VLOGA ODSTRANITVE STEFINA B IN CISTATINA C PRI Nnapredovanju IN METASTAZIRANJU RAKA PRI MIŠJEM MODELU

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MEDNARODNA PODIPLOMSKA ŠOLA JOŽEFA STEFANA
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Doktorska disertacija

THE ROLE OF STEFIN B AND CYSTATIN C KNOCKOUT ON CANCER PROGRESSION AND METASTASIS IN MOUSE MODEL.

Doctoral Dissertation

Mentor: prof. dr. Vito Turk

Somentor: doc. dr. Olga Vasiljeva

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Povzetek

Lizosomski cisteinski katepsini so vključeni tako v številne fiziološke procese kot tudi v različne patološke procese, med drugim tudi v razvoj in napredovanje raka. Proteolitično aktivnost katepsinov regulirajo njihovi endogeni inhibitorji, kot so znotrajcelični stefini ter zunajcelični kininogeni in cistatini. Cistatin C je močan reverzibilni zunajcelični inhibitor cisteinskih katepsinov, medtem ko je stefin B znotrajcelični inhibitor. Spremenjeno izražanje in aktivnost cistatina C in stefina B so potrdili pri različnih tipih raka, vendar njuna vloga pri napredovanju raka še ni povsem jasna.

V prvem delu našega raziskovalnega dela smo se osredotočili na vlogo cistatina C (CstC) in stefina B (StfB) pri rasti in napredovanju raka. V ta namen smo križali miši z izbitim genom za CstC s transgenimi mišmi PyMT ter miši z izbitima genoma za StfB in CstC s transgenimi mišmi PyMT. Tako smo pridobili PyMT transgene miši z izbitim genom za CstC (PyMT;CstC-/-) in PyMT transgene miši z izbitima genoma za StfB in CstC (PyMT;StfB-/-CstC-/-).

Medtem ko je bil CstC difuzno prisoten v mlečni žlezi, ga je bilo v tumorjih manj. Pokazali smo, da odsotnost CstC in StfB pripomore k hitrejšemu nastajanju tumorjev, vendar so tumorji pri 14 tednih starosti manjši v primerjavi s kontrolno skupino. Tako *ex vivo* kot *in vitro* smo določili znižano proliferacijo v tumorjih brez CstC, kar nakazuje na vlogo CstC v signalnih poteh, ki nadzirajo proliferacijo tumorskih celic. V tumorjih brez CstC smo opazili tudi zvišano aktivacijo p38 MAPK in s tem vlogo CstC pri razvoju raka, ki ni odvisna od TGF-β signalne poti. Proteomska analiza sekretoma primarnih PyMT tumorskih celic je pokazala znižanje nivoja vseh izoform 14-3-3 proteinov v sekretomu tumorskih celic z izbitim genom za CstC, kar nakazuje na novo povezavo med vlogami cisteinskih katepsinov, CstC in 14-3-3 proteinov, ki jo bo potrebno dodatno raziskati.

V drugem delu smo preiskovali vlogo stefina B pri imunskem odzivu. Pokazali smo, da se v stimuliranih makrofagih z izbitim genom za StfB izloča manj protivnetnega Il-10, kar nakazuje na vpliv StfB na procese vnetja.

Abstract

Lysosomal cysteine cathepsins are involved in different physiological processes and also in pathological conditions such as cancer and inflammation. Proteolytic activity of cysteine cathepsins is tightly regulated by endogenous inhibitors, including intracellular stefins and extracellular kininogens and cystatins. Cystatin C, a member of the cystatin family, is a potent reversible, tight-binding extracellular inhibitor of cysteine cathepsins expressed in a majority of tissues, whereas stefin B is localized intracellularly in many cells, as well as in macrophages. Altered expressions of cystatin C and stefin B were reported in multiple types of cancer and also in inflammation; however, their exact roles in both pathologies are not clear yet.

In the first part of our study we examined the role of cystatin C (CstC) and stefin B (StfB) in tumor progression and metastasis. We have established a CstC knockout mouse mammary cancer model and a StfB and CstC double knockout mouse mammary cancer model by crossing CstC deficient mice and StfB and CstC double knockout mice with the transgenic polyoma virus middle T oncogene (PyMT) mice that spontaneously develop mammary gland adenocarcinomas. We show that CstC was highly expressed in normal mammary gland tissue while its protein level was reduced in the tumor tissue. We show that ablation of CstC and StfB resulted in earlier development of PyMT tumor nodes. However, despite earlier onset the size of mammary tumors at 14 weeks was smaller in both genotypes compared to wild-type mice. Furthermore, decrease in proliferation of CstC knock-out PyMT tumor cells was demonstrated ex vivo and in vitro indicating a role for this protease inhibitor in signaling pathways that control cell proliferation. An increase in phosphorylated p-38 MAPK was observed in CstC knockout tumors, suggesting a novel function for cystatin C in cancer development, independent of the TGF-β pathway. Moreover, proteomic analysis of the CstC wild-type and knockout PyMT primary cell secretomes revealed a decrease in the levels of 14-3-3 proteins in the secretome of knock-out cells, suggesting a novel link between cysteine cathepsins, cystatin C and 14-3-3 proteins in tumorigenesis, calling for further investigations.

In the second part, we investigated the role of stefin B in immune response. Stimulated StfB knock out macrophages secreted less anti-inflammatory Il-10. The data suggest the increased sensitivity of stefin B deficient macrophages to inflammation.

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Kratice

°C	. . .	stopinje Celzija
3,3-DAB	. . .	3,3-diaminobenzidin
ang	. . .	angleško
Arg	. . .	aminokislina arginin
BMDM	. . .	makrofagi iz kostnega mozga (ang. bone marrow-derived macrophages)
BSA	. . .	goveji serumski albumin
CHX	. . .	cikloheksamid
CstC	. . .	cistatin C
CTSB	. . .	katepsin B
CTSH	. . .	katepsin H
CTSL	. . .	katepsin L
CTSS	. . .	katepsin S
CTSX	. . .	katepsin X
dH_20	. . .	destilirana voda
DMEM	. . .	gojišče, spremenjeno po Dulbeccu (ang. Dulbecco's Modified Eagle Medium)
DMSO	. . .	dimetil sulfoksid
DNA	. . .	deoksiribonukleinska kislina (ang. deoxiribonucleid acid)
DTT	. . .	ditiotreitol
ECL	. . .	povečana kemoluminiscenca (ang. enhanced chemiluminiscence)
EGFR	. . .	receptor epidermalnega rastnega dejavnika
E64	. . .	L-trans-epoksisukcinil-leucilamid(4-gvanidin)butan
EDTA	. . .	etilen diamintetraacetat
ERK	. . .	zunajcelična regulatorna kinaza (ang. extracellular signal-related kinase)
FBS	. . .	fetalni goveji serum
FVB/N	. . .	sev miši FVB/N
GLX	. . .	glutamaks
H2O2	. . .	vodikov peroksid
HEPES	. . .	4-(2-hidroksietil)piperazin-1-etansulfonska kislina
Gly	. . .	aminokislina glicin
IFN-γ	. . .	interferon gama
JNK	. . .	C-Jun N-terminalna kinaza (ang. c-Jun N-terminal kinase)
kDa	. . .	kilodalton, enota molekulske mase
KO	. . .	izbitje (ang. knock-out)
Leu	. . .	aminokislina levcin
LPS	. . .	lipopolisaharid
LTR	. . .	ang. long terminal repeat
MAPK	. . .	z mitogenom aktivirana proteinska kinaza (ang. mitogen-activated protein kinase)
MS	. . .	masna spektrometrija

MS/MS	. . .	tandemska masna spektrometrija
mRNA	. . .	informacijska ribonukleinska kislina (ang. messenger ribonucleic acid)
µl	. . .	mikroliter
µM	. . .	mikromolarna koncentracija
NaDS	. . .	natrijev dodecil sulfat
NP-40	. . .	nonilfenilpolietilen glikol; (ang. nonylphenylpolyethylene glycol)
PBS	. . .	fosfatni pufer (ang. phosphate buffer saline)
PCR	. . .	verižna reakcija s polimerazo (ang. poylmerase chain reaction)
PEG	. . .	polietilen glikol
Phe	. . .	aminokislina fenilalanin
P/S	. . .	penicilin in streptomicin
PI	. . .	propidijev jodid
rcf	. . .	relativna centrifugalna sila (ang. relative centrifugal force)
RNA	. . .	ribonukleinska kislina (ang. ribonucleid acid)
MMTV-PyMT	. . .	ang. Mouse mammary tumor virus-Polyoma virus middle T oncogene
sod.	. . .	sodelavci
StfB	. . .	stefin B
TEMED	. . .	N, N, N', N'-tetrametilendiamin
TGF-β	. . .	tumorski rastni faktor (ang. tumor growth factor) β
TRIS	. . .	tris (hidroksimetil) aminometan-hidroklorid
Val	. . .	aminokislina valin
WT	. . .	divji tip (ang. wild type)
Xaa	. . .	katera koli aminokislina (ang. any aminoacid)
Z-Phe-Arg-AMC	. . .	N-benziloksikarbonil-L-fenilalanin-L-arginil-7-amino kumaril

Poglavje 1

Uvod

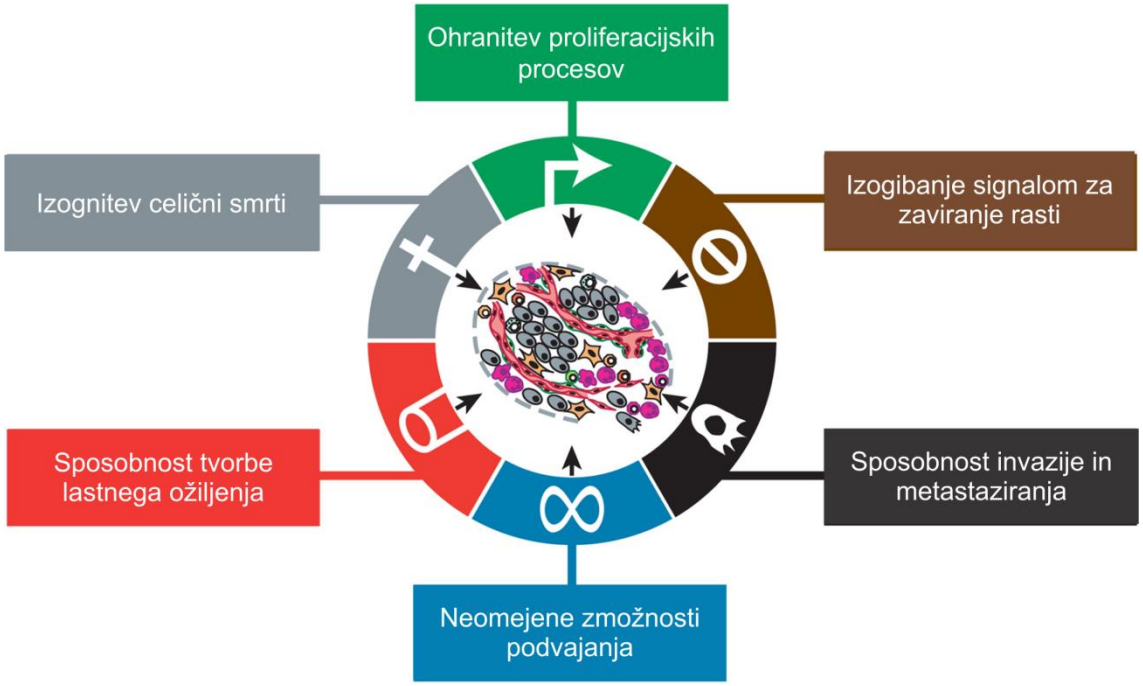
1.1 Rak

Rak spada med glavne povzročitelje smrti na svetu. Rak je bolezen, ki nastane zaradi mutacije genov, ki jih lahko podedujemo ali pa nastanejo za časa življenja. Poleg genetskih dejavnikov na razvoj raka vplivajo tudi zunanji dejavniki, kot so fizikalni kancerogeni (UV, ionizirajoče sevanje), kemični kancerogeni (tobak, arzen, aflatoksini, azbest …) in biološki kancerogeni (virusi, bakterije, paraziti …) (Burrell, McGranahan, Bartek, & Swanton, 2013; Hussain, Hofseth, & Harris, 2003). Pojavnost obolenja za rakom se v svetovnem merilu povišuje zaradi rasti in staranja prebivalstva, pa tudi zaradi vse pogostejših dejavnikov tveganja, kot so kajenje, prekomerna telesna masa, nezdrav način življenja in fizična neaktivnost (Danaei, Vander Hoorn, Lopez, Murray, & Ezzati, 2005). Leta 2012 je za rakom obolelo 14,1 milijona ljudi po vsem svetu, umrlo pa je 8,2 milijona ljudi. Pljučni rak, rak dojk, črevesni rak in rak želodca skupaj predstavljajo več kot 40 % vseh rakavih obolenj (Torre et al., 2015). V svetovnem merilu sta med najpogostejšima vrstama raka pljučni rak pri moških in rak dojke pri ženskah (25,2 % od vseh rakavih obolenj pri ženskah) (Youlden et al., 2012).

Katera koli celica se lahko spremeni v rakavo, zato glede na vrsto tkiva delimo rakava obolenja v 6 skupin. Karcinomi nastanejo iz epitelnih celic. Sem spada 80 % do 90 % vseh primerov raka. Sarkomi nastanejo iz celic opornih in vezivnih tkiv in se pojavljajo v kosteh, hrustancu, maščobnem tkivu in vezivu. Mielomi nastanejo iz celic kostnega mozga. Limfomi so rakavo obolenje limfatičnega sistema. Levkemije so rakava obolenja krvi in krvotvornih organov. Mešani tip raka pa je sestavljen bodisi iz različnih tipov raka znotraj ene skupine bodisi iz različnih skupin rakov (Fritz & World Health Organization., 2000).

Na podlagi številnih študij so prišli do zaključka, da pride pri razvoju raka do šestih glavnih značilnosti:

- ohranitev proliferacijskih procesov,
- neobčutljivost na signale za zaviranje rasti celic,
- izogibanje celični smrti,
- sposobnost invazije in metastaziranja,
- neomejene zmožnosti podvajanja,
- sposobnost tvorjenja lastnega krvnega ožilja (Hanahan & Weinberg, 2011).



Slika 1: Glavne značilnosti raka (Hanahan & Weinberg, 2011).

1.1.1 Tumorsko mikrookolje

Poleg rakavih celic je za razvoj malignega obolenja zelo pomembno tudi t. i. tumorsko mikrookolje. Tumorsko mikrookolje tumorske celice oskrbuje s hranilnimi snovmi in tumorju daje oporo, poleg tega pa tudi omogoča invazijo in metastaziranje tumorja. Tumorsko mikrookolje tvorijo različni tipi celic: imunske celice, fibroblasti, endotelijske celice in periciti; vsi tipi celic vsebujejo različne podtipe (Hanahan & Coussens, 2012). Vsaka od celic mikrookolja ima vsaj eno od glavnih značilnosti raka (Hanahan & Weinberg, 2011). Zaradi svoje protumorske vloge so celice tumorskega mikrookolja tudi pomembne tarče pri zdravljenju raka (Klemm & Joyce, 2015).

1.1.1.1 Ožiljenje raka

Ko velikost tumorja preseže 1–2 mm, se zaradi hipoksije, ki nastane v tumorju, sprožijo procesi, ki omogočijo razvoj tumorskega krvožilnega sistema (Folkman, 1986; Gilkes & Semenza, 2013). Pomembno vlogo pri omogočanju rasti tumorja in metastaziranju tumorskih celic predstavljata tako angiogeneza kot limfangiogeneza (Sundar & Ganesan, 2007). Tako v tumorskih celicah kot v celicah tumorskega mikrookolja pride do zvišanja izražanja različnih aktivatorjev za ožiljenje tumorja, kot so npr. žilni endotelijski rastni faktor A, fibroblastni rastni faktorji in trombocitni rastni faktorji, ki lahko sprožijo proces ožiljenja (Bergers & Benjamin, 2003). Ožilje v tumorjih se razlikuje od ožilja v normalnih tkivih: žile v tumorjih so nepravilnih oblik, se slepo končajo, so razširjene in so namesto hierarhične ureditve v venule, arteriole in kapilare kaotično razporejene. Žile v tumorjih so tudi bolj prepustne, saj so perivaskularne celice slabše povezane z endotelijskimi celicami (Egeblad, Nakasone, & Werb, 2010).

1.1.1.2 Metastaziranje

Metastaze sestavljajo tumorske celice, ki izvirajo iz primarnih tumorjev in se nahajajo v oddaljenih organih. Velika večina smrti zaradi raka je posledica metastaziranja rakavih celic, zato je največja pozornost namenjena raziskavam, ki odpirajo vpogled v molekularne in celične mehanizme, ki omogočajo proces metastaziranja. Medtem ko je vstop tumorskih celic iz primarnega tumorja v krvožilni sistem pogost pojav (zelo majhni tumorji lahko razsevajo na milijone tumorskih celic), pa so le nekatere izmed tumorskih celic sposobne metastaziranja (Nagrath et al., 2007). Med zasevanjem rakavih celic v druge organe rakava celica preide skozi različne zaporedne in med seboj odvisne faze, ki se skupaj imenujejo metastatska kaskada. Po ožiljenju tumorja imajo tumorske celice možnost (i) lokalne invazije v okoliško tkivo. Zaradi prepustnosti krvnih in limfnih žil tumorske celice iz strome (ii) vstopijo v krvožilni sistem (intravazacija). Velika večina tumorskih celic v ožilju hitro propade, le nekatere pa (iii) preživijo in se prenesejo po žilnem sistemu v oddaljene organe. Sledi (iv) izstop tumorskih celic iz krvnega sistema (ekstravazacija), (v) preživetje in prilagoditev na mikrookolje tujega organa ter končno (vi) proliferacija tumorskih celic v oddaljenem organu (kolonizacija) (Fidler, 2003). Vsaka od teh faz se lahko na kateri koli točki prekine (Poste & Fidler, 1980). Tumorske celice lahko po vstopu v oddaljen organ (t. i. metastatska niša) vstopijo v fazo mirovanja kot individualne razsejane tumorske celice ali pa se začnejo deliti in tvorijo makrometastaze (Diepenbruck & Christofori, 2016).

Invazijo in metastaziranje tumorskih celic epitelnega izvora omogoča proces, imenovan epitelijsko-mezenhimski prehod (ang. Epithelial-mesenchymal transition – EMT). Za razliko od tumorskih celic v primarnem tumorju se namreč metastatske tumorske celice morfološko spremenijo: epitelijske markerje nadomestijo mezenhimski (npr. vimentin, N-kadherin), celice pa izgubijo medcelične stike (npr. E-kadherin). Proces EMT spodbudijo različni rastni faktorji, kot so transformni rastni faktor beta, epidermalni rastni faktor, inzulinu podobni rastni faktorji, fibroblastni rastni faktorji, pa tudi zvišana proteolitična aktivnost metaloproteaz matriksa (Christofori, 2006). V novejših raziskavah so ugotovili, da EMT sicer ni nujen za metastaziranje tumorskih celic, vendar so po drugi strani tumorske celice, ki so prešle skozi EMT, odporne na kemoterapijo (Fischer et al., 2015; Zheng et al., 2015).

1.1.2 Značilnosti raka mlečne žleze

Rak mlečne žleze je najbolj pogosto odkrita vrsta raka in glavni povzročitelj smrti zaradi raka med ženskami po vsem svetu. V letu 2012 je za rakom na dojki zbolelo 1,7 milijona žensk, od tega jih je več kot 500 000 umrlo (Torre, et al., 2015). Hude posledice in slabšanje kvalitete življenja imajo tudi zapleti, povezani z rakom na dojki, kot so metastaze v druge organe.

1.1.3 Mišji modeli raka mlečne žleze

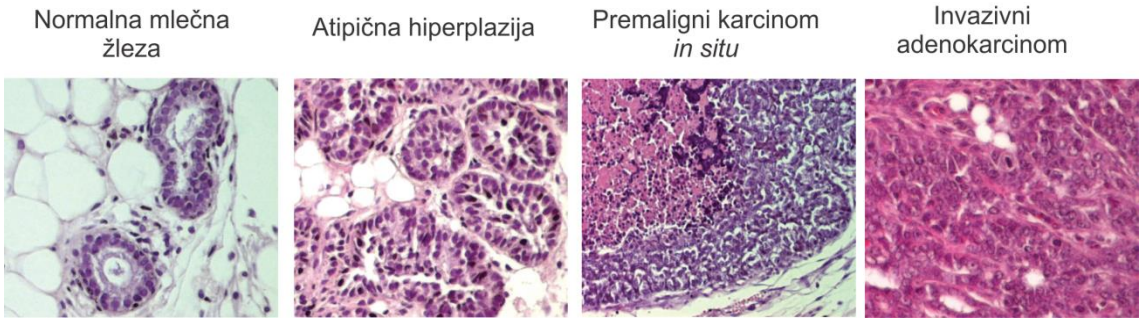
Živalski modeli raka omogočajo vpogled v mehanizme razvoja in zdravljenja rakavih obolenj, zato so pomemben vir novih odkritij v raziskavah rakavih obolenj. Laboratorijska miš (*Mus musculus*) predstavlja enega najboljših modelov pri raziskavah raka zaradi svoje majhne velikosti, hitre reprodukcije, fiziološke in molekularne podobnosti s človekom in v celoti odkritim genomom (Frese & Tuveson, 2007). Trenutno razpoložljive mišje modele za raziskovanje humanega raka mlečne žleze lahko razdelimo v tri glavne skupine: (a) ksenograftni modeli; (b) kemično inducirani, virusno inducirani ali ionizirajoči radiacijsko-inducibilni modeli in (c) genetsko spremenjene miši (transgene miši ali miši z izbitim genom) (Matulka & Wagner, 2005).

1.1.3.1 Mišji model raka mlečne žleze MMTV-PyMT

Transgeni mišji model raka mlečne žleze MMTV-PyMT so leta 1992 razvili Guy in sodelavci. MMTV-PyMT transgeni mišji model je eden najbolj preučenih modelov raka mlečne žleze. MMTV (mišji virus mlečne žleze, ang. mouse mammary tumor virus) promotor inducira razvoj raka le v mlečni žlezi in ne v ostalih organih. MMTV promotor je hormonsko reguliran in se izredno poviša med brejostjo (Pattengale et al., 1989). Šibka stran tega promotorja je, da se izraža v »non-uniform«, mozaičnem vzorcu (Shen & Brown, 2005).

Polioma srednji T antigen (ang. Polyoma middle T antigen – PyMT) je membranski protein, ki ga kodira majhen DNA polioma virus in se ne izraža v humanih tumorskih celicah. Povezan je z različnimi signalnimi potmi, med drugim tudi z družinami *Src* in *ras, c-myc* ter PI3 kinazno potjo, ki so vse spremenjene tudi pri humanem raku dojke (Brison, 1993; Dankort & Muller, 2000; Fluck & Schaffhausen, 2009).

Pri MMTV-PyMT transgenih miših se prične razvoj tumorjev na vseh 10 mlečnih žlezah že zgodaj, v obdobju pubertete, približno od 4 mesecev dalje. Razvoj in napredovanje tumorjev poteka v 4 fazah: hiperplazija, adenom/intraepitelialna neoplazija, zgodnji karcinom in pozni karcinom (Lin et al., 2003). Pri večini živali se do starosti 14 tednov razvijejo metastaze v pljučih, deloma pa tudi v pripadajočih bezgavkah (Francia, Cruz-Munoz, Man, Xu, & Kerbel, 2011; Kim & Baek, 2010). V novejših raziskavah so tumorske celice in mikrometastaze odkrili tudi v kostnem mozgu, in sicer že pri starosti miši 4–6 tednov, ko so primarni tumorji na stopnji hiperplazije. Rezultati nakazujejo zmožnost premalignih celic, da metastazirajo (Husemann et al., 2008). Tudi pri samcih genotipa MMTV-PyMT se razvijejo tumorji mlečnih žlez, le da je razvoj upočasnjen. Pri primerjanju histoloških značilnosti razvoja raka dojke pri ženskah in PyMT modela pri miših so ugotovili veliko podobnosti. Izguba estrogenih in progesteronskih receptorjev ter β1-integrina na eni ter prekomerno izražanje ErbB2/Neu in ciklina D1 na drugi strani je značilnost tako PyMT transgenega modela kot humanega raka dojke, kar še dodatno potrjuje, da je PyMT transgeni mišji model primeren model za proučevanje razvoja raka dojke (Maglione et al., 2001; Qiu et al., 2004).



Slika 2: Razvoj tumorjev pri PyMT mišjem modelu raka mlečne žleze. Povzeto po Vasiljeva in sod. (Vasiljeva et al., 2008).

1.2 Proteaze

Proteaze (proteinaze, peptidaze, proteolitični encimi) so encimi, ki katalizirajo hidrolizo peptidnih vezi proteinskih substratov, kar imenujemo proteoliza (Puente, Sanchez, Overall, & Lopez-Otin, 2003). Proteaze najdemo v vseh živih organizmih. Pri ljudeh predstavljajo 10 % vseh encimov z več kot 800 člani, kot jih navaja baza podatkov MEROPS (Rawlings, Barrett, & Finn, 2016). Proteaze sodelujejo pri nespecifični hidrolizi proteinov, lahko pa tudi selektivno cepijo specifične substrate, kar posledično vpliva na

pomembne biološke procese, kot so oploditev, embrionalni razvoj, razvoj kosti, regulacija celičnega ciklusa, celjenje ran, ožiljenje in apoptoza. Poleg tega proteaze sodelujejo tudi v različnih patoloških procesih, kot so na primer rak, kardiovaskularna in nevrodegenerativna obolenja, osteoporoza in artritis. Proteaze delimo v več razredov: aspartatne, treoninske, serinske, metaloproteaze in cisteinske proteaze.

1.2.1 Cisteinske proteaze

V družino cisteinskih proteaz spada 14 super družin, najbolje raziskana je super družina CA, ki vsebuje družino C1 (papainska družina), kamor spadajo papainu podobne cisteinske proteaze (poddružina C1A). Vse cisteinske proteaze imajo katalitski par, sestavljen iz aminokislinskih preostankov cisteina in histidina v aktivnem mestu. Cisteinski del deluje kot nukleofil, histidin pa sprejme proton (B. Turk, Turk, & Salvesen, 2002).

1.2.1.1 Lizosomski cisteinski katepsini

Ime katepsini izvira iz grščine kathepsein (prebaviti). Ta izraz so v začetku uporabili za proteaze, ki so aktivne v kislem okolju. Danes so katepsini razdeljeni v serinske proteaze (katepsina A in G), aspartatske proteaze (katepsina D in E) in cisteinske proteaze ali cisteinski katepsini. Pri človeku poznamo 11 cisteinskih katepsinov: katepsine B, C, F, H, K, L, O, S, V, W in X (tudi Z ali P), ki spadajo v klan CA in papainsko družino C1A (V. Turk et al., 2012). Značilno za katepsine je, da imajo prisotno aktivno zanko, kjer aminokislinska preostanka cistein in histidin tvorita katalitični par. Katepsini so majhne monomerne molekule velikosti približno 24–30 kDa, izjema je katepsin C, ki je tetrameren encim velikosti približno 200 kDa. Katepsini običajno cepijo substrate za hidrofobnimi ali aromatskimi aminokislinskimi preostanki (Choe et al., 2006) (Tabela 1).

1.2.1.1.1 Lokacija cisteinskih katepsinov

Cisteinski katepsini so sintetizirani v endoplazemskem retikulumu kot neaktivni preproencimi. Kratka N-terminalna presekvenca usmeri proteine v endoplazemski retikulum, kjer signalna peptidaza odstrani signalni peptid. Nastali peptidi se imenujejo proencimi ali zimogeni, ki so katalitično neaktivni, saj propeptidi blokirajo aktivna mesta. nato pa v Golgijevem aparatu poteče glikozilacija, nato pa se prokatepsini prenesejo v endolizosome, kjer se propeptidi bodisi avtokatalitično bodisi z drugimi proteazami odstranijo (Loser & Pietzsch, 2015).

1.2.1.1.2 Regulacija aktivnosti cisteinskih katepsinov

Aktivnost katepsinov je kontrolirana z regulacijo izražanja, posttranslacijskih modifikacij, pH, najpomembnejša pa je regulacija aktivnosti katepsinov z aktivacijo zimogena in inhibicijo z endogenimi inhibitorji (Stoka, Turk, & Turk, 2016; V. Turk, Turk, & Turk, 2001).

1.2.1.1.2.1 Aktivacija zimogena

Kot večina drugih proteaz se tudi katepsini sintetizirajo kot neaktivni preproencimi in se aktivirajo s proteolitično odstranitvijo N-končnega peptida. Proteolitična odstranitev se lahko zgodi bodisi z aktivacijo drugih proteaz (pepsin, katepsin D) bodisi z avtokatalitično aktivacijo pri kislem pH. Avtoaktivacija lahko poteče le pri endopeptidazah, medtem ko katepsina C in X za aktivacijo potrebujeta prisotnost endopeptidaze (Dahl et al., 2001).

Tabela 1: Vrsta aktivnosti in celična specifičnost humanih katepsinov.

Katepsin	Vrsta peptidazne aktivnosti	Celična in tkivna specifičnost
B	endopeptidaza in karboksipeptidaza	splošno prisoten
C	aminodipeptidaza	splošno prisoten
F	endopeptidaza	splošno prisoten
H	endopeptidaza in aminodipeptidaza	splošno prisoten
L	endopeptidaza	splošno prisoten
K	endopeptidaza	osteoklasti, sinovialni fibroblasti
O	ni znano	splošno prisoten
S	endopeptidaza	antigen predstavitvene celice
V	endopeptidaza	timus, testisi
W	ni znano	citotoksični limfociti
X	karboksimonopeptidaza	splošno prisoten

1.2.1.2 Cisteinski katepsini pri raku

Cisteinski katepsini so pogosto zvišani pri različnih vrstah humanega raka, kot so rak dojke, pljuč, možganov, gastrointestinalni rak, rak prostate in melanoma (Reinheckel, Gocheva, Peters, & Joyce, 2008). Najpogosteje so z razvojem raka povezani katepsini B, L, S in H, pa tudi K in X. Katepsinov ne izločajo samo rakave celice, temveč tudi celice mikrookolja, kot so s tumorjem povezani makrofagi (ang. tumor associated macrophages, TAMs), fibroblasti, endotelijske celice, mioepitelne celice, limfociti T, tkivni bazofilci, nevtrofilci in osteoklasti (Mohamed & Sloane, 2006). Cisteinski katepsini vplivajo na napredovanje raka tako z znotrajceličnim kot z zunajceličnim delovanjem (Vasiljeva & Turk, 2008). Med razvojem rakavega obolenja se namreč cisteinski katepsini, ki se v fizioloških pogojih nahajajo v lizosomih, bodisi premaknejo na celično površino bodisi izločajo izven celic. Zaradi hipoksije v tumorskem mikrookolju in z njo povezane glikolize je pH na tem območju rahlo kisel, kar omogoča katepsinom, da se aktivirajo.

Cisteinski katepsini imajo pomembno vlogo pri razgrajevanju komponent zunajceličnega matriksa (npr. laminin, fibronektin, tenascin C, kolagen tipa IV) (Cavallo-Medved et al., 2009) v mikrookolju tumorjev, kar omogoča invazijo in metastaziranje rakavih celic. Poleg zunajceličnega razgrajevanja imajo katepsini vlogo tudi pri znotrajceličnem razgrajevanju komponent zunajceličnega matriksa preko endocitoze. Cisteinski katepsini so sposobni aktivirati tudi druge proteaze, npr. urokinazni plazminogeni aktivator, lahko pa tudi inaktivirajo adhezijske proteine, npr. E-kadherin. Katepsini vplivajo tudi na ožiljenje oz. angiogenezo tumorjev (Mohamed & Sloane, 2006).

Zvišano aktivnost in/ali izražanje cisteinskih katepsinov so določili pri različnih humanih rakavih obolenjih (Olson & Joyce, 2015), med drugim tudi pri raku dojke (Castiglioni et al., 1994; Gabrijelcic et al., 1992; Nouh et al., 2011; Withana et al., 2012; Yano et al., 2001). V humanem raku dojke so v različnih raziskavah potrdili zvišano

izražanje katepsinov B, L, S, in C. Ugotovili so, da je zvišan nivo katepsinov v primarnih tumorjih kazalec za slabo prognozo in za povečano možnost metastaziranja v možgane ali v pljuča (Paik et al., 2006; Sevenich et al., 2014).

Izbitje genov za katepsine B, H, L, S in Z pri miših je povzročilo občutno upočasnitev razvoja raka pri PyMT modelu raka mlečne žleze: transgene miši brez katepsina B so kasneje razvile tumorje, ki so bili manjši v primerjavi s kontrolno skupino. Ugotovili so tudi nadomestno vlogo katepsina X pri tumorskih celicah z izbitim genom za katepsin B ter pomembno vlogo katepsina B tako v tumorskih celicah kot v celicah tumorske strome. Poleg tega izbitje gena za katepsin B povzroči za 65 % zmanjšanje velikosti pljučnih metastaz (Vasiljeva, et al., 2008; Vasiljeva et al., 2006). V istem modelu so pri miših z izbitima genoma za katepsina B in X ugotovili velik časovni zamik v razvoju tumorjev in močno zmanjšanje števila in velikosti pljučnih metastaz (Sevenich et al., 2010). Vsi ti rezultati dokazujejo pomembno vlogo cisteinskih katepsinov pri razvoju raka pri modelu mlečne žleze. Katepsini pomembno vplivajo na razvoj raka tudi pri drugih mišjih modelih, kot so model nevroendokrine neoplazije trebušne slinavke – RIP1-Tag2 (Gocheva, Chen, Peters, Reinheckel, & Joyce, 2010; Gocheva et al., 2006), mišji model črevesnega raka – APC^{min} (Gounaris et al., 2008), model kožnega raka in raka maternične vratu – K14-HPV16 (Dennemarker et al., 2010) in model tumorja mlečne žleze 4T1 (Withana, et al., 2012).

Študije kažejo na pomembno vlogo katepsinov kot tarče za terapije, kar so z več študijami tudi potrdili na mišjih modelih raka (Bell-McGuinn, Garfall, Bogyo, Hanahan, & Joyce, 2007; Joyce et al., 2004; Mikhaylov et al., 2014; Mikhaylov et al., 2011).

1.3 Proteinski inhibitorji cisteinskih proteinaz

Najpomembnejši regulatorji cisteinskih katepsinov so njihovi endogeni inhibitorji: cistatini, tiropini in serpini, ki so kompetitivni, reverzibilni inhibitorji z močno vezavo.

1.3.1 Naddružina cistatinov

Naddružino cistatinov sestavlja velika skupina evolucijsko sorodnih proteinov, ki zavirajo aktivnost papainu podobnih cisteinskih proteaz. Cistatini so prisotni pri živalih, glivah, rastlinah, bakterijah, praživalih in virusih in so razvrščeni v več družin, od katerih so glavne tri stefini, cistatini in kininogeni (Kordis & Turk, 2009; Mussap & Plebani, 2004). Terciarna struktura cistatinov je ohranjena in je sestavljena iz petih β-ploskev, ki se ovijajo okrog petsučne α-vijačnice (t. i. cistatinski zavoj) (Bode et al., 1988; V. Turk, Stoka, & Turk, 2008). Za vezavo na proteaze so pomembni trije segmenti. Prvi je N-terminalna regija, kjer se nahaja glicinski preostanek na mestu 11 (Gly^{11}). Drugi segment, ki se nahaja v centralnem delu, ima najbolj pomembno inhibitorno vlogo in ga sestavljajo aminokisline na mestih 55—59 (Gln^{55}-Xaa-Val-Xaa-Gly^{59}). Tretji segment predstavlja regija s parom prolin-triptofan (par se nahaja pri cistatinih tipa 2 in 3, pri cistatinih tipa 1 se triptofan nadomesti s histidinom ali glicinom) (Abrahamson, Alvarez-Fernandez, & Nathanson, 2003; Machleidt et al., 1989; V. Turk & Bode, 1991). Vsi skupaj tvorijo klin, katerega oblika je komplementarna aktivnemu mestu encima. Glavna razlika med cistatini tipa 1 in tipa 2 je približno 20 aminokislinskih preostankov dolga »pomožna zanka« med 3. in 4. β-ploskvijo pri cistatinih tipa 2. Nekateri cistatini tipa 2 lahko inhibirajo tudi cisteinsko proteazo legumain. Mesto inhibicije se nahaja na nasprotni strani od vezavnega mesta za katepsine: med α-vijačnico in prvo verigo β-ploskve na mestih 39-41 (štetje cistatina C) (Alvarez-Fernandez et al., 1999; Janowski et al., 2001). Cistatini so sicer neselektivni inhibitorji papainu podobnih proteaz, vendar lahko razlikujejo med endo- in

eksopeptidazami. So kompetitivni, reverzibilni inhibitorji, ki tarčne encime inhibirajo v koncentraciji μm do pm (Vito Turk & Turk, 2008).

1.3.1.1 Cistatini tipa 1: Stefina A in B

Cistatini tipa 1 (poddružina 125A po klasifikaciji MEROPS) so enoverižni proteini, niso glikozilirani, nimajo signalnega peptida in so brez disulfidnih vezi. So majhni proteini (~ 100 aminokislin, 11 kDa). Stefina A in B sta prisotna pri ljudeh in različnih sesalcih (miši, podgane, govedo, prašiči). Oba sta močna inhibitorja papaina ter tvorita močne komplekse s katepsini H, L in S. Humani stefin A je v visokih koncentracijah prisoten v koži, medtem ko je stefin B prisoten v večini tkiv in je splošni inhibitor cisteinskih katepsinov v citoplazmi. Pri govedu obstaja tudi stefin C (B. Turk et al., 1993), pri prašičih pa stefin D (Lenarcic, Krizaj, Zunec, & Turk, 1996). Vsi stefini so znotrajcelični inhibitorji, ki se nahajajo v citoplazmi, nahajajo pa se tudi zunajcelično (Abrahamson, Barrett, Salvesen, & Grubb, 1986). Za mišji stefin A je značilno, da obstajajo vsaj tri različne variante, medtem ko pri ljudeh obstaja le ena (Tsui et al., 1993).

1.3.1.1.1 Stefin B

Stefin B je sestavljen iz 98 aminokislinskih preostankov. Je splošni inhibitor v citosolu sesalskih celic. Prisoten je tudi v jedru delečih se celic, kjer se veže na histonske proteine in vpliva na potek celičnega cikla (Ceru et al., 2010). Mutacije v genu za stefin B vodijo v razvoj progresivne miokloničnе epilepsije tipa 1 (EPM1 ali Unverricht-Lundborgova bolezen) pri ljudeh (Pennacchio et al., 1998). Do bolezni pride zaradi povišane apoptoze v možganih, kar vodi v izgubo pomembnih nevronov (Shannon, Pennacchio, Houseweart, Minassian, & Myers, 2002). Bolezenski znaki so napredujoči mioklonični trzljaji, epileptični krči, zmanjšana sposobnost koncentracije in zmanjšana stopnja razumevanja. V celicah pacientov z EPM1 so določili zvišano aktivnost katepsinov (Rinne, Saukko, Jarvinen, & Lehesjoki, 2002).

1.3.1.1.1.1 Miši z izbitim genom za stefin B

Miši z izbitim genom za stefin B (StfB-/-) so l. 1998 razvili Pennacchio in sodelavci (Pennacchio, et al., 1998). Miši razvijejo podobno nevrodegenerativno obolenje kot ljudje, zato služijo kot uporaben model za preučevanje EPM1. Podobno kot pri ljudeh tudi pri miših pride do podobnih patoloških motenj in možganskih poškodb (Shannon, et al., 2002). Povečano izražanje cistatina C v miših z izbitim genom za stefin B je zvišano aktivnost katepsinov zmanjšalo na normalni nivo, klinični znaki bolezni pa so deloma izginili, kar kaže na nevrozaščitno vlogo cistatina C (Kaur et al., 2010).

1.3.1.1.1.2 Stefin B pri raku

V literaturi ni veliko študij o vlogi stefina B pri rakavih obolenjih. Stefin B služi kot biološki kazalec za prognostično napoved pri nedrobnoceličnem raku pljuč pri ljudeh: pacienti z zvišanimi vrednostmi stefina B so imeli večjo možnost za preživetje (Werle et al., 2006). Nasprotno pri hepatoceličnem karcinomu (Lee et al., 2008) in v serumu pacientov z rakom debelega črevesa (Kos et al., 2000) povišan nivo stefina B sovpada s slabšo prognozo. Pri raku pljuč so v rakavem tkivu in v metastazah izmerili povišane koncentracije stefina B v primerjavi z zdravimi pljuči (Ebert et al., 1997). Podobno so tudi v tumorjih pri raku

ovarijev izmerili višjo vrednost stefina B kot v zdravih jajčnikih (Kastelic et al., 1994). Izražanje stefina B je pri humanem raku dojke znižano (Lah et al., 1992; Lah et al., 1997), medtem ko je v PyMT transgenem mišjem modelu povišano (Butinar et al., 2014). Transgene miši seva PyMT z izbitim genom za stefin B razvijejo manjše tumorje v primerjavi s transgenimi mišmi divjega tipa. Do zmanjšanih tumorjev je prišlo zaradi zvišanih območij mrtvih regij v tumorjih, medtem ko razlik v proliferaciji, angiogenezi in metastaziranju pri miših z izbitim genom za stefin B ni bilo. Pri sproženi apoptozi z lizosomotropnim agensom LeuLeu-OMe so opazili povišano stopnjo celične smrti pri tumorskih celicah z izbitim genom za stefin B. Poleg tega izbitje gena za stefin B pri tumorskih celicah vpliva na povečano celično smrt zaradi oksidativnega stresa (Butinar, et al., 2014).

1.3.1.2 Cistatini tipa II: cistatini C, D, E/M, F, G, S, SA in SN

Cistatini (poddružina 125B po klasifikaciji MEROPS) so enoverižni proteini, sestavljeni iz približno 115 aminokislinskih preostankov in veliki od 13 do 15 kDa. Vsebujejo signalni peptid za izločanje skozi celično membrano. Prototip za vse cistatine tipa II je kokošji cistatin C iz beljaka. Vsi cistatini vsebujejo dva intra-molekularna disulfidna mostova in niso glikozilirani. Izjemi sta cistatin E/M in cistatin F, ki sta glikoproteina (Ni et al., 1997; Ni et al., 1998; Sotiropoulou, Anisowicz, & Sager, 1997). Cistatin F vsebuje še en dodatni disulfidni most (Ni, et al., 1998). Cistatini se nahajajo v dokaj visokih koncentracijah v telesnih tekočinah (Abrahamson, et al., 1986). So pomembni endogeni inhibitorji papainu podobnih proteaz, kot so cisteinski katepsini, sesalska asparaginilna endopeptidaza – legumain in parazitskih proteaz (npr. krucipain) (V. Turk, et al., 2008).

Cistatini S, SN, SA

Cistatini tipa »S« so izolirali iz človeške sline. Med sabo so skoraj 90 % identični (Isemura, Saitoh, Sanada, & Minakata, 1991).

Cistatin D

Cistatin D je sestavljen iz 122 aminokislin in 4 disuflidnih mostičkov. Najprej so ga izolirali iz obušesne žleze slinavke, nahaja pa se tudi v slini in v majhnih količinah v solzah (Freije et al., 1991).

Cistatin E/M

Cistatin E/M je sestavljen iz 121 aminokislin in je N-glikoziliran na preostanku 108. Gen za cistatin E/M se za razliko od ostalih cistatinov tipa II ne nahaja na kromosomu 20, ampak na kromosomu 11. Nahaja se v vseh tkivih, še posebej v velikih koncentracijah pa v jetrih, jajčniku in trebušni slinavki (Ni, et al., 1997). Novejše študije trdijo, da se cistatin E/M pri ljudeh nahaja zgolj v epidermalnih keratinocitih in slinskih žlezah, medtem ko je pri miših prisoten v več različnih tkivih (Zeeuwen et al., 2001). Ničelna mutacija cistatina E/M povzroči smrt v mladosti in nepravilno poroženelost epidermisa pri določenih sevih miši (Zeeuwen et al., 2003).

Cistatin F

Cistatin F ali levkocistatin se nahaja v krvnih celicah, celicah T, dendritskih celicah, vranici in hematopoetičnih celicah (Cappello et al., 2004). V različni tipih raka pri miših so izmerili povišane koncentracije cistatina F (Morita, Yoshiuchi, Arakawa, & Nishimura, 1999). Je edini izmed cistatinov, ki je sintetiziran in se izloča kot neaktivni dimer

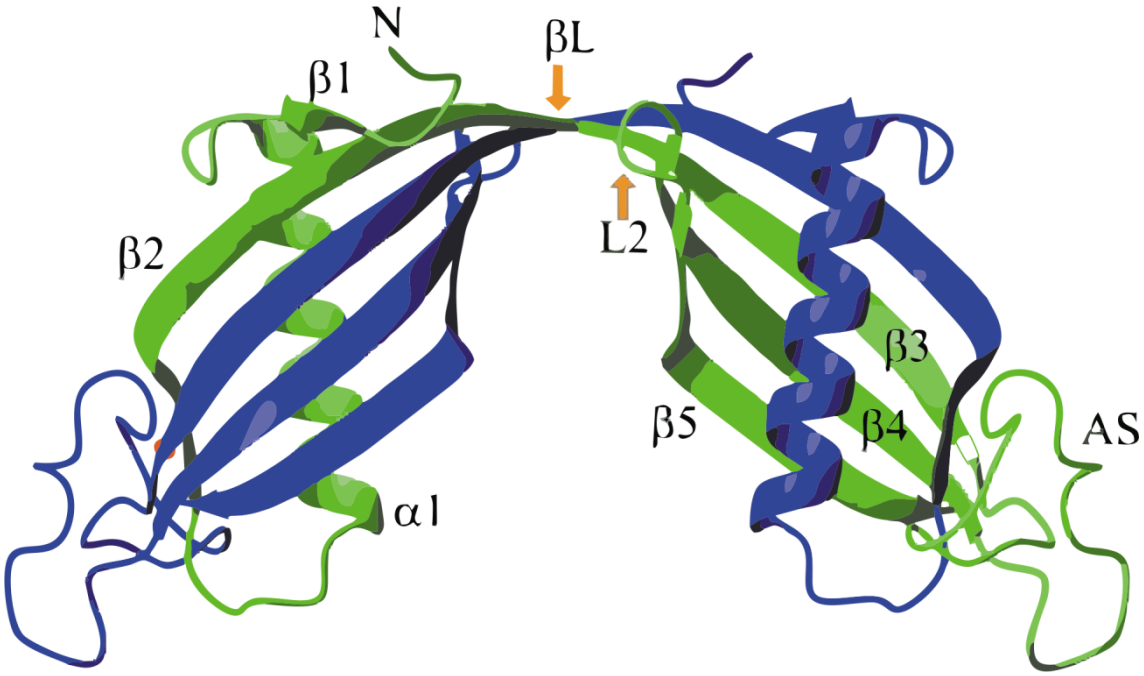
(Cappello, et al., 2004) in postane aktiven kot monomer, ki močno inhibira katepsine F, K, L in V, slabše pa katepsina S in H (Langerholc et al., 2005).

1.3.1.2.1 Cistatin C

Cistatin C je najbolje raziskan cistatin tipa II. Leta 1982 sta Anders Grubb in Helge Löfberg določila njegovo primarno strukturo iz opičje in človeške hipofize (Grubb & Lofberg, 1982). Človeški cistatin C je sestavljen iz 120 aminokislinskih preostankov in ima molekularno težo 15 kDa. Nahaja se na kromosomu 20 in je prisoten v večini tkiv in v telesnih tekočinah. V visokih koncentracijah se nahaja v semenski tekočini (~ 50 mg/l) in cerebrospinalni tekočini (~ 5.8 mg/l) (Abrahamson, et al., 1986). Po dve molekuli cistatina C se med nastajanjem v endoplazmatskem retikulumu začasno povežeta v dimer. Dimer verjetno preprečuje znotrajcelično vezavo cistatina C na cisteinske proteaze. Dimeri cistatina C v sekretornih veziklih z disociacijo razpadejo, posledično se iz celic izločajo samo aktivne monomere cistatina C (Mussap & Plebani, 2004).

Cistatin C se lahko zaradi svoje majhne velikosti prosto filtrira iz krvi skozi ledvične glomerule (Keppler, 2006) in je pomemben kazalec pravilnega delovanja ledvic (Coll et al., 2000; Filler et al., 2005). Cistatin C učinkovito inhibira vse lizosomalne proteaze, ki se izločijo v telesne tekočine. Cistatin C spada med najmočnejše inhibitorje, saj lahko inhibira papain, katepsin L in katepsin S v pM koncentraciji. Je tudi učinkovit inhibitor krucipaina, cisteinskega katepsina, ki se nahaja v parazitu *Trypanosoma cruzi* (Stoka et al., 1995). Poleg cisteinskih katepsinov lahko cistatin C inhibira tudi legumain (B. Turk, et al., 2002).
Poleg svoje inhibitorne vloge ima cistatin C tudi druge fiziološke funkcije. Več študij nakazuje na možno vlogo cistatina C pri imunskem sistemu. Humani cistatin C lahko skozi poškodovano membrano vstopi v celice, okužene z virusi (npr. *Herpes simplex virus, Coronavirus*) in znotrajcelično inhibira cisteinske proteaze (Bjorck, Grubb, & Kjellen, 1990; Collins & Grubb, 1991).

Točkovna mutacija v genu cistatina C, kjer se levcin na mestu 68 zamenja z glutaminom (Leu68Gln-cistatin C), vodi v hereditarno cistatin C amiloidno angiopatijo (ang. Hereditary cystatin C amyloid angiopathy – HCCAA). Takšna oblika cistatina C tvori dimere. Bolezen se pojavlja na Islandiji in se dominantno deduje. Pacienti, oboleli za HCCAA, imajo v mladosti demenco in paralizo zaradi krvavitev v možganih. Obolenje se običajno konča s smrtjo pri starosti okrog 30 let (Olafsson & Grubb, 2000). Histološko se v gladkih mišičnih celicah krvnih žil nahajajo netopni amiloidni plaki, katerih glavni sestavni del je L68Q-cistatin C (Ghiso, Jensson, & Frangione, 1986).



Slika 3: Dimer humanega cistatina C. Prirejeno po (Janowski, et al., 2001).

1.3.1.2.1.1 Miši z izbitim genom za cistatin C

Miši z izbitim genom za cistatin C (CstC-/-) so l. 1999 pripravili Huh in sodelavci. Miši se skotijo zdrave, rastejo enako hitro kot miši divjega tipa in so plodne. Pri histološki analizi različnih tkiv niso opazili razlik med CstC-/- mišmi in mišmi divjega tipa (Huh et al., 1999).

1.3.1.2.1.2 Cistatin C pri raku

O spremenjenem izražanju cistatina C so poročali pri različnih tipih raka (Mulaomerovic et al., 2007; Nakabayashi, Hara, & Shimuzu, 2005). Sokol in sodelavci so pokazali spremenjeno izražanje cistatina C pri 49 % preiskovanih rakavih pacientih, od tega je bilo pri 91 % pacientov izražanje cistatina C znižano, pri 9 % pacientov pa je bilo izražanje cistatina C povišano (Sokol & Schiemann, 2004). Pri raku dojke so z metodo RT-PCR ugotovili znižano izražanje mRNA cistatina C v razmerju do katepsina B. Opazili so nižje izražanje cistatina C v rakavem tkivu v primerjavi z normalnim tkivom, vendar razlike niso bile statistično signifikantne (Yano, et al., 2001). Pri raku prostate pri ljudeh so opazili zmanjšano izražanje cistatina C. Pacienti z zmanjšanim nivojem cistatina C so imeli slabšo prognozo za preživetje (Wegiel et al., 2009). Pri pacientih z melanomo so izmerili nižje vrednosti cistatina C v krvnem serumu kot pri zdravih pacientih (Kos et al., 1997). Tudi pri nedrobnoceličnem karcinomu pljuč so izmerili znižane vrednosti cistatina C v tumorskem tkivu v primerjavi z zdravim pljučnim tkivom, niso pa ugotovili nobene prognostične informacije cistatina C za preživetje pacientov (Werle, et al., 2006). Višje vrednosti cistatina C so bile izmerjene v intersticijski tekočini pacientk z rakom jajčnikov (Kolwijck et al., 2010; Nishikawa et al., 2004) in v serumu pacientov z rakom na pljučih (Chen et al., 2011) ali pri pacientih s kolorektalnim rakom (Kos, Krasovec, et al., 2000). Povišane vrednosti cistatina C v tumorskem tkivu so bile v pozitivni korelaciji s prognozo pacientov (Kos & Lah, 1998), medtem ko so bile povišane vrednosti cistatina C v telesnih tekočinah v povezavi s slabo prognozo pri rakavih pacientih (Kos, Werle, Lah, & Brunner, 2000).

Pri transgenem mišjem modelu kožnega raka K14-HPV 16 so opazili zmanjšano izražanje cistatina C. V zdravi koži je bilo prisotnega največ cistatina C, nato pa se je nivo zmanjševal pri premalignih stanjih in v napredovalem raku. Pri K14-HPV 16 transgenih miših z izbitim genom za cistatin C so opazili hitrejši razvoj raka, povišano izražanje in

aktivnost cisteinskih katepsinov, povišano proliferacijo tumorskih celic in povišan razvoj krvnih žil v tumorskem tkivu (Yu et al., 2010). Pri transgenem mišjem modelu raka pankreasa RIP1-Tag2 z izbitim genom za cistatin C pride do povišane proliferacije tumorskih celic, angiogeneze, razgradnje kolagena in dviga pro-tumorskih citokinov (Wang et al., 2006).

Obratno povezavo med koncentracijo cistatina C in razvojem tumorjev so opisali pri humani glioma celični liniji, kar nakazuje na vlogo cistatina C pri invazivnosti humanih glioblastomskih celic (Konduri et al., 2002).

Pri miših z izbitim genom za cistatin C so pri intravenoznem apliciranju B16-F10 melanomskih tumorskih celic pokazali zmanjšano število metastaz v pljučih glede na kontrolno skupino, medtem ko razlik v rasti podkožnih in intracerebralnih tumorjev ni bilo (Huh, et al., 1999). Vendar so ti rezultati po našem mnenju dvomljive zanesljivosti, saj so število metastaz merili od 0 do 100 ur po injiciranju tumorskih celic, kar je prehitro za razvoj metastaz.

In vitro študija B16 melanomskih tumorskih celic s prekomernim izražanjem cistatina C je pokazala močno zmanjšano metastaziranje celic. Tudi pri *in vivo* študiji so pokazali zmanjšane pljučne metastaze in zmanjšano rast tumorjev (Ervin & Cox, 2005). Podobno so tudi pri miših z lokalno prekomernim izražanjem cistatina C v pljučih in jetrih pokazali močno zmanjšano število pljučnih metastaz po intravenozni aplikaciji humanih fibrosarkomskih tumorskih celic, medtem ko se število metastaz v jetrih ni razlikovalo od kontrolne skupine (Kopitz, Anton, Gansbacher, & Kruger, 2005).

1.3.1.3 Cistatini tipa 3: Kininogeni

Kininogeni so prekurzorji kininov (npr. bradikinin). So veliki, sestavljeni glikoproteini, ki se pretežno nahajajo v krvni plazmi in drugih telesnih tekočinah. So najbolj zapleteno sestavljeni od vseh tipov cistatinov, saj vsebujejo tri cistatinske domene, vsaka od teh domen ima dva disulfidna mostova (Lalmanach, Naudin, Lecaille, & Fritz, 2010). Sposobnost inhibicije cisteinskih proteaz imata le dve od treh cistatinskih domen. Obstajajo trije tipi kininogenov: kininogen z veliko molekulsko maso (L), kininogen z majhno molekulsko maso in T-kininogen, ki se nahaja le pri podganah.

1.4 Transformni rastni faktor β (TGF-β)

Družino transformnih rastnih faktorjev (TGF-β) pri ljudeh predstavlja več kot 30 različnih polipeptidov, ki so strukturno podobni med seboj. Poleg TGF-β izoform v družino spadajo tudi aktivini, inhibini, morfogenetski proteini kosti, rastni diferenciacijski faktorji, nodal in drugi (Massague, 2008). Večina proteinov so povsod prisotni dimerni prohormoni, ki se izločajo v zunajcelični matriks, kjer se aktivirajo (Moustakas & Heldin, 2009). TGF-β faktorji vplivajo na številne celične procese, kot so proliferacija, diferenciacija, migracija in preživetje, ter na različne fiziološke procese, kot so embrionalni razvoj, angiogeneza in celjenje ran. Člani TGF-β imajo vlogo tudi pri razvoju različnih bolezni, kot so bolezni srca in ožilja, bolezni vezivnega tkiva, bolezni kosti in mišic, avtoimune bolezni in rak (Gordon & Blobe, 2008).

1.4.1 Vloga TGF-β pri raku

Vloga TGF-β pri humanem raku je zelo preučena; veliko različnih študij je potrdilo, da so TGF-β signalne poti pri razvoju raka zapletene in imajo nasprotujoče si učinke na napredovanje raka, povzeto v (Bierie & Moses, 2006; Cantelli, Crosas-Molist, Georgouli, & Sanz-Moreno, 2016; Elliott & Blobe, 2005). V zgodnjih fazah raka TGF-β večinoma deluje kot tumorski zaviralec, saj zavira rast tumorskih celic in pospešuje procese apoptoze. V

kasnejših fazah razvoja raka pa TGF-β postane tumorski promotor: pospešuje rast, preživetje, invazijo in metastaziranje tumorskih celic (Ikushima & Miyazono, 2010). TGF-β vpliva tako na rakave celice kot na celice mikrookolja; pri slednjih regulira angiogenezo, sestavo zunajceličnega matriksa in izogibanje imunskemu vplivu (Heldin, Vanlandewijck, & Moustakas, 2012). Da ima TGF-β pomembno vlogo v signalnih poteh pri humanem raku, pove tudi dejstvo, da je TGF-β pri različnih vrstah raka pogosto genetsko spremenjen (Gordon & Blobe, 2008).

TGF-β ima pomembno vlogo tudi pri razvoju raka dojke, saj je njegova aktivnost v tumorjih pogosto višja kot v zdravem tkivu. Nivo izražanja TGF-β je v korelaciji z angiogenezo pri pacientkah z rakom na dojki (de Jong, van Diest, van der Valk, & Baak, 1998). Pri miših so nasprotno ugotovili, da prekomerna ekspresija TGF-β1 v epiteliju mlečnih žlez zavira tvorbo tumorjev (Pierce et al., 1995). Izguba TGF-β receptorja tipa II v mišjem modelu raka mlečnih žlez PyMT pa pospeši razvoj tumorjev in poveča nastanek pljučnih metastaz, kar še potrdi supresorsko vlogo signaliziranja TGF-β v epiteliju mlečnih žlez pri miših (Forrester et al., 2005).

1.4.2 TGF-β in cistatin C

Cistatin C ima poleg inhibitorne vloge tudi vlogo antagonista transformnega faktorja beta (TGF- β) v normalnih in rakavih celicah. Cistatin C namreč preprečuje vezavo TGF-β na TGF-β receptor tip II (TβR-II) (Sokol & Schiemann, 2004). Vlogo cistatina C so potrdili v dveh *in vitro* modelih, kjer so ugotovili, da je cistatin C učinkovito zavrl vpliv TGF-β na epitelno-mezenhimski prehod (EMT) celic in na morfološko transformacijo humanih celičnih linij raka dojk (Sokol, Neil, Schiemann, & Schiemann, 2005). Kasneje so vlogo cistatina C kot antagonista TGF-β potrdili tudi *in vivo* pri mišjem modelu raka mlečne žleze 4T1, kjer je cistatin C učinkovito preprečil angiogenezo v tumorjih ter zaviral vpliv TGF-β na razvoj pljučnih metastaz (Tian & Schiemann, 2009).

Poglavje 2

Namen dela

Izsledki številnih raziskav poročajo o vlogi cisteinskih katepsinov pri razvoju in napredovanju raka, vendar pa obstaja relativno malo raziskav o vlogi endogenih inhibitorjev. Zato smo se odločili za preiskavo vloge znotrajceličnega inhibitorja stefina B in zunajceličnega inhibitorja cistatina C v mišjem modelu raka PyMT. Pri tem bomo uporabili pristop odstranitve obeh inhibitorjev (dvojni »knockout«), kar bo omogočalo preiskavo njunih vlog pri razvoju raka in metastaziranju.

Delovni hipotezi sta bili:

- odstranitev cistatina C pospešuje rast tumorjev in sekundarnih metastaz pri PyMT mišjem modelu raka;
- odstranitev cistatina C in stefina B (dvojni »knockout«) pri PyMT mišjem modelu raka dodatno pospešuje razvoj tumorjev in nastanek sekundarnih metastaz v primerjavi z odstranitvijo le cistatina C.

Namen dela doktorske naloge je bil:

- določiti vpliv odstranitve cistatina C pri PyMT mišjem modelu adenokarcinoma mlečnih žlez na začetek rasti in napredovanje tumorjev, delež proliferacije tumorskih celic, ožiljenje tumorjev, nastanek metastaz v sekundarnih organih ter število in velikost metastaz v pljučih;
- določiti vpliv odstranitve stefina B in cistatina C (dvojni »knockout«) pri PyMT mišjem modelu adenokarcinoma mlečnih žlez na začetek rasti in napredovanja tumorjev, delež proliferacije tumorskih celic, ožiljenje tumorjev, nastanek metastaz v sekundarnih organih ter število in velikost metastaz v pljučih;
- Določiti vpliv odstranitve cistatina C (»knockout«) pri PyMT mišjem modelu eksperimentalih metastaz na začetek rasti tumorja in število ter velikost metastaz v pljučih.

Poglavje 3

Materiali in metode

3.1 Materiali

3.1.1 Kemikalije

- 3,3-DAB (diaminobenzidin; Sigma-Aldrich)
- 3Na-citrat 2H2O (Sigma-Aldrich)
- Amfotericin B raztopina (Sigma-Aldrich)
- Kanadski balzam (Sigma)
- Citronska kislina v prahu (Sigma-Aldrich)
- DMSO (dimetil sulfoksid; Merck)
- DPBS (PAA)
- DTT (Sigma)
- Etanol tehnični 75 %
- E64 (L-*trans*-epoksisukcinil-Leu-4-gvanidinobutilamid) (Bachem)
- Eozin (Sigma-Aldrich)
- Etanol 96 % (Merck)
- Etanol absolutni (Carlo-Erba)
- FBS (fetalni goveji serum; Sigma)
- Goveji serumski albumin (Sigma-Aldrich)
- Glutamax (Gibco-Invitrogen)
- H2O2 30 % (Sigma-Aldrich)
- Ksilol (Sigma-Aldrich)
- Mayerjev hematoksilin (Merck)
- Medij za zamrzovanje tkiv (Jung)
- Mešanica inhibitorjev proteaz (Sigma)
- MgCl2 (Serva)
- Metanol (Merck)
- NaCl (Serva)
- Nitroksolin (Sigma-Aldrich)
- NP-40 (Roche)
- Ocetna kislina absolutna (Applichem)
- Označevalec velikosti proteinov; PageRuler Prestained Protein Ladder (MBI Fermentas)
- Parafin (Leica)
- Penicilin/Streptomicin (Sigma)
- Posneto mleko v prahu (Pomurske mlekarne)
- PEG (polietilen glikol; Fluka)
- Reagent za določanje koncentracije proteinov (Bio-Rad)
- Reagent za kemiluminiscenco ECL (GE Healthcare)
- NaDS (natrijev dodecil sulfat) (Serva)

- SYBR Green (Qiagen)
- SlowFade Gold antifade reagent z DAPI (Invitrogen)
- TEMED (N,N,N', N'-tetrametiletilendiamin) (Merck)
- Triton X-100 (Serva)
- TrypleSelect tripsin (Gibco)
- Trypan Blue (Sigma-Aldrich)
- Tween 20 (Sigma-Aldrich)
- Tris (hidroksimetil)aminometan) (Serva)
- Z-Phe-Arg-AMC (N-benziloksikarbonil-L-fenilalanin-L-arginin-7-amino-4-metilkumarin) (Bachem)

3.1.2 Encimi

- Deoksiribonukleaza I (Sigma-Aldrich)
- Hialuronidaza (Sigma-Aldrich)
- Kolagenaza (Sigma-Aldrich)
- Papain (Sigma-Aldrich)
- Proteinaza K (Fermentas)

3.1.3 Protitelesa

- Primarna kunčja monoklonska protitelesa proti mišjemu cistatinu C (Abcam, ab109508)
- Primarna kunčja poliklonska protitelesa proti mišji aktivni kaspazi-3 (Abcam, ab2302)
- Primarna podganja monoklonska protitelesa proti mišjemu Ki67 (DAKO, M724901)
- Primarna mišja monoklonska protitelesa proti mišjemu β-aktinu (Sigma-Aldrich, A1978)
- Primarna podganja monoklonska protitelesa proti mišjemu CD31 (BD Pharmigen, 550274)
- Primarna kozja poliklonska protitelesa proti mišjemu EGFR (R&D Systems, AF1280)
- Primarna kunčja poliklonska protitelesa proti mišjemu JNK (Cell Signalling Technology, 9252)
- Primarna kunčja poliklonska protitelesa proti mišjemu fosforiliranemu JNK (Cell Signalling Technology, 9251)
- Primarna kunčja poliklonska protitelesa proti mišjima ERK1&2 (Abcam, ab17942)
- Primarna mišja monoklonska protitelesa proti mišjima difosforiliranima ERK1&ERK2 (Sigma-Aldrich, M8159)
- Primarna kunčja poliklonska protitelesa proti mišjemu p38 (Cell Signalling, 9212)
- Primarna kunčja poliklonska protitelesa proti mišjemu fosforiliranemu p38 (Cell Signalling, 9211)
- Sekundarna oslovska anti-podganja protitelesa, konjugirana s Cy3 (Jackson ImmunoResearch, 712-165-150)
- Sekundarna kozja anti-kunčja protitelesa, konjugirana s hrenovo peroksidazo (Jackson ImmunoResearch 111-035-045)
- Sekundarna kozja anti-mišja protitelesa, konjugirana s hrenovo peroksidazo (Jackson ImmunoResearch 115-035-068)
- Sekundarna oslovska anti-kozja protitelesa, konjigurana z Alexa Fluor 488 (Invitrogen A-11055)

3.1.4 Kompleti reagentov

- ApopTag Peroxidase In Situ Apoptosis Detection Kit – TUNEL assay (Millipore, S7100)
- HTS BrdU Cell Proliferation Assay (Calbiochem, HTS01)
- Plasma Protein Membrane Extraction Kit (Abcam ab65400)
- VECTASTAIN ABC Kit Rat IgG (Vector Laboratories, PK-4004)
- VECTASTAIN ABC Kit Rabbit IgG (Vector Laboratories, PK-4001)

3.1.5 Gojišča in pufri za gojenje celic

- Gojišče DMEM z visoko vsebnostjo glukoze in L-glutaminom (Gibco), obogateno z 10 % FBS, 2 mM L-glutaminom (Sigma-Aldrich), 20 enot penicilina (Sigma-Aldrich) in 20 µg/ml streptomicina (Sigma-Aldrich)
- Gojišče Fibrolife® za gojenje celic brez seruma (CellSystems)
- Dulbeccov PBS pufer (PAA)

3.1.6 Laboratorijska oprema

- aparatura za kvantitativni PCR – Q-PCR Mc3005P Real-time PCR system (Agilent, Stratagene products)
- aparatura za obdelavo tkiv in organov Shandon CITADEL 1000 (Shandon)
- aparatura za PCR 2720 Thermal cycler (Applied Biosystems)
- aparatura za prenos western P8DS Penguin (Owl Separation Systems)
- aparatura za pripravo histoloških kock MICROM 350 – 1 in 350 – 2 (Thermo Scientific)
- aparatura za razvijanje filmov SRX-101A (Konica Minolta)
- avtomatska temnica SRX-101A (Konica Minolta)
- avtomatske pipete Eppendorf in Gilson
- centrifuge Eppendorf
- centrifugirke (15 ml in 50 ml) (Falcon)
- filmi Biomax Light film (Kodak)
- filtrski vstavki MWCO 10.000 (Milipore)
- filtrski vstavki MWCO 3.000 (Milipore)
- fluorimeter za mikrotitrske plošče Tecan Infinite M1000 Pro (Tecan)
- grelna plošča Hotplate (Thermo Scientific)
- hemocitometer
- inkubator za celične linije (Binder)
-
- invertni fluorescenčni mikroskop Olympus IX81 (Olympus)
- invertni mikroskop Olympus IX71 (Olympus)
- kadička za agarozno elektroforezo (Owl Separation system)
- kadička za NaDS elektoroforezo (Biorad)
- kadička za prenos Western (Hoefer)
- kriotom Shandon CITADEL (Shandon)
- laminar za delo s celičnimi linijami
- masni spektrometer Orbitrap LTQ Velos (Thermo Scientific), sklopljen z EASY-nanoLCII HPLC enoto Proxeon (Thermo Scientific)
- mikrotom MICROM 315 (Thermo Scientific)
- nitrocelulozna membrana NC45 (Sigma)
- pH meter (Metrel)
- pretočni citometer FACSCalibur (Becton Dickinson)
  ekscitacijska svetloba: 488 nm
  emisijska svetloba, filtri : FL1: 515 – 545 nm
  FL2: 564 – 606 nm
  FL3: > 670 nm

- ultraturrax (IKA, Staufen)
- vodna kopel (Thermo Scientific)

3.1.7 Pufri

BANA pufer
16 mM Na2HPO4
88 mM KH2PO4
3 mM EDTA
0,1 % PEG
pH=6,0

Blokirni pufer endogene peroksidaze
10 % Metanol
3 % H2O2

Citratni pufer
2 mM citronska kislina
7,5 mM Na-citrat
pH 6,0

Elektroforezni pufer za SDS-PAGE
25 mM TRIS
192 mM L-glicin
0,1 % SDS

Fiksativ za organe
4 % Formaldehid v dH2O

Fosfatni pufer
1 mM EDTA
0,1 % (v/v) PEG
1mM DTT
pH 6,0

Lizirni pufer za tkivne homogenate
250 mM TRIS/HCl pH 6,5
10 mM EDTA
0,1 % Triton X-100

Lizirni pufer za študij fosforiliranih proteinov
20 mM Tris-Hcl (pH 7,4)
150 mM NaCl
1mM EDTA
1 % (v/v) NP-40
10 % (v/v) glicerol
1 mM natrijev ortovanedat
10 mM NaF
10 mM glicerofosfat
mešanica proteaznih in fosfataznih inhibitorjev v razmerju 1:100

5-odstotni koncentrirni gel
3,075 ml dH2O
1,25 ml 1,5 mM Tris, pH = 6,8
625 µl 40 % (w/v) akrilamida
50 µl 10 % (w/v) raztopine NaDS

20 µl 10 % (w/v) APS
10 µl TEMED

15-odstotni ločevalni gel
3,75 ml dH_2O
3,65 ml 40 % (w/v) akrilamida
2,5 ml 1,5 mM Tris, pH = 8,8
100 µl 10 % (w/v) raztopine NaDS
40 µl 10 % (w/v) APS
20 µl TEMED

12,5-odstotni ločevalni gel
3,13 ml dH_2O
4,27 ml 40 % (w/v) akrilamida
2,5 ml 1,5 mM Tris, pH = 8,8
100 µl 10 % (w/v) raztopine NaDS
40 µl 10 % (w/v) APS
20 µl TEMED

6-kratni pufer NaDS za nanos vzorcev na PAGE
300 mM Tris
200 mM DTT
100mM NaDS
40 mM EDTA
0,2 % (w/v) bromfenol modro
20 % (v/v) glicerol

PBS pufer
145 mM NaCl
2,5 mM Na2HPO4
7,5 mM NaH2PO4
pH 7,4

PBST pufer
PBS pufer
0,5 % Tween 20

Western prenašalni pufer
25 mM TRIS
192 mM L-glicin
15 mM SDS
20 % Metanol

3.1.8 Mišji model raka mlečne žleze MMTV-PyMT na sevu FVB/N

3.1.8.1 Sev miši FVB/N; divji tip, cistatin C-/- in stefin B-/-

Sev miši FVB/N smo kupili pri firmi The Jackson Laboratory. FVB/N sev omogoča sokrvno parjenje z ožjimi sorodniki z dobro plodnostjo in velikim številom potomcev. Sev je primeren za gensko manipulacijo in transgene modele. Oplojena jajčeca imajo izrazito projedro, kar omogoča mikro-injiciranje DNA (Taketo et al., 1991)(Taketo et al., 1991)[143][143](Taketo et al., 1991)(Taketo et al., 1991)(Taketo, Schroeder et al.

1991)(Taketo et al., 1991)(Taketo et al., 1991)(Taketo et al., 1991). Miši z izbitim genom za stefin B na izvornem sevu C57BL/6J smo dobili od dr. Richarda M. Myersa (Pennacchio, et al., 1998)(Pennacchio et al., 1998)[8282](Pennacchio et al., 1998)(Pennacchio et al., 1998)(Pennacchio, Bouley et al. 1998)(Pennacchio et al., 1998)(Pennacchio et al., 1998) (Pennacchio et al., 1998). Miši z izbitim genom za cistatin C na izvornem sevu C57BL/6J (Huh, et al., 1999) smo dobili od dr. Andersa Grubba.

3.1.8.2 Transgeni mišji model FVB/N MMTV-PyMT

Heterozigotne transgene miši seva FVB/N-TgN(MMTV-PyMT)634Mul (Guy, Cardiff, & Muller, 1992) smo kupili pri firmi The Jackson Laboratory. Za transgene miši je značilen nastanek raka mlečne žleze pri deviških samicah. Nastanek tumorjev poteka z izražanjem onkogena Polyoma virus middle-T (PyMT) v epiteliju izvodil mlečne žleze.

3.1.8.3 Transplantirani alograftni model raka mlečne žleze

Transplantirani alograftni model raka mlečne žleze na FVB/N kongenih miših divjega tipa smo vzpostavili na Institutu »Jožef Stefan«. Na mestu transplantacije primarnih PyMT tumorskih celic se razvije tumor, ne da bi se sprožil imunski odziv. V transplantiranem tumorju se razvije proces angiogeneze, ki tumorju omogoča dostop hranil in kisika, potrebnih za nadaljnjo rast. Transplantirani tumor po 10 dneh doseže povprečno 1–2 mm premera, po 35 dneh pa povprečno 10 mm premera.

3.1.8.4 Genotip uporabljenih miši v poskusih

- Sev miši FVB/N divji tip (FVB;WT)
- Sev miši FVB/N z izbitim genom za cistatin C (FVB;CstC-/-)
- Transgeni sev miši FVB/N MMTV-PyMT divji tip (PyMT;WT)
- Transgeni sev miši FVB/N MMTV-PyMT z izbitim genom za cistatin C (PyMT;CstC-/-)
- Transgeni sev miši FVB/N MMTV-PyMT z izbitima genoma za stefin B in cistatin C (PyMT;StfB-/-CstC-/-)

3.1.8.5 Uporabljene primarne tumorske celice iz miši

- Primarne epitelijske adenokarcinomske celice iz tumorja mlečne žleze MMTV-PyMT; divji tip (PyMT;WT)
- Primarne epitelijske adenokarcinomske celice iz tumorja mlečne žleze MMTV-PyMT; cistatin C-/- (PyMT;CstC-/-)

3.2 Metode

3.2.1 Vzreja laboratorijskih miši

Nastanitev in dobrobit živali sta bili v skladu z Zakonom o zaščiti živali in smernicami FELASA (Federation of European Laboratory Animal Science Associations). Miši so imele dostop do vode in hrane *ad libitum*, dnevno nočni interval se je menjaval na 12 ur. Miši v

poskusih so bile uporabljene v skladu s predpisanim Pravilnikom o pogojih za izvajanje poskusov na živalih Ur. l. RS št. 37/2013, odobrenim s strani Uprave Republike Slovenije za varno hrano, veterinarstvo in varstvo rastlin (UVHVVR). Miši so bile žrtvovane s cervikalno dislokacijo in naknadno izkrvavitvijo. Miši z izbitim genom za stefin B in cistatin C so bile prekrižane na sev miši FVB/N v desetih generacijah. Tako smo dosegli, da so imele miši z izbitim genom za stefin B in cistatin C enako ozadje kot transgene miši MMTV-PyMT. Ko smo prekrižali heterozigotne PyMT transgene samce s samicami z izbitim genom za cistatin C ali stefin B, smo pridobili transgene potomce z izbitim genom za cistatin C oz. stefin B. Po prekrižanju miši z izbitim genom za stefin B in z izbitim genom za cistatin C smo dobili heterozigotne potomce z enim genom za stefin B in enim genom za cistatin C. Po nadaljnjem križanju smo dosegli čisto linijo miši z izbitima genoma za cistatin C in stefin B (dvojni »knock-out«). Pri križanju PyMT transgenega samca z izbitima genoma za cistatin C in stefin B s samico z izbitima genoma za cistatin C in stefin B smo pridobili PyMT transgene potomce z izbitima genoma za cistatin C in stefin B (PyMT;StfB-/-CstC-/-). Dovoljenja za delo z laboratorijskimi živalmi so bila izdana s strani Ministrstva za kmetijstvo, gozdarstvo in prehrano, Uprava Republike Slovenije za varno hrano, veterinarstvo in varstvo rastlin. Številke dovoljenj: 34401-14/2008/8, 34401-28/2012/4, U34401-20/2015/5.

3.2.2 Spremljanje napredovanja rasti tumorjev

Pri starosti miši 28 dni smo samicam trikrat tedensko pričeli digitalno palpirati vseh 10 mlečnih žlez, pri čemer nismo poznali genotipa samic, z namenom detekcije tumorjev mlečne žleze. Premer tumorjev smo s kljunastim merilom izmerili 30 dni po posamični detekciji. Pri starosti miši 14 tednov smo živali žrtvovali, odvzeli tumorje in jih stehtali.

3.2.3 Priprava alograftnega ortotopično transplantiranega mišjega modela raka mlečne žleze v kongenem sevu miši FVB/N

Za alograftni mišji model smo uporabili 8-10 tednov stare kongene miši FVB/N divjega tipa (FVB/WT) in FVB/N miši z izbitim genom za cistatin C (FVB/CstC-/-). Po tri različne primarne PyMT;WT in PyMT;CStC-/- tumorske celice smo prešteli in enako število celic istega genotipa združili v en skupni vzorec. Celice smo nato centrifugirali in resuspendirali v ustreznem volumnu gojišča DMEM brez dodanega FBS v končni koncentraciji 7,5 x 10^6 celic v 1 ml. 200 µl celične suspenzije smo aplicirali v maščobno blazinico leve abdominalne mlečne žleze mišim prejemnice. Nastale so 4 skupine: 8 FVB;WT miši je prejelo PyMT;WT tumorske celice, 7 FVB;CstC-/- miši je prejelo PyMT;WT tumorske celice, 9 FVB;WT miši je prejelo PyMT;CstC-/ tumorske celice in 8 FVB;CstC-/- miši je prejelo PyMT;CstC-/- tumorske celice. 14 dni po aplikaciji tumorskih celic smo s palpiranjem pričeli spremljati razvoj tumorja. Meritve velikosti tumorjev smo opravljali vsak drugi dan s kljunastim merilom. 35 dni po transplantaciji smo živali usmrtili in odvzeli tumorje in ostale organe.

3.2.4 Odvzem organov

Po žrtvovanju miši smo odvzeli naslednje organe: tumorje ali mlečne žleze, aksilarne bezgavke, pljuča, srce, jetra, vranico in ledvici za pripravo primarnih tumorskih celic, patohistološke analize, tkivne lizate in izolacijo mRNA.

3.2.4.1 Organi – parafinske rezine

Odvzete organe smo 48–72 ur inkubirali v 4 % formalinu, nato še v 75 % etanolu. Nato smo organe vstavili v kasete za obdelavo tkiv in organov in s pomočjo aparature Shandon Citadel 1000 vzorce pripravili za vstavitev v parafinske kocke.

3.2.4.2 Organi – krio rezine

Vse organe, namenjene za krio-rezine, smo obdali z medijem za zamrzovanje tkiv, jih potopili v tekoči dušik do popolne zamrznitve in nato shranili na -80 °C.

3.2.4.3 Organi za izolacijo mRNA

Vse organe, namenjene za izolacijo mRNA, smo potopili v tekoči dušik do popolne zamrznitve in nato shranili na -80 °C.

3.2.5 Histomorfometrija

Za določitev celokupnega deleža in velikosti metastaz v pljučih smo pripravili 5 µm debele parafinske rezine pljuč na treh različnih globinah, pri čemer je bila vsaka globina od prejšnje oddaljena za 100 µm. Parafinske rezine smo nato pobarvali z metodo barvanja po hematoksilinu in eozinu. Povprečno število in velikost metastaz smo določili s pomočjo svetlobne mikroskopije na invertnem mikroskopu Olympus IX81 in z uporabo računalniške meritve površine pljučnih metastaz in pljuč (Cell^{f}). Iz meritev pljučnih metastaz in površine pljučnega krila smo izračunali povprečno velikost metastaz v mm^2 in povprečno število metastaz na mm^2 površine pljuč.

3.2.6 Imunohistokemija in imunofluorescenca

3.2.6.1 Imunohistokemija

Organe smo narezali na 5 µm debele parafinske rezine. Rezine tumorjev smo pripravili iz miši genotipa PyMT;WT, PyMT;CstC-/- in PyMT;StfB-/-CstC-/- in iz tumorjev miši genotipa FVB;WT in FVB;CstC-/-, ki smo jim transplantirali tumorske celice. Postopek barvanja smo pričeli z deparafinizacijo s ksilolom in rehidracijo v padajočih koncentracijah ezanola (100 %, 96 %, 70 % in 50 %). Morebitne sterične ovire tkivnih antigenov smo odstranjevali s segrevanjem v mikrovalovni pečici v citratnem pufru. Po ohlajanju smo reakcijo na preparatu ustavili z blokirnim pufrom, nato pa smo preparat inkubirali še z blokirnim serumom. Sledila je inkubacija preparatov s primarnimi protitelesi v vlažni komori preko noči na 4 °C . Kunčja primarna protitelesa proti cistatinu C smo redčili 1:500, podganja primarna protitelesa proti faktorju Ki67 smo redčili 1:20. Za detekcijo primarnih protiteles smo uporabili Vectastain Elite ABC kit (Vector Laboratories) po navodilih proizvajalca. Nato smo rezine inkubirali s 3,3 DAB (Sigma-Aldrich) ter jedra pobarvali s hematoksilinom. Po barvanju s hematoksilinom smo preparat spirali v banjici pod tekočo vodo, nato smo ga dehidrirali z naraščajočimi koncentracijami etanola (50 %, 70 %, 96 % in 100 %) ter bistrili v ksilolu. Na preparat smo kanili kapljico kanadskega balzama ter nanj položili krovno stekelce. Tako pripravljene preparate smo opazovali s svetlobnim mikroskopom.
Za določitev fragmentacije DNA (metoda TUNEL) smo uporabili kit ApopTag Peroxidase In Situ apoptosis detection kit (Millipore) po navodilih proizvajalca.

3.2.6.2 Imunofluorescenca

Imunofluorescenco smo določali na 5 μm debelih zamrznjenih tumorskih rezinah. Določili smo ožiljenje v tumorjih s pomočjo endotelijskega kazalca CD31 v koncentraciji 1:100. Primarna protitelesa smo detektirali s sekundarnimi protitelesi, konjugiranimi s fluoroforjem Cy3. Nato smo tumorske rezine zaščitili in pobarvali jedra z uporabo SlowFade Gold antifade reagenta s fluoroforjem DAPI.

3.2.7 Mikroskopija

Za določitev deleža delečih se celic s faktorjem Ki67 smo z mikroskopom Olympus IX71 in programsko opremo Olympus Cell^F pod vidno svetlobo in povečavo 40 x naredili 20 naključnih slik. Te slike smo s pomočjo programa ImageJ analizirali in določili delež delečih se celic. Enako metodo smo uporabili za določitev deleža fragmentirane DNA (TUNEL). Za določitev deleža angiogeneze v tumorjih (kazalec CD31) smo uporabili fluorescentni mikroskop Olympus IX81 in programsko opremo Olympus Cell^F. Za fluorofor Cy3 smo uporabili vzbujevalni filter 516–555 nm in emisijski filter 574–648 nm. Za fluorofor DAPI smo uporabili filter 360–370 in emisijski filter 420–460 nm. Slike smo kvantitativno obdelali s pomočjo programa ImageJ in določili delež angiogeneze v tumorjih.

3.2.8 Priprava tumorskih lizatov

Zamrznjene primarne tumorje smo v lizirnem pufru homogenizirali s pomočjo homogenizatorja Ultrathurrax (IKA) in nato homogenate centrifugirali 10 min pri 4 °C na 10.000 g. Po centrifugiranju smo odvzeli supernatante in z metodo po Bradfordu (Bio-Rad) izmerili celokupno proteinsko koncentracijo.

3.2.8.1 Priprava tumorskih lizatov za detekcijo fosforiliranih proteinov

Tumorske lizate smo pripravili v pufru z dodatkom mešanice proteaznih inhibitorjev (1:100) in mešanice fosfataznih inhibitorjev (1:100).

3.2.9 Kvantitativni PCR v realnem času

Celokupno RNA smo izolirali iz zamrznjenih tumorjev z uporabo kompleta reagentov Allprep DNA/RNA/Protein Mini Kit (Qiagen) in TURBO DNA free kit (Ambio) za odstranitev genomske DNA. Celokupno RNA smo izolirali iz zamrznjenih PyMT tumorjev z uporabo kompleta reagentov Allprep DNA/RNA/Protein Mini Kit (Qiagen) in TURBO DNA free kit (Ambio) za odstranitev genomske DNA. Kvaliteto RNA smo preverili spektrofotometrično z določitvijo razmerja absorbance A260/A230 in A260/A280. Integriteto RNA smo potrdili na 1,2 % elektroforeznem gelu. Nato smo z RNA naredili obratno transkripcijo v cDNA z uporabo naključnih nanomernih začetnih oligonukleotidov iz kita Precision nanoScriptTM Reverse Transcription (PrimerDesign Ltd.). Nivo izražanja mRNA za katepsine B (CTSB), H (CTSH), L (STCL), S (CTSS), in X/Z (CTSX) smo detektirali s SYBR green kemijo in 2x Precision MasterMix (PrimerDesign Ltd.). Za referenčna gena smo s pomočjo kita GeNorm reference gene selection (PrimerDesign Ltd.) izbrali evkariontski iniciatorski translacijski faktor 4A2 (ang. Eukaryotic translation initiation factor 4A2 - Eif4a2) in sukcinat dehidrogenazni kompleks, podenota A (ang. Succinate dehydrogenase complex, subunit A – SDHA). Iz normaliziranih vrednosti smo nato izračunali relativno izražanje mRNA posameznih genov. Kvantitativni PCR v realnem času smo opravili na aparaturi Mx3005P Real-time PCR system (Agilent) pod naslednjimi

pogoji: 10 minut na 95 °C (denaturacija), 40 ciklov 15 sekund na 95 °C in 1 min na 60 °C (prileganje in podaljševanje). Po vsakem ciklu je bila določena denaturacijska krivulja. Začetni oligonukleotidi so bili narejeni tako, da izključujejo prepis intronov (PrimerDesign Ltd.) z naslednjimi nukleotidnimi zaporedji:
- CTSB, 5'-TACTTGCTGTGGTATCCAGTGTG-3' (naprej) in 5'-ATGGTGTATGGTAAGCAGCCTAC-3' (nazaj)
- CTSH, 5'-TTTGATGTATAAAAGTGGCGTCTA-3' (naprej) in 5'-GAGTTTTTCACAATCCAGTAGAGT-3' (nazaj).
- CTSL, 5'-AAGCCATCCGTCTCTCCAG-3' (naprej) in 5'-CGTTTCTTGACAAGCCAATATTTATTC-3' (nazaj)
- CTSS, 5'-CTGTTCTTGACAAGCCAATATTTATTC-3' (naprej) in 5'-AGCCAGTAATCTTTGCCATCA-3' (nazaj)
- CTSX/Z, 5'-CTATGCCAATGGTCCCATCAG-3' (naprej) in 5'-CCTGGTCCTGGTGCTCAG-3' (nazaj)

Relativen nivo izražanja tarčnih genov smo izračunali glede na njihovo učinkovitost pri PCR v realnem času.

3.2.10 Priprava primarnih tumorskih celic

Primarne MMTV-PyMT tumorske celice smo izolirali po metodi, opisani v (Vasiljeva, et al., 2006). Izolirali smo tumorske celice genotipa PyMT;WT in PyMT;CstC-/-. Celice smo gojili v gojišču za gojenje celic DMEM, ki smo mu dodali 10 % fetalnega bovinega seruma (FBS, Sigma), 2 mM L-glutamina (Sigma-Aldrich), 20 enot penicilina in 20 μg/ml streptomicina (Sigma-Aldrich). Celice smo gojili v inkubatorju v nadzorovani atmosferi, nasičeni z vodno paro in 5 % CO_2 pri 37 °C. Celice smo uporabljali največ do 4. pasaže.

3.2.10.1 Delo s primarnimi celičnimi kulturami

3.2.10.1.1 Priprava celotnega celičnega lizata

Po inkubaciji v mediju Fibrolife® smo celice tripsinizirali in centrifugirali 5 minut pri 400 rcf. Nato smo jih dvakrat sprali s hladnim PBS. Celicam smo dodali ustrezen volumen hladnega fosfatnega pufra z dodatkom 1 % NP-40. V primeru, da smo celične lizate uporabili za detekcijo fosforiliranih proteinov, smo dodali tudi mešanico inhibitorjev proteaz in fosfataz v razmerju 1:100 (v/v) ter jih inkubirali na ledu 30 minut. Celice smo nato sonificirali, lizate pa centrifugirali 10 minut pri 10.000 rpm in 4 °C, nato smo supernatante prenesli v novo epico in jih bodisi takoj uporabili bodisi kratkotrajno shranili pri -80 °C.

3.2.10.1.2 Izolacija membranskih proteinov iz primarnih tumorskih celic

Proteine plazemske membrane smo izolirali iz primarnih PyMT tumorskih celic z uporabo Plasma Protein Membrane Extraction Kit (Abcam).

3.2.10.1.3 Analiza sinteze DNA z metodo BrdU

Primarne tumorske celice smo gojili na ploščah s 96 jamicami v koncentraciji 10^5 celic/ml. Celice smo 4 ure inkubirali z BrdU, nato pa smo po navodilih proizvajalca nivo vključitve BrdU v DNA izmerili na čitalcu mikrotitrskih plošč Tecan infinite M1000 Pro (Tecan) pri vzbujevalni valovni dolžini 450 nm in emisijski valovni dolžini 540 nm.

3.2.11 Priprava primarnih mišjih makrofagov iz kostnega mozga

Usmrčeni miši smo sterilno odvzeli stegnenico in golenico, odstranili mišično tkivo in sterilno odrezali sklep. Kostni mozeg smo spirali iz kosti z brizgo, napolnjeno s toplim gojiščem, in zbirali celice kostnega mozga v sterilni petrijevki. Celično suspenzijo smo centrifugirali 5 minut pri 400 rcf. Po centrifugiranju smo celično suspenzijo resuspendirali v 10 mL svežega ogretega gojišča DMEM, obogatenega z 20 % FBS-HI, 1 % glutaminom in 1 % streptamicinom/penicilinom, ter dodali 40 ng/ml rekombinantnega stimulirajočega dejavnika za makrofagne kolonije (M-CSF) ali 10 % filtriranega gojišča mišje fibroblastne celične linije L929, ki izločajo M-CSF. Po sedmih dneh je potekla popolna diferenciacija monocitov v makrofage. Diferencirane makrofage smo tripsinizirali in nasadili za poskus.
Za pridobitev gojišča, bogatega z M-CSF, smo nasadili 4,7 x 10^5 celic L929 v 55 ml gojišča DMEM, obogatenega z 10 % FBS-HI, 1 % glutaminom in 1 % streptamicinom/penicilinom, v posodo T-75 (75 cm2) za gojenje celic. Celice smo pustili rasti v inkubatorju 7 dni (brez menjave gojišča). Sedmi dan smo gojišče odvzeli, ga centrifugirali, filtrirali skozi filter z velikostjo por 0,45 µm in gojišče nato uporabili za diferenciacijo mišjih makrofagov.

3.2.11.1 Sprožitev vnetnega odziva *in vitro*

Primarne makrofage smo nasadili na plošče (1 x 10^6 celic / jamico) za pripravo lizatov ali v mikrotitrske plošče (1 x 10^5 celic / jamico) za merjenje citokinov v gojišču in jih inkubirali preko noči. 80–90 % konfluentne celice smo najprej 18 ur stimulirali z LPS (v končni koncentraciji 100 ng/mL) ali z IFN-γ (v končni koncentraciji 100 U/mL), nato smo celice sprali s PBS in dodali LPS ter inkubirali nadaljnjih 18 ur.

3.2.11.2 Merjenje koncentracije IL-10 v celičnem gojišču z encimsko imunskim testom ELISA

Za merjenje citokina IL-10 v gojišču smo uporabili komercialni komplet reagentov za indirekten test ELISA ter ga izvedli po navodilih proizvajalca (Abcam, ab108870). Absorbanco smo izmerili na čitalcu mikrotitrskih plošč Tecan infinite M1000 Pro (Tecan) pri valovni dolžini 450 nm. Koncentracijo IL-10 v vzorcih smo določili iz umeritvene krivulje.

3.2.12 Merjenje katepsinske aktivnosti

Tumorskim lizatom smo določili katepsinsko aktivnost s hidrolizo sintetičnega substrata Z-Phe-Arg-AMC. Mlečne žleze ali primarne tumorje smo homogenizirali v lizirnem pufru. 20 ug vzorca smo nanesli na plošče s 96 jamicami ter dodali 0,1 M fosfatni pufer z DTT. Za kontrolo razgradnje substrata s cisteinskimi substrati smo uporabili papain (pozitivna kontrola). Za kontrolo specifičnosti razgradnje substrata smo vzorce inkubirali z inhibitorjem E64 v končni koncentraciji 10 µM (negativna kontrola). Ploščo smo inkubirali 15 minut pri 37 °C. Kinetiko hidrolize smo merili 10 min na čitalcu mikrotitrskih plošč Tecan infinite M1000 Pro (Tecan) pri vzbujevalni valovni dolžini 370 nm in emisijski valovni dolžini 460 nm.
Pri merjenju katepsinske aktivnosti v supernatantu PyMT primarnih celičnih kultur smo tumorske celice obeh genotipov gojili na ploščah z 12 jamicami (7,5x10^5 celic na jamico). Celice smo nato 2 uri inkubirali v PBS pufru. Po inkubaciji smo 50 µl PBS nanesli na plošče s 96 jamicami, dodali 0,1 M fosfatni pufer z DTT in postopek nadaljevali, kot je opisano zgoraj.
Pri merjenju katepsinske aktivnosti v celičnih lizatih smo pripravili celične lizate iz primarnih PyMT tumorskih celic, pomerili smo proteinsko koncentracijo lizatov in nanesli 40 µg proteinov na plošče s 96 jamicami ter dodali 0,1 M fosfatni pufer z DTT. Postopek smo nadaljevali, kot je opisano zgoraj.

3.2.13 Poliakrilamidna gelska elektroforeza v prisotnosti NaDS

Poliakrilamidno gelsko elektroforezo z dodanim denaturantom natrijevim dodecilsulfatom (NaDS-PAGE) smo uporabili pod redukcijskimi pogoji (v prisotnosti DTT) za ločevanje proteinov iz tkivnih lizatov, celičnih lizatov ali supernatantov celičnih kultur. Vzorcem smo pred nanosom na gel dodali nanašalni pufer z NaDS in DTT ter jih termično obdelali (5 minut pri 100 °C). Za referenčne velikosti proteinov smo nanesli proteinske standarde. Elektroforeza je potekala v elektroforeznem pufru v aparaturi za elektroforezo pri konstantnem toku 30 mA/gel.

3.2.14 Detekcija proteinov s prenosom western

Prenos western je metoda prenosa proteinov iz poliakrilamidnega gela na nitrocelulozno membrano s pomočjo električnega toka. Proteine iz preiskovanih vzorcev smo po končani elektroforezi iz NaDS-PAGE (natrijev dodecil sulfat poliakrilamidna gelska elektroforeza) gela prenesli na polivinilidin fluoridno membrano (PVDF, Serva) s pomočjo električnega polja v aparaturi za prenos western (Hoefer). Čas prenosa je bil 120 min pri konstantnem električnem toku 250 mA v western prenašalnem pufru. Po končanem prenosu proteinov smo prosta mesta na membrani blokirali z inkubacijo v blokirnem pufru (5 % mleko v prahu v PBST) pri sobni temperaturi 45 min. Po blokadi smo membrane inkubirali s primarnimi protitelesi preko noči na stresalniku pri 4 °C. Uporabili smo primarna monoklonska kunčja protitelesa proti cistatinu C (Abcam, 1:5000), primarna poliklonska kunčja protitelesa proti stefinu B (Abcam, 1:1000), primarna poliklonska kunčja protitelesa proti JNK (Cell Signaling, 1:1000), primarna poliklonska kunčja protitelesa proti fosforiliranemu JNK (Cell Signaling, 1:1000), primarna poliklonska kunčja protitelesa proti ERK1&2 (Abcam, 1:1000), primarna monoklonska mišja protitelesa proti difosforiliranima ERK1&2 (Sigma-Aldrich, 1:1000), primarna poliklonska kunčja protitelesa proti p38 MAPK (Cell Signaling, 1:1000), primarna poliklonska kunčja protitelesa proti p38 MAPK (Cell Signaling, 1:1000), primarna poliklonska kozja protitelesa proti EGFR (R&D, 1:1000). Za kontrolo nanosa proteinov smo uporabili primarna monoklonska mišja protitelesa proti aktinu (Sigma-Aldrich, 1:5000).
Po inkubaciji s primarnimi protitelesi smo nevezana protitelesa na membrano spirali 3x5 min s PBST. Nato smo membrane inkubirali 45 minut pri sobni temperaturi v sekundarnih protitelesih (sekundarna kozja anti-kunčja protitelesa, 1:5000 v PBST, sekundarna kozja anti-mišja protitelesa, 1:7000 v PBST). Po inkubaciji smo membrane spirali 4x15 min v PBST. Za detekcijo proteinov smo uporabili sistem ECL (GE Healthcare) po navodilih proizvajalca.

3.2.15 Pretočna citometrija

Primarne celične kulture smo sprali s PBS in jih odlepili od podlage z disociacijsko raztopino za celice. Celice smo nato centrifugirali, jih sprali s PBS ter jih resuspendirali v 100 ul hladnega 3 % BSA v PBS. Celice smo za 15 minut pustili na ledu, nato smo dodali primarna poliklonska kozja protitelesa proti EGFR (R&D, 1: 250), inkubirali 45 min na ledu, centrifugirali in sprali s hladnim 3 % BSA. Nato smo dodali še sekundarna protitelesa (1:250) in inkubirali v temi 45 minut. Celice smo ponovno centrifugirali, sprali pelet z mrzlim PBS ter pomerili na pretočnem citometru. Poskus smo delali v triplikatu, v poskus smo vključili negativno kontrolo (celice, ki smo jim dodali samo sekundarna protitelesa).

3.2.16 Priprava vzorcev za masno spektrometrijo

Enako število primarnih PyMT tumorskih celic smo nacepili na plošče in gojili v kompletnem gojišču DMEM 24 ur. Nato smo plošče s celicami sprali s PBS in 24 ur inkubirali v DMEM mediju brez seruma. Po inkubaciji smo medije zbrali in jih skoncentrirali v filtrskih vstavkih z MCW 3000 Da. Vzorcem supernatanta smo dodali nanašalni pufer z NaDS in DTT ter jih termično obdelali (5 minut inkubacije pri 100 °C). Proteine v supernatantih smo nato ločili na 12,5 % NaDS-PAGE gelih. Barvanju s Comassie Brilliant Blue je sledilo razbarvanje z raztopino za razbarvanje (25 mM NH4HCO3, 50 % acetonitril) do primerne intenzivnosti proteinskih prog. Sledila je rehidracija v reducirajoči raztopini (10 mM DTT, 25 mM NH4HCO3). Po 45-minutni inkubaciji na 65 °C smo reducirajočo raztopino zamenjali z alkilacijsko (55 mM jodoacetamid, 25 mM NH4HCO3). Nato smo koščke gela sprali s 25 mM NH4HCO3 in jih dehidrirali z acetonitrilom. Sledila je tripsinizacija koščkov preko noči pri 37 °C v tripsinizacijskem pufru (25 mM NH4HCO3, 1 µg tripsina). Po inkubaciji smo peptide ekstrahirali z raztopino 50 % acetonitrila in 5 % mravljične kisline. Vzorce smo analizirali z masnim spektrometrom LTQ Orbitrap Velos, sklopljenem z EASY-nanoLCII HPLC enoto Proxeon. Peptide smo nanesli na C18 lovilno kolono (Proxeon Easy-ColumnTM, dolžina 2 cm, notranji premer 100 µm, 5 µm 120 Å, C18-A1 kroglice) in jih ločili na 10 cm dolgi analitski koloni C18 PicoFritTM AQUASIL (notranji premer 75 µm, 5 µm 120 Å, C18 kroglice) s pretokom mobilne faze 300 nl/min. Sledila je elucija peptidov z 90-minutnim linearnim gradientom 5–40 % topila (0,1 % mravljinčna kislina v acetonitrilu). Spektre MS/MS smo pridobili s CID fragmentacijo devetih najbolj intenzivnih prekurzorskih ionov. Z dinamičnim izključevanjem smo vsak spekter peptida, ki se je enkrat ponovil, za 60 sekund izločili iz postopka snemanja. Za iskanje po bazi podatkov in kvantifikacijo s spektralnim štetjem smo uporabili proteomski programski paket MaxQuant (različica 1.5.3.8) (Cox & Mann, 2008) s pomočjo UniProtKB. Kot stalno modifikacijo smo uporabili karbamidometilacijo cisteinov, medtem ko je bila metioninska oksidacija opredeljena kot variabilna modifikacija. Za relativno kvantifikacijo proteinov v vzorcih smo uporabili metodo spektralnega štetja. Število spektrov znotraj istega genotipa smo povprečili glede na štiri biološke ponovitve. Razmerje med številom spektrov (*angl.* spectral count ratio – SCR) smo izračunali kot število povprečnih MS/MS spektrov proteina v vzorcih genotipa brez cistatina C, ulomljeno s številom povprečnih MS/MS spektrov proteina v vzorcih genotipa divjega tipa. Število MS/MS spektrov vseh proteinov smo med primerjavo podatkov povečali za ena, da smo se izognili deljenju s številom 0. Kot proteine s spremenjeno prisotnostjo v tretiranem vzorcu smo obravnavali le tiste, ki se jim je SCR spremenil za vsaj 1,5-krat glede na negativno kontrolo. Dodaten pogoj je bilo najmanj 10 MS/MS spektrov v vseh štirih bioloških ponovitvah v vzorcih PyMT;WT.

3.2.17 Statistična analiza

Vsi kvantitativni rezultati so predstavljeni kot srednje vrednosti s standardno napako aritmetične sredine. Za ugotavljanje statističnih razlik smo uporabljali t test (z dvodelno porazdelitvijo in dvovzorčno neenako varianco), $\chi 2$ Chi-square test, za analizo Kaplan-Meier grafičnega prikaza smo uporabili log-rank test. Kot statistično signifikantno razliko smo upoštevali, če je bila vrednost *; $p < 0,05$, **; $p < 0,01$ in ***; $p < 0,001$.

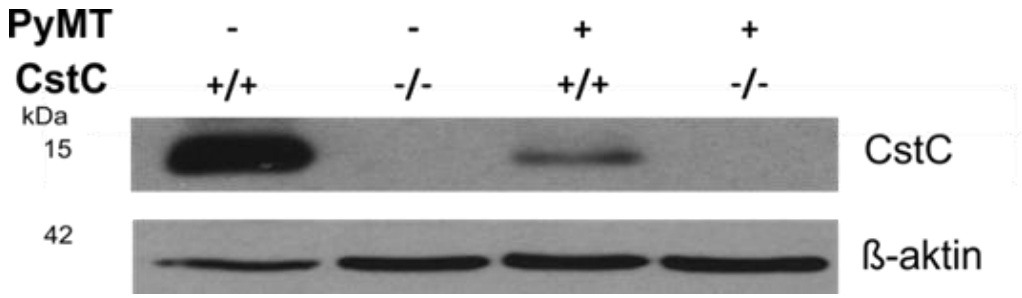
Poglavje 4

Rezultati

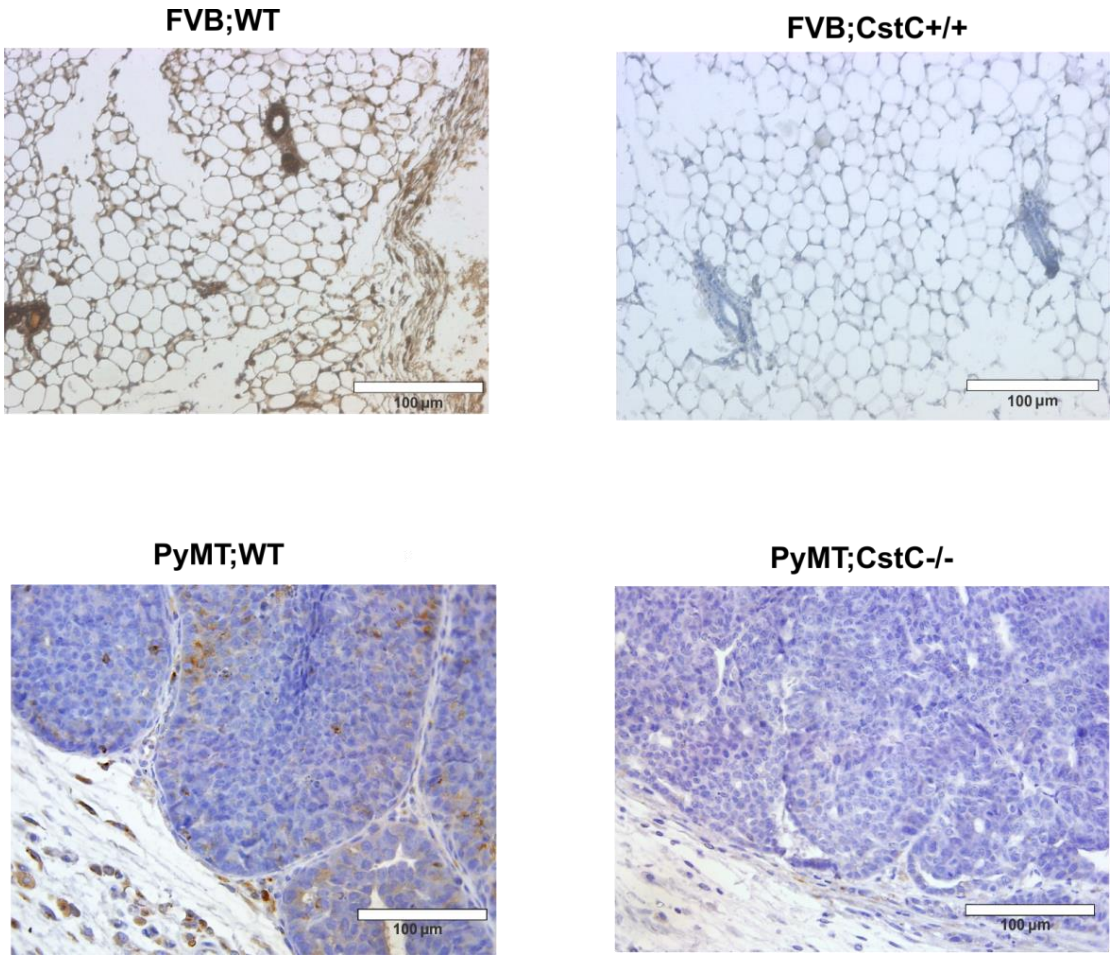
4.1 Analiza PyMT mlečno žleznih karcinomov

4.1.1 V tumorjih mlečnih žlez je manj cistatina C kot v zdravih mlečnih žlezah

Preverili smo izražanje in lokacijo cistatina C v mlečnih žlezah in v tumorjih pri miših divjega tipa in pri miših z izbitim genom za cistatin C. V totalnih lizatih mlečnih žlez in v tumorskih lizatih obeh genotipov smo s prenosom Western določili izražanje cistatina C. Ugotovili smo, da je izražanje cistatina C v tumorjih nižje kot v mlečnih žlezah (Slika 4). Histološka analiza mlečnih žlez in tumorjev ustreznega genotipa je pokazala, da je cistatin C v mlečnih žlezah prisoten difuzno, medtem ko je v tumorjih cistatin C pretežno izražen na zunanjih robovih tumorskega tkiva in v tumorskem mikrookolju (Slika 5).
Prisotnost stefina B smo določili v mlečnih žlezah in tumorjih. Ugotovili smo zvišano izražanje stefina B v tumorjih v primerjavi z mlečnimi žlezami, neodvisno od genotipa (Slika 6).



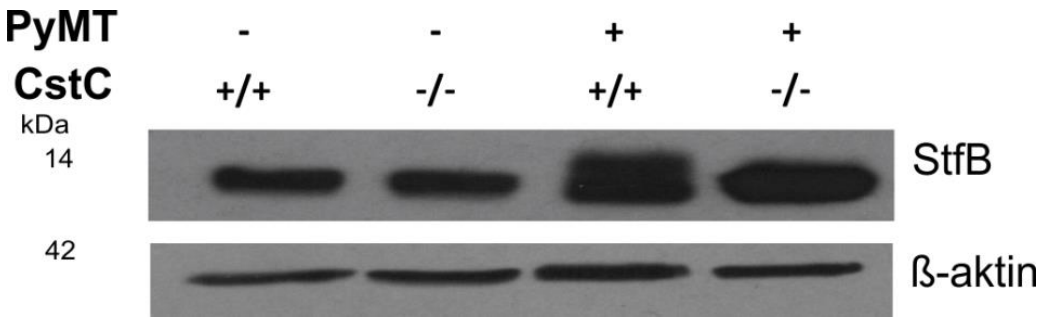
Slika 4: Izražanje cistatina C v normalnih mlečnih žlezah in v tumorjih mlečnih žlez pri 14 tednov starih miših. Po prenosu Western se vidi znižano izražanje cistatina C v PyMT tumorjih v primerjavi z mlečno žlezo. Na sliki so prikazani totalni lizati mlečne žleze ali tumorja v naslednjem vrstnem redu: FVB;WT, FVB;CstC-/-, PyMT;WT in PyMT;CstC-/-. Aktin je služil kot kontrola količine nanesenega proteina.



Slika 5: Imunohistokemijska detekcija cistatina C v mlečnih žlezah in v tumorjih.

Reprezentativne slike rezin mlečnih žlez (FVB;WT in FVB;CstC-/-) ter tumorjev (PyMT;WT in PyMT;CstC-/-). Rjavo je obarvan cistatin C, modro so obarvana jedra celic. Skala na slikah predstavlja 100 µm.

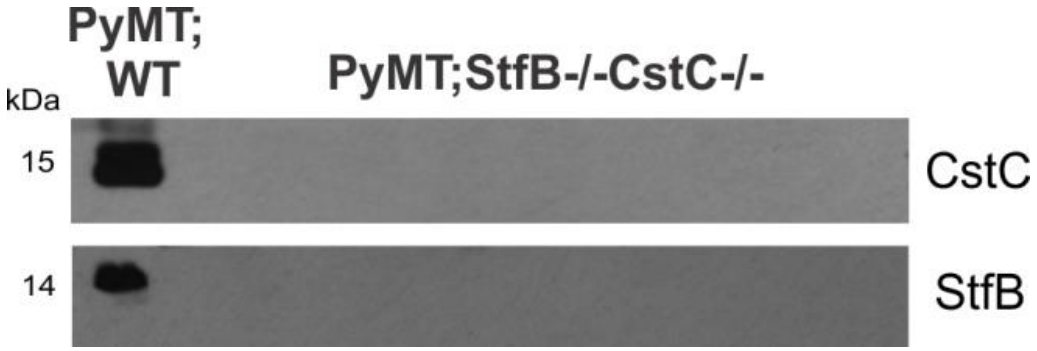
4.1.2 V tumorjih mlečnih žlez je več stefina B kot v zdravih mlečnih žlezah



Slika 6: Izražanje stefina B v normalnih mlečnih žlezah in v tumorjih mlečnih žlez. Po prenosu Western je opazno zvišano izražanje stefina B v PyMT tumorjih v primerjavi z mlečno žlezo. Na sliki so prikazani totalni lizati mlečne žleze ali tumorja v naslednjem vrstnem redu: FVB;WT, FVB;CstC-/-, PyMT;WT in PyMT;CstC-/-. Aktin je služil kot kontrola količine nanesenega proteina.

4.1.3 Dokaz odsotnosti cistatina C in stefina B v tumorjih miši z izbitima genoma za stefin B in cistatin C

V primarnih tumorjih PyMT;StfB-/-CstC-/- smo preverili prisotnost stefina B in cistatina C (Slika 7). Potrdili smo odsotnost obeh proteinov v tumorskih lizatih.



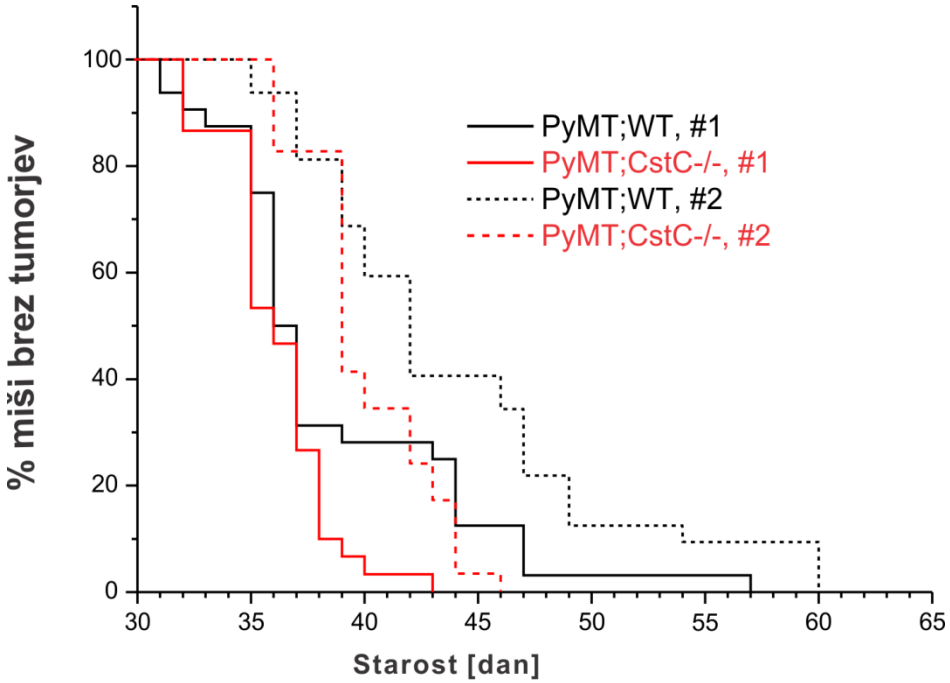
Slika 7: Izražanje cistatina C in stefina B v tumorskih lizatih. Po prenosu Western v tumorjih PyMT;StfB-/-CstC-/- ni prisotnega cistatina C niti stefina B. Tumorski lizat PyMT;WT je služil kot pozitivna kontrola prisotnosti obeh proteinov.

4.1.4 Primerjava rasti tumorjev

4.1.4.1 Cistatin C in stefin B vplivata na hitrost razvoja tumorjev

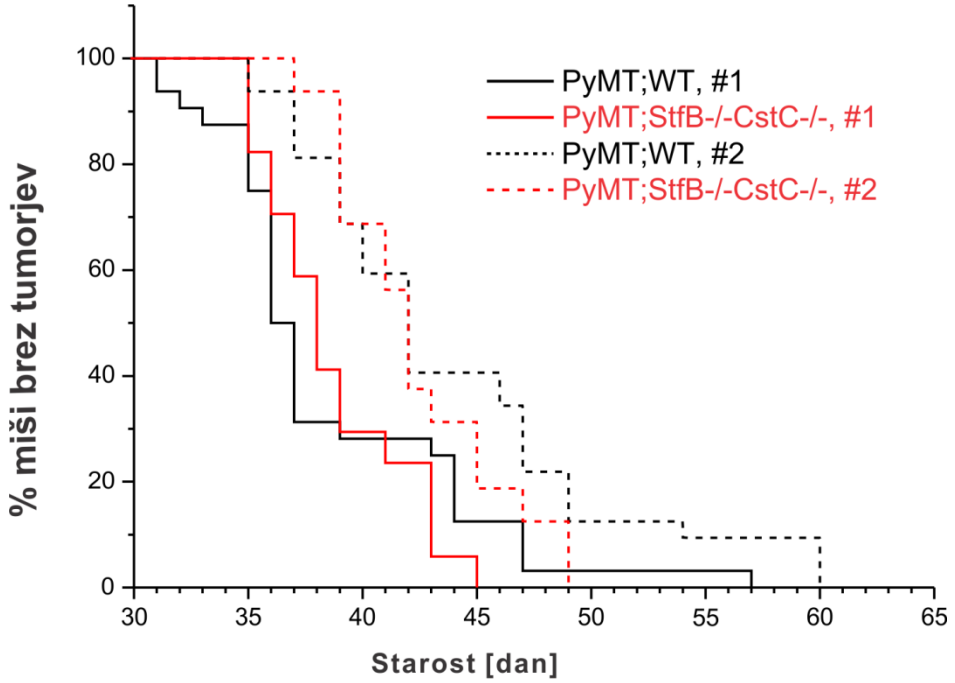
Da bi ugotovili vpliv cistatina C in stefina B na razvoj raka pri PyMT mišjem modelu, smo primerjali rast tumorjev pri genotipih PyMT;WT, PyMT;CstC-/- ter PyMT;StfB-/-CstC-/-. Hitrost razvoja tumorjev smo spremljali pri samicah pri vseh 10 mlečnih žlezah od starosti 28 dni dalje. Miši smo palpirali 3x tedensko. Pričetek rasti tumorjev smo določili, ko smo

detektirali prvi posamični tumor pri živali. Tumorji se pri PyMT;CstC-/- miših razvijejo pri starosti 46 dni, kar je 14 dni prej kot pri PyMT;WT tumorjih. Pri miših z izbitim genom za cistatin C je opažena statistično signifikantna razlika v začetku rasti tumorjev (Slika 8).



Slika 8: Kaplan-Meierjev graf za miši divjega tipa in za miši brez cistatina C. Prvi (#1) in drugi (#2) zaznani tumorji pri skupini miši PyMT;CstC-/- (n=30) so nastali po 14 dni bolj zgodaj v primerjavi s kontrolno skupino PyMT;WT (n=32). #1;$P<0.05$ in #2;$P<0.01$. Statistična analiza je bila narejena z uporabo testa log-rank.

Tudi pri transgenih miših z izbitima genoma za stefin B in cistatin C se tumorji razvijejo hitreje v primerjavi s PyMT;WT tumorji. Vsi tumorji se razvijejo pri starosti 49 dni, kar je 11 dni bolj zgodaj v primerjavi s PyMT;WT tumorji, vendar 3 dni kasneje kot PyMT;CstC-/- tumorji (Slika 9).

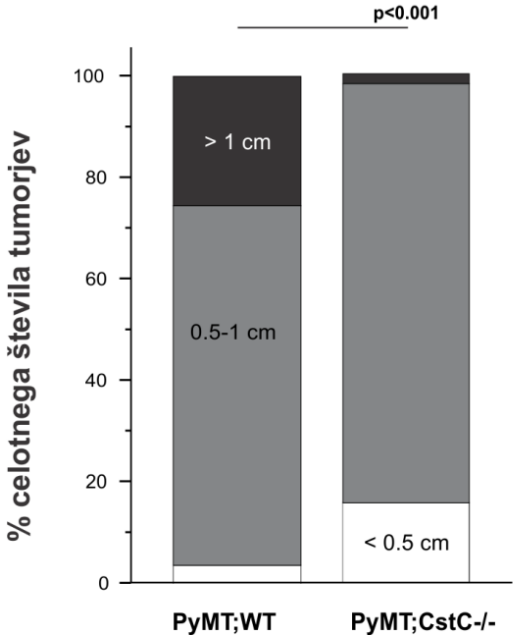


Slika 9: Kaplan-Meierjev graf za miši divjega tipa in za miši brez stefina B in cistatina C.

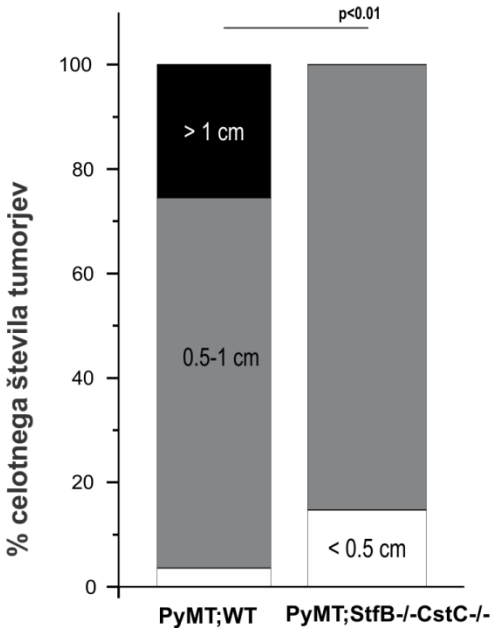
Prvi (#1) in drugi (#2) zaznani tumorji pri skupini miši PyMT;StfB-/-CstC-/- (n=17) so nastali 12 dni in 11 dni bolj zgodaj v primerjavi s kontrolno skupino PyMT;WT (n=32). Statistična analiza je bila narejena z uporabo testa log-rank.

4.1.4.2 Tumorji z izbitima genoma za cistatin C in stefin B so manjši in lažji

Poleg hitrosti nastanka tumorjev smo spremljali tudi rast tumorjev. 30 dni po posamezni detekciji tumorjev smo izmerili njihovo velikost in jih glede na premere razdelili v tri skupine: majhni tumorji: < 0,5 cm, srednje veliki tumorji: 0,5–1 cm, veliki tumorji: > 1 cm. Primerjava rezultatov je pokazala, da so tumorji PyMT;CstC-/- manjši od tumorjev kontrolne skupine. Ugotovili smo, da je bilo skoraj 90 % tumorjev PyMT;CstC-/- srednje velikosti in 15,4 % majhne velikosti, le 5,4 % tumorjev pa je bilo večjih od 1 cm. V kontrolni skupini je več kot četrtina tumorjev presegla velikost 1 cm, tumorjev, manjših od 0,5 %, je bilo le 3,6 %, tumorjev srednje velikosti pa je bilo 70,9 % (Slika 10). Iz teh podatkov lahko sklepamo, da ima cistatin C vlogo pri rasti tumorjev.
Podobne rezultate smo dobili tudi pri tumorjih z izbitima genoma za stefin B in cistatin C: nobeden od tumorjev PyMT;StfB-/-CstC-/- ni dosegel velikosti 1 cm, temveč je bilo 85,3 % tumorjev srednje velikosti, ostali (14,7 %) pa so bili manjši od 0,5 cm (Slika 11).

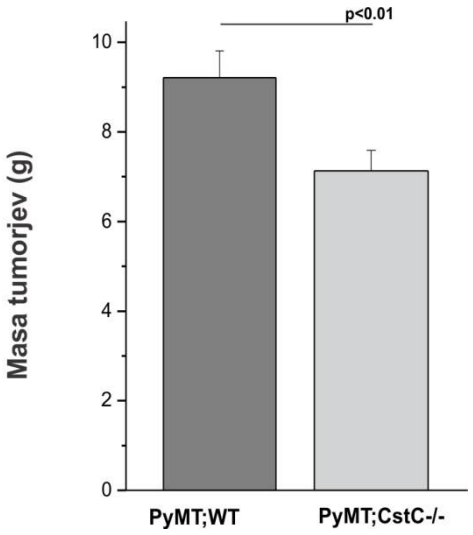


Slika 10: Velikost tumorjev PyMT;WT in PyMT;CstC-/-. 30 dni po posamični detekciji smo tumorje izmerili in glede na velikost razdelili v 3 skupine. Število analiziranih tumorjev je bilo za cistatin C: PyMT;WT (n=19) in PyMT;CstC-/- (n=26). Statistično analizo smo naredili z uporabo Chi-square testa. P<0.001.

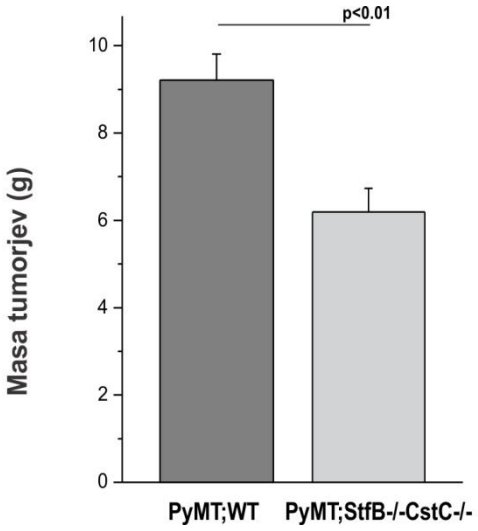


Slika 11: Velikost tumorjev PyMT;WT in PyMT;StfB-/-CstC-/-. 30 dni po posamični detekciji smo tumorje izmerili in glede na velikost razdelili v 3 skupine. Število PyMT;WT analiziranih tumorjev je bilo 19, število PyMT;StfB-/-CstC-/- pa 18. Statistično analizo smo naredili z uporabo Chi-square testa. P<0,01.

Podatke o velikosti tumorjev smo potrdili s težo tumorjev, kjer smo odstranili vseh 10 tumorjev in jih stehtali. Ugotovili smo, da imajo tumorji PyMT;CstC-/- za skoraj četrtino nižjo maso (7,1 g) kot tumorji kontrolne skupine (9,2 g) (Slika 12).
Tumorji z izbitima genoma za stefin B in cistatin C so bili še manjši: njihova masa je bila 5,8 g, kar je 36,9 % manj v primerjavi z maso PyMT;WT tumorjev in za 18,3 % manj v primerjavi z maso PyMT;CstC-/- tumorji (Slika 13).



Slika 12: Povprečna masa tumorjev PyMT;WT in PyMT;CstC-/-. Skupna masa vseh 10 tumorjev pri PyMT;WT (n=23) in PyMT;CstC-/- (n=26) miših pri starosti 14 tednov. Statistično analizo smo naredili z uporabo t-testa. $P < 0,01$.

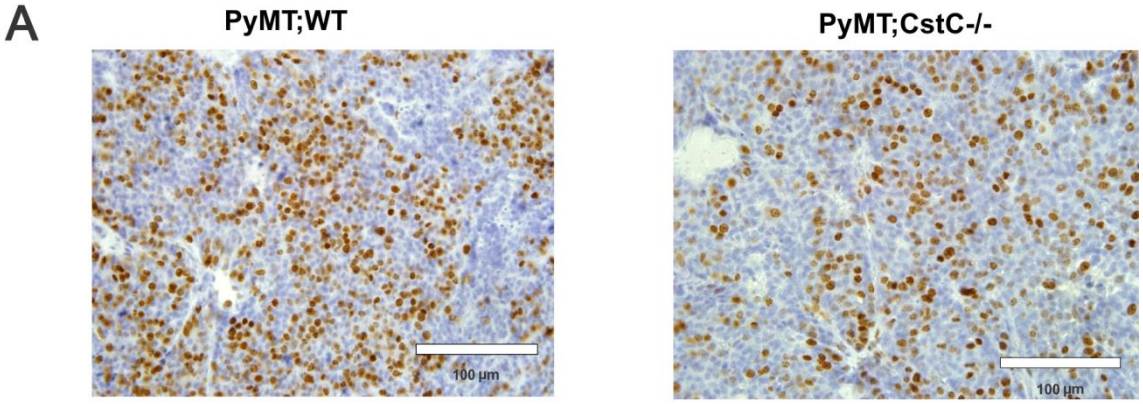


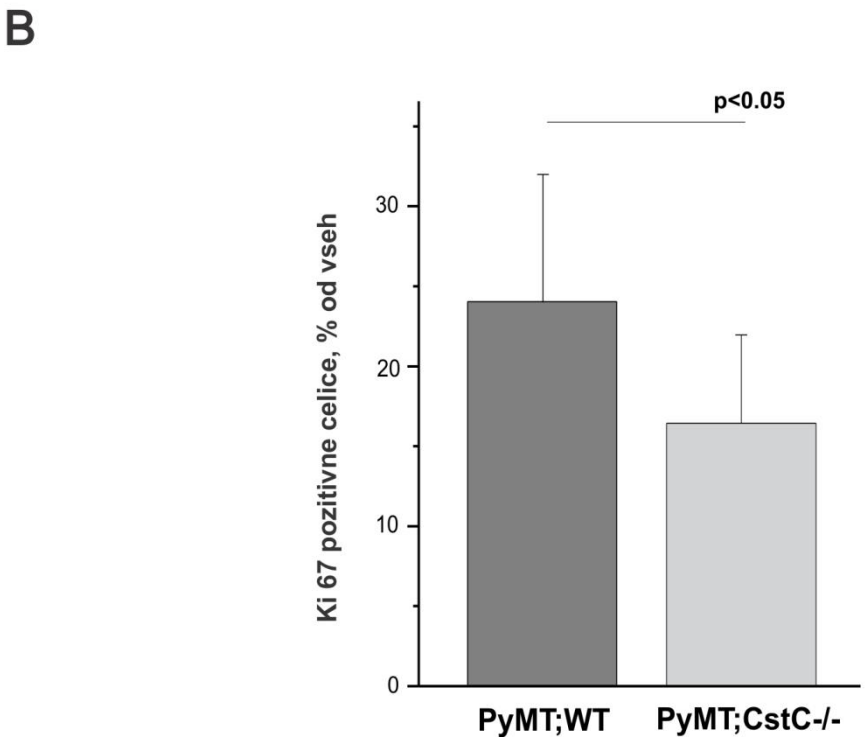
Slika 13: Povprečna masa tumorjev PyMT;WT in PyMT;StfB-/-CstC-/-. Skupna masa vseh 10 tumorjev pri PyMT;WT (n=23) in PyMT;StfB-/-CstC-/- (n=12) miših pri starosti 14 tednov. Statistično analizo smo naredili z uporabo t testa. $P<0,01$.

4.1.5 Vpliv cistatina C na proliferacijo, celično smrt in angiogenezo v PyMT tumorjih

4.1.5.1 V tumorjih brez cistatina C se celice manj delijo

Celično proliferacijo v tumorjih smo kvantificirali s pomočjo imunohistokemijskega barvanja za kazalec Ki67 (Slika 14). Analiza je pokazala, da je pri PyMT;CstC-/- tumorjih delež delečih se celic manjši v primerjavi s PyMT;WT tumorji. Medtem ko je pri PyMT;WT tumorjih delež delečih se celic 24 %, je pri PyMT;CstC-/- tumorjih ta delež 16,4 %, kar je 7,6 % manj. Rezultati kažejo na to, da bi cistatin C lahko vplival na proliferacijo tumorskih celic v PyMT tumorjih (Slika 14).

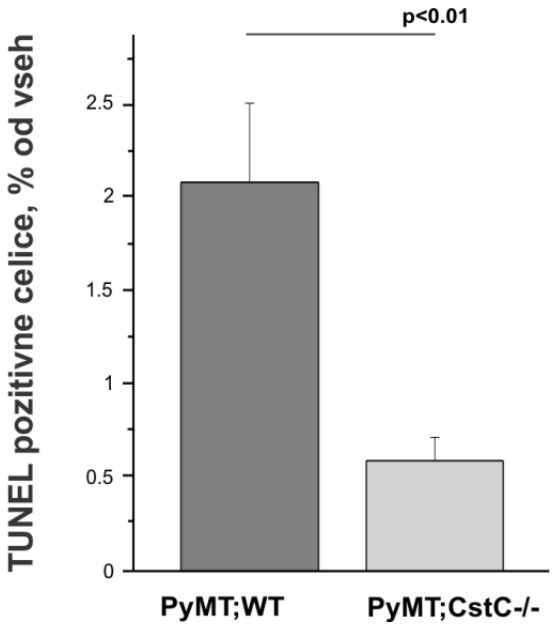




Slika 14: Vpliv cistatina C na proliferacijo. Imunohistokemijska detekcija označevalca Ki67 pri 14 tednov starih PyMT;WT in PyMT;CstC-/- miših. (A) Reprezentativni sliki za posamezni genotip s 40 x povečavo, skala na sliki predstavlja 100 μm. (B) Graf za kvantifikacijo Ki67 pozitivnih celic, predstavljen kot odstotek pozitivnih celic glede na celotno število celic pri PyMT;WT (n=11) in PyMT;CstC-/- (n=12), predstavljena kot srednja vrednost s standardno napako. Ki67 označevalec je bil izračunan na podlagi 20 naključnih slik s pomočjo računalniškega programa ImageJ. Statistično analizo smo naredili z uporabo t-testa. $P < 0,05$.

4.1.5.2 Cistatin C vpliva na umiranje tumorskih celic

Razlog za manjše tumorje bi lahko bila tudi povišana celična smrt v tumorjih. S tem namenom smo s pomočjo označevanja koncev fragmentirane DNA zaradi celične smrti z dUTP (metoda TUNEL) določili delež celične smrti. Opazili smo, da je pri PyMT;CstC-/- tumorjih delež fragmentirane DNA 0,6 %, medtem ko je pri PyMT;WT tumorjih delež fragmentirane DNA 2,1 % (Slika 15). Razlika je bila statistično signifikantna.

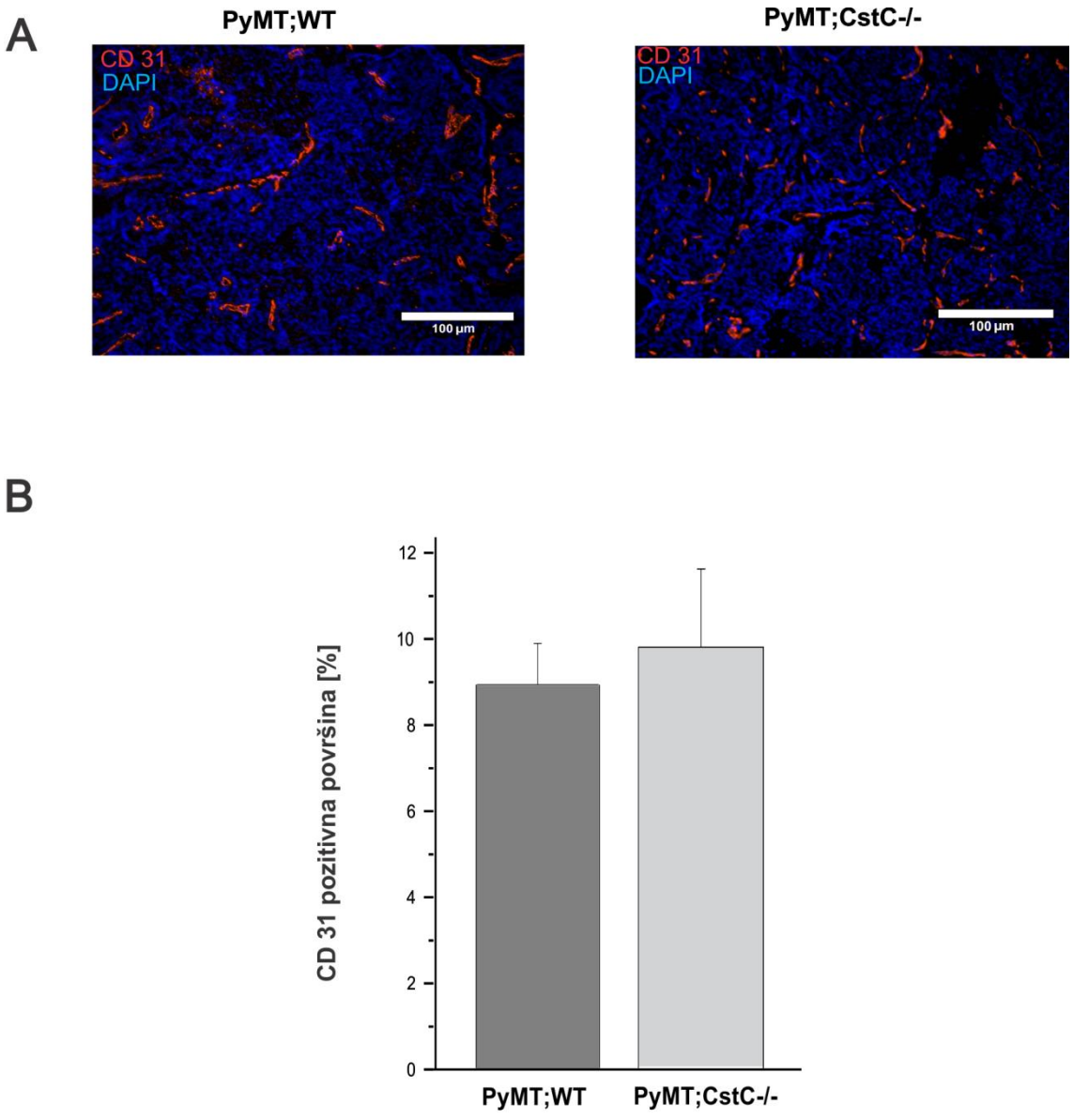


Slika 15: Vpliv cistatina C na celično smrt v tumorjih. Imunohistokemijska detekcija mrtvih celic v tumorjih s pomočjo barvanja TUNEL pri PyMT;WT in PyMT;CstC-/- miših pri 14 tednih. Delež mrtvih celic je bil določen s pomočjo slik celotne površine tumorja in računalniško vodenega programa ImageJ pri PyMT;WT (n=9) in PyMT;CstC-/- (n=9) miših. Statistično analizo smo naredili z uporabo t-testa. $P<0,01$.

4.1.5.3 Odstranitev cistatina C ne vpliva na ožiljenje tumorjev

Razlog za manjše tumorje pri PyMT;CstC-/- miših je lahko tudi slabše ožiljenje tumorjev. V tumorjih se namreč razvije lastno ožilje, ki tumorske celice preskrbuje s hranilnimi snovmi in kisikom. Vpliv cistatina C na ožiljenje tumorjev smo preverili s pomočjo

imunofluorescenčnega barvanja tumorskih rezin na endotelijski kazalec CD31 (Slika 16). Mikroskopska analiza slik je pokazala, da so oblika, velikost in število žil v tumorjih enake, kar nakazuje, da odsotnost cistatina C ne vpliva na formacijo ožiljenja v tumorjih.

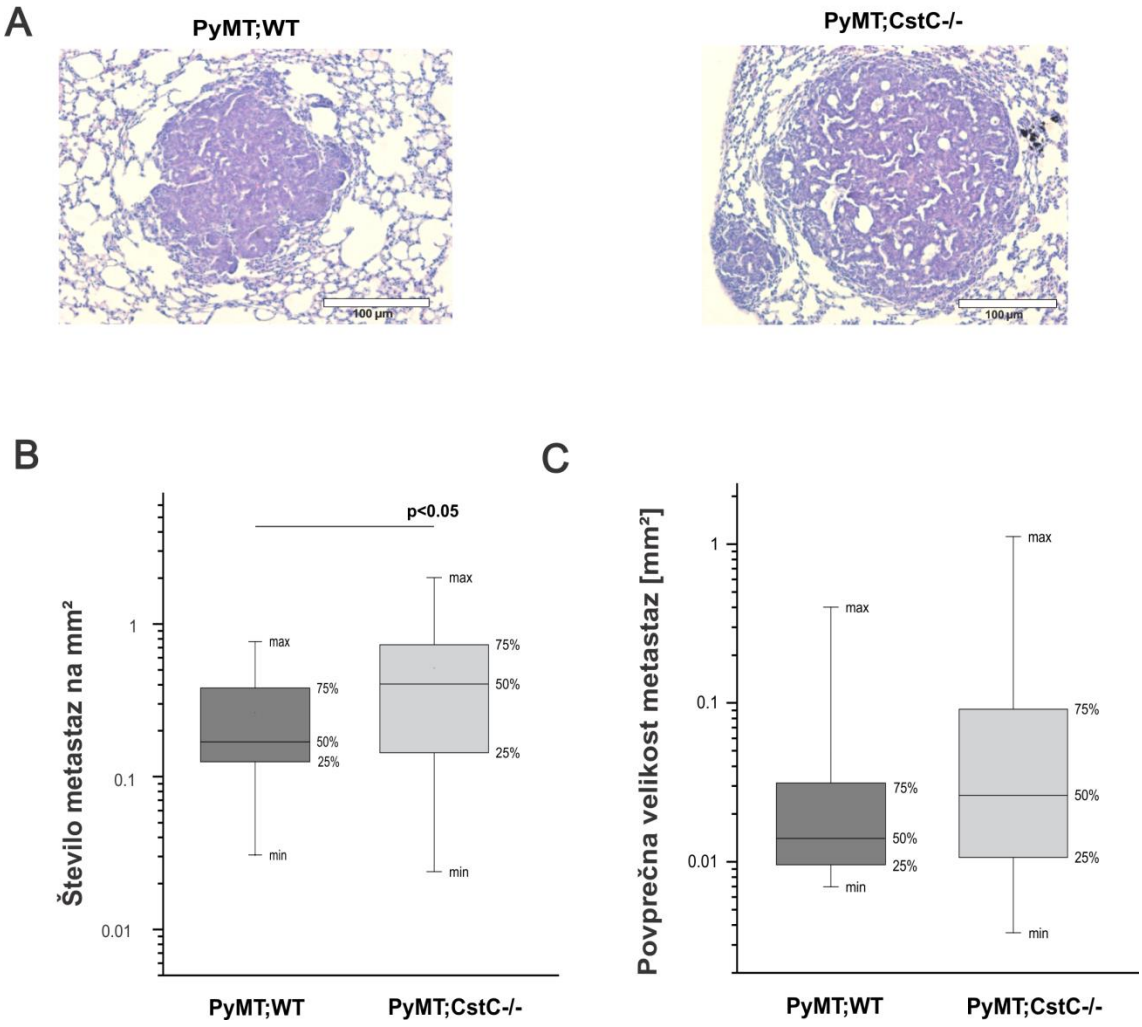


Slika 16: Vpliv cistatina C na angiogenezo. (A) Reprezentativni sliki imunofluorescentnega barvanja tumorjev na endotelijski kazalec CD31 (rdeče obarvanje) pri PyMT;WT in PyMT;CstC-/- tumorjih. (B) Kvantifikacija CD31 pozitivnih regij kot delež celotne tumorske površine pri PyMT;WT (n=6) in PyMT;CstC-/- (n=6) tumorjih. Statistično analizo smo naredili z uporabo t-testa.

4.1.6 Nastanek pljučnih metastaz

Za transgeni mišji model raka mlečne žleze je značilno, da tumorske celice pri starosti 14 tednov skoraj 100 % metastazirajo v pljuča (Guy, et al., 1992). Z namenom, da bi ugotovili morebitne razlike v velikosti in številu pljučnih metastaz pri obeh genotipih, smo naredili

histomorfometrijo s hematoksilinom in eozinom pobarvanih rezin pljučnih kril na treh različnih globinah pljučnega tkiva z namenom določitve števila in velikosti metastaz na mm^2 pljučnega krila pri 14 tednov starih miših (Slika 17). Metastaze so bile prisotne pri vseh analiziranih miših, vendar je bilo pri skupini PyMT;WT število pljučnih metastaz v primerjavi s PyMT;CstC-/- skupino na mm^2 pljučnega krila statistično signifikantno manjše. Pljučne metastaze so pri PyMT;CstC-/- skupini večje kot pri PyMT;WT skupini, vendar razlika ni statistično signifikantna. Iz teh rezultatov lahko zaključimo, da ima cistatin C pri metastaziranju zaščitno vlogo, še najbolj možno zaradi inhibicije cisteinskih proteaz.



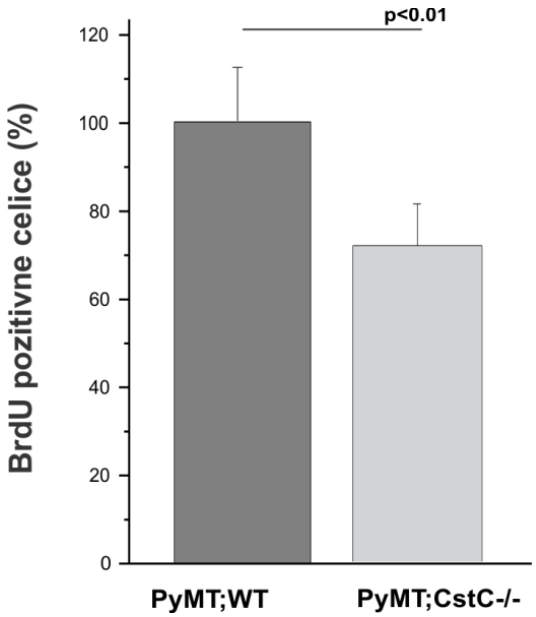
Slika 17: Vpliv cistatina C na nastanek pljučnih metastaz. (A) Reprezentativni sliki pljučnih metastaz pri obeh genotipih. 10x povečava, skala na sliki predstavlja 100 µm. Histomorfometrična določitev (B) števila metastaz na površino pljuč in (C) povprečna velikost pljučnih metastaz pri PyMT;WT (n=18) in PyMT;CstC-/- (n=22) miših. Statistična analiza je bila narejena z uporabo t-testa.

4.2 Analiza primarnih PyMT tumorskih celic WT in CstC-/-

Da bi ugotovili vlogo cistatina C pri proliferaciji in katepsinski aktivnosti, smo analizirali primarne PyMT tumorske celice, pridobljene iz 14 tednov starih PyMT;WT in PyMT;CstC-/- tumorjev.

4.2.1 Vpliv cistatina C na proliferacijo tumorskih celic

Poleg imunohistokemijske določitve proliferacije v tumorjih smo tudi *in vitro* analizirali proliferacijo PyMT tumorskih celic s pomočjo BrdU kompleta reagentov, kjer se BrdU vstavi v genom celic, ki se delijo, nato pa se vstavljene BrdU meri s fluorimetričnim čitalcem plošč Tecan Infinite M1000 Pro. Rezultati kažejo, da je pri PyMT;CstC-/- tumorskih celicah proliferacija nižja v primerjavi s PyMT;WT tumorskimi celicami. Pri PyMT;CstC-/- celicah je delež BrdU pozitivnih celic za 29 % nižji kot pri PyMT;WT celicah (Slika 18). Rezultat je v skladu z rezultati imunohistokemijskega barvanja na Ki67 kazalec v tumorskem tkivu.

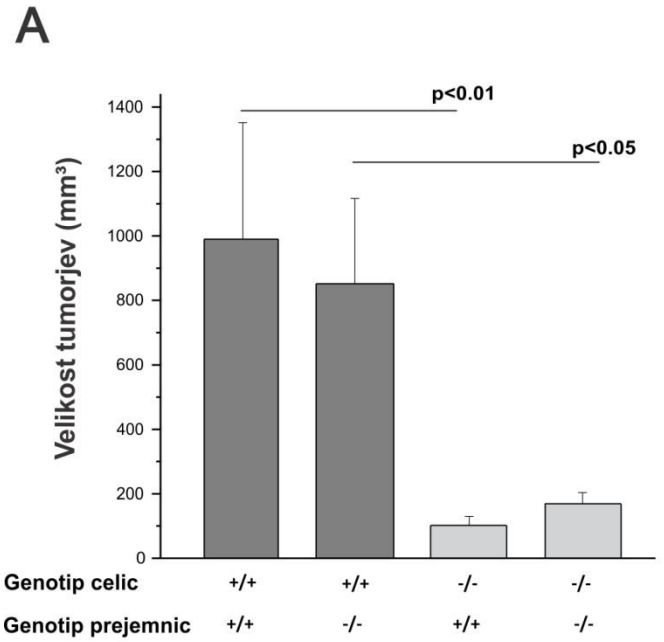


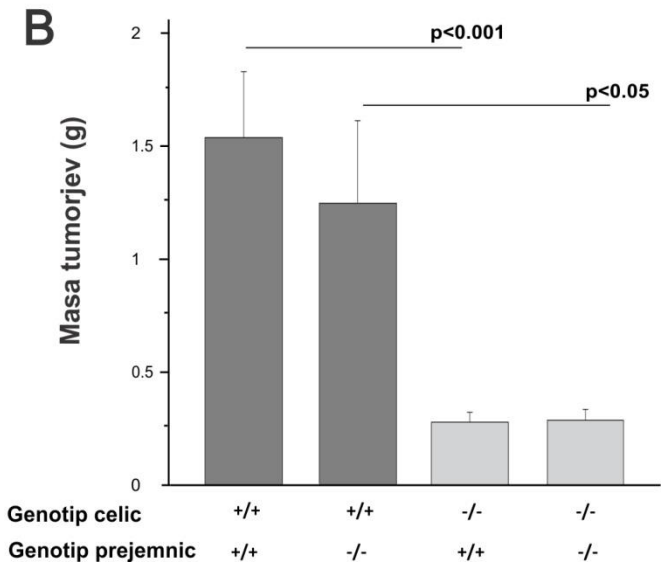
Slika 18: Znižana proliferacija primarnih PyMT tumorskih celic z izbitim genom za cistatin C. Celična proliferacija PyMT;WT (n=5) in PyMT;CstC-/- (n=5) primarnih tumorskih celic, določena z BrdU. Statistično analizo smo naredili z uporabo t-testa.

4.2.2 Tumorske celice brez cistatina C tvorijo manjše tumorje

Z namenom preučevanja rasti tumorskih celic *in vivo* in s tem vpliva cistatina C na rast in razvoj tumorjev smo pripravili ortotopični model s transplantacijo tumorskih celic obeh genotipov v levo trebušno mlečno žlezo samic obeh genotipov. Dva tedna po implantaciji smo pričeli z merjenjem premerov tumorjev in nato nadaljevali z merjenjem trikrat tedensko. 35 dni po transplantaciji smo miši usmrtili, tumorje pa izrezali, izmerili njihovo

velikost in jih stehtali. Ugotovili smo, da so bili tumorji, ki so zrasli iz PyMT;CstC-/- tumorskih celic, veliki povprečno 135 mm^3 (102 mm^3 pri FVB;WT prejemnicah in 169 mm^3 pri FVB;CstC-/- prejemnicah). Tumorji, ki so zrasli iz PyMT;WT celic, pa so bili veliki povprečno 921 mm^3 (990 mm^3 pri FVB;WT prejemnicah in 852 mm^3 pri FVB;CstC-/- prejemnicah) (Slika 19 A).
Tumorje smo tudi stehtali. Rezultati so bili primerljivi z meritvami velikosti tumorjev. Povprečna masa tumorjev, ki so izvirali iz PyMT;WT celic in FVB;WT prejemnic, je bila 1,54 g (1,24 g pri FVB;CstC-/- prejemnicah). Povprečna masa tumorjev, ki so izvirali iz PyMT;CstC-/- celic in FVB;WT prejemnic, pa je bila 0,28 g (0,29 g pri FVB;CstC-/- prejemnicah) (Slika 19 B).
Ugotovili smo, da iz tumorskih celic brez cistatina C zrastejo veliko manjši tumorji, neodvisno od genotipa miši prejemnic.

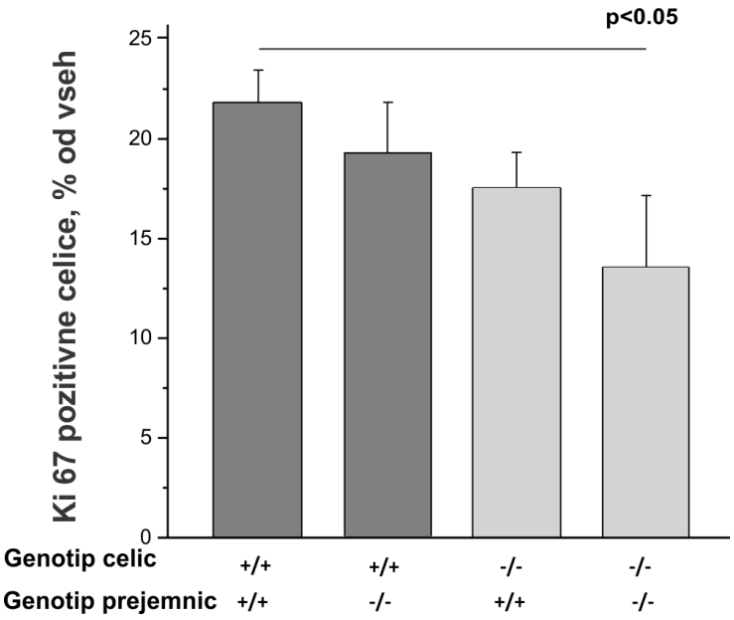




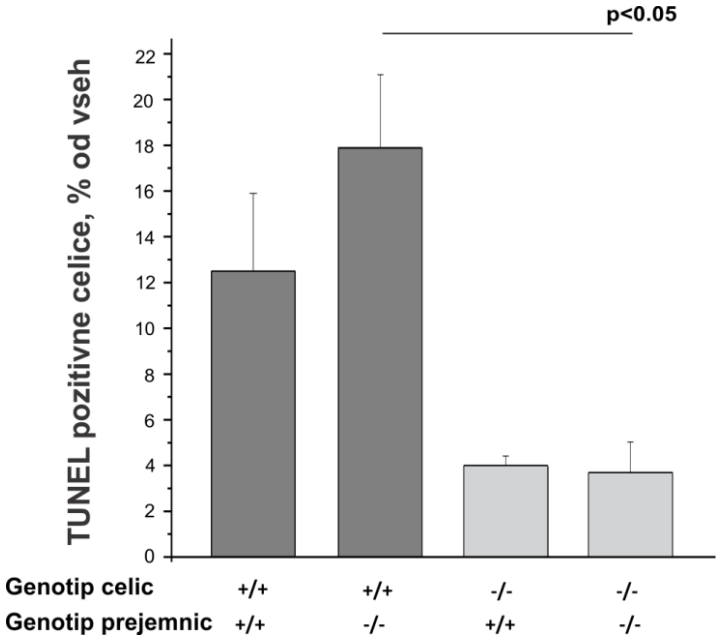
Slika 19: Ortotopična transplantacija z intramamarno aplikacijo PyMT tumorskih celic obeh genotipov v kongene miši prejemnice obeh genotipov. (A) Velikost transplantiranih tumorjev iz PyMT;WT in PyMT;CstC-/- tumorskih celic, apliciranih v FVB;WT in FVB;CstC-/- miši (velikost vsake skupine: ≥ 7). Statistično analizo smo naredili z uporabo t-testa. (B) Masa transplantiranih tumorjev iz PyMT;WT in PyMT;CstC-/- celic, apliciranih v FVB;WT in FVB;CstC-/- miši. Statistično analizo smo naredili z uporabo t-testa.

4.2.2.1 Transplantirani model

V transplantiranih tumorjih smo preverili tudi delež kazalca proliferacije Ki67 ter delež mrtvih celic z metodo TUNEL. Rezultati nakazujejo podoben trend razvoja tumorjev kot smo zaznali pri analizi primarnih tumorjev iz transgenih miši. Transplantirani tumorji, katerih izvor so PyMT;CstC-/- celice, se manj delijo in tudi manj umirajo kot transplantirani tumorji iz PyMT;WT celic (Slika 20 in Slika 21).



Slika 20: Delež Ki67 pozitivnih celic v transplantiranih tumorjih. Kvantifikacija Ki67 pozitivnih celic kot odstotek glede na celotno število celic pri transplantiranih tumorjih, predstavljena kot srednja vrednost s standardno napako. Ki67 označevalec je bil izračunan na podlagi 20 naključnih slik s pomočjo računalniškega programa ImageJ. Za vsako skupino smo kvantificirali najmanj 4 tumorje. Statistično analizo smo naredili z uporabo t-testa.

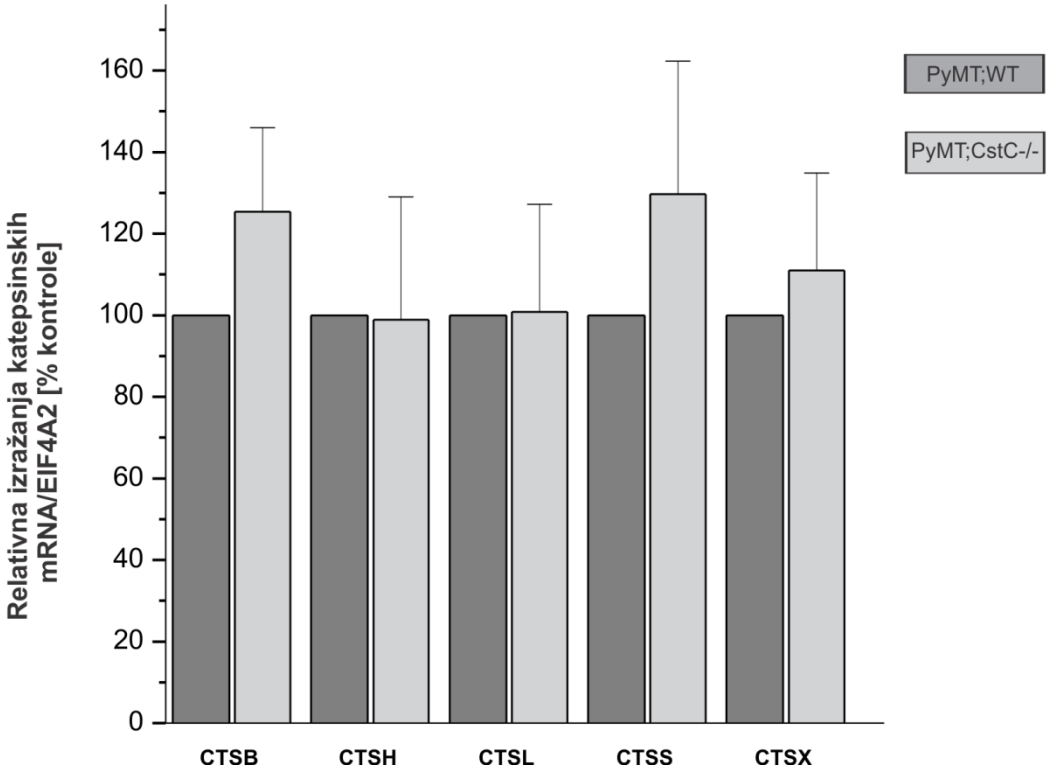


Slika 21: Delež mrtvih celic v transplantiranih tumorjih. Imunohistokemijska detekcija mrtvih celic s pomočjo barvanja TUNEL pri transplantiranih tumorjih. Delež mrtvih celic je bil določen s pomočjo slik celotne površine tumorja in računalniško vodenega programa ImageJ pri treh tumorjih za vsako skupino. Statistično analizo smo naredili z uporabo t-testa.

4.3 Izražanje in aktivnost katepsinov

4.3.1 Relativno izražanje katepsinov B, H, L, S in X se med genotipoma ne razlikuje

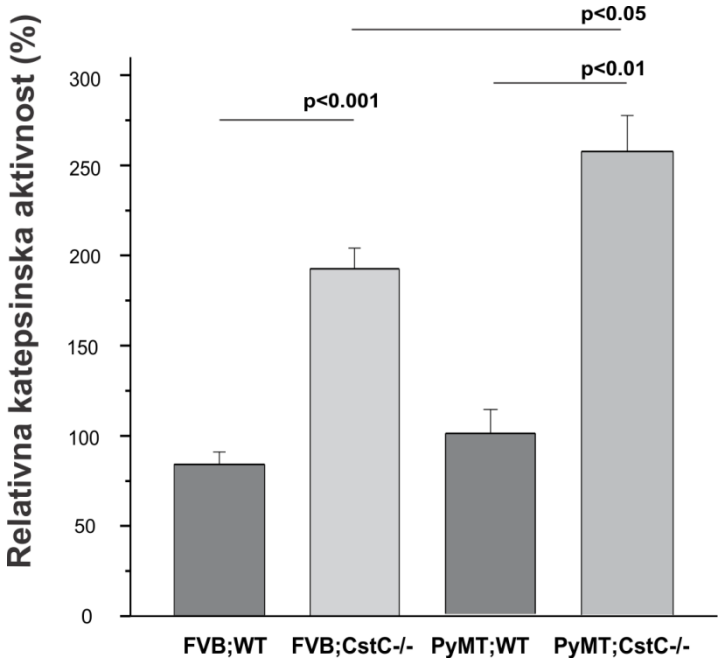
Zaradi pomembne inhibitorne vloge cistatina C smo preverili nivo izražanja genov za cisteinske katepsine B, H, L, S in X. Z verižno reakcijo s polimerazo v realnem času smo določili izražanje informacijske RNA (mRNA) v tumorjih obeh genotipov, vendar razlik med genotipoma nismo opazili (Slika 22).



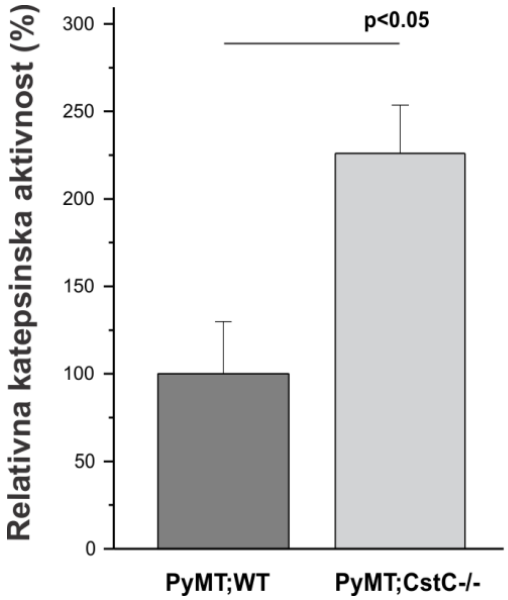
Slika 22: Izražanje cisteinskih katepsinov v tumorjih. Izražanje cisteinskih katepsinov v PyMT;WT (n= 4) in PyMT;CstC-/- (n= 4) tumorjih. Primerjava mRNA katepsina B (CTSB), katepsina H (CTSH), katepsina L (CTSL), katepsina S (CTSS) in katepsina X/Z (CTSX) med genotipoma z RTQ-PCR. Podatki so predstavljeni kot odstotek izražanja od kontrole (100 %).

4.3.2 Katepsini so v tumorjih brez cistatina C bolj aktivni

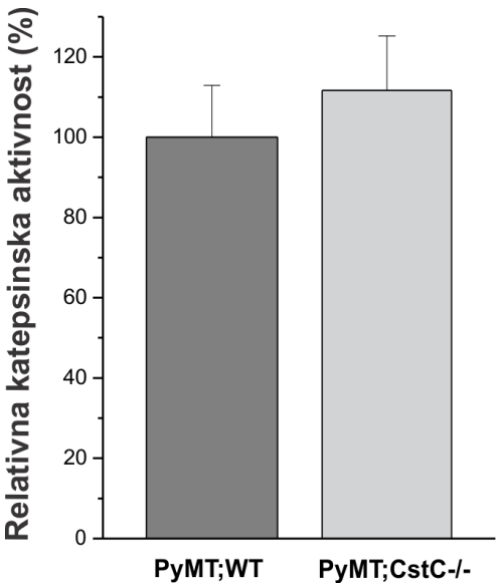
Z namenom preučitve inhibitorne vloge cistatina C smo v tkivnih homogenatih mlečnih žlez in tumorjev merili katepsinsko aktivnost. Izrazito povišanje hidrolize katepsinskega substrata je bilo prisotno tako v mlečni žlezi kot v tumorju z izbitim genom za CstC (Slika 23). S tem smo pokazali povišano katepsinsko aktivnost v tumorskem tkivu. Zaradi prisotnosti cistatina C v zunajceličnem prostoru smo izmerili tudi aktivnost katepsinov v mediju tumorskih celic (Slika 24). V skladu s pričakovanji se je izkazalo, da odsotnost cistatina C povzroči povišano katepsinsko aktivnost v zunajceličnem prostoru. Poleg zunajcelične aktivnosti katepsinov smo preverili tudi njihovo aktivnost v lizatih tumorskih celic obeh genotipov (Slika 25). Ugotovili smo, da se katepsinska aktivnost ne razlikuje pri obeh genotipih. S tem smo dodatno potrdili izvencelično inhibitorno aktivnost cistatina C.



Slika 23: Relativna katepsinska aktivnost v mlečnih žlezah in tumorjih. Relativna katepsinska aktivnost v tkivnih homogenatih mlečnih žlez (FVB;WT n=4, FVB;CstC-/- n=4) in tumorjev (PyMT;WT n=4, PyMT;CstC-/- n=4). Aktivnost je bila merjena kot hidroliza substrata Z-Phe-Arg-AMC. Statistična analiza je bila narejena z uporabo t-testa.



Slika 24: Relativna katepsinska aktivnost v mediju primarnih tumorskih celic. Relativna katepsinska aktivnost je bila merjena kot hidroliza substrata Z-Phe-Arg-AMC v mediju primarnih tumorskih celic genotipa PyMT;WT (n=3) in PyMT;CstC-/- (n=3). Statistična analiza je bila narejena z uporabo t-testa.

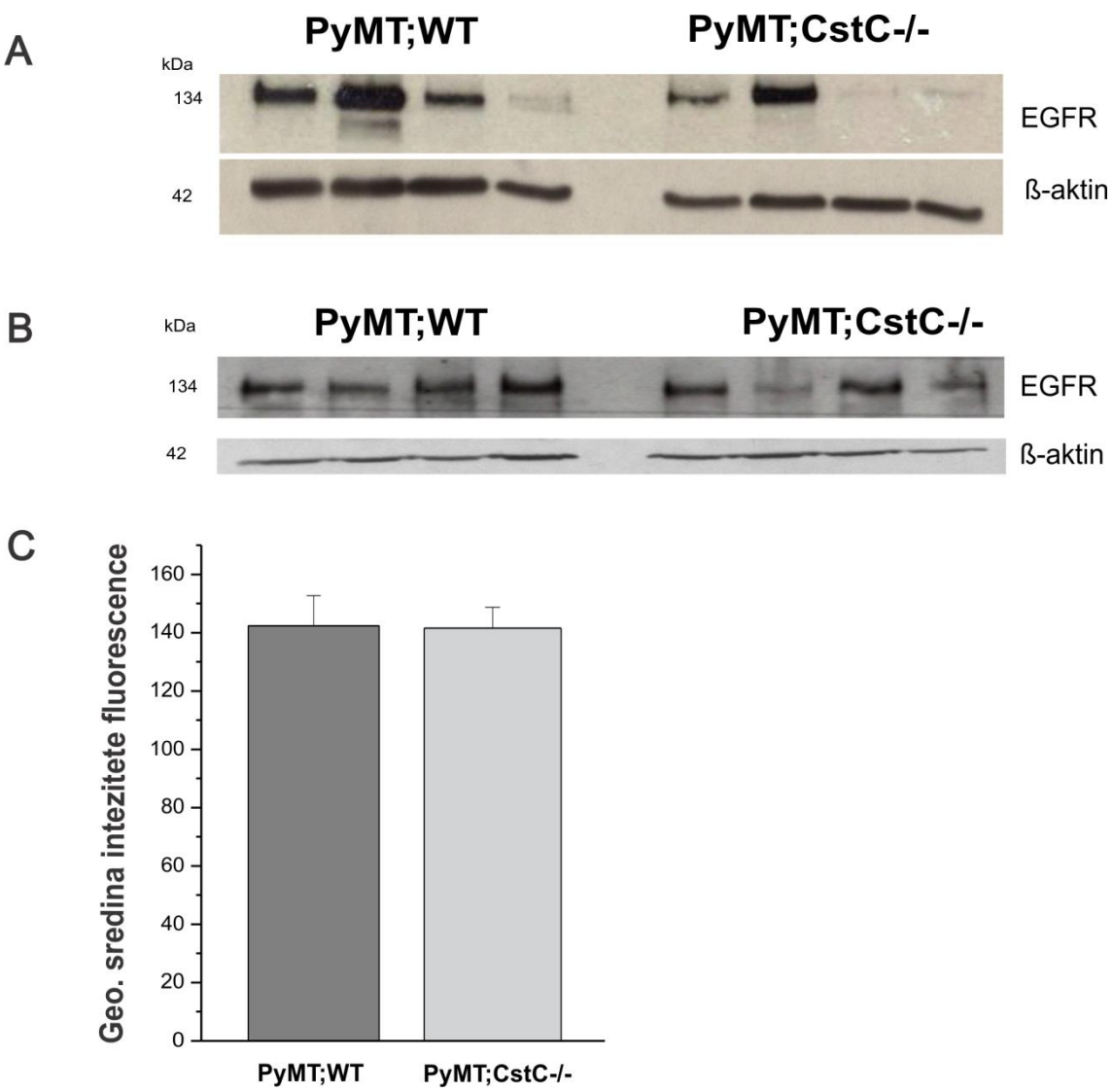


Slika 25: Relativna katepsinska aktivnost v homogenatih primarnih tumorskih celic.

Relativna katepsinska aktivnost je bila merjena kot hidroliza substrata Z-Phe-Arg-AMC v homogenatih primarnih tumorskih celic genotipa PyMT;WT (n=3) in PyMT;CstC-/- (n=3). Statistična analiza je bila narejena z uporabo t-testa.

4.4 Izražanje EGFR

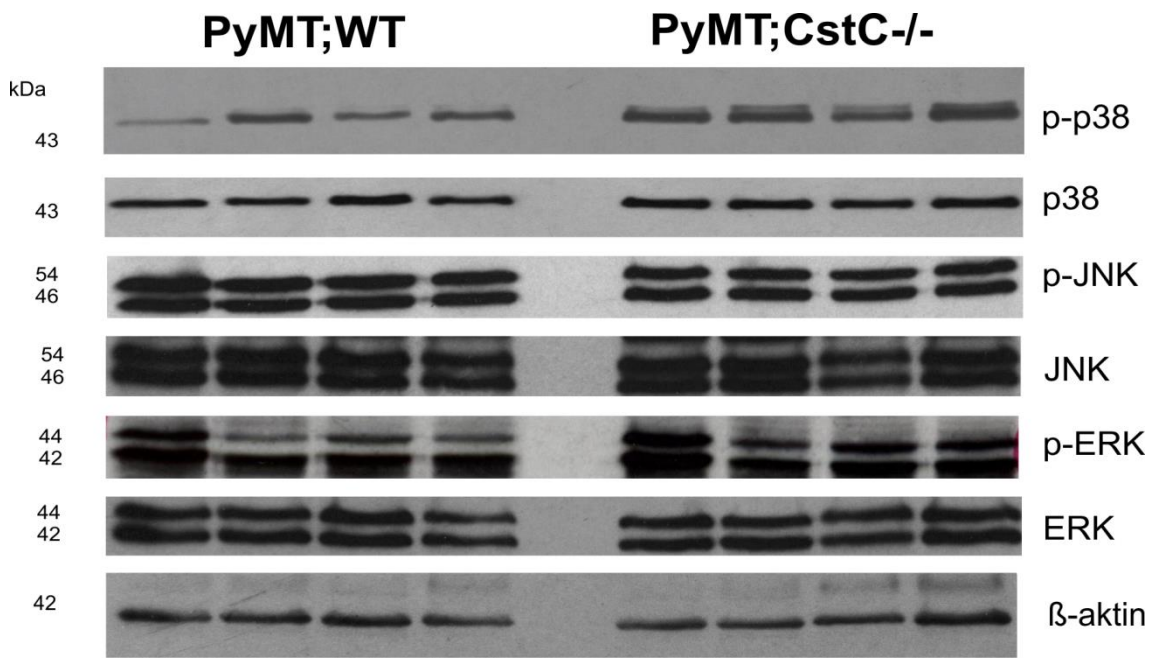
Nedavno so katepsine opisali kot zmožne odcepitve zunajceličnih domen transmembranskih receptorjev, med drugim tudi receptor za epidermalni rastni faktor, imenovan EGFR (Sobotic et al., 2015). Zato nas je zanimalo, če se povišana aktivnost katepsinov zaradi odsotnosti cistatina C odrazi v povišani cepitvi zunajcelične domene EGFR. Preverili smo razlike na tumorskih homogenatih in na izoliranih membranah iz primarnih tumorskih celic, vendar nismo zaznali razlik med genotipoma. Tudi z metodo pretočne citometrije nismo potrdili razlik pri vezavi protiteles na membrano primarnih tumorskih celic pri obeh genotipih (Slika 26).



Slika 26: Primerjava izražanja EGFR med genotipoma. EGFR smo detektirali v (A) homogenatih PyMT;WT (n=4) in PyMT;CstC-/- (n=4) primarnih tumorjev ter v (B) izoliranih plazemskih membranah iz primarnih tumorskih celic PyMT;WT (n=4) in PyMT;CstC-/- (n=4). (C) Prisotnost EGFR smo z metodo pretočne citometrije merili v primarnih tumorskih celicah PyMT;WT (n=3) in PyMT;CstC-/- (n=3). Rezultati so predstavljeni kot povprečna geometrična sredina intenzitete fluorescence treh neodvisnih poskusov.

4.5 Izražanje članov MAPK družine

Z namenom odkritja vzroka za manjšo proliferacijo tumorskih celic v tumorjih in tumorskih celicah brez cistatina C smo preučili izražanje članov družine z mitogenom aktiviranih proteinskih kinaz, in sicer ERK, JNK in p38 (Slika 27). Ugotovili smo povišano izražanje aktivne oblike p38 in ERK-1.



Slika 27: Prisotnost celokupnih in fosforiliranih proteinov iz družine MAPK. Totalni p38, fosforilirani p38, totalni JNK, fosforilirani JNK, totalni ERK in fosforilirani ERK smo detektirali v PyMT;WT (n=4) in PyMT;CstC-/- (n=4) tumorskih lizatih s prenosom Western. Aktin je služil kot kontrola količine nanesenega proteina.

4.6 Analiza medija primarnih tumorskih celic z masno spektrometrijo

Poleg detekcije proteinov s protitelesi smo z metodo masne spektrometrije in z uporabo spektralnega štetja identificirali proteine v supernatantu primarnih tumorskih celic. Zanimalo nas je morebitno povišano ali znižano izražanje proteinov v medijih PyMT;CstC-/- celic v primerjavi s PyMT celicami divjega tipa. Od 2207 identificiranih proteinov, je imelo zgolj 21 proteinov nekoliko zmanjšano izražanje v PyMT;CstC-/- celicah. Eno tretjino teh proteinov predstavljajo izoforme 14-3-3 proteinov.

Tabela 2: Seznam detektiranih proteinov v mediju iz primarnih PyMT tumorskih celic. Povprečno masno število posameznega proteina je povprečje 4 različnih vzorcev za posamezen genotip.

Ime proteina	Uniprot	Št. peptidov	Povprečno masno število		Razmerje WT/KO
			PyMT; WT	PyMT; CstC-/-	
Cistatin C	P21460	9	46	0	46
14-3-3 protein sigma	O70456	21	46	20	2,31
Proteasom podenota alfa tip -7	Q9Z2U0	14	24	12	2
Proteasom podenota alfa tip -5	Q9Z2U1	11	22	11	1,98
Plazma proteaza C1 inhibitor	P97290	17	14	7	1,93
14-3-3 protein eta	P68510	18	19	10	1,9
14-3-3 protein teta	F6YY69	19	27	14	1,89
14-3-3 protein beta/alfa	Q9CQV8	15	17	9	1,83
Citoplazemska tioreduksin reduktaza 1	Q9JMH6	20	18	10	1,78
Stresni protein HSP 90-alfa	P07901	48	59	34	1,72
Plastin-2	Q61233	42	48	28	1,71
Fosfoglicerat mutaza 1	Q9DBJ1	16	41	24	1,71
14-3-3 protein epsilon	P62259	24	123	74	1,67
Inhibitor metaloproteaze 1	P12032	11	53	32	1,65
Protein S100-A6	P14069	7	37	23	1,62
Proteasom podenota alfa tip -3	O70435	15	19	12	1,6
14-3-3 protein gama	P61982	20	38	24	1,57
Protein visoko mobilne skupine B1	P63158	15	31	20	1,56
Heterogeni jedrni ribonukleoprotein A1	Q5EBP8	17	34	22	1,55
14-3-3 protein zeta/delta	P63101	21	64	42	1,52
Prehodna jedrna endoplazemska ATPaza	Q01853	42	38	25	1,52
Profilin-1	P62962	11	71	47	1,5

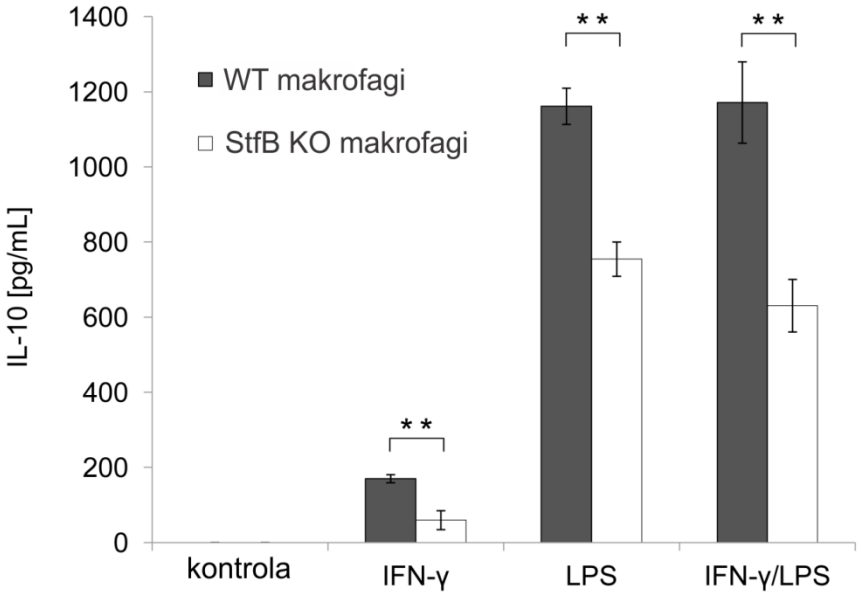
4.6.1 14-3-3 proteini

Tabela 3: Seznam članov družine 14-3-3, detektiranih z masno spektrometrijo. Povprečno masno število posameznega proteina je povprečje 4 različnih vzorcev za posamezen genotip.

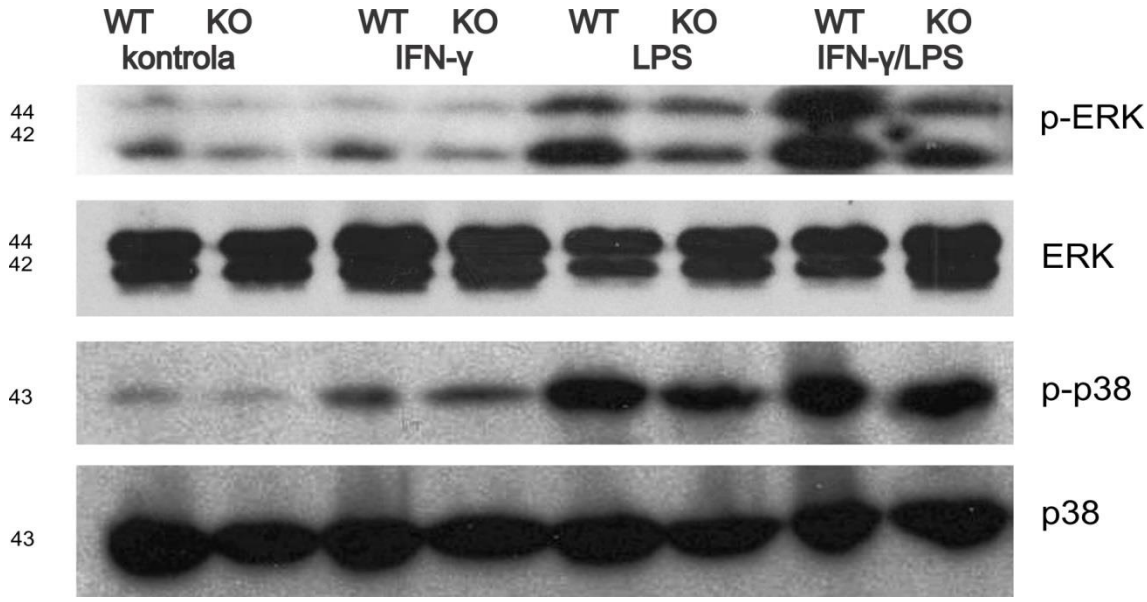
Ime proteina	Uniprot	St. peptidov	Povprečno masno število		Razmerje WT/KO
			PyMT; WT	PyMT; CstC-/-	
14-3-3 protein beta/alfa	Q9CQV8	15	17	9	1,89
14-3-3 protein gama	P61982	20	38	24	1,58
14-3-3 protein zeta/delta	P63101	21	64	42	1,52
14-3-3 protein epsilon	P62259	24	123	74	1,66
14-3-3 protein eta	P68510	18	19	10	1,9
14-3-3 protein teta	P68254	19	27	14	1,93
14-3-3 protein sigma	O70456	21	46	20	2,3

4.7 Vloga stefina B pri vnetju

V drugem delu smo preverjali vlogo stefina B pri naravnem vnetnem odzivu. Primarne mišje makrofage, pridobljene iz kostnega mozga (ang. bone marrow - derived macrophages, BMDM), smo stimulirali z lipopolisaharidom (LPS) ali interferonom gama (IFN-γ). Nato smo s testom ELISA določili koncentracijo protivnetnega citokina interlevkina 10 (IL-10) v gojišču. IL-10 negativno regulira izražanje provnetnih citokinov in ščiti organizem pred prekomernim vnetnim odzivom. Po stimulaciji makrofagov z LPS smo v gojišču izmerili visoke koncentracije IL-10, medtem ko so bile koncentracije IL-10 po stimulaciji z IFN-γ zelo nizke. Ugotovili smo, da je v gojišču stimuliranih makrofagov z izbitim genom za stefin B manj protivnetnega IL-10 (Slika 28).
V celičnih lizatih stimuliranih BMDM pa smo določili fosforilacijo kinaz ERK in p38. V makrofagih je namreč izražanje IL-10 odvisno od aktivacije z mitogenom aktiviranih proteinskih kinaz (MAPK) ERK in p38 (Lucas, Zhang, Prasanna, & Mosser, 2005; Ma et al., 2001). Ob stimulaciji z LPS smo v makrofagih divjega tipa določili izrazito povišan nivo fosforiliranega ERK in v manjši meri p38 v primerjavi z makrofagi z izbitim genom za stefin B (Slika 29).



Slika 28: Tvorba proteina IL-10 v primarnih makrofagih. Primarne makrofage smo 18 ur stimulirali z LPS ali IFN-γ ali pa smo jih preko noči stimulirali z IFN-γ, sprali in nato dodali LPS za 18 ur. S testom ELISA smo določili koncentracije IL-10 v gojišču.



Slika 29: Vpliv stefina B na fosforilacijo kinaz ERK in p38. Primarne makrofage smo stimulirali z IFN-γ ali z LPS 1 uro oziroma predinkubirali z IFN-γ, po eni uri sprali in dodali LPS za nadaljnjo 1 uro. V celičnih lizatih smo z imunodetekcijo določili nivo fosforilacije proteinov. Detekcija nefosforiliranih proteinov je služila za kontrolo nanosa in kontrolo izražanja celokupnega proteina.

Poglavje 5

Razprava

Aktivnost proteaz je v celicah in v organizmih skrbno regulirana, saj lahko nepravilna proteoliza privede do različnih patoloških sprememb, med drugim tudi do razvoja raka (B. Turk, 2006). Da bi lahko bolje razumeli vlogo proteaz in uspešno razvili nove strategije zdravljenja, je nujno poznati vlogo proteaz v zapletenih mehanizmih, ki vodijo do razvoja in napredovanja bolezni. Eden glavnih mehanizmov, ki uravnavajo delovanje proteaz, je inhibicija njihove aktivnosti z endogenimi proteinskimi inhibitorji. Izražanje proteaz in njihovih inhibitorjev pa je v bolezenskih procesih pogosto spremenjeno zaradi zapletenih povezav med tumorskimi celicami in njihovim mikrookoljem (Hanahan & Coussens, 2012).

V tej doktorski disertaciji smo ovrednotili vpliv izgube dveh endogenih inhibitorjev cisteinskih proteaz cistatina C in stefina B na razvoj in napredovanje raka mlečnih žlez pri PyMT mišjem modelu raka. Cistatin C je glavni zunajcelični inhibitor cisteinskih katepsinov, medtem ko stefin B zavira aktivnost proteaz znotraj celic (Vito Turk & Turk, 2008). Oba proteina se izražata v vseh tkivih. Prva preiskovana skupina je sestavljala PyMT transgene miši z izbitim genom za cistatin C (PyMT;CstC-/-), druga skupina pa je bila sestavljena iz PyMT transgenih miši z izbitima genoma tako za stefin B kot tudi za cistatin C, t. i. dvojni »knockout« (PyMT;Stfb-/-CstC-/-). Obe skupini smo primerjali s PyMT transgenimi mišmi divjega seva (PyMT;WT).

Pri analizi izražanja cistatina C v tumorjih in mlečnih žlezah smo ugotovili, da je cistatina C manj v tumorjih kot v zdravih mlečnih žlezah pri starosti 14 tednov (slika 4). Ugotovili smo tudi, da je bil cistatin C v tumorjih prisoten le na robovih tumorjev in v tumorski stromi, medtem ko ga v sredini tumorskega tkiva ni bilo (slika 5), kar nakazuje na znižanje ravni izražanja cistatina C v PyMT tumorskem modelu, kar smo potrdili tudi s prenosom western. Znižano izražanje cistatina C so opisali pri različnih vrstah raka, npr. pri raku prostate, kjer je znižana prisotnost cistatina C korelirala s slabšo preživetveno prognozo (Wegiel, et al., 2009). Pri karcinomu pljuč znižana vrednost cistatina C v primerjavi z zdravim tkivom ni imela vpliva na prognozo pacientov (Werle, et al., 2006). Tudi pri mišjem modelu K14-HPV 16 so opazili zniževanje prisotnosti cistatina C med malignim razvojem kožnega raka. Pri K14-HPV 16 miših z izbitim genom za cistatin C so opazili hitrejši razvoj kožnih tumorjev zaradi zvišane celične proliferacije in angiogeneze tumorjev ter znižane celične smrti v tumorskem tkivu (Yu, et al., 2010). Nasprotno smo v PyMT tumorjih divjega tipa in v tumorjih brez cistatina C opazili višjo prisotnost stefina B kot v mlečnih žlezah obeh genotipov (slika 6). Povišano izražanje stefina B je značilno za več vrst raka, npr. za hepatocelični karcinom (Lee, et al., 2008), rak pljuč (Ebert, et al., 1997) in rak jajčnikov (Kastelic, et al., 1994).

Zaradi odsotnosti glavnega zunajceličnega inhibitorja cisteinskih katepsinov cistatina C smo preverili izražanje in aktivnost le-teh. Med genotipoma nismo opazili razlik v izražanju katepsinov B, H, L, S in X na mRNA nivoju v primarnih tumorjih (slika 22). Vendar je izguba izražanja cistatina C pri PyMT transgenih miših pričakovano povzročila zvišanje aktivnosti cisteinskih katepsinov tako v tumorjih kot v mediju primarnih PyMT

tumorskih celic (slika 23 in slika 24). Nasprotno se aktivnost katepsinov v celičnih lizatih med genotipoma ni razlikovala (slika 25). Rezultati potrjujejo vpliv cistatina C na zunajcelično aktivnost katepsinov, medtem ko na znotrajcelično aktivnost katepsinov odsotnost cistatina C ni imela vpliva.

Izguba izražanja cistatina C in višja aktivnost cisteinskih katepsinov pri PyMT transgenih miših je skladno s pričakovanji povzročila hitrejši razvoj tumorjev, saj so tumorji nastali 14 dni bolj zgodaj v primerjavi s PyMT;WT skupino (slika 8). Ta rezultat je v skladu z zunajcelično aktivnostjo cistatina C pri zaviranju aktivnosti cisteinskih katepsinov. Znano je namreč, da se cisteinski katepsini izločajo v zunajcelični prostor tumorskega mikrookolja, kjer pospešujejo razvoj in napredovanje raka preko zapletenih mehanizmov, kot so cepitev, procesiranje ali odcepljanje signalnih molekul in proteinov, ki sestavljajo zunajcelični matriks (Olson & Joyce, 2015). Zaradi rahlo kislega pH v tumorskem mikrookolju cisteinski katepsini lahko ohranijo svojo aktivnost in tam vplivajo na razvoj in napredovanje raka, npr. z degradacijo kolagena tipa IV, fibronektina in komponent laminina na bazalni membrani (Bromme & Wilson, 2011). Odsotnost cistatina C tako cisteinskim katepsinom omogoči vpliv na hitrejši razvoj tumorjev.

Tudi pri miših z izbitima genoma za stefin B in cistatin C so tumorji nastali hitreje kot pri kontrolni PyMT;WT skupini (slika 9), vendar nekaj dni počasneje kot pri PyMT;CstC-/- skupini. Tumorji pri transgenih miših z izbitim genom za stefin B so se razvijali počasneje od kontrolne skupine (Butinar, et al., 2014). Morda je ravno odsotnost stefina B razlog za počasnejše razvijanje tumorjev PyMT;StfB-/-CstC-/- v primerjavi s PyMT;CstC-/- tumorji.

Kljub hitrejšemu pojavu tumorjev je na drugi strani prišlo do zmanjšanja rasti tumorjev in velikosti tumorjev pri PyMT;CstC-/- miših v primerjavi s PyMT;WT mišmi v obdobju 30 dni po detekciji in do starosti miši 14 tednov, ko smo miši žrtvovali (slika 10 in slika 12). Enak odziv je bil prisoten tudi v PyMT;StfB-/-CstC-/-skupini z dvojnim izbitjem inhibitorjev (slika 11 in slika 13). Rezultati so bili zelo presenetljivi, saj je vloga katepsinov kot pospeševalcev razvoja in napredovanja tumorjev že dolgo znana (Sloane et al., 1982). Pomembnost vloge katepsinov so potrdili na več različnih modelih raka z izbitjem genov za določen(e) katepsin(e). Izbitje gena za katepsin B pri PyMT mišjem modelu raka mlečne žleze je tako povzročilo močno zmanjšanje rasti in velikosti tumorjev (Vasiljeva, et al., 2008; Vasiljeva, et al., 2006). Transgeno izražanje humanega katepsina B v PyMT modelu je povzročilo povišan razvoj tumorjev in metastaz, kar potrjuje protumorsko funkcijo katepsina B (Sevenich et al., 2011). Izbitje gena za katepsin Z je povzročilo povečano velikost tumorjev pri 10. in 14. tednu starosti, sočasno izbitje genov za katepsina B in Z pa je povzročilo močan protitumorski učinek pri PyMT tumorskem modelu (Sevenich, et al., 2010). Podobno je tudi izbitje genov za katepsine B, L, H, S ali Z precej zmanjšalo tumorigenezo pri tumorskem modelu raka trebušne slinavke (Akkari et al., 2014; Gocheva, et al., 2010; Gocheva, et al., 2006). Po drugi strani pa novejše študije dokazujejo, da imajo lahko katepsini tudi zaviralno vlogo pri razvoju raka (Olson & Joyce, 2015). Tako je izbitje gena za katepsin L povzročilo povišan razvoj tumorjev in metastaz pri K14-HPV16 transgenem mišjem modelu preko modulacije protein kinaze B (Akt) in mitogen-aktivirane proteinske kinaze (Dennemarker, et al., 2010). Pri istem modelu so ugotovili, da odstranitev gena za katepsin C vodi do zaviranja razvoja raka, medtem ko odstranitev gena za katepsin B ni imela nobenih posledic na razvoj raka (Ruffell et al., 2013).

Poleg že dolgo znane vloge katepsinov pri napredovanju raka so nedavno odkrili tudi njihovo novo vlogo. Večina od katepsinov lahko namreč deluje kot proteaze, ki cepijo zunajcelične domene membranskih proteinov (ang. shedding), med drugim tudi pleksine ali receptor za epidermijski rastni faktor, kar lahko pojasnjuje zaviralno vlogo katepsinov pri razvoju raka (Sobotic, et al., 2015). Rezultati nakazujejo na možno dvojno vlogo cisteinskih katepsinov pri razvoju raka: kot promotorji pri razvoju raka na eni strani ali kot zaviralci razvoja raka na drugi strani.

Glavnina umrljivosti pri raku dojke ni posledica razvoja primarnih tumorjev, temveč nastanka sekundarnih metastaz v druge organe, najpogosteje v pljuča, kosti in jetra (Weigelt, Peterse, & van 't Veer, 2005). Zdravljenje metastaz je tudi glavni izziv in cilj pri razvoju novih oblik zdravljenja rakavih obolenj (Wan, Pantel, & Kang, 2013). PyMT mišji model raka mlečne žleze omogoča vpogled v nastanek metastaz, saj pri starosti 14 tednov pride do 100 % prisotnosti metastaz v pljučih (Lin, et al., 2003) V naši raziskavi smo ugotovili, da je odsotnost cistatina C povzročila statistično signifikantno zvišanje števila pljučnih metastaz, metastaze pa so bile nekoliko večje kot pri kontrolni skupini (slika 17). Povišano število in velikost metastaz je v skladu z našimi pričakovanji, saj je zaradi odsotnosti zunajceličnega inhibitorja cistatina C močno povišana zunajcelična proteoliza. S tem je posledično omogočena invazija tumorskih celic v sekundarne organe, kar bi se lahko odrazilo v povišanem razvoju metastaz.

V nadaljevanju raziskave smo največ pozornosti namenili odkrivanju razlogov za manjše tumorje pri PyMT;CstC-/- skupini. Obstaja več možnosti za manjše tumorje: poleg manjše proliferacije tumorskih celic ali nižje stopnje ožiljenosti tumorjev je po drugi strani možna tudi višja celična smrt. Pri določanju celične smrti v tumorjih smo presenetljivo opazili statistično signifikantno znižanje deleža mrtvih celic v PyMT;CstC-/- tumorjih (slika 15). Rezultat se razlikuje od rezultata pri PyMT tumorjih z izbitim genom za stefin B, pri katerih so opazili velika območja mrtvih celic (Butinar, et al., 2014). Stefin B je intracelični inhibitor in ima pomembno vlogo pri razvoju odpornosti celic na oksidativni stres in apoptozo, sproženo z reaktivnimi kisikovimi zvrstmi (ang. Reactive oxygen species – ROS) (Lehtinen et al., 2009). Vloga cistatina C pri celični smrti je sicer opisana kot zaščitna (npr. pri nevronih) (Nishio, Yoshida, Nishiyama, Hatanaka, & Yamada, 2000), vendar povezava med cistatinom C in celično smrtjo pri raku ni znana. Po drugi strani pa smo pri tumorskih celicah z izbitim genom za cistatin C izmerili močno znižanje proliferacijskega kazalca Ki67 v primarnih tumorjih in BrdU proliferacije v primarnih tumorskih celicah (slika 14 in slika 18). Oba rezultata nakazujeta na možno vlogo cistatina C na regulacijo proliferacijske aktivnosti v tumorskih celicah, s tem pa tudi na vlogo cistatina C, ki je drugačna od vloge stefina B pri PyMT mišjem tumorskem modelu raka mlečne žleze. Pri PyMT tumorskih celicah z izbitim genom za stefin B namreč niso opazili razlik v proliferaciji (Butinar, et al., 2014). Hipotezo smo potrdili z ortotopično transplantacijo primarnih PyMT tumorskih celic obeh genotipov v miši prejemnici obeh genotipov. Ugotovili smo signifikantno manjše tumorje, ki so izvirali iz PyMT;CstC-/- tumorskih celic ne glede na genotip miši prejemnic (slika 19). Rezultat nam dodatno dokazuje vpliv cistatina C na tumorske celice in ne na celice tumorskega mikrookolja. Cistatinu C v več študijah pripisujejo vlogo pri proliferaciji celic, neodvisno od vpliva na aktivnost cisteinskih katepsinov. Leta 1989 so pokazali, da inkubacija mišjih fibroblastov NIH/3T3 skupaj s kokošjim cistatinom zviša nivo proliferacije fibroblastov (Sun, 1989). Tudi podganji cistatin C je pozitivno vplival na proliferacijo podganjih mezangijskih celic, kar so opisali kot primer avtokrine regulacije (Tavera et al., 1992). Vpliv cistatina C na proliferacijo celic so opisali tudi pri nevronskih zarodnih celicah, kjer so ugotovili, da cistatin C skupaj s fibroblastnim rastnim faktorjem 2 (FGF-2) ohranja preživetje in proliferacijo nevronskih zarodnih celic (Taupin et al., 2000). Vpliv na proliferacijo tumorskih celic so opisali tudi pri drugih cistatinih tipa II, npr. pri cistatinu M in cistatinu SN. Ugotovili so, da utišanje cistatina M pri MDA-686LN tumorski celični liniji vodi do večje invazije in mobilnosti tumorskih celic preko cisteinsko-proteazne poti ter tudi povzroči hiperproliferacijo tumorskih celic preko neznanih mehanizmov (Vigneswaran et al., 2006). Podobno cistatin M pri humani celični liniji raka dojke MDA-MB-435S zniža proliferacijo tumorskih celic ter njihovo migracijo, invazijo in adhezijo na endotelijske celice (Shridhar et al., 2004; Song et al., 2006). Tudi cistatin SN ima vlogo pri celični proliferaciji pri raku želodca. Utišanje gena za cistatin SN je povzročilo znižanje proliferacije tumorskih celic, medtem ko je bila katepsinska aktivnost povišana (Choi et al., 2009). Kljub mnogim raziskavam, ki dokazujejo vpliv cistatinov na proliferacijo celic, pa je do sedaj zelo malo znanega o mehanizmih.

Razlog za znižano proliferacijo pri tumorskih celicah brez cistatina C bi lahko bil tudi v tem, da zunajcelična povišana aktivnost cisteinskih katepsinov vodi do cepitve zunajceličnih domen, med drugim tudi receptorja epidermijskega rastnega faktorja (EGFR) (Sobotic, et al., 2015). Zvišana prisotnost EGFR pogosto povzroča večjo rast tumorjev pri različnih tipih raka, zato je EGFR pomembna tarča pri zdravljenju raka tarčno zdravljenje (Nicholson, Gee, & Harper, 2001; Seshacharyulu et al., 2012). V ta namen smo preverili izražanje receptorja EGFR v tumorskih lizatih in v membranah, izoliranih iz primarnih tumorskih celic (slika 26A, slika 26B). Rezultati niso pokazali razlik med izražanjem EGFR pri PyMT;WT in PyMT;CstC-/- genotipih (slika 26C). Ravno tako tudi z metodo pretočne citometrije nismo ugotovili razlik v prisotnosti EGFR na površini tumorskih celic.

Poleg inhibicije cisteinskih katepsinov in legumaina cistatin C vpliva tudi na delovanje TGF- β. Cistatin C deluje tako, da preprečuje vezavo TGF-β na TGF- β receptor tipa II in s tem zavira signalno pot TGF-β pri normalnih in rakavih celicah (Sokol & Schiemann, 2004). Kasneje so to odkritje potrdili tudi *in vivo* pri mišjem modelu raka mlečne žleze. V poskusu so poleg kontrolnih 4T1 tumorskih celic uporabili transfecirane 4T1 tumorske celice, ki so izražale humani cistatin C in transfecirane 4T1 tumorske celice, ki so izražale mutirani cistatin C, ki je ohranil le zmožnost inhibicije TGF-β signalne poti, ne pa tudi inhibicije aktivnosti cisteinskih katepsinov. Pri obeh transfeciranih tumorskih celicah so opazili znižano rast tumorjev in manj metastaz ter znižano aktivnost nekaterih efektorjev TGF- β: Smad2, p38 MAPK in ERK-1/2 v primerjavi s kontrolnimi 4T1 tumorskimi celicami, ki so izločale le majhno količino endogeno prisotnega cistatina C (Tian & Schiemann, 2009). TGF- β namreč aktivira različne signalne poti, ki lahko vplivajo tudi na proliferacijo rakavih celic in s tem na rast tumorjev (Chapnick, Warner, Bernet, Rao, & Liu, 2011; Ikushima & Miyazono, 2010; Massague, 2008). Prisotnost in aktivnost teh efektorjev smo preverili tudi v naši študiji. V PyMT;CstC-/- tumorjih smo opazili povišano fosforilacijo p38 MAPK (slika 27). Glede na aktivacijo proteinov, ki pomembno vplivajo na proliferacijo celic, bi pričakovali tudi večje tumorje. Vendar aktivacija teh proteinov ni sprožila mehanizmov, ki bi vodili v zvišano proliferacijo, rast in metastaziranje primarnih tumorskih celic. Ti rezultati nakazujejo, da je bil nivo cistatina C v PyMT tumorjih morda prenizek, da bi presegel prag, potreben za aktivirana proteina p38 MAPK in ERK-1, da sprožita regulacijo proliferacije celic (Murphy, MacKeigan, & Blenis, 2004). Pri PyMT tumorjih smo namreč opazili močno znižanje izražanja cistatina C v primerjavi z normalnim tkivom mlečne žleze pri starosti 14 tednov. Ugotovili smo tudi, da je bil cistatin C v tumorjih prisoten le na robovih tumorjev in v tumorski stromi, medtem ko ga v sredini tumorskega tkiva ni bilo, kar nakazuje na znižanje ravni izražanja cistatina C v PyMT tumorskem modelu. Znižano izražanje cistatina C so opazili tudi pri drugih vrstah raka, npr. pri raku prostate (Wegiel, et al., 2009), raku dojke (Yano, et al., 2001) in med malignim razvojem kožnega raka (Yu, et al., 2010).

Z namenom da bi določili možne alternativne mehanizme, ki regulirajo proliferacijsko aktivnost tumorskih celic pri PyMT tumorskem modelu, smo z masno spektrometrijo analizirali supernatante primarnih celičnih kultur obeh genotipov. Med 22 različnimi proteini, ki so bili vsaj za 1,5-krat povišani pri genotipu divjega tipa z masnim številom najmanj 10 pri vsakem vzorcu divjega tipa (tabela 2), so tretjino proteinov predstavljale izoforme družine 14-3-3 (tabela 3). 14-3-3 proteini so družina ohranjenih dimernih proteinov v evkariontskih organizmih. Pri sesalcih obstaja 7 izoform, ki imajo pomembno vlogo pri regulaciji različnih celičnih procesov, med drugim tudi rast, preživetje in diferenciacijo celic (Hermeking, 2003). Zvišano izražanje 14-3-3 proteinov je bilo povezano z napredovanjem tumorjev pri različnih vrstah raka (Tzivion, Gupta, Kaplun, & Balan, 2006). Obstaja tudi povezava med 14-3-3 proteini in produkti PyMT onkogena. Fosforilacija serina 257 namreč omogoča povezavo med PyMT antigenom in 14-3-3 proteini (Cullere et al., 1998). Člani 14-3-3 proteinov se običajno nahajajo znotraj celic, v nekaterih nedavnih študijah pa so potrdili tudi njihovo zunajcelično delovanje (Ghaffari,

Li, Kilani, & Ghahary, 2010; Maksymowych & Marotta, 2014; McLean, Moylan, Horrell, & Andrade, 2015). Pri vseh izoformah smo opazili znižanje nivoja proteinov v mediju PyMT;CstC-/- celic, najbolj opazno znižanje pa je bilo pri 14-3-3β izoformi. 14-3-3β protein ima pomembno vlogo pri rasti tumorjev in napredovanju raka. Izražanje 14-3-3β izoforme je bilo povišano pri veliko tipih tumorjev, kot je Kaposijev sarkom (Zeng et al., 2010; Zeng et al., 2007), rak na želodcu (Tseng et al., 2011), papilarni karcinom ščitnice (Sofiadis et al., 2012), hepatocelični karcinom (Liu et al., 2011; Tang, Lv, Sun, Han, & Zhou, 2016), pljučni rak (Qi, Liu, Qiao, & Martinez, 2005) in glioma (Liang et al., 2009). Ugotovili so, da prekomerno izražanje 14-3-3β v hepatocelularnem karcinomu povzroči večjo pojavnost metastaz v obdobju 5 let od pojava primarnega tumorja in znižanje preživetja bolnikov (Liu, et al., 2011). Nedavno so potrdili tudi zunajcelično vlogo 14-3-3β proteina v humanih epitelijskih celicah pljuč, kjer je 14-3-3β povzročil zvišano izražanje proteina MMP-1 (Asdaghi et al., 2012). V sekretomu PyMT tumorskih celic nismo odkrili MMP-1a, mišjega ortologa proteina MMP-1, vendar se lahko aktivirajo druge molekularne poti, ki so pospešile razvoj PyMT tumorjev. V literaturi nismo zasledili povezave med 14-3-3β izoformo in rakom na dojki, je pa znana povezava med 14-3-3ζ pri napredovalem raku na dojki (Neal et al., 2009). Prekomerno izražanje izoforme 14-3-3ζ pri transgenem modelu raka mlečne žleze je povzročilo tumorigenezo in napredovanje raka zaradi povišane proliferacije tumorskih celic (Rehman et al., 2014). Na podlagi teh podatkov lahko domnevamo, da bi lahko znižanje nivoja proteinov 14-3-3, ki smo ga opazili pri medijih PyMT;CstC-/- tumorskih celic, vodilo do zmanjšane tumorigeneze pri PyMT tumorskem modelu.

Če povzamemo, naši rezultati kažejo, da izguba cistatina C negativno vpliva na proliferacijo tumorskih celic, kar vodi v zmanjšano rast primarnih tumorjev v PyMT tumorskem modelu. Ta rezultat je očitno posledica zapletenih povezav med aktivnostjo cisteinskih katepsinov, cistatinom C, 14-3-3 proteini in TGF-β signalnimi potmi. Večina raziskav v zadnjem času se osredotoča na inhibicijo proteazne aktivnosti. Rezultati teh raziskav so pridobljeni iz modelov z izbitim genom za določeno proteazo ali pa z inhibicijo določenih proteaz. Le redke študije se osredotočajo na povišano aktivnost proteaz, do katere pride v povezavi z neuravnovešenostjo aktivnosti endogenih inhibitorjev. Kljub znani vlogi cisteinskih katepsinov kot promotorjev napredovanja raka naša raziskava vpliva zunajceličnega inhibitorja cistatina C in znotrajceličnega inhibitorja stefina B odkriva nove vloge cisteinskih katepsinov pri raku. Pomembno je namreč poudariti možno vlogo cisteinskih katepsinov kot zaviralcev razvoja raka, saj so tudi pri matriks metaloproteazah (MMP), pomembnih pospeševalcih razvoja raka, po neuspešnem kliničnem preizkusu splošnega inhibitorja MMPjev (Coussens, Fingleton, & Matrisian, 2002) ugotovili zaviralno funkcijo nekaterih MMP (MMP8 in MMP9) na razvoj raka (Balbin et al., 2003; Coussens, Tinkle, Hanahan, & Werb, 2000; Gutierrez-Fernandez et al., 2008).

Pri proučevanju vloge stefina B pri vnetju smo se osredotočili na odziv primarnih makrofagov iz kosti na vnetje. Negativni regulator imunskega odziva, protivnetni citokin IL-10 inhibira transkripcijo in translacijo številnih provnetnih citokinov, zmanjša predstavitev antigenov in aktivacijo limfocitov T (Saraiva & O'Garra, 2010). Zanimalo nas je, kako odsotnost stefina B vpliva na izražanje protivnetnega citokina IL-10. V gojišču aktiviranih makrofagov z izbitim genom za stefin B smo določili nižji nivo protivnetnega citokina IL-10 (slika 28), medtem ko so bili provnetni citokini povišani (Maher et al., 2014). Aktivacija kinaz ERK in p38 vodi v fosforilacijo histona 3 na promotorju gena za IL-10 in s tem prepis gena (Lucas, et al., 2005). Določili smo signifikantno zmanjšan nivo fosforilirane kinaze ERK ter v manjši meri nivo fosforilirane p38 v aktiviranih makrofagih z izbitim genom za stefin B (slika 29). Ugotovili so tudi znižan nivo fosforilacije transkripcijskega faktorja STAT-3 v aktiviranih makrofagih z izbitim genom za stefin B (Maher, et al., 2014). Sklepamo, da je sinteza IL-10 v našem sistemu regulirana preko kinaz MAP in transkripcijskega faktorja STA-3, vendar podrobnega mehanizma, kako stefin B vpliva na obe signalni poti, ne poznamo. Po drugi strani so v aktiviranih

makrofagih z izbitim genom za cistatin C določili zvišane vrednosti IL-10 v primerjavi z makrofagi divjega tipa (Frendeus, Wallin, Janciauskiene, & Abrahamson, 2009). Medtem ko je stefin B znotrajcelični inhibitor, se cistatin C nahaja zunajcelično; poleg tega je znano, da se sinteza cistatina C v aktiviranih makrofagih ne poveča (Suzuki et al., 2000), medtem ko je sinteza stefina B v aktiviranih makrofagih višja (Maher, et al., 2014). Iz rezultatov lahko zaključimo, da stefin B pomembno uravnava imunski odziv.

Zaključki

Doktorska disertacija prinaša nova spoznanja o vplivu stefina B in cistatina C na razvoj in napredovanje raka mlečne žleze pri PyMT transgenem mišjem modelu. Prišli smo do naslednjih zaključkov:

Izbitje genov za cistatin C in stefin B vpliva na razvoj in rast tumorjev mlečne žleze, saj se tumorji začnejo razvijati prej, vendar so po končanem razvoju manjši v primerjavi s tumorji divjega tipa. Odsotnost cistatina C v manjši meri vpliva tudi na proces metastaziranja.

Odsotnost cistatina C pomembno vpliva na aktivnost cisteinskih katepsinov v tumorjih in v mediju primarnih tumorskih celic.

Odsotnost cistatina C vpliva na proliferacijo tumorskih celic in na aktiviranje MAPK p38, medtem ko na tumorsko mikrookolje nima vpliva.

Z masno spektrometrijo smo ugotovili, da celice z izbitim genom za cistatin C izločajo manj 14-3-3 izoform kot celice divjega tipa, kar bi lahko vodilo do zmanjšane tumorigeneze pri PyMT tumorskem modelu.

Odsotnost stefina B zmanjša sintezo in izločanje protivnetnega citokina IL-10 zaradi manjše aktivacije ERK in p38, ki sta med drugim odgovorna za prepis gena Il10.

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Decreased IL-10 expression in stefin B-deficient macrophages is regulated by the MAP kinase and STAT-3 signaling pathways

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ABSTRACT

Innate immune responses are tightly regulated to avoid excessive activation and subsequent inflammatory damage to the host, and interleukin-10 (IL-10) plays a crucial role in preventing inflammation. Stefin B (cystatin B) is an endogenous inhibitor of cysteine proteinases. In stefin B-deficient bone marrow-derived macrophages (BMDMs), we detected an increase in the induction of the LPS-induced pro-inflammatory signal nitric oxide (NO) but decreased IL-10 expression. The phosphorylation of ERK and p38 MAP-kinases was significantly decreased in stefin B-deficient macrophages, as was STAT-3 phosphorylation. These findings show that stefin B influences the expression of anti-inflammatory IL-10 in response to the TLR4 agonist LPS.
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1. Introduction

The cystatins are endogenous, reversible and tight-binding inhibitors of the papain (C1) and legumain (C13) families of the cysteine proteases and have significant similarities in the amino acid sequence and in the protein structure [1,2]. Stefin B (cystatin B) is an endogenous cysteine cathepsin inhibitor localized in the cytosol and nucleus, where it interacts with histones and cathepsin L [3]. A loss-of-function mutations in the stefin B gene are found in patients with the Unverricht–Lundborg disease (EPM1), but its physiological function in the pathogenesis of the disease is not known [4,5]. Studies of stefin B-deficient mice show that lack of this inhibitor is associated with the signs of cerebellar granular cell apoptosis, progressive ataxia and myoclonic seizures [6]. It was demonstrated that stefin B deficiency sensitizes the cells to the oxidative stress-induced cell death [7,8]. In the past, several studies examined the influence of cystatins on nitric oxide (NO) and cytokine synthesis, predominantly type II cystatins induce NO secretion in IFN-γ-primed mouse macrophages [9,10].

IL-10 is a potent anti-inflammatory cytokine that is crucial for dampening the inflammatory response after pathogen invasion and acts to protect the host from excessive inflammation [11]. The anti-inflammatory function of IL-10 is demonstrated by the phenotype of IL-10-deficient mice that develop severe inflammatory bowel disease due to the spontaneous chronic inflammation [12,13]. During acute inflammation, IL-10 is primarily produced by the macrophages and dendritic cells [14] and it functions to limit the expression of pro-inflammatory cytokines and chemokines [15]. The production of IL-10 in LPS-stimulated macrophages is dependent on the signal transducer and activator of transcription (STAT-3) transcription factor [16] and two mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase (ERK) and p38 [17–19].

In this study we examined how does the lack of stefin B influence NO synthesis and IL-10 expression in mouse primary macrophages. We detected increased NO production in stefin B-deficient BMDMs, whereas the down-regulation of IL-10 synthesis was due to the significantly decreased MAP kinase ERK and STAT-3 activation in stefin B-deficient BMDMs. These results indicate that stefin B is involved in immunomodulation and the signaling pathways determined in stefin B-deficient BMDMs could be important in the pathology of EPM1.

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2. Materials and methods

2.1. Reagents and cell culture

LPS from *Escherichia coli* (*E. coli* 055:B5, Sigma, St. Louis, USA) was used at final concentration of 100 ng/ml. Recombinant mouse Interferon-γ (IFN-γ) (eBioscience, San Diego, CA, USA) was used at a final concentration of 100 U/ml. Antibodies used in Western blotting were purchased from Abcam (Cambridge, UK). Anti-stefin B (ab53725), anti-iNOS (ab3523), anti-ERK 1/2 (ab17942), anti-phospho-STAT3 Y705 (ab76315), anti-STAT3 (ab50761), from Sigma (St. Louis, USA) anti-diphosphorylated ERK 1/2 (M8159), from Cell Signaling (Danvers, MA, USA) anti-phospho-p38 (9211) and anti-p38 (9212). Anti-cathepsin L antibodies were described in [20]. Recombinant mouse M-CSF was purchased from eBioscience (San Diego, CA, USA) and used at a final concentration of 40 ng/ml.

2.2. Mice

Stefin B (cystatin B)-deficient mice were created as described previously [6], provided by Dr. R.M. Myers, Stanford University and bred in our local colony. Mice (8–12 weeks of age) used in this study were wild type (WT) and stefin B-deficient (KO), fully backcrossed to FVB/N background. Stefin B heterozygous mice were regularly intercrossed in order to prevent genetic drift. All mouse studies were conducted in accordance with protocols approved by the Veterinary Administration of the Republic of Slovenia (VARS) and the government Ethical Committee. Procedures for animal care and use were in accordance with the "PHS Policy on Human Care and Use of Laboratory Animals" and the "Guide for the Care and Use of Laboratory Animals".

2.3. Preparation of macrophages

Mouse primary bone marrow-derived macrophages (BMDMs) were obtained by *ex vivo* differentiation from mouse bone marrow progenitors in the presence of M-CSF over 7 days, as previously described [21]. Cells were kept in DMEM supplemented with 20% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine and M-CSF.

2.4. Nitric oxide measurements

Nitric oxide (NO) release was determined by measuring nitrite by the Griess reaction [22] in the BMDM supernatants. Cells were cultured in 96-well plates, stimulated with LPS and IFN-γ for 18 h or IFN-γ-primed overnight, washed, and then stimulated with LPS for 18 h. Supernatants were collected and mixed with Griess solutions. The absorbance was measured at 545 nm on a microplate reader (Tecan Infinite, Männedorf, Switzerland).

2.5. Enzyme-linked immunosorbent assay (ELISA)

Cells were cultured in 96-well plates, stimulated with LPS and IFN-γ for 18 h or IFN-γ-primed overnight, washed, and then stimulated with LPS for 18 h. Murine IL-10 cytokine was measured by ELISA (Abcam, ab108870) in cell culture supernatants after indicated stimulations according to the manufacturer's instructions. The absorbance was read on a microplate reader (Tecan Infinite, Männedorf, Switzerland) at a wavelength of 450 nm. Sample concentrations were calculated from the standard curve.

2.6. Whole cell lysates preparation and Western blot analysis

Monolayer BMDMs at 80% confluence were first washed with ice-cold PBS and then scraped into the Nonidet P-40 lysis buffer (20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol, 1 mM sodium orthovanadate, 10 mM NaF, 10 mM β-glycerophosphate), supplemented with the Complete protease inhibitor coctail (Sigma) and the Phosphatase inhibitor coctail (Sigma, St. Louis, USA). Cells were incubated on ice for 30 min. The lysates were then clarified by centrifugation at 13 000×*g* for 10 min at 4 °C, and the protein concentrations of the cell lysates were measured using the Bradford reagent (Bio-Rad). The lysates were subjected to electrophoresis on 12.5% SDS–PAGE gels followed by Western blotting as described previously [8]. The proteins were visualized with ECL (GE Healthcare) according to the manufacturer's instructions.

2.7. Quantitative real-time PCR

Total RNA was isolated from the BMDMs after stimulation with LPS using PureLink RNA mini kit (Ambion), followed by DNase treatment using TURBO DNA-free kit (Ambion). cDNA was synthesized from RNA with Precision nanoScript Reverse Transcription kit (Primerdesign Ltd) using random nanomer primers. The following primers designed by Primerdesign Ltd were used for the real-time quantitative PCR: IL-10 (*Il10*) sense: 5′-CATACTGCTAACCGAC TCCTTAAT-3′ and anti-sense: 5′-GGGCATCACTTCTACCAGGTA-3′. Real-time PCR reaction was run on Mx3005P system (Agilent, Stratagene products) using the following thermal profile: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Additionally, melting curve (55–95 °C) was performed at the end of each run. The mRNA expressions of target genes were normalized to the expression of *Gapdh* and *B2m*. The mRNA expression of genes was calculated considering their real-time PCR efficiencies using REST (Relative Expression Software Tool) 2009 and presented as a fold increase, relative to the unstimulated cells (control). The mRNA expression levels of genes in control samples were normalized to 1.0.

2.8. Confocal microscopy

BMDMs were seeded on cover slides, stimulated with IFN-γ and LPS and fixed with 4% paraformaldehyde in PBS (pH 7.2) for 10 min and permeabilized with 0.1% Triton X-100 for additional 5 min. Cathepsin L was labeled with mouse anti-cathepsin L monoclonal antibody N135 [20] and stefin B with rabbit anti-stefin B polyclonal antibodies. Fluorescence microscopy was performed using Carl Zeiss LSM 510 confocal microscope. Secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 546 and rhodamine were excited with an argon (488 nm) or He/Ne (543 nm) laser and emission was filtered using a narrow band LP 505–530 nm (green fluorescence) and 560 nm (red fluorescence) filter, respectively. Carl Zeiss LSM image software 3.0 (Correlation Plots) was used to evaluate co-localization between the two labeled proteins (i.e., between red and green fluorescence signals).

2.9. Statistical analysis

Average results are presented as the mean ± S.D. from the number of assays indicated. Statistical significance of the results was determined using unpaired Student's *t*-test, assuming unequal variances. $^{*}P < 0.05$, $^{**}P < 0.01$.

3. Results

3.1. Stefin B is up-regulated in classically activated mouse macrophages

Previously studies showed that the gene transcripts for stefin B were significantly up-regulated upon differentiation of monocytes into the macrophages [23]. We determined increased stefin B

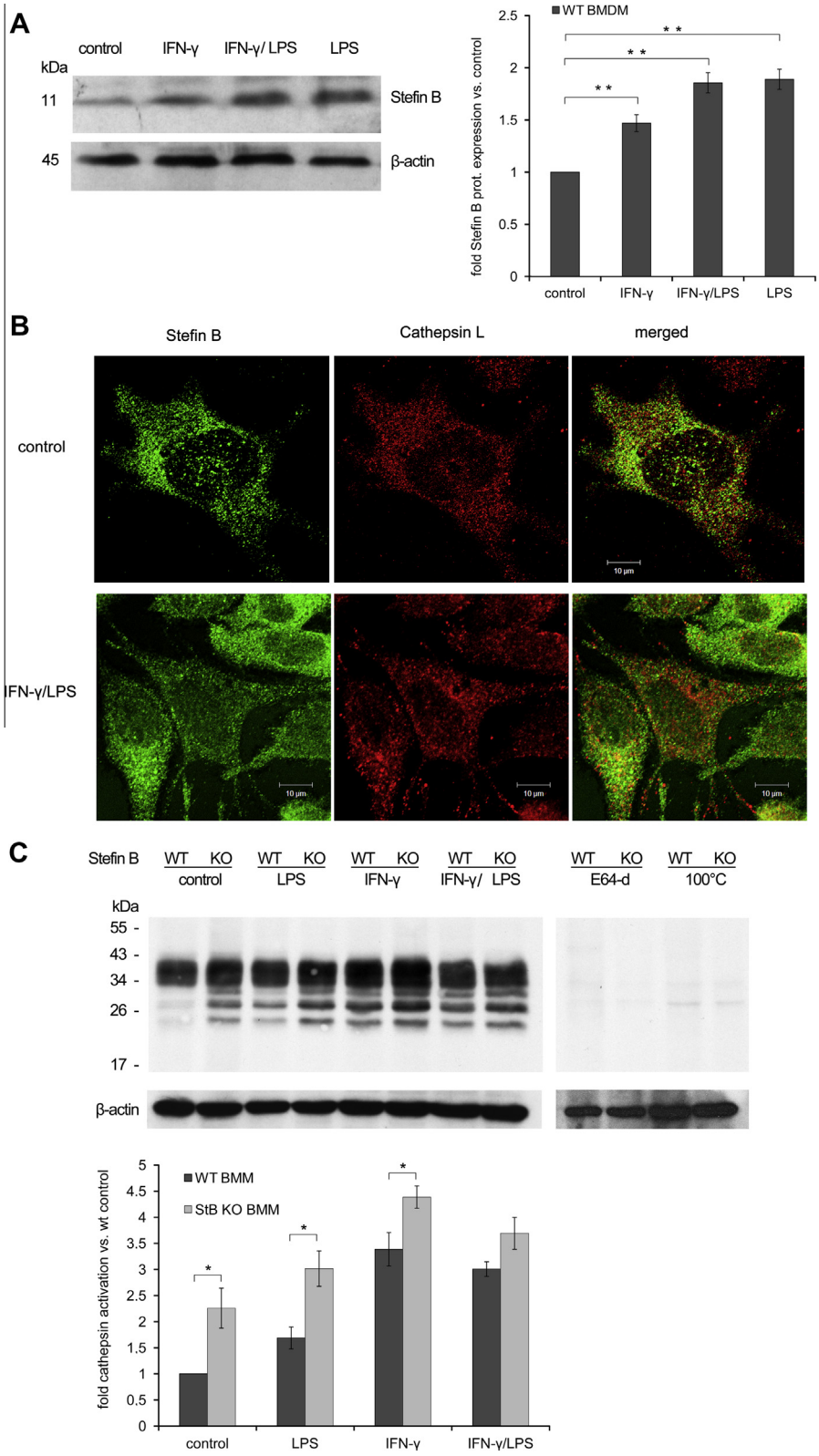


Fig. 1. Stefin B is up-regulated in activated macrophages. (A) Mouse primary macrophages were stimulated with LPS (100 ng/ml), IFN-γ (100 U/ml) for 18 h and IFN-γ primed for 18 h prior to LPS challenge. Stefin B expression was determined by Western blotting of total cell lysates. The signals were quantified by densiometry analysis using ImageJ program. The results represent the mean value of the fold increase ± S.D. relative to the normalized WT BMDM negative control of three independent experiments with 3 different biological samples. $^{**}P < 0.01$. (B) Cells were left untreated or primed overnight with 100 U/ml IFN-γ and treated with 100 ng/ml LPS for 18 h. After indicated treatments, cells were stained with anti-cathepsin L antibodies (red), and anti-stefin B antibodies, fixed and labeled with Alexa conjugated secondary antibodies, as described in Section 2. Scale bar: 10 µm. (C) Cathepsin activity was measured after LPS (100 ng/ml), IFN-γ (100 U/ml) and IFN-γ priming followed by LPS stimulation in WT and Stefin B KO BMDMs. Cell lysates were prepared and labeled with 20 µM DCG-04 active site directed probe for 1 h at 37 °C. Probe-labeled cathepsins were visualized by streptavidin affinity blotting. Incubation of these cell lysates with the cysteine-protease inhibitor E-64 and temperature denaturation completely abolish cathepsin activity. The signals were quantified by densiometry analysis using ImageJ program. The results represent the mean value of the fold increase ± S.D. relative to the normalized WT BMDM negative control of 3 independent experiments, 3 different biological samples. $^{*}P < 0.05$.

expression in IFN-γ primed and LPS stimulated macrophages (Fig. 1A). Although its expression was up-regulated in classically activated BMDMs, we could not detect any co-localization with the lysosomal cathepsin L (Fig. 1B). Studies by Rinne et al. showed that the reduced stefin B inhibitory activity, due to reduced expression in lymphoid cells of EPM1 patients, resulted in a marked increase of general cathepsin activity [24]. In line with that study we determined increased overall cathepsin activity in untreated and activated stefin B-deficient BMDMs compared to WT (Fig. 1C). As expected, we observed no cathepsin activity in the negative controls. The above data indicate that in the cytosol stefin B may act as an emergency inhibitor by inhibiting the cathepsins that are released from the lysosomes upon damage, or it could have alternative functions in the cytosol [25].

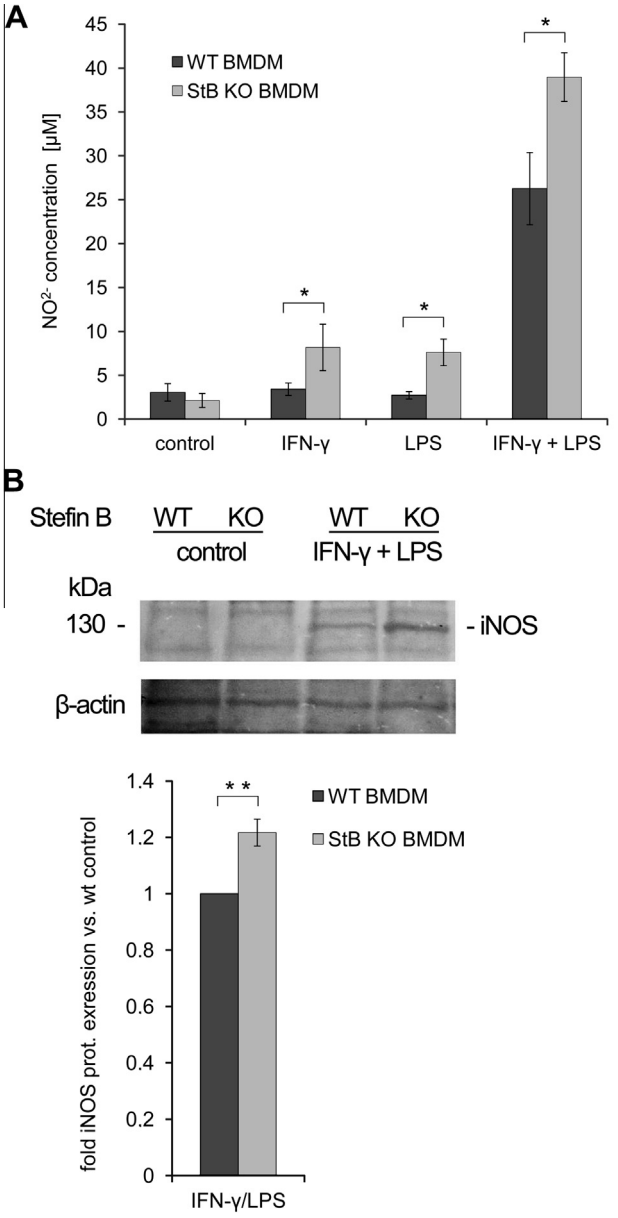


Fig. 2. Stefin B-deficiency results in elevated NO production and iNOS expression in BMDMs. (A) Nitric oxide (NO) release was determined by the Griess reaction in the BMDM supernatants. Cells were cultured in 96-well plates, stimulated with IFN-γ (100 U/ml), LPS (100 ng/ml) for 18 h or primed with IFN-γ (100 U/ml), overnight, followed by LPS (100 ng/ml) stimulation for 18 h. The absorbance was measured at 545 nm. Data were obtained from three independent experiments performed in triplicate, with 3 different biological samples, and the results are presented as means ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$. (B) Cells were left untreated or primed with IFN-γ- (100 U/ml and stimulated with LPS (100 U/ml). Whole cell lysates were separated by SDS–PAGE and analysed by Western blots with iNOS specific antibodies. The signals were quantified by densiometry analysis using ImageJ program. The results represent the mean value of the fold increase ± S.D. relative to the normalized WT BMDM negative control of 3 independent experiments, with 3 different biological samples. $^{**}P < 0.01$.

3.2. Classically activated stefin B-deficient BMDMs produce increased levels of NO due to increased levels of inducible nitric oxide synthase (iNOS) expression

Previous studies by Verdot et al. showed that the cystatins have immune-modulatory functions, independent of cathepsin inhibition [9]. We investigated the effect of stefin B deficiency on NO production in mouse BMDMs. In line with the finding that LPS is a powerful synergistic agent of NO induction [26], we also determined up-regulation of the NO production in BMDMs when primed with IFN-γ and stimulated with LPS. Moreover, stefin B-deficient BMDMs secrete significantly higher levels of NO compared to WT BMDMs (Fig. 2A). In macrophages, the inducible nitric oxide synthase (iNOS), is the enzyme responsible for the NO production in the immune defense against pathogens. To examine whether the increased levels of NO in stefin B-deficient BMDMs are resulting from an increased iNOS protein expression, Western blot analysis was performed on the IFN-γ-primed and LPS-treated lysates. As shown in Fig. 2B, stefin B-deficient BMDMs have increased iNOS expression, when compared to WT BMDMs (Fig. 2B). The increased production of NO suggests the pro-inflammatory phenotype of stefin B-deficient mice.

3.3. Stefin B deficiency in BMDMs modulates LPS-induced IL-10 production

Previous studies indicated that cystatins induce pro- and anti-inflammatory cytokines in macrophages [9,10]. First, we analysed the expression of IL-10, a potent anti-inflammatory cytokine that has pleiotropic effects in immune regulation and inflammation. Notably, we have detected a significantly lower relative IL-10 mRNA expression levels in LPS-stimulated stefin B-deficient BMDMs, when compared to WT (Fig. 3A). Next, the IL-10 protein expression was evaluated in cell culture supernatants of stefin B-deficient and WT BMDMs stimulated with IFN-γ and LPS. We determined decreased IL-10 expression in cell culture supernatants from stefin B-deficient BMDMs compared to WT BMDMs (Fig. 3B), the result correlates well with the lower relative IL-10 mRNA expression levels detected in stefin B-deficient BMDMs.

3.4. Decreased IL-10 expression is due to decreased MAPK ERK1/2 and STAT-3 activation in stefin B-deficient BMDMs

The expression of IL-10 in BMDMs is dependent on two mitogen-activated protein kinases (MAPK), extracellular signal-regulated kinase (ERK) and p38, therefore we analyzed the activation of ERK1/2 and p38. BMDMs were primed with IFN-γ, stimulated with LPS and then analyzed by Western blots. In stefin B-deficient BMDMs we detected significantly decreased levels of phosphorylated ERK 1/2 in all samples, however, the phosphorylation of p38 was significantly lower only in LPS-stimulated stefin B-deficient BMDMs, when compared to WT BMDMs (Fig. 4A). In order to further define the intracellular signaling pathways in stefin B-deficient BMDMs we analyzed the activation of STAT-3 transcription factor in cell lysates generated after IFN-γ and LPS stimulation. Decreased phosphorylation of STAT-3 at Tyrosine 705 was detected in stefin B-deficient than in WT BMDMs (Fig. 4B), in agreement with the decreased IL-10 expression.

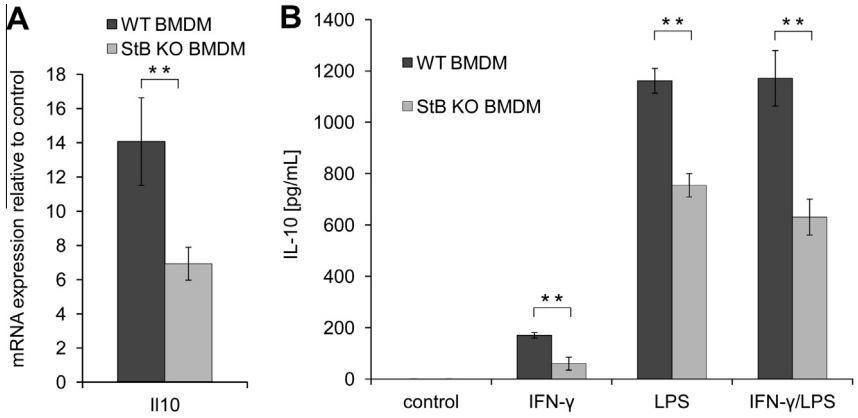


Fig. 3. IL-10 gene and protein expression is down-regulated in stefin B-deficient BMDMs. (A) BMDMs were stimulated with LPS (100 ng/ml) for 4 h and mRNA expression was determined relative to unstimulated BMDMs. mRNA expression was normalised to the reference genes *Gapdh* and *B2m* and presented as a fold increase over control samples (unstimulated BMDMs) that were normalised to 1. Data were obtained from three independent experiments performed in triplicate, with 3 different biological samples; and the results are presented as means ± S.D. $^{**}P < 0.01$. (B) Cells were cultured in 96-well plates, stimulated with LPS (100 ng/ml), IFN-γ (100 U/ml) for 18 h or primed with IFN-γ (100 U/ml) overnight, followed by LPS (100 ng/ml) stimulation for 18 h. Murine IL-10 was measured in cell culture supernatants by ELISA, as described in Section 2. Data shown are representative of at least three independent experiments, with 3 different biological samples.

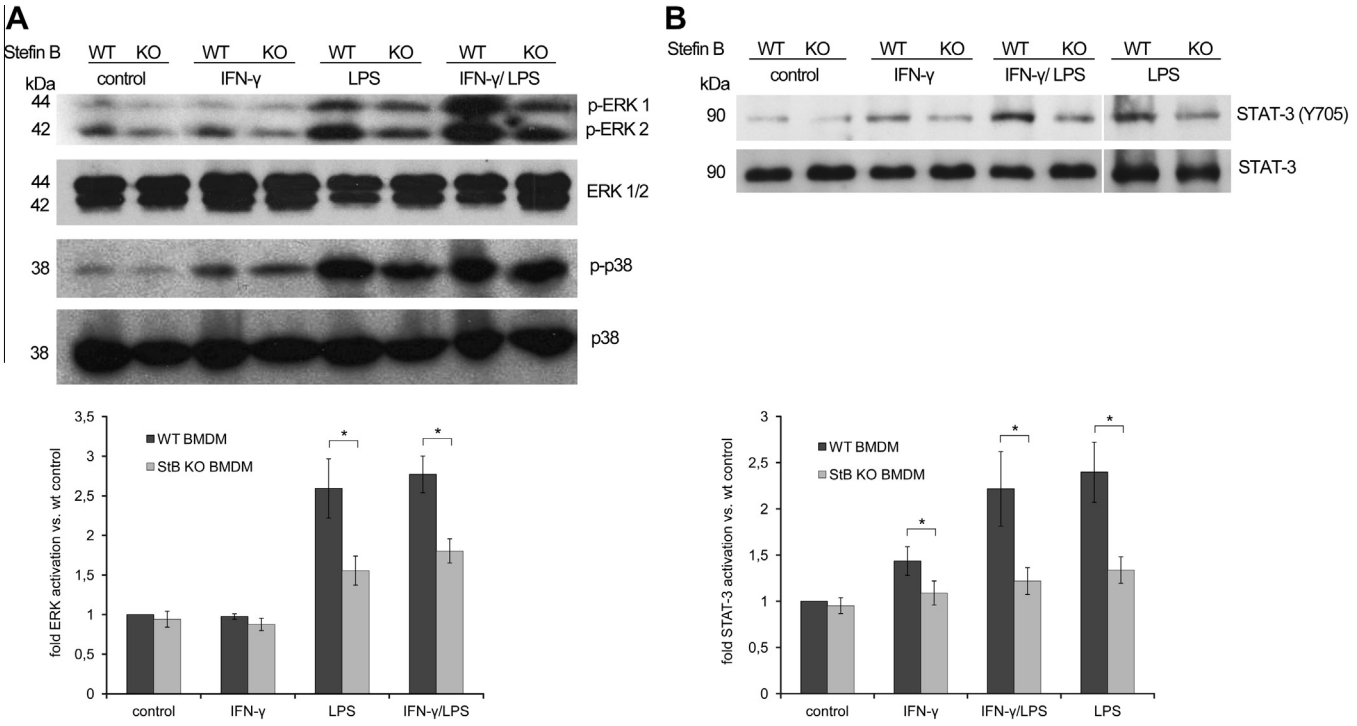


Fig. 4. Decreased activation of ERK and p38 kinases and STAT-3 in IFN-γ and LPS-activated stefin B-deficient BMDMs. (A and B) BMDMs were treated with LPS (100 ng/ml), IFN-γ (100 U/ml) for 1 h, or primed with IFN-γ (100 U/ml) for 1 h, and subsequently stimulated with LPS (100 ng/ml) for 1 h. Whole cell lysates were subjected to SDS–PAGE followed by Western blotting. Proteins of interest were detected using phospho-specific Ab against ERK 1/2, p38 and STAT-3 (Tyr 705). Total ERK 1/2, p38 and STAT-3 were detected as controls. The signals were quantified by densiometry analysis using ImageJ program. The results represent the mean value of the fold increase ± S.D. relative to the normalized WT BMDM negative control of 3 independent experiments, with at least 3 different biological samples. $^{*}P < 0.05$.

The protein levels of ERK, p38 of and STAT-3 remained unchanged, suggesting that the differences in the phosphorylation were due to the activation and not protein expression.

4. Discussion

In the present study we examined the signaling pathways leading to the increased NO and decreased IL-10 production in stefin B-deficient BMDMs. We demonstrated that down-regulation of the IL-10 synthesis is due to the decreased activation of the MAP kinases ERK 1/2, p38 and STAT-3 in stefin B-deficient BMDMs. These data provide underlying molecular mechanisms for pro-inflammatory phenotype reported previously in stefin B-deficient mice [6]. The mechanisms that we observed in stefin B-deficient BMDMs could be compared to the signaling events in macrophages triggered by filarial cystatins, particularly AvCystatin [27] and to a some extent to mouse cystatin C [28].

Early study showed that the addition of chicken cystatin to IFN-γ-primed mouse macrophages increased NO production, TNF-α and IL-10 expression, independently of cystatin inhibitory activity towards cathepsins [9]. In our present work we showed that IFN-γ and LPS-activated stefin B-deficient BMDMs produced

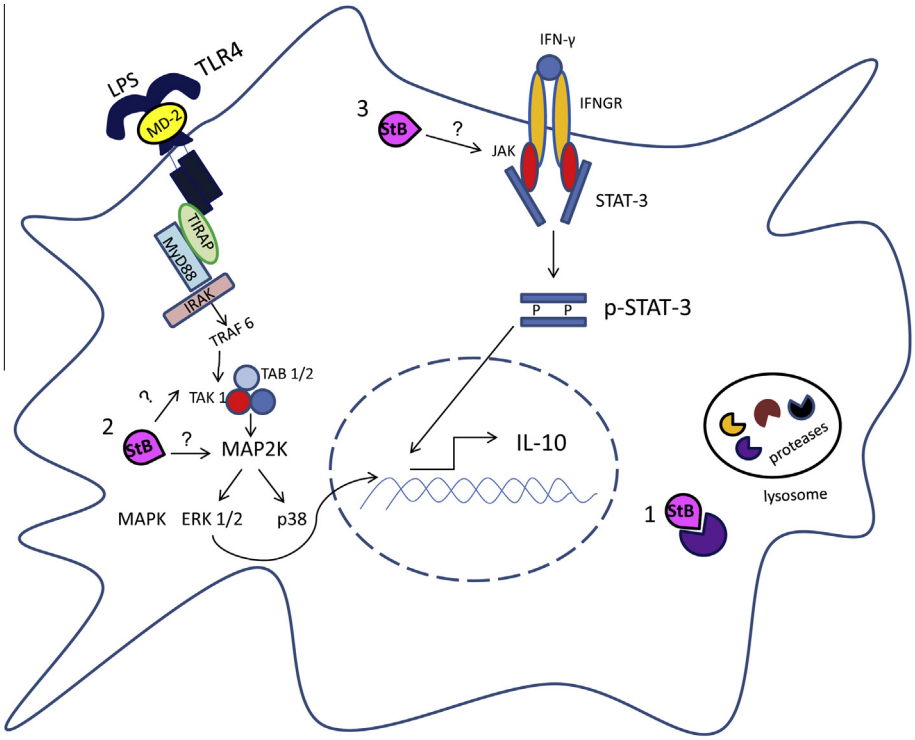


Fig. 5. Proposed stefin B action upon LPS and IFN-γ stimulation in BMDM. (1) Stefin B acts as a cytosolic emergency cathepsin inhibitor and prevents excessive proteolysis. (2) Stefin B increased activation levels of ERK as we determined higher levels of phosphorylated ERK in WT BMDM in comparison to StB KO BMDM. We propose that stefin B might act on MAP2K or upstream. (3) Stefin B was implicated in STAT-3 activation either by promoting its phosphorylation by JAK or by preventing its dephosphorylation. Both signaling pathways lead to IL-10 gene expression, however, precise stefin B role remains elusive. MAP2K; mitogen-activated protein kinase kinase, MAPK; mitogen-activated protein kinase, StB; stefin B.

higher amounts of NO, and expressed more iNOS than WT BMDMs. The result is comparable with the finding of Frendeus et al., showing that the IFN-γ-stimulated macrophages from cystatin C-deficient mice produced higher levels of NO than WT control macrophages [28].

We further showed that upon LPS stimulation stefin B-deficient BMDMs have significantly lower relative IL-10 mRNA expression than WT BMDMs, and as a consequence produced lower levels of IL-10 protein. IL-10 expression has been studied by several groups, (for a review see [16]). In macrophages, the promoter region of the IL-10 gene contains binding sites for the transcription factors STAT-3 and Sp1 transcription factor (SP1) [29,30]. The two transcription factors are controlled by MAP kinases: ERK and p38 [31]. In our present work we determined decreased ERK phosphorylation in stefin B-deficient macrophages, as well as decreased STAT-3 phosphorylation upon IFN-γ and LPS stimulations. In contrast to our results, Frendeus et al. reported that macrophages lacking the extracellular cystatin C expressed higher levels of IL-10 mRNA upon IFN-γ stimulation [28], suggesting that the two cystatins may regulate IL-10 synthesis differently**.** However, not only the localization of cystatin C, but also the cystatin C expression in the activated immune cells is different from that of stefin B. The SAGE studies reported that LPS stimulations resulted in up-regulation of stefin B expression in human monocytes, whereas cystatin C expression was not affected [32].

Our results are in accordance with the recent report regarding the role of nematode cystatins in the activation of macrophages [10,27]. Klotz et al. demonstrated that filarial cystatin, AvCystatin influenced the host macrophages by activation of ERK 1/2 and p38, that further lead to the expression IL-10 [27]. In addition, AvCystatin induced phosphorylation of the transcription factors CREB and STAT-3 by phospho-ERK dependent pathway. Application of AvCystatin strongly reduced colitis in the mouse model, by up-regulating IL-10 [33]. Previous studies demonstrated that stefin B interacted with the STAT-1 in monocyte-derived and placental macrophages [34]. However, the recent study from the same group was unable to confirm the interaction of stefin B with STAT-1 [35]. They showed that stefin B interacted with several different proteins in HIV-1-infected macrophages, among others also two proteins involved in JAK/STAT signaling pathway [35]. Since stefin B influences the activation of STAT-1 [34] and STAT-3 (our results), we propose that it likely interferes with signaling pathway upstream of STAT-1 and STAT-3. The precise mechanism by which stefin B influences JAK/STAT signaling is yet to be determined.

The anti-inflammatory function of stefin B is demonstrated in the pro-inflammatory phenotype of stefin B-deficient mice, that have increased expression of apoptosis and glial activation genes [36]. IL-10 plays an essential role in mediating inflammatory processes not only in the cells of immune system, but also in the brain [37]. It was reported to increase the survival of cerebellar granule cells by blocking caspase-3-like activity [38]. Therefore we propose that the decreased IL-10 expression in stefin B-deficient mice could contribute to the increased apoptosis in the cerebellum in EPM1.

Collectively, our study demonstrates that stefin B-deficiency in BMDMs leads to the decreased expression of the anti-inflammatory cytokine IL-10 (Fig. 5) and into the increased pro-inflammatory NO secretion. The data suggest that the pro- inflammatory phenotype of stefin B-deficient could be a consequence of decreased IL-10 expression.

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The authors declare that there are no conflicts of interest.

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Cystatin C deficiency suppresses tumor growth in a breast cancer model through decreased proliferation of tumor cells

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ABSTRACT

Cysteine cathepsins are proteases that, in addition to their important physiological functions, have been associated with multiple pathologies, including cancer. Cystatin C (CstC) is a major endogenous inhibitor that regulates the extracellular activity of cysteine cathepsins. We investigated the role of cystatin C in mammary cancer using CstC knockout mice and a mouse model of breast cancer induced by expression of the polyoma middle T oncoprotein (PyMT) in the mammary epithelium. We showed that the ablation of CstC reduced the rate of mammary tumor growth. Notably, a decrease in the proliferation of CstC knockout PyMT tumor cells was demonstrated *ex vivo* and *in vitro*, indicating a role for this protease inhibitor in signaling pathways that control cell proliferation. An increase in phosphorylated p-38 was observed in CstC knockout tumors, suggesting a novel function for cystatin C in cancer development, independent of the TGF-β pathway. Moreover, proteomic analysis of the CstC wild-type and knockout PyMT primary cell secretomes revealed a decrease in the levels of 14-3-3 proteins in the secretome of knock-out cells, suggesting a novel link between cysteine cathepsins, cystatin C and 14-3-3 proteins in tumorigenesis, calling for further investigations.

INTRODUCTION

Proteases play crucial roles in multiple steps of tumor progression and metastasis. Among the proteases associated with tumorigenesis and tumor progression, lysosomal cysteine proteases or cysteine cathepsins have gained major attention due to their high proteolytic potential under the more acidic conditions of the tumor microenvironment [1-3]. Although their main functions are associated with lysosomes, cysteine cathepsins can be secreted into the extracellular milieu, which is of particular importance in pathological processes, including cancer [4]. Numerous studies have shown a correlation between the increased proteolytic activity of cysteine cathepsins and neoplastic transformation, tumor invasion and metastasis through highly regulated and complex processes, including remodeling of the extracellular matrix, processing, or shedding of cell adhesion molecules, as well as growth factors and cytokines in the tumor microenvironment, leading to tumor spread [1-3, 5]. In different types of cancer, increased expression and proteolytic activity of lysosomal cathepsins have been correlated with poor cancer prognosis [6-10]. Moreover, cysteine cathepsins, in particular cathepsins B and L, were also found to promote migration and invasion of tumor cells [11-13]. In addition, they were suggested to participate in the proteolytic cascade activation [14, 15], which may all contribute to their tumor-promoting roles.

Protease activity is regulated by multiple mechanisms, with the most important being zymogen activation and inhibition by endogenous protein inhibitors [4, 16]. The best-known endogenous inhibitors of cysteine cathepsins are cystatins. Cystatins are further classified into three families: type 1 cystatins or stefins, which are intracellular inhibitors (stefins A and B); type 2 cystatins, which are extracellular inhibitors (cystatins C, D, E/M, F, S, SA and SN); and type 3 cystatins or kininogens, which are primarily found in blood plasma [17]. Type 2 cystatins contain a signal peptide that is responsible for secretion across the cell membrane, thereby representing an important regulatory mechanism for the extracellular activity of cysteine cathepsins. Cystatin C is the best-characterized type 2 cystatin and the most important extracellular inhibitor of cathepsins [18]. Cystatin C is a ubiquitously expressed secretory protein and a tight-binding inhibitor of lysosomal cysteine proteases, which inhibits their targets with nM (cathepsin B, asparaginyl endopeptidase (legumain)) to sub-pM (cathepsin L) affinity [17, 19, 20]. In addition to its role in physiological processes by controlling extracellular proteolysis via inhibition of cysteine proteases, cystatin C has been associated with different pathological conditions, such as neurodegenerative disorders, inflammatory and cardiovascular diseases, and cancer [21-23]. Increased levels of cystatin C in tumor tissues have been correlated with a favorable prognosis for cancer patients, whereas higher levels of cystatin C in body fluids have been associated with poor prognosis [23]. However, altered expression of cystatin C was reported in different types of cancer, although the levels varied depending on the cancer type. While reduced expression of cystatin C was observed in skin during the development of epithelial cancer [24], an increase in cystatin C expression was reported in ovarian cancer [25]. Interestingly, an inverse correlation between the cystatin C levels and tumor grade was reported in glioma, suggesting a role for cystatin C in the invasiveness of human glioblastoma cells [26]. In addition, higher levels of cystatin C have been detected in the cyst fluid of patients with ovarian cancer [27] and in the sera of patients with lung [28] or colorectal cancer [29]. Moreover, cystatin C was identified as a novel antagonist of transforming growth factor β (TGF-β) type II receptor (TβR-II), which prevents the binding of this receptor to TGF-β, thereby inhibiting TGF-β signaling in normal and malignant cells [30], suggesting a role for cystatin C apart from the inhibition of cathepsins. Taken together, the precise role for cystatin C in tumor progression is not fully elucidated and remains controversial. Therefore, to reveal the function of cystatin C at different stages of tumor progression and metastasis *in vivo*, we employed a mammary tumor virus (MMTV) Polyoma Middle T (PyMT) transgenic mouse model of mammary cancer [31] and crossed it with cystatin C-deficient mice [32]. The data demonstrated that the depletion of cystatin C in the PyMT breast cancer model reduced tumor growth and proliferation, suggesting a novel function for cystatin C in cancer development and progression.

RESULTS

Generation and characterization of PyMT transgenic mice with CstC deficiency

CstC-deficient mice (CstC-/-) were backcrossed to the FVB/N genetic background for more than 10 generations. These mice did not show any gross phenotype at 14 weeks of age and were further crossed with FVB/PyMT transgenic mice, resulting in two cohorts of female mice that were hemizygous for the PyMT transgene: PyMT;CstC+/+ and PyMT;CstC-/-.

First, the expression level of CstC was analyzed in PyMT tumor and normal mammary glands of 14-week-old mice using Western blotting. Interestingly, while CstC was highly expressed in normal mammary gland tissue, its protein level was substantially reduced in the tumor tissue (Figure 1A). Furthermore, immunohistochemical staining for CstC in PyMT tumors revealed that CstC was predominantly localized in the tumor stroma and at the edge of tumor nodes, and much less expression was observed in the tumor core, whereas homogeneous staining of CstC was observed in mammary glands (Figure 1B and Supplementary Figure 1). This observation suggests the down-regulation of CstC expression during the malignant transformation of the mammary epithelium, consistent with previous reports on decreased CstC expression in patient tumor tissues [33, 34]. Furthermore, these data suggest that due to the reduced cystatin C levels, cathepsins may escape internal control, resulting in elevated extracellular cathepsin activities.

To evaluate this idea, we next investigated the effect of CstC ablation on the activity of cysteine cathepsins in normal mammary glands and mammary tumors by monitoring the hydrolysis of the general cathepsin fluorogenic substrate Z-Phe-Arg-AMC in the lysates of tumors and normal mammary tissues of both genotypes (Figure 1C). A significant increase in the hydrolysis of Z-Phe-Arg-AMC was observed in both CstC-deficient tissues compared to wild-type controls, consistent with the protective role of CstC. Moreover, Z-Phe-Arg-AMC hydrolysis was much higher in CstC-deficient PyMT tumor lysates compared to CstC-deficient normal mammary tissue, consistent with the elevated cathepsin activity in malignant tissue. Consistently, addition of human recombinant CstC to the tumor lysates significantly decreased cathepsin activity (Supplementary Figure 2).

Progression and metastasis of CstC depleted PymT-induced mammary carcinomas

To further investigate the role of CstC ablation on tumor development and growth, the volumes of the first two palpable mammary tumors were measured at 30 days after the first tumor detection and grouped according to their diameter into three categories: small (<5 mm),

medium (5-10 mm) and large (>10 mm) (Figure 1D). Surprisingly, PyMT;CstC-/- tumors were significantly smaller compared to tumors from PyMT;CstC+/+ mice. This observation was confirmed *ex vivo*, where the cumulative weight of all 10 mammary tumors from mice at the age of 14 weeks was measured (Figure 1E). The weight of tumors from PyMT;CstC-/- mice was 7.1 g, which is 23 % lower compared to tumors from PyMT;CstC+/+ mice, which had an average tumor weight of 9.2 g. These findings further suggest that CstC exhibits pro-tumorigenic activity in the PyMT mammary cancer model.

We next addressed how CstC depletion affects metastasis development and growth. In the MMTV;PyMT mouse model, pulmonary metastases occur in most animals at the age of 14 weeks. We therefore assessed the number and cumulative size of pulmonary metastases using computer-assisted histomorphometry in sequential histological sections of lungs from both experimental groups of mice at the age of 14 weeks. Compared to the PyMT;CstC+/+ group, the number of lung metastases in the PyMT;CstC-/- group calculated per 1 mm^2 of lung tissue was significantly higher (0.51 in PyMT;CstC-/-,

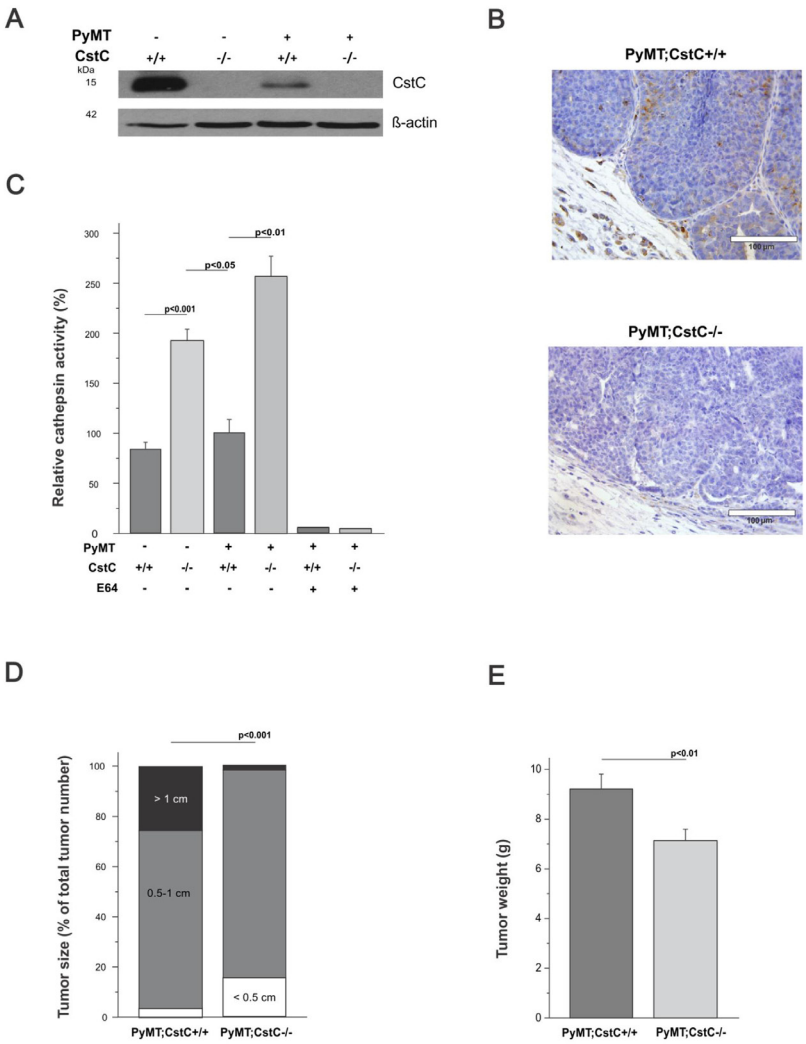


Figure 1: Suppression of PyMT-induced tumor growth in cystatin C deficient female mice. (A) Expression of cystatin C evaluated by Western blot analysis in normal mammary glands (FVB;CstC+/+, FVB;CstC-/-) and PyMT-induced mammary carcinomas (PyMT;CstC+/+, PyMT;CstC-/-) at 14 weeks of age. β-actin was used as the internal loading control. **(B)** Detection of cystatin C by immunohistochemistry staining of PyMT;CstC+/+ and PyMT;CstC-/- tumor sections. Scale bar, 100 μm. **(C)** Relative cathepsin activity determined in homogenates of tumors (PyMT;CstC+/+ n=4 and PyMT;CstC-/- n=4) and mammary glands (FVB;CstC+/+ n=4, FVB;CstC-/- n=4). Activity was measured as Z-Phe-Arg-AMC hydrolysis in the presence or absence of E64. Statistics were analyzed using Student`s t-test. **(D)** Size of mammary tumors 30 days after first detection of the individual tumor node. The first 2 individual mammary tumors in each female were analyzed; thus, 35 and 33 individual tumors were included in the PyMT;CstC+/+ and PyMT;CstC-/- groups, respectively. The Chi-square test was used for statistical analyses. P<0.001. **(E)** The cumulative tumor weight per mouse for all mammary tumors was calculated for PyMT;CstC+/+ (n=23) and PyMT;CstC-/- (n=26) mice at 14 weeks of age. Statistics were analyzed using Student's t-test. P<0.01.

0.26 in PyMT;CstC+/+) (Figure 2A). However, although there was a trend towards an increase in the average size of lung metastases in CstC-deficient PyMT mice, no statistically significant difference was detected between the two cohorts of mice (Figure 2B).

Effect of CstC ablation on tumor cell proliferation, cell death and angiogenesis

We next analyzed whether CstC ablation affects major processes associated with tumor progression, tumor cell proliferation, cell death and angiogenesis in late-stage primary PyMT tumors. First, cell proliferation of mammary tumors isolated from PyMT;CstC-/- and PyMT;CstC+/+ mice was evaluated by immunohistochemical detection of the proliferating factor Ki67 (Figure 3A). Consistent with the reduced size of PyMT;CstC-/- tumors (Figure 1E), the Ki67 proliferation index was reduced in PyMT;CstC-/- tumors (16.4 %) compared to PyMT;CstC+/+ tumors (24 %) (Figure 3B). In addition to the lower rate of tumor cell proliferation, reduced tumor size may also result from increased cell death or the reduced vascularization of tumors. Therefore, we next quantified the cell death of cancer cells in primary PyMT tumors using terminal dUTP nick-end labeling (TUNEL) staining as a measure of DNA fragmentation, which is directly related to apoptosis. Although the fraction of TUNEL-positive cells in PyMT primary tumors was low (2 % in average), apoptotic cell death was lower in CstC-deficient tumors compared to wild-type controls (Figure 3C). This was confirmed by quantification of active caspase 3 in primary PyMT tumors from CstC-deficient mice compared to wild-type controls (Supplementary Figure 3). We next evaluated the potential impact of CstC ablation on the vascularization of PyMT tumors by immunofluorescence staining of endothelial cells in primary tumor sections by measuring the levels of CD31, a known marker of angiogenesis. Notably, the quantification of CD31+ cells revealed no genotype-dependent differences in the mean vessel density between PyMT; CstC-/- and PyMT; CstC+/+ tumors (Supplementary Figure 4A and 4B). Taken together, these data suggest a role for cystatin C in tumor cell proliferation, but not in apoptotic cell death or angiogenesis, potentially explaining the significant reduction of PyMT tumors in CstC-deficient mice.

To better understand the role of cystatin C in tumor cell proliferation at the molecular level, we next investigated the level of protease activity and the proliferation rate of the primary PyMT;CstC-/- and PyMT;CstC+/+ tumor cells. First, the activities of the cathepsins were measured in conditioned media of isolated primary PyMT tumor cells of both CstC genotypes with fluorogenic substrate Z-Phe-Arg-AMC. A significant increase in substrate cleavage was detected in PyMT;CstC-/- tumor cells compared to wild-type PyMT tumor cells, providing evidence for the presence of extracellular cysteine cathepsins in PyMT cells (Figure 3D). Furthermore, the BrdU assay performed on primary PyMT tumor cells revealed a significant decrease in the proliferation index of PyMT;CstC-/- tumor cells (approximately 30 %) compared to PyMT;CstC+/+ tumor cells (Figure 3E), thereby confirming the Ki67 proliferation data obtained from the analysis of the primary tumor tissues.

However, because it is difficult to explain the effect on tumor cell proliferation solely by cathepsin inhibition, we next addressed the potential role for CstC as an antagonist of transforming growth factor β (TGF-β)

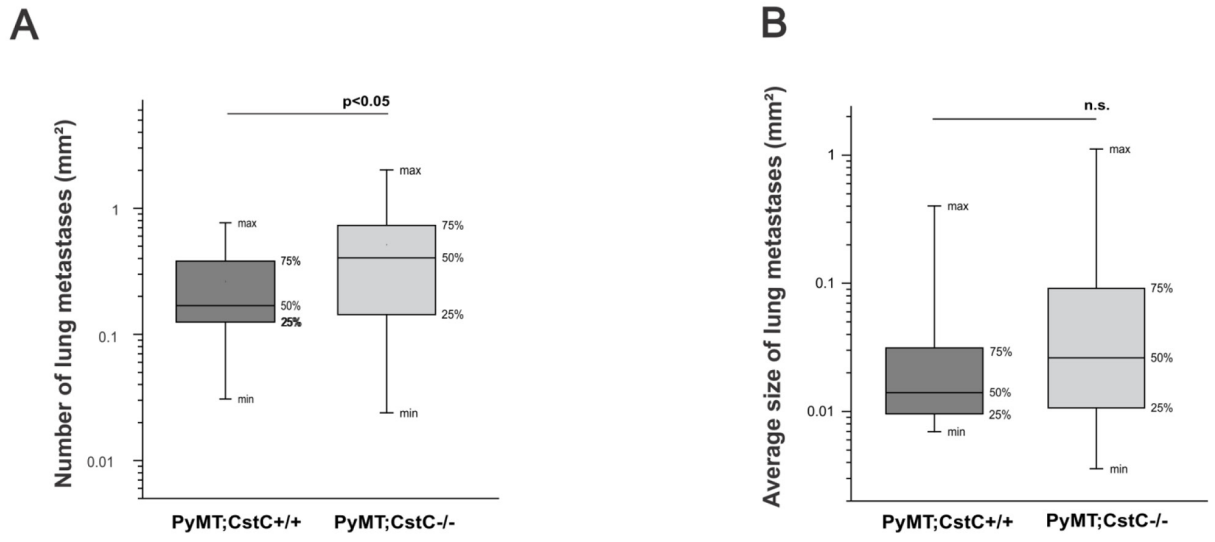


Figure 2: Effect of cystatin C deficiency on pulmonary metastases of PyMT mammary tumors. Histomorphometric analysis of **(A)** number and **(B)** average size of lung metastasis in PyMT;CstC+/+ (n=18) and PyMT;CstC-/- (n=22) female mice at age of 14 weeks. Statistics were analyzed using Student's t-test.

signaling [35]. We therefore examined whether the absence of CstC expression affected the activation of mitogen-activated protein kinases (MAPKs), p38, JNK and ERK, which are associated with the TGF-β signaling [36, 37], by Western blot analysis of primary tumor lysates to monitor the activation status of these signaling molecules (Figure 4A). Notably, the phosphorylation and activation of p38 MAPK was increased in PyMT tumors deficient for CstC compared to their control counterparts (Figure 4B), supporting the idea that CstC acts as an antagonist of transforming growth factor β (TGF-β) signaling in normal and malignant cells [38]. However, whether the activation of p38 signaling pathways in PyMT may induce down regulation of tumor cell proliferation remains unknown.

Impact of CstC from mouse breast cancer cells on proliferation and growth of PyMT tumors

Another factor that can critically affect the development and progression of cancer is the crosstalk

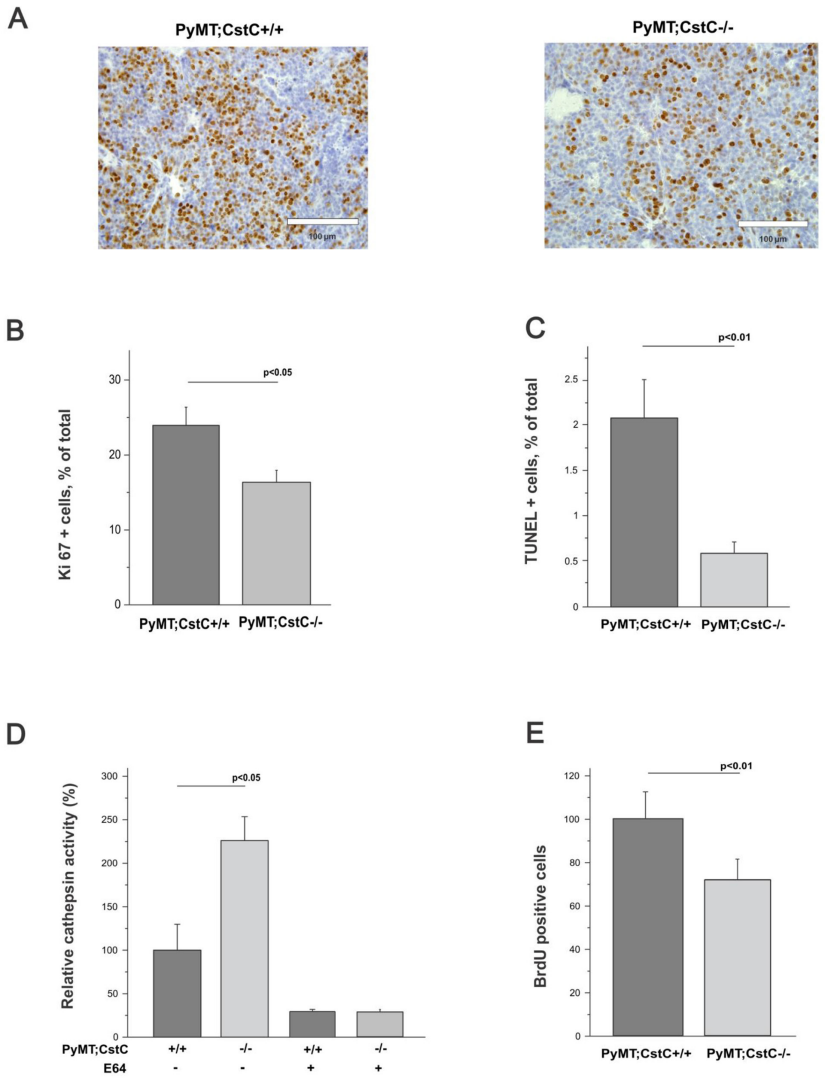


Figure 3: Effect of cystatin C deficiency on proliferation, cell death and vessel density. (A) Cell proliferation in primary tumors determined through immunodetection of Ki67 in PyMT;CstC+/+ and PyMT;CstC-/- mice at 14 weeks of age. Representative images for each genotype are shown under 40x magnification. **(B)** Quantification of Ki67-positive cells as a percentage of total cells in PyMT;CstC+/+ (n=11) and PyMT;CstC-/- (n=12) tumors. Ki67 index was calculated from 20 high-power fields per tumor through computer-assisted data analysis using ImageJ software. Statistics were analyzed using Student's t-test. P<0.05. **(C)** Quantification of TUNEL-positive cells as a percentage of total cells from each of PyMT;CstC+/+ (n=9) and PyMT;CstC-/- (n=9) tumors of 14 weeks old mice. TUNEL+ index was calculated from 10 high-power fields per tumor by computer-assisted data analysis using ImageJ software. Statistics were analyzed using Student's t-test. P<0.01. **(D)** Relative cathepsin activity determined in cell culture medium of primary tumor cells (PyMT;CstC +/+, n=3, PyMT;CstC-/-, n=3). Activity was measured as Z-Phe-Arg-AMC hydrolysis in the presence or absence of E64. Statistics were analyzed using Student's t-test. **(E)** Percentage of BrdU incorporation into nuclei of primary tumor cells (PyMT;CstC+/+, n=5, PyMT;CstC-/-, n=5). Statistics were analyzed using Student's t-test. P<0.01.

between tumor cells and the tumor stroma [39, 40]. Since CstC is expressed and present in both the tumor cells and stromal compartments (Figure 1A) of primary PyMT tumors, we developed an orthotopic tumor model, in which primary PyMT;CstC-/- and PyMT;CstC+/+ syngeneic mouse breast cancer cells were implanted into the mammary fat pad of female recipient mice of both CstC genotypes (Figure 5A). Starting at two weeks post implantation, the tumor diameters were measured three times per week during a 20-day observation period. Notably, tumors from mice originating from PyMT;CstC-/- tumor cells were significantly smaller and their weight was significantly lower than that of PyMT;CstC+/+ tumor cell donors, independent of the recipient CstC genotype (Figure 5B and 5C). Taken together, this result suggests that the reduced proliferation of PyMT;CstC-/- tumor cells could result in decreased tumor growth *in vivo*, consistent with the findings on the inhibition of tumor progression in transgenic PyMT mouse breast cancer model deficient for CstC.

Collectively, these data suggest that CstC from tumor cells, and not the stroma, plays an important role in the observed tumor growth phenotype of both transgenic and orthotopic implantation mouse models. To further investigate the potential mechanism of CstC ablation on the decreased proliferation of PyMT tumor cells, we performed a proteomic analysis of the PyMT tumor cell secretome. Conditioned media samples from PyMT; CstC-/- and PyMT; CstC+/+ tumor cells isolated from tumor-bearing mice were analyzed using mass spectrometry.

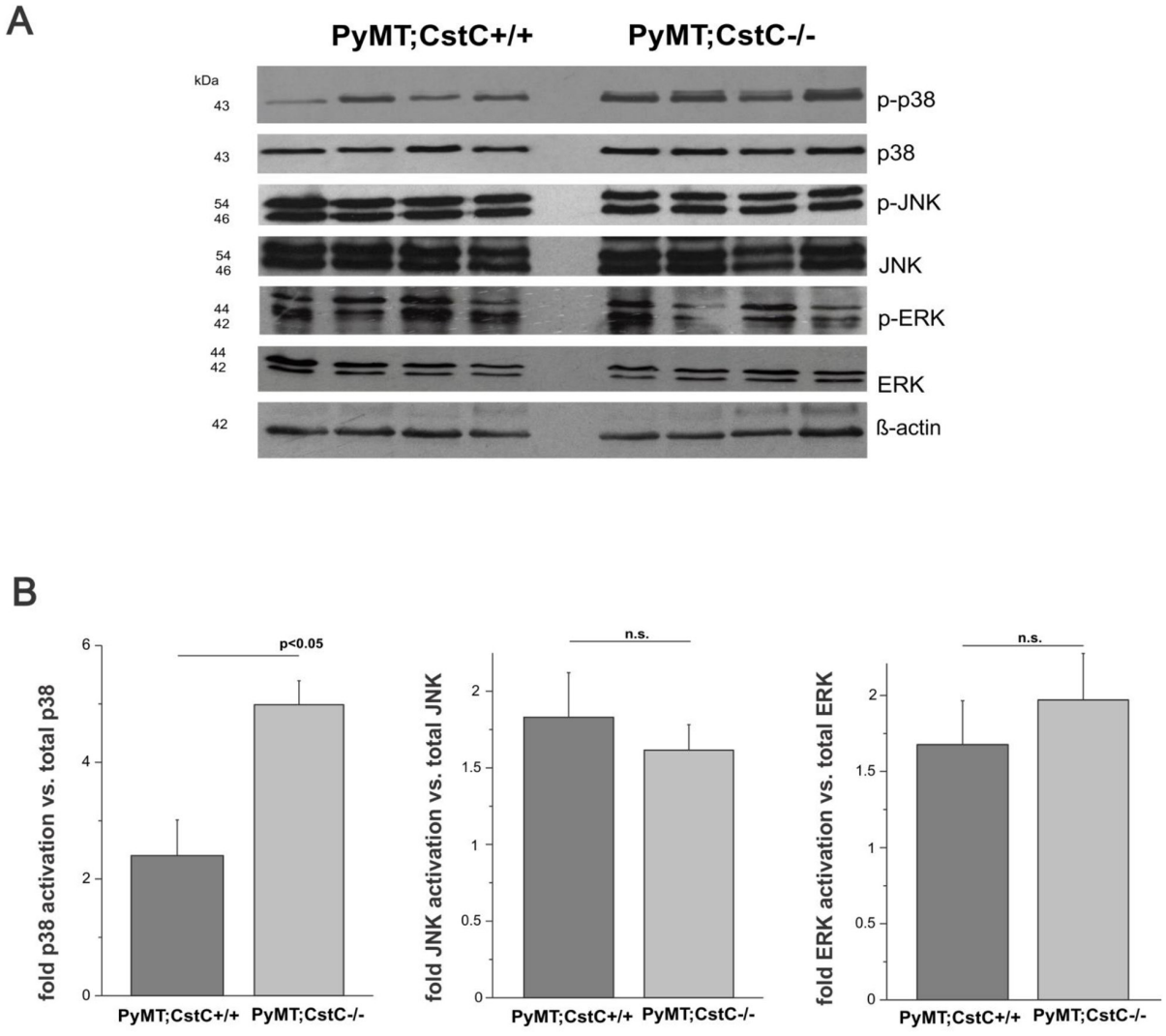


Figure 4: Phosphorylation of MAPK family. (A) Westernblot analysis of phosphorylated p38, total p38, phosphorylated JNK and total JNK, phosphorylated ERK and total ERK in lysates of representative tumors from 4 PyMT;CytC+/+ and 4 PyMT;CstC-/- mice. β-actin served as a loading control. **(B)** Quantification of phosphorylated p38 MAPK, JNK and ERK signals by densitometry analysis using ImageJ program. The results represent the mean value of the fold increase ± S.D. relative to the amount of total p38 MAPK, JNK and ERK proteins, respectively. The experiment was performed on 4 different biological samples per genotype. Statistics were analyzed using Student's t-test. $P<0.05$.

The relative quantification of secreted proteins by spectral counting confirmed that CtsC is secreted from PyMT tumor cells in addition to several cysteine cathepsins, such as cathepsins B, Z(X), S and L1(V) and asparaginyl endopeptidase [41] (Table 1). Whereas the lack of CstC was confirmed in PyMT;CstC-/- cell-conditioned medium, no difference in cysteine cathepsin levels was detected in cells of both genotypes. The latter funding was also confirmed by evaluation of their mRNA, protein expression and intracellular activity level (Supplementary Figures 5 and 6). Notably, the largest difference between the CstC knockout and wild-type tumor cell secretomes was observed for the 14-3-3 proteins, which were decreased in knockout animals (Table 1). 14-3-3 adaptor proteins have a wide range of roles in cell signaling and are also known to be present in the extracellular

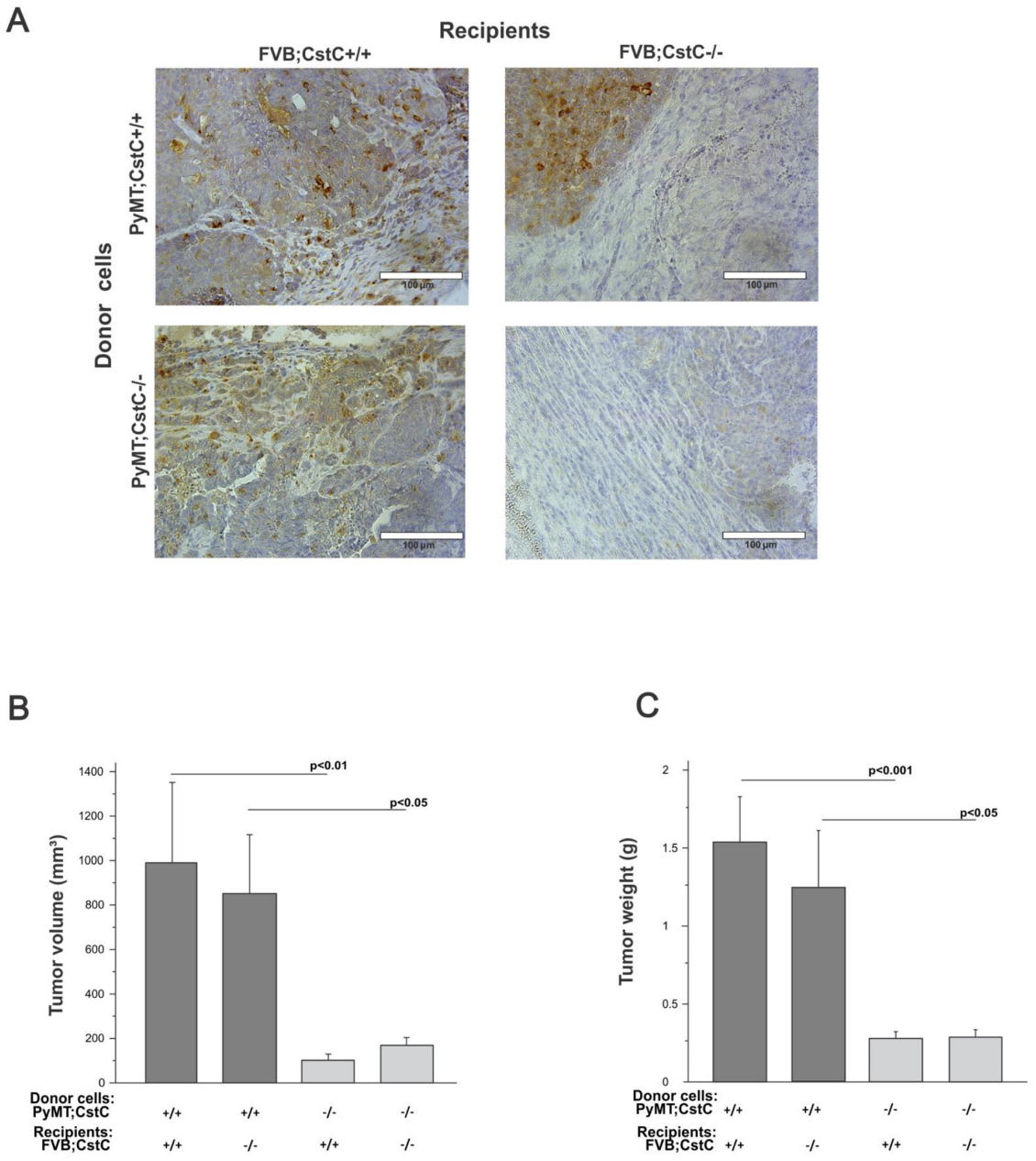


Figure 5: Orthotopic transplantation through intra-mammary injection of PyMT cells of both cystatin C genotypes into congenic recipients of both cystatin C genotypes. (A) Detection of cystatin C by immunohistochemistry staining of orthotopical transplanted tumor sections. Scale bar, 100 µm. **(B)** Tumor volume of tumors from PyMT;CstC+/+ and PyMT;CstC-/- tumor cells, injected into FVB;CstC+/+ or FVB;CstC-/- female mice (number of each group: ≥7). Statistics were analyzed using Student's t-test. **(C)** Tumor weight of tumors from PyMT;CstC+/+ and PyMT;CstC-/- tumor cells, injected into FVB;CstC+/+ or FVB;CstC-/- female mice (number of each group: ≥7). Statistics were analyzed using Student's t-test.

Table 1: List of proteins identified in conditioned media from primary PyMT tumor cells with differential expression of CstC

Protein name	Uniprot accession	Number of peptides	MS/MS count		Fold difference
			PyMT; CstC+/+	PyMT; CstC-/-	
Cystatin C	P21460	9	46	0	
Cathepsin B	P10605	17	42	44	0.96
Cathepsin L(V)	P06797	15	34	36	0.94
Cathepsin Z(X)	Q9WUU7	12	16	18	0.89
Legumain	O89017	12	9	9	1.00
14-3-3 protein beta/alpha	Q9CQV8	15	17	9	1.89
14-3-3 protein epsilon	P62259	24	123	74	1.66
14-3-3 protein eta	P68510	18	19	10	1.90
14-3-3 protein gamma	P61982	20	38	24	1.58
14-3-3 protein sigma	O70456	21	46	20	2.30
14-3-3 protein theta	P68254	19	27	14	1.93
14-3-3 protein zeta/delta	P63101	21	64	42	1.52

Conditioned media of primary PyMT tumor cells from 4 different transgenic mice of each genotype were used.

environment where they regulate biological activities with relevance for tissue homeostasis and diseases, such as cancer [42]. Therefore, proteolysis of 14-3-3 proteins could represent an alternative mechanism for their down-regulation that would affect their function, including their reported role in stimulation of matrix metalloproteinase (MMP) activity [43]. To test this idea we incubated recombinant 14-3-3β protein with active cathepsins B, L, and S *in vitro* under defined conditions and analyzed the reaction at different time points. The data revealed that 14-3-3β protein can be specifically cleaved by cathepsins L and S, and to a lesser extent by cathepsin B (Figure 6), suggesting a novel mechanism for cysteine cathepsin's tumor-suppressive role.

DISCUSSION

Protease activity is tightly regulated within cells and organisms, and inappropriate proteolysis can play a major role in the development of pathological conditions, such as cancer [44]. To better understand the function of proteases and successfully develop novel therapeutic strategies, it is necessary to understand the role of proteases in the complex sequence of events leading to the development of pathology and its progression. One of the major regulating mechanisms of proteases is the inhibition of their activity by endogenous protein inhibitors [16, 17]. The expression of proteases and their inhibitors in tumors is frequently dysregulated, which is often based on complex interactions between tumor cells and their microenvironment [45] and metabolic changes in the tumor microenvironment such as hypoxia and acidosis [46]. In the present study, we evaluated the impact of the depletion of the extracellular cysteine cathepsin inhibitor cystatin C on the development and progression of breast cancer using a PyMT mammary tumor model. Cysteine cathepsins, as well as legumain, a cysteine endopeptidase of the asparaginyl endopeptidase family, are reportedly secreted into the extracellular milieu by tumor or tumor associated cells of the tumor microenvironment, where they promote tumor progression and metastasis through complex processes involving cleavage, processing, or shedding of signal molecules and extracellular matrix proteins [10, 47]. Notably, while the ablation of CstC expression in PyMT transgenic mice increased the activity of cysteine cathepsins, as detected in the conditioned medium of primary PyMT tumor cells, a significant reduction in tumor growth and tumor volume was detected in PyMT;CstC-/- mice compared to CstC wild-type PyMT transgenic mice.

Although this finding is a bit surprising, as cathepsins were long believed to be tumor promoting proteases, it is now clear that they can also play "antagonistic" tumor-promoting roles, depending on the tissue and the type of cancer studied [10]. For example, the ablation of cathepsin B (Ctsb) in the PyMT model resulted in a significant decrease in mammary tumor growth and volume [2, 48]. In support of the pro-tumorigenic function of cathepsin B, the transgenic expression of human

Ctsb promoted progression and metastasis in the same mammary cancer transgenic model [49]. Interestingly, although the depletion of another lysosomal cysteine protease, cathepsin Z (Ctsz; also called cathepsin X/P), significantly increased the sizes of PyMT tumors on weeks 10 and 14, profound synergistic anticancer effects were observed in mice after the combined loss of Ctsb and Ctsz [3]. Similarly, the ablation of cathepsins B, L, H, S or Z substantially reduced tumorigenesis in the pancreatic islet tumor model, although their individual roles clearly differ [50-52]. However, the deficiency of cathepsin L (Ctsl) enhanced tumor progression and metastasis in the K14–HPV16 transgenic mouse model of epidermal carcinogenesis through modulation of protein kinase B/Akt and mitogen-activated protein kinase pathways [53]. The removal of the cathepsin C gene in the same tumor model had a tumor suppressing effect, whereas the ablation of cathepsin B had no effect [54]. In addition, the functions of multiple cathepsins as sheddases that cleave extracellular receptors, such as plexins and EGFR, were recently uncovered, further supporting their tumor suppressing roles [55].

Taken together, these observations indicate a potential dual role for cysteine proteases in tumor progression. Consistently, a decrease in PyMT tumor progression was observed upon depletion of stefin B, the general cytosolic cysteine cathepsin inhibitor [56]. In contrast to CstC, stefin B is an intracellular inhibitor that plays an important role in the development of resistance of cells to oxidative stress and reactive oxygen species

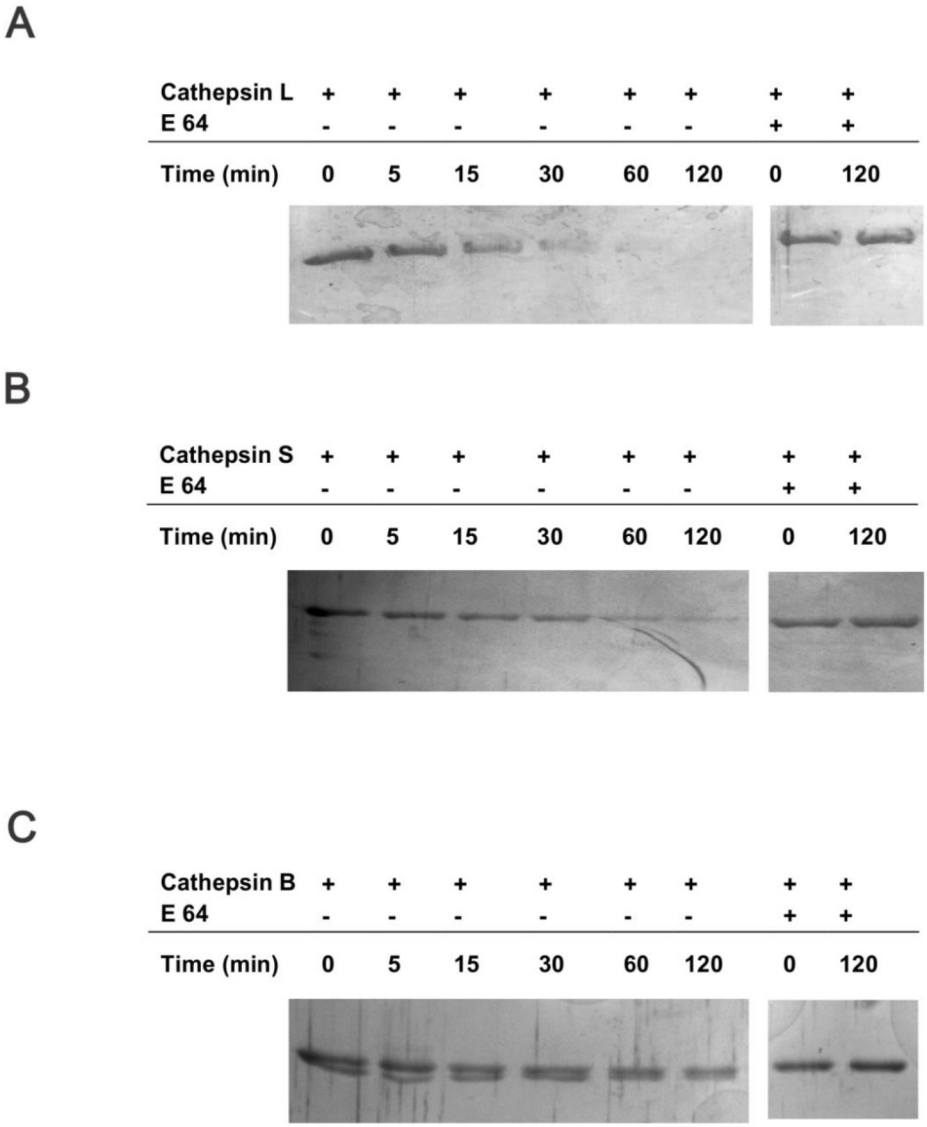


Figure 6: 14-3-3β protein is a target substrate for cathepsins L, S and B. *In vitro* assessment of proteolytic cleavage of 14-3-3β protein by cathepsins L, S and B. Human 14-3-3β protein was incubated at pH 6 and 37 °C with cathepsins **(A)** L, **(B)** S and **(C)** B and the level of 14-3-3β protein was evaluated at different time points in the presence or absence of E64.

(ROS)-mediated apoptosis [56, 57]. However, whereas no change in the proliferation of PyMT tumor cells *in vivo* and *in vitro* was detected in StfB-/- PyMT mice, a significant decrease in the Ki67 and BrdU proliferation index was measured in CstC knock-out PyMT primary tumors and isolated PyMT cells, indicating a potential role for cystatin C in the regulation of the proliferative activity of tumor cells. This hypothesis was confirmed by the decreased tumor growth in an orthotopic transplantation breast cancer model, providing additional evidence that the role of CstC is associated with tumor cells and is not related to stromal cell effects. Cystatin C was identified as a novel TGF-β type II receptor (TβR-II) antagonist that prevents the binding of this receptor to TGF-β and, consequently, inhibits TGF-β signaling in normal and malignant cells [38]. Subsequently, this observation was confirmed *in vivo*, demonstrating a reduction in tumor growth and metastasis upon use of the 4T1 mouse breast cancer cells overexpressing CstC [58]. Interestingly, while the increase in the phosphorylation and activation of p38 MAPK was detected in PyMT breast cancer models deficient for CstC, the corresponding increase in tumor cells proliferation, growth or metastasis was not observed. This finding suggests that the expression level of CstC was not sufficiently high enough in the PyMT model to overcome the threshold needed for phosphorylated p38 MAPK regulation of the expression of downstream genes [59]. This idea is supported by the significant decrease in CstC expression detected in the PyMT tumors compared to normal breast tissue (Figures 1A and B), providing evidence for the downregulation of CstC in the PyMT mammary tumor model. This observation is consistent with previously reported evidence of the decreased cystatin C expression in prostate cancer [34] and breast cancer [60] as well as during malignant progression in skin cancer [24]. Although elevated expression of another type 2 cystatin, cystatin M, was also found to be associated with malignant progression [61, 62], its expression was not detected by proteomic analysis in either PyMT cell lysates or conditioned tumor cell media, thereby excluding the possibility of the compensatory effect.

However, using a mass spectrometry-based approach, we identified a potential alternative mechanism for the regulation of the proliferative activity of tumor cells in the PyMT mammary cancer model through proteolytic degradation of 14-3-3 proteins. The 14-3-3 proteins comprise a family of highly conserved dimeric proteins in eukaryotic organisms and have seven isoforms in humans that play crucial roles in regulating multiple cellular processes, including cell growth, survival and differentiation [63]. Increased expression of 14-3-3 proteins was associated with tumor progression of various malignancies [64]. Furthermore, the association of 14-3-3 proteins with PyMT oncogene products has previously been reported and demonstrated as being dependent on phosphorylation at serine 257 [65, 66]. Among the 14-3-3 proteins, especially the 14-3-3β protein plays a vital role in tumor growth and progression. As such, 14-3-3β isoform expression was up-regulated in multiple types of cancer, such as Kaposi's sarcoma [67, 68], gastric cancer [69], papillary thyroid carcinoma [70], hepatocellular carcinoma [71, 72], lung cancer [73] and gliomas [74]. Studies have shown that 14-3-3β overexpression in primary hepatocellular carcinoma predicts a higher 5-year incidence of subsequent metastasis and correlates with a worse 5-year overall survival [71]. The extracellular function of 14-3-3β protein was demonstrated in human lung epithelial cells, leading to enhanced MMP-1 expression [75]. Although no MMP-1a, the mouse ortholog of human MMP1, was detected in the secretome of PyMT cells, other molecular pathways can be activated by the extracellular form of the 14-3-3β protein, promoting the progression of mammary cancer in the PyMT breast cancer model. Furthermore, 14-3-3β protein was shown to regulate the transcriptional activity of ERα in a ligand-dependent manner and to increases the breast cancer cell proliferation [76]. It was shown that overexpression of the 14-3-3ζ isoform promoted mammary tumorigenesis and progression via enhanced neoplastic cell proliferation in a transgenic breast cancer model [77]. Moreover overexpression of this isoform of the 14-3-3 protein correlated with the advanced stage breast cancer and was determined to be an independent prognostic factor for reduced disease-free breast cancer survival [78]. Importantly, we have demonstrated that cathepsins L, S and B can cleave the 14-3-3β protein, suggesting that CstC might protect the 14-3-3 proteins from degradation by cysteine cathepsins, thereby providing an additional explanation for the potential tumor suppressor role of these proteases.

Taken together, these results show that CstC depletion negatively affects tumor cell proliferation, resulting in the decreased growth of tumors in an orthotopic mouse model of breast cancer as a consequence of the complex interplay between cysteine cathepsins activity, cystatin C, 14-3-3 proteins and TGF-beta signaling pathways. Currently, most studies focusing on the inhibition of protease activity have examined the application of protease inhibitors and/or protease knockout models. However, few research programs are aimed at a better understanding of the excessive protease activities associated with an imbalance of the activities of endogenous inhibitors. Thus, despite the well-known role of cysteine cathepsin as promoters of tumor progression, the present study on the ablation their major extracellular inhibitor enabled us to uncover novel functions of cysteine cathepsins in the tumor microenvironment that should be considered when designing novel protease inhibition strategies for cancer treatment.

MATERIALS AND METHODS

Animals

Mice were used in accordance with the Administration of the Republic of Slovenia for food safety, veterinary and plant protection. Procedures for animal care and experiments were in accordance with the "Guide for the Care and Use in Laboratory Animals". Cystatin C (CstC-/-) knockout mice were established on C57BL/6 strain in 1999 [32]. The CstC-/- knockout mice on C57BL/6 strain were kindly provided by dr. Anders Grubb (Lund University, Lund, Sweden). CstC-/- mice were backcrossed for more than 10 generations to the FVB transgenic mouse strain expressing PyMT under the control of the MMTV long-terminal repeat promoter (FVB/N-TgN (MMTVPyVT)634-Mul). After the intercross, wild type and CstC-deficient tumor bearing mice were named PyMT;CstC+/+ and PyMT;CstC-/-, respectively. Maintenance and breeding of the animals used in this study were performed in accordance with Slovene law for animal protection as published in May 2013.

Tumor progression study

At 30 days of age, female mice were examined three times a week for the development of mammary tumors by palpation in a genotype-blinded fashion, and 30 days after detection of the first tumors, the diameters of the tumors were measured with a digital caliper. At the age of 14 weeks, the mice were euthanized, and all 10 tumors were excised, measured and weighted.

Immunohistochemistry and immunofluorescence staining

Left thoracic tumors of PyMT;CstC-/- and PyMT;CstC+/+ mice were collected and fixed in 10 % Neutral-buffered Formalin overnight and then processed for paraffin embedding. Formalin fixed, paraffin embedded samples of tumors were sectioned (5 µm) and affixed to glass slides. The slides were deparaffinized and rehydrated through graded alcohol to distilled water, followed by antigen retrieval with citrate buffer, pH 6.0. Endogenous peroxidase was quenched with 0.3 % Hydrogen Peroxide. The sections were stained with antibodies against CstC (Abcam, Cambridge, UK; ab 109508, rabbit monoclonal, dilution 1:500), Ki67 (Dako, Carpinteria, CA, USA; M724901, rabbit monoclonal, dilution 1:50), CD31 (BD Pharmingen, San Diego, CA, USA; 550274, rabbit monoclonal, dilution 1:50) and active caspase 3 (Abcam, Cambridge, UK; ab 2302, rabbit polyclonal, dilution 1:200). Dead cells in tumor sections were determined using the ApopTag Peroxidase In situ apoptosis detection Kit (Millipore, Billerica, MA, USA). Detection utilized the Vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA), and staining was visualized with DAB (Sigma-Aldrich, Steinheim, Germany). The slides were counterstained with hematoxylin, dehydrated, cleared and cover-slipped. The stained sections were imaged using an IX81 bright-field microscope (Olympus, Tokyo, Japan) and Imaging Software for Life Science Microscopy Cell. Tissue sections of each tumor were histologically examined using proliferation factor Ki67, cell death and tumor vascularization through CD31 as described [48]. For Ki67 data assessment, 20 fields per tumor were selected at random using the x40 objective and quantified using ImageJ software. For vascularization, samples were co-stained and mounted in SlowFade Gold antifade reagent with DAPI (Invitrogen, Carlsbad, CA, USA) and examined with a fluorescence microscope (Olympus IX 81, Olympus, Tokyo, Japan) with Imaging Software for Life Science Microscopy Cell.

For the volumetric measurement of lung metastases the paraffin-embedded lungs were cut into 5 µm thick sections from three different planes. The sections were stained with hematoxylin and eosin. The average sizes of metastasis were measured by computer-assisted measurement using Cellquest software (Olympus, Tokyo, Japan).

Isolation of primary tumor cells from pymT-induced mammary carcinomas

Primary PyMT tumor cells were isolated and cultured as described [2]. Only primary tumor cells with a low passage number (< 4 passages total) were used for experiments. All primary tumor cells were maintained in DMEM (Gibco, (Life Technologies), Paisley, UK), supplemented with 10 % fetal bovine serum (Sigma-Aldrich, St. Louise, MO, USA), 2 mM L-glutamine (Sigma-Aldrich, St. Louise, MO, USA), 20 U of penicillin and 20 µg/ml of streptomycin (Sigma-Aldrich, St. Louise, MO, USA). Cultured cells were maintained at 37˚C in a humidified 5 % CO_2 atmosphere.

Analysis of DNA synthesis using the BrdU incorporation method

The BrdU cell proliferation kit was purchased from EMD Millipore Corporation (Billerica, MA, USA). Primary tumor cells were cultured in 96-well plates at the density of 10^5 cells/ml. Cells were labeled with BrdU for 4 h, and levels of BrdU incorporation were determined according to the manufacturer's instructions using a Tecan Safire (Tecan, Gröding, Austria) microplate reader at a wavelength of 450 nm and subtracting absorbance measured at 540 nm.

Orthotopic transplantation

Primary tumor cells from three different PyMT;CstC+/+ and PyMT;CstC-/- female donors were isolated. The cells were counted, and equal amounts of

cells from each animal per genotype were mixed in serum-free medium to a concentration of 7.5 million cells/ml, and 200 µl of the cell suspension was injected into the mammary fat pad of left abdominal mammary gland of 8-week-old FVB;CstC+/+ and FVB;CstC-/- recipient female mice. A total of 4 groups were established: 8 FVB;CstC+/+ mice received PyMT;CstC+/+ cells, 7 FVB;CstC-/- mice received PyMT;CstC+/+ cells, 9 FVB;CstC+/+ mice received PyMT;CstC-/- cells, and 8 FVB;CstC-/- mice received PyMT;CstC-/- cells. Palpation and a tumor volume measurement were conducted at 14 days after the tumor cell injection and continuously repeated three times a week. At 35 days after tumor cell injection, the animals were euthanized, diameters of tumors were measured, and tumors were excised and weighed.

Cathepsin activity assay

The activity of cathepsins was detected by the hydrolysis of the fluorogenic substrate Z-Phe-Arg-4-methyl-coumarin-7-amide (Z-Phe-Arg-AMC, Bachem, Bubendorf, Switzerland). Mammary glands or primary tumors were homogenized in lysis buffer (250 mM TRIS, 10 mM EDTA 0.1 % Triton, pH 6.8), using an Ultrathurrax (IKA, Staufen, Germany). A total of 20 µg of each sample was mixed in a 96-well plate with 0.1 M phosphate buffer (1 mM EDTA, 0.1 % (v/v) PEG, 1 mM DTT, pH 6) in the presence or absence of cysteine cathepsin inhibitor E64 at a final concentration of 10 µM. Recombinant human cystatin C was produced as described [79] and was added to tumor lysates at a final concentration of 10 µM. After 15 minutes of incubation at 37 °C, substrate was added to a final concentration of 30 µM and its hydrolysis was continuously measured for 15 min using a Tecan Safire (Tecan, Gröding, Austria) plate reader at excitation and emission wavelengths of 370 and 460 nm, respectively.

When measuring the cathepsin activity in conditioned media of PyMT primary tumor cells, tumor cells of both genotypes were cultured in 12-well plates at a density of $7.5x10^5$ cells per well. The cells were incubated for 2 h at 37˚ C in PBS buffer (Lonza, Verviers, Belgium). After incubation, 50 µl of PBS was mixed in a 96-well plate with 0.1 M phosphate buffer, and the experiment was continued as described above.

Immunoblotting

Equal amounts of proteins of tumor tissue or mammary glands were resolved on 12.5 % SDS-PAGE electrophoresis gels and electrotransferred to polyvinylidene difluoride membranes. The blots were probed with rabbit anti-CstC antibodies (Abcam, Cambridge, UK; ab 109508, dilution 1:5000). For the detection of different mitogen- activated protein kinases, antibodies against JNK (Cell Signaling Technology, Danvers, MA, USA; 9252, rabbit polyclonal, dilution 1:1000), phosphorylated JNK (Cell Signaling Technology, Danvers, MA, USA; 9251, rabbit polyclonal, dilution 1:1000), ERK1&2 (Abcam, Cambridge, UK; ab17942, rabbit polyclonal, dilution 1:1000), diphosphorylated ERK1&2 (Sigma-Aldrich, St Louis, MO, USA; M8159, mouse monoclonal dilution 1:10000), p38 MAPK (Cell Signaling Technology, Danvers, MA, USA; 9212, rabbit polyclonal, dilution 1:1000), phosphorylated p38 MAPK (Cell Signaling Technology, Danvers, MA, USA; 9211, rabbit polyclonal, dilution 1:1000), cathepsin L (prepared in-house; rabbit polyclonal, dilution 1:1000) [80]), cathepsin S (R&D Systems, Minneapolis, MN, USA; AF 1183, goat polyclonal, dilution 1:2000), cathepsin B (prepared in-house; mouse polyclonal, dilution 1:1000) [81] and legumain (Abcam, Cambridge, UK; ab183028) were used. For loading control anti-β-actin antibody (Sigma-Aldrich, St Louis, MO, USA; A1978, mouse monoclonal, dilution 1:5000) was used. Secondary antibodies were used at 1:5000 dilutions. Tissue lysates were prepared as previously described [82].

Quantitative real-time PCR

Total RNA was isolated from flash-frozen PyMT mammary tumors with an PureLink RNA Mini kit (Ambion, Life Technologies, Foster City, CA, USA) and treated with TURBO DNA-free kit (Ambion, Life Technologies, Foster City, CA, USA) for genomic DNA elimination. RNA quality (A260/230 and A260/280 ratios) was assessed spectrophometrically, whereas RNA integrity was confirmed by 1.2% gel electrophoresis. RNA was further reverse transcribed into cDNA with a random nanomer primers using Precision nanoScript 2 Reverse Transcription kit (Primerdesign Ltd, Southampton, UK). mRNA expression levels of cathepsin B, cathepsin H, cathepsin L, cathepsin S and cathepsin X were detected by SYBR green detection chemistry using 2 X Precision MasterMix (Primerdesign Ltd). Eukaryotic translation initiation factor 4A2 (EIF4A2) was selected as the most stable expressed reference gene by geNorm reference gene selection kit (Primerdesign Ltd), and normalized values were used to calculate the relative mRNA expression of individual genes. Quantitative real-time PCR reaction was run on Mx3005P Real-time PCR system (Agilent, Stratagene products, Waldbronn, Germany) using the following conditions: 95 °C for 10 min (an initial denaturation step) followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min (annealing and extension). In addition, a melting curve (55–95 °C) was performed at the end of each run. The relative expression ratios of target genes were calculated considering their real-time PCR efficiencies. Intron spanning gene-specific primers (Primerdesign Ltd) were as follows: cathepsin B, 5'-TACTTGCTGTGGTATCCAGTGTG-3' (forward) and 5'-ATGGTGTATGGTAAGCAGCCTAC-3' (reverse);

cathepsin H, 5'-TTTGATGTATAAAAGTGGCGTCTA-3' (forward) and 5'-GAGTTTTTCACAATCCAGTAG AGT-3' (reverse); cathepsin L, 5'-AAGCCATCCGTC TCTCCAG-3' (forward) and 5'-CGTTTCTTGACA AGCCAATATTTATTC-3' (reverse) cathepsin S, 5'-CTGTTCTTGACAAGCCAATATTTATTC-3' (forward) and 5'-AGCCAGTAATCTTTGCCATCA-3' (reverse); and cathepsin X, 5'-CTATGCCAATGGTCCCATCAG-3' (forward) and 5'-CCTGGTCCTGGTGCTCAG-3' (reverse).

Proteomic analysis of PyMT conditioned medium

Conditioned medium of primary PyMT tumor cells was generated by 24-hour incubation of cells in serum-free medium (Gibco, (Life Technologies), Paisley, UK). The obtained conditioned medium was subsequently concentrated 100-fold using Centricon filter units with 3000 Da cut-off (Millipore, Billerica, MA, USA). The samples were separated on a 12.5 % SDS-PAGE gel (Lonza, Verviers, Belgium). The gel was stained with Coomassie Brilliant Blue, and each protein lane was cut into nine samples and destained using 25 mM NH_4HCO_3 (Fluka Biochemica, Buchs, Switzerland) in 50 % acetonitrile (JT Baker). Gel separated proteins were reduced with 10 mM DTT in 25 mM NH4HCO3 (Fluka Biochemica, Buchs, Switzerland), followed by alkylation with 55 mM iodoacetamide in the same buffer. Gel pieces were washed in 25 mM NH_4HCO_3 (Fluka Biochemica, Buchs, Switzerland) and dehydrated with acetonitrile. Trypsinization was performed overnight at 37 °C by the addition of 1 µg of trypsin (Promega, WI, Madison, USA) diluted in 80 µl of 25 mM NH_4HCO_3. Digested peptides were extracted from the gel with 50 % acetonitrile/ 5 % formic acid. The samples were analyzed with LTQ Orbitrap Velos (Thermo Fisher Scientific, Waltham, MA, USA) mass spectrometer coupled to a Proxeon-nanoLC. Peptides were loaded on a C18 EASY trapping column (Proxeon EASY-Column™; Thermo Fisher Scientific, Waltham, MA, USA) and separated on a C18 PicoFrit AQUASIL analytical column (New Objective, Woburn, MA, USA) at a flow rate of 300 nl/min. Elution was performed with a 90 min acetonitrile gradient from 5 % to 40 %. Nine most intense precursor ions in each full scan were selected for CID fragmentation. Dynamic exclusion was set at a repeat count of 1 with exclusion duration of 60 s. Database searches were performed with MaxQuant software, version 1.5.3.8 [83], using UniProtKB derived mouse reference proteome. The carbamidomethylation of cysteine was fixed, and the oxidation of methionine was set as a dynamic modification. Relative quantification of identified proteins was performed using spectral counting where proteins with at least 1.5-fold difference in spectral count and at least 10 detected spectra were considered as significantly different [84].

In vitro cleavage of recombinant human 14-3-3β-protein with recombinant human cathepsins L, S and B

Recombinant human cathepsins L, S and B were expressed in the methylotrophic yeast expression system *P. pastoris* (Invitrogen, Carlsbad, CA, USA) or in the *E. coli* expression system according to standard protocols [85, 86]. Pure mature proteins were titrated with the broad spectrum cysteine cathepsin inhibitor E-64 yielding active concentrations of 120 µM, 48 µM and 96 µM for cathepsin B, L and S, respectively. The experiment was performed in phosphate buffer, containing 100 mM Na_2HPO_4, 1 mM EDTA and 1 mM DTT, pH 6.0. Recombinant cathepsins were added to the 14-3-3β-protein (AbFrontier, Seul, Korea) at 1:100 enzyme/substrate molar ratio. Samples were then incubated at 37°C up to 120 min. As a negative control, E64 at a final concentration of 20 µM was added to block cathepsin activity. Samples were ran on 12.5 % SDS-PAGE electrophoresis gels and silver stained as described [87].

Statistical analysis

Quantitative data are presented as the means ± S.E. The differences were compared using Student's t-test. Proportions were compared using the Chi-square test. When P-values were $p<0.05$, differences were considered statistically significant.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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