Univerza v Mariboru

MEDICINSKA FAKULTETA

Doktorska disertacija

UČINEK RASTNIH FAKTORJEV IZ AVTOLOGNE TROMBOCITNE PLAZME IN ANTAGONISTOV TGF-β NA PROLIFERACIJO IN DIFERENCIACIJO SKELETNOMIŠIČNIH CELIC

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Mentor: doc. dr. Matjaž Vogrin

Somentor: prof. dr. Marjan Slak Rupnik

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KAZALO TABEL

KAZALO SLIK

UČINEK RASTNIH FAKTORJEV IZ AVTOLOGNE TROMBOCITNE PLAZME IN ANTAGONISTOV TGF-β NA PROLIFERACIJO IN DIFERENCIACIJO SKELETNO-MIŠIČNIH CELIC

POVZETEK

Uvod

Regeneracija skeletne mišice po poškodbi je omejena s tvorbo brazgotinskega tkiva, počasnim celjenjem in relativno visoko verjetnostjo ponovitve poškodbe. Terapija, ki temelji na pripravkih avtolognih trombocitnih preparatov (*angl. platelet-rich plasma*; PRP), je v zadnjem času postala izjemno popularna, predvsem v primeru poškodb sklepnih vezi in mišičnih tetiv. Za zdravljenje mišičnih poškodb se PRP še ne uporablja, poglavitni pomislek je, da med ostalim vsebuje tudi rastni faktor TGF-β, ki je pomemben dejavnik brazgotinjenja v skeletnomišičnem tkivu.

V raziskavi smo želeli prirediti učinek PRP-ja za uporabo na skeletnem mišičju. Učinek rastnega faktorja TGF-β smo zavirali z njegovim zaviralcem s ciljem izločitve potencialno neželenega učinka v procesu mišične regeneracije.

Materiali in metode

Zaradi pomanjkanja znanstvenih temeljev o terapiji z avtologno trombocitno plazmo smo zasnovali predklinično raziskavo, v kateri smo uporabili humano, CD56-pozitivno mioblastno celično linijo. Po dodajanju PRP-ja, dekorina (zaviralca TGF-β) in njune kombinacije v gojilni medij smo preučevali stopnjo proliferacije mioblastov, njihove metabolne aktivnosti, profil izražanja fibrotičnih citokinov in ekspresijo miogenih regulatornih faktorjev. Z uporabo slikovne pretočne citometrije smo analizirali razmerje med hibernirajočimi, aktiviranimi in diferencirajočimi mioblasti ter rezultate primerjali med posameznimi skupinami. Dodatno smo opravili še vizualno analizo izražanja dezmina z uporabo visokoločljivostnega konfokalnega mikroskopa ter ugotavljali stopnjo formacije večjedrnih miotubulov.

Rezultati

Ugotovili smo, da pride v mioblastnih celičnih kulturah pod vplivom PRP-ja do značilnega znižanja izražanja fibrotičnih citokinov, povečanja stopnje proliferacije in viabilnosti celic. Obenem pride prav tako do povečane ekspresije miogenih regulatornih faktorjev, kar pripomore k pomembnemu pomiku v miogeni diferenciaciji mioblastov. V kombinaciji PRP-ja z dekorinom smo zaznali pomembne dodatne sinergistične učinke.

Razprava

Izsledki raziskave dokazujejo, da v pogojih *in vitro* PRP ne samo potencialno znižuje brazgotinjenje, temveč tudi pripomore k učinkovitejši mišični regeneraciji, še posebej v kombinaciji z zaviralcem TGF-β.

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EFFECT OF GROWTH FACTORS RELEASED FROM AUTOLOGOUS PLATELET-RICH PLASMA AND TGF-β ANTAGONISTS ON SKELETAL CELL MUSCLE PROLIFERATION AND DIFFERENTIATION

ABSTRACT

Introduction

Regeneration of skeletal muscle after injury is limited by scar formation, slow healing time, and a high recurrence rate. A therapy based on platelet-rich plasma (PRP) became a promising lead for tendon and ligament injuries in recent years, however concerns have been raised that PRP derived TGF-β could contribute to fibrotic remodelling in skeletal muscle after injury.

In our study we wanted to customize the effects of PRP for use in therapy of muscle injuries by blocking the activity of TGF-β with its inhibitor.

Methods

Due to the lack of scientific grounds for a PRP-based therapy, we have designed a pre-clinical study using a *human* CD56 positive myoblast cell line and evaluated the potential of PRP both alone and in combination with decorin (a TGF-β inhibitor), to alter myoblast proliferation, metabolic activity, cytokine profile, and expression of myogenic regulatory factors. Imaging flow-cytometry enabled us to create a valuable picture on the ratio of quiescent, activated and terminally committed myoblasts in treated versus control cell populations. Finally high-resolution confocal microscopy revealed the potential of PRP and decorin to stimulate the formation of polynucleated myotubes.

Results

PRP has been shown to down-regulate fibrotic cytokines, increase cell viability and proliferation, enhance expression of myogenic regulatory factors, and contribute to a significant myogenic shift during differentiation. When combined with decorin further synergistc effects have been identified.

Discussion

Our results suggest that *in vitro* PRP could not only prevent fibrosis but also improve muscle regeneration, especially when combined with a TGF-β inhibitor decorin.

UDK: 616.74-003.93+576.385.5:615.35(043.3)

KRAJŠAVE

KRAJŠAVA	POMEN
ACD-A	kisla citronska dekstroza (angl. acid citric dextrose anticoagulant)
ActIIA in B	aktivinski receptor tipa I in II
ALK	aktivinska receptorju podobna kinaza (angl. activin receptor-like kinase)
AT II	angiotenzin II
BMP	kostne morfogenetske beljakovine (angl. bone morphogenetic proteins)
COX	ciklooksigenaza (angl. cyclooxygenase)
CPC	cement kalcijevega fosfata (angl. calcium phosphate cement)
DCN	dekorin (angl. decorin)
DLC	diamantu podoben ogljik (angl. diamond-like carbon)
DMR	dejavnik miogene regeneracije
DNK	deoksiribonukleinska kislina
EDTA	etilendiamintetraocetna kislina
ERK	signalno uravnavane kinaze (angl. signal-regulated kinases)
ET-1	endotelin-1
EU	Evropska unija
FLRG	s folistatinom povezani gen (angl. follistatin-related gene)
FOTM	miši s prekomernim izražanjem folistatina (angl. follistatin-overexpressing transgenic mice)
FSH	folikle stimulirajoči hormon
GDF	diferenciacijski rastni faktor (angl. growth differentiation factor)
IFN-γ	interferon gama
IGF-1	inzulinu podoben rastni faktor 1 (angl. insuline-like growth factor 1)
JNK	c-Jun N-terminalne kinaze
KOPB	kronična obstruktivna pljučna bolezen
LAP	z latenco povezani peptid (angl. latency-associated peptide)

LTBP	latentni transformirajoči protein (angl. latent transforming growth factorbinding protein)
M6P	manoza-6-fosfat
MAPK	z mitogenom aktivirana protein kinaza (angl. mitogen-activated protein kinase)
MRF	regulatorni dejavniki miogeneze (angl. myogenic regulatory factors)
MRS	magnetnoresonančna spektroskopija
MSTN	miostatin
MTT	3-(4,5-dimetiltiazol-2-Yl)-2,5-difeniltetrazolijev bromid
Myf-5	miogeni faktor 5
MyoD	MyoD, dejavnik miogene regeneracije
NAC	N-acetilcistein
Pax3	gen iz Paired boX družine
PRP	s trombociti bogata plazma (angl. platelet-rich plasma)
RICE	mirovanje, hlajenje, kompresija, dvig uda (angl. rest, ice, compression elevation)
TAK	s TGF-β-aktivirane kinaze
TbR	TGF-β receptor (I in II)
TGF-β	transformirajoči rastni faktor β (angl. transforming Growth Factor β)
TRAF	faktor, povezan z receptorjem tumorje nekrotizirajočega faktorja (angl. tumor necrosis factor receptor-associated factor)
VEGF	žilni endotelni rastni faktor (angl. vascular endothelial growth factor)
α-SMA	alfa gladkomišični aktin (angl. alpha smooth muscle actin)
β-TCP	β-trikalcijev fosfat (angl. β-tricalcium phosphate)

1. UVOD

1.1. Skeletno mišičje

Skeletno mišičje lahko pri odraslem človeku predstavlja 40 % telesne teže in je tako eno izmed najobsežnejših tkiv v telesu (1). Mišice so v celoti ovite v zunanjo vezivno ovojnico – epimizij, ki v nekoliko bolj odebeljeni obliki ovija tudi mišične tetive in skrbi predvsem za zmanjševanje trenja med posameznimi mišicami.

Sama mišica, ki se na koncih preko mišičnih tetiv pripenja na koščene strukture, je sestavljena iz več mišičnih snopov oz. fasciklov (slika 1a), ovitih v perimizij - posebno vezivno ovojnico. Med njo in membrano mišičnih vlaken je plast zunajceličnega matriksa, ki tvori bazalno membrano ali lamino. Ta ima pomembno vlogo pri razvoju in regeneraciji skeletnih mišic (1).

Skeletnomišične celice so velike, večjedrne celice, ki nastanejo s fuzijo več enojedrnih prekurzorskih celic. Celična membrana se v primeru skeletnih mišic imenuje sarkolema, obdaja pa jo notranja mišična ovojnica - endomizij. Znotraj sarkoleme, v citoplazmi oz. sarkoplazmi, pa se nahajajo številne v miofilamente povezane kontraktilne beljakovine, ki se združujejo v vzdolžno potekajoče miofibrile (2).

1.2. Mehanizmi mišičnih poškodb

Mišične poškodbe so lahko posledica množice različnih vzrokov: poškodbe med športno in delovno aktivnostjo, poškodbe med medicinskimi posegi in poškodbe ostalih vzrokov. Glede na mehanizem nastanka jih delimo na neposredne in posredne. Med neposredne štejemo laceracije in kontuzije, med slednje pa popolna in nepopolna mišična pretrganja (3).

Aktualna klasifikacija mišičnih poškodb glede na klinično simptomatiko razlikuje med blagimi, zmernimi in resnimi poškodbami. Pri blagih poškodbah, kjer pride do pretrganja le majhnega števila mišičnih vlaken, sta značilna samo manjše otekanje in bolečina, brez izgube v funkciji mišice ali omejenosti gibanja. Pri zmernih poškodbah sta oteklina in bolečina večji, značilni pa sta tudi delna izguba funkcije in omejenost gibanja. Pri resnih, kjer gre za popolno prekinitev mišičnih vlaken vzdolž prečnega prereza mišice, pride do popolne izgube mišične funkcije (4,5). Mišična natrganja glede na resnost delimo v tri razrede (tabela 1) (2).

Razred	Klinična manifestacija
I	• pretrganje majhnega števila mišičnih vlaken z minimalnim otekanjem ter bolečino • minimalna izguba mišične moči brez omejenosti gibanja
II	• večja mišična poškodba • delna izguba mišične moči in omejenost gibanja
III	• popolno pretrganje preko celotnega prečnega preseka mišice • popolna izguba mišične funkcije

Tabela 1: Delitev mišičnih natrganj glede na resnost klinične slike. (3)

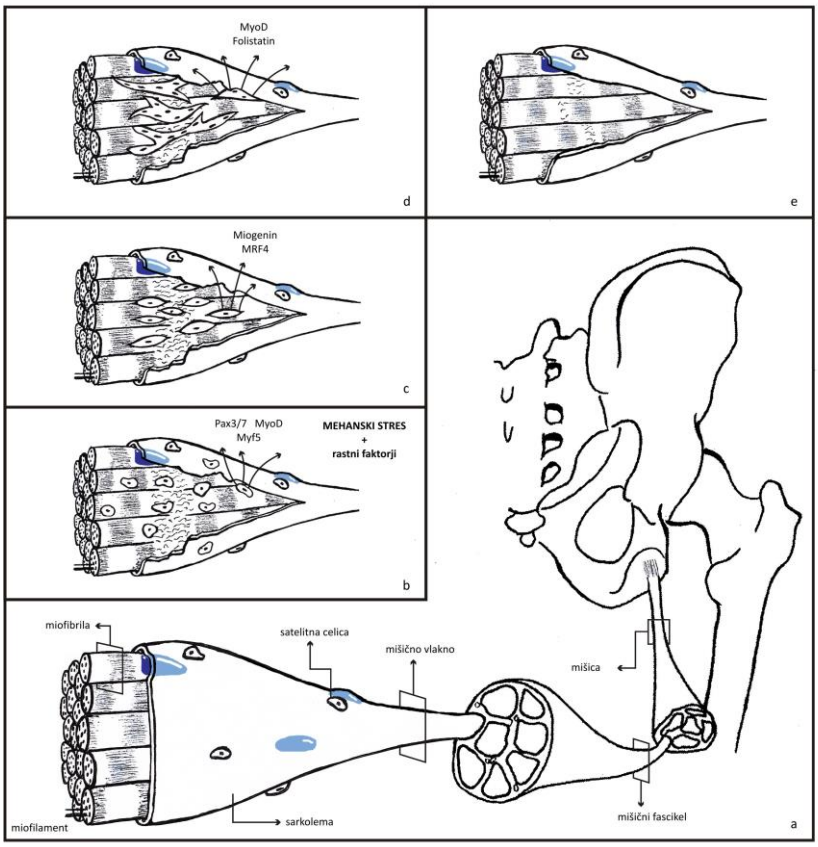
1.3. Patofiziološko dogajanje po mišični poškodbi

V zadnjih letih je prišlo do mnogih odkritij celičnih in molekularnih mehanizmov mišične regeneracije po poškodbi (6–8). Takoj po poškodbi pride med degeneracijo tkiva do porušenja homeostaze kalcija v poškodovanih vlaknih, razgradnje miofibril in sarkoleme. Ob tem ostaneta endomizij in bazalna membrana večinoma nepoškodovana in tvorita ti. endomizijski tulec, znotraj katerega kasneje potekajo procesi regeneracije (1).

Proces celjenja sestavlja zaporedje prekrivajočih se faz: (1) *hemostaza*, ki se navadno prične s tvorbo krvnega strdka in degranulacijo trombocitov; (2) *akutna vnetna faza* s kontrakcijo mišičnih vlaken, tvorbo edema in celično smrtjo; in (3) *remodelacijska faza*, v kateri pride do ponovne vzpostavitve tkivne arhitekture. V to fazo je vključenih mnogo različnih celic, predvsem pomembni pa so miofibroblasti, ki so odgovorni za tvorbo fibrotičnega tkiva.

Ker se pretrgana sarkolema hitro po poškodbi ponovno pričvrsti na endomizij, pride ob poškodbi le do lokalne nekroze poškodovanih mišičnih celic, medtem, ko ostali deli preživijo (9). Poškodovano tkivo odstranijo makrofagi, ki obenem tudi izločajo rastne dejavnike za aktivacijo satelitskih celic. Te so regenerativne enojedrne matične celice mišičnega tkiva, ki normalno ležijo med bazalno lamino in plazemsko membrano mišičnih vlaken (10). Najprej se preobrazijo v mioblaste, ki nato izločajo mišično-specifične proteine in končno dozorijo v mišična vlakna s periferno ležečimi jedri (7) (slika 1).

Poseben dogodek v procesu mišične diferenciacije je izražanje vrste genov in faktorjev miogeneze (11). Specifični dejavniki miogene regeneracije (DMR) se izražajo izključno v skeletnih mišicah in uravnavajo proces mišičnega razvoja (12) (slika 2b-d). Igrajo pomembno vlogo pri uravnavanju več drugih genov, ki so vključeni v proces miogeneze, od preobrazbe mezodermalnih celic v mišično linijo, do diferenciacije somatskih celic in končne diferenciacije miocitov v zrele miofibrile (13).



Slika 1: Vloga satelitskih celic pri mišični regeneraciji po poškodbi. (a) anatomija zdrave skeletne mišice z neaktivnimi satelitskimi celicami; (b) mehanski stres (poškodba) in sproščanje rastnih dejavnikov iz makrofagov povzročita aktivacijo satelitskih celic, ki pričnejo izražati proteine miogeneze – ti stimulirajo nadaljnjo proliferacijo; (c) mioblasti v zgodnji diferenciacijski fazi izražajo miogenin in MRF4 - faktorja, ki vzpodbujata nadaljnjo diferenciacijo in fuzijo enojedrnih celic; (d) večjedrni miotubuli pričnejo v pozni diferenciacijski fazi izražati faktorje, ki vplivajo na končno fuzijo in diferenciacijo v miotubule ter zrele miofibrile; (e) kljub samoregenerativni zmožnosti mišičnega tkiva je del končnega izida tudi fibroza, ki je vzrok delne izgube mišične funkcije.

DMR so prepisovalni dejavniki, ki jih delimo v dve funkcionalni skupini, primarne in sekundarne. Primarna DMR, MyoD in Myf-5, sta ključna za determinacijo skeletnih mioblastov. Sekundarna DMR, miogenin in MRF4, delujeta kasneje v poteku regeneracije, najverjetneje kot diferenciacijska faktorja (12). Aktivirane satelitske celice sprva izražajo le MyoD oziroma le Myf5, čemur sledi koekspresija obeh. Po proliferacijski fazi se z izražanjem miogenina in MRF4 prične faza diferenciacije (11,14).

Kljub temu, da so skeletne mišice sposobne samoregeneracije, je proces celjenja navadno nepopoln. Nepopolna regeneracija ima za posledico izgubo mišične funkcije in večje tveganje za ponovitev poškodbe na mestu prve poškodbe (15,16). Proces mišičnega celjenja namreč vključuje kompleksno ravnovesje med regeneracijo mišičnih vlaken in tvorbo brazgotinskega tkiva (6).

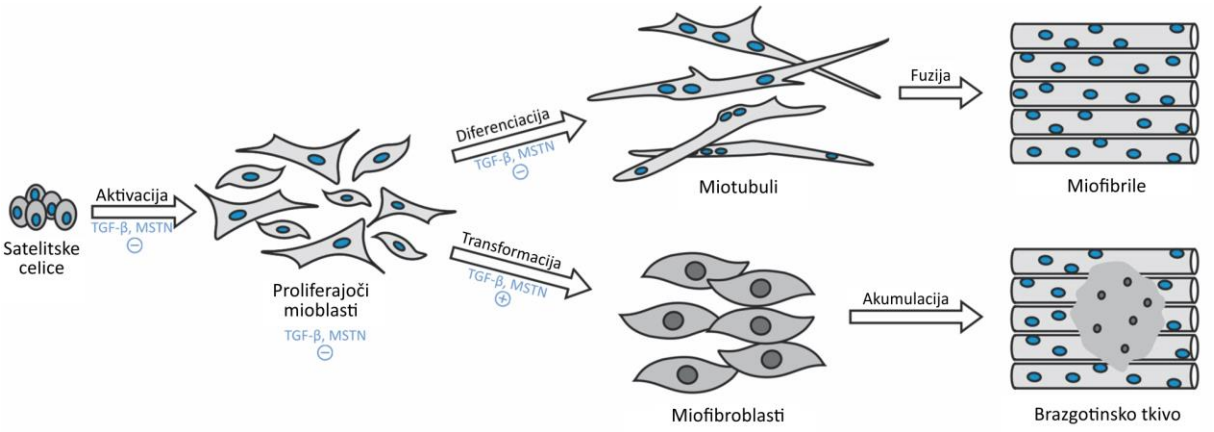
1.4. Tvorba brazgotinskega tkiva

Faktorji TGF-β (*angl. Transforming Growth Factor*-β) predstavljajo družino citokinov, ki ima številne biološke aktivnosti pri celjenju ran. Biološke aktivnosti TGF- β so povzete v tabeli 2. Tri glavne izooblike, ki jih najdemo pri sesalcih, TGF-β1, TGF-β2, in TGF-β3, lahko izloča večina celic, ki so aktivno udeležene pri celjenju ran, pri čemer so najpomembnejše celice trombociti (17).

Biološka aktivnost TGF-β
Vzpodbujanje proliferacije mezenhimskih celic
Uravnavanje aktivnosti endotelijskih celic in fibroblastov
Pospeševanje tvorbe zunajceličnega matriksa
Vzpodbujanje endotelijske kemotakse in angiogeneze
Zaviranje proliferacije makrofagov in limfocitov
Zaviranje diferenciacije satelitskih celic

Tabela 2: Aktivnost TGF-β, povzeto po Borrione in sodelavci (3)

Medtem, ko je med raziskovalci že široko sprejeto dejstvo, da je TGF-β močan aktivator fibroze v ledvicah, jetrih, srcu in pljučih (18–20), se je v zadnjem času podobno pokazalo tudi za njegovo aktivnost pri celjenju poškodovane skeletne mišice. Igra namreč pomembno vlogo pri tvorbi fibroze in indukciji miofibroblastne diferenciacije miogenih celic (21,22) (slika 2). Številna poročila kažejo na povečano tvorbo TGF-β1 kot odziv na poškodbo ali bolezen. Posledica povečane tvorbe tega citokina naj bil tudi glavni vzrok tkivne fibroze (18,21).



Slika 2: Proces regeneracije skeletne mišice. Med regeneracijo pride do aktivacije satelitskih celic, ki se diferencirajo v mioblaste. Ti proliferirajo in se bodisi diferencirajo naprej v večjedrne miotubule, bodisi transformirajo v miofibroblaste. V regulaciji teh procesov imata pomembno vlogo rastna faktorja TGF-β in miostatin (MSTN) (označeno s +/-).

V številnih raziskavah, kjer so zavirali aktivnosti TGF-β se je pokazalo, da to lahko vodi v zmanjšanje satelitskih celic, zmanjšanje nalaganja kolagena in posledično zmanjšanje tkivnega brazgotinjenja. Uporaba zaviralcev TGF-β pri incizijskih ranah pri podganah se je izkazala za učinkovito proti kožnemu brazgotinjenju (11). Vendar pa še ni popolnoma jasno, ali TGF-β deluje sam ali so za razvoj mišične fibroze potrebni še kakšni drugi dejavniki.

Nedavne raziskave so pokazale, da naj bi bil v razvoj mišične fibroze vpleten tudi miostatin (MSTN) (23,24) (slika 2). V zadnjih letih je zanimanje za ta rastni faktor, ki ga prav tako uvrščamo v družino faktorjev TGF-β, izrazito poraslo, saj naj bi imel pomembne učinke na mišično presnovo in uravnavanje velikosti mišičnih vlaken kot odziv na različna fiziološka in patofiziološka stanja (25,26). MSTN, imenovan tudi GDF8 (*angl. Growth Differentiation Factor 8*) zavira rast in diferenciacijo mišičnih vlaken (27) ter se izraža specifično v razvijajočih se in zrelih skeletnomišičnih celicah (25). Preko uravnavanja mobilnosti makrofagov zavira aktivnost satelitskih celic med mišično regeneracijo in prav tako zavira razmnoževanje mioblastov ter njihovo diferenciacijo (28,29). V razvijajočih se mišičnih celicah miostatin znižuje tvorbo MyoD, zgodnjega označevalca mišične regeneracije, kot tudi izražanje genov Pax3 in Myf5, ki kodirata transkripcijske regulatorje celične proliferacije (30). Glavne funkcije miostatina so povzete v tabeli 3.

Glavne biološke funkcije miostatina
Zaviranje aktivnosti satelitskih celic
Kontrola gibanja makrofagov
Zniževanje koncentracije MyoD
Zaviranje proliferacije transkripcijskih regulatorjev
Zaviranje proliferacije in diferenciacije mioblastov
Uravnavanje velikosti mišičnih vlaken

Tabela 3: Biološka aktivnost miostatina

Izguba gena za miostatin zaradi naravnih mutacij se pri govedu, psih in ljudeh pokaže predvsem v povečani mišični masi (31). Jarvninen je s sodelavci leta 2007 prav tako poročal o učinku uporabe nevtralizirajočega monoklonalnega protitelesa na povečanje mišične mase pri miših brez drugih pomembnih stranskih učinkov (9). Ta metoda se je v nedavnih kliničnih preizkušanjih izkazala za varno, vendar pa je višina odmerka zaenkrat odvisna od ravni kožne preobčutljivosti (32).

Blokiranje signalne transdukcijske poti MSTN s specifičnimi zaviralci in gensko manipulacijo je pokazalo izrazit učinek povišanja mišične mase (33,34). Zaviranje te signalne poti se v principu lahko doseže na tri različne farmakološke načine: blokiranje genskega izražanja MSTN (»knocking out«, inaktivacija MSTN gena z »antisense« tehnologijo z virusom); blokiranje sinteze proteina MSTN; in blokiranje receptorja za MSTN (majhne molekule, specifična blokirajoča protitelesa) (35).

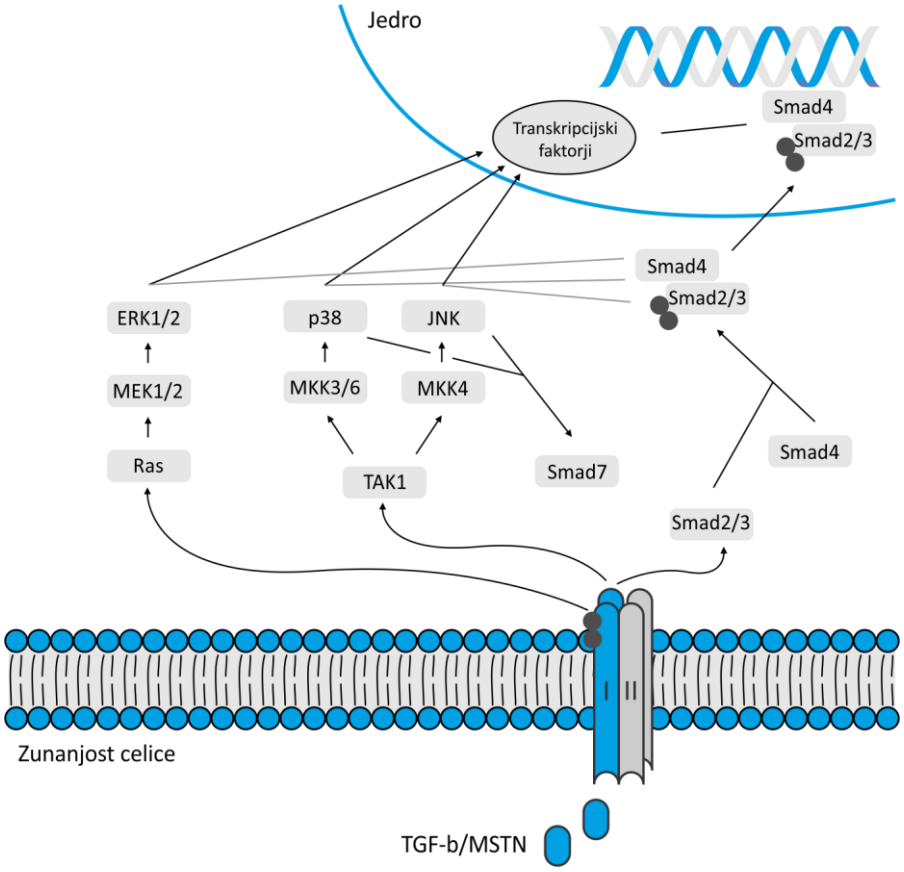
1.5. Signalizacijske poti TGF-β

Družina TGF-β igra ključno vlogo v normalni fiziologiji in patogenezi številnih tkiv. Glede na širokopaletne učinke v različnih tkivih lahko disregulacija signalizacijskih kaskad TGF-β vodi do razvojnih defektov ali bolezni (36). Za mnoge citokine iz družine TGF-β je bilo dokazano, da igrajo pomembno vlogo pri regulaciji mišične rasti in atrofije, med njimi pa zaenkrat najbolje razumemo delovanje tistih, ki imajo pomemben vpliv na skeletno mišičje: TGF-β1, z mitogeni aktivirane proteinske kinaze (angl. mitogen-activated protein kinase – MAPK) in MSTN (37).

Družina citokinov TGF-β sestoji iz različnih signalnih molekul, vključno z izooblikami TGF-β (1 do 3), MSTN, kostnim morfogenim proteinom (angl. Bone morphogenic protein – BMP 1 do 20), rastnimi in diferenciacijskimi faktorji (angl. Growth and differentiation factors – GDF), aktivini (A in B), inhibini (A in B) in anti-Müllerjevim hormonom (36). TGF-β se v zunajceličnem prostoru vežejo na t.i. receptorje Smad na celični membani, ti pa signal prenesejo preko membrane v citoplazmo in nato preko efektorskih molekul v celično jedro. Glede na vključenost receptorjev Smad delimo citokine iz družine TGF-β v dve skupini: skupino TGF-β, katere signalizacija poteka preko efektorskih molekul Smad 2 in 3, in skupino BMP, katere signalizacija poteka preko Smad 1, 5 in 8. Družina TGF-β ima kot omenjeno pomembno vlogo v embriološkem razvoju, homeostazi zrelih tkiv in patogenezi različnih bolezni. Dokazano je bilo, da ima prav specifično vlogo pri uravnavanju proliferacije, diferenciacije, celične smrti, migracije, remodelacije zunajceličnega matriksa, funkciji imunskega sistema in invaziji tumorske rašče (38).

TGF-β1 nastane po tem, ko pride do znotrajceličnega cepljenja prekurzorja v neaktivni kompleks sestavljen iz zrelega dela TGF-β1 in prekurzorskega peptida, imenovanega z latenco povezani peptid (angl. latency-associated peptide – LAP) (39). Ta neaktivni kompleks TGF-β1-LAP tvori še večji kompleks z latentnim transformirajočim proteinom (angl. latent transforming growth factor binding protein – LTBP), ki direktno veže in sprošča TGF-β1 iz zunajeličnega matriksa. LTBP-4 specifično uravnava sekvestracijo in razpoložljivost TGF-β1 ter vezavo z njegovim receptorjem (40). Do cepljenja TGF-β1 pride zaradi delovanja proteaz, kot so plazmin, trombin, plazemske transaglutaminaze in endoglikozilaze oz. preko fizične interakcije LAP-ov z ostalimi beljakovinami (39). Do aktivacije pride zunaj celic, nakar se lahko TGF-β1 veže s svojima receptorjema tipa I (TbR-II) in tipa II (aktivinska receptorju podnobna kinaza (angl. activin receptor-like kinase – ALK) 5). Aktivni receptor tipa II fosforilira in aktivira receptor tipa I, kar povzroča direktno fosforilacijo proteinov Smad2 in/ali Smad3, ki pričnejo transdukcijo signala preko od katenina β odvisne signalne poti, t. i. kanonične kaskade (41) (slika 3). Po fosforilaciji receptorja Smad namreč pride do tvorbe kompleksa z znotrajceličnim

mediatorjem Smad4, ki se translocira v celično jedro in se direktno veže na DNK (38). K uravnavanju tega procesa pomembno vplivata tudi inhibitorna proteina Smad6 in 7. Smad7 je vključen v signalizaciji obeh skupin in tekmuje z receptorji Smad za interakcijo z receptorjem tipa I, medtem ko Smad6 sodeluje samo v signalizacijski poti BMP in tekmuje s proteinom Smad4 za vezavo na Smad1 (5).



Slika 3: Signalizacijske poti TGF-β1 in MSTN: Po tem, ko se TGF-β1 ali MSTN veže na receptor tipa I in II, pride do fosforilacije receptorja in posledično fosforilacije efektorjev v celični citoplazmi. V kanonični signalizacijski poti receptor tipa I fosforilira Smad2/3, ki nato veže Smad4 in se translocira v jedro, kjer deluje kot transkripcijski faktor. V nekanonični poti receptor tipa I fosforilira proteine vključene v aktivacijo kinaz MAPK, ki uravnavajo transkripcijske faktorje in proteine Smad, bodisi preko direktnih interakcij, bodisi posredno preko različnih proteinov (povzeto po Buijs s sodelavci in Lindsay s sodelavci (42,43)).

Signalizacijska pot TGF-β1 pa poteka tudi preko indukcije nekanonične signalizacijske poti, ki vključuje MAPK (slika 3). Družina MAPK sestoji iz izoform zunajceličnih signalno-uravnavanih kinaz (angl. signal-regulated kinases – ERKs), c-Jun N-terminalnih kinaz (JNK) in p38. Mehanizmi aktivacije MAPK, povzročene s strani TGF-β1 in posledični biološki procesi so celično specifični (44). Generalno gledano pa se v Smad signalizacijski poti receptor tipa I poveže z adapterskimi proteini, Shc in faktorjem povezanim z receptorjem za tumorje nekrotizirajoči faktor 6 (angl. tumor necrosis factor receptor-associated factor (TRAF 6), kar privede do aktivacije Ras in kinaze aktivirane s TGF-β 1 (TAK) in posledično ERK oz. JNK ter signalizacijske poti p38 (45). Potrebno je dodati, da lahko MAPK modulira tudi s TGF-β1-povzročeno signalizacijo in fosforilacijo Smad proteinov neodvisno od TGF-β1 ter prečne povezave med kanoničnimi in nekanoničnimi signalnimi potmi TGF-β (44,45) (Slika 3).

Signalizacija MSTN, ki se tipično izraža v skeletnem mišičju, prav tako poteka preko signalne poti TGF-β (46). MSTN nastaja kot prekurzorski protein, ki ga procesirajo furinske proteaze do nastanka propeptida. Po proteolitičnem dogajanju ostaja biološko aktivni MSTN nekovalentno vezan na propeptid, pri čemer slednji vzdržuje neaktivno, latentno stanje kompleksa (47,48). MSTN je uravnavan tudi s strani zunajceličnih vezavnih proteinov: folistatina, s folistatinom povezanega genskega proteina (angl. Follistatin-related gene – FLRG) in z rastnim in diferencirajočim faktorjem povezanega serumskega proteina 1 (angl. growth and differentiation factor-associated serum protein – GASP) (47,49–51). Ko aktivni MSTN ni vezan na propeptid ali vezavne proteine povzroča signalizacijo z vezavo na aktivinske receptorje tipa II, ActRIIA ali ActRIIB (52,53). Aktivacija receptorja tipa I (ALK5 in v manjšem obsegu ALK4) vodi v fosforilacijo receptorjev Smad2 in 3 (53). MSTN prav tako povzroča aktivacijo signalizacijske poti MAPK preko od Smad odvisnih in neodvisnih mehanizmov (54–56).

1.6. Zaviralci tvorbe brazgotinskega tkiva

Spontana regeneracija po poškodbi mišice je zaradi fibrotične infiltracije navadno počasen in nepopoln proces. To končno tudi omejuje proces obnavljanja in kontraktilno funkcijo mišice (6). Čim uspešnejše celjenje po poškodbi je izrazitega pomena za obnavljanje mobilnosti in kakovosti bolnikovega življenja. Medicina tako nosi relativno veliko breme odgovornosti po doseganju učinkovitejšega procesa mišične regeneracije, ki bi po eni strani pospešil in olajšal obnovo mišičnih vlaken, po drugi pa v največji meri omejil proces brazgotinjenja.

Navkljub klinični pomembnosti mišičnih poškodb, terapija pogosto ne sloni na trdnih znanstvenih temeljih, temveč na splošno sprejetem konzervativnem zdravljenju po principu RICE (Rest, Ice, Compression, Elevation), adjuvantnem fizioterapevtskem zdravljenju in občasno sočasnem predpisovanju nesteroidnih protivnetnih zdravil, katerih uporaba pa se je v zadnjem času izkazala za potencialno kontroverzno. Nesteroidna protivnetna zdravila naj bi namreč z zaviranjem vnetnega procesa še dodatno pripomogla k tkivnemu brazgotinjenju (57,58).

Odkritje in identifikacija DMR omogoča raziskovalcem nov vpogled v proces mišične regeneracije in spremljanje posameznih faz v tem procesu. Boljše razumevanje procesov mišične regeneracije je ključno pri iskanju novih molekularnih tarč ter snovanju molekularne terapije. Tako v *in vitro* kot *in vivo* poskusih je bilo pokazano, da lahko učinkovine z antifibrotičnim delovanjem pomembno vplivajo na zmanjšanje brazgotine, tako samostojno kot v kombinaciji različnih učinkovin. Različne učinkovine, vključno s folistatinom, dekorinom, suraminom, relaksinom, manozo-6-fosfatom, interferonom gama, zaviralci angiotenzinskih receptorjev in N-acetilcisteinom, so pokazale pomemben zaviralni učinek na TGF-β in miostatin in tako na razvoj fibroze in regeneracijo skeletnih mišic. Kljub temu, da še nobena

izmed učinkovin ni bila preizkušena na ljudeh, pa utegne v prihodnosti njihov učinek značilno spremeniti terapijo mišičnih poškodb.

1.6.1. Dekorin

Dekorin je proteoglikan in se nahaja v zunajceličnem matriksu vseh s kolagenom bogatih tkiv (59). Igra pomembno vlogo v uravnavanju bioaktivnosti različnih rastnih faktorjev, obenem pa deluje tudi kot direktna signalna molekula za različne celice (60). Znano je, da dekorin veže vse izooblike TGF-β in zavira njihovo aktivnost s sekvestriranjem v zunajceličnem matriksu (61) in s tem fosforilacijo receptorjev Smad. Opisan je bil tudi nevtralizirajoč učinek dekorina na miostatin tako v fibroblastih kot mioblastih, kot tudi njegova vloga pri celični proliferaciji in diferenciaciji zaradi vezave različnih rastnih faktorjev in interakcije s kolagenom, fibronektinom in trombospondinom ter posledičnim vplivom na brazgotinske procese (52). Fukushima je s sodelavci v svoji raziskavi dokazal negativni učinek dekorina na stimulirajoče lastnosti TGF-β na rast in diferenciacijo miofibroblastov *in vitro*, kar se je pokazalo z manjšim obsegom razvoja fibroze in boljšo mišično regeneracijo ter močjo (62). Nedavna poročila govorijo tudi o učinkovitosti injiciranega dekorina v natrgano mišico pri procesu celjenja in skoraj popolni obnovi mišične moči po poškodbi (21,62,63). Ob zmanjševanju obsega fibroze dekorin vzpodbuja celično diferenciacijo s povečanjem izražanja folistatina in genov vključenih v miogenezo (MyoD) (64). Dekorin tako predstavlja obetavno »novo« molekulo s pomembno vlogo v signalni poti TGF-β in miostatina (24,65,66).

1.7. Avtologna trombocitna plazma

Med raziskovalci je splošno sprejeto mnenje, da predstavlja razumevanje fizioloških mehanizmov celjenja na molekularni ravni jedro razvoja novih pristopov pri zdravljenju poškodb. Med novimi uveljavljenimi metodami za doseganje hitrejšega tkivnega celjenja se v zadnjih letih pojavlja koncept avtologne trombocitne plazme, katere bistvo predstavljajo avtologni koncentrirani trombociti, ki so najbogatejši vir rastnih faktorjev v telesu (67).

V prejšnjih poglavjih omenjene faze tkivnega celjenja potekajo v prisotnosti vrste citokinov in rastnih faktorjev, ki uravnavajo celično funkcijo preko neposrednih interakcij z zunajceličnimi deli transmembranskih celičnih receptorjev (68). Ker predstavljajo trombociti glavni vir rastnih faktorjev v krvnih strdkih, se je ideja njihovega koncentriranja uveljavila relativno hitro. V enostavnem in hitrem postopku lahko tako pridobimo veliko količino rastnih faktorjev, ki fiziološko delujejo na različne signalne poti, odgovorne za tkivno celjenje.

Avtologna trombocitna plazma je bioaktivna komponenta celotne krvi, pri kateri gre za od ostalih krvnih celic ločene trombocite in plazmo (69). Ker se po principu mehanskega ločevanja celic s centrifugiranjem celice med seboj ločijo, to omogoča koncentriranje trombocitov v vnaprej določeni količini krvne plazme. Od tod izhaja sprejet angleški izraz »platelet-rich plasma« (PRP), ki označuje avtologni preparat s trombociti bogate plazme.

Ob *ex vivo* ali *in vivo* aktivaciji trombociti sprostijo beljakovine, ki se nahajajo v granulah alfa in delta znotraj citoplazme in imajo široko paleto boioloških učinkov (tabela 4). Medtem, ko je normalna vrednost trombocitov v človeški krvi 150.000-400.000/µl, 1 µl PRP tipično vsebuje *vsaj* 1 milijon trombocitov (70).

Rastni faktor	Biološka aktivnost
Transformirajoči rastni faktor β (TGF-β)	• stimulacija proliferacije mezenhimskih celic • uravnavanje mitogeneze endotelijskih celic in fibroblastov • vzpodbujanje tvorbe zunajceličnega matriksa • stimulacija kemotakse endotelijskih celic in angiogeneze • zaviranje proliferacije makrofagov in limfocitov • zaviranje proliferacije in diferenciacije satelitskih celic
Fibroblastni rastni faktor (FGF)	• stimulacija proliferacije fibroblastov • vzpodbujanje proliferacije satelitskih celic • zaviranje diferenciacije satelitskih celic • stimulacija mitogeneze mezenhimskih celic
Trombocitni rastni faktor (PDGF)	• stimulacija mitogeneze mezenhimskih celic • stimulacija kemotakse fibroblastov in mitogeneze • stimulacija proliferacije satelitskih celic • zaviranje diferenciacije terminalnih mioblastov
Epidermalni rastni faktor (EGF)	• stimulacija kemotakse endotelijskih celic in angiogeneze • stimulacija migracije in proliferacije fibroblastov • zaviranje apoptoze satelitskih celic
Žilni endotelni rastni faktor (VEGF)	• stimulacija mitogeneze endotelijskih celic • stimulacija migracije endotelijskih celic • povečevanje permeabilnosti žilja • stimulacija migracije mioblastov • zaviranje apoptoze mioblastov
Inzulinu podobni rastni faktor 1 (IGF-1)	• vzpodbujanje mitogeneze mezenhimskih celic • vzpodbujanje sinteze kolagena • stimulacija kemotakse in mitogeneze fibroblastov • stimulacija proliferacije in fuzije mioblastov • zaviranje apoptoze mioblastov

Tabela 4: Rastni faktorji v granulah alfa trombocitov in njihova biološka aktivnost (3).

Po sprostitvi se lahko citokini prosto vežejo na transmembranske receptorje na površinah lokalnih ali cirkulirajočih celic. S tem povzročijo kaskado znotrajcelične signalizacije, s posledičnim izražanjem beljakovin odgovornih za celično kemotakso, sintezo matriksa in proliferacijo. Tkivno regeneracijo preko angiogeneze ter tvorbe zunajceličnega matriksa in kolagena medtem uravnavajo avtokrini in

parakrini učinki rastnih faktorjev (70). Učinek množice rastnih faktorjev iz trombocitov v obliki PRP se je izkazal kot bistveno večji v primerjavi z uporabo posameznih rastnih faktorjev, saj se je njihovo delovanje v različnih fazah procesa celjenja izkazalo za sinergistično (3). Poleg tega je priprava trombocitnega preparata v primerjavi z izolacijo ali rekombinantno proizvodnjo posameznih rastnih faktorjev enostavnejša in cenovno ugodnejša.

1.7.1. Učinki avtologne trombocitne plazme

Spoznanje o klinični vrednosti avtologne trombocitne plazme izvira iz kliničnih opažanj, kot so izboljšano celjenje kostnine in protivnetni učinek po oralnih in maksilofacialnih aplikacijah (71,72), kvalitetnejše celjenje in zmanjšanje brazgotine po rekonstruktivnih posegih (73–75), kožnih ulkusih in očesnih poškodbah (76–78). Protivnetni in antibakterijski učinki so posledica prisotnosti trombocitov, v krvi prisotnih brezjedrnih celic, katerih glavna vloga je uravnavanje primarne hemostaze. Po koagulaciji sprostijo rastne faktorje in ostale citokine, ki imajo pomembno vlogo pri tkivnem celjenju (74,79). Pred leti je Afaha s sodelavci poročal tudi o protibolečinski vlogi trombocitov, najbrž zaradi delovanja njihovih anti-nociceptivnih peptidov (81).

1.7.2. Avtologna trombocitna plazma v športni in ortopedski medicini

Enostavnost priprave, biološka varnost in široka uporabnost preparatov, ki temeljijo na koncentriranih trombocitih, so vzbudili veliko zanimanje tudi med športnimi zdravniki in ortopedi. Eno izmed zanimivejših področij raziskav in uporabe PRP predstavljajo poškodbe mišičnih tetiv. Te namreč same po sebi niso velik porabnik energije, imajo s tem nizko metabolno raven in posledično počasno celjenje ob poškodbah. Molloy je s sodelavci pokazal, da lahko rastni faktorji po zunanji aplikaciji učinkovito izboljšajo celjenje (82). V drugi raziskavi je Anitua s sodelavci ugotovil, da rastni faktorji iz PRP povečajo proliferacijo humanih tenocitov (83). V primeru poškodb Ahilovih tetiv pri ovcah je po večkratnih aplikacijah PRP v mišično tetivo prišlo do povečanega števila celic in izrazitejše angiogeneze ob odsotnosti nastajanja brazgotinskega tkiva (84).

Uporaba PRP ima svoje mesto tudi pri zdravljenju sklepnih nepravilnosti. Večja biološka razpoložljivost trombocitnih rastnih faktorjev dokazano izboljša prekrvljenost presadka pri rekonstrukciji križnih vezi v kolenskem sklepu, kar ima za posledico hitrejšo remodelacijo (83). Dobre rezultate daje tudi intraartikularna aplikacija PRP med artroskopskim zdravljenjem avulzije sklepnega hrustanca kolena (85), medtem ko zdravljenje hrustančnega defekta celotne debeline pri zajčjih modelih privede do izboljšanih mehanskih lastnosti hrustanca (86). Potencial PRP se kaže tudi pri zdravljenju osteoartrotičnih sklepnih sprememb, saj naj bi intraartikularna aplikacija zviševala koncentracijo endogene hialuronske kisline (87).

Več trombocitnih rastnih faktorjev je že bilo preizkušenih tudi pri celjenju mišic. *In vitro* rezultati učinkov *posameznih* rastnih faktorjev na skeletno mišičje so variabilni, kljub temu pa je moč trditi, da imajo nekateri pomembno vlogo pri regeneraciji in končni mišični moči po poškodbi (88). Rastni faktorji skupaj z makrofagi in produkti regulacijske poti ciklooksigenaze 2 (COX-2) uravnavajo vnetno fazo celjenja skeletnega mišičja (89). Inzulinu podobni rastni faktor 1 (angl. Insuline-like growth factor 1 - IGF-1 in fibroblastni rastni faktor (angl. Fibroblast growth factor – FGF) sta v primeru mišjega modela mišične laceracije izboljšala celjenje mišice in hitrost krčenja ter tetanično mišično moč (90). V primeru podganjega modela kontuzije mišice gastroknemius je po aplikaciji PRP prišlo do povečane aktivacije satelitskih celic in tvorbe debelejših mišičnih vlaken (91). Do hitrejšega mišičnega celjenja je prišlo tudi pri vrhunskih športnikih zdravljenih z ultrazvočno vodeno aplikacijo PRP v samo mišico, saj se je pri teh čas okrevanja skrajšal za polovico (92).

Kljub poročilom o uspehu pri uporabi PRP za zdravljenje mišičnih poškodb, so se sočasno pojavili tudi dvomi o takšni uporabi, ki naj bi vodila v obsežnejše brazgotinsko celjenje mišice (3). Te hipoteze temeljijo predvsem na opaženih povišanih koncentracijah rastnega faktorja TGF-β po injiciranju PRP v mišice, za katerega pa so eksperimentalni podatki v preteklih raziskavah že pokazali, da je močno povezan z razvojem brazgotine. Dodatni pomisleki so se pojavili zaradi prisotnosti nevtrofilcev v preparatih PRP, saj lahko provnetne proteaze in kisle hidrolaze, ki jih izločajo, delujejo kot citotoksični agensi in povzročijo dodatno sekundarno poškodbo tkiva (93).

Randomiziranih kontrolnih raziskav, ki bi potrdile vlogo PRP pri mišičnih poškodbah, še ni bilo (70), prav tako so dosedanje raziskave na ljudeh vključevale premajhne vzorce, da bi to predstavljalo dovolj močno statistično orodje za oceno dejanske učinkovitosti PRP pri zdravljenju mišičnih poškodb (94).

1.8. Funkcionalne celične kulture

Celične kulture v raziskavah celične rasti, diferenciacije, proliferacije, izražanja genov, citotoksičnosti, tvorbe rekombinantnih proteinov in reimplantacije že desetletja s pridom uporabljajo znanstveniki različnih strok. Velik pomen imajo pri razumevanju znotrajceličnih aktivnosti, genomike, proteomike in medceličnih interakcij. Glavna prednost celičnih kultur pred *in vivo* sistemi je zmožnost nadzora nad fizikalnim in kemičnim okoljem ter fiziološkimi pogoji, pod katerimi jih gojimo. Medtem ko so tkivni vzorci do velike mere heterogeni, predstavljajo celice, gojene v kulturi relativno homogeno skupino (95). V vsaki podkulturi replikati ostajajo enaki, z ohranjenimi lastnostmi tudi skozi več generacij. Zaradi te enakosti predstavljajo tudi poenostavljen model za statistične obdelave.

Izvajanje eksperimentov na celičnih kulturah ima ekonomske prednosti, saj je pri teh potrebna manjša količina reagentov, ki jih nanašamo direktno na celice. Prav tako ni sistemskih izgub na račun izločanja ali porazdelitve reagentov v ostala, nepreučevana tkiva (95).

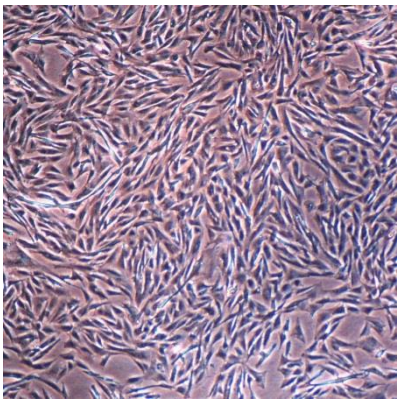
1.8.1. Skeletnomišične kulture v biokemijskih raziskavah

Celične kulture imajo pomembno vlogo tudi pri endokrinoloških in ortopedskih raziskavah. V zadnjih desetletjih je v raziskavah na celičnih kulturah prišlo do pomembnih odkritij različnih celičnih in tkivnih procesov, regeneracije in učinkov različnih materialov na specifična tkiva. Celične kulture pridobljene iz skeletnega mišičja postajajo vse pomembnejše v raziskavah procesov regeneracije po poškodobah in razvijajočih se mišic, predvsem kot komplementarni modeli poskusom *in vivo*, čeprav so takšne raziskave, v katerih so sočasno poskuse izvajali *in vitro* ter *in vivo*, v glavnem bile izvedene na celičnih kulturah nehumanega izvora.

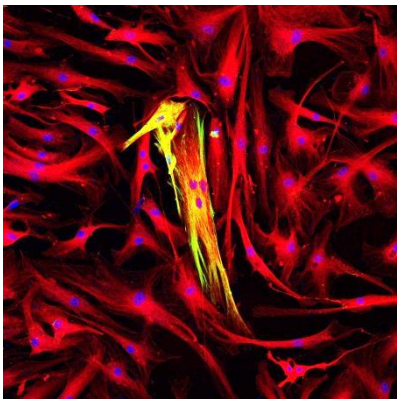
Skeletnomišične celice so glavna komponenta skeletnih mišic. Pomembno vlogo imajo ne samo pri gibanju, pokončni drži in proizvajanju toplote, temveč tudi pri vzdrževanju homeostaze in uravnavanju presnove. Skeletno mišičje je eno izmed tkiv za najenostavnejše gojenje v velikih količinah, saj ga je kot takšnega veliko in ga je tudi relativno enostavno pridobiti iz živih bitij (96). Skeletnomišične celice v kulturi so predstavljale jedro raziskav genetskih mišičnih bolezni (97), kot potencialen srčnomišični presadek (98), uporabljene so bile v raziskavi učinkov nizko gravitacijskega okolja na mišičje (99) in kot glavne tarče delovanja inzulina pri sladkorni bolezni (100,101). Vse pomembnejše postajajo tudi v raziskavah mišične rasti, razvoja in regeneracije po poškodbi. Moderne tehnologije za karakterizacijo, kot je konfokalna laserska mikroskopija, pretočna citometrija, analiza deoksiribonukleinske kisline (DNK) itd. omogočajo podrobnejši vpogled v celično in tkivno dogajanje kot kadarkoli prej (slika 2).

Slika 4: Sodobna biotehnologija omogoča boljši in bolj detajlen vpogled v celice, gojene v kulturah

a.) skeletnomišične celice rastejo v kulturi znotraj stekleničke z gojilnim medijem.



c.) Primarna celična kultura humanih skeletnomišičnih celic pod svetlobnim mikroskopom.



c.) Kultura humanih skeletnomišičnih celic pod konfokalnim laserskim mikroskopom. z barvanimi jedri (modra), α-tubulinom (rdeča) in dezminom (zelena). Mioblasti so se združili v velik, centralno ležeč miotubul.

Identifikacija različnih genov in transkricpijskih faktorjev vključenih v proliferacijo in diferenciacijo celic je vodila v boljše razumevanje procesov regeneracije, pri kateri prihaja do fuzije enojedrnih celic v večjedrne miotubule. Skeletnomišične kulture tako predstavljajo uporaben in relevanten model za

proučevanje teh procesov in razvoj novih terapevtskih strategij. To je razvidno tudi iz tabele 5, ki povzema več raziskav in korelacijo eksperimentalnih rezultatov, pridobljenih iz *in vivo* modelov s skeletnomišičnimi celičnimi kulturami.

Tabela 5: Pregled raziskav, v katerih so bili sočasno opravljeni *In vitro* in *In vivo* poskusi

Raziskava	**Objekt raziskave**	**Celična kutlura**	**Rezultati *in vitro***	**živalski/humani model**	***Rezultati in vivo***
Allen et al, 2001 (102)	Učinek vsadkov, prekritih z diamantu-podobnim karbonom (DLC) na skeletnomišični sistem	Osteosarkomska (SaOS-2 in MG-63), gojena na polistirenskih ploščicah, prekritih z DCL	• brez neželenih učinkov prekritja na celično morfologijo • brez razlik v citotoksičnosti • brez razlik v aktivnosti alkalne fosfataze • brez razlik v tvorbi kolagena tipa I • DLC prekritja so biokompatibilna	Sprague-Dawley podgane, kirurška intramuskularna vsaditev vzorcev z DLC prekritjem; ovce mešane pasme, kirurška vsaditev v stegnenice	• brez tkivnih sprememb v barvi v okolici vsadka • brez razlik v debelini fibrotične plasti v okolici vsadka • DLC prekritja so biokompatibilna
Wang et al, 2011 (103)	Proliferacija in diferenciacija humanih tenocitov kot odziv na trobmocitno plazmo (PRP)	Humani tenociti, pridobljeni z biopsijo, gojeni s PRP	• pomembno zvišanje celične proliferacije • brez razlike v viabilnosti celic • povečana tvorba kolagena • povečano izražanje markerjev tenocitov	Balb/C miši, vsaditev difuzijskih komor s tenociti, predhodno gojenimi z 10% PRP	• povečanje števila kolagenskih vlaken z D-trakovi • brez razlik v izražanju tenocitnih markerjev
Zhu et al., 2007 (24)	Vloga TGF-β, miostatina (MSTN) in dekorina (DCN) pri uravnavanju rasti skeletne mišice	PP1 fibroblasti iz mišjih skeletnih mišic, gojeni z MSTN;; C2C12 mioblasti, gojeni z DCN in MSTN	• povišano izražanje α-SMA v fibroblastih in mRNA za prokolagen po aplikaciji MSTN; • MSTN stimulira diferenciacijo C2C12 celic miofibroblaste • TGF-β stimulira izražanje MSTN in obratno v C2C12 celicah • DCN nevtralizira učinek MSTN v PP1 in C2C12 celicah	Wild-type miši in $MSTN^{-/-}$ miši; laceracija mišice	• 38,8% večji premer mišičnih progenitornih celic pri $MSTN^{-/-}$ miših • manj brazgotinskega vezivnega tkiva v mišicah $MSTN^{-/-}$ miši • povišano izražanje dekorina pri $MSTN^{-/-}$ miših
Li et al, 2004 (21)	Indukcija diferenciacije miogenih celic v fibrotične celice poškodovane mišice s TGF-β	C2C12 mioblasti, gojeni s TGF-β; C2C12 mioblasti, transfecirani s plazmidom, vključujočim gen za TGF-β, gojeni z dekorinom	• povišano izražanje α-SMA, vimentina in fibronektina • znižano izražanje dezmina, MyoD in miogenina • znižanje fibrotičnih markerjev po aplikaciji dekorina	Navadne miši, injiciranje TGF-β; transplantacija C2C12 celic , ki prekomerno izražajo TGF-β, v mišice	• diferenciacija enojedrnih celic celic v fibrotične celice in brazgotino • dekorin zmanjša fibrotično diferenciacijo

Chan et al., 2005 (104)	Preprečevanje nastanek brazgotine po poškodbi, s suraminom z zaviranjem aktivnosti TGF-β	Fibroblasti iz mišjih skeletnih mišic, gojeni s suraminom	• TGF-β stimullira proliferacijo fibroblastov • povečanje števila regenerajočih myofibril po aplikaciji suramina	Miši divjega tipa, povzročitev natrganja mišice in intramuskularna aplikacija suramina	• zmanjšanje obsega brazgotinskega tkiva • povečanje fast-twitch tetanične mišične moči
Ishida et al., 2006 (105)	Regenerativni učinki trombocitne plazme (PRP) na meniskusne celice	Meniskusne celice iz japonskih belih zajcev, gojene v prisotnosti PRP	• povečana viabilnost celic • znižano izražanje agrekana • povišano izražanje biglikana in dekorina	Japonski beli zajci, povzročitev poškodbe meniskusa, intraartikularna aplikacija PRP	• boljše celjenje meniskusov
Zhu et al, 2011 (106)	Vloga folistatina pri mišični regeneraciji, angiogenezi in fibrinogenezi po poškodbi	C2C12 mioblasti, gojeni s folistatinom, miostatin om (MSTN), aktivinom A in TGF-β	• TGF-β zavira miogeno diferenciacijo mioblastov • folistatin preprečuje zaviranje miogeneze zaradi TGF-β • folistatin stimulira miogeno diferenciacijo mioblastov	Miši divjega tipa in miši s prekomernim izražanjem folistatina (FOTM), povzročitev delne mišične laceracije	• manj miostatina pri miših FOTM • povišano izražanje $CD31^+$ kapilaram podobnih struktur pri miših FOTM • povečan premer regenerajočih mišičnih vlaken pri miših FOTM • zmanjšano nalaganje kolagena in razvoja fibrotičnega področja
Bates et al, 2006 (17)	Antifibrotični učinki manoze-6-fosfata (M6P) po poškodbi mišične tetive	Primarne kulture iz ovojnice zajčje tetive, epitenon in endotenon, gojene s TGF-β in M6P	• zmanjšanje s TGF-β povzročene tvorbe kolagena	Zajčje tetive fleksorjev; transekcija in takojšnje zašitje; injiciranje M6P na mesto poškodbe	• en sam intraoperativni odmerek M6P izboljša pooperativno gibljivost
Foster et al, 2003 (107)	Antifibrotični učinki interferona γ (IFNγ) pri regeneraciji skeletnega mišičja	Primarni fibroblasti iz skeletnega mišičja, gojeni z IFNγ; C2C12 mioblasti, transfekcija s plazmidom, ki vsebuje gen za humani TGF-β (CT celice), gojeni z IFNγ	• IFNγ zavira fibroblaste in rast CT celic • IFNγ znižuje izražanje profibrotičnih beljakovin (α-SMA, vimentin) pri CT celicah	Miši divjega tipa, laceracija mišica in intramuskularna aplikacija IFNγ	• zmanjšanje fibrotičnega področja v mišici, zdravljeni s IFNγ • boljša mišična regeneracija po IFNγ • izboljšane fiziološke lastnosti mišice po IFNγ
Bedair et al, 2008 (108)	Antifibrotični učinki zaviranja receptorja angiotenzina II (AT II) pri regeneraciji skeletne mišice	3T3 fibrolbasti, PP2 primarni mišični fibroblasti in C2C12 mioblasti, gojeni z angiotenzinom II	• AT II nima učinka na proliferacijo 3T3, PP2 ali C2C12 celic • AT II zniža tvorbo TGF-β v fibroblastih	Imunokompetentne miši, mišična laceracija, intramuskularna aplikacija zaviralca receptorja AT II	• povečanje števila regenerativnih vlaken • manjše področje fibroze znotraj področja poškodbe
Larson-Meyer et al., 2001 (109)	Odnos med in vivo in in vitro meritvami eksudativne presnove skeletnih mišic	Humane mišice, pridobljene z biopsijami; markerji	• odstotek tipa IIa oksidativnih vlaken in aktivnost citratne sintetaze	27 nediabetičnih premenopauzalnih žensk, izvedba vzdržljivostne	• MRS markerji korelirajo z odstotkom oksidativnih

		oksidativne kapacitete (mitohondrijska funkcija)	korelirata z MRS markerji • aktivnost COX šibko korelira z markerji MRS	vadbe; magnetno resonančna spektroskopija (MRS)	vlaken tipa IIa in aktivnostjo citratne sintetaze • MRS marker šibko korelirajo z aktivnostjo COX
Shemyakin et al., 2011 (110)	Zmanjšanje privzema glukoze skeletnih mišic zaradi endotelina 1 (ET-1)	Humane satelitske celice, izolirane I skeletnih mišic (biopsija), diferencirane v miotubule, gojene z ET-1	• ET-1 zniža privzem glukoze pri 24 urah • ET-1 zviša z inzulinom stimulirano Akt fosforilacijo	9 moških z neodzivnostjo na inzulin; infuzija fiziološke raztopine in ET-1; ocena toka krvi in vazodilatacije na podlakti	• ET-1 zniža privzem glukoze na podlakti za 39% • ET-1 zniža tok krvi na podlakti za 36 % • ET-1 učinkuje na vazodilatacijo

1.8.2. Omejitve celičnih kultur

Kljub spodbudnim eksperimentalnim rezultatom pridobljenim na celičnih kulturah in njihovi korelaciji z *in vivo* modeli (tabela 5) je ob odločanju o uporabi eksperimentalnega modela potrebno upoštevati tudi omejitve dela na celičnih kulturah. Pri teh so namreč sistemski homeostatski mehanizmi, ki so značilni za modele *in vivo*, odsotni, zaradi česar je celični metabolizem sicer bolj konstanten, a morebiti tudi manj reprezentativen.

Zaradi mnogih ogrožajočih kontaminantov, kot so bakterije ali glive, ki rastejo hitreje od živalskih in človeških celic, gojenje le-teh zahteva stroge aseptične pogoje. Ker celice iz večceličnih organizmov niso prilagojene življenju v izolaciji, jim je poleg tega navadno potrebno zagotavljati tudi kompleksno okolje za simulacijo normalnih naravnih pogojev.

Naslednja omejitev se pojavi pri količini materiala, ki je potreben za tvorbo relativno majhne količine tkiva. Stroški povezani z gojenjem celic v kulturi so približno 10-krat višji v primerjavi z uporabo modela *in vivo*. Tako morajo biti v primeru velikih zahtev po tkivih razlogi za uporabo celičnih kultur še posebej utemeljeni (95).

Neredko se zgodi, da se fenotipske lastnosti celic v kultur izgubijo. Pri tem dogodku imenovanem dediferenciacija, ki ga je moč delno preprečiti z gojenjem celic v mediju brez seruma, je sorodne lastnosti gojenih celic s tistimi iz izvornega tkiva težko opredeliti. Posledično občasno prihaja do napačnih identifikacij celičnih linij. Heterogenost hitrosti rasti in diferenciacijska kapaciteta lahko v različnih celičnih pasažah povzročata variabilnost celic, celo v primeru kratkoročnih netransformiranih celic (95).

Ob upoštevanju vseh omejitev in etičnih, ekonomskih, logističnih ter proceduralnih prednosti uporabe celičnih kultur kot eksperimentalnih modelov, le-te predstavljajo temelj biokemijskih raziskav tudi v prihodnosti. Področje uporabe je kot v ostalih kliničnih področjih široko tudi v ortopedskih raziskavah,

od bioproizvodnje, razvoja zdravil, toksikoloških testiranj in tkivnega inženirstva. Za pričakovati je tudi porast uporabe celičnih kultur humanega izvora, ki bodo s še bolj reprezentativnimi eksperimentalnimi rezultati pripomogle k boljšemu razumevanju regenerativnih procesov, kompatibilnosti biomaterialov in razvoju zdravil.

2. NAMEN DELA IN HIPOTEZE

Mišičnoskeletne poškodbe predstavljajo v športni medicini velik problem. Zaradi pogostosti teh poškodb v populaciji samo v Združenih državah Amerike posredne in neposredne stroške mišičnoskeletnih poškodb v literaturi ocenjujejo na več kot 800 milijard USD letno, kar je približno 8 % bruto domačega proizvoda (BDP). Do podobnih izsledkov so leta 2009 prišli v evropski raziskavi 23 držav članic, kjer naj bi več kot 44 milijonov prebivalcev trpelo za dalj časa trajajočimi težavami zaradi mišičnoskeletnih vzrokov, ki so botrovali tudi njihovi odsotnosti z dela (6). Te poškodbe zavzemajo večji delež fizične neopravilnosti kot katerokoli drugo bolezensko stanje. Stroški povezani z obravnavo mišičnoskeletnih poškodb pa so bili samo v letu 2009 v državah Evropske unije (EU) ocenjeni na 240 milijard evrov (111).

V poškodovani mišici sicer prihaja do spontane regeneracije, vendar pa je ta proces nepopoln zaradi brazgotinjenja, ki pomeni razraščanje zunajceličnega matriksa in nalaganje kolagena. Mišične poškodbe so tako pogosto vzrok pomembnih obolevnosti, vključno z zgodnjimi funkcionalnimi in strukturnimi deficiti, kontrakcijskimi poškodbami, mišičnimi atrofijami in bolečino. Novo znanje, ki bo omogočilo boljšo obravnavo skeletnomišične poškodbe, lahko značilno dvigne raven kvalitete življenja in zniža družbeno-finančno breme zdravljenja teh poškodb {Citation}.

Cilj doktorske naloge je bil dokazati statistično pomembno razliko v proliferaciji in miogeni diferenciaciji različnih skupin v kulturi rastočih mioblastov. Tem smo v gojilni medij dodajali različne v prvi točki navedene učinkovine s predhodno dokazanimi biološkimi aktivnostmi in to v različnih koncentracijah. Pridobljene strukturne in funkcionalne parametre smo primerjali tako med testnimi skupinami kot tudi s kontrolno skupino. Na podlagi rezultatov laboratorijskih študij smo izoblikovali teoretske podlage za morebitne nadaljnje klinične raziskave.

Testirali smo naslednje hipoteze:

- rastni faktorji iz trombocitne avtologne plazme (PRP) privedejo po aplikaciji v kulturi gojenih mioblastov do spremembe izražanja markerjev celične regeneracije, in sicer:
    - povečanja pozitivnih markerjev (MyoD, miogenin) in
    - povečanja izražanja negativnih markerjev oz. negativnih dejavnikov celične regeneracije (miostatin, TGF-β);
- antagonist TGF-β zavira delovanje TGF-β, ki je ena izmed komponent avtologne trombocitne plazme (PRP), zato predpostavljamo, da pride po hkratni aplikaciji rastnih faktorjev iz trombocitne avtologne plazme in antagonista TGF-β do povečanega izražanja prej omenjenih

pozitivnih markerjev celične regeneracije in znižanega izražanja prej omenjenih negativnih dejavnikov celične regeneracije;

- po hkratni aplikaciji rastnih faktorjev iz trombocitne avtologne plazme in antagonista TGF-β v celično kulturo pride v primerjavi s kontrolo do obsežnejše proliferacije in miogene diferenciacije mioblastov.

3. MATERIALI IN METODE

3.1. Celične kulture mioblastov

Celice mioblastov smo izolirali iz mišičnega tkiva bolnikov med standardnim operativnim posegom rekonstrukcije sprednje križne vezi po kolenski poškodbi. Uporabili smo vzorce treh posameznikov, pri katerih smo z anamnezo in kliničnim pregledom izključili genetsko okvaro ali predispozicijo za obolenje skeletnega mišičja.

Med posegom po protokolu žrtvujemo tetivo mišice semitendinosus, iz katere je po odvzemu potrebno odstraniti mišično tkivo, ki se že med posegom zavrže. Ta del tkiva speremo z 2 ml tripsina in gojilnim medijem Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, Grand Island, ZDA), z dodanim 5% fetalnim telečjim serumom (Lonza, Basel, Švica), L-glutaminom (Sigma), penicilinom in streptomicinom. Stekleničko postavimo v inkubator, dokler se celice ne pritrdijo na podlago, kar opazujemo pod svetlobnim mikroskopom. Celične linije rutinsko gojimo v 25 cm^2 stekleničkah za celične kulture (Corning, New York, ZDA) pri 37 °C vlažne atmosfere s 5 % CO_2 do tvorbe konfluentnega monosloja celic. Pred poskusom celice nanesemo na testne ploščice opisane pri biokemijskih poskusih.

3.2. Trombocitna plazma - PRP

Trombocitno plazmo (*angl.* Platelet-rich plasma; PRP), s koncentriranimi trombociti dobimo po tem, ko bolniku odvzamemo 50 ml krvi in jo po dodatku kisle citronske dekstroze (ACD-A, antikoagulant) v razmerju 10:1 vstavimo v plazma separator Megellan (Medtronic Biologic Therapeutics and Diagnostics, Minneapolis, MN, ZDA) – napravo, ki s centrifugiranjem loči eritrocite od s trombociti bogate in s trombociti revne plazme. Na ta način pridobimo 5 ml plazme, s koncentracijo trombocitov, ki je 3-krat višja od njihove koncentracije v periferni krviPreparat petkrat zamrznemo in odmrznemo, s čimer povzročimo razpad trombocitov in sproščanje rastnih faktorjev (112). Raztopino centrifugiramo 10 minut na 1500 obratih/minuto, nakar odlijemo supernatant rastnih faktorjev in jih prefiltriramo skozi filter velikosti 0,45 µm in nato še 0,22 µm. Na ta način pridobimo 1,5 ml raztopine, ki jih v različnih koncentracijah (20 %, 10 % in 5 %) uporabimo za nanos na celične kulture.

3.3. Dekorin - zaviralec TGF-β

Dekorin (R&D Systems, Minneapolis, MN, ZDA) smo uporabili v treh različnih koncentracijah, in sicer 10 ng/ml , 25 ng/ml in 50 ng/ml. Kot suplement celičnemu mediju smo ga dodajali bodisi samostojno, bodisi v kombinaciji s PRP s ciljem nevtralizacije učinka TGF-β.

3.4. Test MTT

S testom mitohondrijske aktivnosti (angl. MTT assay) merimo aktivnost reducirajočih encimov, ki po reakciji rumeno tetrazolno barvilo reducirajo v vijolično formazansko. V glavnem ga uporabljamo za oceno metabolne aktivnosti celic kot tudi za določanje citotoksičnosti različnih snovi in materialov, ki stimulirajo ali zavirajo celično rast in viabilnost (113,114). Za izvedbo testa na testno ploščo s 96 vodnjaki nagojimo mioblaste, ki jim v gojilni medij dodajamo različne koncentracije preizkušanih učinkovin, nakar plošče postavimo v inkubator. Čez 48 ur monosloj nagojenih celic speremo in v vsako izmed testnih ploščic dodamo 200 µL gojilnega medija DMEM (Dullbecco's Modified Eagle Medium) brez fenol rdečega, z antibiotikom in L-glutaminom ter 20 µl rumene raztopine MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide). Ploščico za 5 minut postavimo na vibracijsko ploščo, da se barvilo MTT dobro vmeša v medij. Celice na ploščici nato 3 ure inkubiramo pri 37 °C in 5 % CO_2. Za tem medij odlijemo in v ploščice dodamo 100 µL 0,04 % HCl in izopranola, s čimer raztopimo formazin, metabolni produkt MTT-ja. Ploščico ponovno za 5 minut položimo na vibracijsko ploščo, inkubiramo 15 – 20 min in s spektrofotometrom izmerimo absorbanco pri 570 nm.

S testom smo ugotavljali potencialno citotoksičnost oz. metabolno aktivnost v kulturi gojenih celic po tem, ko so bile v gojilni medij dodane različne koncentracije rastnih faktorjev iz avtologne trombocitne plazme (5 %, 10 %, 20 % eksudat PRP) in dekorina (10 ng/ml , 25 ng/ml in 50 ng/ml). Za vsako učinkovino smo v vseh koncentracijah opravili po tri testiranja.

Učinkovina	**Preizkušane koncentracije**		
Dekorin	50 ng/ml	25 ng/ml	10 ng/ml
PRP	20 %	10 %	5 %

Tabela 6: Učinkovine in koncentracije, uporabljene pri testiranju MTT in celični viabilnosti s kristal vijoličnim.

3.5. Viabilnost celic

Kristal vijolično je triarilmetansko barvilo s širokim spektrom uporabe, v biomedicinskih znanostih predvsem v namen histološkega barvanja in razvrščanja bakterij po Gramovi metodi. V biokemijskih

poskusih kristal vijolično uporabljamo tudi za oceno viabilnosti celic, saj na ta način med seboj ločimo nežive celice, ki se z barvilom ne obarvajo, od obarvanih živih celic.

Za izvedbo testa na tesno ploščo s 96 vodnjaki nagojimo mioblaste, ki jim v gojilni medij dodajamo navedene koncentracije preizkušenih učinkovin, nakar plošče postavimo v inkubator. Čez 48 ur monosloj celic speremo in v vsako izmed testnih ploščic dodamo 100 µl 0,1 % kristal vijoličnega. Po dveh minutah inkubacije na sobni temperaturi ploščice speremo pod tekočo destilirano vodo in preko noči pustimo, da se posušijo. Nato v vsako testno ploščico dodamo 100 µl 10 % ocetne kisline in celotno ploščo za 5 minut položimo na vibracijsko ploščo. S spektrofotometrom izmerimo absorbanco pri 595 nm. Za vsako učinkovino v vseh koncentracijah smo opravili po tri testiranja.

3.6. Encimski imunski testi

Encimski imunski test (angl. Enzyme-Linked ImmunoSorbent Assay; ELISA) je biokemijska metoda za detekcijo protiteles oz. antigenov v vzorcu. Encim vezan na protitelesa povzroči spremembo barve substrata in detekcijo prisotnosti preiskovanega protitelesa oz. antigena. Za detekcijo citokinov po dodajanju učinkovin na celične kulture smo uporabili od proizvajalca pripravljene sete za izvedbo direktnega imunskega testa, in sicer za TGF-β1 (Invitrogen Co., Camarillo, ZDA) in MSTN (USC Life Science Inc., Wuhan, Kitajska). Mioblaste z dodatkom učinkovin v določenih koncentracijah smo pred izvedbo testa 48 ur gojili v inkubatorju. Za vsako učinkovino v vseh koncentracijah smo opravili po tri testiranja.

3.7. Miogena diferenciacija

Med regeneracijo skeletnomišičnega tkiva po poškodbi aktivirane satelitske celice dozorijo v mioblaste, ki se nato bodisi diferencirajo v miocite, bodisi transformirajo v miofibroblaste. Slednji so iz vidika funkcionalnosti mišičnega tkiva in varnosti v smislu možnosti za ponovitev poškodbe v procesu celjenja nezaželeni. Po aplikaciji preiskovanih učinkovin na celične kulture smo opazovali obseg miogene diferenciacije mioblastov in s tem ugotavljali njihov dejanski regenerativni potencial.

Mioblaste smo nagojili na prekatna objektna stekelca z gostoto 10.000 celic/prekat in ploščice s 48-prekati z gostoto 5.000 celic/prekat. Po 24 urah smo v posamezne prekate aplicirali tiste preizkušane učinkovine, ki so v prejšnjih testiranjih pokazali dejansko vrednost in potencialno pomembno vlogo v procesu mišične regeneracije (tabela 7). Po imunskem barvanju smo celice pripravili za pretočno

citometrično analizo in mikroskopiranje, kjer smo opazovali specifične površinske celične markerje in označevalce miogeneze.

Učinkovina	Koncentracija
Dekorin	25 ng/ml
PRP	20%, 10%
PRP + dekorin	10%, 25 ng/ml

Tabela 7: Učinkovine in njihove koncentracije, uporabljene za teste miogene diferenciacije mioblastov.

3.7.1. Konfokalna laserska mikroskopija

Celice smo nagojili na prekatnih objektnih stekelcih in jih tako kultivirali pri 37 °C v vlažnem okolju v celičnem inkubatorju do tvorbe konfluentnega celičnega monosloja. Celicam smo nato dodali 10 % eksudat PRP, dekorin v koncentraciji 25 ng/ml ali kombinacijo obeh in jih tako inkubirali še 5 dni. Za tem smo celice fiksirali s CellFix-om (BD Pharmingen, Heidelberg, Nemčija) in permeabilizirali z 0,1 % Triton X ter PBS (Sigma-Aldrich Chemie GmbH, Schnelldorf, Nemčija). Za barvanje smo uporabili primarna mišja protitelesa proti dezminu, zajčja anti-mouse FITC ter kozja anti-rabbit Cy3 (vsa Abcam, Cambridge, Velika Britanija) po protokolih podanih s strani proizvajalca. V zadnjem koraku smo z barvanjem z DRAQ5 (Abcam) prikazali jedra. Optično smo slike posneli z laserskim konfokalnim mikroskopom Leica TCS SP5 II (Leica Microsystems Gmbh., Manheim, Nemčija). Zajemali smo v fokalni ravnini celičnih jeder in absolutnem centru objektnih stekelc, saj je bila gostota celic tam največja. Fluorescenco smo vzbujali z laserji valovnih dolžin 488 nm za FITC (dezmin), 561 nm za Cy3 (alfa-tubulin) in 633 nm za DRAQ5 (jedra). Emisije so bile zaznane z detektorji v območjih 495 – 515 nm za FITC, 570 – 625 za Cy3 in 705 – 730 nm za DRAQ5. Za analizo zajetih slik smo z uporabo programskega orodja Adobe Photoshop in funkcijo »color selection« napravili histrogramsko analizo ter med skupinami primerjali število slikovnih elementov, pozitivnih za dezmin.

3.7.2. Pretočna citometrija z lasersko mikroskopijo – Image Stream X

Kontrolne in z učinkovinami kultivirane celične kulture smo tripsinizirali z 0,25 % raztopino tripsin-EDTA (Sigma-Aldrich Chemie GmbH, Schnelldorf, Nemčija), jih prenesli v polipropilenske epruvete, sprali s PBS (Sigma-Aldrich Chemie GmbH, Schnelldorf, Nemčija) in fiksirali s CellFix (BD Pharmingen, Heidelberg, Nemčija) ter permeabilizirali z 0,1 % Triton X ter PBS (Sigma-Aldrich Chemie GmbH, Schnelldorf, Nemčija). Po protokolih proizvajalcev smo barvali površinske celične markerje CD56, ter markerja miogene diferenciacije miobastov MyoD in miogenin (tabela 8). V analizi smo z uporabo

pretočnega citometra Image Stream X (Amnis Corporation, Seattle, USA) zajeli 5000 dogodkov v vsakem vzorcu pri 40-kratni povečavi.

Barvilo	Proizvajalec	Antigen
CD56-APC konjugat	BD Pharmingen, Heidelberg, Nemčija	CD56
Goat MyoD in goveji anti-goat IgG PE konjugat	Santa Cruz GmbH, Heidelberg, Nemčija	MyoD
AlexaFluor 488	R&D Systems, Abingdon, UK	Myogenin

Tabela 8: Barvila, uporabljena za pretočno citrometrijo.

Barvili AlexaFluor 488 in konjugat PE smo vzbujali z laserjem valovne dolžine 488 nm, konjugat APC pa z laserjem valovne dolžine 658 nm. Fluorescenčna detekcija je potekala z detektorji v območjih 480-560 nm, 560-595 nm in 660-745 nm. Trojno barvane celice (CD56, MyoD in miogenin) so imele za kontrolo tri enojno barvane celice zaradi kompenzacije fluorescence med posameznimi kanali slike. Analizo fotografij smo napravili s programsko opremo IDEAS (Amnis Corporation, Seattle, USA). Izmed dogodkov zaznanih v analizi smo hierarhično izločili dovolj velike, da smo odstranili debris, ter med njimi tiste, ki so bile v optičnem fokusu. Pri tako izbranih celicah smo analizirali izražanje markerjev MyoD in miogenin oz. njuno odsotnost ob pozitivnem površinskem markerju CD56.

3.8. Statistično ovrednotenje podatkov

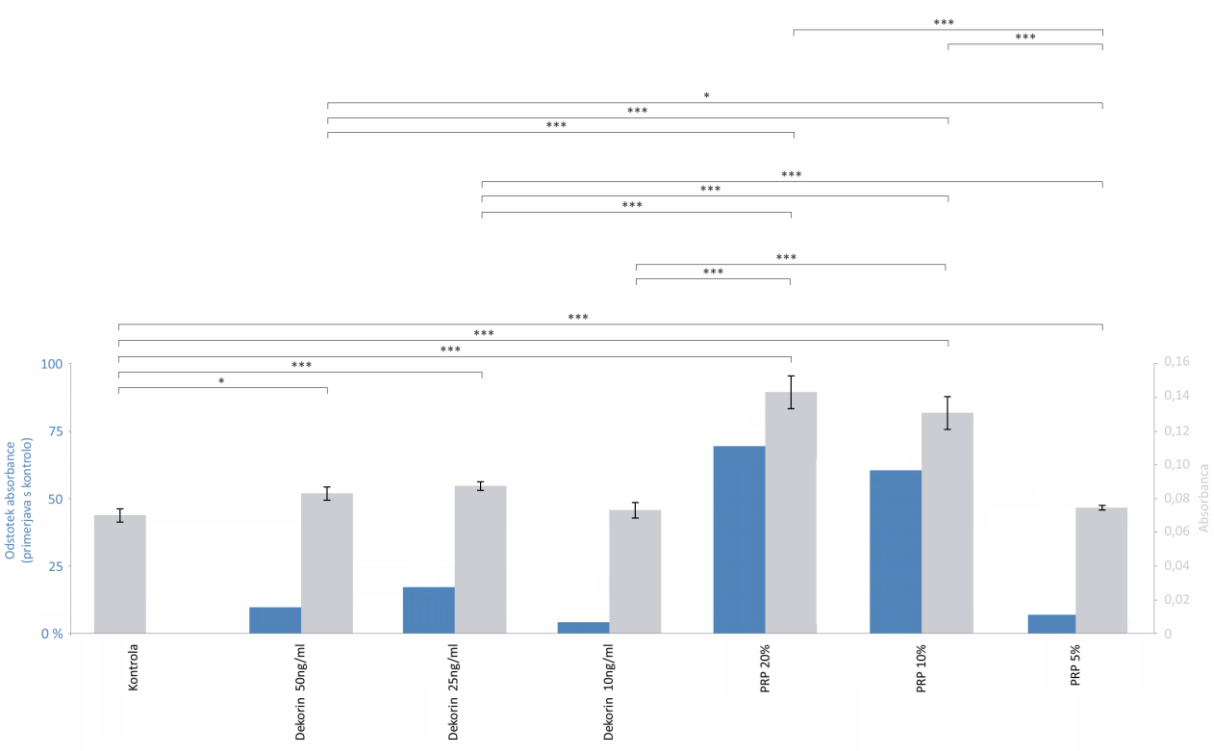
V vseh skupinah smo sprva izvedli Shapiro-Wilk test zavoljo ugotovitve porazdelitve podatkov, ki je bila v vseh primerih normalna. Nato smo primerjave med skupinami izvedli s parametričnimi testi v dveh korakih. V prvem smo ugotavljali razlike med vsemi skupinami s testom ANOVA. V primeru ugotovljenih razlik smo pri *post hoc* primerjavah uporablili Bonferronijev popravek za število parnih primerjav. Pri analizi podatkov, pridobljenih s pretočno citometrijo z lasersko mikroskopijo smo uporabili test hi-kvadrat, saj smo za vsako celico iz obsežne skupine posamezne kombinacije, ugotavljali prisotnost oz. odsotnost določenega markerja. Za mejo statistične značilnosti smo izbrali vrednost $p<0,05$, razen, kjer je označeno drugače.

4. REZULTATI

4.1. Test MTT

S testom MTT smo ugotavljali metabolno aktivnost celic v kulturah, ki smo jim v gojilni medij dodajali preizkušane učinkovine v različnih koncentracijah. Do najvišjega povišanja mitohondrijske aktivnosti je prišlo v primeru 10 % in 20 % koncentracije PRP, saj je bila ta v primerjavi s kontrolno skupino višja za več kot 80 % ($p<0,001$, slika 5). Omenjeni koncetraciji sta imeli večji učinek na omenjeno celično aktivnost tudi v primerjavi s skupinami, ki so prejele dekorin ($p<0,001$). Sicer se je kot najaktivnejša koncentracija dekorina izmed vseh izkazala srednja (25 ng/ml), pri kateri je v primerjavi s kontrolno skupino prišlo do povišanja metabolne aktivnosti celic za 17,1 % ($p<0,001$), med tem, ko je pri višji koncentraciji (50 ng/ml) prišlo do 11,1 % povišanja ($p<0,05$). Omeniti velja, da med samima višjima koncetracijama PRP ni bilo statistično pomembne razlike. Na podlagi pridobljenih rezultatov smo nadaljnja testiranja izvajali brez 5 % koncentracije PRP in najvišje koncentracije dekorina (50 ng/ml).

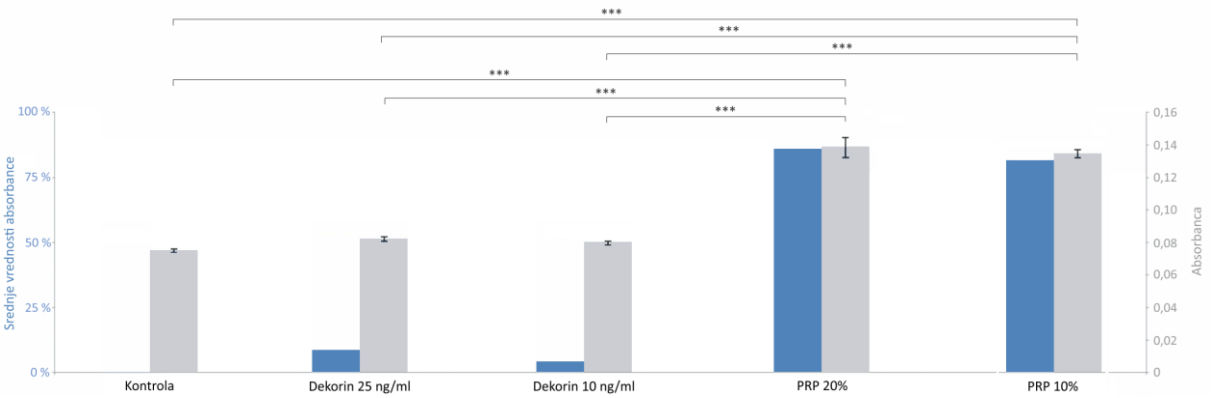
($p<0,001$).



Slika 5: Rezultati testa MTT po treh opravljenih meritvah, izraženi z absolutnimi vrednostmi absorbance (v sivi barvi; ±SD) in razmerji povprečnih vrednosti absorbance v primerjavi s kontrolo (v modri barvi; tip primerjave (vzorec-kontrola)/kontrola)x100 v skupinah s suplementom eksudatov PRP in dekorina. ANOVA, *$p<0,05$, ***$p<0,001$.

4.2. Viabilnost celic

Test s kristal vijoličnim smo opravili zaradi ocene viabilnosti mioblastov, po tem ko smo jih pod vplivom rastnih faktorjev in dekorina inkubirali 10 dni (slika 6). Dekorin v koncentracijah 10 ng/ml in 25 ng/ml ni imel statistično pomembnega vpliva v primerjavi s kontrolo, med tem ko sta obe koncentraciji PRP privedli do več kot 70 % povečanja v številu viabilnih celic ($p<0,001$), tako v primerjavi s kontrolno, kot obema skupinama z dekorinom, brez statistično pomembne razlike med skupinama z različnima koncentracijama PRP.

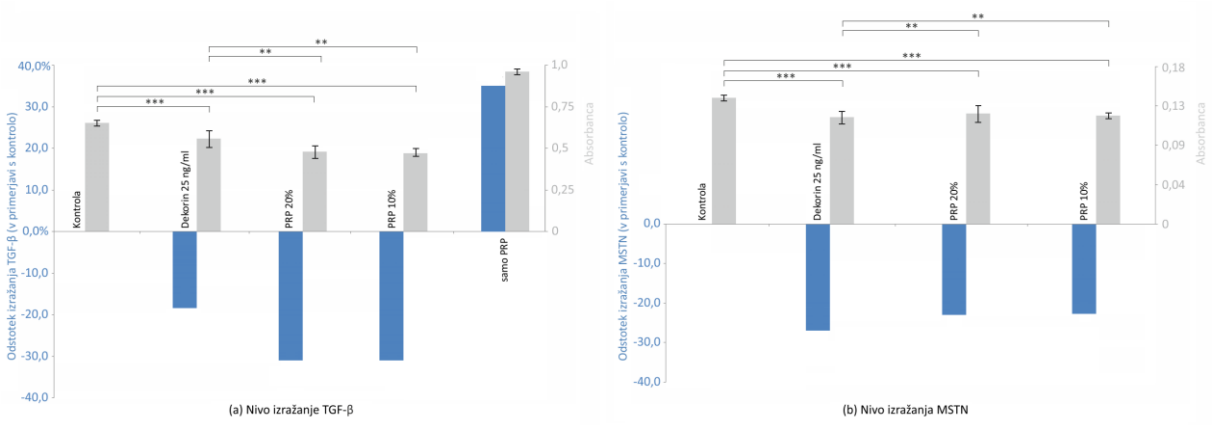


Slika 6: Viabilnost mioblastov po sedmih dneh inkubacije z gojilnim medijem DMEM, suplementiranim z dekorinom 25 ng/ml, dekorinom 10 ng/ml, 20 % eksudatom PRP in 10 % eksudatom PRP. Napravljeni so bili po trije neodvisni testi, rezultati so prikazani z absolutnimi vrednostmi absorbance (v sivi barvi; ±SD) in razmerji povprečnih vrednosti absorbance v primerjavi s kontrolo (v modri barvi; tip primerjave (vzorec-kontrola)/kontrola)x100 v skupinah s suplementom PRP eksudatov in dekorina. ANOVA; *** $p<0,001$ v primerjavi s kontrolo.

4.3. Encimski imunski testi

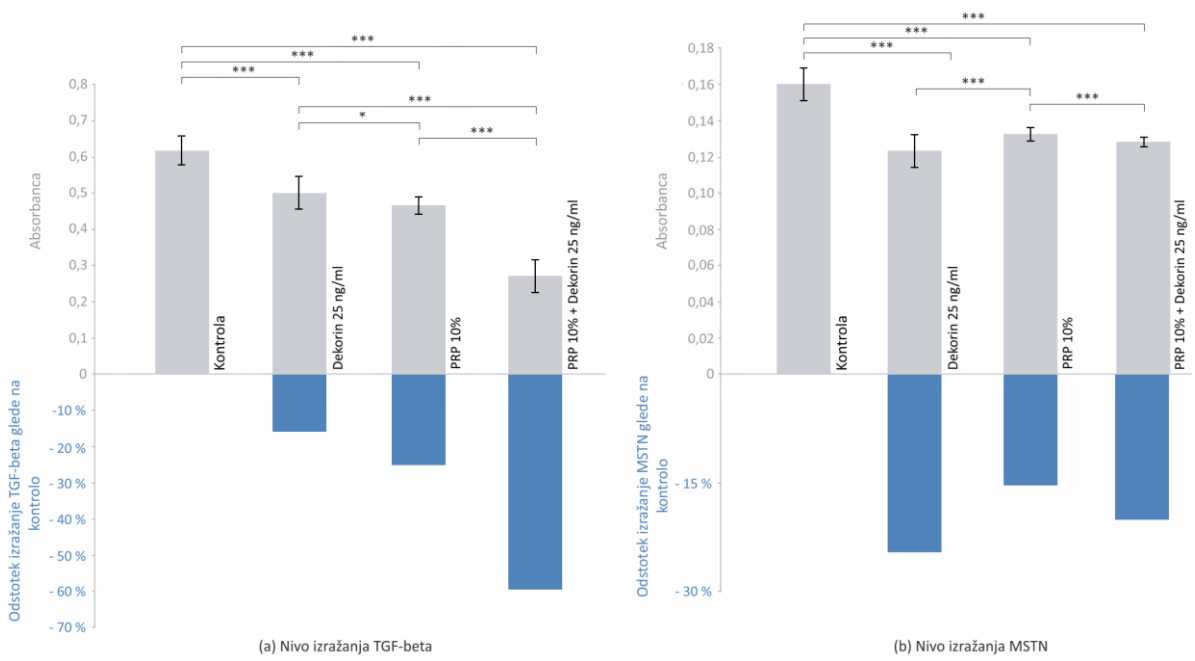
Z encimskimi imunskimi testi smo ugotavljali izražanje dveh ključnih citokinov pri regulaciji tvorbe brazgotinskega tkiva. Ob 10 % in 20 % koncentraciji PRP smo na podlagi rezultatov testa MTT in celične viabilnosti uporabili srednjo koncentracijo dekorina (25 ng/ml) . V primeru slednjega se je izraženost TGF-β v primerjavi s kontrolnimi skupinami znižala za več kot 15 % ($p<0,001$) (slika 7a), vendar pa je ta upad kljub temu bil signifikantno nižji kot v obeh skupinah, ki smo jima v gojilni medij dodali različni koncentraciji PRP ($p<0,01$, v primerjavi z dekorinom). Oba eksudata PRP sta namreč privedla do več kot 30 % znižanja v primerjavi s kontrolo ($p<0,001$). Koncentracija TGF-β v vzorcu PRP, ki ga nismo nanesli na v kulturi rastoče celice je bila več kot 30 % višja ($p<0,001$) kot v kontrolni skupini celičnih kultur, kar potrjuje, da PRP predstavlja nezanemarljiv eksogeni vir tega v procesu mišične regeneracije nezaželenega citokina.

Podobno je prav tako prišlo do znižanja ekspresije MSTN v vseh skupinah, ki smo jim dodali PRP in dekorin (slika 7b). V primeru slednjega je v primerjavi s kontrolno skupino prišlo do 28,4 % znižanja ($p<0,001$), med tem, ko je bila koncentracija MSTN v skupini, ki smo ji dodali PRP, v primerjavi s kontrolno nižja za 23,2 % ($p<0,001$). Med obema koncentracijama PRP-ja nismo zaznali statistično pomembne razlike v vplivu na izražanje MSTN. Koncentracije MSTN v samem eksudatu PRP nismo merili, saj gre v tem primeru za rastni dejavnik, ki se specifično nahaja v skeletnem mišičju in v krvnem obtoku ni prisoten.



Slika 7: Ekspresija TGF-β in MSTN. Mioblasti v kulturi po inkubaciji z DMEM gojilnim medijem, suplementiranim z dekorinom 25 ng/ml, 20 % eksudatom PRP in 10 % eksudatom PRP. Ekspresija citokinov je bila izmerjena po 48 urah inkubacije. (a) Ekspresija TGF-β. (b) Ekspresija MSTN. Napravljeni so bili po trije neodvisni testi, rezultati so prikazani in izraženi z absolutnimi vrednostmi absorbance (v sivi barvi; ±SD) in razmerji povprečnih vrednosti absorbance v primerjavi s kontrolo (v modri barvi; tip primerjave (vzorec-kontrola)/kontrola)x100 v skupinah s suplementom PRP eksudatov in dekorina. ANOVA; ** $p<0,01$ v primerjavi s kontrolo, ** $p<0,001$ v primerjavi z dekorinom. V skupini PRP ni bilo nasajenih celic, namenjena je bila kot kontrola za dokaz prisotnosti in koncentracije TGF-β v samem preparatu PRP.

Ker med obema koncentracijama eksudatov PRP ni bilo statistično pomembnih razlik v ekspresiji profibrotičnih citokinov, smo ob podatkih, pridobljenih tudi s testom MTT in viabilnosti celic testiranja nadaljevali z nižjo koncentracijo (10 %). Po tem, ko smo celicam v gojilni medij dodali kombinacijo 10 % eksudata PRP in dekorina v koncentraciji 25 ng/ml, je prišlo do 59,9 % ($p<0,001$) znižanja ekspresije TGF-β v primerjavi s kontrolno skupino, med tem, ko je bilo izražanje v primerjavi s skupino, ki je prejela samo PRP, nižja za 26 % ($p<0,001$) (slika 8). Ob tem je dekorin v primerjavi s kontrolo znižal koncentracijo tega fibrotičnega citokina za 18 % ($p<0,001$). Nivo MSTN se je v največji meri znižal v skupinah, ki so prejele dekorin oz. kombinacijo učinkovin, in sicer za 24 % oz. 20 % v primerjavi s kontrolo ($p<0,001$), brez statistično pomembne razlike med omenjenima skupinama. V skupini, ki je prejela samo PRP je prišlo v primerjavi s kontrolo prišlo do več kto 15 % znižanja v izražanju MSTN.

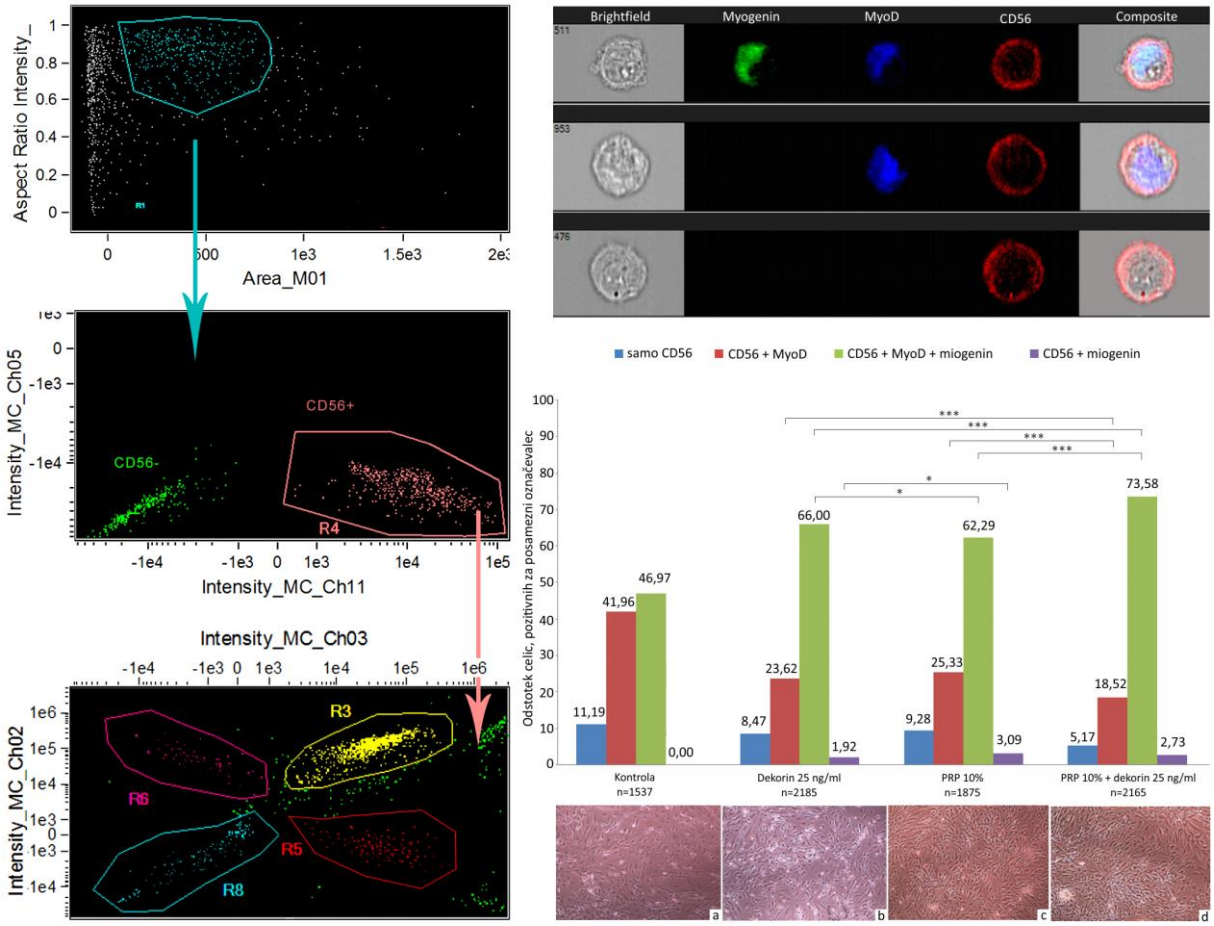


Slika 8: Ekspresija TGF-β in MSTN. Napravljeni so bili po trije neodvisni testi za vsak citokin. Rezultati so prikazani z absolutnimi vrednostmi absorbance (v sivi barvi; ±SD) in razmerji povprečnih vrednosti absorbance v primerjavi s kontrolo (v modri barvi; tip primerjave: (vzorec-kontrola)/kontrola)x100 v skupinah s suplementom PRP, dekorina in njune kombinacije. ANOVA; *p<0,05, ***p<0,001.

4.4. Pretočna citometrija z lasersko mikroskopijo – Image Stream X

Z analizo s pomočjo pretočne citometrije smo ugotavljali razlike v miogeni diferenciaciji med kontrolno skupino in skupinami, ki smo jim v gojilni medij dodali samo PRP, samo dekorin ali kombinacijo obeh. V vsaki izmed skupin smo zajeli in analizirali 5.000 celic, ki so bile pozitivne za površinski celični marker CD56. Celice smo nato hierarhično razporedili glede na sočasno izražanje MyoD in/ali miogenina, ki sta markerja zgodnje oz. pozne faze miogene diferenciacije (slika 9).

V skupni, ki je prejela PRP, je prišlo do 39,1 % povišanja števila celic, pozitivnih za miogenin, v primerjavi s kontrolno skupino (p<0,001). Izmed vseh celic v skupini je bilo 3,1 % takšnih, ki so izražale samo miogenin (brez MyoD), med tem, ko takšnih celic v kontrolni skupini ni bilo. Gre za celice, ki so v terminalni fazi miogene diferenciacije in kot takšne pri regeneracije mišice izjemnega pomena. Ob tem je v skupini PRP prišlo do znižanja števila neaktivni satelitskih celic (pozitivnih samo za CD56), in sicer za petino (p<0,001). Celice, ki so pozitivne tako za MyoD kot miogenin predstavljajo populacijo, pri kateri je prišlo do pomembnega premika v smeri zdravih miocitov v procesu miogeneze, vendar ti še niso popolnoma dozoreli.

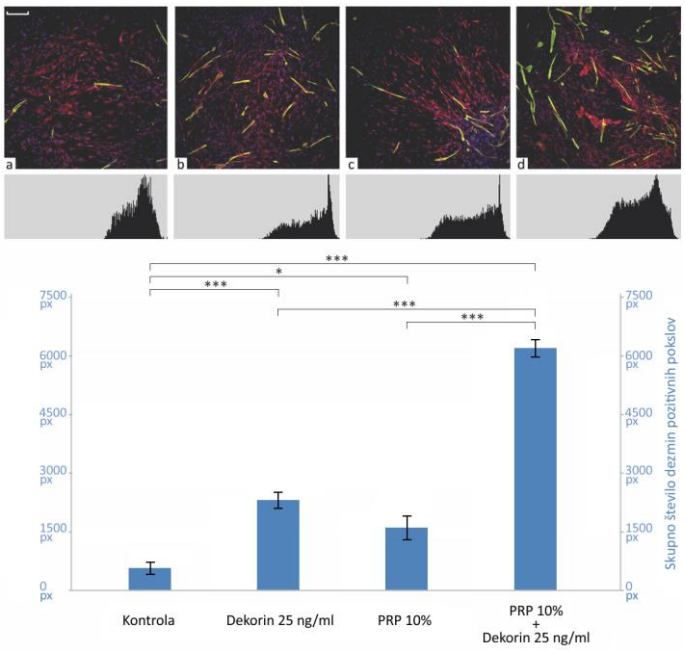


Slika 9: Miogena diferenciacija mioblastov. Analiza dejavnikov miogene regeneracije s slikovno pretočno citometrijo. V vsakem vzorcu je bilo 5000 celic, ki so bile hierarhično razdeljene v skupine glede na prisotnost specifičnih markerjev (zgoraj desno). Zgoraj levo: izbira celic glede na velikost in obliko – izločanje debrija; sredina levo: podskupina prej določenih celic, ki so v fokalni ravnini in pozitivni za površinski celični marker CD56; spodaj levo: prisotnost MyoD in/ali miogenina v CD56-pozitivnih celicah z meritvijo intenzivnosti vsake posamezne celice. Spodaj desno: Rezultati izraženi kot razmerja odstotkov med celicami, ki so pozitivne samo za marker CD56, tistimi, ki dodatno vsebujejo še MyoD in tistimi, ki izražajo še miogenin, in to v suplementiranih in kontrolnih skupinah populacij celic. Merilo = 50 µm. Pearson Hi-kvadrat *p<0,05 in ***p<0,001.

Med tem, ko je dekorin povzročil le majhen dodaten pozitiven pomik v smeri miogene diferenciacije (a statistično pomemben), pa je do večjega učinka prišlo v primeru kombinacije dekorina in PRP. Izražanje MyoD in miogenina se je povečalo za dodatnih 16,7 % v primerjavi s skupinama, ki sta prejeli samo PRP ali samo dekorin ($p<0,001$) ob hkratnem znižanju števila celic, ki izražajo zgolj MyoD. V primerjavi s kontrolno skupino je kombinacija dekorina in PRP tako privedla do povišanega izražanja MyoD in miogenina za 60,4 % ($p<0,001$) in znižanja števila neaktiviranih satelitskih celic (pozitivnih samo za CD56) za 46,2 % ($p<0,001$).

4.5. Konfokalna laserska mikroskopija

S ciljem vizualizacije v kulturah gojenih celic smo z uporabo konfokalnega laserskega mikroskopa zajeli mikrografije posameznih vzorcev. Celice smo pred tem obarvali z barvili za jedra, α-tubulin za vizualizacijo celične citoplazme in dezmina, intermediarnega filamenta, ki je eden izmed ključnih markerjev diferenciacije mioblastov (115,116). V primerjavi s kontrolno skupino smo opazili statistično pomembno razliko v pozitivni regulaciji izražanja dezmina v vseh skupinah ($p<0,05$ za skupino, ki je prejela PRP, $p<0,001$ za skupino, ki je prejela dekorin in $p<0,001$ za skupino, ki je prejela PRP in dekorin) (slika 10). Med tem ko med skupinama, ki sta prejeli samo PRP in samo dekorin ni bilo statistično pomembne razlike, smo v skupni, ki je prejela obe učinkovini, opazili več kot 3-kratno povišanje izražanja dezmina v primerjavi s skupinama, katerim sta bila dodana bodisi samo PRP bodisi samo dekorin ($p<0,001$).



Slika 10: Miotubuli z izraženim dezminom. Imunofluorescenčno barvanje jedra (modro), α-tubulina (rdeče) in dezmina (zeleno), prikazano v zgornjem delu v združenih slikah. Napravljeni so bili po trije neodvisni testi za vsako terapevtsko skupino, rezultati so izraženi s srednjimi vrednostmi števila za dezmin pozitivnih pikslov (±SD) v skupinah s suplementom PRP, dekorina in njune kombinacije. ANOVA; * $p<0,05$, *** $p<0,001$ Merilo = 200 µm.

5. RAZPRAVA

Rastni faktorji iz PRP izboljšajo viabilnost in povečajo proliferacijo mioblastov in vitro

Kljub temu, da do danes ni bila napravljena še nobena randomizirana prospektivna raziskava, ki bi potrjevala vlogo PRP-ja pri terapiji skeletnomišičnih poškodb, se le-ta kljub temu široko uporablja v primeru poškodb mišičnih tetiv in sklepnih vezi. V zadnjem času prihaja do vse večjih teženj po uporabi PRP-ja tudi v primeru mišičnih poškodb (3,117), vendar pa se v literaturi med ostalim pojavlja pomislek o varnosti takšne terapije, saj PRP vsebuje rastni faktor TGF-β, ki pa je ključni dejavnik tkivnega brazgotinjenja. Kljub temu naši rezultati kažejo nasprotno. S pozitivnim učinkom na metabolno aktivnost mioblastov v kulturi in stopnjo viabilnosti nismo samo izključili potencialno škodljivega učinka PRP zaradi prisotnosti TGF-β, temveč dokazali pozitiven učinek tako na celični metabolizem kot na proliferacijo. Ti rezultati dodobra korelirajo z rezultati iz prejšnjih že objavljenih raziskav, ki so pokazale pozitivne učinke PRP in nekaterih posameznih rastnih faktorjev v procesu regeneracije skeletne mišice (88–91,118,119). Ti učinki so bili statistično pomembno večji v primerjavi z dekorinom, ki pa zaenkrat velja za najmočnejšega regulatorja brazgotinjenja in miogene diferenciacije v procesu mišične regeneracije (11,24,64,65).

Rastni faktorji iz PRP znižajo ekspresijo TGF-β in MSTN v kulturah mioblastov in vitro

Kljub temu, da PRP predstavlja pomemben vir TGF-β, se koncentracija tega rastnega faktorja v kulturi mioblastov po gojenju s suplementom PRP zniža, presenetljivo tudi v primerjavi z dekorinom, enim izmed najmočnejših zaviralcev TGF-β (62). Mehanizem takšnega rezultata zaenkrat ostaja nepojasnjen, najverjetneje pa gre za sinergističen učinek ostalih rastnih faktorjev, ki privedejo do končnega celokupnega znižanja koncentracije TGF-β.

MSTN proizvajajo skeletno mišične celice in navadno ni prisoten v preparatih koncentriranih avtolognih trombocitov. Kljub temu pa je prisoten v krvnem obtoku in se v omenjenih preparatih na ta način teoretično lahko pojavi. Vendar pa je koncentracija MSTN po gojenju mioblastov s suplementom PRP upadla oz. skoraj dosegla raven znižanja, ki jo povzroči dekorin. Sicer v literaturi ni moč najti pomisleka zaradi z MSTN povzročene fibroze po intramuskularnem injiciranju PRP, pa vendar daje ta ugotovitev pomemben podatek o mišični regeneraciji pod vplivom avtolognih rastnih faktorjev.

Sicer že sam PRP znižuje koncentracijo TGF-β v kulturi mioblastov, pa vendar je z dodatkom dekorina moč doseči še dodatno zaviranje aktivnosti tega rastnega faktorja. Po ko-kultivaciji s PRP in dekorinom

pride namreč do statistično pomembnega dodatnega znižanja izražanja TGF-β, kar kaže na pomemben sinergističen učinek obeh učinkovin.

PRP in dekorin promovirata miogeno diferenciacijo mioblastov In vitro

V procesu mišične regeneracije pride do aktivacije satelitskih celic, ki se iz stanja mirovanja ob povečani ekspresiji DMR diferencirajo v miofibroblaste. Del populacije satelitskih celic ostane v stanju mirovanja in neaktivnosti, s tem pa predstavljajo vir regeneracijskih celic za morebitne procese celjenja v prihodnosti. Ves ta proces je močno reguliran s strani TGF-β in MSTN (25,31,120–122). V raziskavi smo poleg znižanja števila neaktiviranih prekurzorskih celic v skupinah zdravljenih s PRP oz. dekorinom ter njuno kombinacijo v primerjavi s kontrolno skupino opazili tudi znižanje števila nezrelih aktiviranih celic, ki od DMR izražajo samo MyoD. Na ta račun smo obenem opazili statistično pomembno povečanje števila celic, ki so ob MyoD hkrati izražale tudi miogenin, kar je značilnost kasnejše faze miogene diferenciacije. V tem primeru je sinergističen učinek PRP in dekorina očiten, saj je kombinacija privedla ne le do povečanega števila aktiviranih satelitskih celic, temveč glede na izražanje dejavnikov miogene regeneracije, MyoD in miogenina, tudi do pomembnega pomika v smeri miogene diferenciacije.

Pokazali smo tudi, da pride po gojenju celic ob dodajanju PRP in dekorina do izrazitega povišanja izražanja dezmina, ki je mišično-specifičen intermediarni filament, značilen za zgodnjo oz. pozno fazo miogeneze (123). Ekspresija dezmina je uravnavana s strani MyoD in miogenina in je kot prvi protein citoskeleta, ki se izraža v procesu miogeneze, eden izmed ključnih pokazateljev miogene diferenciacije (124,125). Ti rezultati dobro korelirajo s povišanim izražanjem dejavnikov miogene regeneracije, ki smo jo dokazali s pomočjo pretočne citometrije in služijo kod dodaten dokaz miogenega premika v smeri terminalne diferenciacije mioblastov.

6. OMEJITVE RAZISKAVE IN MOŽNOSTI NADALJNJIH RAZISKAV

Ob vzpodbudnih rezultatih, ki smo jih pridobili v opravljeni raziskavi, pa je potrebno omeniti tudi omejitve le-te in jih upoštevati pri sami interpretaciji. Nezanemarljivo je kot prvo dejstvo, da nismo posebej opravili analize sestave PRP, ki smo ga uporabili ob kultivaciji gojenih celic. V poglavju o metodah smo sicer opisali postopek priprave, pri katerem je v literaturi zapisano, kakšno koncentracijo trombocitov in s tem rastnih faktorjev pridobimo pri prav takšnem postopku s prav takšnim aparatom. Sicer je to pomembno vprašanje in glede na zanimive rezultate, ki smo jih pridobili, gre za zanimivo področje za prihodnje raziskave s ciljem ugotoviti, katera sestavina PRP nosi dominanten učinek pri vzpodbujanju miogeneze.

Ob tem ostajajo odprta tudi nekatera druga vprašanja, med njimi morda celo najpomembenjše o učinkovitosti in racionalnosti uporabe enkratnega bolusa koncentriranih trombocitov v živem organizmu, pri katerem pride takoj po aplikaciji tako do sproščanja rastnih faktorjev kot tudi do njihovega izplavljanja iz »tarčnega« tkiva. Omenjena problematika zagotovo predstavlja izziv za razvoj raznih bioloških nosilcev, ki bi hkrati skrbeli za postopno sproščanje rastnih faktorjev v regenerirajoče se tkivo in jih določen čas tudi zadrževali v njem. Neodgovorjeni ostajata tudi vprašanji o točni koncentraciji preparata PRP in optimalnem režimu aplikacije v klinični uporabi, na kateri bodo morale v prihodnje odgovoriti raziskave sprva z uporabo živalskih modelih. Zavedati se je tudi potrebno, da izsledki študij na celičnih modelih ne ponujajo popolnega vpogleda v učinek rastnih faktorjev in ostalih učinkovin, predvsem na račun odsotnosti homeostatskih in ostalih parakrinih mehanizmov, značilnih za večcelične eksperimentalne modele *in vivo*.

Podobno velja za dekorin, pri katerem pa gre za rekombinantni glikoprotein, katerega uporaba je zaenkrat striktno omejena na neklinčna raziskovanja. V prihodnjih raziskavah bo potrebno temeljito preučiti tako optimalne koncentracije kot časovni režim aplikacije te učinkovine. Ob tem je treba upoštevati tudi dejstvo, da je dekorin kot zaviralec miostatina na seznamu prepovedanih substanc mednarodne protidopinške agencije in bo tako ostalo vsaj tako dolgo, dokler ne bo ustreznih dokazov o tem, da njegova lokalna aplikacija v mišično tkivo nima potencialnih sistemskih učinkov.

Dodaten problem pri klinični uporabi dekorina predstavlja njegova potencialna imunogenost. Kljub temu, da so z rekombinantno tehnologijo pridobljene učinkovine v svojih sekvencah izredno podobni izvornim človeškim proteinom, pa je pri njihovi uporabi zmeraj možen pojav razvoja protiteles (126). Primerjava med prašičjim in rekombinantnim humanim inzulinom je na primer pokazala, da kljub temu, da je slednji manj imunogen, povzroča tvorbo protiteles pri 44% bolnikov s sladkorno boleznijo (127).

Imunski pojavi se lahko kažejo bodisi kot alergijske reakcije bodisi vodijo degradacijo apliciranega rekombinantnega zdravila in s tem znižanjem učinka oz. potrebo po večanju odmerka.

7. ZAKLJUČEK

Z raziskavo smo testirali tri zastavljene hipoteze, pri čemer smo eno potrdili delno, druge v celoti:

- Potrdili smo hipotezo, da rastni faktorji iz trombocitne avtologne plazme po aplikaciji v kulturo gojenih mioblastov privedejo do povečanega izražanja pozitivnih markerjev celične regeneracije (MyoD, miogenin)
- Zavrgli smo hipotezo, da pride zaradi TGF-β, enega izmed vsebujočih rastnih faktorjev v avtologni trombocitni plazmi, hkrati tudi do povečanega izražanja negativnih dejavnikov celične regeneracije (miostatin, TGF-β);
- Potrdili smo hipotezo, da antagonist TGF-β zavira delovanje TGF-β, ki je ena izmed komponent avtologne trombocitne plazme, zaradi česar pride po hkratni aplikaciji rastnih faktorjev iz trombocitne avtologne plazme in antagonista TGF-β do sinergističnega učinka - povečanega izražanja pozitivnih markerjev celične regeneracije in znižanja negativnih dejavnikov celične regeneracije;
- Potrdili smo hipotezo, da pride po hkratni aplikaciji rastnih faktorjev iz trombocitne avtologne plazme in antagonista TGF-β v celično kulturo do sinergističnega učinka - obsežnejše proliferacije in miogene diferenciacije mioblastov.

Aktivacija satelitskih celic in miogena diferenciacija proliferirajočih mioblastov sta dva ključna koraka v učinkoviti regeneraciji skeletnega mišičja. Naše ugotovitve govorijo v prid temu, da utegnejo preparati avtologne trombocitne plazme igrati pomembno vlogo kot potencialna terapevtska možnost pri zdravljenju mišičnih poškodb, kljub dejstvu, da predstavljajo signifikanten dodaten vir TGF-β. V kombinaciji z dekorinom, antagonistom tega citokina lahko njegov učinek še dodatno zaviramo. Naša raziskava predstavlja ne samo vpogled v molekularne mehanizme in učinke PRP na skeletno mišičnih celicah, temveč ponuja novo možnost v snovanju biološke terapije mišičnih poškodb. Kljub temu, da so za potrditev dejanske vloge PRP pri zdravljenju mišičnih poškodb potrebne še nadaljnje raziskave *in vivo*, pa na podlagi naše raziskave kaže, da utegne kombinacija PRP in dekorina imeti svoje mesto pri doseganju učinkovitejše mišične regeneracije.

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Hvala!

10.PRILOGE

10.1. Članka kot sestavni del doktorske disertacije

1.01 Izvirni znanstveni članek

KELC, Robi, TRAPEČAR, Martin, SLAK RUPNIK, Marjan, VOGRIN, Matjaž. Platelet-rich plasma, especially when combined with a TGF-β inhibitor promotes proliferation, viability and myogenic differentiation of myoblasts In Vitro. Plos ONE, 10(2): e0117302. doi:10.1371/journal.pone.0117302. **IF=3,53**

1.02 Pregledni znanstveni članek

KELC, Robi, TRAPEČAR, Martin, VOGRIN, Matjaž, CENCIČ, Avrelija. Skeletal muscle derived cell cultures as potent models in regenerative medicine research. Muscle & nerve, ISSN 1097-4598. [Online ed.], Apr. 2013, vol. 47, iss. 4, str. 477-482. **IF=2.31**

PLOS | ONE

RESEARCH ARTICLE

Platelet-Rich Plasma, Especially When Combined with a TGF-β Inhibitor Promotes Proliferation, Viability and Myogenic Differentiation of Myoblasts *In Vitro*

Robi Kelc[1]*, Martin Trapecar[2], Lidija Gradisnik[2], Marjan Slak Rupnik[2], Matjaz Vogrin[1]

1 Department of Orthopaedic Surgery, University Medical Center Maribor, Maribor, Slovenia, **2** Institute of Physiology, Faculty of Medicine, University of Maribor, Maribor, Slovenia

* robi.kelc@gmail.com

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Abstract

Regeneration of skeletal muscle after injury is limited by scar formation, slow healing time and a high recurrence rate. A therapy based on platelet-rich plasma (PRP) has become a promising lead for tendon and ligament injuries in recent years, however concerns have been raised that PRP-derived TGF-β could contribute to fibrotic remodelling in skeletal muscle after injury. Due to the lack of scientific grounds for a PRP -based muscle regeneration therapy, we have designed a study using human myogenic progenitors and evaluated the potential of PRP alone and in combination with decorin (a TGF-β inhibitor), to alter myoblast proliferation, metabolic activity, cytokine profile and expression of myogenic regulatory factors (MRFs). Advanced imaging multicolor single-cell analysis enabled us to create a valuable picture on the ratio of quiescent, activated and terminally committed myoblasts in treated versus control cell populations. Finally high-resolution confocal microscopy validated the potential of PRP and decorin to stimulate the formation of polynucleated myotubules. PRP was shown to down-regulate fibrotic cytokines, increase cell viability and proliferation, enhance the expression of MRFs, and contribute to a significant myogenic shift during differentiation. When combined with decorin further synergistc effects were identified. These results suggest that PRP could not only prevent fibrosis but could also stimulate muscle commitment, especially when combined with a TGF-β inhibitor.

Introduction

Musculoskeletal injuries that result in the necrosis of muscle fibres are frequently encountered in clinical and sports medicine [1,2]. Despite their clinical significance, current therapeutic options remain rather conservative and include the R.I.C.E. (rest, ice, compression, elevation) principle or the controversial therapy using corticosteroids as well as non-steroidal anti-inflammatory drugs [3].

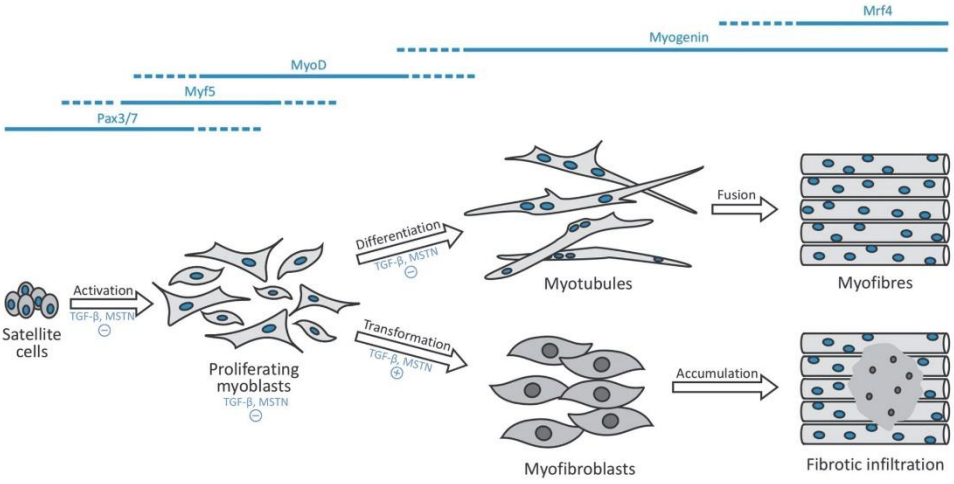

Figure 1. Schematic representation of muscle regeneration on the regulatory level. During skeletal muscle regeneration various MRFs are being expressed (in blue). Satellite cells differentiate into myoblasts which proliferate and either further differentiate into polynucleated myotubules or transform into myofibroblasts. TGF-β and MSTN play an important role in inhibiting/stimulating these steps (marked with +/- symbols).

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Injured skeletal muscle has regenerative capacities and can repair spontaneously; however, this process is often incomplete because of overgrowth of the extracellular matrix and the deposition of collagen, which leads to significant fibrous scarring [4,5,6]. Fibrotic remodelling further limits the functionality of the muscle and represents a significant risk factor for the injury to recur.

Platelet-rich plasma (PRP), an autologous platelet concentrate, has gained popularity for the therapy of tendon and ligament injuries [7] despite few and limited relevant scientific reports about its equivalent therapeutic efficiency. PRP is isolated by the centrifugation of whole blood, obtained from an individual, allowing extraction of thrombocytes rich in granules containing various growth factors [8]. As such, the patient-derived endogenous preparation is theoretically perfect to be administered locally at the site of the tissue injury. Several individual PRP-derived growth factors have positive regenerative effects in muscle healing [9,10,11,12]; nonetheless, PRP-derived TGF-β could potentially stimulate fibrosis as shown previously [1,13]. Because of its presence in PRP, its application into skeletal muscle raises concerns due to the risk of even greater fibrotic remodelling of the tissue [14].

Fibrotic effects of TGF-β are balanced by decorin, a component of the extracellular matrix of all collagen-containing tissues [15]. It has been shown that decorin inhibits both TGF-β as well as myostatin (MSTN), which is another, skeletal muscle-specific, member of the TGF-β superfamily [16,17,18,19,20]. Both cytokines up-regulate the expression of each other [19] and inhibit the activation of satellite cells [14,21,22], myoblast proliferation [19,23] as well as their myogenic differentiation [13,24,25]. Alternatively they promote fibroblast commitment [1,5,19] (Fig. 1). We hypothesise that by the simultaneous use of the anitfibrotic agent decorin with PRP we might reduce TGF-β-dependent fibrotic scaring while at the same time stimulating muscle regeneration via the introduction of homologous growth factors.

A typical feature during muscle differentiation are transient changes in the expression levels of various muscle specific transcription factors [26] (Fig. 1). Myogenic regulatory factors (MRFs) such as Pax3/7, Myf5, MyoD, myogenin and Mrf4 are expressed exclusively in skeletal muscle [27] and govern the expression of multiple genes during myogenesis [28,29]. MyoD is required for the determination of skeletal myoblasts whereas myogenin acts later in the program, likely as a terminal differentiation factor [27]. Although it has been shown that decorin alters the expression of MRFs in skeletal muscle after injury, such a mode of action has not yet been studied with PRP. Furthermore, despite the results from two recent studies showing positive effects of PRP on skeletal muscle proliferation, no data about TGF-β and MSTN expression has been provided [30,31].

In our study we used a human CD56 positive myoblast cell line and evaluated the potential of PRP alone and in combination with decorin, a TGF-β inhibitor to alter myoblast proliferation, metabolic activity, TGF-β and myostatin activation and expression of myogenic regulatory factors (MRFs). Imaging flow cytometry enabled us to create a valuable and unique insight into the ratio of quiescent, activated and terminally committed single myoblasts in treated versus control cell populations. Finally high-resolution confocal microscopy validated the potential of PRP and decorin to stimulate the formation of desmin-expressing polynucleated myotubes.

Materials and Methods

Cell culture isolation and cultivation

We used the human $CD56^+$ positive myoblast cell line (hMC) [32,33] previously characterized and stored at the Institute of Physiology, University of Maribor. The hMC cell line was maintained in DMEM (Life Technologies Ltd, Paisley, UK) including 100 Units/ml of penicillin, 1 mg/ml of streptomycin and 2 mmol of L-glutamine, as well as 20% bovine serum (Life Technologies Ltd, Paisley, UK) at 37°C in an atmosphere of 5% CO_2. The medium was routinely changed every three days to the point of experimentation. Furthermore, during experiments DMEM was used without serum to exclude the influence of contained growth factors and to simulate conditions after injury as previously described by Li et al [30], while PRP and decorin (R&D Systems, Minneapolis, MN, USA) were added to non-control wells.

Preparation of PRP-derived growth factors

PRP derived from various donors was prepared as previously described by Doucet et al. and Schallmoser et al. [34,35,36]. All participants provided their written consent to participate in the study. The consent procedure and the study were approved by the Slovenian national ethics committee and the institutional review board at the University Medical Centre Maribor. One hundred ml of blood was collected from five single blood donations. Blood was drawn into a tube containing 10 mL Acid Citrate Dextrose (ACD-A) anticoagulant. Five mL of PRP was then prepared using a Magellan Plasma Separator (Medtronic, Minneapolis, USA) according to the manufacturer's protocol. Furthermore, we froze the PRP units to-80°C, without further manipulation. This was followed by thawing of the units in a water bath at 37°C until ice clots disappeared. We repeated this process 5 times in order to lyse the platelets and release the growth factors. Mechanical lysis was the method of choice in order to preserve chemically unhampered samples. The solution was centrifuged for 10 minutes at 1,500 rpm, and the supernatant was used for ultrafiltration (0.22 μm filter size) in order to remove any residual particles as these tend to aggregate and may induce alloimmunization. One point five mL of suspension containing growth factors was diluted with DMEM (Sigma-Aldrich, Grand Island, USA) in order to make 5%, 10% and 20% solutions of PRP-derived growth factors.

Viability of treated cell cultures

To determine the effect of PRP-derived growth factors and decorin on the viability of hMC we performed a Tetrazolium [3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide] (MTT) assay. The cells were seeded at 10,000 cells/well in 96-well plates at 37°C in a humidified CO_2 incubator until they were confluent. DMEM prepared with different concentrations of PRP exudates (5%, 10%, and 20%) and decorin (10 ng/mL, 25 ng/mL, and 50 ng/mL) were added to the cells, which were further cultured for 4 hours. MTT 5 mg/mL (Sigma) was used for the quantitative determination of cell viability as previously described [37,38].

Proliferative ability of exposed cell cultures

To perform the cell proliferation assay, hMCs were separately seeded at 10,000 cells/well in 96-well plates at a concentration of 30 viable cells per well. DMEM prepared with different concentrations of PRP exudates (5%, 10%, and 20%) and decorin (10 ng/mL, 25 ng/mL, and 50 ng/mL) were added and incubated for 7 days at 37°C in an atmosphere of 5% CO_2. After incubation we stained the cells with crystal-violet and measured the absorbance at 595 nm.

Enzyme-Linked Immunosorbent Assay

Cells were seeded at 10,000 cells/well on 96-well plates. After 48 hours of incubation with 10% and 20% PRP-exudate and decorin (25 μg/mL) the TGF-β and MSTN levels in supernatants were assayed using commercially available enzyme-linked immunosorbent kits (Invitrogen Co., Camarillo, USA and USC Life Science Inc., Wuhan, China) following the manufacturer's protocol. Additionally, we measured the TGF-β concentration in a PRP exudate sample only to show its presence before applying it to the culture. Triplicates were performed for all assays.

Myogenic differentiation

For myogenic differentiation, myoblasts were seeded in 4-well chamber slides at 10,000 cells/well and 48-well plates at 5,000 cells/well. After 24 hours PRP-exudate, decorin and their combination was added to DMEM without bovine serum, while no drug was added to the control group. After immunostaining, the cells were prepared for microscopical examination and Image Stream single-cell analysis to evaluate the expression of cell surface markers such as CD56 and the myogenic markers MyoD, Myogenin and desmin as further described below.

Confocal imaging. Immunocytochemical staining was performed as previously described [39]. Briefly, the cells were seeded in 4-well chamber slides and cultured with a 10% PRP exudate, decorin 25 ng/mL and their combination. After 4 days the cells were fixed, permeabilized and stained with primary and secondary antibodies against desmin (FITC), α-tubulin (goat-anti rabbit IgG Cy3), and nuclei (DRAQ5), all obtained from Abcam (Cambridge, UK). Triplicates were performed for the analysis. Optical images were acquired at the center of chamber slides where the cell density is at its highest using a Leica TCS SP5 II confocal microscope (Leica Microsystems Ltd., Mannheim, Germany) and analyzed using Photoshop CS6 (Adobe, San Jose, USA), where a "Color Range" tool was used in combination with a histogram palette to count the pixels that correspond to desmin-positive areas in an image.

Imaging multicolor flow cytometry. In order to quantitatively determine myogenic differentiation of cultured myoblasts, we analyzed the cells using the imaging flow cytometer ImageStreamX (Amnis Corporation, Seattle, USA). After harvesting, followed by permeabilization, we stained the cells simultaneously with antibodies against CD56 (CD56-APC conjugate; BD Pharmingen, Heidelberg, Germany), MyoD (goat MyoD and bovine anti-goat IgG PE conjugate; Santa Cruz GmbH, Heidelberg, Germany) and myogenin (AlexaFluor 488; R&D

Systems, Abingdon, UK) according to the manufacturer's protocols. We acquired cell images of 5,000 events per sample at 40 x magnification using 488 nm and 658 nm lasers and fluorescence was collected using three spectral detection channels. For triple-stained cells (CD56, MyoD and Myogenin), three single-stained controls were used to compensate fluorescence between channel images. Cell images were analyzed with the IDEAS image-analysis software (Amnis). First, we gated the aspect ratio versus cell area to isolate a population of single cells on a bivariate plot. Cells within the focal plane were further selected using a two-dimensional plot of image contrast versus root-mean-squared (rms) gradient. We scouted for the presence of MyoD and myogenin exclusively in CD56 positive cells by measuring the intensity of each probe. Results are expressed as percentage ratios between the cells positive for CD56 only, those additionally positive for MyoD and those expressing also myogenin or myogenin alone in each treated and untreated population of cells, respectively.

Statistical analysis

Data collected was analyzed using the Statistical Package for Social Sciences (SPSS) version 16. All of the results from this study are expressed as the mean +/- S.D. The differences between means were considered statistically significant if $p < 0.05$. Comparison between groups was made by a Student's t test, two-way ANOVA for multiple comparisons and Bonferroni post-hoc analysis. A Chi^2 test was used to analyze the results from image flow cytometry. SPSS software was used for statistical analysis.

Results

PRP enhances viability of hMCs

The mitochondrial activity of cells, as determined by the MTT assay, was significantly increased ($p<0.001$) after exposure to tested concentrations of PRP exudates. Similarly, viability was elevated in all tested concentrations of decorin, except 50 ng/ml (Fig. 2). PRP 20% and 10% exudates enhanced the viability of cells to more than 400% when compared to the control ($p<0.001$). The viability of cells treated with PRP exudates was also significantly higher when compared to both decorin concentrations of 25 ng/ml and 50 ng/ml ($p<0.001$) whereas there was no significant difference between 10% and 20% PRP exudate concentrations.

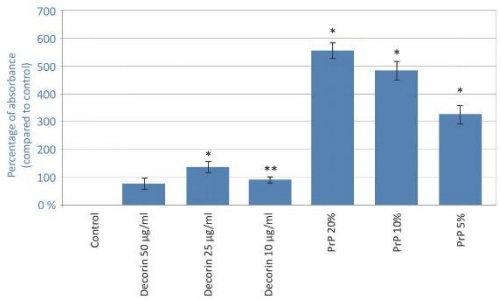

Figure 2. MTT assay. Three independent tests were performed and the results were expressed by the mean ratios (%, ± SD) of absorbance in treated wells to those in control wells. ANOVA, *$p<0.001$, **$p<0.05$ compared to control.

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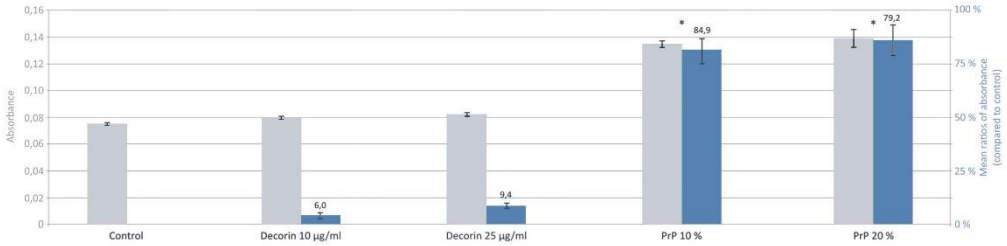

Figure 3. Myoblast proliferation rate. The figure shows the proliferation of myoblasts after 7 days of incubation with DMEM supplemented with decorin 10 ng/ml, decorin 25 ng/ml, 10% PRP exudate and 20% PRP exudate. Three independent tests were performed and the results are expressed by the absolute absorbance values (in blue) and mean ratios (%, ± SD) of absorbance in treated wells to those in control wells (in grey). ANOVA, *p<0.001 compared with control.

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PRP enhances the proliferative ability of hMC

We performed a crystal violet assay to determine the proliferative rate of cells incubated for 7 days with two different decorin and PRP exudate concentrations (Fig. 3). Both decorin concentrations did not show a significant effect on cell proliferation compared to the control; however, cultivation with both PRP exudates leads to a 5-fold increase in cell proliferation ($p<0.001$) with no significant differences between them.

PRP and decorin—both and in combination down-regulate TGF-β and MSTN expression by hMCs

Decorin was shown to down-regulate the expression of TGF-β when compared to the control by more than 15% (0.552±0.014 vs. 0.676±0.008 $p<0.001$) but significantly less than both 10% and 20% PRP exudate concentrations (0.466±0.0.017 and 0.467±0.00027 $p<0.005$). Tested PRP exudates significantly down-regulated TGF-β expression by more than 30% ($p<0.001$) (Fig. 4). The pure PRP exudate sample is more than 30% (0.913±0.011 vs. 0.676±0.008, $p<0.001$) richer in TGF-β concentration than a control sample of cultivated muscle cells confirming that PRP represents an exogenous source of TGF-β.

Similarly, the MSTN expression levels were significantly down-regulated by both decorin and PRP exudates (Fig. 4). MSTN levels of cells treated with decorin were decreased by 28.4% ($p<0.001$) and 23.1% by PRP ($p<0.001$) when compared to the control group. There was no significant difference between both PRP exudate concentrations withregard to MSTN expression.

Since no significant differences in cell viability and cytokine expression of 10% and 20% PRP exudates were found, we performed further experiments with a lower exudate concentration (10%). When cultivating cells with a combination of decorin 25 ng/ml and 10% PRP exudate, the TGF-β cytokine expression was further down-regulated by 59.9% when compared to the control and twice as much when treated with PRP exudate or decorin alone (Fig. 5). MSTN levels were decreased by 24% compared to the control but were not significantly lower than in cells treated with PRP exudate or decorin alone.

PRP and decorin act synergistically towards myogenic differentiation of hMCs

ImageStream analysis revealed the differences in myogenic differentiation between the control, PRP and decorin treated groups, as well as their combinations. We captured and analyzed

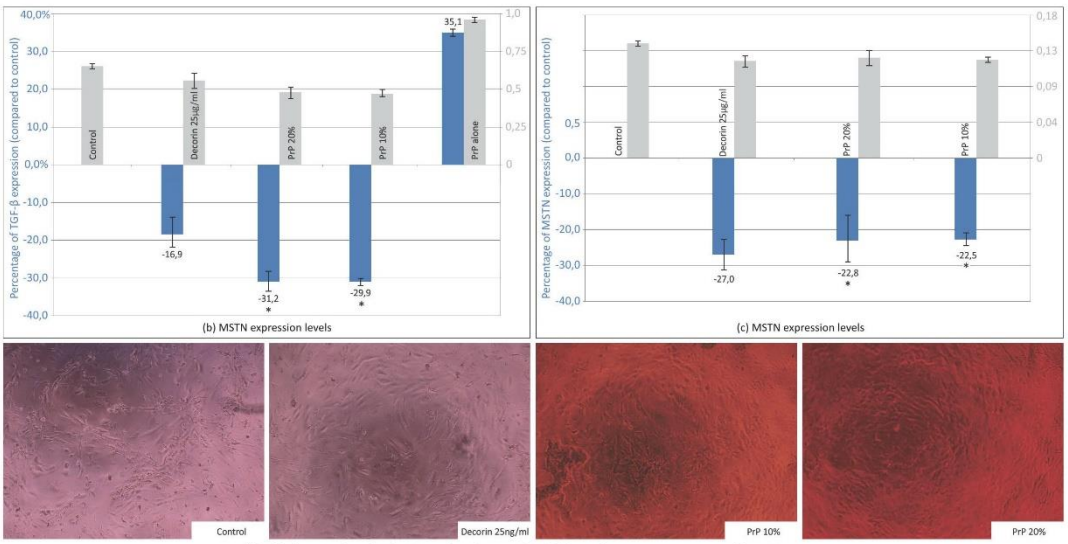

Figure 4. TGF-β and myostatin expression. (a) Cultured myoblasts incubated with DMEM and supplemented with decorin 10 ng/ml, decorin 25 ng/ml, 10% PRP exudate and 20% PRP exudate (ELISA). Cytokine expression was measured after 48 hours of incubation. (b): TGF-β expression. (c): MSTN expression. Three independent tests were performed and the results were expressed by the absolute absorbance values (in grey) and mean ratios (%, ± SD) of absorbance in treated wells to those in control wells (in blue). ANOVA, $p<0.001$ compared with control, *$p<0.005$ compared with decorin. In PRP group, no cells were growing in the well in order to determine the TGF-β content in PRP.

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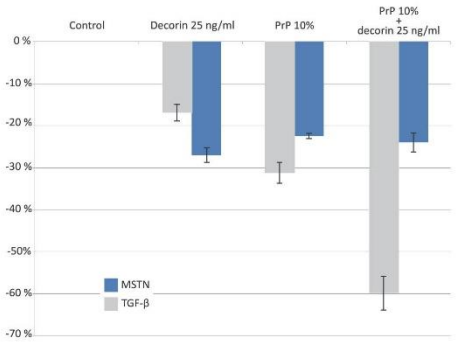

Figure 5. TGF-β and MSTN expression (ELISA). Three independent tests were performed for each cytokine and the results are expressed by the mean ratios (%) of absorbance in treated wells to those in control wells. ANOVA, $p<0.001$ compared to control.

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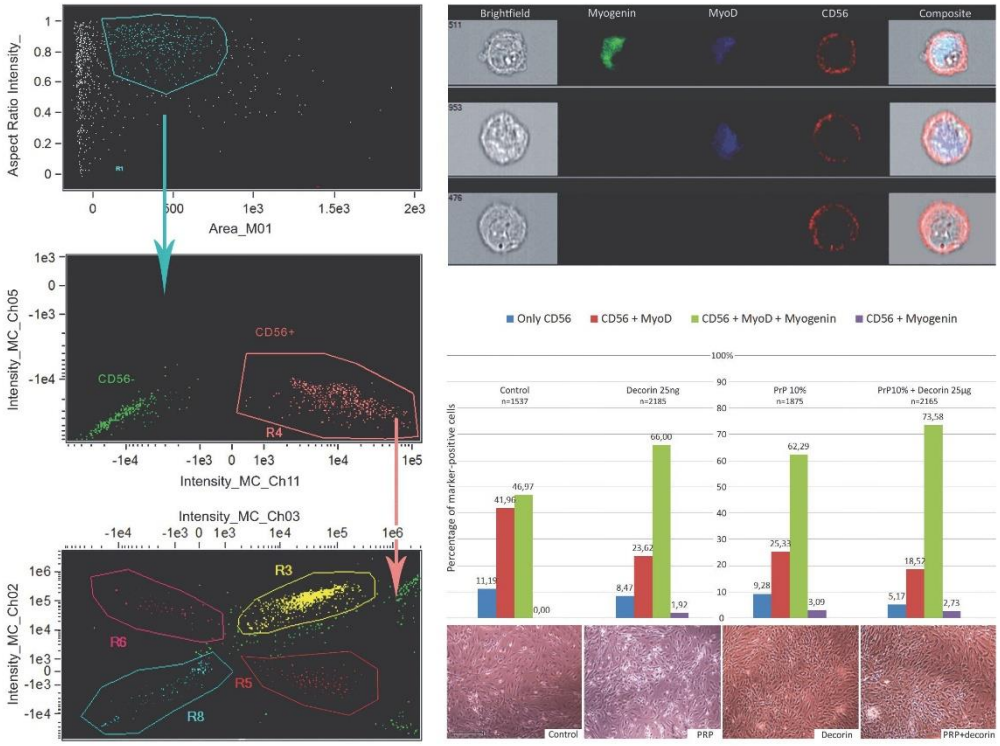

Figure 6. Myogenic differentiation of hMC. Each sample consisted of 5,000 cells that were hierarchically gated according to the expression of specific markers (top right). Top left: Aspect ratio versus cell area gated on a bivariate plot in order to isolate single cells; middle left: subgroup of previously gated cells in focal plane positive with CD56 surface marker selected using a two-dimensional plot of image contrast versus root-mean-squared (rms) gradient; bottom left: presence of MyoD and/or myogenin exclusively in CD56 positive cells by measuring the intensity of each probe. Bottom right: Results are expressed as percentage ratios between cells positive only for CD56, those additionally positive for MyoD and those expressing also myogenin or myogenin alone in each treated as well as each untreated population of cells. Scale bar = 50 µm.

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5,000 cells of each population positive for CD56. Further cells were hierarchically gated according to co-expression of CD56 and MyoD and/or myogenin as markers of early and late stages of differentiation (Fig. 6).

In the PRP-treated group 39.1% more myogenin positive cells were detected compared to the control. Moreover, there was a 3.09% increase in cells positive only for myogenin, whereas no such cells were found in the control cell population. The population of cells positive only for myogenin is considered as fully differentiated and capable of fusion into myotubes as well as future mucle fibers and is thus of great importance for muscle regeneration. At the same time 20.6% fewer cells remained quiescent (positive only for CD56). Cells positive for both MyoD and myogenin represent the population that shifted significantly towards mature myocites during myogenesis but are not yet fully committed.

While decorin alone led only to a slightly increased differential shift when compared to PRP, it was shown to exert a synergistic effect with PRP. The combination led to a 16.7%

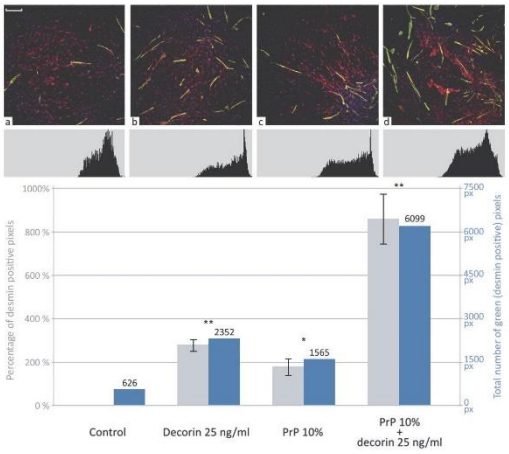

Figure 7. Desmin expressing myotubules. Immunofluorescence staining for merged stained nuclei (blue), α-tubulin (red), and desmin (green). Three independent tests were performed and the results are expressed by the mean values of "Selective color" pixel count (in grey) and mean ratios (%, ± SD) of pixel count in the treated group to those in control groups (in blue). ANOVA, * $p<0.01$, ** $p<0.005$ compared with the control. (a) control, (b) decorin-treated group, (c) PRP-treated group, (d) PRP and decorin-treated group. Evident differences can be seen in the polynucleated myotubules count and in desmin expression among the control and PRP and/or decorin treated groups. Scale bar = 200 μm.

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increase in cells positive for both myogenin and MyoD (compared to PRP alone). When compared to the control, the combination led to a 60.4% increase in cells positive for myogenin and MyoD and a 46.2% decrease among quiescent cells (only $CD56^+$).

In order to visualize the cultured cells and study the presence of desmin-containing myocites we took photomicrographs using a confocal laser microscope. We stained the nuclei, α-tubulin, to visualize the cell cytoplasm, and desmin, an intermediate filament as one of the key markers of myogenic differentiation of myoblasts [39,40]. A color selection tool and histogram analysis were performed using Adobe Photoshop to count the pixels as desmin positive (Fig. 7). A statistically significant up-regulation of desmin expression ($p<0.01$ for the PRP treated group, $p<0.005$ for the decorin and PRP + decorin treated groups) was present in all therapeutic groups when compared to the control. While no significant difference was found between the PRP and decorin-treated groups, their combination led to a more than 3-fold increase ($p<0.005$) of desmin expression when compared to single bioactives.

Discussion

Despite the limited number of relevant reports confirming its value, platelet-rich plasma (PRP) as a source of autologous growth factors is widely used in therapy of tendinopathies and ligament injuries. Recently, increasing tendencies [41,42] to use PRP to improve skeletal muscle regeneration after injury, raise concerns especially because of one PRPs specific growth factor TGF-β, which is known to impair the process of muscle regeneration. However, our study suggests the opposite. By improving the metabolic activity of myoblasts, we not only excluded the potential cytostatic effect of PRP, but also showed its positive effect on the viability as well as the proliferation of hMC. These findings correlate well with a few previous publications about the positive effects of PRP

and various individual growth factors on skeletal muscle regeneration [9,10,11,12,30,43]. The inhibitory effects on TGF-β caused by PRP were also significantly higher when compared to decorin, which was identified as a powerful regulatory agent of muscle regeneration [17,19,20,26].

Although PRP represents a significant source of TGF-β, its overall expression in hMC was down-regulated and was surprisingly greater when compared to hMC treated with decorin, which is one of the most potent TGF-β antagonists [44]. The mechanism behind such an effect remains unknown. It seems as though there is a synergistic connection between multiple PRP-derived growth factors responsible for the outcome and this will be the focus of our future studies.

MSTN is produced rather by skeletal muscle cells and is normally not present in autologous platelet concentrates. Although MSTN does circulate in the blood and may therefore theoretically appear in the preparation no concerns about MSTN-induced fibrotic remodelling after intramuscular PRP injection are found in the literature. However, our data shows that MSTN expression by hMC was down-regulated when treated with PRP in comparison to non-treated cells and almost reached the regulatory level of decorin which is believed to be one of the strongest inhibitors of MSTN activity.

PRP itself down-regulates TGF-β expression in hMC and further reduction is possible with additional inhibition of TGF-β by decorin. After co-cultivation of hMC with PRP and decorin, a 60% decrease in TGF-β expression was identified indicating their synergistic effect.

During muscle regeneration satellite cell progenitors are being activated from the quiescent state followed by the expression of MRFs, while some of the satellite cells remain inactive to provide a further regenerative scaffold. The whole process is mediated by TGF-β and MSTN [45,46] [47,48,49]. Advanced single-cell analysis showed a significant increase of cells expressing MyoD and/or myogenin, which are both MRFs characteristic for myogenic differentiation. Again, synergism of PRP and decorin is evident as the combination of both leads not only to a duplicated count of active satellite cells, but also to a significant shift in myogenic terminal differentiation.

We also showed an evident increase in desmin expression, as well as polynuclear cell count after PRP and decorin treatment. Desmin is a muscle-specific intermediate filament protein expressed early and late in the myogenic program and accumulated during myogenesis *In vitro* [50]. Its expression is directly controlled by MyoD and myogenin and is, as the first expressed cytoskeletal protein during myogenesis, one of the key markers of muscle commitment [51,52]. Our findings correlate with the up-regulation of MyoD and myogenin activation detected using imaging flow cytometry, and serve as additional proof of myogenic shifts towards terminal myoblast differentiation.

Conclusion

Activation of satellite cells and myogenic differentiation of proliferating myoblasts are two crucial steps for effective skeletal muscle regeneration. Our findings suggest that preparations of autologous growth factors might act as a relevant therapeutic option for skeletal muscle injuries, despite the fact that they represent an additional source of TGF-β. In combination with TGF-β antagonist decorin, the effect of this cytokine can be eliminated. This study presents not only new mechanistic insights into the effects of PRP, but also a possible new therapeutic approach for injured skeletal muscle. Although human studies are due to take place in order to confirm the *In vivo* value of our findings, it seems that decorin-supplemented PRP therapy can be rationalized.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: RK MSR LG MT. Performed the experiments: RK MT LG. Analyzed the data: RK MT. Contributed reagents/materials/analysis tools: MV. Wrote the paper: RK MT MSR MV.

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INVITED REVIEW

SKELETAL MUSCLE–DERIVED CELL CULTURES AS POTENT MODELS IN REGENERATIVE MEDICINE RESEARCH

ROBI KELC, MD,[1] MARTIN TRAPECAR, MSc,[2] MATJAZ VOGRIN, MD, PhD,[1] and AVRELIJA CENCIC, PhD[2]

[1] Department of Orthopaedic Surgery, University Medical Center Maribor, Ljubljanska Ulica 5, Maribor SI-2000, Slovenia
[2] Department of Biochemistry and Nutrition, Faculty of Medicine, University of Maribor, Maribor, Slovenia

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ABSTRACT: Cell cultures have been used extensively by many scientists in recent decades to study various cell and tissue mechanisms. The use of cell cultures has many advantages over use of *in vivo* experimental models, but there are also limitations. As skeletal muscle–derived cell cultures become more commonly utilized in studies of muscle regeneration processes the question of their relevance in experimentation is highlighted with regard to *in vivo* experimental models. This article reviews studies that have been performed simultaneously in *in vivo* and *in vitro* experiments on skeletal muscle and assesses the correlation of results. Although they seem to correlate, no such studies on humans have been performed so far.

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For many years cell cultures have been used extensively by biologists, physiologists, pharmacists, geneticists, biochemists, nutritionists, and other scientists. These cultures are used to study the mechanisms and control of growth, differentiation, proliferation, and gene expression; to test toxicity and screen drugs; for recombinant protein production; and for purposes of reimplantation. Because *in vitro* platforms do not simulate the complex cell–cell and cell–matrix interactions that are crucial for regulating cell behavior *in vivo*,[1] and because of the strict regulatory restrictions limiting the use of animal models,[2] human-based tissue-like constructs for disease modeling and drug testing have been developed extensively in recent decades.

Cell cultures were first devised at the end of the 19th century, when Wilhelm Roux removed a section of the medullary plate of an embryonic chicken and maintained it in a warm saline solution for several days. In 1907, Harrison was the first to study the behavior of animal cells free of systemic variations that might arise *in vivo*, both during normal homeostasis and under the stress of experimentation, when he demonstrated the growth of frog nerve cell processes in a medium of clotted lymph.[3] About 40 years later the first permanent cell line was developed by Earle from subcutaneous mouse tissue.[4] One of the first significant applications of cell cultures arose from the observation that poliovirus could be cultured in simian and human kidney cells as opposed to nerve tissue. Soon after live attenuated vaccines for polio were developed, they were also produced in kidney cell lines.[5] In the 1960s, Chinese hamster ovarian cells were introduced and grown in a cultured monolayer, thereby offering a good basis for biological and medical research. The development of technologies to economically express proteins from mammalian cells began in the 1970s and 1980s. In the last 3 decades we have witnessed a >6-fold increase in publications describing cell culture experiments (Fig. 1).

Cell cultures have been of great importance in understanding intracellular activity and flux, genomics, proteomics, and cell–cell interactions over a wide field of applications (Table 1). Their major advantages over *in vivo* systems include the ability to control the physiochemical environment and physiological conditions. Although tissue samples are invariably heterogeneous, cultured cell lines assume a homogeneous constitution after 1 or 2 passages.[6] At each subculture, replicate samples are identical, and their shared characteristics may be perpetuated over several generations. Because of virtually identical replicates, simplification of statistics is another advantage of cell lines as experimental models.

Experiments using cell cultures also have an economic benefit. Usually fewer reagents are needed, as they can be applied directly into the culture and at lower concentrations. There is no systemic excretion or loss due to distribution to other tissues. Economic benefit is also apparent through use of screening tests, with many variables and replicates with additional legal, moral, and ethical questions of animal experimentation being avoided.[6–8]

SKELETAL MUSCLE–DERIVED CELL CULTURES IN BIOCHEMICAL RESEARCH

Cell cultures also have an important role in endocrine and orthopedic research. In recent decades,

Abbreviations: ARB, angiotensin receptor blocker; COX-2, cyclooxygenase-2; DCN, decorin; FOTM, follistatin-overexpressing transgenic mice; MRF, muscle regeneratory factor; MRF4, muscle regeneratory factor 4; MSTN, myostatin; Myf5, protein, a member of MRF family; MyoD, protein, a member of MRF family; NSAID, non-steroid anti-inflammatory drug; RJMC, skeletal muscle cell line; TGF-β, transforming growth factor-beta; α-SMA, alpha-smooth muscle actin; IFN-γ, interferon-gamma

Key words: cell culture; experimental model; *in vitro*; *in vivo*; skeletal muscle cell

Additional Supporting Information may be found in the online version of this article.

Correspondence to: R. Kelc; e-mail: robi.kelc@uni-mb.si

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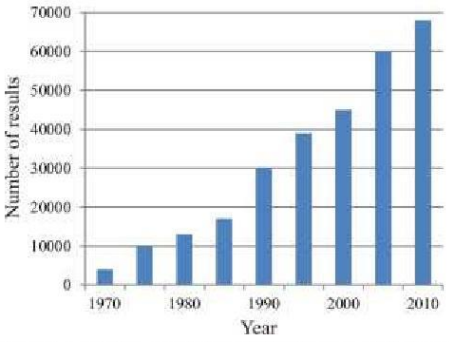

FIGURE 1. Number of results in PubMed for 'cell culture' from 1970 showing an increase in research on *in vitro* models.[6] [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

studies using cell cultures have led to important discoveries of different cell and tissue processes, regeneration, and effects of exogenous materials on specific tissues. Skeletal muscle–derived cell cultures have been the subject of increased interest in studies of regeneration processes in injured and developing muscles, especially when complementary to experiments on *in vivo* models. However, such studies using both *in vivo* and *in vitro* experiments have been performed mainly using non-human cell cultures.

Skeletal muscle plays an important role not only in body movements, postural support, and heat production, but also in maintenance of homeostasis and metabolic regulation. Skeletal muscle is one of the easiest tissues to culture in large amounts, because it is plentiful and readily obtainable from a wide variety of species, including humans (Fig. 2).[9] As such, these tissues have been used in studies of genetic muscule diseases[10] and

Table 1. Role of cell cultures in basic research[6]

Intracellular activity	DNA transcription
	Protein synthesis
	Energy metabolism
	Drug metabolism
	Cell cycle
	Differentiation
	Apoptosis
Intracellular flux	RNA processing
	Hormone receptors
	Metabolite flux
	Calcium mobilization
	Signal transduction
	Membrane trafficking
Genomics	Genetic analysis
	Transfection
	Transformation
	Immortalization
	Senescence
Proteomics	Gene products
	Cell phenotype
	Metabolic pathways
Cell-cell interaction	Morphogenesis
	Paracrine control
	Cell proliferation
	Kinetics
	Metabolic cooperation
	Cell adhesion and motility
	Matrix interaction
	Invasion

cardiac grafting,[11] in microgravity experiments to study the effects of low-gravity environments on muscles,[12] and, as major target cells of insulin action, in studies on their role in diabetes mellitus.[13,14] In recent years, skeletal cell muscle cultures have been used in studies of muscle growth, regeneration, and repair during development and after injury. Using modern technology techniques, such as confocal microscopy, flow cytometry, and DNA protein analysis, can offer

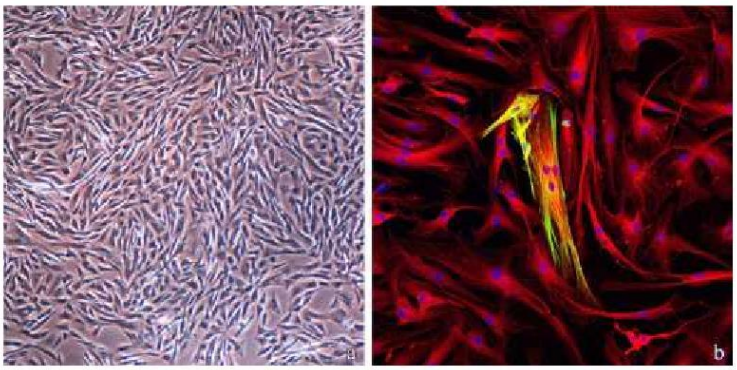

FIGURE 2. Isolated human skeletal myoblasts suitable for *in vitro* experimentation. (**a**) Primary culture of human skeletal muscle cell under light microscope. (**b**) Human skeletal muscle cell culture under confocal microscope with stained nuclei (blue), α-tubulin (red), and desmin (green). Myoblasts have fused into a large, centrally located multinucleated myotube. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

greater detailed insight into cellular physiology. Identification of genes and transcription factors involved in muscle proliferation and differentiation has led to better understanding of skeletal muscle regeneration, where the fusion of mononucleated cells to form multinucleated myotubes represents a central event.

A typical feature during muscle differentiation is the variation in expression of various genes along with myogenic factors.[15] Sequence-specific myogenic regulatory factors (MRFs) are expressed exclusively in skeletal muscle and regulate the process of muscle development.[16]

It is the role of MRFs to govern the expression of multiple genes in myogenesis, from the engagement of mesodermal cells in the muscle lineage to the differentiation of somatic cells and the terminal differentiation of myocytes into myofibers.[17] MRFs consist of a group of transcription factors that have been divided into 2 functional groups. The primary MRFs, MyoD and Myf-5, are required for determination of skeletal myoblasts; the secondary MRFs, myogenin and MRF4, act later, likely as differentiation factors.[16] Discovery and identification of these MRFs offer researchers new insights into the process of muscle regeneration and tracking its individual phases. Detailed understanding of molecular mechanisms has a key role in developing novel biological targets and therapies. Recently, *in vitro* systems capable of inducing vigorous contraction of skeletal muscle cells have been developed and have attracted considerable attention. Until recently, analyses of some specific mechanistic details could only have been performed using whole animal experiments, because conventional cell culture systems lack the muscle contractile activity required for physiologically relevant energy expenditure and mechanical stress.[18] This has been shown to be of a great importance in studies of insulin- and exercise-induced glucose uptake by skeletal muscle,[19,20] where contracting human skeletal muscle cell cultures were established by applying electrical pulse stimulation. Such cell-based assays will be beneficial for decreasing costs while leading to a more accurate predictive capacity in drug discovery.[18]

For *in vitro* models of skeletal muscle to be adequate they need to mimic *in vivo* models and cellular behavior. Muscle fibers undergo force in a longitudinal direction under normal physiological conditions *in vivo* during growth and exercise, and it is also desirable for *in vitro* systems to simulate such a pattern.[21] However, the various types of stretching systems available differ with regard to producing uniaxial or multiaxial[22–24] stretch, which is of great importance to consider when investigating pathophysiological responses in different experiments.

CORRELATION BETWEEN *IN VITRO* AND *IN VIVO* RESULTS

Over the last 30 years, a number of *in vitro* experimental models have been developed that show similarities with muscle *in vivo*[21] (see Table S1 in supplementary material). In 2003, Li et al.[25] studied the induction of differentiation of myogenic cells into fibrotic cells by transforming growth factor-beta (TGF-β) in injured skeletal muscles of normal mice. Simultaneously, they cultured C2C12 murine myoblast cells with TGF-β and used live mice for intramuscular injection of TGF-β.

In vitro, they reported an increase of profibrotic markers α-SMA, vimentin, and fibronectin, and a decrease in myogenic markers of muscle regeneration, MyoD and myogenin. After the injection of TGF-β into muscles of mice, mononucleated cells differentiated into fibrotic cells, which resulted in scar tissue formation.

In both experiments they also added decorin (DCN), a recombinant protein with a possible ability to antagonize the effects of TGF-β, and showed a decrease in fibrosis-related proteins *in vitro* and fibrotic differentiation *in vivo*.

Also in 2003, Foster et al.[26] tested the antifibrotic effects of interferon-gamma (IFN-γ) in regenerating skeletal muscles. They used primary muscle-derived murine fibroblasts and C2C12 myoblasts transfected with a plasmid containing human TGF-β gene (CT cells), both cultured with IFN-γ. In mice, they performed muscle laceration followed by intralesional application of IFN-γ. In the CT cells they noticed a remarkable down regulation of fibrosis-related proteins by IFN-γ, as well as inhibition of fibroblasts and overall growth of CT cells. In a group of mice treated with IFN-γ after muscle laceration, they showed a decrease in fibrotic area, an increase in regeneration, and improved physiological properties of injured muscles.

Zhu et al.[27] studied the role of TGF-β, myostatin (MSTN), and DCN in skeletal muscle growth regulation in wild-type MSTN$^{-/-}$ mice and on PP1 fibroblasts from murine C2C12 skeletal muscle myoblasts. After culturing both cell lines with MSTN they witnessed an increase in α-SMA and procollagen mRNA expression in PP1, and MSTN stimulation of differentiation of C2C12 cells into myofibroblasts. Adding DCN, they neutralized the effects of MSTN in both PP1 and C2C12 cells. After lacerating muscles in wild-type MSTN$^{-/-}$ mice they showed larger diameters of muscle progenitor cells in comparison to normal mice, with less fibrotic connective tissue and elevated DCN expression.

To study antifibrotic effects of angiotensin II receptor blockade (ARB) in regenerating skeletal muscle, Bedair et al.[28] used a 3T3 murine fibroblast line, PP2 primary muscle fibroblast isolates,

and the C2C12 myoblast line, all cultured with angiotensin II. They did not find any difference in cell proliferation in any of the cell lines, but there was a significant increase in the production of TGF-β in fibroblasts. In an *in vivo* experiment, they used immunocompetent mice for muscle laceration, followed by ARB injection at the site of the injury. This, on the other hand, showed the increase in regenerating myofibers and less fibrosis within the zone of injury.

In 2005, Chan et al.[29] tried to prevent scar tissue formation in skeletal muscle after injury by antagonizing TGF-β with suramin. They used fibroblasts from murine skeletal muscle and cultured them with suramin. In 1 of the control groups they showed that TGF-β stimulates fibroblast proliferation. *In vitro*, suramin increased the number of regenerating myofibers.

In vivo, the research team induced strain injuries to normal mice and injected suramin into the muscle immediately after injury. Later, they reported a decrease in fibrotic tissue and decreases in fast-twitch and tetanic muscle strength.

Recently, Zhu et al.[30] again used C2C12 myoblasts, this time to test the role of follistatin in muscle regeneration, angiogenesis, and fibrinogenesis after muscle injury. They cultured the C2C12 cell line with MSTN, follistatin, activin A, and TGF-β, and found out that TGF-β inhibits myogenic differentiation of myoblasts. Results show that follistatin prevented TGF-β from inhibiting myogenesis and thereby stimulated myoblasts to undergo myogenic differentiation. To confirm these results in an *in vivo* model, they used normal and follistatin-overexpressing transgenic mice (FOTM) to perform a partial muscle laceration. Later in regeneration, they found less MSTN in FOTM with greater expression of $CD31^+$ capillary-like structures, larger diameter regenerating muscle fibers, and smaller collagen deposition and fibrotic area development.

To study the controversial role of nonsteroidal anti-inflammatory drugs (NSAIDs) after muscle injury, some attempts have been made to assess their effects on skeletal muscle regeneration. Bondesen et al. performed an *in vitro* experiment with a COX-2 inhibitor and showed a slower proliferation and maturation rate of differentiated myogenic precursor cells and thus delays in regenerative myogenesis.[31,32] In a muscle laceration mouse model observed by another research group, the *in vivo* data were in agreement with *in vitro* results, with expression of higher levels of TGF-β1, delayed muscle regeneration, and higher fibrosis deposition.[33]

To date, in all studies that have performed *in vitro* and *in vivo* experiments on skeletal muscles, animal models were used to see the correlation of both groups of experiments. However, as it is clear that animal models are not relevant for human studies, at the Department of Biochemistry and Nutrition of the Faculty of Medicine and University Medical Centre Maribor, we have established a novel, stable human skeletal muscle cell culture RJMC that can be readily differentiated.[34,35] The RJMC cell line was established from skeletal muscles derived from a healthy adult male by using a limiting dilution technique. Cells are positive for α-actin and myostatin. The cell line is functional, because it responds with proliferation to treatment with follistatin and other muscle-cell growth-stimulating factors. Recently, Trapecar et al. (manuscript in review) evaluated the potential of carbonated mineral water to enhance muscle proliferation. The study was simultaneously performed in a three-dimensional model of the gut linked with the RJMC muscle cell line and on human volunteers who had regularly consumed mineral water daily. Comparison of the *in vitro* and *in vivo* data showed a remarkable correlation between the results, thus proving the potential of human-derived muscle cell models.

LIMITATIONS

Despite good experimental results acquired from cell cultures and their correlation with *in vivo* models, there are still some limitations that have to be considered when deciding on the experimental model. Because there are some systemic components involved in homeostatic regulation *in vivo* that are missing in cell cultures, the cellular metabolism may be more constant *in vitro* than *in vivo*, but may therefore not be as representative.

Because of the many common contaminants, such as bacteria and yeasts, which grow more rapidly than animal and human cells, it is essential for culture techniques to be carried out under strict aseptic conditions. Cells from multicellular organisms are not accustomed to live in isolation and therefore need the provision of a complex environment that simulates normal conditions.

Another major disadvantage of cell cultures is the amount of effort and material needed for production of relatively little tissue. The expense of producing cells in culture is estimated to be about 10 times that of using animal tissue. Consequently, if large amounts of tissue are required, the reasons for providing them by culture must be very compelling.[6]

Sometimes the phenotypic characteristics of the original tissue disappear in cell culture. This effect, known as dedifferentiation, can be partially prevented by serum-free selective media. In cases where dedifferentiation occurs, however, the

relation of the cultured cells to functional cells in the original tissue is difficult to know. Due to this unwanted event, misidentification of cell lines as a consequence of cross-contamination or stock control errors is possible. Continuous cell lines have an unstable aneuploidy chromosomal constitution and therefore face the problem of instability. Heterogeneity in growth rate and the capacity to differentiate can result in variability of cells from 1 passage to the next, even in the case of short-term cultures of untransformed cells.[6]

FUTURE PROSPECTS

The previously mentioned muscle regulatory factors that control regeneration processes and muscle growth represent not only relevant molecular targets in therapies of different pathological conditions but also a valuable source for characterization of cell types and stages of regeneration. As such, they will enable development of stable and functional human skeletal muscle cell lines and, together with appropriate methods, investigation of the impact of new potential bioactive compounds.

New approaches and technology using high-end cell-based assay systems enables researchers to explore different physiological processes and delve further into the details. The commonly accepted C2C12 myoblast cell line can be used to monitor skeletal muscle cell contraction and activity as well as many other parameters. Efforts have been described to generate *in vivo*–like structures and functions of skeletal muscle, such as myotube alignment control,[36–39] substrate stiffness modulation,[36,40,41] and three-dimensional construct of myotube creation.[42,43] It has recently been shown how a Ca^{2+} transient induced by electrical pulse stimulation accelerates assembly of functional sarcomeres and stimulates contractile activity of the cells. Kaji et al. upgraded previous systems by designing electrical stimulation of C2C12 myotubes cultured on a porous membrane substrate modified with an atelocollagen membrane that has a muscle tissue-like stiffness and induces the assembly of sarcomeres in the myotubes during the differentiation from myoblasts.[18] A porous alumina membrane–based cell culture device masked by an elastic film allows electric current generated between electrodes to geometrically condense at the hole of the film and to perpendicularly pass through cells, resulting in their effective depolarization to produce vigorous contraction of the myotubes on the membrane.[18] With a proven correlation between contractility of the myotubes and glucose uptake, such systems represent a promising new tool in biochemical research.

The engineering of functional skeletal muscle tissue substitutes is a promising new potential treatment of various muscule diseases and injuries[42] although human functional myoblast cultures are much needed. Microengineering technologies used in the development of physiologically relevant *in vitro* tissue models enable more complex tissue cultures to be patterned on-chip.[44] To perform specific studies on skeletal muscle tissue models, these require *in vivo*–like structure and coculture with primary human skeletal cells with motor neurons.[44] Such models offer the ability to control stiffness and muscle contraction, either by a laminar stream of agrin,[45] a chemical secreted by neurons, atelocollagen and electrical stimulation of myotubes,[18] or by an integrated microelectrode array with aligned myotubes on a fibrin gel sheet.[46] A combination of new culturing techniques, OMICS tools, systemic biology, and integration of new microscopic approaches will guide the future of muscle research.

CONCLUSIONS

When considering the limitations as well as the ethical, economical, logistical, and procedural advantages of cell cultures as experimental models, it is clear they have a bright future in biochemical research. Application of experimental cell cultures is wide ranging, from bioproduction, drug development, and toxicology testing to tissue engineering. Experimental use of human-derived cell cultures is expected to emerge with even more representative results while offering further insight into human regeneration and repair processes, biomaterial compatibility, and drug development. To provide relevant results from *in vitro* experiments, studies done simultaneously on humans *in vivo* would be extremely valuable.

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10.2. Življenjepis

Osebni podatki	**Službeni naslov** Oddelek za ortopedsko kirurgijo Univerzitetni klinični center Maribor Ljubljanska ulica 5, 2000 Maribor **Domači naslov** Filipičeva 23, 2000 Maribor **e-pošta** robi.kelc@gmail.com **Datum rojstva:** 24.7.1983
Izobrazba	**Doktorat znanosti na področju Biomedicinske tehnologije** (2010-2016) Medicinska fakulteta Univerze v Mariboru **Doktor medicine** (2004-2010) Medicinska fakulteta Univerze v Mariboru **Univerzitetni diplomirani medijski komunikolog** (2002-2010) Fakulteta za elektrotehniko, računalništvo in informatiko Univerze v Mariboru **Gimnazijski maturant** (1998-2002) Prva gimnazija Maribor **Osnovna šola** (1990-1998) Osnovna šola Janka Padežnika Maribor
Zaposlitev	**Specializant ortopedske kirurgije** (2012 -) Univerzitetni klinični center Maribor **Asistent, Anatomija s histologijo in emrbriologijo** (2010 -) Inštitut za anatomijo, Medicinska fakulteta Univerze v Mariboru
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10.3. Osebna bibliografija za obdobje 2009-2016

ČLANKI IN DRUGI SESTAVNI DELI

1.01 Izvirni znanstveni članek

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5. KELC, Robi, TOPOLOVEC, Janja. Trendi uporabe in razvoja bioloških zdravil za zdravljenje malignih obolenj = Trends of usage and development of biological drugs for treatment of malignancies. Medicinski razgledi, ISSN 0025-8121. [Tiskana izd.], 2012, letn. 51, št. 2, str. 145-157. [COBISS.SI-ID 4351551]

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MONOGRAFIJE IN DRUGA ZAKLJUČENA DELA

2.11 Diplomsko delo

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IZVEDENA DELA (DOGODKI)

3.15 Prispevek na konferenci brez natisa

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56. VOGRIN, Matjaž, KELC, Robi. Effects of a platelet gel on early graft revascularization and knee stability after anterior cruciate ligament reconstruction : XXXII World Congress of Sports Medicine Sports medicine, the challenge for global health: Quo Vadis?, Roma, 27-30 September 2012. Roma, 2012. [COBISS.SI-ID 4416063]

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Urednik

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68. VOGRIN, Matjaž (urednik, recenzent), KRAJNC, Zmago (urednik), KELC, Robi (urednik). Nujna stanja in vnetja v ortopediji : zbornik predavanj. Maribor: Univerzitetni klinični center, 2013. 1 optični disk (CD-ROM). ISBN 978-961-6909-19-8. [COBISS.SI-ID 1988260]

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10.4. Izjava doktorskega kandidata

UNIVERZA V MARIBORU

MEDICINSKA FAKULTETA

IZJAVA DOKTORSKEGA KANDIDATA

Podpisani Robi Kelc, vpisna številka 30808796

izjavljam,

da je doktorska disertacija z naslovom »Učinek rastnih faktorjev iz avtologne trombocitne plazme in antagonistov TGF-β na proliferacijo in diferenciacijo skeletnomišičnih celic«:

- rezultat lastnega raziskovalnega dela,
- da predložena disertacija v celoti ali v delih ni bila predložena za pridobitev kakršnekoli izobrazbe po študijskem programu druge fakultete ali univerze,
- da so rezultati korektno navedeni in
- da nisem kršil avtorskih pravic in intelektualne lastnine drugih.

Podpis doktorskega kandidata:

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