aggregates was visualized with SEM and TEM after 24 h incubation. Thin extensions from the cell membrane were observed, adhering to amelogenin either on the plastic surface or to spheres on top of the cells (Figs. 2a, b and 3a). TEM sections also displayed a condensation of the cell membrane, visualized by a relatively increased electron-dense staining of the plasma membrane in areas of close proximity to amelogenin (Fig. 3b).

Further, cellular uptake of amelogenin was visible for NHDF in the SEM micrographs, shown both with membrane-enclosed spheres (Fig. 2d) as well as spherical protrusions of the cell surface (Fig. 2c), having similar size and shape as amelogenin spheres and most likely representing internalized protein spheres. Aggregates of the protein were

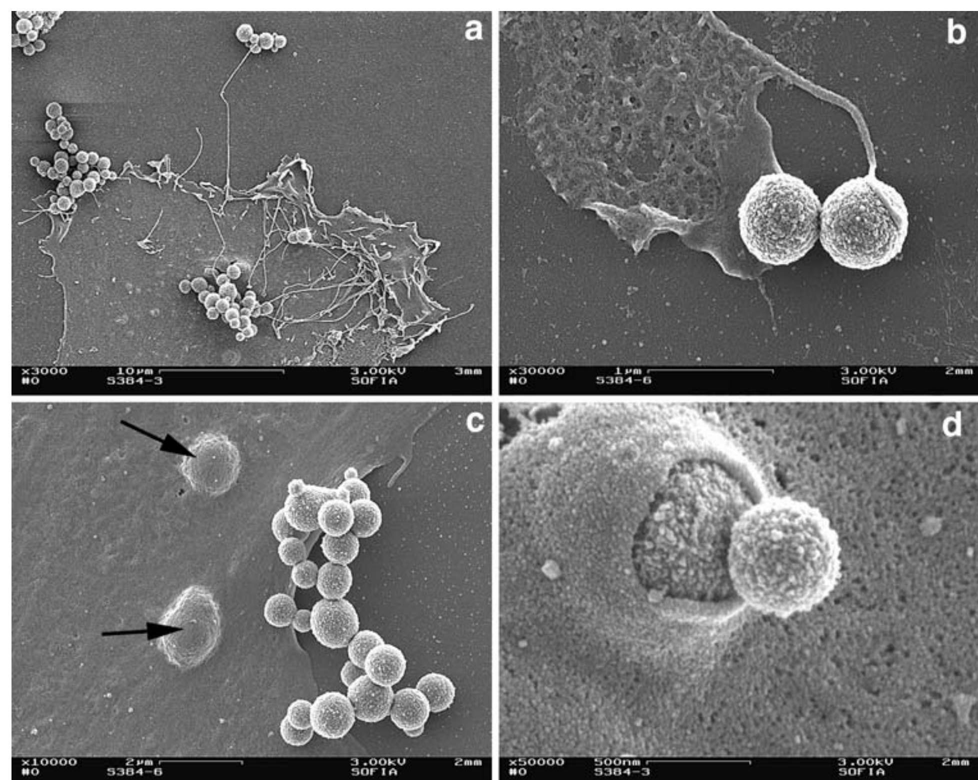

Fig. 2 SEM micrographs displaying the interaction of NHDF with amelogenin, showing extensions adhering to the protein at **a** 3000 and **b** 30,000 times magnification after 24 h incubation and also displaying possible internalization of amelogenin of NHDF, showing **c** protrusions (*arrows*) under the cell membrane in similar size and shape as amelogenin spheres, and **d** possible uptake of amelogenin after 24 h incubation, showing a larger sphere that is partially enveloped by the cell membrane and a relatively smaller sphere

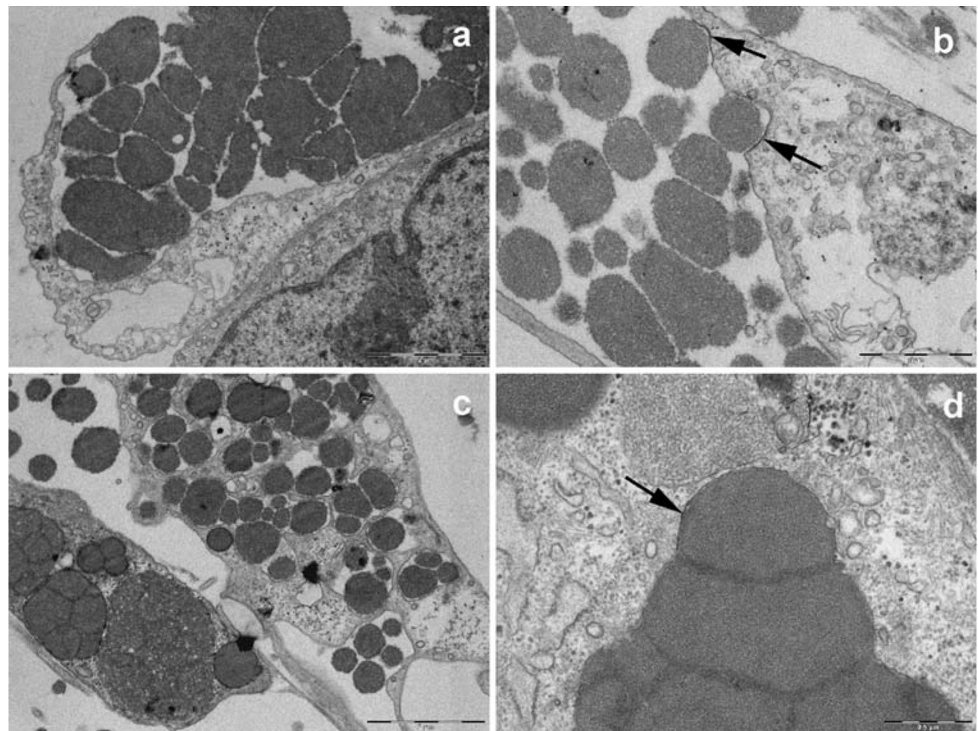

Fig. 3 TEM micrographs displaying NHDF after 24 h treatment with amelogenin, showing **a** extension adhering to the protein (scale bar = 2 μm) and **b** condensations (*arrows*) of the membrane in close proximity to amelogenin (scale bar = 1 μm). Also, details of the internalization of amelogenin with NHDF after 24 h incubation, showing **c** the internalized amelogenin (scale bar = 2 μm) and **d** a thin membrane (*arrow*) surrounding the densely packed spheres (scale bar = 0.5 μm)

present where amelogenin displayed structures typically between 0.5 and 1 μm (Fig. 2d). The uptake of amelogenin was verified in TEM, occurring in high degree for many cells in microscopic sections (Fig. 3c). A thin membrane was observed surrounding the internalized amelogenin, in which the enclosed protein was found to be both tightly packed or having a less dense structure (Fig. 3c, d).

Fibroblasts remodel the surrounding ECM partly by phagocytosis and ingest both collagen and fibronectin-coated beads in the size-range of amelogenin spheres in vitro [34–36]. Thus, amelogenin could also be subject to endocytosis for this intent. The cellular uptake of amelogenin has previously been detected in osteoblasts through clathrin-coated pits [25]. Interactions between amelogenin and the transmembrane proteins LAMP1 and CD63, involved in endocytosis have also been reported, where CD63 is known to form complexes with integrins [37]. Thus, the found interaction and uptake of amelogenin by human dermal fibroblasts demonstrated here could be part of the remodelling activity of fibroblasts and probably involve integrin interactions.

3.3 Gene microarray

The gene microarray studies showed that amelogenin treatment of normal dermal fibroblasts caused more than threefold up or down-regulation (log change >1.6) of 856 genes out of the 28,869 genes represented in the Affymetrix GeneChip® Human Gene 1.0 ST Array. Of these, 507 genes were regulated after 48 h and 676 genes after 72 h. A total of 327 of the involved genes showed similar regulation at both time points, whereas 180 genes were uniquely regulated after 48 h and 349 genes were only regulated after 72 h.

An eightfold regulation (log change >3.0) was observed for 486 genes of which most are involved in cellular growth, migration, survival and differentiation (Table 2). Several of these genes are also involved in canonical pathways, especially in the hepatic fibrosis pathway and IL-6 and FGF signalling (Table 3). The microarray studies displayed no changes in integrin gene expression above the threefold cut off level after 48 or 72 h of EMD exposure. However, a significant (>3-fold) increase in gene expression for some ECM proteins, e.g. several collagens and fibronectin was observed at both time points. The previously reported increase in VEGF, TGF-β1 and MMP-2 secretion from EMD stimulated NHDF cells [17, 18] could not be confirmed on the gene level at the chosen cut off level and time points, suggesting that these factors are mainly regulated at a higher cellular level and/or is part of a secondary response not detectable in the present experimental set-up.

These results support earlier observations in other gene expression and gene micro-array studies on the effect of amelogenin on human periodontal ligament fibroblasts [21, 38–40] and on primary human osteoblasts [25]. To sum up, amelogenin treatment seems to influence key pathways in connective tissue repair and regeneration.

Table 2 Number of genes, sorted by function, regulated by amelogenin more than eightfold (log change >3.0) in Affymetrix experiments

Molecular and cellular functions	Total number of genes regulated	Number of genes regulated after 48 h	Number of genes regulated after 72 h	Number of genes regulated both at 48 and 72 h
Growth and proliferation	166	76 (36)	130 (90)	40
Cell survival	121	39 (24)	97 (82)	15
Cell differentiation	103	53 (23)	80 (50)	30
Migration	99	47 (19)	80 (52)	28
Signalling and interaction	64	26 (0)	64 (38)	26
Cell cycle	61	11 (3)	58 (50)	8
Gene expression	35	35	0 (0)	0
Small molecule biochemistry	10	10	0 (0)	0
Amino acid metabolism	3	3	0 (0)	0
Total number of genes[a]	550	218 (98)	424 (336)	132

Genes per functions are listed according to Ingenuity analysis. Figures in parenthesis indicate number of genes unique to that particular time point

[a] Some genes participate in several functions

Table 3 Number of genes, sorted by the most involved canonical pathways, regulated by amelogenin more than eightfold (log change >3.0) in Affymetrix experiments

Top canonical pathways involved	Total number of genes regulated[a]	Number of genes regulated after 48 h	Number of genes regulated after 72 h	Number of genes regulated both at 48 and 72 h
Hepatic fibrosis pathway	13/131	5 (2)	11 (8)	3
IL-6 signalling	9/91	1 (1)	8 (7)	1
FGF signalling	7/84	7 (0)	7 (0)	7
LXL/RXR activation	6/81	3 (3)	6 (3)	3
NF-κB signalling	9/143	9 (9)	9 (9)	9
Nicotinate metabolism	7/129	0 (0)	7 (7)	0
Phenylalanine metabolism	4/107	0(0)	4 (4)	0
Cytochrome p450	5/215	5 (3)	3 (0)	3
Glycerolipid metabolism	3/144	3 (0)	3 (0)	3
Coagulation system 2/215	2/35	2 (0)	2 (0)	2
Fatty acid metabolism	3/189	3 (0)	3 (0)	3
Axon guidance signalling	12/386	12(7)	5(0)	5
Eicosanoid signalling	4/84	4 (3)	1 (0)	1
TR/RXR activation	4/92	4 (3)	1 (0)	1
Acute phase response signalling	8/172	0 (0)	8 (8)	0
G2/M DNA damage check	4/43	0 (0)	4 (4)	0
SAPK/JNK signalling	4/93	4 (4)	0 (0)	0
IL-4 signalling	3/68	3 (3)	0 (0)	0

Pathway participations are listed according to Ingenuity analysis. Figures in parenthesis indicate number of genes unique to that particular time point

[a] Number of regulated genes/Total number of genes known to be involved in that particular pathway

3.4 Fibroblast proliferation

The effect of amelogenin on fibroblast proliferation was studied by culture of NHDF for up to 7 days. Cells were counted in separate wells every 24 h. Overall cell growth was assessed by area under the curves (AUC) from days 1 to 7. Amelogenin increased the proliferation significantly with 42% at 100 µg/ml ($P = 0.022$) and 112% with 1000 µg/ml ($P < 0.0001$) compared to medium control (Fig. 4). The in vitro stimulatory effect of amelogenin on

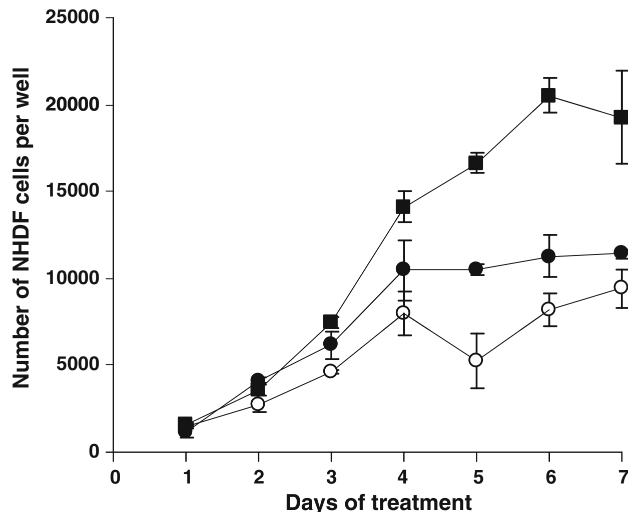

Fig. 4 Proliferation of normal human dermal fibroblasts (NHDF) in medium without (*open circles*) or with amelogenin at 100 µg/ml (*filled circles*) and 1000 µg/ml (*filled squares*) over 7 days in 96-well plates. At indicated time points, cells were counted. Overall cell proliferation, estimated by AUC from days 1 to 7, was 34250 ± 484 cells*day for medium control, 48833 ± 4049 cells*day for 100 µg/ml amelogenin and 72700 ± 2421 cells*day for 1000 µg/ml amelogenin. Mean ± SEM ($n = 3$)

proliferation of human dermal fibroblasts is in accordance to earlier findings on fibroblasts derived from other tissues [14, 17, 18, 24].

4 Conclusions

Amelogenin is an extracellular matrix protein with alleged positive effects on the regeneration of periodontal tissues as well as on healing of chronic cutaneous wounds. The present results provide possible mechanisms for the reported positive effects of amelogenin on wound healing. Amelogenin induced the expression of genes crucial for migration, differentiation, growth and survival of normal human dermal fibroblasts. The effect of amelogenin on cells was exemplified by a significantly increased cell growth over 7 days. Ultrastructural observations showed that both solitary and aggregated micrometer-sized spherical structures were attached to the plasma membrane and also internalized. It is hypothesized that the observed effects are, at least partly, mediated by integrin adhesion to and uptake of amelogenin structures.

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