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Modulacija morfologije osteogenih stanica pomoću ECM liganada i derivata caklinskog matriksa

Modulation of Osteogenic Cell Morphology by ECM Ligands and Enamel Matrix Derivative

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Sažetak

Preduvjet za uspješnu regeneraciju parodontnog tkiva jest odgovarajuća aktivacija populacije za to odgovornih stanica, poput osteoblasta. U tom su slučaju stanična adhezija i sazrijevanje usko povezani s morfologijom stanica i f-aktinskom organizacijom citoskeleta. Mogućnost pojačavanja parodontalnog cijeljenja pomoću pojedinih komponenti izvanstaničnog matriksa (ECM) i derivata caklinskog matriksa dobro je dokumentirana u literaturi. **Svrha rada**: Svrha ovog istraživanja bila je testirati učinak ECM-bjelančevina, kolagena tipa I, laminina-1 te komercijalnog proizvoda EMD-a na morfološku i citoskeletnu organizaciju osteogenih stanica. **Ispitanici i postupci:** Tijekom promatranja analizirano je ukupno 2450 osteogenih stanica iz pet različitih staničnih linija (četiri primarnih i jedne komercijalne) kultiviranih na pojedinim supstratima, a analizirali su ih tri neovisna promatrača. Nakon bojenja za f-aktin staničnog citoskeleta i automatizirane CLSM-vizualizacije, stanice su podijeljene u tri skupine ovisno o njihovim morfološkim svojstvima (nezrele, prijelazne i zrele). Osim deskriptivne analize obavljena je bila i multivarijantna logička regresija radi identificiranja odgovarajućih parametara koji utječu na staničnu morfologiju i organizaciju citoskeleta. Rabljeni pojedinačni ligandi kolagena i laminina te posebice EMD poticali su stvaranje zrelog osteogenog fenotipa, premda su bile uočene i određene razlike među korištenim staničnim linijama. **Rezultati:** Analiza morfologije i citoskeleta pouzdan je način skupljanja prvih podataka o biokompatibilnosti i bioaktivaciji stanica na različitim supstratima. Naši rezultati upućuju na mogući potencijal istraživanih liganada u pojačavanju osteogenoga staničnog pričvrstka i sazrijevanju te time i pomaganju cijeljenja parodontnog tkiva.

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Ključne riječi
osteoblasti; citoskelet; parodontne bolesti; izvanstanični matriks; caklinski matriks, proteini

Uvod

Uspješna regeneracija intraoralnih koštanih i parodontnih defekata zahtijeva reakciju tkiva domaćina s regrutiranjem i aktivacijom (i zaštitom) odgovarajućih susjednih populacija stanica. Za stvaranje novoga mineraliziranog koštanog tkiva i reorganizaciju potpornih tkiva zuba nužne su osteogene stanice (1). U tom su kontekstu postavljeni temelji terapijskih strategija *vođene regeneracije tkiva* (GTR-a) (2-5). Ako se koristimo aloplastičnim biomaterijalima kao membranama ili zamjenama za kost, one moraju zadovoljavati sužene kriterije, u prvom redu biokompatibilnost. Osim toga, potrebna je nekakva vrsta bio-aktivacije kako bi se potaknulo sazrijevanje. Kao mogući rizični čimbenici za GTR identificirana je nedovoljna integracija tkiva i/ili bakterijska kolonizacija aloplastičnih materijala (6).

Preduvjet za regeneraciju tkiva dobro su uravnotežene interakcije stanica i izvanstaničnog matriksa (ECM-a). Kod koštanog tkiva sastav ECM-a ima važnu regulacijsku zadaću za osteogene stanice poput adhezije, pokretljivosti, umnažanja i sazrijevanja (7-9). Osim neizostavnog kolagena, laminini kao sastavni dijelovi bazalne membrane, imaju dodatnu važnu zadaću u koštanom cijeljenju (1, 8, 10, 11).

Premda pročišćene koštano morfogenetske bjelančevine nisu prihvaćene u svakodnevnoj rutinskoj parodontnoj terapiji, korištenje derivata caklinskoga matriksa (EMD-a) je kao terapijska mogućnost u literaturi dobro dokumentirana (4, 12 – 18). Biološki aktivni sastojci identificirani u caklinskom matriksu sastoje se od niza bjelančevina poput amelogenina, tufelina, ameloblastina i enamelina te također proteaze koje su prije toga imale ulogu u razvoju zuba i potpornih tkiva (19-23). Jedini ECM koji je dostupan na tržištu jest svinjski Emdogain ® (Straumann, Švicarska). Emdogain ® je kiseli ekstrakt svinjskoga caklinskog matriksa i sadržava uglavnom amelogeninsku frakciju. Uporabom te biomimetički aktivne bjelančevine ustanovljena su, i klinički i radiološki, dugoročna poboljšanja kliničkog stanja, poput smanjenja koštanih defekata, dubine sondiranja i poboljšanja razine pričvrtska (4, 14, 18, 24). Na životinjskim modelima uočeno je poboljšanje parodontne regeneracije, osteogeneza i angiogeneza (25, 26). Nedavno tiskan revijalni članak na staničnoj je i molekularnoj razini sažeo učinke EMD-a na: (1) stanični pripoj, širenje i kemotaksiju; (2) proliferaciju stanica i preživljavanje; (3) izražaj transkripcijskih čimbenika; (4) izražaj čimbenika rasta, citokine, sastavne dijelove izvanstaničnog

Introduction

Successful regeneration of intra-oral osseous and periodontal defects requires adequate host tissue reactions with recruitment and subsequent activation (and protection) of relevant adjacent cell populations. Indispensable for the formation of new mineralised bony tissue and re-organisation of tooth supporting structures are osteogenic cells (1). In this context, fundamental therapeutic strategies in the field of 'guided tissue regeneration' (GTR) have been introduced (2-5). However, in case of utilisation of alloplastic biomaterials like occlusive membranes or bone substitute materials, the applied materials have to meet stringent requirements, first of all biocompatibility. Furthermore, some kind of bioactivation with promotion of cell maturation is desirable. Insufficient tissue integration and/or bacterial colonisation of the alloplastic materials have been identified as potential risk factors for GTR (6).

Prerequisite for tissue regeneration are well-balanced cell to extracellular matrix (ECM) interactions. In bony tissue, the composition of the ECM plays a regulatory role for osteogenic cell functions like adhesion, motility, proliferation and differentiation (7-9). Besides the ubiquitous protein collagen, laminins as components of the basement membrane play an additional important role in bone healing (1, 8, 10, 11).

While the use of purified growth factors like bone morphogenetic proteins has not been established in daily routine periodontal therapy, the application of enamel matrix derivative (EMD) is an evidence-based therapeutic option (4, 12-18). The biological active ingredients identified in enamel matrix consist of a variety of specific matrix proteins like amelogenin, tuftelin, ameloblastin and enamelin as well as proteases, contributing a pivotal role in the development of teeth and supporting structures (19-23). The only EMD currently commercially available is the porcine derived Emdogain® (Straumann, Switzerland). Emdogain® represents an acidic extract of porcine enamel matrix and contains mainly the amelogenin fraction. A positive long term effect on bony defects, probing depth and attachment level could be documented both clinically or radiographically after application of these biomimetric active proteins (4, 14, 18, 24). In animal models, an improvement of periodontal regeneration, osteogenesis and angiogenesis has been observed (25, 26). On a cellular and molecular level, a recent systematic review summarises effects of EMD on: (1) cell attachment, spreading, and chemotaxis; (2) cell prolif-

matriksa i ostale makromolekule; i (5) izražaj molekula uključenih u remodeliranje kostiju za različite vrste stanica, uključujući epitelne, gingivalne fibroblaste, stanice parodontnog ligamenta, cementoblaste i osteogene (20). Zaključeno je da svi ti podaci daju čvrstu potporu tezi da EMD poboljšava cijeljenje rana i stvaranje novoga parodontnog tkiva. Podaci o osteogenim stanicama potanko dokazuju da se povećava preživljavanje i proliferacija stanica u dozom ovisnom obliku (27, 28), zatim povećani su stanični pripoj (29) i pokretljivost stanica (7). Schwartz i suradnici pokazali su da EMD utječe na rane faze sazrijevanja stanica zahvaljujući stimulaciji stanične proliferacije preko aktivnosti alkalne fosfataze, a u kasnijim fazama stimulacijom izražaja osteokalcina (30). Drugi autori potvrdili su primarnu ulogu EMD-a u diferencijaciji osteogenih stanica (29, 31). Ako proučimo raspoloživu literaturu, doći ćemo do pretpostavke da utjecaj EMD-a djelomice ovisi o promatranim vrstama stanica. Na kraju, mora se reći da je učinak EMD-a na osteoblastne stanice više stimulatoran negoli inhibitoran.

Aktinski citoskelet je spoj između izvanstaničnog matriksa i unutarstanične reakcije (32). Vitalno je važan za održavanje stanične homeostaze i nezamjenjiv za staničnu adheziju i migraciju (33-35). Osim toga, regulacija prenošenja signala za proliferaciju i staničnu diferencijaciju u unutrašnjost stanice obavlja se preko aktinskoga signalnog puta od izvanstaničnog matriksa do odgovarajućih unutarstaničnih odjeljaka (36). Mnogobrojna istraživanja pokazala su da su spomenuta svojstva osteoblastnih stanica, poput adhezije i diferencijacije, usko povezana s njihovom karakterističnom staničnom morfologijom (37, 38). Osteogene stanice kultivirane na «biološki aktivnim» biokompatibilnim površinama predstavljaju izdužene stanice vretenastog fenotipa s razvojem lamelipodiza i filopodiza, a kultivacija na manje kompatibilnih površina rezultira stvaranjem okruglastih stanica (37-40).

Mnoga istraživanja otkrila su usku povezanost između f-aktinske organizacije citoskeleta, stanične adhezije i osteogenog sazrijevanja (diferencijacije) sa stvaranjem matriksa (39, 41, 42). U tim istraživanjima stanični fenotip i organizacija citoskeleta potanko su opisani i kategorizirani (okrugli stanični oblik s niskom organizacijom citoskeleta nasuprot polariziranim stanicama sa srednjim stupnjem organizacije citoskeleta te proširenim stanicama s visokoorganiziranom strukturom citoskeleta). U nedavnim istraživanjima Titushkin i Cho (2007.) tvrde da diferencijaciju osteogenih stanica prati povećanje

eration and survival; (3) expression of transcription factors; (4) expression of growth factors, cytokines, extracellular matrix constituents, and other macromolecules; and (5) expression of molecules involved in the regulation of bone remodelling for different cell types including epithelial cells, gingival fibroblasts, periodontal ligament cells, cementoblasts and osteogenic cells (20). It was concluded that the analysed data provided strong evidence for EMD to support wound healing and new periodontal tissue formation. For osteogenic cells in detail, it has been suggested that EMD promotes cell viability and cell proliferation in a dose-dependent manner (27, 28), furthermore cell attachment (29) and cell motility (7) were increased. Schwartz et al. showed that EMD affects early states of cell maturation by stimulating cell proliferation and alkaline phosphatase activity as well as late states by stimulating osteocalcin expression (30). Other authors confirmed the principal promotion of osteogenic cell differentiation by EMD (29, 31). A summarization of the current literature, to include divergent results, leads to the assumption that the effects of EMD depends to a degree on the investigated cell type. Nevertheless, the overall effect of EMD on osteoblastic cells is stimulatory rather than inhibitory.

The actin-cytoskeleton imparts as connective link between extracellular matrix and intercellular reactions (32). It is of vital importance to maintain cellular homeostasis and indispensable for cell adhesion and migration (33-35). Furthermore, over the actin-supported signalling pathway between extracellular matrix and intracellular compartments, the outside-in signalling for proliferation and cellular differentiation is regulated (36). Various studies show that the mentioned osteoblastic cell attributes adhesion and differentiation are closely associated with characteristic cell morphologies (37, 38). Osteogenic cells cultivated on “biological active“ biocompatible surfaces represented a stretched, spindle-shaped phenotype with development of lammelipodias and filopodias, whereas less compatible surfaces resulted in rather roundish cell forms (37-40).

Various studies reveal a close correlation between f-actin cytoskeletal organisation, cell adhesion and osteogenic cell maturation (differentiation) with associated matrix production (39, 41, 42). In these studies, cell phenotypes and cytoskeletal organisation were expediently categorised (round cell shape with low cytoskeletal organisation vs. polarisized cell shape with intermediate cytoskeletal organisation vs. spreaded cell shape with highly so-

interakcije citoskeletnih membrana, što je tipična značajka zrelih osteoblasta. Te modulacije su u vezi s remodelacijom aktinskih citoskeletnih debelih (pojedinačnih) niti kod osteogenih prekursora u tanje vlaknaste mreže u zrelim osteoblastima (43).

Zbog toga se određivanje morfologije stanica i organizacije citoskeleta može iskoristiti kao pokazatelj za dobivanje prvih naznaka utjecaja funkcionalnih supstrata na pojačano sazrijevanja osteogenih stanica.

Svrha ovog istraživanja bila je odrediti utjecaj klasičnih pojedinačnih funkcionalnih ECM-liganada (kolagen tip I, laminin-1) te komercijalnog EMD-a (Emdogain®) na osteogeni stanični pripoj i sazrijevanje pomoću procjene morfologije stanica i arhitekture citoskeleta. U skladu s relevantnom literaturom testirali smo pretpostavku da pospješuju razvoj i diferencijaciju osteoblastnog fenotipa.

Materijali i metode

Stanične linije i kultura stanica

Istraživane su komercijalna osteoblastna stanična linija dobivena iz kosti kuka (line "P") HHOB-c (Fa. Promocell, Heidelberg, Njemačka) i četiri primarne humane stanične linije (linije "K", "N", "S", "T"). Posljednje navedene stanične linije dobivene su iz viška koštanog materijala tijekom rutinskih kirurških operacija (ortognatske) na Odjelu za oralnu i maksilofacijalnu kirurgiju Sveučilišta Johannes Gutenberg u Mainzu. U skladu s odredbama lokalnoga Etičkog povjerenstva, uzorci su bili anonimni i nisu bili zabilježeni nikakvi osobni podaci o pacijentima. Pacijenti nisu imali nikakve poremećaje koštane homeostaze (osteoporozu ili ostale bolesti kostiju poput: neoplazmi, sistemskih infekcija, steroidnih lijekova, terapija radijacijom i sl.). U skupini su bile tri žene i jedan muškarac u dobi od 25 do 40 godina. Uzorci kostiju temeljito su oprani u fosfatima puferiranoj otopini (PBS, PAA Laboratories, Pasching, Austrija) i zasađeni u petrijeve posudice sa standardnim medijem za kultivaciju osteoblasta uz 10% fetalnoga telećeg seruma (FCS, Gibco Invitrogen, Karlsruhe, Njemačka), Dulbecco's modified Eagle's medium (DMEM, Gibco Invitrogen), deksametazon (100 nmol/l, Serva Bioproducts, Heidelberg, Njemačka), L-glutamin (Gibco Invitrogen) i streptomicin (100 mg/ml, Gibco Invitrogen). Kul-

phisticated cytoskeletal organisation). In a recent investigation, Titushkin and Cho (2007) stated that osteogenic cell differentiation goes along with an increase in membrane-cytoskeleton interaction which is typical for mature osteoblasts. These modulations are related to remodelling of the actin cytoskeleton from thick (solitary) stress fibers in osteogenic precursor cells into a thinner filamentous network in mature osteoblasts (43).

As an implication, capturing of cell morphology and cytoskeletal organisation can be identified as a helpful approach to get an idea on the influence of functionalised substrates on the promotion of osteogenic cell maturation.

The aim of the present study was to determine the influence of classical solitaire functional ECM-ligands (collagen type I, laminin-1) as well as commercially available EMD (Emdogain®) on osteogenic cell attachment and maturation by assessing cell morphology and cytoskeletal architecture. In concordance with the relevant literature, we tested the assumption that EMD promotes the development of a differentiated osteogenic phenotype.

Materials and Methods

Cell lines and cell culture

A commercial hipbone-derived osteoblastic cell line (line "P") HHOB-c (Fa. Promocell, Heidelberg, Germany) and four primary human cell lines (lines "K", "N", "S", "T") were examined. The latter were obtained from excess material of routine surgical (orthognatic) procedures at the Department of Oral and Maxillofacial Surgery, Johannes Gutenberg-University of Mainz. In accordance to the regulations of the local ethics committee, no patient-related data were recorded and the samples were anonymised. The patients were free of systemic or local alterations of bone homeostasis (osteoporosis or other bone diseases, neoplasies, systemic infections, steroidal medication, radiation therapy etc.). The patients were three female and one male patient, age ranging from 25 to 40 years. The bone specimens were thoroughly washed in phosphate-buffered saline solution (PBS, PAA Laboratories, Pasching, Austria) and seeded onto culture dishes in standard osteoblast cultivation medium, consisting of 10% fetal calf serum (FCS, Gibco Invitrogen, Karlsruhe, Germany), Dulbecco's modified Eagle's medium (DMEM, Gibco Invitrogen), dexamethasone (100 nmol/l, Serva Bioproducts, Heidelberg, Germany), L-glutamin (Gibco Invitrogen) and streptomycin (100 mg/ml, Gibco Invitrogen). Culti-

tura stanica bila je obavljena na 37°C u konstantno vlažnoj atmosferi uz 95% zraka i 5% CO_2.

Prije početka našeg istraživanja svih pet staničnih linija kvalitativno je karakterizirano korištenjem imunohistokemijskog izražaja alkalne fosfataze (AP-a) i osteokalcina (obilježen streptavidin–biotin/horseradish peroksidazom). Presađivanja redovito ovise o njihovim karakteristikama rasta uz pomoć 0,25% trypsina (Seromed Biochrom KG, Berlin, Njemačka).

Svi pokusi bili su obavljeni između trećeg i petog presađivanja. Predmetna stakalca presvučena odgovarajućim ligandima (pogledati ispod) kultivirana su s različitim osteogenim staničnim linijama (gustoća sađenja 2 x 10^4/ml). Nakon 48 sati kultivacije u standardnim uvjetima, bila je obavljena fiksacija i specifično bojenje za citoskelet (pogledati ispod).

Testirani supstrati i premazi

Kao predstavnici pojedinačnih ECM-bjelančevina korišteni su: kolagen tip I (humana koža; Sigma, St.Louis, SAD) i laminin-1 (humana placenta; Sigma, St.Louis, SAD). Od komercijalnih EMD-a uporabljen je bio Emdogain® (Straumann, Basel, Švicarska). Sve komponente otopljene su u PBS-u kojemu je dodan 1 mmol $CaCl_2$ te prilagođena koncentracija na 100 mg/ml. Kao kontrola uporabljen je kravlji serumski albumin (BSA, Sigma, St.Louis, SAD) s koncentracijom od 10 mg/ml. Kao što je već istaknuto, sterilizirana predmetna stakalca promjera 2 cm (Hecht-Assistant, Sondheim, Njemačka) inkubirana su s 200 μl odgovarajuće otopine jedan sat na temperaturi od 37 stupnjeva kako bi se omogućila precipitacija bjelančevina na staklenu površinu (7). Topivi ostatci blago su isprani PBS-om. Potencijalno slobodna vezna mjesta blokirana su pomoću 1-postotne otopine BSA 30 minuta na sobnoj temperaturi. Kao negativna kontrola rabila su se neprepariranа predmetna stakalca. Kultura stanica na tako pripremljenim različitim predmetnim stakalacima nastavljena je njihovim postavljanjem u 90 mm petrijeve posudice (Greiner Holding AG, Kremsmuenster, Austrija).

Vizualizacija staničnog citoskeleta i kategorizacija stanične morfologije

Nakon kultivacije od 48 sati stanice su paraformaldehidom (4%) fiksirane na predmetnim stakalcima na sobnoj temperaturi 10 minuta te su učinjena propusnima uz pomoć 0,1% Triton-X-a tijekom 3 minute. Obavljeno je bilo i specifično bojenje za citoskeletni f-aktin pomoću BODIPY® FL Phalla-

vation was carried out at 37°C in a constant humidified atmosphere of 95% air and 5% CO_2.

Before our investigations, all five cell lines were qualitatively characterized by immunohistochemical expression of alkaline phosphatase (AP) and osteocalcin (labelled streptavidin–biotin/horseradish peroxidase). Cells were passaged at regular intervals depending on their growth characteristics using 0.25% trypsin (Seromed Biochrom KG, Berlin, Germany).

All trials were taken out between the 3rd and 5th passage. Glass slides coated with the respective ligands (see below) were cultivated with the different osteogenic cell lines (seeding density of 2 x 10^4/ml). After 48h of cultivation under constant culture conditions fixation and cytoskeleton-specific staining was performed (see below).

Test substrates and coating

As representatives of solitaire ECM-proteins, collagen type I (human skin; Sigma, St.Louis, USA) and laminin-1 (human placenta; Sigma, St.Louis, USA) were applied. As commercially available EMD, Emdogain® (Straumann, Basel, Switzerland) was utilised. All components were dissolved in PBS supplemented with 1mmol $CaCl_2$ and adjusted to a concentration of 100 mg/ml. Bovine serum albumin (BSA, Sigma, St.Louis, USA) at a concentration of 10mg/ml served as the control. As previously described, sterilized glass slides, diameter 2cm, (Hecht-Assistant, Sondheim, Germany) were incubated with 200μl of the respective stock solutions for 1h at 37°C, thus allowing the proteins to precipitate on the glass surface (7). Soluble remnants were removed by rinsing gently with PBS. Potential remaining free adhesion sites were blocked with a solution of 1% BSA for 30 minutes at room temperature. Untreated glass slides served as negative control. After placing the differently prepared glass slides in 90mm tissue culture petri dishes (Greiner Holding AG, Kremsmuenster, Austria) further cultivation was performed.

Visualisation of the cell cytoskeleton and categorisation of cell morphology

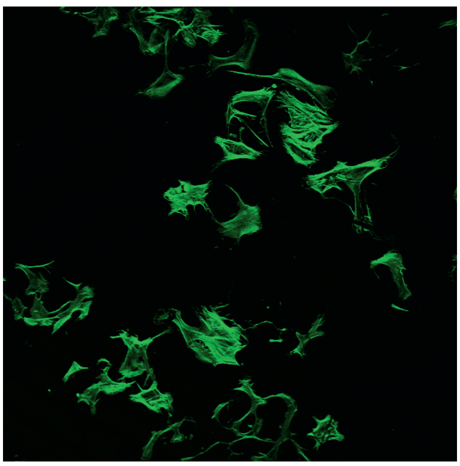
After a cultivation period of 48 hours the cells were fixed on the glass slides with paraformaldehyd (4%) at room temperature for 10 minutes and permeabilized with 0,1% Triton-X for three minutes. Specific staining for the f-actin cytoskeleton was done with BODIPY® FL Phallacidin (Molecu-

cidina (Molecular Probes, Carlsbad, SAD) prema uputama proizvođača. Vizualizacija je obavljena laserskim konfokalnim skening-mikroskopom (CLSM: Leica TCS SP2 X1, Wetzlar, Njemačka). Poticanje zelene fluorescentne boje učinjeno je u fluorescentnom modu rada mikroskopa pomoću plavog argonskog lasera valne duljine λ=488nm. Za detekciju se rabio *Acusto Optic Beam Splitter* (AOBS) u detekcijskom kanalu pri Langpassu >500 nm. Korištenjem *Fluotar* 10x04 uljnog objektiva (Leica, Wetzlar, Njemačka) bilo je moguće snimiti područja veličine 1,5 x 1,5 mm (Slika 1.). Razlučivost skeniranih slika jest 1024x1024 piksela. Nakon automatizirane detekcije 25 dodirnih skeniranih područja (5 x 5), bilo ih je moguće spojiti u krajnje slike veličine 7,5 x 7,5 mm (Slika 2.).

Prema opisanoj metodi Sinha i suradnika (42) te El-Amina i njegovih kolega (39), obavili smo deskrip-

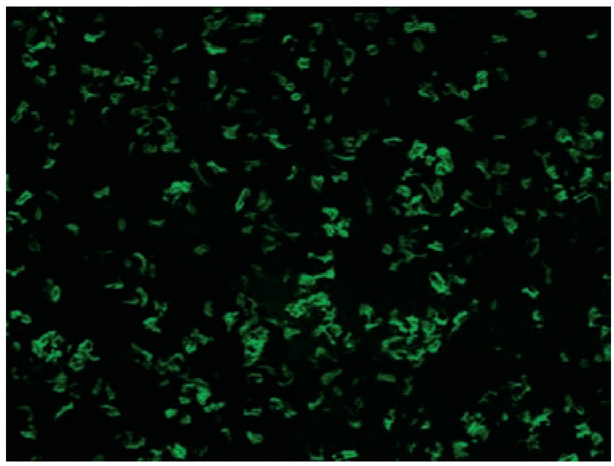
lar Probes, Carlsbad, USA) according to manufactures guidelines. Visualisation was performed by confocal laser scanning microscopy (CLSM: Leica TCS SP2 X1, Wetzlar, Deutschland). The excitation of the green fluorescenting dye was done in the fluorescence mode of the microscope with a blue argon laser at a wave length λ=488nm. For detection, an *Acusto Optic Beam Splitter* (AOBS) in a detection channel at Langpass >500nm was utilised. With a *Fluotar* 10x04 oil-objective (Leica, Wetzlar, Deutschland) it was possible to scan a detail of 1,5 x 1,5 mm (Fig. 1). The resolution of the scanned images was 1024 x 1024 pixels. After automated detection of 25 adjacent scanning areas (5 x 5), it was possible to merge them to a complete image of 7.5 x 7.5 mm (Fig. 2).

According to the method of Sinha et al. (42) and El-Amin et al. (39), we performed a descriptive cat-



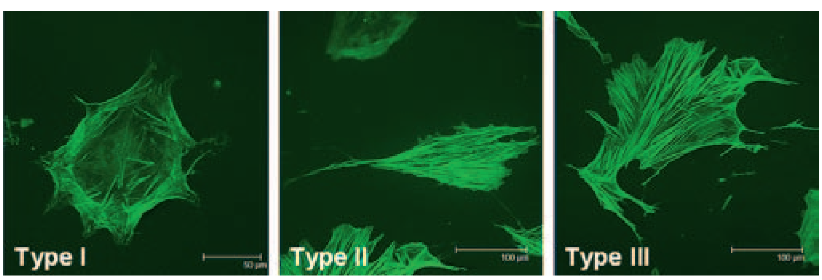
Slika 1. Osteogene stanice "S" stanične linije nakon 48-satne kultivacije na kolagenu tipa I. Phallacidinsko bojenje aktinskog citoskeleta (originalno povećanje 10x; dužina ruba: 1,5mm).

Figure 1 Osteogenic cells of line "S" after 48h cultivation time on collagen type I. Phallacidin staining of the actin cytoskeleton (original magnification 10x; edge length: 1.5mm).



Slika 2. Osteogene stanice "S" linije nakon 48-satne kultivacije na kolagenu tipa I. Složena slika od 25 pojedinačnih slika (originalno povećanje 10x; dužina ruba: 7,5mm).

Figure 2 Osteogenic cells of line "S" after 48h incubation on collagen type I. Merged image of 25 single pictures (original magnification 10x; edge length: 7.5mm).



Slika 3. Primjer osteogenih stanica podtipa I, II i III (phallacidinsko bojenje, originalno povećanje 40 x). Obratiti pozornost na različita povećanja koja odgovaraju mjernoj oznaki omjera.

Figure 3 Exemplary representation of osteogenic cells of subtype I, II and III (phallacidin staining, original magnification 40x). Note the different digital enlargements with corresponding scale bars.

tivnu kategorizaciju stanične morfologije i citoskeleta u trima maturacijskim podskupinama (Slika 3.):

- tip I (nezreo): okrugle, male stanice (≈ 50μm) - aktinski citoskelet je slabo diferenciran sa stresnim nitima na rubovima stanica;
- tip II (prijelazan): dugačke, vretenaste stanice (>50μm) te stvaranje perifernih aktinskih stresnih niti;
- tip III (zreo): velike stanice (> 100μm) uz mnoge diferencirane filopode (stanične izdanke) i lamelopode i visokoorganizirani citoskelet s tankom, sofisticiranom (složenom) mrežom aktina.

Ocjenjivanje stanične morfologije i statistička analiza

Obojene i CLSM-zabilježene stanice bile su podvrgnute daljnjoj statističkoj procjeni ako su zadovoljavale sljedeće uvjete:

- cijela stanica oštro je ocrtana i struktura dobro vidljiva (u fokusu nema artefaktnog preklapanja slojeva);
- izolirani položaj stanice (malo stanica-stanica kontakata, bez staničnog nakupljanja).

Počevši od gornjega lijevog kuta složene slike, prvih 100 stanica (za svaku staničnu liniju i svaki supstrat) koje su zadovoljavale navedene kriterije uključivanja, pobrojeno je redom (1 - 100). Uvrštavanje stanica u definirane skupine (tip I - III) obavila su polukvantitativno tri neovisna promatrača. Radi «kalibracije», oni su na preliminarnim testovima uvježbavali prepoznavanje ključnih značajki za svrstavanje stanica u navedene skupine.

Statistička analiza bila je obavljena u Institutu za medicinsku biostatistiku, epidemiologiju i informatiku (IMBEI) Sveučilišta Johannes Gutenberg u Mainzu. Korišten je programski paket SPSS-a (SPSS Inc., Chicago, SAD). Samo stanice sa 100% podudarnom klasifikacijom među promatračima (međupromatračko preklapanje) uporabljene su za daljnju obradu. Za analizu utjecaja pojedinih važnih čimbenika (supstrata, staničnih linija) na staničnu morfologiju i organizaciju citoskeleta, rabila se multivarijantna logistička regresija. Kao referentnu polaznu točku odredili smo neutralni supstrat BSA i staničnu liniju «K» koja je imala prosječno najmanji stupanj sazrijevanja. Sve dobivene vrijednosti na razini p<0,05 smatrale su se statistički velikima.

Rezultati

U staničnoj liniji «T» je, zbog artefakata i stanične agregacije, jasna identifikacija i procjena samostalnih stanica bila moguća na 450 od 500 naci-

egorisation of cell morphology and cytoskeleton into three maturation subtypes (Fig. 3):

- type I (immature): round, small cells (≈ 50μm), the actin cytoskeleton is poorly differentiated with stress fibers at the margin of the cell,
- type II (intermediate): long, spindle shaped cells (>50μm) with formation of peripheral actin stress fibers,
- type III (mature): large cells (> 100μm) with many differentiated filopodias and lamellipodias and a highly organized cytoskeleton with a fine, sophisticated actin network.

Evaluation of cell morphology and statistical analysis

The stained and CLSM-recorded cells were subjected to further statistical evaluation if they met the following inclusion criteria:

- the whole cell is displayed in a sharp manner (in focus, no overlay caused by artefacts etc.),
- isolated position of the cell (low cell-cell-contacts, no cellular aggregation).

Beginning from the upper left side of a merged image, the first 100 cells (for each cell line and each substrate) which fulfilled the listed inclusion criteria where numbered consecutively (1 - 100). The assignment of the cells into the above defined categories (type I - III) was conducted in a semi-quantitative manner by three independent observers. In preliminary tests, the observers were trained about the relevant features of the respective cell categories in order to achieve a “calibration”.

The statistical analysis was performed by the Institute of Medical Biostatistics, Epidemiology and Informatics (IMBEI), Johannes Gutenberg-University Mainz. The software program SPSS (SPSS Inc., Chicago, USA) was utilised. Only cells with 100% agreement between the observers (inter-observer concordance) were further processed. For the analysis of potential influence factors (substratum, cell line) a multivariate logistic regression on cell morphology and cytoskeleton organisation was performed. As references we defined the neutral substrate BSA and the cell line “K”, which showed the lowest overall maturation. All p-values <0.05 were considered to be significant.

Results

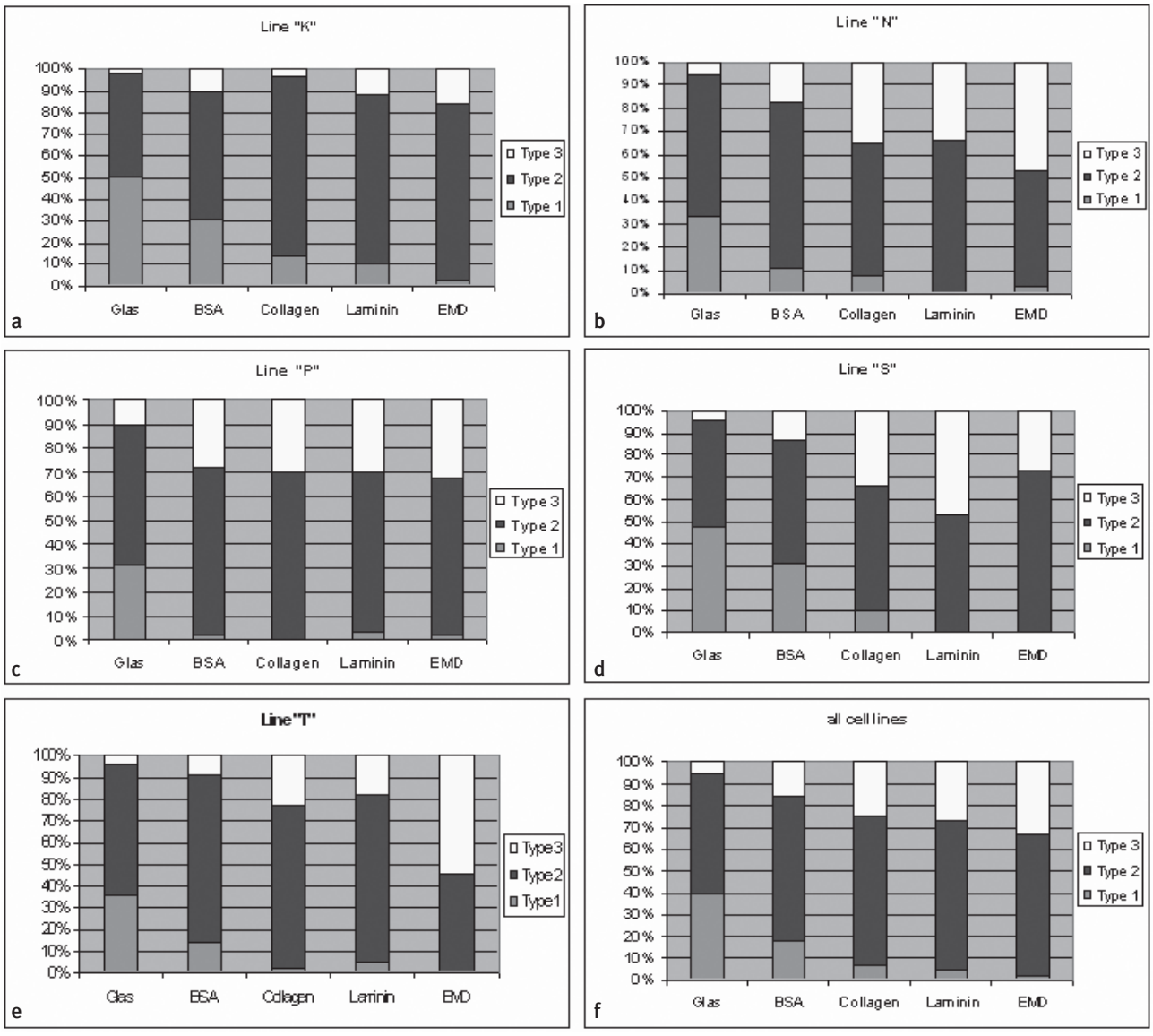
Due to the artefacts and cell aggregation, a clear identification and evaluation of solitaire cells was only possible for 450 of the aimed 500 cells in cell

ljanih stanica. U pet staničnih linija svaki neovisni promatrač analizirao je ukupno 2450 stanica. Od toga broja je 1507 stanica (60,3%) zadovoljavalo uvjet međupromatračkog podudaranja. Između različitih staničnih linija omjer se kretao od 58,0% (linija "S") do 66,2% (linija "K").

Slike od 4a do 4e pokazuju omjere svakog maturacijskog podtipa (tipovi I-III) za analiziranu staničnu liniju i odgovarajući supstrat. Na Slici 4f su rezultati za cijelu populaciju (n=1507). Među svim staničnim linijama najveći udjel tip-I staničnog fenotipa pronađen je u negativnoj kontroli (netretirana predmetna stakalca). Kretao se od 31,4% (linija "P") do 50,8% (linija "K").

line "T". For all 5 cell lines, a total of 2450 cells were analysed by independent observers. Out of this, 1507 cells (60.3%) met the criterion of inter-observer concordance. Between the different cell lines, the ratio ranged from 58.0% (line "S") to 66.2% (line "K").

Figures 4a-4e show the ratio of each maturation subtype (types I-III) for the analysed lines cultivated on the respective substrates. Figure 4f shows the results for the whole population (n=1507). For all cell lines, the majority of the type-I cell phenotype was found on the negative control (untreated glass). It ranged from 31.4% (line "P") to 50.8% (line "K").



Slike od 4a do 4f. Omjeri različitih podtipova zrelosti stanica (I: nezrela, II: prijelazna, III: zrela) za testirane supstrate. Slike od 4a do 4e prikazuju različite istraživane stanične linije, a na Slici 4f je ukupna analizirana stanična populacija (n=1507).

Figures 4a - 4f Ratios of the different maturation subtypes (I: immature, II: intermediate, III: mature) for the investigated substrates. Figures 4a - 4e display the different investigated cell lines, figure 4f displays the cumulative cell population (n=1507).

Najviše je stanica, bez obzira na supstrat i staničnu liniju, imalo obilježja podtipa II. Jedine iznimke bile su stanice linije "K" na staklu (50,8% podtip I) i linija "T" na EMD (54,5% podtip III).

U negativnoj kontroli stakla opazili smo ukupno manje staničnog fenotipa tipa III (raspon od 1,5% za liniju "K" do 10,0% za liniju "P"). U usporedbi s BSA-kontrolnom skupinom supstrata, za pojedinačne ECM-bjelančevine kolagen i laminin te EMD, pronađene su više vrijednosti tipa III staničnog fenotipa. Jedina iznimka identificirana je u liniji «K» s nižim vrijednostima za kolagen. Za kolagen je omjer tipa III staničnog fenotipa bio od 3,1% za liniju "K" do 34,9% za liniju "N", za laminin omjer je bio od 11,.8% za linju "K" do 47,2% za liniju "S", te za EMD omjer je iznosio od 15,4% za liniju "K" do 54,5% za liniju "T".

Na Slici 5. su kumulativni rezultati za sve ispitane supstrate na pet istraženih staničnih linija. Općenito je bio visok udjel staničnog fenotipa tipa II (raspon od 56,% za liniju "S" do 69,% za liniju "K"). Ukupan omjer za tip II staničnog fenotipa u svim staničnim linijama iznosio je 64,6%. Omjer za tip III bio je u rasponu od 8,% za liniju "K" do 26,% za liniju "N", uz ukupni omjer od 19,9% za sve stanične linije.

Primjenjujući logističku regresiju, izračunat je «rizik» za odgovarajuće supstrate – u odnosu prema BSA-u, bez obzira na to je li stanica pokazivala svojstva tipa I, II ili III (Tablica 1.). Prema tome je izračunata vjerojatnost omjera (Exp(B)) kao mjere. Negativna kontrola stakla je statistički znatno »podupirala» izražaj podtipa I, a razvoj podtipa III bio je jako «inhibiran». Pojedinačne ECM-bjelančevine kolagen i laminin te EMD pojačale su izražaj podtipa III (Eksp(B) kolagen: 1,745; Eksp(B) laminin: 2,181; Eksp(B) EMD: 2,491) i znatno smanjile podtip I.

Regardless of substratum and cell line the majority of the cells expressed subtype II. The only exceptions were assessed for line "K" on glass (50.8% subtype I) and line "T" on EMD (54.5% subtype III).

For the negative control glass we observed a collective lower expression of type III cell phenotypes (ranging from 1.5% for line "K" to 10.0% for line "P"). Compared to the substratum control group BSA, for the solitaire ECM-proteins collagen and laminin as well as for EMD, higher levels of type III cell phenotypes were found. The only exception was identified for line "K" with lower values for collagen. For collagen, the ratio of type III cell phenotypes ranged from 3.1% for line "K" up to 34.9% for line "N", for laminin, the ratio ranged from 11.8% for line "K" up to 47.2% for line "S" and for EMD, the ratio ranged from 15.4% for line "K" up to 54.5% for line "T".

Figure 5 shows the cumulative results of all tested substrates for the investigated 5 cell lines. In general, a high proportion of type II cell phenotypes (ranging from 56.9% for line "S" up to 69.9% for line "K") was found. The ratio of type II cell phenotypes for all lines amounted 64.6%. The ratio of type III cell phenotypes ranged from 8.5% for line "K" up to 26.6% for line "N" with a total ratio of 19.9% for all cell lines.

Employing logistic regression, a "risk-"calculation for the respective substrates - referred to BSA - was performed, whether a cell was likely to express cell phenotypes I, II or III (Table 1). Therefore, odds ratio (Exp(B)) was calculated as a measure. The negative control glass "supported" the expression of subtype I in a significant manner, whereas the development of subtype III was significantly "inhibited". The solitaire ECM-proteins laminin and collagen as well as EMD led to a significant higher expression of subtype III (Exp(B) collagen: 1.745; Exp(B) laminin: 2.181; Exp(B) EMD: 2.491) with a respective significant down regulation of subtype I.

Slika 5. Omjeri različith podtipova tipova stanične zrelosti (I: nezrela, II: prijelazna, III: zrela) za različite stanične linije na svim testiranim supstratima.

Figure 5 Ratios of the different maturation subtypes (I: immature, II: intermediate, III: mature) for the different investigated cell lines on all tested substrates.

Tablica 1. Rezultati logističke regresije korištene za procjenu stanične morfologije i organizacije citoskeleta za odgovarajuće skupine (tipovi I – III) ovisno o supstratu BSA i staničnoj liniji "K". (df = stupnjevi slobode; Exp(B) = omjer vjerojatnosti)

Table 1 Results of the logistic regression aimed on cell morphology and cytoskeleton organisation for the referring categories (types I – III) in dependence of substrate BSA and cell line "K". (df = degrees of freedom; Exp(B) = odds ratio)

Varijable • Variables	Analiza rizika tip I • Risk analysis Type I	df	Značajnost • Significance	Exp(B)	Analiza rizika tip II • Risk analysis Type II	df	Značajnost • Significance	Exp(B)	Analiza rizika tip III • Risk analysis Type III	df	Značajnost • Significance	Exp(B)
Svi supstrati • All substrates		4	< 0.001			4	< 0.001			4	< 0.001	
Staklo • Glass		1	0.000	3.175		1	0.002	0.606		1	0.000	0.337
Kolagen • Collagen		1	0.000	0.315		1	0.420	1.153		1	0.009	1.745
Laminin		1	0.000	0.205		1	0.667	1.079		1	0.000	2.181
EMD		1	0.000	0.077		1	0.688	1.074		1	0.000	2.491
Sve stanične linije • All cell lines		4	0.000			4	0.012			4	0.000	
Line N		1	0.000	0.389		1	0.066	0.73		1	0.000	4.037
Line P		1	0.123	0.264		1	0.251	0.821		1	0.000	4.066
Line S		1	0.000	0.706		1	0.002	0.589		1	0.000	3.685
Line T		1	0.000	0.406		1	0.893	0.997		1	0.000	2.626

U usporedbi s referentnom staničnom linijom "K", stanične linije "N", "S" i "T" imale su mnogo niži omjer tipa I staničnog fenotipa, a kod stanične linije "S" nađen je dodatno i dosta niži omjer tipa II staničnog fenotipa. U usporedbi sa staničnom linijom "K", kod svih drugih linija bio je zabilježen pojačan izražaj tipa III (iz Eksp(B) linija "T": 2,626 do Eksp(B) linija "P": 4,066).

Rasprava

Preduvjet za uspješnu i pravodobnu parodontnu regeneraciju jest dobro uravnotežena interakcija odgovarajućih stanica na mjestu oštećenja. Za stanice osteogene linije opisani su specifični procesi koji se događaju u određenom redoslijedu (1): *osteokondukcija* (uraštanje koštane strukture u strukturu implantata ili grafta) i (2) *novo stvaranje kostiju* (proliferacija i diferencijacija osteogenih stanica) (1). *In vitro* pridruživanje određenih staničnih svojstava, poput stanične pokretljivosti, proliferacije i diferencijacije, služi kao pokazatelj staničnog sazrijevanja: u slijedu sazrijevanja osteogenih stanica smanjuju se stanična pokretljivost i proliferacija, a povećava diferencijacija, što rezultira stvaranjem zrelih postmitotičkih osteoblasta (44-46). Osim toga, brza i učinkovita stanična adhezija obvezatna je za daljnju diferencijaciju stanica. U tom kontekstu mnoga su istraživanja jasno dodijelila određenu staničnu morfologiju i organizaciju citoskeleta određenim stupnjevima sazrijevanja osteogenih stanica i biokompatibilnosti supstrata (37-42). Prema metodi Sinha i suradnika (42) te El-Amina i njegovih kolega (39), mi smo u ovom istraživanju klasificirali morfolo-

Compared to the reference cell line "K", cell lines "N", "S" and "T" showed a significant lower ratio of type I cell phenotypes, for cell line "S" a significant lower ratio of type II cell phenotypes was found additionally. Compared to line "K", for all cell lines an increased expression of cell type III was recorded (from Exp(B) line "T": 2.626 up to Exp(B) line "P": 4.066).

Discussion

Precondition for successful and timely periodontal tissue regeneration is a well balanced interaction of relevant cell populations at the defect site. For the osteogenic cell lineage in special a distinct sequence of (1) *osteoconduction* (the growth of bony tissue into the structure of an implant or graft) and (2) *de novo bone formation* (osteogenic cell proliferation and differentiation) has been described (1). *In vitro*, assignment of specific osteogenic cell attributes like cell motility, cell proliferation and cell differentiation serve as an indicator of the state of cell maturation: in consecutive osteogenic maturation stages cell motility and proliferation decreases while cell differentiation increases, finally resulting in postmitotic mature osteoblasts (44-46). Furthermore, a fast and plane cell adhesion is mandatory precondition for further cell differentiation. In this context, various studies clearly assigned cell morphology and cytoskeletal organisation to distinct osteogenic cell maturation stages and substrate biocompatibility (37-42). According to the method of Sinha et al. (42) and El-Amin et al. (39) in this observational study, we categorized osteogenic cell morpholo-

giju i organizaciju osteogenih stanica u tri podtipa, ovisno o stupnju sazrijevanja stanica.

Loša strana te metode jest ovisnost o promatračevoj subjektivnosti. Osim toga, preporučeno 100-postotno međupromatračko preklapanje rezultiralo je isključivanjem mnoštva stanica (943 stanice ili 39,7%) iz daljnje statističke analize. Automatizirani kompjutorski algoritmi za procjenu i analizu staničnе morfologije i organizacije citoskeleta već postoje (47, 48) i možda bi mogli biti korisni u obavljanju tih pokusa. Nažalost, ti automatizirani algoritmi zahtijevaju visoke standarde pripreme uzoraka i jako su osjetljivi na površinske, fiksacijske i procesne artefakte. Poluautomatizirana metoda opisana u našoj studiji uspjela je kompenzirati takve artefakte za mišljenje promatrača o obliku i boji.

Naši rezultati upućuju na to da na istraživanim staničnim linijama "N", "S" i "T" pojedinačni ECM ligandi: kolagen i laminin te derivati caklinskog matriksa (EMD-a) pomažu dobro priljubljeno diferenciranje u osteogeni fenotip već nakon 48 sati, što je premašilo staklo i BSA. To pokazuje da ti supstrati pozitivno djeluju na staničnu adheziju i sazrijevanje.

Multivarijantna logistička regresija je kod svih ispitanih stanica dodatno potvrdila te nalaze (bez obzira na staničnu liniju) s najvećim stupnjem sazrijevanja za EMD (Eksp(B) EMD: 2,491, Eksp(B) laminin: 2,181, Eksp(B) kolagen: 1,745). Naši se rezultati slažu s onima iz literature (8, 9, 20, 27-29, 31). Osim toga, u našem radu su ispitivani ligandi pokazali da potiču i staničnu pokretljivost, premda kod EMD-a u manjoj mjeri nego kod kolagena i laminina (7). Činjenica da je stanična migracija obično prije diferencijacije (44), podupire pretpostavku da osteogene stanice na EMD-u predstavljaju više diferencirane stanice, a time i manje pokretnu subpopulaciju.

Bez obzira na ispitivane ligande, multivarijantna logistička regresija otkrila je da linija "K" općenito ima najmanje zreo osteogeni fenotip, a linije "N" i "P" pokazuju naveću tendenciju prema zrelim osteoblastima. Ti nalazi podupiru hipotezu da se diferencirani učinci EMD-a mogu povezati sa stanjem sazrijevanja istraživane staničnе linije (49) i ističu potrebu za poznavanjem intrinzičnih svojstava stanica za interpretaciju rezultata kultura stanica (50).

Pažljivo prenošenje naših *in vitro* podataka u *in vivo* kliničke situacije pokazuje potencijal istraživanih liganada u podupiranju parodontne regeneracije, te ističe osteogeni stanični pričvrstak i sazrijevanje

gy and cytoskeletal architecture into three subtypes, reflecting the stage of cell maturation.

A drawback of the utilised methodology is the subjective observer-dependence. Furthermore, the presupposed 100% inter-observer concordance resulted in an exclusion of a larger cell population (943 cells or 39.7%) from further statistical analysis. Computer-based, automated algorithms to assess and analyse cell morphology and cytoskeletal organisation have already been established (47, 48) and might be an useful completion of this trial. However, such automated algorithms demand high processing standards have been proven to be vulnerable to surface-, fixation- and processing artefacts. The semi-automated analysis routine described in the present study was able to compensate such artefacts by the observer's abstract opinion of shape and colour.

Our results show for the investigated cell lines "N", "S" and "T" that the solitaire ECM ligands collagen and laminin as well as enamel matrix derivative (EMD) promote a well-attached, differentiated osteogenic phenotype after 48 hours, which exceeded glass and BSA. This indicates a promotion of cell adhesion and cell maturation for these substrates.

Multivariate logistic regression of all investigated cells (regardless cell line) additionally underlined these findings with highest cell maturation for EMD (Exp(B) EMD: 2.491, Exp(B) laminin: 2.181, Exp(B) collagen: 1.745). Our results are concordant with current literature (8, 9, 20, 27-29, 31). Furthermore, in a recent observation by our group, the investigated ligands proved to promote osteogenic cell motility as well, however for EMD to a lower extend than for collagen and laminin (7). The fact that cell migration generally precedes cell differentiation (44) underlines the assumption that the osteogenic cells on EMD represent a more differentiated and therefore less mobile subpopulation.

Regardless the investigated ligands, multivariate logistic regression revealed for line "K" generally the least mature osteogenic phenotype, whereas lines "N" and "P" showed the highest tendencies towards mature osteoblasts. These findings support the hypothesis that differential effects of EMD can be well related to the maturation state of the investigated cell line (49) and emphasizes the need of knowledge of intrinsic cell properties for the interpretation of cell culture results (50).

A careful translation of our *in vitro* data into the *in vivo* clinical situation highlights the potential of the investigated ligands to support periodontal re-

na mjestu oštećenja. To se odnosi kako na pročišćeni EMD tako i na primjenu kolagenih membrana te potvrđuje dosadašnje kliničke spoznaje (5, 18).

Zaključak

Prve naznake biokompatibilnosti i bioaktivacije različitih supstrata mogu se zapaziti tijekom analize stanične morfologije i organizacije citoskeleta dobivene iz jednostavnih operativnih postupaka. Ako prihvatimo djelomično heterogene rezultate za različite istraživane stanične linije, naša zapažanja jasno upućuju na potencijal kako pojedinačnih ECM liganada (kolagen tipa I, laminin-1), tako i EMD-a u podupiranju osteogene stanične adhezije i sazrijevanja. Rezultatima se možemo koristiti kao osnovom za daljnja opsežna istraživanja *in vitro* i *in vivo*.

generation by enhancing osteogenic cell attachment and maturation at the defect site. This applies to both the use of purified EMD as well as the employment of occlusive collagen membranes and supports respective clinical observations (5, 18).

Conclusion

First indications of biocompatibility and bio-activation of different substrates can be observed using analysis of cell morphology and cytoskeletal organisation derived from low operative intense procedures. Accepting the partly heterogeneous results for the different investigated cell lines, our findings clearly indicate the potential of both solitaire ECM ligands (collagen type 1, laminin-1) as well as EMD to promote osteogenic cell adhesion and maturation. The obtained results might serve as basis for further detailed *in vitro* and *in vivo* studies.

Abstract
The precondition for successful periodontal regeneration is adequate activation of relevant cell populations like osteogenic cells. Here, cell adhesion and maturation are closely associated with cell morphology and f-actin cytoskeletal organisation. The potential of solitaire extracellular matrix (ECM) components as well as enamel matrix derivative (EMD) to enhance periodontal healing is well documented. **Objective:** The aim of the study was to test the impact of the ECM proteins collagen type 1 and laminin-1 as well as commercially available EMD on osteogenic cell morphology and cytoskeletal organisation. **Material and methods:** In an observational study, a total of 2450 osteogenic cells of 5 different cell lines (4 primary ones and 1 commercial one) cultivated on the respective substrates were analysed by 3 independent observers. After staining for the f-actin cytoskeleton and automated CLSM visualisation, cells were assigned to 3 different categories depending on morphological cell attributes (immature vs. intermediate vs. mature). Besides descriptive analysis, a multivariate logistic regression was performed to identify relevant influence parameters on cell morphology and cytoskeletal organisation. **Results:** The applied solitaire ligands collagen and laminin and especially EMD promoted a mature osteogenic phenotype. Nevertheless, considerable differences between the investigated cell lines could be identified as well. Analysis of cell morphology and cytoskeletal organisation offers a reliable method of acquiring the first hints of biocompatibility and bio-activation on different substrates. **Conclusion:** Our results highlight the potential of the investigated ligands to support periodontal regeneration by enhancing osteogenic cell attachment and maturation.

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References

1. Davies JE, Hosseini MM. Histodynamics of endosseous wound healing. In: Davies JE, editor. Bone engineering. Toronto: em squarred inc.; 1999. p. 1-14.
2. Cortellini P, Pini Prato G, Tonetti MS. Periodontal regeneration of human infrabony defects. I. Clinical measures. J Periodontol. 1993;64(4):254-60.
3. Sculean A, Chiantella GC, Windisch P, Arweiler NB, Brecx M, Gera I. Healing of intra-bony defects following treatment with a composite bovine-derived xenograft (Bio-Oss Collagen) in combination with a collagen membrane (Bio-Gide PERIO). J Clin Periodontol. 2005;32(7):720-4.
4. Sculean A, Schwarz F, Miliauskaite A, Kiss A, Arweiler N, Becker J, et al. Treatment of intrabony defects with an enamel matrix protein derivative or bioabsorbable membrane: an 8-year follow-up split-mouth study. J Periodontol. 2006;77(11):1879-86.
5. Sculean A, Nikolidakis D, Schwarz F. Regeneration of periodontal tissues: combinations of barrier membranes and grafting materials - biological foundation and preclinical evidence: a systematic review. J Clin Periodontol. 2008;35(8 Suppl):106-16.
6. Tonetti MS, Prato GP, Cortellini P. Factors affecting the healing response of intrabony defects following guided tissue regeneration and access flap surgery. J Clin Periodontol. 1996;23(6):548-56.

7. Klein MO, Reichert C, Koch D, Horn S, Al-Nawas B. In vitro assessment of motility and proliferation of human osteogenic cells on different isolated extracellular matrix components compared with enamel matrix derivative by continuous single-cell observation. Clin Oral Implants Res. 2007;18(1):40-5.
8. Roche P, Goldberg HA, Delmas PD, Malaval L. Selective attachment of osteoprogenitors to laminin. Bone. 1999;24(4):329-36.
9. Liu G, Hu YY, Zhao JN, Wu SJ, Xiong Z, Lu R. Effect of type I collagen on the adhesion, proliferation, and osteoblastic gene expression of bone marrow-derived mesenchymal stem cells. Chin J Traumatol. 2004;7(6):358-62.
10. Timpl R, Brown JC. The laminins. Matrix Biol. 1994;14(4):275-81.
11. Boskey AL, Paschalis E. Matrix proteins and biomineralization. In: Davies JE, editor. Bone engineering. Toronto: em squarred incorporated; 1999. p. 44-63.
12. Cortellini P, Tonetti MS. A minimally invasive surgical technique with an enamel matrix derivative in the regenerative treatment of intra-bony defects: a novel approach to limit morbidity. J Clin Periodontol. 2007;34(1):87-93.
13. Heijl L, Heden G, Svärdström G, Ostgren A. Enamel matrix derivative (EMDOGAIN) in the treatment of intrabony periodontal defects. J Clin Periodontol. 1997;24(9 Pt 2):705-14.
14. Pietruska MD. A comparative study on the use of Bio-Oss and enamel matrix derivative (Emdogain) in the treatment of periodontal bone defects. Eur J Oral Sci. 2001;109(3):178-81.
15. Sculean A, Schwarz F, Becker J, Brecx M. The application of an enamel matrix protein derivative (Emdogain) in regenerative periodontal therapy: a review. Med Princ Pract. 2007;16(3):167-80.
16. Sculean A, Schwarz F, Chiantella GC, Arweiler NB, Becker J. Nine-year results following treatment of intrabony periodontal defects with an enamel matrix derivative: report of 26 cases. Int J Periodontics Restorative Dent. 2007;27(3):221-9.
17. Tonetti MS, Lang NP, Cortellini P, Suvan JE, Adriaens P, Dubravec D, et al. Enamel matrix proteins in the regenerative therapy of deep intrabony defects. J Clin Periodontol. 2002;29(4):317-25.
18. Trombelli L, Farina R. Clinical outcomes with bioactive agents alone or in combination with grafting or guided tissue regeneration. J Clin Periodontol. 2008;35(8 Suppl):117-35.
19. Bègue-Kirn C, Krebsbach PH, Bartlett JD, Butler WT. Dentin sialoprotein, dentin phosphoprotein, enamelysin and ameloblastin: tooth-specific molecules that are distinctively expressed during murine dental differentiation. Eur J Oral Sci. 1998;106(5):963-70.
20. Bosshardt DD. Biological mediators and periodontal regeneration: a review of enamel matrix proteins at the cellular and molecular levels. J Clin Periodontol. 2008;35(8 Suppl):87-105.
21. Bosshardt DD, Nanci A. Hertwig's epithelial root sheath, enamel matrix proteins, and initiation of cementogenesis in porcine teeth. J Clin Periodontol. 2004;31(3):184-92. 22. Fincham AG, Moradian-Oldak J, Simmer JP. The structural biology of the developing dental enamel matrix. J Struct Biol. 1999;126(3):270-99.
23. Veis A. Amelogenin gene splice products: potential signaling molecules. Cell Mol Life Sci. 2003;60(1):38-55.
24. Sculean A, Kiss A, Miliauskaite A, Schwarz F, Arweiler NB, Hannig M. Ten-year results following treatment of intrabony defects with enamel matrix proteins and guided tissue regeneration. J Clin Periodontol. 2008;35(9):817-24.
25. Shimizu-Ishiura M, Tanaka S, Lee WS, Debari K, Sasaki T. Effects of enamel matrix derivative to titanium implantation in rat femurs. J Biomed Mater Res. 2002;60(2):269-76.
26. Donos N, Sculean A, Glavind L, Reich E, Karring T. Wound healing of degree III furcation involvements following guided tissue regeneration and/or Emdogain. A histologic study. J Clin Periodontol. 2003;30(12):1061-8.
27. Guida L, Annunziata M, Carinci F, Di Feo A, Passaro I, Oliva A. In vitro biologic response of human bone marrow stromal cells to enamel matrix derivative. J Periodontol. 2007;78(11):2190-6.
28. Schwarz F, Rothamel D, Herten M, Sculean A, Scherbaum W, Becker J. Effect of enamel matrix protein derivative on the attachment, proliferation, and viability of human SaOs(2) osteoblasts on titanium implants. Clin Oral Investig. 2004;8(3):165-71.
29. Rincon JC, Xiao Y, Young WG, Bartold PM. Enhanced proliferation, attachment and osteopontin expression by porcine periodontal cells exposed to Emdogain. Arch Oral Biol. 2005;50(12):1047-54.
30. Schwartz Z, Carnes DL Jr, Pulliam R, Lohmann CH, Sylvia VL, Liu Y, et al. Porcine fetal enamel matrix derivative stimulates proliferation but not differentiation of pre-osteoblastic 2T9 cells, inhibits proliferation and stimulates differentiation of osteoblast-like MG63 cells, and increases proliferation and differentiation of normal human osteoblast NHOst cells. J Periodontol. 2000;71(8):1287-96.
31. He J, Jiang J, Safavi KE, Spångberg LS, Zhu Q. Emdogain promotes osteoblast proliferation and differentiation and stimulates osteoprotegerin expression. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2004;97(2):239-45.
32. Carpenter CL. Actin cytoskeleton and cell signaling. Crit Care Med. 2000;28(4 Suppl):N94-9.
33. Yamada KM. Integrin signaling. Matrix Biol. 1997;16(4):137-41.
34. Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, et al. Cell migration: integrating signals from front to back. Science. 2003;302(5651):1704-9.
35. Zamir E, Geiger B. Components of cell-matrix adhesions. J Cell Sci. 2001;114(Pt 20):3577-9.
36. Pienta KJ, Coffey DS. Nuclear-cytoskeletal interactions: evidence for physical connections between the nucleus and cell periphery and their alteration by transformation. J Cell Biochem. 1992;49(4):357-65.
37. Kim HJ, Kim SH, Kim MS, Lee EJ, Oh HG, Oh WM, et al. Varying Ti-6Al-4V surface roughness induces different early morphologic and molecular responses in MG63 osteoblast-like cells. J Biomed Mater Res A. 2005;74(3):366-73.
38. Park BS, Heo SJ, Kim CS, Oh JE, Kim JM, Lee G, et al. Effects of adhesion molecules on the behavior of osteoblast-like cells and normal human fibroblasts on different titanium surfaces. J Biomed Mater Res A. 2005;74(4):640-51.
39. El-Amin SF, Lu HH, Khan Y, Burems J, Mitchell J, Tuan RS, et al. Extracellular matrix production by human osteoblasts cultured on biodegradable polymers applicable for tissue engineering. Biomaterials. 2003;24(7):1213-21.
40. Klein MO, Tapety F, Sandhöfer F, Götz H, Schäfer B, Wagner W, et al. Visualization of vital osteogenic cells on differently treated titanium surfaces using Confocal Laser Scanning Microscopy (CLSM) – a pilot study. Z Zahnärztl Impl. 2006(2):145-53.
41. Shah AK, Lazatin J, Sinha RK, Lennox T, Hickok NJ, Tuan RS. Mechanism of BMP-2 stimulated adhesion of osteoblastic cells to titanium alloy. Biol Cell. 1999;91(2):131-42.

42. Sinha RK, Morris F, Shah SA, Tuan RS. Surface composition of orthopaedic implant metals regulates cell attachment, spreading, and cytoskeletal organization of primary human osteoblasts in vitro. Clin Orthop Relat Res. 1994;(305):258-72.
43. Titushkin I, Cho M. Modulation of cellular mechanics during osteogenic differentiation of human mesenchymal stem cells. Biophys J. 2007;93(10):3693-702.
44. Aubin JE. Advances in the osteoblast lineage. Biochem Cell Biol. 1998;76(6):899-910.
45. Aubin JE. Bone stem cells. J Cell Biochem Suppl. 1998;30-31:73-82.
46. Malaval L, Liu F, Roche P, Aubin JE. Kinetics of osteoprogenitor proliferation and osteoblast differentiation in vitro. J Cell Biochem. 1999;74(4):616-27.
47. Krtolica A, Ortiz de Solorzano C, Lockett S, Campisi J. Quantification of epithelial cells in coculture with fibroblasts by fluorescence image analysis. Cytometry. 2002;49(2):73-82.
48. Lichtenstein N, Geiger B, Kam Z. Quantitative analysis of cytoskeletal organization by digital fluorescent microscopy. Cytometry A. 2003;54(1):8-18.
49. Dean DD, Lohmann CH, Sylvia VL, Cochran DL, Liu Y, Boyan BD, et al. Effect of porcine fetal enamel matrix derivative on chondrocyte proliferation, differentiation, and local factor production is dependent on cell maturation state. Cells Tissues Organs. 2002;171(2-3):117-27.
50. Balls M, Coecke S, Bowe G, Davis J, Gstraunthaler G, Hartung T, et al. The Importance of Good Cell Culture Practice (GCCP). ALTEX. 2006;23 Suppl:270-3.