mgr Mateusz Gielata

Charakterystyka inwazyjnych komórek raka piersi z wykorzystaniem nowatorskiego systemu reporterowego alfa katuliny.

Rozprawa na stopień doktora nauk medycznych i nauk o zdrowiu

w dyscyplinie nauki medyczne

Promotor: **dr hab. Agnieszka Kobielak prof. ucz.**

Centrum Nowych Technologii

Uniwersytet Warszawski

Obrona rozprawy doktorskiej przed Radą Dyscypliny Nauk Medycznych

Warszawskiego Uniwersytetu Medycznego

Warszawa 2023 r.

Słowa kluczowe: potrójnie ujemny nowotwór piersi, alfa katulina, przerzutowanie, tranzycja epitelialno-mezenchymalna, MCAM, komórki inwazyjne, mimikra naczyniopodobna

Keywords: triple negative breast cancer, alpha-catulin, metastasis, epithelial to mesenchymal transition, MCAM, invasive cells, vascular mimicry

Najszczersze podziękowania kieruję do mojej promotor dr hab. Agnieszki Kobielak za nieocenione wsparcie, cenne uwagi, ogrom wiedzy przekazanej w trakcie wykonywania badań i cudowną atmosferę pracy.

Pragnę również podziękować wszystkim obecnym i byłym członkom Laboratorium Biologii Molekularnej Nowotworów w Centrum Nowych Technologii UW za pomoc, wsparcie oraz wspaniałą atmosferę pracy. Szczególne podziękowania kieruję do dr Kamili Karpińskiej, z którą wspieraliśmy się i współpracowaliśmy przez cały okres trwania doktoratu.

Pracę dedykuję mojej ukochanej żonie Alicji i Rodzicom, bez Was nie byłoby mnie tutaj dzisiaj.

Spis treści

Wykaz publikacji stanowiących pracę doktorską

1. Gielata M, Karpińska K, Pieczonka T, Kobielak A. **Emerging Roles of the α-Catenin Family Member α-Catulin in Development, Homeostasis and Cancer Progression.** Int J Mol Sci. 2022 Oct 8;23(19):11962. doi: 10.3390/ijms231911962. PMID: 36233261; PMCID: PMC9569426. Praca przeglądowa.
   Impact Factor: 6,208; Punkty ministerialne: 140
2. Gielata M, Karpińska K, Gwiazdowska A, Boryń Ł, Kobielak A. **Catulin reporter marks a heterogeneous population of invasive breast cancer cells with some demonstrating plasticity and participating in vascular mimicry.** Sci Rep. 2022 Jul 25;12(1):12673. doi: 10.1038/s41598-022-16802-2. PMID: 35879327; PMCID: PMC9314412. Praca oryginalna.
   Impact factor: 4,997 ; Punkty ministerialne: 140

Sumaryczny IF = 11,205

Sumaryczne punkty ministerialne = 280

Finansowanie badań

Niniejsza rozprawa doktorska została sfinansowana ze środków pochodzących z następujących projektów:

1. Preludium 19, NCN; Charakterystyka wolno krążących komórek nowotworowych raka piersi z wykorzystaniem sekwencjonowania RNA pojedynczych komórek.
   Nr projektu: 2020/37/N/NZ5/03069
   Kierownik projektu: mgr Mateusz Gielata

2. Opus 19, NCN; Transkrypcyjna i funkcjonalna charakterystyka inwazyjnych komórek raka piersi izolowanych przy użyciu nowatorskiego systemu reporterowego *in vivo*.
   Nr projektu: 2020/37/B/NZ5/03950
   Kierownik projektu: dr hab. Agnieszka Kobielak prof. ucz.

Wykaz stosowanych skrótów

ER – receptor dla estrogenu (ang. Estrogen Receptor)

PR – receptor dla progesterone (ang. Progesterone Receptor)

TNBC – potrójnie ujemny nowotwór piersi (ang. Triple Negative Breast Carcinoma)

CTNNAL1 – gen kodujący alfa katulinę (ang. Alpha-catenin-like 1, alpha-catulin gene)

CD146 – cząsteczka adhezyjna komórek czerniaka (ang. MCAM, melanoma cell adhesion molecule)

HNSCC – nowotwory głowy i szyi wywodzące się z tkanki nabłonkowej (ang. Head and Neck Squamous Cell Carcinoma)

EMT – przejście epitelialno-mezenchymalne (ang. Epithelial-to-Mesenchymal Transition)

MET – przejście mezenchymalno-epitelialne (ang. Mesenchymal-to-Epithelial Transition)

VM – mimikra naczyniopodobna (ang. Vascular Mimicry)

CSC – nowotworowe komórki macierzyste (ang. Cancer Stem Cells)

BCSC – komórki macierzyste nowotworu piersi (ang. Breast Cancer Stem Cells)

Her2 – receptor ludzkiego naskórkowego czynnika wzrostu 2 (ang. Human Epidermal Growth Factor Receptor 2)

IKK-β – ang. inhibitor of nuclear factor kappa-B kinase subunit beta

SNAIL1/2 – ang. Zinc finger protein SNAI1/2

ZEB1/2 – ang. Zinc Finger E-Box Binding Homeobox 1/2

TGF-β – transformujący czynnik wzrostu beta (ang. Transforming Growth Factor beta)

VEGF-1 – czynnik wzrostu śródbłonka naczyniowego (ang. Vascular Endothelial Growth Factor)

ECM – macierz pozakomórkowa (ang. Extracellular Matrix)

HGF – czynnik wzrostu hepatocytów (ang. Hepatocyte Growth Factor)

RNAseq – sekwencjonowanie RNA nowej generacji (ang. Next Generation RNA sequencing)

α-SMA – białko aktyny (ang. Alpha Smooth Muscle Actin)

Streszczenie w języku polskim

Rak piersi jest obecnie najczęściej diagnozowanym nowotworem u kobiet na świecie. Spośród różnych podtypów to potrójnie negatywny nowotwór piersi ma najniższą średnią przeżywalność z powodu bardzo często występujących przerzutów i braku dostępnej terapii celowanej. EMT jest procesem odgrywającym kluczową rolę podczas rozwoju zarodkowego, jednakże rola tego procesu została również potwierdzona w progresji nowotworowej i procesie przerzutowania. Obecnie trwa dyskusja nad nie binarnością tego zjawiska, wydaje się, że komórki mogą występować nie tylko w formie epitelialnej lub mezenchymalnej, a w wielu stanach pośrednich charakteryzujących się cechami macierzystych komórek nowotworowych. W przypadku raka piersi utrata adhezji międzykomórkowej jest kluczowa dla rozpoczęcia procesu przerzutowania. W trakcie trwania EMT komórki obniżają ekspresję apikalnych oraz bazopodstawnych, epitelialno specyficznych białek, co skutkuje wzrostem ekspresji białek typowo mezenchymalnych. Taka tranzycja skutkuje zwiększoną ruchliwością komórek. Zaobserwowane zostało, że obniżenie ekspresji białka epitelialnego α-kateniny korelowało ze wzrostem ekspresji białka α-katuliny i przejściem epitelialno mezenchymalnym. Ponadto ekspresja α-katuliny była zwiększona w komórkach na froncie inwazyjnym nowotworu płaskonabłonkowego głowy i szyi. Jednakże funkcja α-katuliny w progresji nowotworów piersi nie była do tej pory badana.

W niniejszej pracy doktorskiej na początek skupiłem się na przeglądzie literaturowym na temat słabo poznanego białka α-katuliny. Jest to białko należące do super rodziny białek winkuliny. W pracy tej wykazałem wysoką homologię sekwencji jak i struktury pomiędzy α-katuliną, α-kateniną i winkuliną. Opisałem ponadto bardzo ograniczoną, ale jednocześnie znaczącą listę białek, które wchodzą w interakcje z α-katuliną jak Lbc Rho GEF, dystrobrewina, IKK-β czy kinaza NEK. α-katulina pełni istotną funkcję w stanach zapalnych, odporności na apoptozę, starzeniu komórkowym a także w reorganizacji cytoszkieletu komórkowego, progresji nowotworów i EMT. Dodatkowo α-katulina odgrywa kluczową rolę w trakcie rozwoju embrionalnego, ponieważ jest niezbędna dla prawidłowej neurulacji. W pracach naukowych udowodniono podwyższony poziom ekspresji α-katuliny w raku piersi, płuc czy prostaty. Pomimo licznych doniesień

opisujących udział α-katuliny w migracji i inwazji komórek nowotworowych, mechanizm molekularny prowadzący do takiego fenotypu jest nadal bardzo słabo poznany.

W mojej drugiej oryginalnej publikacji wykazałem, że ekspresja α-katuliny zachodzi w ludzkich tkankach nowotworu piersi i poziom tej ekspresji koreluje z zaawansowaniem choroby nowotworowej. Ponadto kiedy wyciszyłem ekspresję α-katuliny w komórkach nowotworowych zaobserwowałem spadek potencjału inwazyjności komórek w modelu trójwymiarowym. Następnie rozwinąłem nowy system reporterowy katuliny w celu śledzenia i możliwości izolacji najbardziej inwazyjnych komórek raka piersi, a następnie możliwości poddania ich analizie sekwencjonowania RNA. Wysoka ekspresja α-katuliny koreluje z ekspresją genów zaangażowanych w migrację komórkową, a także może być odpowiedzialna za modulację właściwości adhezyjnych komórek nowotworowych i ich interakcję z naczyniami krwionośnymi. Barwienia immunohistochemiczne guzów ksenograficznych potwierdziły wyniki otrzymane przez sekwencjonowanie RNA. Dzięki temu wykazałem, że system reporterowy katuliny oznacza nie tylko inwazyjne komórki nowotworowe, ale także rzadką populację wysoce plastycznych komórek nowotworowych, które eksprymują endotelialne markery takie jak MCAM i uczestniczą w mimikrze naczyniowej. Udział inwazyjnych nowotworowych komórek o podwyższonej ekspresji katuliny, w tworzeniu struktur przypominających naczynia krwionośne umożliwiadostawę substancji organicznych do wnętrza guza i prawdopodobnie poprzez ułatwianie wnikania komórek nowotworowych do krwioobiegu może początkować proces przerzutowania. Ponadto wykazałem, że wyciszenie ekspresji α-katuliny w komórkach nowotworowych wpływało na ich zmniejszony potencjał inwazyjności i zmniejszoną macierzystość nowotworową poprzez deregulację ekspresji markera macierzystości komórek raka piersi – CD44.

Wykorzystując system reporterowy katuliny udało mi się wyizolować i scharakteryzować plastyczne, wysoce inwazyjne komórki nowotworowe biorące udział w mimikrze naczyniopodobnej i przerzutowaniu. α-katulina odgrywa kluczową rolę w interakcji komórek nowotworowych i mikrośrodowiska, modulując właściwości adhezyjne komórek. Zwiększona plastyczność komórek nowotworowych, wysoce eksprymujących α-katulinę może być konieczna do tranzycji w struktury endotelialne, udział w tworzeniu mimikry naczyniopochodnej i finalnie w przerzutowaniu komórek nowotworowych. W mojej pracy doktorskiej wyraźnie wykazałem iż α-katulina jest kluczowa dla potencjału

nowotworzenia i inwazyjności komórek raka piersi. Jest ona markerem specyficznym charakteryzującym plastyczne komórki nowotworowe przechodzące przez stany pośrednie EMT. Poznanie pełnej złożonej biologii białka α-katuliny może prowadzić do poznania nowych skutecznych celów terapeutycznych w różnych typach nowotworów.

Abstract

Characterization of highly invasive breast cancer cells using novel catulin reporter system

Breast cancer is currently the most widely diagnosed cancer in women worldwide. Triple negative breast cancer has the poorest survival ratedue to high metastatic potential and no targeted treatment is available at this time. EMT is a process playing a pivotal role during development, however it is also associated with cancer progression and metastasis. It has been implicated that this phenomenon is non-binary, meaning that there may be some interstates of cells between epithelial and mesenchymal features possessing cancer stem-like properties. In breast cancer progression, loss of intracellular adhesion is crucial for metastatic disease. In the process of EMT, progressive downregulation of apical and basolateral, epithelial specific proteins is accompanied by re-expression of mesenchymal specific proteins. The switch results in increased motility of the cells. It was observed that decrease in the expression of the epithelial protein α-catenin correlated with an increase in the expression of the α-catulin protein and EMT. In addition, the expression of α-catulin was increased in the cells at the invasive front of head and neck squamous cell carcinoma. However, the function of α-catulin in the progression of breast cancer has not been studied so far.In this dissertation, I first focused on reviewing the published data regarding poorly described protein α-catulin. It is a member of the vinculin superfamily proteins. I showed high homology in sequence between α-catulin, α-catenin and vinculin. I described the limited yet representative number of binding partners of α-catulin like Lbc Rho GEF, dystrobrevin, IKK-β, NEK kinase. α-catulin has been shown to be important in inflammation, apoptotic resistance, cytoskeletal reorganization, senescence resistance, cancer progression, and EMT. α-catulin plays a pivotal role during embryonic development as it is necessary for proper neurulation. In cancer progression α-catulin has been shown to be upregulated in highly invasive breast, lung, and prostate carcinoma. Despite multiple reports describing α-catulin as an important factor contributing to cancer cell migration and invasion, the exact molecular mechanism leading to this phenotype remains unclear.

In my second publication, I showed that α-catulin is expressed in human breast cancer samples, and its expression correlates with the cancer progression. Moreover, when I knocked down α-catulin, invasive potential of cells in 3D model was highly decreased. Then I developed a novel reporter catulin system to track highly invasive breast cancer

cells, and I was able to isolate those cells from xenograft transplants and perform RNAseq analysis. Expression of catulin in the invasive cancer cells correlated with the expression of genes involved in migration, and also could be important in the modulation of adhesive properties of cancer cells and their interactions with the vasculature. Immunohistochemical staining of xenographic tumors confirmed the results obtained by RNA sequencing. Thus, I demonstrated that the catulin reporter system not only labels invasive tumor cells, but also a rare population of highly plastic tumor cells that express endothelial markers such as MCAM and participate in vascular mimicry.Participation of catulin expressing tumor cells in forming vascular structures enables a flow of nutrients to the tumor mass and may facilitate the entry of cancer cells into the vascular system leading to the metastatic spread. When simultaneously knocking down α-catulin I observed modulation of adhesive properties of tumor cells and a high decrease in the invasive and cancer stemness potential, what resulted in decreased breast cancer stemness marker – CD44. It means that by utilizing a novel catulin reporter system, I could isolate and characterize highly invasive breast cancer cells of high plasticity that participate in vascular mimicry and metastasis.

Concluding, α-catulin may play a pivotal role in the interaction between cancer cells and the microenvironment, modulating the adhesive properties of cancer cells. Increased plasticity of cancer cells highly expressing α-catulin may be of high demand for the cells to change into endothelial like and participate in vascular mimicry and then in metastasis. In this dissertation, I clearly show that α-catulin is crucial in breast cancer tumorigenesis and the invasiveness process. It is clearly a specific marker of plastic cancer cells undergoing different intermediate EMT states. Deciphering the complex nature of α-catulin may lead to finding potentially novel therapeutic targets in various types of carcinomas.

Wstęp

Nowotwory piersi – ich charakterystyka i klasyfikacja

Rak piersi jest najczęściej występującym typem nowotworów u kobiet na świecie [1]. Jest odpowiedzialny za 23% przypadków spośród wszystkich typów nowotworów [2]. Typowymi objawami są wyczuwalne palpitacyjnie grudki guzowate w piersi, zmiana barwy skóry na piersi, wysięk sutkowy, zmiana kształtu całej piersi [3]. Głównymi czynnikami ryzyka zachorowania na tę chorobę są: czynniki genetyczne takie jak np. mutacje w genach *BRCA1, BRCA2* i inne, płeć, wiek, brak karmienia piersią, czynniki hormonalne endogenne i egzogenne, otyłość i styl życia przede wszystkim nadmierne spożycie alkoholu i palenie papierosów [4]. Klasyfikacja nowotworów piersi jest bardzo złożona i składa się z oceny histopatologicznej, w której ocenia się czy dany nowotwór jest luminalny czy bazalny, naciekający czy nienaciekający, stopień zróżnicowania komórek nowotworowych względem komórek normalnych i klasyfikacji TNM, w której ocenia się wielkość guza, zajęcie okolicznych węzłów chłonnych i występowanie przerzutów [5]. Innym rodzajem klasyfikacji jest określenie statusu receptorowego komórek nowotworowych. Wyróżniamy nowotwory ER pozytywne i/lub PR pozytywne, Her2 pozytywne i nowotwór potrójnie ujemny, gdzie komórki nie wykazują ekspresji żadnego z powyższych markerów. O ile w przypadku komórek ER/PR/Her2 pozytywnych istnieje szereg terapii celowanych wykorzystujących przeciwciała monoklonalne, to w przypadku nowotworu potrójnie ujemnego, który stanowi ok 20% wszystkich przypadków, nie ma dostępnej żadnej terapii, stąd przeżywalność z tym typem nowotworu jest najniższa [6]. Podtyp potrójnie ujemny według klasyfikacji molekularnej pokrywa się z typem bazalnym, jednakże komórki te są dużo mniej zróżnicowane, dużo bardziej agresywne i często mają mutację w genie *BRCA1* [7]. O ile umiejscowienie guza pierwotnego bardzo ułatwia możliwość całkowitej eliminacji masy guza poprzez mastektomię, to zgony następują najczęściej na skutek częstych nawrotów choroby i bardzo wysokiej częstotliwości przerzutowania do kości, wątroby, płuc i mózgu [8]. Jedyną opcją leczenia potrójnie negatywnego raka piersi jest obecnie resekcja guza i ogólna chemioterapia, a częstotliwość występowania przerzutów jest nadal bardzo wysoka [9], dlatego ważne jest lepsze zrozumienie biologii potrójnie ujemnego nowotworu piersi, a także lepsze poznanie molekularnego podłoża procesu przerzutowania komórek nowotworowych.

Rola EMT w rozwoju, regeneracji i progresji nowotworów

EMT jest procesem komórkowym, w którym dochodzi do zmiany fenotypu i zachowania komórek z epitelialnego na mezenchymalny. Tranzycja ta jest często odpowiedzią na sygnały pochodzące z mikrośrodowiska [10]. Komórki w stanie epitelialnym charakteryzują się stabilnymi połączeniami międzykomórkowymi, polaryzacją wierzchołkowo-podstawną i interakcjami z błoną podstawną. Podczas EMT dochodzi do deregulacji ekspresji różnych genów i modulacji post-translacyjnych, które prowadzą do obniżenia ekspresji markerów epitelialnych, co prowadzi do zwiększonej ekspresji genów charakterystycznych dla stanu mezenchymalnego [11]. Komórki te charakteryzuje tylno-przednia polaryzacja, morfologia podobna do fibroblastów i zwiększona ruchliwość komórkowa [12]. Po raz pierwszy EMT zostało opisane przez naukowców badających wczesną embriogenezę [13]. Już na bardzo wczesnym etapie rozwoju zarodkowego, bo już na etapie implementacji zarodka i tworzenia się łożyska można zaobserwować proces EMT. Komórki trofoektodermy, będące prekursorami cytotrofoblastu, poddawane są EMT w celu ułatwienia inwazji endometrium i późniejszego prawidłowego zakotwiczenia łożyska, umożliwiając jego funkcję w wymianie składników odżywczych i gazowych [14]. Komórki przechodzą EMT w celu przemieszczenia się i migracji w trakcie procesów tworzenia nowego organizmu. Docierając do miejsca docelowego przechodzą natomiast proces odwrotny MET gdzie z powrotem nabierają cech epitelialnych i są w stanie nawiązać interakcje z błoną podstawną [15]. To tylko jeden z przykładów udziału EMT w rozwoju zarodkowym. Późniejsza gastrulacja również związana z procesem EMT jest kontrolowana przez kanoniczną ścieżkę sygnałową Wnt [16]. EMT w rozwoju zarodkowym to jeden z trzech podtypów EMT. Innym typem EMT jest to związane z regeneracją, gojeniem ran i zwłóknieniem organów. Zdarzenia EMT typu drugiego występują jako część procesu związanego z naprawą, w którym komórki nabłonkowe różnicują się w nowe komórki podobne do fibroblastów w celu odbudowy tkanek po urazie i stanie zapalnym. Komórki te wykazują morfologię specyficzną dla nabłonka i markery molekularne, takie jak cytokeratyna i E-kadheryna, ale jednocześnie eksprymują białko supresorowe ferroptozy 1, markery mezenchymalne i α- SMA. EMT typu drugiego jest związane ze stanem zapalnym i kończy się po jego redukcji [17]. Zwłóknienie natomiast pojawia się w wielu organach jako patologiczny mechanizm naprawy na skutek chronicznego stanu zapalnego, gdzie EMT doprowadza do powstania

miofibroblastów z komórek nabłonkowych i ich nadmiernej akumulacji prowadząc do zaburzenia prawidłowego funkcjonowania wątroby, płuc czy nerek [18]. Trzecim typem EMT jest ten związany z progresją nowotworów. Epitelialne komórki nowotworowe tracą polaryzację i połączenia komórkowe, nabierając mezenchymalnych właściwości, ze zwiększoną ruchliwością i inwazyjnością [19]. Komórki w stanie epitelialnym eksprymują E-kadheryne, cytokeratyny, okludynę i klaudynę, przechodząc EMT ekspresja tych białek zanika, a pojawia się zwiększona ekspresja wimentyny, N-kadheryny czy fibronektyny. Wimentyna jest eksprymowana na wysokim poziomie w wielu nowotworach nabłonkowych, w tym raku piersi, raku prostaty, czerniaku i raku płuc, a jej ekspresja jest determinantem wzrostu guza, inwazji i złego rokowania [20]. Proces EMT jest kontrolowany przez kaskadę czynników transkrypcyjnych [21]. Najbardziej poznanymi regulatorami EMT są TWIST1, TWIST2, SNAIL1, SNAIL2, ZEB1, i ZEB2. Hamują one ekspresję E-kadheryny i promują w ten sposób przejście do stanu mezenchymalnego [22]. Ponadto ścieżka sygnałowa TGF-β/Smad również odgrywa kluczową rolę w indukcji EMT, zwłaszcza w nowotworach piersi. TGF-β oddziałuje także na inne ścieżki sygnałowe jak Notch, Wnt/β-katenina, NF–κB, i RTK w celu podtrzymania mezenchymalnego fenotypu agresywnych komórek raka piersi [23]. Istnieje coraz więcej dowodów na to, że tylko niewielka część komórek najbardziej agresywnych może przetrwać i tworzyć przerzuty. Ta subpopulacja komórek jest zdolna do wzrostu, inwazji i samo odnawiania się, a zatem wykazuje właściwości komórek macierzystych, stąd nazywane są one nowotworowymi komórkami macierzystymi (CSC) [24]. CSC są w stanie przetrwać nawet po usunięciu pierwotnego guza. Może to być spowodowane mutacjami i deregulacją szlaków epigenetycznych, które sprzyjają przeżyciu i mogą wynikać z oddziaływań mikrośrodowiska, które wymusza ich ewolucję genetyczną. Mikrośrodowisko guza może dostarczać sygnałów regulujących samoodnowę, EMT i procesy homeostatyczne takie jak stan zapalny, niedotlenienie i angiogeneza, które regulują wejście CSC w stan uśpienia, albo promowanie reaktywacji CSC, które inicjują przerzuty [25]. W przypadku nowotworów piersi BCSC do tej pory charakteryzowane były jako CD44+/CD24- i ALDH1 pozytywne [26]. Jednakże lepsze poznanie natury BCSC jak i natury procesu EMT doprowadziły do konkluzji, że jest to proces niebinarny i komórki mogą występować w wielu różnych formach hybrydowych EMT [27]. Ta plastyczność komórek nowotworowych jest procesem kluczowym w kontekście agresywności i przerzutowania nowotworów, stąd wymaga dokładniejszego zbadania i charakterystyki.

Unaczynienie nowotworów i mimikra naczyniopodobna

Rozrastająca się masa guza pierwotnego doprowadza do stanu niedotlenienia i głodu komórek znajdujących się w jej wnętrzu. Limitowane unaczynienie wnętrza guza pierwotnego ogranicza dostęp zarówno składników odżywczych niezbędnych dla poprawnego metabolizmu komórek nowotworowych jak i tlenu. Dawniej uważano, że nowotwór pierwotny, aby przetrwać indukuje w mikrośrodowisku angiogenezę wtórną, gdzie nowe naczynia rozrastają się z już obecnych naczyń krwionośnych w samej masie guza lub w bliskim sąsiedztwie [28]. Próby leczenia nowotworów inhibitorami specyficznymi dla komórek śródbłonka naczyń okazały się jednak mieć ograniczone rezultaty. To skłoniło naukowców do myślenia, że musi istnieć inny mechanizm pozwalający guzom pierwotnym dostarczyć niezbędne składniki odżywcze. Już w 1999 roku pokazano, że komórki nowotworowe same potrafią stworzyć struktury przypominające naczynia krwionośne, jednakże zupełnie przy braku udziału komórek endotelialnych [29]. Proces ten został nazwany mimikrą naczyniopodobną (VM), jest niezależny od angiogenezy i składa się z komórek nowotworowych i błony podstawnej [30]. Na podstawie badań nad rakiem piersi udowodniono, że struktury naczyniopodobne złożone wyłącznie z komórek nowotworowych są w stanie połączyć się z pobliskim endogennym unaczynieniem i tworząc mikrocyrkulację w skuteczny sposób zaopatrują guz pierwotny w niezbędne substancje odżywcze [31]. Wraz ze wzrastającą wiedzą na temat nowotworowych komórek macierzystych, koncept CSC i mimikry naczyniopodobnej zaczęły się ze sobą łączyć. Dobrym przykładem są badania nad nowotworami piersi, gdzie wykazano, że po wstrzyknięciu do myszy NOD.Scid komórek macierzystych ludzkiego raka piersi, unaczynienie wewnątrz guza było ludzkiego pochodzenia, co potwierdza udział komórek CSC w tworzeniu struktur naczyniopodobnych [32]. Również w badaniach nad glejakami wykazano, że komórki macierzyste glejaka, charakteryzujące się fenotypem CD133+, są w stanie różnicować do komórek endotelialnych [33]. Wiemy również, że ścieżka sygnałowa VEGF-1 jest zaangażowana i promuje proces mimikry naczyniopodobnej [34]. Jako, że teoria o powstawaniu CSC jest bardzo blisko powiązana z procesem EMT, jego udział również został potwierdzony w tworzeniu się struktur naczyniopodobnych w guzie nowotworowym. Komórki tworzące VM wysoce eksprymują ZEB1 co prowadzi do obniżonej ekspresji E-kadheryny i nadekspresji wimentyny w tych komórkach, a wyłączenie ekspresji Twist1 prowadzi do zahamowania procesu VM [35,

36]. Dlatego podsumowując, wiele badań potwierdza, że EMT jest niezbędne do indukcji komórek epitelialnych guza pierwotnego w kierunku komórek mezenchymalnych, a co za tym idzie niezróżnicowanych nowotworowych komórek macierzystych. EMT indukując proces tworzenia CSC jest włącznikiem procesu mimikry naczyniopodobnej. W procesie tym również ważny jest fakt występowania warunków beztlenowych wewnątrz guza i udział czynnika HIF-1a w procesie VM, a także regulacja CSC i EMT poprzez szlaki sygnałowe NF-κB, PI3K/Akt/mTOR, Notch, Wnt/β-katenina i Hedgehog [37]. Jako, że leki hamujące powstawanie nowych naczyń krwionośnych mają niewystarczające działanie to globalnie wykorzystanie wiedzy na temat EMT i CSC jest postulowane jako przyszłość terapii przeciwnowotworowych.

Alfa-Katulina i uzasadnienie połączenia publikacji w jeden cykl

Obniżony poziom ekspresji E-kadheryny i Alfa-Kateniny jest często obserwowany w litych guzach pierwotnych i w większości przypadków może być markerem ostrego przebiegu choroby nowotworowej z bardzo złym rokowaniem [38, 39]. W laboratorium wykonywania niniejszej pracy doktorskiej już wcześniej zaobserwowano, iż spadek ekspresji alfa-kateniny koreluje ze wzrost ekspresji nowego homologu alfa-kateniny – alfa-katuliny [40]. Zauważono również, że ekspresja katuliny jest najwyższa na inwazyjnym froncie nowotworu w modelu HNSCC, a jej podwyższona ekspresja korelowała z przejściem komórek ze stanu epitelialnego do mezenchymalnego. Ponadto wyciszenie ekspresji katuliny w komórkach HNSCC drastycznie zmniejsza potencjał komórek do migracji i inwazji zarówno w modelu *in vitro* jak i *in vivo* [41]. Alfa katulina jest białkiem bardzo słabo poznanym. Wiemy, że odgrywa ona bardzo ważne funkcje w embriogenezie, homeostazie organizmu i przede wszystkim jest specyficznym markerem dla najbardziej inwazyjnych komórek nowotworowych. W związku z tym w niniejszej rozprawie doktorskiej w pierwszej kolejności skupiłem się na dokładnym przeglądzie literaturowym i głębokiej analizie i charakterystyce tego białka co zostało opisane w publikacji nr 1. Następnie w moich badaniach laboratoryjnych wykorzystałem fakt, iż podwyższony poziom ekspresji alfa katuliny jest specyficzny dla inwazyjnych komórek nowotworowych i wygenerowałem nowy system reporterowy wyznakowujący te najbardziej agresywne komórki w systemie ludzkiego, potrójnie ujemnego nowotworu piersi. Udało mi się

scharakteryzować transkryptom tych komórek, a także zaobserwować proces VM i określić udział katuliny w powstawaniu struktur naczyniopodobnych. Wyniki zostały szczegółowo zaprezentowane w publikacji nr 2.

Założenia i cele pracy

Cel główny:

Celem głównym badań w pracy doktorskiej była charakterystyka mało poznanego białka alfa-katuliny na podstawie wszystkich dostępnych publikacji, a następnie wykorzystanie ekspresji tego białka jako markera komórek inwazyjnych potrójnie ujemnego nowotworu piersi w celu wyizolowania tych komórek w systemie *in vivo* i analizy ich heterogenności, transkryptomu a także ich udziału w procesie mimikry naczyniopodobnej.

Cele szczegółowe:

- Analiza wszystkich dostępnych prac naukowych poprzez przeszukanie bazy danych Pubmed za pomocą słów kluczowych alpha-catulin, catulin, CTNNAL1, a następnie charakterystyka tego białka i opisanie właściwości strukturalnych, wszystkich białek wchodzących w interakcję z katuliną, udział katuliny w rozwoju zarodkowym, homeostazie organizmu, a także w procesach nowotworzenia.
- Wygenerowanie nowego systemu reporterowego znakującego inwazyjne komórki ludzkich linii TNBC poprzez ekspresję GFP z promotora genu *CTNNAL1*. Następnie izolacja komórek najbardziej inwazyjnych i poddanie ich analizie sekwencjonowania RNA nowej generacji.
- Jednoczesna analiza komórek ludzkich linii TNBC w systemie *in vivo* z wyłączonym genem *CTNNAL1*, ich izolacja i poddanie analizie sekwencjonowania RNA nowej generacji.
- Zbadanie wpływu wyciszenia alfa-katuliny w komórkach linii TNBC w systemie 3D na ich migrację, potencjał inwazyjności i potencjał macierzystości (CSC).
- Określenie udziału najbardziej inwazyjnych komórek nowotworowych i alfa-katuliny w tworzeniu struktur naczyniopodobnych.

Publikacja nr 1

International Journal of
Molecular Sciences

MDPI

Review

Emerging Roles of the α-Catenin Family Member α-Catulin in Development, Homeostasis and Cancer Progression

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Abstract: α-catulin, together with vinculin and the α-catenins, belongs to the vinculin family of proteins, best known for their actin-filament binding properties and crucial roles in cell-cell and cell-substrate adhesion. In the past few years, an array of binding partners for α-catulin have surfaced, which has shed new light on the possible functions of this protein. Despite all this information, the molecular basis of how α-catulin acts in cells and controls a wide variety of signals during morphogenesis, tissue homeostasis, and cancer progression remains elusive. This review aims to highlight recent discoveries on how α-catulin is involved in a broad range of diverse biological processes with an emphasis on cancer progression.

Keywords: α-catulin; CTNNAL1; catenin; invasion; epithelial-mesenchymal transition; EMT; vascular mimicry

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Citation: Gielata, M.; Karpińska, K.; Pieczonka, T.; Kobielak, A. Emerging Roles of the α-Catenin Family Member α-Catulin in Development, Homeostasis and Cancer Progression. *Int. J. Mol. Sci.* **2022**, *23*, 11962. https://doi.org/10.3390/ijms231911962

Academic Editor: Laura Paleari

Received: 14 September 2022
Accepted: 6 October 2022
Published: 8 October 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

1. Introduction

Homeostasis in healthy tissues strongly depends on cadherin- and integrin-mediated, cell-to-cell and cell-to-extracellular matrix (ECM) adhesion, respectively [1]. Both types of adhesion are crucial for maintaining tissue architecture and sensing and responding to changes in their environments. Cadherins are transmembrane glycoproteins that mediate calcium-dependent cell-cell adhesion. Through their homophilic binding interactions, cadherins play a role in cell-sorting mechanisms, conferring adhesion specificities on cells. The regulated expression of cadherins also controls cell polarity and tissue morphology. Classical cadherins are located at adherens junctions and are characterized by five homologous repeats at the extracellular domain. In contrast, the intracellular classical cadherin cytoplasmic domain (CCD) binds armadillo family proteins β-catenin (Ctnnb1) and p120ctn (Ctnnd1). The interaction with β-catenin links cadherins to α-catenin and the actin cytoskeleton, whereas p120ctn is involved in cadherin turnover. By regulating contact formation and stability, cadherins play a crucial role in tissue morphogenesis and homeostasis [1].

The adhesion of cells to the extracellular matrix (ECM) is mainly mediated by integrins, which undergo a conformational change upon activation to recruit structural and signaling molecules. Thus, integrins not only mechanically couple the cytoskeleton to the ECM but also transmit molecular signaling cascades to regulate cellular functions in response to extracellular cues [1].

During tissue morphogenesis, wound healing or pathological alterations in diseases like cancer, the ability of cells to rapidly and reversibly change adhesive properties is a key feature. This cell plasticity is driven by the programs of the epithelial–mesenchymal transition (EMT) and mesenchymal–epithelial transition (MET), both of which play essential roles during normal embryogenesis and tissue homeostasis [2]. However, the aberrant activation of these processes can also drive different stages of cancer progression, including invasion, cell dissemination, metastatic colonization, and secondary tumor outgrowth [3]. EMT

enables physically connected epithelial cells to disassociate their characteristic classical cadherin/catenin cell-cell contacts, lose their apical-basolateral polarity, and increase expression and activity of integrins displaying leading-edge asymmetry to become motile and mesenchymal-like and capable of degrading the basement membrane. The events occurring during EMT include the downregulation of cytokeratins and E-cadherin, epithelial-specific markers, and an increase in mesenchymal markers, such as fibronectin, N-cadherin, and vimentin. Transcription factors, including Snail1/Snail, Snail2/Slug, Twist, and ZEB1, are well known to be involved in the orchestration of EMT. Cell–cell adhesion and cell–ECM sites contain overlapping functional constituents containing common and distinct proteins. The crosstalk between these adhesion sites is crucial to coordinate cell migration with dynamic interactions between cells. Because both integrins and cadherins associate with the cytoskeleton and many common signaling molecules, it is likely that the cell–ECM and cell–cell adhesion processes mediated by these two types of receptors act in a coordinated manner in regulating cellular functions [4]. Changes in expression or mutations of these proteins, especially cadherins, catenins, and integrins, are frequently associated with diseases ranging from developmental defects to carcinogenesis and metastasis [5–7]. It is well established that two significant hallmarks of cancer, namely loss of cell-to-cell adhesion and anchorage-independent growth, are both dependent on cell adhesion molecules. Vinculin and α-catenin are two related proteins that play crucial roles in those processes [4,8]. However, the function of their recently characterized homolog α-catulin is still poorly understood. α-catulin is a protein whose name is composed of "α-cat", which comes from α-catenin, and "ulin", which comes from vinculin as it is a homolog of α-catenin protein belonging to the vinculin superfamily. Despite the sequence homology and shared superfamily with α-catenin, α-catulin's localization and functions appear to differ. Multiple reports describe α-catulin as an important factor contributing to cancer cell migration and invasion; however, the exact molecular mechanism leading to this phenomenon remains unclear. A growing number of reported α-catulin-interacting partners and new connections imply even more complex regulatory functions for this protein. This review aims to highlight recent discoveries emphasizing how α-catulin is involved in the coordination of a network of signals and actin cytoskeleton regulation.

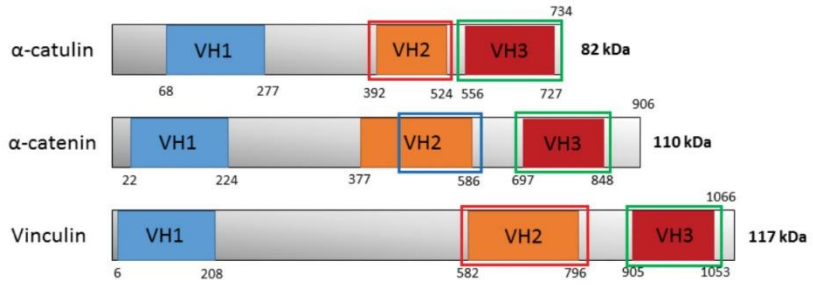
2. α-Catulin—A Member of the α-Catenin Family

Whereas all other catenins (β-catenin, plakoglobin and p120 catenin) share relatively high sequence homology and belong to the Armadillo family of proteins, vinculin and α-catenins differ in sequence and structural organization and form the vinculin family [9] together with the recently characterized homolog α-catulin [10]. Although it is well known that α-catenin is necessary for cadherin-catenin-mediated cell-cell adhesion, and vinculin is important for integrin-mediated cell-ECM adhesion and cell-cell adhesion, the function of α-catulin is still not well understood. α-catulin (catenin alpha like 1) protein is encoded by the *CTNNAL1* gene located on chromosome 9 in loci q31–32 (Figure 1B) positions 108,942,569–109,013,522 in a minus-strand orientation, resulting in a base length of 70,954 (https://www.genecards.org/cgi-bin/carddisp.pl?gene=CTNNAL1 (accessed on 5 September 2022)). The protein is 734 amino acids long and weighs 81,896 Da (https://www.uniprot.org/uniprotkb/Q9UBT7/entry (accessed on 5 September 2022)). However, two other alternative splicing isoforms have been described, one with substitution in positions 714–734 [10] and another with missing aa in positions 397–480 [11]. There is no 3D structure for α-catulin that has been deposited in the PDB file. The only available structure is the one predicted by AlphaFold (https://alphafold.ebi.ac.uk/ (accessed on 5 September 2022)) which still has a poor structural prognosis in some locations (Figure 1C). mRNA of α-catulin is widely expressed in the human body. It has been reported to be expressed in the thymus, prostate, testes, ovary, small intestine, colon [12], skeletal muscle, lung, heart, and placenta [10]. The human protein atlas also confirms that α-catulin protein is widely found in the human body, interestingly having the highest score in endocrine tissues, female and muscle tissues (proteinatlas.org). In 2002, Park

et al. demonstrated that when using the Blast tool and analyzing the *CTNNAL1* sequence, they reported having high similarity to α-catenin. The BESTFIT similarity alignment of α-catulin with its closest human homolog α-catenin showed 27% identity, and alignment with vinculin showed almost 20% identity (Figure 1A). They also showed that α-catulin is characterized by an extra 16 N-terminal amino acids not present in mammalian α-catenins. α-catulin and α-catenin homology is represented by two blocks; the first homology sequence is between α-catulin residues 18–524 and αN-catenin positions 2–504. The following sequence is a region of 110 amino acids present in α-catenin that is omitted in α-catulin. The second homologous block extends α-catulin residues 525–734 [12]. α-catulin also shows high sequence similarity with vinculin, hence being categorized as a part of the vinculin superfamily of proteins. The homology with vinculin, however, is lower, reaching 21%. The similarity is essentially high in the N-terminal domain of the protein, shown to have putative binding sites for β-catenin, talin, and α-actinin [10]. Amphipathic helices in the C-terminal region corresponding to α-catenin contain potential binding sites for the actin cytoskeleton. This region also contains potential binding sites for ZO-1, the protein important for tight junctions [10,12], another type of intercellular adhesion complex that forms the border between the apical and basolateral cell surface domains in polarized epithelia and controls paracellular permeability. Despite the sequence homology between α-catulin and α-catenin, their subcellular localization pattern is different as shown by Park et al. α-catulin localized to both the membrane-rich (pellet) fraction and the soluble (cytosolic) fraction, whereas α-catenin was found to localize almost exclusively to the membrane-rich fraction. They confirmed those results with two different experiments, one with high-speed fractionation into cytosolic and membrane-rich fractions followed by Western blotting, and the second with Myc-tagged α-catulin (pcDNA Myc:α-catulin) and indirect immunofluorescence. Despite the above-mentioned characteristics of α-catulin, it is still very poorly characterized.

A

	α-catulin	α-catenin	Vinculin
α-catulin	100	27.09	19.15
α-catenin	27.09	100	25.84
Vinculin	19.15	25.84	100



B

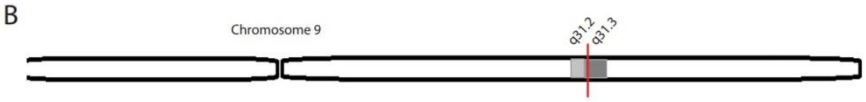


Figure 1. *Cont.*

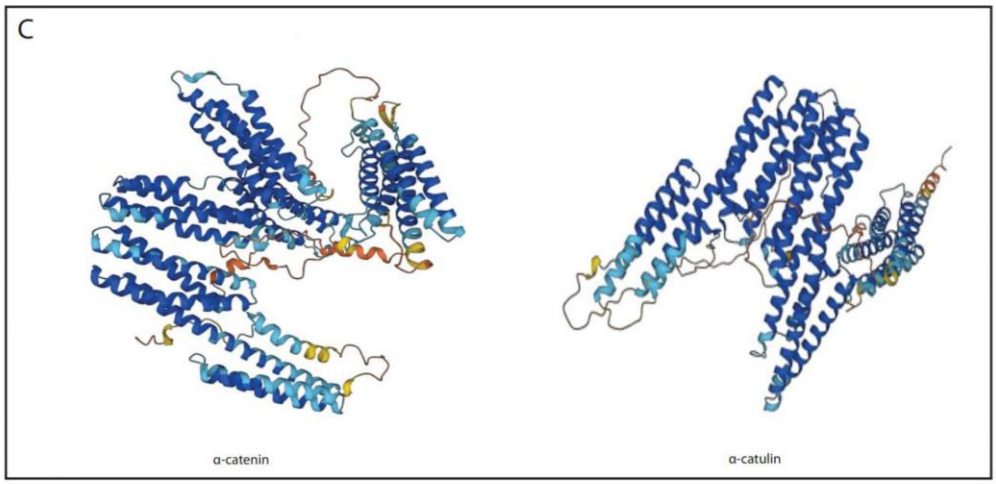


Figure 1. Structural features of α-catulin. (**A**) Table represents amino acid sequence similarities (%) between α-catulin, α-catenin and vinculin. α-catulin shares 27.09% homology with α-catenin and 19.15% with vinculin. (**B**) Schematic representation of α-catulin (CTNNAL1) gene on chromosome 9 locus 31.3. (**C**) Scheme shows the predicted 3D structure of α-catulin and α-catenin protein by AlphaFold.

3. Binding Partners of α-Catulin

One of the first described interacting partners of α-catulin is Lbc Rho guanine nucleotide exchange factor. Rho guanine nucleotide exchange factor (GEF) functions for the RhoA small GTPase protein [13]. RhoA is inactive when bound to the GDP, but when acted on by the Rho GEFs, GDP can be released, and GTP might be attached, leading to the activation of RhoA. Furthermore, active RhoA can bind to and activate distant effectors or enzymes. Interestingly, in this particular case, RhoA is a major regulator of the cell actin cytoskeleton [14]. One of the GEFs specific for Rho is a DH domain containing Lbc oncogenic product GEF [15,16]. All Lbc Rho GEF forms possess common C-terminal regions following DH domain cassette [17]. Park et al. showed a direct association between Lbc Rho GEF and α-catulin using three independent systems: yeast two-hybrid interaction, direct binding in vitro, and complex formation in mammalian cells. The required site of interaction within the Lbc C-terminal region was mapped to the ~253-residue IDR (intrinsically disordered region). They also determined that the α-catulin site required for the interaction lies in the N-terminal residues 34–524. Coexpression of α-catulin and wt-Lbc led to increased GTP-Rho formation in cooperative action. This implies that α-catulin is an upstream regulator of Rho. Overall, the authors conclude that α-catulin acts as a scaffold protein for Lbc Rho GEF and facilitates Lbc-induced Rho signals [12,17].

α-Catulin has also been shown to interact with the dystrophin complex through direct interaction with dystrobrevin in *C. elegans*. This interaction is conserved and also present in mouse skeletal muscles [18]. Dystrophin has been known as a cause of Duchenne muscular dystrophy, yet dystrophin usually functions in protein complexes known as dystrophin-associated protein complex (DAPC) [19]. It had been previously shown that mutations in the CTNNAL1 gene lead to the interruption of DAPC localization near dense bodies [20]. In the above-mentioned publication, the reciprocal action of α-catulin with dystrobrevin was validated by co-immunoprecipitation as well as by mass spectrometry and yeast two-hybrid screen. The authors observed an increase in α-catulin expression levels in the skeletal muscle of dystrophin-deficient mice, where dystrophin-associated

protein complex is disassembled, and the link between the costamere and the sarcolemma is absent. To bind α-catulin, dystrobrevin requires a C-terminus as well as an α-helix H2 proximal to the C-terminal region [18]. Similar results have been obtained in other studies. Lyssand et al. showed that the C-terminal domain of dystrobrevin recruits α-catulin to the α_{1D}-AR signalosome. Adrenergic receptors (ARs) and G protein-coupled receptors (GPCR) are important regulators of cardiovascular system function. Their function revolves around increasing blood pressure and promoting vascular remodeling. [21]. Sequence analysis revealed that, similar to α-catenin, α-catulin has a putative binding domain for β-catenin; therefore, a group led by Deniz Toksoz took a closer look into this interaction, mapping it to the N-terminal 163 amino acids of the protein [21,22]. When performing co-immunoprecipitation, they noticed that α-catulin indeed co-precipitates with β-catenin, but the amount of α-catulin associated with β-catenin appeared to be smaller than that of α-catenin associated with β-catenin. Given that endogenously in cells, the pool of β-catenin is naturally bound to α-catenin, these results were not surprising. α-catulin might associate with a different fraction of β-catenin than α-catenin does. There might be other pools of β-catenin, such as tyrosine phosphorylated β-catenin, in which protein interactions are altered [21,23–25]. Here, the authors additionally proposed the antiproliferative role of α-catulin, as it attenuates cyclin D1 transcription, leading to decreased cyclin D1 protein levels. They also observed that expression of α-catulin had a negative impact on cancer cell colony formation ability, leading to the statement that α-catulin modulates endogenous growth signaling pathways [21,22]. As β-catenin functions at the adherens junctions and also acts in the nucleus after stabilization of a pool of β-catenin in response to the upstream Wnt signals, it is crucial to further investigate the catulin-β-catenin interaction. Another α-catulin interacting protein was reported in the publication by Wiesner et al. in 2008. It was shown that α-catulin can modulate the NF-κB pathway by binding to IKK-β [21,26]. The NF-κB pathway plays a pivotal role in a variety of biological processes like innate and adaptive immune responses, tissue differentiation and apoptosis [21,27,28]. The targets of NF-κB include its own inhibitors IκBα and IκBβ [21,29]. Different extracellular stimuli activate the IκB kinases IKK-α and -β, which phosphorylate IκBα, which results in the degradation of IκBα and translocation of NF-κB to the nucleus [21,30]. Wiesner et al. provided evidence that α-catulin binds to IKK-β by immunoprecipitation. Moreover, they limited the interaction site in α-catulin to its C-terminal 87 amino acids. As α-catulin binds to IKK-β in the C terminus and to Lbc Rho GEF in the N-terminus, the authors claim that it may allow simultaneous stimulation of both pathways, being a bridge between those two. As there is evidence that both the NF-κB and RhoA signaling pathways play multiple roles in tumorigenesis, cell migration, invasion, and escape from apoptosis, α-catulin, as a linker of those two pathways, might serve as a crucial clinical target [21,26,31]. The interaction of α-catulin with Lbc, dystrophin complex and other proteins and resulting pathways activation have been represented in Figure 2.

Finally, α-catulin has been described as an interactor protein of human NEK1 protein kinase. NEK kinases are involved in regulating diverse cellular processes like the cell cycle, mitosis, cilia formation, and the DNA damage response and the etiology of polycystic kidney disease (PKD). α-catulin has been described as one of the 11 new binding proteins of NEK1. Moreover, it has been proven to interact with both regulatory and kinase domains (NRD and NKD) [32]. Interestingly, aberrant expression of NEKs appears to be involved in the initiation, maintenance, progression and metastasis of cancer and is associated with a poor prognosis [33]. A better understanding of NEK1 kinase interaction with α-catulin may lead to more successful clinical trials of NEK inhibitors.

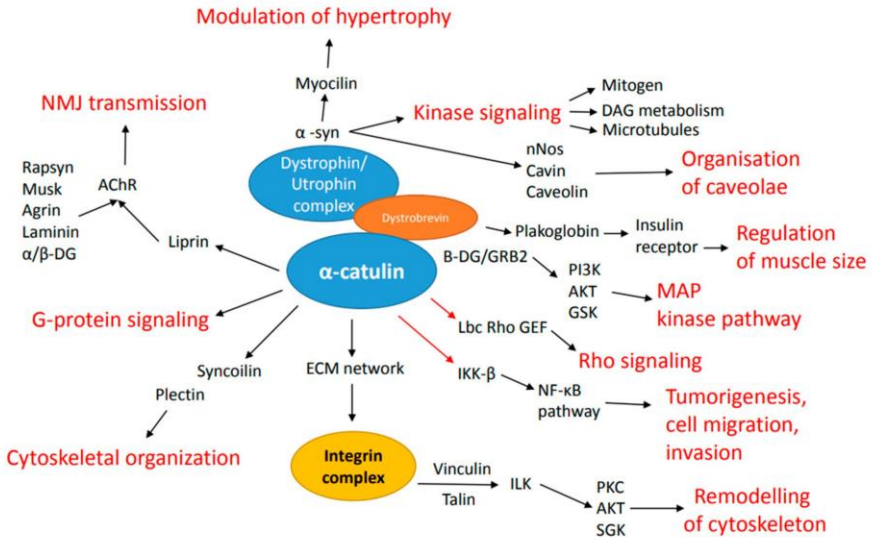


Figure 2. Overview of the function of α-catulin in dystrophin complex. In blue circles, α-catulin and dystrophin complex are shown. The interaction occurs via dystrobrevin, highlighted in orange. Shown are distinct interactors of the complex as well as direct interactors of α-catulin. Enlarged is also integrin complex, having interactions indirectly via ECM and impacting cytoskeleton remodeling. Highlighted in red are key pathways and functions resulting from either interaction of the complex or α-catulin directly.

4. α-Catulin and Its Function during Development

The plethora of interacting proteins indicates that α-catulin may play essential roles in various vital regulatory processes. Thus far, the important role of α-catulin has been shown in the process of neurulation during mouse development [34], where cell-cell and cell-ECM interactions are constantly under remodeling to enable proper architecture and function of forming tissues. The actin-cytoskeleton and actomyosin contractility integrated at the cell-cell and cell-ECM adhesions cooperatively are crucial to shape the cells and tissues [35–37]. The adherens junctions are required for the transmission of force across an epithelium, and the actomyosin cortex, which spans the apical surface of an epithelium, transitions between elongation and active state of actin nucleation while still attached to the adherens junction, allowing for apical constriction, which is crucial, for example, during neurulation [37–39]. It is important that actomyosin machinery is located at the right place and time to generate the required force to pull the neural folds together [40]. Interestingly, α-catulin was shown to participate in the apical actomyosin network regulation by serving as a scaffold protein that may be important for properly directing Rho family GTPase signaling during neurulation. α-catulin-deficient mice show neural tube (NT) closure defects. They are embryonically lethal with massive disorganization of their neuroepithelium, extra bending, absence of apically localized actin filaments, nestin and phosphorylated myosin, and inappropriate basement membrane assembly due to very low expression of its components: laminin and fibronectin. The neuroepithelium of α-catulin deficient mice lack apically localized actin filaments and P-Mlc, which typically correlate with proper Rho-dependent cell constriction. In vitro studies performed in a three-dimensional model of MDCK cells showed that α-catulin is localized specifically at the apical parts of cells membranes and is important for proper cell polarization, organization of actomyosin cytoskeleton, stabilization of

intercellular junction as well as distribution of active Rho A. Taken together, data collected both from in vivo mouse model and in vitro 3D studies indicated a pivotal role of alpha-catulin protein in neurulation during embryonic development, as it can act as a scaffold for RhoA in apical parts of cells, which results in correct spatial activation of downstream myosin to influence actin-myosin dynamics and the stability of cell-cell junctions, which allows generating the appropriate tension needed for the apical constriction of cells and proper bending of the neural plate [34].

5. Role of α-Catulin in Homeostasis

In the last decade, numerous studies have also demonstrated the importance of α-catulin in the maintenance of tissue homeostasis. A-catulin was reported to play potential functions in hematopoietic stem cells (HSCs), bronchial epithelium, muscles and intestine [18,20,41–45]. In hematopoietic stem cells, α-catulin is expressed only in a specific population of 0.02% of bone marrow hematopoietic cells. Generation of a mouse model with green fluorescent protein (GFP) knocked-in into the α-catulin locus allowed to show that α-catulin together with c-kit marks the population of cells that possess the long-term multilineage reconstitution ability of bone marrow after irradiation [41]. In addition, the distribution of α-catulin$^+$ c-kit1$^+$ cells indicates that HSCs are more common in the central marrow than near the bone surface [41,42]. Even though α-catulin proved to be a great marker for HSC visualization in the bone, the exact function of this protein in those cells was not established.

Furthermore, high expression of α-catulin was also detected in bronchial epithelium under ozone-stressed conditions. Results from this study suggest that elevated α-catulin expression may be a protective response aimed at maintaining airway epithelial integrity [43].

Moreover, in neuromuscular junctions, dystrobrevin utilizes α-catulin for proper neurotransmitter receptor (AChR) clustering on myotubes, indicating its important role in a synaptic machinery organization [44]. As an anchor protein that locates potassium channels and neurotransmitter receptors in specific nanodomains, α-catulin plays a key role in the physiological processes related to the neurosecretion as well as excitation of neurons and muscles. Dysfunction of this important protein may be linked to muscular and neurological disorders [20,44]. It has also been reported that α-catulin ortholog is a critical cytoskeletal regulator in C.elegans, crucial for the proper localization of calcium-dependent potassium channels in both neurons and muscles. In muscles, α-catulin, via the dystrophin complex, binds the calcium-dependent potassium channels near L-type calcium channels. In turn, in neurons, α-catulin controls the localization of the potassium channels independently of the dystrophin complex [20,21,46]. The interaction with dystrophin complex seems to be the best characterized so far for α-catulin.

Recent studies performed on Chinese patients with Hirschsprung disease revealed that α-catulin can be attributed to genetic factors or gene-gene interaction networks responsible for enteric neuronal dysfunction [45]. Interestingly, catulin expression was observed in the enteric innervation of newborn mice [34].

6. α-Catulin in Cancer Invasion and Metastasis

Even though α-catulin is overall a very poorly described protein, its participation in cancerogenesis and influence on cancer cell invasion and metastasis has been reported and researched in many papers. Both structural and signaling functions of α-catulin may play a role in cancer progression. As mentioned above, α-catulin in the N-terminal region contains binding sites for β-catenin, talin, α-actinin, and the actin cytoskeleton. This suggests that it may function as a cytoskeletal linker protein that is able to modulate cell migration [8]. Cell migration is a process that plays a pivotal role in carcinogenesis and participates in the metastasizing of cancer cells. Metastasis is a complex phenomenon that occurs in all types of cancers and is responsible for death [47]. It is based on the fact that cancer cells escape from the primary tumor, migrate, enter the lumen of blood and lymphatic vessels and reach distant organs, where they can repopulate the tumor mass [48,49]. Cancer cells need to ac-

quire a mesenchymal phenotype in the process called epithelial-to-mesenchymal transition (EMT) [50]. EMT is a phenomenon where cells downregulate proteins involved in apical cell-cell contact and adherence junction formation, such as E-cadherin and α-catenin, and start upregulating proteins specific for mesenchymal features of the cell, such as N-cadherin and vimentin, which results in the enhanced motility of the cells. This switch between relatively stable cell-cell contacts and an increase in motility is crucial for cancer invasion [51]. It has been observed that when α-catenin, a cell-cell junction protein, is conditionally lost in the epithelium, cells begin to demonstrate increased proliferation rates, migrative properties, and the squamous cell carcinoma (SCC) phenotype [52]. Using microarray analysis to compare mouse α-catenin cKO keratinocytes, which failed to form cell–cell junctions, and WT epithelial cells, it was observed that α-catulin is highly upregulated in the cells with increased motility and mesenchymal phenotypes [52]. This data suggested the participation of α-catulin in cancer progression and was further investigated by our group in a model of human head and neck squamous cell carcinoma (hHNSCC), which is a very aggressive tumor type and accounts for more than 450,000 malignancies diagnosed each year. Despite new treatment options, patients are still faced with a very high rate of recurrence and metastatic disease, with a 5-year survival rate of only 50% [53–55]. It was shown that α-catulin is upregulated in the metastatic cells in the xenotransplant in vivo model and also in vitro in the hSCC (human squamous cell carcinoma) cell line after EMT induction. Moreover, α-catulin is highly expressed at the invasion front and in migrating, metastatic streams of cells in human samples of HNSCC and in higher grades of tumor samples when compared with normal mucosa epithelium [56]. Most importantly, ablation of α-catulin in hSCC cells decreased the ability of these cells to migrate and invade in vitro and decreased their metastatic potential in vivo [56]. Given that the expression of α-catulin not only correlates with tumor grade, but also appears to be involved in the regulation of the invasive character of the HNSCC cells, it suggests that α-catulin may represent a novel yet critical mediator of oral cancer progression. As this type of cancer usually spreads locally, utilizing collective migration, α-catulin could be important for spatiotemporal fine-tuning of Rho GTPases within a group of cancer cells to control divergent cell-cell and cell-ECM adhesion as well as cytoskeletal functions to achieve cellular coordination and mechanocoupling. This is one of the options that will require further testing to better understand the role of catulin in the process of HNSCC invasion. As α-catulin expression and function correlated with the early onset of hSCC cell invasion, our group used the human α-catulin promoter fragment driving GFP expression to develop a reporter system. This unique system, for the first time, allowed us to isolate in vivo a small population of invasive cells at the human tumor invasion front [57]. After verifying the reporter system, we showed that cells highly expressing GFP driven from α-catulin expression localize at the invasion front in a spheroid model of hSCC cells. Additionally, this system marked the cells with higher migratory, invasive, and tumorigenic potential in vitro in the 3D model. Cells highly expressing α-catulin were also observed in a small population of invasive cells at the tumor front in the in vivo model of head and neck squamous cell carcinoma. Expression of GFP under α-catulin promoter correlated with the loss of an epithelial marker, E-cadherin expression, indicative of ongoing EMT. The reporter system allowed for isolation and transcriptional characterization of those highly invasive cells, providing a list of deregulated genes that are involved in cellular movement, ILK and integrin signalling, as well as axonal guidance signalling [57]. This functional genomic study of the purified population of invasive cells revealed enrichment in genes involved in cellular movement and invasion, providing a molecular profile of HNSCC invasive cells. Interestingly, this profile overlapped partially with the expression of signature genes related to partial EMT available from single-cell analysis of human HNSCC specimens [58]. This comparison strengthens the idea that α-catulin in this type of cancer might be important for spatiotemporal regulation of Rho GTPases within a group of cancer cells to control dynamic plasticity and crosstalk between cadherin-mediated cell-cell contact and integrin-dependent cell-ECM adhesion, which is crucial during collective invasion and

migration. Further research on catulin revealed that its role in cancer progression is not only limited to HNSCC specifically, which utilizes collective invasion for local spread. It was recently published that α-catulin is also expressed in human breast cancer samples and triple-negative breast cancer cell lines, and its expression correlates with tumor progression [59]. Breast cancer is now the most common cancer worldwide [60], and the worst outcome is presented by triple-negative breast cancer [61]. Knockdown of α-catulin in triple-negative human breast cancer cell lines MDA-MB-231 and HCC1806 revealed a decrease in the invasion capability of those cells in 3D spheroid model assays [59]. The use of a catulin-GFP-promoter-based reporter system in a 3D spheroid model of triple-negative breast cancer cell lines showed that the most invading cells co-express α-catulin and known EMT marker vimentin. Transcriptional profiling of GFP-positive cells isolated from tumors that formed after injection of a catulin-GFP triple-negative breast cancer cell line disclosed the list of deregulated genes involved in cellular movement and invasion and, interestingly, migration of endothelial cells [59]. Top pathways deregulated in the α-catulin GFP + cells involved epithelial adherens junction signaling and remodeling of epithelial adherens junctions. Special attention was paid to genes involved in the vasculature, as it was observed that tumor areas enriched in GFP+ cells presented visible dense vasculature. Surprisingly, some cells highly expressing GFP co-expressed MCAM (CD146), an endothelial marker but also a cellular surface receptor of different ligands, are actively involved in signaling in the numerous physiological and pathological processes involving metastases of different cancer types. Cells highly expressing GFP and co-expressing MCAM formed vasculogenic structures resembling vessels. This suggests that α-catulin marks highly invasive breast cancer cells that are characterized by increased plasticity and might participate in the process of vascular mimicry, allowing cancer cells to metastasize [59]. In addition, ablation of α-catulin in the in vivo model resulted in decreased tumor size and decreased stemness potential of cancer cells with lowered expression of CD44, which is known to be enriched in breast cancer (BC) stem cells [59]. These data implicate that α-catulin might play an important role in cancer type-specific tumor-microenvironment interplay. Moreover, it may be involved in the inflection of adhesive properties of tumor cells. The possible mechanism of increased α-catulin expression in invasive cancer cells might be explained by the research performed by Cassandri et al. [62]. They showed that zinc-finger protein 750 (ZNF750) is a negative regulator of the migration and invasion of breast cancer cells. It functions as a repressor of a prometastatic transcriptional program. This transcriptional program was shown to express genes that are involved in focal adhesion and extracellular matrix interactions with an emphasis on CTNNAL1. They showed that the expression of CTNNAL1 and LAMB3 contradictorily correlated with ZNF750 expression in a breast cancer model. ZNF750 recruits epigenetic modifiers KDM1A and HDAC1 to the promoter region of the α-catulin gene, which affects histone marks and trans activates these genomic sites. Additionally, they also showed gene expression analysis in cancer patient datasets that indicated ZNF750 and its targets to be negative prognostic factors in breast cancer [62]. In 2011, Fan et al. published a paper confirming the previously described participation of α-catulin in tumorigenesis. They showed that α-catulin expression is elevated in oral cancer cells versus normal cells. They also found that the knockdown of α-catulin resulted in the accumulation of cell populations in S and G2/M cell-cycle phases with decreased cyclin A and cyclin B1 expression. α-catulin knockdown induced cellular senescence as the major phenotype of cell death in two oral cancer cell lines, OC2 and A549. In patients, α-catulin expression correlated with tumor size, whereas α-catulin knockdown supressed tumorigenicity in xenograft models. Knockdown of α-catulin in cancer cells bearing either wild-type or mutant p53 was sufficient to trigger DNA damage response and eventually induce cellular senescence in vitro [63]. In addition to structural functions and regulation of actin cytoskeleton during cancer invasion and migration, α-catulin enhances cancer metastasis by influencing signaling pathways. Liang et al. showed that α-catulin expression correlates with the cell invasiveness potential in vitro and metastatic potential in vivo. It occurs via an ILK/NF-κB/integrin network where α-catulin directly interacts with ILK, which in turn

activates the ILK/Akt/NF-κB signaling pathway and upregulates fibronectin and integrin $\alpha_v\beta_3$. α-catulin as an integrin signaling adaptor might play a pivotal role in regulating integrin-mediated cellular functions via binding to ILK [64]. Later, the same scientific group focused on the participation of α-catulin in cancer stemness and EMT. They found that cells overexpressing α-catulin have genes such as *FGF2*, *BMI1*, *ALDH1A3*, *POU5F1* and *NANOG* upregulated. Additionally, high expression of α-catulin was required to maintain stemness in a lung cancer model, and klf5 was indicated as a new interacting protein that plays an important role in stemness maintenance by cooperating with α-catulin to enhance the transcription of *POU5F1* and *NANOG*. Knockdown of klf5 in cells overexpressing α-catulin abolished the sphere formation capacity. α-catulin not only interacts with klf5 but also protects this protein by blocking the WWP1-mediated proteasomal degradation of KLF5 [65]. The participation of α-catulin in cancer cell migration and invasion has also been proven in a melanoma cancer model. Kreiseder et al. showed that α-catulin is highly expressed in melanoma cells, resulting in reduced E-cadherin and increased N-cadherin expression. Upregulation of α-catulin promotes expression of EMT markers Snail/Slug and Zeb1/2; in addition, α-catulin regulated PTEN and RKIP, inhibitors of the NF-κB pathway. They also found MCAM, plakoglobin, and occludin to be altered in α-catulin-deficient cells. Their results further confirmed that α-catulin is not only responsible for the downregulation of E-cadherin but is also required for melanoma invasion by the upregulation of MMP 2 and 9 and the activation of ROCK/Rho [66]. They further studied the role of α-catulin and, in 2015, published a paper showing that α-catulin is responsible for the chemoresistance of melanoma cells to cisplatin. This reduction in cisplatin-mediated apoptosis of melanoma cancer cells is due to the fact that α-catulin is responsible for NF-κB, AP-1 activation and ERK phosphorylation, and, in the case of knockdown of α-catulin, the cisplatin-mediated apoptosis was shown to be enhanced [67].

7. Conclusions

α-catulin, together with vinculin and α-catenins, belongs to the vinculin family of proteins, best known for their actin-filament binding properties and crucial roles in cell-cell and cell-substrate adhesion; however, despite sequence homology, α-catulin seems to have independent roles. α-catulin has been shown to be important in inflammation, apoptotic resistance, cytoskeletal reorganization, senescence resistance, cancer progression, and EMT. Multiple binding proteins of α-catulin revealed in recent years suggest a molecular hub function, integrating a cytoskeleton with a number of signaling pathways. Unfortunately, the molecular mechanisms of α-catulin action are still poorly characterized and need further investigation, especially in the field of cancer progression.

Increased α-catulin expression was observed in the invading front of squamous cell carcinoma, and its depletion led to decreased invasion and metastasis in a xenograft transplant mouse model. α-catulin was also reported to be upregulated in a highly invasive non-small cell lung cancer cell line, as well as in breast and prostate cancer. Despite multiple reports describing α-catulin as an important factor contributing to cancer cell migration and invasion, the exact molecular mechanism leading to this phenotype remains unclear.

As α-catulin depletion was shown to have a strong effect on RhoA signaling and the actomyosin cytoskeleton arrangement, it will be crucial to further investigate the role of α-catulin in spatial RhoA distribution during cell migration. Further experiments encompassing mass spectrometry are currently underway in our laboratory in order to identify potential α-catulin interaction partners contributing to the front-rear stabilization of migrating cancer cells.

A question also remains about potential α-catulin and cadherin interactions. The role of α-catulin in the process of EMT and the switch between relatively stable cell-cell contacts and an increase in motility, which is crucial for cancer invasion, is of great interest. Catulin expression was reported to be upregulated when α-catenin, a cell-cell junction protein, was conditionally lost in the epithelium, which was accompanied by an increased proliferation rate and migrative properties. Therefore, further investigations

at the structural, cellular, and functional levels are also needed to understand the exact sequence of molecular interactions and conformational changes operating between the cadherin/β-catenin/α-catenin complex and α-catulin and F-actin and tension-dependent remodeling of cell-cell adhesion.

An analysis of α-catulin dynamics using high-resolution live imaging should help us to map α-catulin's localisation and interactions in time and space. These could bring us closer to solving how α-catulin orchestrates adhesion and the actin cytoskeleton.

As α-catulin is broadly expressed and plays multiple physiological functions both during development and adult life, direct therapeutic strategies towards silencing its gene may not be applicable. On the other hand, targeted disruption of signaling pathways originating or ending at α-catulin may be a more promising therapeutic target.

Author Contributions: M.G. contributed: literature review and manuscript writing; K.K. contributed: literature review and manuscript writing; T.P. contributed: literature review and manuscript writing; A.K. contributed: literature review and manuscript writing. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by the Polish National Science Center (NCN) grants: 2015/17/B/NZ5/02551; 2017/25/B/NZ5/01597 and 2020/37/B/NZ5/03950 to A. Kobielak.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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Catulin reporter marks a heterogeneous population of invasive breast cancer cells with some demonstrating plasticity and participating in vascular mimicry

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Breast cancer is the most commonly diagnosed cancer in women worldwide. The activation of partial or more complete epithelial–mesenchymal transition in cancer cells enhances acquisition of invasive behaviors and expands their generation of cancer stem cells. Increased by EMT plasticity of tumor cells could promote vascular mimicry, a newly defined pattern of tumor microvascularization by which aggressive tumor cells can form vessel-like structures themselves. VM is strongly associated with a poor prognosis, but biological features of tumor cells that form VM remains unknown. Here we show that catulin is expressed in human BC samples and its expression correlates with the tumor progression. Ablation of catulin in hBC cell lines decreases their invasive potential in the 3D assays. Using a novel catulin promoter based reporter we tracked and characterized the small population of invasive BC cells in xenograft model. RNAseq analysis revealed enrichment in genes important for cellular movement, invasion and interestingly for tumor-vasculature interactions. Analysis of tumors unveiled that catulin reporter marks not only invasive cancer cells but also rare population of plastic, MCAM positive cancer cells that participate in vascular mimicry. Ablation of catulin in the xenograft model revealed deregulation of genes involved in cellular movement, and adhesive properties with striking decrease in CD44 which may impact stemness potential, and plasticity of breast cancer cells. These findings show directly that some plastic tumor cells can change the fate into endothelial-like, expressing MCAM and emphasize the importance of catulin in this process and breast cancer progression.

Abbreviations

BC	Breast cancer
EMT	Epithelial–mesenchymal transition
VM	Vascular mimicry
GFP	Green fluorescent protein
MCAM	Melanoma cell adhesion molecule
ER	Estrogen receptor
PR	Progesterone receptor
Her2	Human epidermal growth factor receptor 2
hHNSCC	Human head and neck squamous cell carcinoma
GEF	Guanine exchange factor
GTP	Guanosine-5'-triphosphate

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CSC	Cancer Stem Cells
CatGFP	Catulin GFP reporter plasmid
KD	Knock down
Ctrl	Control

Breast cancer is the most commonly diagnosed cancer in women worldwide[1], and accounts for 23% of all cancer cases[2]. Breast cancers are categorized into three main groups based on cellular markers reflecting available targeted therapies: Estrogen receptor (ER) or progesterone receptor (PR) positive, Her2 positive and triple negative breast cancer with no ER, PR and Her2 expression having no standard treatment option and the poorest survival[3]. Majority of breast cancer related deaths are a consequence of late diagnosis resulting in resistant to treatment, metastatic disease. The EMT plays a role in many developmental processes, and is also associated with many solid tumors progression. Therefore, pharmacological intervention of this process may represent a crucial therapeutic target. Unfortunately, it is challenging to observe EMT in vivo, in human carcinomas. In addition recent studies indicate high heterogeneity within tumor cells undergoing epithelial-mesenchymal transition and exhibiting different phenotypes: epithelial, mesenchymal, or one or more hybrid epithelial-mesenchymal phenotypes within the same tumor[4–7]. This behavior has been reported across different cancer types, and implicated in multiple processes associated with metastasis and appearance of cancer cell subpopulations with most plastic stem cell-like properties. Therefore, labeling, isolation and functional characterization, including cancer stem cell potential, and genetic signature of heterogeneous populations of cancer cells undergoing transient and reversible partial EMT process is very challenging. During breast cancer progression, and metastasis the reduction of intercellular adhesion is one of the critical steps[8]. The process of epithelial to mesenchymal transition is characterized by progressive redistribution or downregulation of apical, and basolateral epithelial-specific tight, and adherens junction proteins, including E-cadherin and α-catenin, and re-expression of mesenchymal molecules like Vimentin and N-cadherin. This switch between relatively stable cell–cell contacts and increase in the motility is necessary for invasion[9]. The important factors playing a role in the EMT process are *TWIST1* and *TWIST2,* responsible for inducing transformation alone or in a cooperation with TGFβ, Wnt, Notch, etc.[10]. Suppression of the E-cadherin transcription is known to be regulated by the Snail1 and Snail2 as well as *Zeb1* and *Zeb2* genes[11]. Downregulation of E-cadherin, and α-catenin is a prognostic marker of poor clinical outcome in majority of solid tumors[12,13]. We observed previously, that loss of cell–cell junction protein α-catenin was accompanied by upregulation of the expression of a new α-catenin homologue, α-catenin-like 1 (α-catulin, catulin)[8]. We also described previously that catulin is highly expressed at the invasion front of human head and neck squamous cell carcinoma (HNSCC). The upregulation of catulin correlated with the transition of tumor cells from an epithelial to mesenchymal state. On the other hand, knockdown of catulin in hHNSCC cell lines dramatically decreased the migratory and invasive potential of those cells in vitro and metastatic potential in vivo, in the mouse model. Interestingly, analyses of tumors deficient in catulin showed that its ablation prevented tumor cells from invading the surrounding stroma[14]. Catulin has not been well characterized so far. It was shown to act as a scaffold for the Rho GTPase signaling complex by interacting with the Lbc-Rho GEF[15–17]. Catulin has also been shown to interact with the IKK-b and Lbc to promote tumor cell migration, and resistance to apoptosis[18]. Moreover, it has been shown that catulin knockdown reduces NF-κB, and AP-1 activity, diminishes ERK phosphorylation in malignant melanoma cells, and sensitizes them to treatment with chemotherapeutic drugs[19]. Catulin has also been proven to interact with dystrophin in the dystroglycan-dystrophin/utrophin complex, where dystroglycan mediates cell-ECM adhesion[20–23]. Our laboratory has also described that catulin plays an important role during mouse neural tube closure by acting as a scaffold for RhoA distribution, resulting in proper spatial activation of myosin to influence actin-myosin dynamics, and tension at cell–cell adhesion[24]. Additionally, it has been reported that catulin plays a critical role in cancer metastasis by activating the ILK-mediated Akt-NF-κB-αvβ3 signaling axis[25]. As catulin shows high similarities in structure to vinculin, and α-catenin in N-terminal region that contains binding sites for β-catenin, talin, α-actinin, and actin cytoskeleton[26] it may act as a cytoskeletal linker protein that modulates adhesive properties, motility and invasive properties of cancer cells.

There is a growing evidence that only a minority of metastasizing cells may persist and form metastases[27–29]. This subpopulation of cells is able to grow, invade, and self-renewal, and exhibit stem-like properties, therefore those cells are called Cancer Stem Cells (CSCs)[30]. CSCs are able to survive even after removal of the primary tumor. This might be due to the mutations and deregulation of epigenetic pathways that favor survival and those may arise from the microenvironment that forces their genetic evolution[31,32]. Tumor microenvironment may provide signals which regulate self-renewal, epithelial-mesenchymal transition, and homeostatic processes such as inflammation, hypoxia and angiogenesis which regulate either entering of CSCs in a dormant state or promoting the reactivation of CSCs that initiate metastasis[33]. Endothelial cells of blood vessels are crucial in cancer progression that is further than delivering oxygen and nutrients[34–36]. Solid tumors that have outgrown beyond a few cubic millimeters to receive nutrients to progress need to induce tumor angiogenesis. Tumor angiogenesis requires development of new blood vessels from established vascular beds and the process is very complex[37–39]. Angiogenesis in tumorigenesis is always pathological, resulting in immature vessels with irregular structure. This hostile environment may facilitate the EMT process and result in the progression and metastases of the primary tumor[40,41]. On the other hand, vasculogenic mimicry is the process where tumor cells mimic endothelial cells and form blood vessel like channels[42–44]. It has been reported among others in breast cancer solid tumors. This process can facilitate cancer cell migration, invasion and metastatic potential as well as resistance to therapies[45,46]. However, the process of tumor cells participation in vascular mimicry is not fully understood yet and needs further investigating.

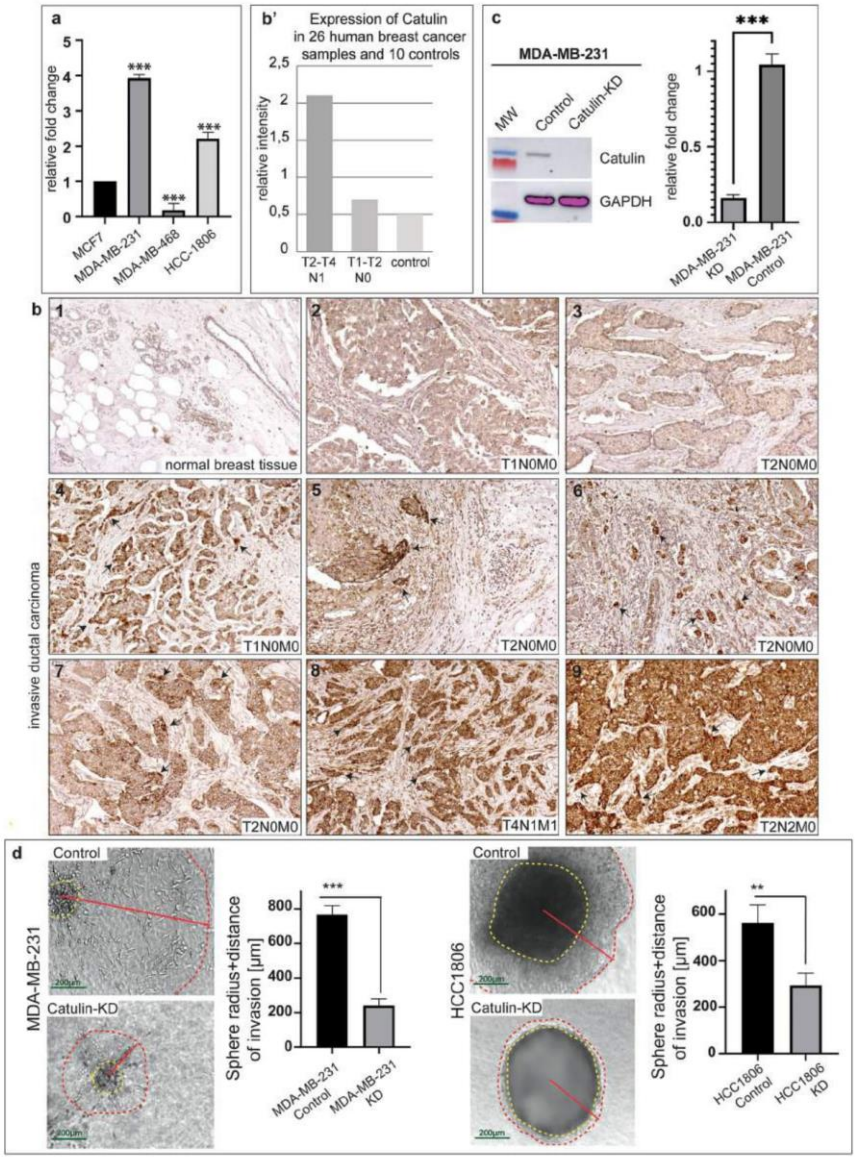
Here we observe that catulin is expressed in human breast cancer samples, and cell lines, and its expression correlates with the tumor progression. Ablation of catulin in human triple negative breast cancer cell lines

decreases their invasive potential in the 3D spheroid, and invasion chip assays. Therefore, to track and characterize the population of invasive breast cancer cells we took advantage of catulin expression and developed a novel catulin promoter based reporter system with green fluorescent protein (GFP) in TNBC cell lines. We verified this system in vitro in 3D spheroid model where catulin-GFP expression correlated with expression of known EMT marker Vimentin. Injection of cells stably expressing catulin GFP reporter plasmid into the immunocompromised mice allowed us to track, isolate and characterized the small population of invasive BC cells. RNAseq analysis revealed enrichment in genes important for cellular movement, cell invasion and interestingly for tumor-vasculature interactions. By analyzing the tumors, we discovered that catulin reporter system marks not only invasive cancer cells enriched at the tumor-stroma border and around newly formed vasculature but also rare population of highly plastic MCAM positive cancer cells that participate in vascular mimicry. We also showed that ablation of catulin in hBC cell lines decreased significantly the invasive potential of those cells in the 3D assays. Analysis of tumor cells in the xenograft model after catulin ablation revealed deregulation of genes involved in cellular movement and adhesive properties. We also observed decrease in membrane localized CD44 protein, which may impact stemness potential and plasticity of breast cancer cells. We confirmed the decrease in CD44 membrane fraction by performing FACS (fluorescence-activated cell sorting) analysis of catulin control and deficient tumor cells. These findings show high intratumoral heterogeneity with population of cells marked by catulin reporter characterized by increased invasiveness as well as plasticity of tumor cells which results in the change of fate into endothelial-like, expressing among other markers, MCAM. Our data confirm the growing number of evidence that cancer cells rarely undergo complete EMT and rather exist in a continuum of E/M intermediate states, preserving high levels of plasticity[47].

Results

Catulin is expressed in human breast cancer samples, and cell lines, and its expression correlates with the tumor progression. To investigate the level of catulin expression in the triple negative breast cancer cell lines, we used: MDA-MB-231, MDA-MB-468, HCC1806, and non-triple negative MCF-7 cell line as a control. RT-qPCR analysis was performed using pure RNA isolated from those cell lines. As MDA-MB-231 and HCC1806 showed the highest expression level of catulin, we selected those two cell lines for further studies with a main focus on MDA-MB-231 cells (Fig. 1a). To determine catulin protein level in human BC samples, and to check if its expression correlates with tumor progression, we performed immunohistochemical analysis using commercially available tissue array BR8014 from US Biomax (Fig. 1b). It showed different levels of expression in a tissue array consisting of 26 cases of hBC samples (all represented in duplicates), and 10 cancers adjacent, or normal breast tissue samples. There is a minimal catulin expression in the normal epithelium (Fig. 1b1). Low grade tumors, with no lymph nodes involved, showed relatively low expression level of catulin (Fig. 1b2–3). Interestingly, in some samples, stages T1–T2, catulin expression was observed in tumor cells or small clusters of cells, invading tumor stroma (arrows in Fig. 1b4–7). High level of catulin expression was noticed in stages T2–T4, especially in samples with lymph nodes involved (arrows in Fig. 1b8–9). In Fig. 1b', average expression of catulin in all 26 cases of hBC samples, and 10 cancers adjacent or normal breast tissue samples are shown. Tumor samples were divided into two groups: first, advanced group in stages between T2–T4 with lymph nodes involved—N1, second group less advanced in stages between T1–T2 without lymph nodes involved—N0. The highest expression of catulin was noticed in stages T2–T4 with lymph nodes involved, 4 times higher compared to stages T1–T4 without lymph nodes involved (Fig. 1b' and Sup. Fig. 1b). Tumor grade, as provided by the manufacturer, is indicated in Sup. Fig. 1b. These suggest that catulin protein level is important for highly aggressive breast cancer cells and its expression correlates with the tumor progression.

Ablation of catulin in hBC cell lines decreases their invasive potential in the 3D spheroid assay. Seeing that catulin is particularly upregulated in highly invasive triple negative breast cancer cell lines, and its expression correlated with the tumor progression, we wanted to investigate if catulin knockdown would influence invasive potential of those cells in vitro. To obtain stable TNBC cell lines deficient in catulin, we generated lentivirus containing catulin-specific shRNA to knockdown catulin in BC cell lines MDA-MB-231 and HCC1806. We were able to successfully knockdown (KD) catulin in the MDA-MB-231 (G85) and HCC1806 (G85), compared to cells transduced with the non-silencing control (GNS) on the RNA and protein level (Fig. 1c and Sup. Fig. 1a). To evaluate the invasive potential in vitro we used 3D spheroid assays. Formed spheres were allowed to invade matrigel-collagen stroma, and distance of invasion was calculated. We noticed, that for both MDA-MB-231, and HCC1806 cell lines, morphology of G85 KD spheres remained unchanged, however the distance of invasion was impaired in G85 KD spheres compared to GNS controls (Fig. 1d). Mean sphere distance of invasion for MDA-MB-231 GNS control cells was close to 800 µm compared to 200 µm for G85 KD cells, and for HCC1806 GNS control cells 550 µm compared to 300 µm for G85 KD cells (Fig. 1d). Those results correlate with previous findings where SCC cells with downregulated catulin level were also unable to efficiently migrate and invade in vitro in 2D migration and invasion assays[14]. To confirm the results in the independent assay, we took the advantage of 3D cell culture invasion chips. 10^6 of MDA-MB-231 G85 KD and GNS control cells were seeded in the media channel of a chip. At the time point 0 h we observed that GNS cells group together, and gather close to the medium-matrigel stroma barrier compared to G85 KD cells that were dispersed in medium (Fig. Supl. 1c). After 96 h we calculated the amount of cells that passed the barrier, and invaded matrigel stroma. As shown in the Fig.Supl. 1c total mean of the number of invasive cells was estimated around 140 for GNS control, and around 50 for G85 KD cells. In addition, classical in vitro migration, and invasion assays using this cell line, again showed that catulin ablation decreased the ability of these cells to migrate, and invade (Supplementary Fig. S2) in[14]. All these data suggest that catulin is an important factor determining invasive potential in human triple negative breast cancer cell lines in vitro and lack of this protein halts their invasive potential.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MCF7
MDA-MB-231
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HCC-1806
b'
Expression of Catulin in 26 human breast cancer samples and 10 controls
relative intensity
T2-T4 N1
T1-T2 N0
control
c
MDA-MB-231
MW
Control
Catulin-KD
Catulin
GAPDH
relative fold change
MDA-MB-231 KD
MDA-MB-231 Control
b
normal breast tissue
T1N0M0
T2N0M0
T2N0M0
T1N0M0
T2N0M0
T2N0M0
T2N0M0
T4N1M1
T2N2M0
invasive ductal carcinoma
d
Control
Catulin-KD
MDA-MB-231
Sphere radius+distance of invasion [µm]
MDA-MB-231 Control
MDA-MB-231 KD
HCC1806
HCC1806 Control
HCC1806 KD

◂**Figure 1.** Catulin is expressed in triple negative breast cancer cell lines and in high grade human breast cancer tissue and its ablation in hBC cell lines decreases their invasive potential in the 3D spheroid assay. (**a**), RT-qPCR shows that catulin is upregulated in MDA-MB-231 and HCC1806 cell lines and not in MDA-MB-468 compared to control MCF-7 cell line. (**b**), immunohistochemical analyses show catulin expression in a tissue array slide panel BR8014 (Biomax) consisting of 26 cases of human breast cancer samples (all represented in duplicates) and 10 cancers adjacent or normal breast tissue samples. Tissue origin and tumor grade, as provided by the manufacturer, is indicated. 1–9, representative images are shown. Arrows point out the positive invasive tumor cells. (**b'**), Average expression of catulin in all 26 cases of human breast cancer samples and 10 cancers adjacent or normal breast tissue samples are shown. Tumor samples were divided into two groups: one, advanced group in stages between T2–T4 with lymph nodes involved—N1, second group less advanced in stages between T1–T2 without lymph nodes involved—N0. (**c**), RT-qPCR shows significant stable knockdown of catulin expression in MDA-MB-231 cells (t-test, $p < 0.005$). The results were confirmed on a protein level on Western Blot analysis resulting in no catulin protein in knockdown samples. Presented is cropped gel picture, an original view of the gel is included in Supplementary Fig. S1d). (**d**), 3D spheroid assay shows impaired invasive properties of MDA-MB-231 and HCC1806 knockdown cells compared to control and the calculated distance of invasion is shown on charts and marked with red line. Yellow dotted line represents the sphere body while the red dotted line circles around the invasion zone.

Labeling and tracking of invasive breast cancer cells using novel catulin promoter based reporter system. We described previously that catulin marks the invasive front of SCC, and that upregulation of catulin expression correlates with the transition of cells from epithelial to mesenchymal morphology[8,14]. Moreover, we have already shown that catulin expression correlates with BC progression and cancer cells invasive potential. Taken together, these data suggest that catulin might be a great marker of invasive, and motile cancer cells. To mark those cells, we developed a novel catulin promoter based reporter system, in which GFP expression is driven directly from catulin short promoter (Fig. 2a). After stable transfection of MDA-MB-231 cell line with catulin reporter plasmid, followed by puromycin selection, GFP fluorescence of established MDA-MB-231 CatGFP cell line was analyzed using flow cytometry. We gated for GFP + signal comparing reporter cell line to corresponding original MDA-MB-231 cell line as a negative control (representative results are shown in Fig. 2b). Around 20% of total cells (40% of parent) highly expressed GFP signal, coming from catulin promoter, and this percentage was stable in culture. To test the correlation of GFP fluorescence with catulin expression level, we performed qPCR analysis of catulin expression in sorted MDA-MB-231GFP + and MDA-MB-231GFP- populations (Fig. 2b'). This analysis confirmed enrichment of catulin expression in MDA-MB-231GFP + population. To verify how heterogeneous, the catulin expression is in the MDA-MB-231 cell line, we performed immunostaining of cells using antibodies against catulin and beta-catenin (general epithelial marker). As expected, we observed differences in the level of catulin expression (Fig. 2c). To check in vitro the localization of cells with GFP expression in the reporter system, we used 3D sphere invasion model, and immunostained the spheres with GFP antibody, and known EMT marker, Vimentin as well as cell–cell adhesion, and Wnt signaling pathway marker, β-catenin (Fig. 2d). The most outer cells, located at the invasion front of the sphere show the strongest GFP signal, and this signal correlates with the expression of EMT marker, Vimentin (arrows in Fig. 2d, lower panel). In both cases GFP and Vimentin signal is much weaker in the sphere body, in the center (dashed line). The strongest expression of GFP signal is also visible in the cells invading into the matrigel from the reporter sphere stained with β-catenin (arrows in Fig. 2d, top panel). In this case GFP signal is also much weaker in the sphere body, whereas β-catenin expression is quite uniform (dashed line). These data support the idea that novel Catulin-GFP reporter system marks invasive and motile cells in vitro, in the 3D model.

Genetic signature of Catulin GFP positive breast cancer cells by RNAseq analysis revealed enrichment in genes important for migration, invasion and tumor-vasculature interactions. As we developed a functioning reporter system marking invasive BC cells, we wanted to check the genetic signature of those cells. We used in vivo xenograft model, where the MDA-MB-231-Cat-GFP reporter cell line was injected into the mammary fat pad of 5 Nod.Scid mice. Primary tumors were formed after 8 weeks and exhibited patchy signal for the GFP. Interestingly, visible vasculature was located in the GFP positive areas of the tumor mass (arrows in Fig. 3a). Tumors after dissection were collected, either for further immunohistochemical analysis, or sorted in order to collect GFP + and GFP − fraction for RNAseq analysis. To correlate localization of invasive cells (GFP +) with known EMT marker Vimentin, and epithelial marker CD44, we performed immunohistochemical staining of tumors formed after the injection of the MDA-MB-231-Cat-GFP reporter cell line. Strong GFP fluorescence, indicating catulin GFP reporter expression, was observed in the areas of tumor-stroma interface, especially in the cells invading surrounding stroma (arrows in Fig. 3c,d and e). On the other hand, Vimentin was expressed relatively broadly in the tumor (stars in Fig. 3d), not only at the tumor invasion front (arrows in Fig. 3d,e). Catulin GFP + cells marked smaller population of cancer cells with the majority of them co-expressing Vimentin (arrows in Fig. 3d, d' and e, e'), and the level of Vimentin expression in GFP positive cells, at the tumor invasion front, was much higher than in the tumor center. The strong correlation of the Vimentin and GFP expression is presented in magnified areas of tumor cells invading into the stroma (arrows in Fig. 3d'), and into the muscles (arrows in Fig. 3e'). This shows high heterogeneity within tumor mass with Catulin-GFP reporter, marking tumor cells with the highest Vimentin expression. Co-staining of CatulinGFP reporter derived tumor with the human specific CD44 antibody also shows similar pattern of GFP expression, with majority of GFP + cells localized at the tumor stroma interface (Fig. 3c and c'). To determine the genetic signature of the catulin GFP + cells we isolated, and FACS sorted GFP + CD44 + and GFP-CD44 + cells from 3

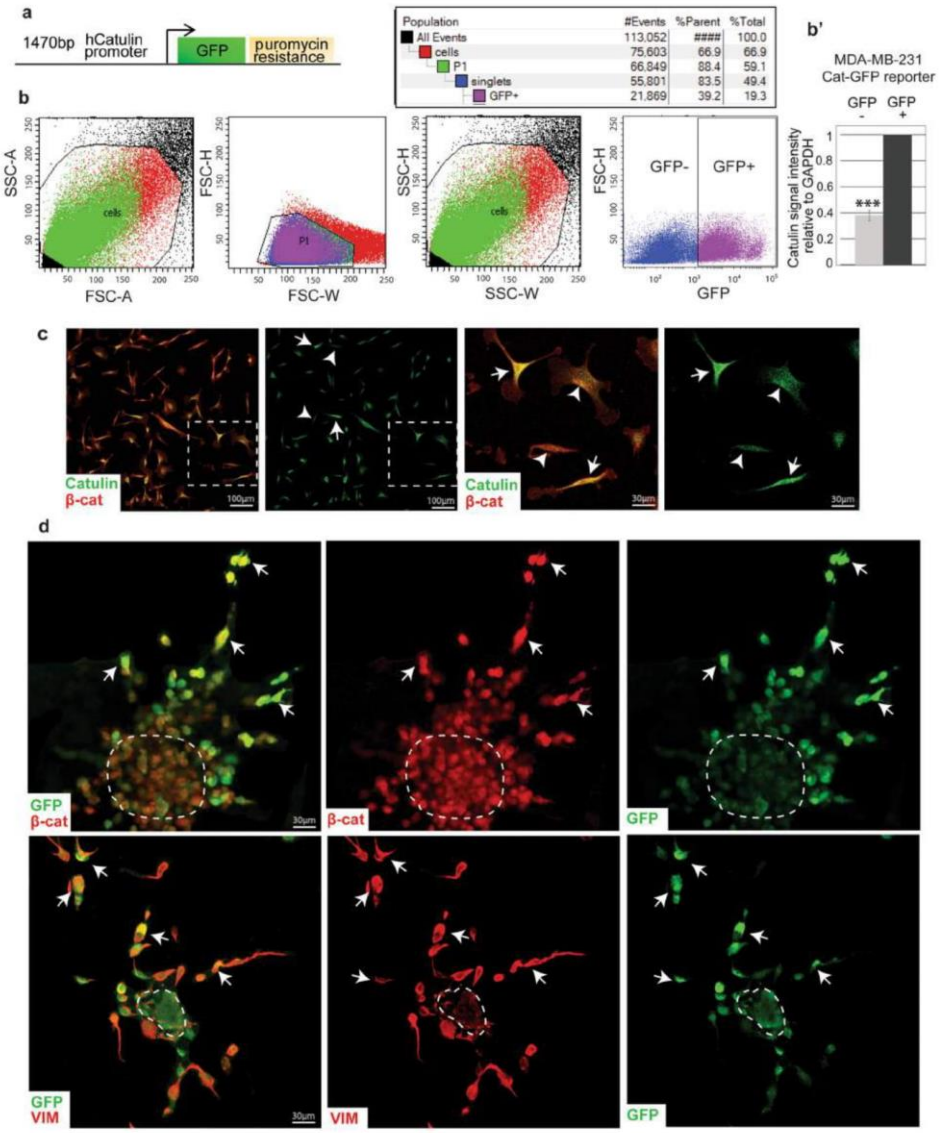
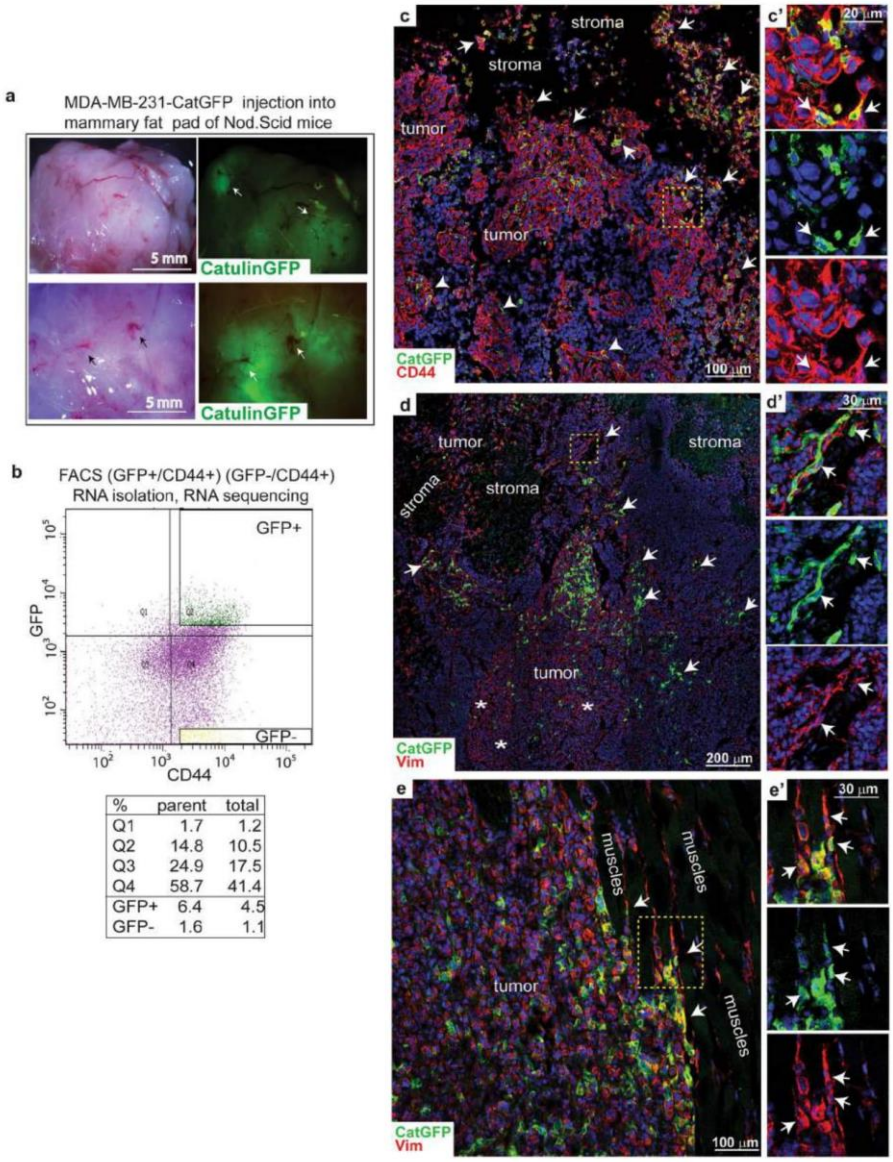


Figure 2. Development of catulin promoter based novel reporter system to label and track population of invasive cancer cells of breast cancer. (**a**), Shown is the graphical representation of catulin promoter novel reporter system where GFP expression is driven from catulin promoter expression. (**b**), Flow cytometry analysis of MDA-MB-231 cells transfected with catulin promoter reporter system. Gating strategy set to determine population of GFP positive cells. (**b'**), RT-qPCR shows difference in catulin expression level in sorted MDA-MB-231-Cat-GFP + and MDA-MB-231-Cat-GFP- cells (t-test, $p < 0{,}005$). (**c**), Immunostaining of MDA-MB-231cells using antibodies against catulin and beta-catenin, dashed squares in left panels indicate magnified panels on the right side. Arrows indicate cells with strong catulin expression, whereas arrowheads indicate cells with lower catulin expression. (**d**), Spheres formed from MDA-MB-231-Cat-GFP reporter cell line were analyzed on immunofluorescence. In all sets there was GFP stained in green and in red stained was β-catenin and Vimentin. On the right side one can find the magnification of spheres with green and red signal colocalization. Arrows indicate invasive cells spreading into the matrigel, whereas dashed line indicates sphere body.

independent tumors formed after the injection of the MDA-MB-231-Cat-GFP reporter cell line (Fig. 3b) using CD44 as an additional tumor cell marker to increase sort purity. RNAseq analysis was performed comparing RNA isolated from GFP + /CD44 + versus GFP−/CD44 + sorted cells, and proper function of the reporter system and sorting strategy was confirmed by the appearance in the RNAseq data CTNNAL1 gene in Cat-GFP + /CD44 + population as an internal control (log2FC 0.43, *p*val 0.04). Comparison of RNA from GFP + / CD44 + versus GFP-/CD44 + cells revealed a list of 1856 upregulated, and 1617 downregulated genes ($p < 0.05$). The minimum log2-fold change considered to be significant change in the expression between the two populations was set up at log2FC 0.43, $p\text{val} \geq 0.05$). Principal component analysis (PCA) and Volcano plot of RNAseq analysis for the MDACatGFP + /CD44 + (GFPp), and the MDACatGFP −/D44 + (GFPn) sorted populations is presented in Supplementary Fig. 3a. The Ingenuity Pathway Analysis (Qiagen), revealed cellular movement to be deregulated as one of the top 5 molecular and cellular functions. It includes 813 deregulated molecules (Fig. 4a). Interestingly, genes upregulated in this category were included in subcategories revolving around cell movement, invasion of cells, and migration of endothelial cells, where particularly interesting were genes like *SAA1, ENG, CDH5, PECAM1, DIO2, NOS3, MMP9* being responsible for tumor vascularization process (Fig. 4b). Then we analyzed top physiological system development and functions. Top 5 categories included cardiovascular system development and function, having 501 molecules deregulated (Fig. 4c). Subcategory of development of vasculature revealed same upregulated genes that were shown in Fig. 4b, *SAA1, ENG, CDH5, PECAM1, DIO2, NOS3, MMP9,* and additionally *WT1, TIE1, GATA2,* and others (Fig. 4d). Upstream analysis showed *Vegf, HGF* and *MYC* among others as a potential upstream regulators of observed gene changes (Fig. 4e). Interestingly, top pathways deregulated in the catulin GFP + cells involved epithelial adherens junctions signaling and remodeling of epithelial adherens junctions (Fig. 4f) with upregulation of Wnt pathway transcription factors *Tcf7, Lef1* as well as *Fgf1 and Met* (Fig. 4g). These data suggest that increased expression of catulin in the invasive cancer cells correlates with the expression of genes involved in migration, and also could be important in the modulation of adhesive properties of cancer cells and their interactions with vasculature.

Invasive breast cancer cells express endothelial marker MCAM, and participate in vascular mimicry. As our RNAseq analysis of the catulin reporter GFP + breast cancer cells revealed enrichment in genes important for tumor-vasculature interactions, we stained isolated tumor samples with antibody against PECAM (CD31)—well known endothelial marker. We noticed that indeed some of the Catulin GFP + cells localize in the areas rich in endothelial structures, stained in red (stars in Fig. 5a) attaching themselves to the endothelial walls at first glance. Interestingly, some of the GFP + cancer cells co-expressed GFP and endothelial marker PECAM (arrows in Fig. 5a and a'). Next we focused on the MCAM (CD146) which was also upregulated in RNAseq data, because CD146 is not only an adhesion molecule on endothelial cells, but also a cellular surface receptor of different ligands, actively involved in signaling in the numerous physiological and pathological processes. Overexpression of CD146 can be observed in most of malignancies, and is implicated in the progression of cancers, especially vascular, and lymphatic metastasis. Interestingly, we observed that marked by GFP invasive breast cancer cells were enriched at the proximity of the CD146 expressing vascular structures (arrows in Fig. 5b). Sometimes, we observed that those GFP + cancer cells expressed both GFP and CD146 and even participated in the formation of single layered vascular like structures (arrows in Fig. 5b', c and d). We also noticed GFP + /MCAM − cancer cells that participated in vascular mimicry together with GFP + /MCAM + cells, as marked by arrowheads in Fig. 5c. This suggests, that invasive cancer cells can participate in vascular mimicry process, and also with increased plasticity start to exhibit endothelial fate and express endothelium specific markers like MCAM, resembling vascular structures.

Signaling pathways affected by the decrease of catulin expression in BC. To better understand how the ablation of catulin in tumors influence the capability of those cells to invade, change adhesive properties, and plasticity we performed RNAseq analysis of tumor cells isolated using FACS, after injection of NOD. Scid mice with the MDA-MB-231 G85 (catulin KD) and the GNS (Ctrl) cell lines (Fig. 6). Principal component analysis (PCA) and Volcano plot of RNAseq analysis for the MDA-MB-231 G85 (catulin KD) and the GNS (ctrl) sorted populations is presented in the Supplementary Fig. 3b. We performed functional annotation of the microarray data using Ingenuity Pathways Software to identify the biological functions that were significantly represented in the catulin-deficient tumors. When we compared catulin KD versus control group, we got a list of 730 downregulated (internal control *CTNNAL1* gene downregulated (log2fold change − 2459) and 528 upregulated genes (Fig. Supl. 3b). Ingenuity Pathway analysis revealed that cellular movement was on the top of 5 deregulated molecular, and cellular functions (Fig. 6a). The list of 30 most downregulated genes in catulin knock down cells included genes involved in cellular movement (*NGFR, XIRP1, MYO1F, ACTC1,* etc.), cellular adhesion (*DSCAM, NTM, PCDG1, ICAM2* etc.), cell-ECM interaction (*MMP3, COL1A2, PDLIM2,* etc.), EMT (*MEGF6*) and migration of cancer cells (*SERINC2, MIR503HG, HSPB1,* etc.) (Fig. 6b). Those results emphasize the role of catulin in cellular movement, EMT, interactions with microenvironment, and progression of cancer, as downregulation of this gene resulted in deregulation of genes important for those processes. As we were mostly interested in the upregulated genes in the invasive catulin reporter GFP + cells, we wanted to evaluate if any of these genes appear in the downregulated gene set from catulin KD cells. We used Biovenn web tool to compare and visualize those sets of genes[48]. 75 genes (catulin related) appeared to be both highly upregulated in the reporter Catulin GFP + invasive tumor cells and highly downregulated in catulin KD tumor cells. From those 75 genes we selected 21 to be involved in vascularization, TNBC development and EMT related. As shown in the Fig. 6c we found internal control *CTNNAL1* gene to be present in both groups. Interestingly, in these 75 genes many were responsible for tumor vascularization process—*CNN1, ESM1, ADAMTSL4, RCAN1, EDN1, JMJD6, JUP.* Some were specific for TNBC development—*CPA4, SIX2, NEU3, LYN,* and some were specific for



%	parent	total
Q1	1.7	1.2
Q2	14.8	10.5
Q3	24.9	17.5
Q4	58.7	41.4
GFP+	6.4	4.5
GFP-	1.6	1.1

◂**Figure 3.** Catulin-GFP reporter system marks the population of tumor cells at the invasive front in the xenograft model of breast cancer enriched with the vasculature. (**a**), MDA-MB-231-Cat-GFP reporter cell line was injected into the mammary fat pad of Nod.Scid mice. Tumors were imaged for GFP. Arrows show the vasculature network enrichment. (**b**), Tumors formed after injection of MDA-MB-231-Cat-GFP cells were sorted for GFP +/CD44 + cells and GFP −/CD44 +. RNA was isolated and used for RNAseq analysis. (**c**), Isolated tumors after injection of MDA-MB-231-Cat-GFP reporter cell line into the mammary fat pad of Nod.Scid mice were cut and stained for GFP (green), and epithelial marker –CD44 (red). Nuclei were stained with DAPI (blue). Arrows indicate cells highly expressing catulin (GFP +) at the tumor-stroma invasive front. Arrowheads point out cells expressing catulin (GFP +) in the patches of tumor cells located deeper in the tumor but still mixed with the stroma. In the yellow bracket marked is the area enlarged on (**c'**)—where cells highly expressing GFP are shown and marked with arrows to be simultaneously expressing CD44 (red). (**d**), Isolated tumors after injection of MDA-MB-231-Cat-GFP reporter cell line into the mammary fat pad of Nod.Scid mice were cut and stained for GFP (green), and mesenchymal marker -Vimentin (red). Nuclei were stained with DAPI (blue). Arrows indicate cells highly expressing catulin (GFP +) at the tumor-stroma invasive front. Asterisks point out broad vimentin expression. In the yellow bracket marked is the area enlarged on (**d'**)—where cells highly expressing GFP are shown and marked with arrows to be simultaneously highly expressing signal from vimentin (red). (**e**), Demonstrated is the border of the tumor and muscles, enriched in GFP + cells. In the yellow bracket marked is the area enlarged on (**e'**)—where GFP + cells squeezed in between muscle cells also co-express red signal from vimentin (marked with arrows).

inducing cancer migration, EMT and metastasis—*MEGF6, SERINC2, RHEBL1, FBLIM1, CXCL8, CKS2*. This suggests that catulin might play a role in the vascularization process, TNBC development, cancer cell migration and metastasis.

Catulin influences breast cancer cells adhesive properties and stem cell potential. High expression of CD44 and low expression of CD24 are well-known breast cancer stem cells markers[49]. To further investigate the possible increase in the plasticity of catulin expressing cells we wanted to verify if CD44 expression correlates with the expression of catulin in vivo. Therefore, we analyzed tumors formed after injection of NOD. Scid mice with the MDA-MB-231 G85 (catulin KD) and the GNS (Ctrl) cell lines. We observed small difference in the tumor size between WT and Catulin KD (Fig. 7a). The slides with sections cut from those tumors, were immunostained using human specific CD44 antibody. The level of CD44 expression is different in tumor cells, however most of the control tumor cells are positive for CD44 expression (Fig. 7a' upper panel). However, when we compared catulin depleted tumors (G85) with control (GNS) we noticed decrease in the CD44 expression (Fig. 7a', lower panel). As breast cancer stem cells are characterized as CD44 + /CD24 − we trypsinized the MDA-MB-231 G85 and GNS spheres, stained them with CD44 and CD24 specific antibodies, and analyzed by flow cytometry. As it can be seen in the Fig. 7b, there is a tremendous downshift in CD44 localized at the membrane in the catulin ablated cells, while the CD24 expression remains similar in both groups. These data suggest that knockdown of catulin correlates with the decrease in the CD44, and may impact stemness potential of breast cancer cells. To verify that this deregulation of CD44 expression is linked to impaired stemness potential, we performed sphere serial re-plating assay. In this method spheres, every 7 days are subjected to serial passage allowing to determine the number of cancer stem cells with each passage[50]. As it can be seen in the Fig. 7c, sphere forming capacity for G85 (catulin KD) number of spheres dropped dramatically over the time, compared to GNS control. Similar results were obtained for the HCC1806 cell line (Fig. Supl 2). On the other hand, growth curve performed using MDA-MB-231 G85 and GNS cells in the 2D culture conditions didn't show any difference in proliferation (Fig. 7d) indicating that the function of catulin might be important for proper adhesion and/or apical polarity regulation as described previously[24]. Together these results suggest that catulin might be involved in modulation of cellular adhesive properties which is important for stem cell function.

Discussion

Tumor progression, invasion and metastatic dissemination is a very complex process requiring vast variety of transcriptional and protein function changes. Epithelial-to-mesenchymal transition allows to destabilize cadherin/catenin cell–cell contacts and transform epithelial state of a cell into mesenchymal one. It would not be possible without cell-stroma interactions. Catulin, α-catenin homologue has been previously described to be upregulated at the invasion front of human squamous cell carcinoma resulting in an increased motility and progression of those tumors[14]. Here we observe that catulin is highly expressed in the triple negative breast cancer cell lines. Moreover, elevated catulin expression was noticed in human tissue array and its expanding level correlated with the tumor progression. Additionally, knockdown of catulin in TNBC cell lines resulted in a decreased migratory and invasive potential on 3D spheroid model and in invasion chip assay what correlates with the previously described results performed on HNSCC cells in our laboratory[14]. It has also been previously shown that catulin promotes cell migration and invasion of lung carcinoma cells. Catulin interacts directly with ILK, what activates ILK/Akt/NF-κB signaling pathway. This led to the upregulation of fibronectin and integrin $\alpha_v\beta_3$ what resulted in cancer metastasis[25]. Same signaling cascade occurs probably in breast carcinoma progression. Hence inhibiting of catulin expression leads to decreased motility and invasiveness of TNBC cells. Basing on the fact that the higher invasive potential of cancer cells the higher catulin expression and the fact that depleting catulin decreases aggressiveness of those cells we propagate that catulin is a great marker for invasive breast cancer cells. By developing novel reporter system based on catulin promoter-GFP we were able to characterize and track highly invasive cells. We demonstrated that this population of breast cancer cells exhibit mesenchymal

a

Top molecular and cellular functions	p-value range	# mol
Cell death and survival	4,42E-11 - 5,70E-43	1130
Cell cycle	4,82E-11 - 5,02E-36	677
Cellular assembly and organization	4,13E-11 - 4,30E-29	819
Cellular function and maintenance	3,43E-12 - 4,30E-29	604
Cellular movement	2,13E-11 -1,72E-27	813

b

MDA-CatGFP+ vs GFP- upregulated genes
Molecular and cellular function - cellular movement

cell movement		invasion of cells		migration of endothelial cells	
IL7R	5,274	SAA1	5,018	SAA1	5,018
SAA1	5,018	ESM1	3,557	TNFSF15	4,925
TNFSF15	4,925	ENG	3,449	SH2D2A	3,469
ABI3	4,83	CDH5	3,341	ENG	3,449
CNN1	4,277	PECAM1	3,273	CDH5	3,341
DCDC2	3,941	MMP1	3,212	PECAM1	3,273
SH2D1A	3,469	WT1	2,976	DIO2	3,019
ENG	3,449	IL11	2,696	NOS3	2,503
CDH5	3,341	TERT	2,656	FGF13	2,425
PECAM1	3,273	KISS1	2,521	PRKCZ	2,397
MMP1	3,212	NOS3	2,503	IL1B	2,278
SPNS2	3,209	FGF13	2,425	ADGRA2	2,084
ACTC1	3,1	AGAP2	2,419	MMP9	2,036
DIO2	3,019	PRKCZ	2,397	IGF2	1,949
WT1	2,976	ZNF503	2,356	GATA2	1,865
NLRP10	2,914	RNF43	2,336	ECSCR	1,842
TIE1	2,742	DKK3	2,305	BMP6	1,834
IL11	2,696	COCH	2,289	SEMA3E	1,731
ADRB1	2,585	IL1B	2,278	AS1PR3	1,658
KISS1	2,521	SEMA7A	2,131	EGFL7	1,445
NOS3	2,503	HOXB9	2,077	F11R	1,421
FGF13	2,425	MMP9	2,036	MCAM	1,409
AGAP2	2,419	ANXA8	2,023		
PRKCZ	2,397	DSP	1,981		
KCNK2	2,313	IGF2	1,949		
DKK3	2,305	FGFR4	1,894		
COCH	2,289	BMP6	1,834		
IL1B	2,278	MYH10	1,815		
MNX1	2,269	ADAM12	1,751		
SEMA7A	2,131	CCNA1	1,659		
ADGRA2	2,084	S1PR3	1,658		
HOXB9	2,077	FERMT3	1,644		
MMP9	2,036	SPHK1	1,639		
		CTSV	1,536		
		LCP1	1,522		
		XDH	1,521		
		SRGN	1,509		
		AURKA	1,509		
		ARHGEF2	1,508		
		F11R	1,421		
		MCAM	1,409		

log2 fold change

c

Top physiological system development and function	p- value range	# mol
Organismal survival	6,35E-11 - 5,49E-41	845
Cardiovascular system development and functiontion	2,13E-11 - 3,75E-22	501
Embryonic development	1,58E-11 - 6,71E-21	397
Organismal development	5,56E-11 - 6,710E-21	966
Tissue morphology	6,34E-11 - 2,40E-20	656

d

MDA-CatGFP+ vs GFP- upregulated genes
Cardiovascular system development

development of vasculature	
SAA1	5,018
TNFSF15	4,925
CNN1	4,277
ESM1	3,557
ENG	3,449
CDH5	3,341
PECAM1	3,273
MMP1	3,212
PLCB1	3,07
WT1	2,976
TIE1	2,742
TERT	2,656
ADRB1	2,585
NOS3	2,503
FGF13	2,425
IL1B	2,278
PTK7	2,189
WNK4	2,168
SEMA7A	2,131
ADAMTS6	2,103
ADGRA2	2,084
MMP9	2,036
DYSF	1,974
IGF2	1,949
GATA2	1,865
ECSCR	1,842
BMP6	1,834

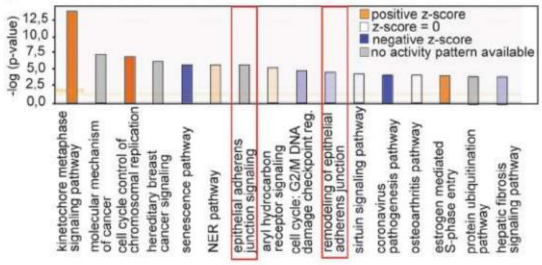
log2 fold change

e

UPSTREAM ANALYSIS

selected upstream regulators	predicted activation state	activation z-score
TP53	inhibited	-3.324
NUPR1	inhibited	-6.500
TGFB1	inhibited	-2.278
ERBB2	activated	4.053
E2F1	activated	3.324
Vegf	activated	2.284
HGF	activated	2.235
MYC	activated	5.709
ESR1	activated	2.451
TCF4	activated	2.251

f



g

MDA-CatGFP+ vs GFP-
epithelial adherens junction signaling

upregulated genes		downregulated genes	
ACTC1	3,1	TCF4	3,53
MYH10	1,815	TUBA1A	0,834
TCF7	1,747	ACVR2A	0,823
ARPC5L	0,905	PTPRM	0,814
TUBG1	0,723	EPN2	0,797
TUBA1C	0,714	TCF7L2	0,794
TUBB4B	0,708	BMPR2	0,791
LEF1	0,695	BAIAP2	0,783
MYH3	0,661	NECTIN3	0,762
FGF1	0,64	ACVR1	0,746
NECTIN1	0,546	CTNND1	0,659
SSX2IP	0,546	LMO7	0,496
MYL6B	0,531	MAGI1	0,482
WASF1	0,517	CTNNB1	0,473
RRAS2	0,492	PTEN	0,462
TUA1B	0,495	IQGAP1	0,443
TUBB6	0,494		
MET	0,466		

log2 fold change

◂**Figure 4.** RNAseq and Ingenuity Pathway Analysis of invasive cancer cells (GFP+) reveals enrichment in genes responsible for cell movement and vasculogenesis in cancer. (**a**), Top 5 deregulated categories of molecular and cellular functions. In the red bracket enlightened is the most relevant cellular movement function. (**b**), listed are genes upregulated (log2 fold change > 1.4) in categories of cell movement, invasion of cells and migration of endothelial cells. (**c**), Top 5 deregulated categories of physiological system development and function. In the red bracket, enlightened is the cardiovascular system development and function. (**d**), Upregulated genes involved in development of vasculature. (**e**), Upstream analysis revealed top molecular upstream regulators with predicted activation state and z-score. (**f**), Top deregulated canonical pathways. In red brackets marked are pathways involved in adherens junction signaling and remodeling important for tumor progression. Orange indicates activation of the pathway; blue indicates inhibition and gray indicates no activity pattern available for this particular pathway in IPA. Gradient in colors is reflecting the level of activation or inhibition. (**g**), Listed are genes upregulated and downregulated in epithelial adherens junction signaling pathway with log2 fold change > 0.4.

as well as cancer stem cell features, like high CD44 expression in vitro in spheroid model. Very interesting was the finding that knockdown of catulin results in the significant attenuation of CD44 signal on the membrane, when analyzing cells on flow cytometry and in tumor tissue from xenograft transplant. These results suggest that this fraction of invasive cells highly expressing catulin and CD44 + may constitute so called Cancer Stem Cells—a subpopulation of tumor cells having tumor-initiating properties as well as being able to reconstitute the cellular heterogeneity typical of their tumors of origin[51], which we are planning to test further. Potential role of catulin in stem cells, in this case in the adult stem cells, was shown in the work of S.J. Morrison group. They showed the localization of haematopoietic stem cells (HSCs) which reside in a perivascular niche using a green fluorescent protein (GFP) knock-in for the catulin gene in mice, discovering that catulin(GFP) is expressed by only 0.02% of bone marrow haematopoietic cells, including almost all HSCs[52]. It will be interesting to further test the role of catulin in adhesive properties of stem cells and cancer stem cells. Our RNAseq data also indicated elevated level of MMP-9 expression in Catulin GFP + cells. It has been observed that CD44 co-localizes with active MMP-9, what promotes degradation of extracellular matrix and facilitates invasion[53]. This might be one of the mechanisms driving high invasive potential of those cells however interaction between catulin and CD44 pathway needs further investigating, in terms of cancer propagation. Transcriptional profiling of invasive triple negative breast cancer cells, basing on our novel reporter system, revealed significant upregulation of genes involved in cell motility, invasive potential and angiogenesis. Many of the genes we listed above, have been already associated with tumor vascularization propagation in other types of cancer—*PECAM1, ESM1, CD146, CDH5, ENG, ICAM4, PTN, FGF1, ANKRD1, GATA2, KDR, NOS3, SERPINE1, TIE1*[54,55]. Interestingly when we compared genes upregulated in the invasive TNBC cells with downregulated genes from catulin KD samples, we got a list of 75 genes including genes specific for vasculogenesis *CNN1, ESM1, ADAMTSL4, RCAN1, EDN1, JMJD6, JUP.* Some were specific for TNBC development—*CPA4, SIX2, NEU3, LYN,* and some were specific for inducing cancer migration, EMT and metastases—*MEGF6, SERINC2, RHEBL1, FBLIM1, CXCL8, CKS2.*

We recently used the same reporter system in the head and neck squamous cell carcinoma model, which is characterized by different way of invasion and spreading than breast cancer. HNSCC spreads mostly by loco-regional invasion, moving in clusters, very often along innervation[56]. Molecular profiling of CatulinGFP + reporter cells from the HNSCC model also provides a list of genes associated with cells movement and invasion. Interestingly transcriptional profile of CatulinGFP + reporter cells from HNSCC model overlapped with the expression of signature genes from single cell analysis of human HNSCC specimens, related to partial EMT, so hybrid between epithelial and mesenchymal state rather than complete EMT. In addition, we also observed upregulation of genes with adhesive properties, involved in the axonal guidance like L1CAM, Neuropilin-1, semaphorins, and ephrins, emphasizing potential interactions of cancer cells and neuronal components of the stroma[56].

Our data from both systems implicate that catulin may play a pivotal role in cancer type specific tumor-microenvironment interaction, being involved in modulation of adhesive properties of tumor cells. The possible mechanism of increased catulin expression in invasive cancer cells comes from recent paper by Cassandri et al.[57]. They showed that zinc-finger protein 750 (ZNF750) is a negative regulator of the migration, and invasion of breast cancer cells by repressing a prometastatic transcriptional program, which includes genes involved in focal adhesion and extracellular matrix interactions, such as LAMB3 and CTNNAL1. The expression of CTNNAL1 and LAMB3 inversely correlated with ZNF750 expression in breast cancer. ZNF750 is responsible for recruitment of the epigenetic modifiers KDM1A and HDAC1 to the promoter regions of LAMB3 and CTNNAL1, affecting histone marks and trans activating these genomic sites. Importantly, they also showed gene expression analysis in cancer patient datasets which indicated that ZNF750, and its targets were negative prognostic factors in breast cancer.

The change in adhesive properties, resulting in increased plasticity of those cells might be also important for the switch of tumor cells into endothelial phenotype and participation in the vascular mimicry. Two different concepts at first glance which are Cancer Stem Cells and Vascular Mimicry start to merge together having influence on each other. It has been described that Cancer Stem Cells stimulate Vascular Mimicry in the micro-environment of the tumor, by differentiating of endothelium cells together with cancer cells lining up to form brunching lumen and tubes mimicking vascular network allowing extensive nutrition to the tumor mass[58]. Here we clearly show that catulin plays a pivotal role in breast cancer tumorigenesis and invasiveness process. It is a great marker of heterogeneous population of invasive breast cancer cells and by interacting with different cancer stem cell and adherens junction molecules it may be a significant linker between different pathways leading to enhanced cancer cell motility. That is why deciphering the complex nature of this protein is very important. Moreover, here we show that invasiveness of cancer cells is a very complex process linking cancer stem cell theory

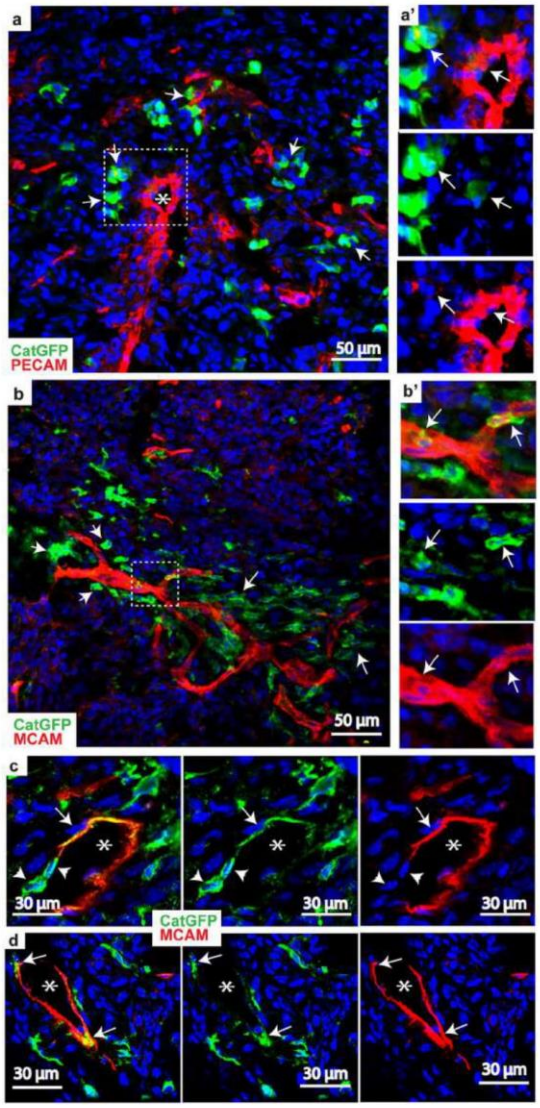


Figure 5. Catulin positive breast cancer cells localize in the areas enriched in vasculature and coexpress endothelial marker—MCAM (CD146). (**a**), Immunostaining of tumor isolated after injection of MDA231-Cat-GFP cells. In green shown are highly invasive cancer cells localized in the areas enriched in vasculature stained with endothelial specific marker PECAM in red. Nuclei are stained with DAPI (blue). With arrows marked are GFP positive cells and with asterisk blood vessel. (**a'**), Enlarged, is the area presented in white bracket. With arrows marked are cells both expressing GFP and PECAM. (**b**), Immunostaining of tumor isolated after injection of MDA231-Cat-GFP cells. In green shown are cancer cells localized in the areas enriched in vasculature stained with endothelial specific marker MCAM in red. Nuclei are stained with DAPI (blue). With arrows marked are GFP positive cells. (**b'**), Enlarged, is the area presented in white bracket. With arrows marked are cells both expressing GFP and MCAM. (**c**,**d**), Two examples of vascular mimicry. With arrows marked are cells expressing both GFP + (green) and endothelial marker MCAM (red). Arrowheads indicate GFP + cells which are MCAM negative but still participate in vascular mimicry. With stars marked is the lumen of the vasculature.

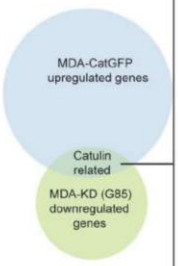
a

Top molecular and cellular functions	p-value range	# molecules
Cellular movement	2,21E-07 – 9,23E-24	328
Cell death and survival	1,45E-07 – 8,23E-23	411
Cell cycle	1,87E-07 – 1,07E-17	233
Cellular development	2,04E-0,7 – 1,08E-16	430
Cellular growth and proliferation	2,04E-0,7 – 1,08E-16	405

b MDACat KD (G85) vs MDACatCtrl (GNS) downregulated genes

gene symbol	summary	MDA231-G85-vs-GNS log2fold	MDA231-G85-vs-GNS pvalue
RELN	secreted extracellular matrix protein, controls cell-cell interactions	-4,641	3,10E-06
NGFR	nerve growth factor receptor, regulates cell migration and gene expression	-3,304	0,004
DSCAM	member of the immunoglobulin superfamily of cell adhesion molecules (Ig-CAMs)	-3,282	0,005
FAT3	FAT atypical cadherin 3	-2,690	0,006
MMP3	matrix metalloproteinase (MMP) family, involved in the breakdown of extracellular matrix	-2,609	0,003
XIRP1	protects actin filaments during depolymerization	-2,597	2,83E-05
NTM	(GPI)-anchored cell adhesion molecules	-2,560	0,003
CTNNAL1	actin cytoskeleton dynamic, migration and invasion	-2,459	6,63E-31
CCL26	secreted protein involved in immunoregulatory and inflammatory processes	-2,403	2,48E-06
MYO1F	molecular motor protein that generates force on actin filaments	-2,397	0,005
PCDH1	found at cell-cell boundaries	-2,377	0,001
ACTC1	actins, highly conserved proteins that are involved in various types of cell motility	-2,039	0,001
PROM2	at the basal epithelial cells, involved in the organization of plasma membrane microdomains	-2,019	4,32E-06
ELFN2	extracellular Leucine Rich Repeat And Fibronectin Type III Domain Containing 2	-2,006	1,32E-05
CCL20	secreted protein involved in immunoregulatory and inflammatory processes	-1,951	0,001
COL1A2	extracellular matrix protein	-1,927	0,021
MEGF6	promotes EMT	-1,926	0,008
CNN1	actin filament-associated regulatory protein	-1,920	0,015
KRT17	type I intermediate filament chain keratin 17	-1,769	0,030
EPHB3	ephrins mediate numerous developmental processes, particularly in the nervous system	-1,716	0,005
ADAMTSL2	member of the ADAMTS a disintegrin and metalloproteinase with thrombospondin motifs	-1,631	0,032
ARC	Activity Regulated Cytoskeleton Associated Protein	-1,631	1,74E-05
EREG	secreted peptide hormone and member of the epidermal growth factor (EGF) family	-1,601	0,013
COL11A2	extracellular matrix protein	-1,536	0,009
KRT34	member of the keratin gene family	-1,515	0,025
PRDM6	repressor, involved in regulation of vascular smooth muscle cells contractile proteins	-1,400	0,038
RHOB	role in actin organization and cell migration, also implicated in membrane trafficking	-1,238	0,007
ICAM2	member of the intercellular adhesion molecule (ICAM) family	-1,236	0,006
IL1A	member of the interleukin 1 cytokine family	-1,196	0,004
NEBL	actin binding protein	-1,179	0,014

c



Catulin (Ctnnal1) related	Function
Ctnnal1	Cancer cell invasion (internal control)
CNN1	Enhances breast cancer vascularization
ESM1	Endothelial cell specific molecule
CPA4	Triple negative breast cancer specific
MEGF6	Promotes EMT
ADAMTS4L	Important for angiogenesis
SERINC2	Promotes migration of cancer cells
RHEBL1	Required for the early stage of mammary tumor progression
TSPYL5	Suppressor of p53
SIX2	Maintains stemness of breast cancer cells
RCAN1	Stimulated by VEGF, RCAN1 regulates gene expression, cell migration and tubular morphogenesis
FBLIM1	Modulates cell adhesion and motility
PDLIM2	Promotes cell migration and adhesion through interactions with the actin cytoskeleton
CXCL8	Critical for cancer progression and metastasis, regulates cancer stem cells proliferation
EDN1	Vasoconstrictor present in endothelium
CKS2	Promotes progression of certain cancers via positive control of proliferation, invasion and migration
MIR503HG	Regulates cancer invasion and progression
NEU3	Marker of BC
LYN	Highly expressed in TNBC
JMJD6	Has a role in angiogenesis, the process of vessel formation, whereas further roles of JMJD6 in pathophysiological processes were implicated, such as mammary tumorigenesis
JUP	Plays a pivotal role in the regulation of cell-cell adhesion

Figure 6. RNAseq and Ingenuity Pathway analysis of catulin KD tumor cells reveals deregulated genes involved in cellular movement. (**a**), Top 5 molecular and cellular functions show cellular movement to be highly deregulated in case of catulin KD. (**b**), List of genes highly downregulated in catulin KD cells derived from tumors. In the red bracket highlighted is catulin as an internal control. (**c**), Biovenn analysis of a common set of genes to be both highly upregulated in catulin reporter GFP + cells and highly downregulated in catulin KD tumor cells. Set of catulin related genes listed on the right side of the panel.

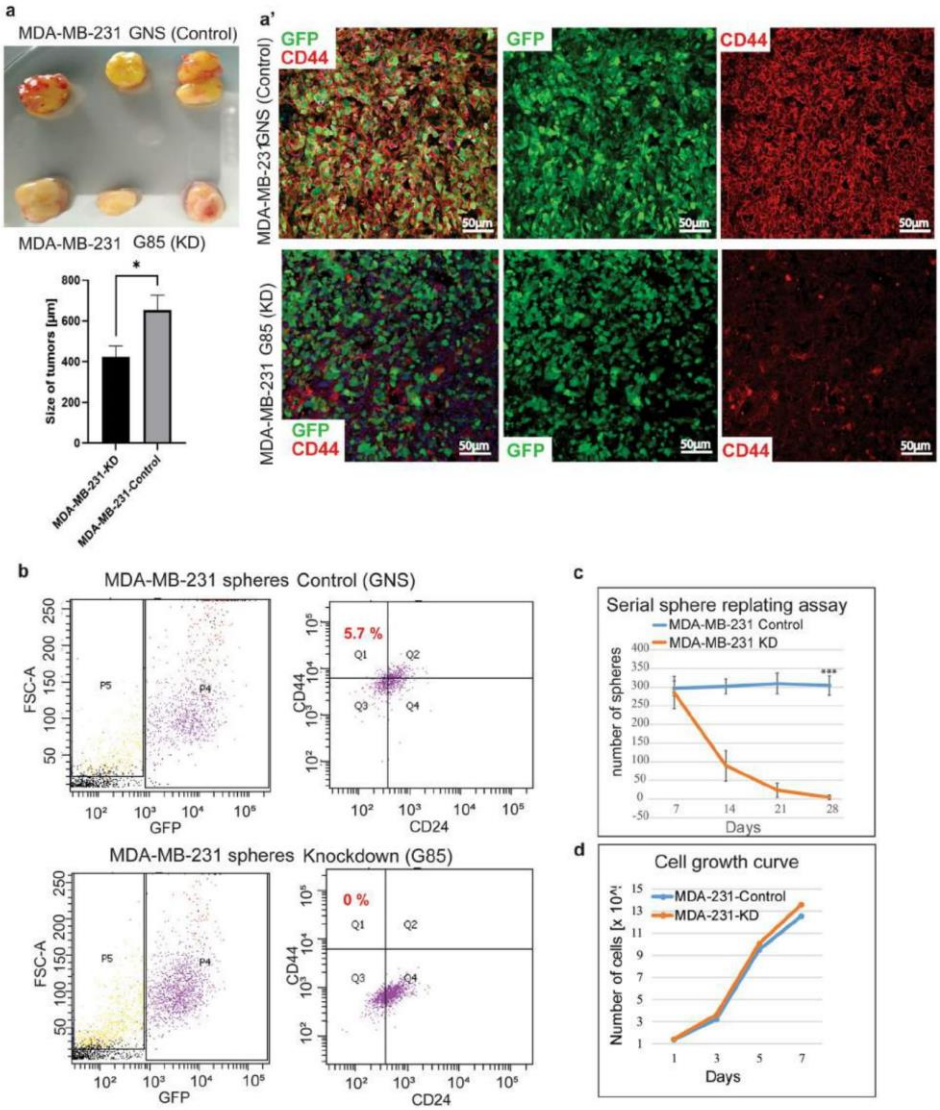


Figure 7. Catulin influences breast cancer stem cell potential. (**a**), Tumors formed after injection of MDA-MB-231-Cat-GNS (control) and MDA-MB-231-G85 (catulin KD) cells. Tumors size was measured and data are presented as a graph. (**a'**), Tumors formed after injection of MDA-MB-231-Cat-GNS (control) and MDA-MB-231-G85 (catulin KD) cells were cut and stained for GFP (green) and CD44 (red). (**b**), Flow cytometry analysis of breast cancer stem cells markers CD44 + /CD24 – of MDA-MB-231 GNS (control) and G85 (catulin KD) sphere cells (the sort was repeated twice). (**c**), Sphere serial replating assay of MDA-MB-231-Cat-GNS (control) and MDA-MB-231-G85 (catulin KD) cells (done in triplicate). Sphere forming capacity presented as a number of spheres generated on each time point. (**d**), Growth curve of MDA-MB-231-Cat-GNS (control) and MDA-MB-231-G85 (catulin KD) cells (done in triplicate).

with vascular mimicry and EMT. While a number of currently used cancer therapeutics are effective inhibitors of angiogenesis, developing a new class of vascular mimicry specific inhibitors could allow for the treatment of angiogenesis-resistant tumors, inhibit cancer metastasis and improve patient survival. Therefore, further investigating in cancer research needs broader perspective combining those processes together.

Materials and methods

Generation of stable cell lines and cell culture. MDA-MB-231 and MCF7 cell lines were cultured in DMEM High Glucose (Biowest #L0102-500) supplemented with 10% FBS (Biowest, #S181S-500), 100 IU penicillin and 100 µg/mL streptomycin (Biowest #L0018-100). HCC1806 cells were cultured in RPMI-1640 (Biowest #L0500-500) supplemented with 10% FBS (Biowest, #S181S-500), 1 mM sodium pyruvate (Gibco), 100 IU penicillin and 100 µg/mL streptomycin (Biowest #L0018-100). MDA-MB-468 were cultured in L-15 supplemented with 10% FBS (Biowest, #S181S-500), 100 IU penicillin and 100 µg/mL streptomycin (Biowest #L0018-100). HCC1806, MDA-MB-231 and MDA-MB-468 cell lines were obtained from the American Type Culture Collection (ATCC) and authenticated by ATCC with tests such as short tandem repeat profiling (STR profiling). All cells were maintained in a humidified atmosphere at 37 °C with 5% CO2. pGIPZ lentiviral shRNA clones (two independent shRNA sequences against human catulin) are available from Open Biosystems and were packaged according to manufacturer's protocol. See Supplementary Materials and Methods for clone details. At 48 h, post-transduction, cells were selected with puromycin to establish stable cell lines. The number of living cells was calculated by Trypan Blue staining using EVETM Automatic Cell Counter (Nano EnTek, South Korea). Cell line was regularly tested for mycoplasma contamination using a PCR-based method (Young et al., 2010).

Plasmid stable transfection. To generate stable alpha-catulin promoter GFP reporter cell lines MDA-MB-231 and HCC1806 cells were transfected with GLuc-ON Promoter Reporter Clone (GeneCopoeia # HPRM14050-PF02) construct using Lipofectamine 3000 Transfection Reagent (Thermo Fisher Scientific #L3000001) according to manufacturer's instructions. See Supplementary Materials and Methods for clone details. At 48 h' post-transfection, cells were selected with puromycin to establish stable cell lines. Fluorescence was examined under confocal microscope LSM 700 (Zeiss).

RNA isolation, cDNA synthesis and Real-Time Quantitative PCR (RT-qPCR). RNeasy Mini Kit (Qiagen, #74,106) was used to isolate total RNA from MDA-MB-231 catulin KD and control cells and HCC1806 KD and control cells, according to manufacturer's instructions. With the usage of DeNovix DS-11 Spectrophotometer concentration and purity of RNA was established. cDNA was synthetized according to the PrimeScript RT Master Mix (TAKARA BIO INC. #RR036A) protocol (1 µg of RNA was used). Catulin gene expression levels were quantified by RT-qPCR. Each reaction mixture was composed of as follows: 1 µl of 20-times diluted cDNA, 6.25 µl of Fast SG qPCR Master Mix (2x) (EurX, #E0411) and 0.5 µM oligonucleotide primers (listed in section Supplementary Material and Methods) in a total volume of 12.5 µl. Reactions were performed in triplicates on the LightCycler 480 II (Roche) in the following conditions: 40 s at 95 °C, followed by 42 amplification cycles (95 °C for 15 s, 60 °C for 15 s, 72 °C for 15 s). Catulin expression level was normalized to GAPDH. Primers are provided in Supplementary Materials and Methods. Statistical significance was determined using student *t*-test. $p < 0.05$ was considered statistically significant. FACS sorted cells were immediately collected in RNAprotectR cell reagent (QIAGEN). Then they were centrifuged and resuspended in RLT buffer according to manufacturer's protocol. RNeasyR Micro Kit (QIAGEN). Was used to extract total RNA from those cells. RNAseq analysis was performed by the Next-Generation Sequencing core facility in the Centre of New Technologies, University of Warsaw with the usage of NovaSeq 6000 system (Illumina).

Expression data analysis. The expression and data analysis were performed as described in[56]. The RNA-seq libraries were paired-end sequenced using NovaSeq 6000 at the CeNT's Genomics Core Facility. The resulting reads were trimmed; Illumina adapters were removed using trimmomatic v0.36. Quality of individual fastq files was assessed by FastQC. Remaining rRNAs were removed using sortmeRNA v3.03 and then mapped to hg38 reference transcriptome (GRCh38.p13 gencode v34) using STAR aligner. Due to the contamination of the samples with mouse transcripts and after careful principal component analysis, 1 control sample was removed from the downstream analysis. Such aligned reads were then quantified using Salmon v0.13.1 Differentially expressed genes (DEGs) were then identified using DESeq2. Only genes of adjusted p-value less than 0.05 were considered.

Statistical analysis. Statistical significance was determined using student *t*-test. $p < 0.05$ was considered statistically significant.

Western blot. Cells from the 10 cm plate were scraped with the usage of ice cold PBS, centrifuged and resuspended in 200 µL of lysis buffer containing 0.15 M NaCl, 1% Triton X100, 0.05 M Tris, and protease inhibitor cocktail III (Calbiochem). Cells were multiply passed through a 26 g needle, incubated on ice for 30 min, and then centrifuged at 14,000 × g for 15 min at 4 °C. Supernatant containing proteins was then collected and separated on 4–12% NuPAGE Novex Bis–Tris gels (Invitrogen) and transferred onto a nitrocellulose membrane with a semi-dry Biorad transfer system for 40 min at 70 mA. Membranes were then blocked in a 5% skim milk diluted in TBS-T (TBS with 0.1% Tween-20) for 1 h at RT on a gentle shaker. Primary antibody in 5% skim milk in 0.1% TBS-T was then added to the membranes and incubated on a gentle shaker overnight at 4 °C. Membranes were washed 3 times with TBS-T and secondary antibodies (peroxidase conjugated) were added in 0.1%

TBS-T. Membranes were then incubated for 1 h at room temperature on a gentle shaker. Antibody list is included in Supplementary Materials and Methods. Amersham Imager 600 RGB was used to visualize the blots.

Sphere formation and invasion assay. Cells transduced with non-silencing and catulin specific shRNA and cells transfected with catulin promoter reporter system were used for sphere invasion assay. 1000 serum free cells were seeded in 200 µl of sphere formation medium [DMEM/F12 (Biowest), 1% B27 supplement (Gibco), 20 ng/mL EGF (Invitrogen) and 20 ng/mL FGF(Invitrogen)] in 96 well Corning Costar ultra-low attachment plate and allowed to form spheres for 72 h. Then spheres were mixed with 1:3 matrigel (Corning)—collagen (Corning) solution and plated on Millicell EZ Slide 8-well glass (Merck Millipore). Incubation and invasion was allowed for 48 h. Immunostainings were performed according to Elia., Lippincott-Schwartz., 2009. Sphere invasion assays were repeated independently 3 times. Images were taken using confocal microscope LSM 700 (Zeiss). A one-way ANOVA test was applied to calculate the statistical significance for distance of invasion considering data from all 3 experiments where $P<0.05$).

Chip invasion assay. Cells transduced with non-silencing and catulin specific shRNA were used for chamber invasion assay. 3D Cell Culture Chips (#Dax-1, Aim Biotech) were used for invasion assays. 10^6 of cells were counted and resuspended in serum free medium and were added into media channel of invasion chip. Medium containing chemoattractant (20% FBS) was added to the opposite media channel. In between those two channels there was a hydrogel channel containing 1:3 matrigel (Corning)—collagen (Corning) solution. Chips were maintained in a humidified atmosphere at 37 °C with 5% CO2. Invasion was allowed for 0, 24, 48, 72, 96 h. Invasion assays were repeated independently 3 times. Images were taken using fluorescence microscope Axio Observer (Zeiss). A one-way ANOVA test was applied to calculate the statistical significance for distance of invasion considering data from all 3 experiments where $P<0.05$).

Xenograft transplants. 5×10^5 of MDA-MB-231 cells transduced with non-silencing and catulin specific shRNA and cells transfected with catulin promoter reporter system, suspended in medium mixed 1:1 with matrigel (Corning) were injected into 4th mammary gland of NOD.CB17/Prkdcscid/scid/Rj mice, using isoflurane as a general anesthetic. Each variant of cells was injected in 5 repeats. Tumors were allowed to form for 4–9 weeks before sacrificing and collecting the primary tumor from each subject. According to the protocol, mice were euthanized with CO_2 following a cervical dislocation. Briefly, tumors used for RNA isolation were first dissected from the mouse, minced into small pieces, incubated in collagenase (1000U/mL) for 1 h at 37 °C, washed in PBS, trypsinized (0.25% trypsin- EDTA from Gibco) for 20 min at 37 °C, and FACS sorted for GFP + and GFP- cells using a BD Biosciences FACSAria cell sorter. All experiments and procedures were preapproved by the First Warsaw Local Ethics Committee for Animal Experimentation nr 214/2017, 26 April 2017. The animals were treated in accordance with the EU Directive 2010/63/EU for animal experiments, and the ARRIVE guidelines (https://arriveguidelines.org).

Indirect immunofluorescence detection. Tumors formed in our xenografts were resected washed with PBS and embedded in OCT. Then they were sectioned at 12 µM with the usage of Leica Cryostat for indirect immunofluorescence detection of various markers. At first samples were fixed in 4% paraformaldehyde for 10 min, washed with PBS and permeabilized in PBS-T (PBS with 0.1% Triton X-100) for 10 min. Then the tissues were blocked in 0.1% BSA, 2.5% NGS (normal goat serum), 2.5% NDS (normal donkey serum) in PBS-T for 1 h at RT. Primary antibodies were diluted in 0.1% BSA in PBS-T and incubated overnight at 4 °C. Descriptions and dilutions of primary antibodies are described in Supplementary Materials and Methods. Secondary antibodies conjugated with Alexa Fluor 488 or 594 were diluted 1:500 in blocking solution and incubated for 1 h at RT. Confocal microscope LSM 700 (Zeiss) was used to take pictures.

Immunohistochemistry. The immunohistochemical staining analysis for Catulin was performed on tissue microarray slide panel BR8014 (Biomax, Derwood, MD, USA). The slide consists of breast cancer and normal tissue microarray, containing 30 cases of carcinoma (26 invasive ductal carcinomas, 4 invasive lobular carcinoma), 5 each of adjacent normal tissue and normal tissue, duplicate cores per case. The procedure was performed as described previously in Karpinska et al., 2021[56]. Briefly, the slide was deparaffinized and rehydrated and after blocking the endogenous peroxidase activity in 0.3% H2O2 for 3 min, the slides were blocked in a blocking buffer (0.1% gelatin, 0.1% BSA, 2.5% donkey serum, 2.5% goat serum, and 0.3% Triton X in PBS) for 1 h at room temperature. Slides were then incubated with a primary antibody in 0.1% BSA in 0.1% PBS-T ON at 4 °C. Next day, the slides were incubated in appropriate biotin-conjugated secondary antibody (1:100) (Vector Labs, Burlingame, CA, USA) for 1 h in the blocking buffer, followed by the incubation in the prepared Vectastain A + B solution (2 drops of A, 2 drops of B, 2.5 mL PBS 1X) for 30 min at RT. Reactions were developed using the diaminobenzi-dine (DAB) reagent as the chromogenic substrate (SK-4100; Vector). The sections were counterstained with 5 × diluted haematoxylin, mounted in 80% glycerol and examined under a light microscope.

Spheres serial replating assay. Cells transduced with non-silencing and catulin specific shRNA were used for sphere serial replating assay. 20,000 serum free cells were seeded in 1 ml of sphere formation medium [DMEM/F12 (Biowest), 1% B27 supplement (Gibco), 20 ng/mL EGF(Invitrogen) and 20 ng/mL FGF(Invitrogen)] in 6 well Corning Costar ultra-low attachment plates. Cells were allowed to form spheres for 7 days. After 7 days, spheres were trypsinized and counted and calculated by Trypan Blue staining using EVETM Automatic Cell Counter (Nano EnTek, South Korea). 20,000 of cells were then plated again in 1 ml of sphere formation medium

and allowed for the next 7 days to form spheres again. Whole process was repeated 3 times until day 28th was reached. The results were calculated as sphere forming capacity = number of spheres/number of cells plated [%]. A one-way ANOVA test was applied to calculate the significance of the number of spheres considering data from all 3 experiments where $P < 0.05$).

Ethics approval and consent to participate. All experiments were preapproved by the LKE (local ethic committee) at the University of Warsaw.

Data availability

The data regarding manuscript are presented in main figures of the article. The datasets generated during the current study are not publicly available due to continuation of the project and preparation of the next publication. Additional data obtained in this study are available on request from the corresponding author.

Received: 21 January 2022; Accepted: 15 July 2022
Published online: 25 July 2022

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Acknowledgements
To Dr. J. Palatini, MSc Eng. D. Adamska and Msc M. Szelag from Genomic Core Facility at CeNT, University of Warsaw for RNAseq analysis.

Author contributions
M.G. design the work, acquired the data, analysed and interpret the data, wrote the main manuscript text, prepared figures; K.K. contibuted to Figs. 2, 3 and 7 in FACS analysis; A.G. participated in mouse work; Ł.B. contributed to RNAseq data analysis; A.K. : design the work, acquired the data, analysed and interpret the data, wrote the main manuscript text, corrected figures. All authors reviewed the manuscript.

Funding
This work was funded by the Polish National Science Center (NCN) Grants: 2020/37/N/NZ5/03069 to M. Gielata, 2017/25/B/NZ5/01597 and 2020/37/B/NZ5/03950 to A. Kobielak.

Competing interests
The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-022-16802-2.

Correspondence and requests for materials should be addressed to A.K.

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Podsumowanie i wnioski

W publikacji nr 1 udało mi się dokonać kompleksowego, szczegółowego opisu dotychczasowego stanu wiedzy na temat bardzo słabo poznanego białka alfa-katuliny. Najważniejsze wnioski płynące z tej publikacji:

- Alfa-katulina i alfa-katenina należą do grupy białek wchodzących w składrodziny winkuliny. W przeciwieństwie do szerokiej wiedzy na temat alfa-kateniny, która jest niezbędna do adhezji komórkowej, oraz winkuliny istotnej dla adhezji komórek z ECM za pośrednictwem integryn i adhezji komórka-komórka, funkcja alfa-katuliny nadal nie jest dobrze poznana.
- W publikacji pokazałem, że mimo wysokiej homologii sekwencji alfa-katuliny z alfa-kateniną i winkuliną, lokalizacja komórkowa jest odmienna. Alfa-katulina znajduje się zarówno we frakcji błonowej i cytozolowej, a alfa-katenina znajdywana jest jedynie we frakcji błonowej komórki.
- Jednym z lepiej opisanych białek wchodzących w interakcje z katuliną jest Lbc Rho GEF, jeden z czynników wymiany nukleotydów guaninowych. Alfa-katulina funkcjonuje jako rusztowanie dla białka Lbc Rho GEF, będąc regulatorem aktywnego RhoA co skutkuje regulacją cytoszkieletu aktynowego.
- Alfa-katulina odgrywa kluczową rolę w trakcie embriogenezy na etapie neurulacji. Działając jako rusztowanie dla RhoA w wierzchołkowych częściach komórki prowadzi do prawidłowej przestrzennej aktywacji miozyny wpływając na dynamikę aktynowo-miozynową i stabilność połączeń międzykomórkowych co gwarantuje poprawne wygięcie płytki nerwowej w trakcie neurulacji.
- Interakcje alfa-katuliny z dystrobrewiną, wchodzącą w skład kompleksu dystrofiny, są kluczowe dla poprawnego łączenia się neurotransmiterów w miotubach w płytce ruchowej, odgrywając kluczową rolę w neurosekrecji, pobudzenia neuronów i mięśni.

Najwięcej uwagi poświęca się udziałowi alfa-katuliny w procesie nowotworzenia. Jako, że katulina jest cytoszkieletowym łącznikiem, może ona wpływać na migrację komórek nowotworowych. W różnych modelach nowotworowych wykazano wysoki poziom ekspresji katuliny, zwłaszcza w najbardziej inwazyjnych komórkach, w których aktywując różne ścieżki sygnałowe może promować inwazyjność nowotworów.

Pomimo powyższych doniesień na temat alfa-katuliny, nadal istnieje wielka potrzeba lepszego poznania biologii tego białka. Wiemy, że alfa-katulina jest zaangażowana w progresję nowotworów, lecz dokładny mechanizm molekularny jest nadal nieznany. Kluczowe będzie również zbadanie dokładnego wpływu alfa-katuliny na przestrzenną dystrybucję białka RhoA w trakcie migracji komórkowej. Znalezienie wszelkich potencjalnych białek wiążących się z katuliną powinno dać znacznie więcej odpowiedzi.

W publikacji nr 2 wykorzystałem fakt występowania podwyższonej ekspresji alfa-katuliny w najbardziej inwazyjnych komórkach nowotworowych i generując nowy system reporterowy promotora katuliny zademonstrowałem heterogenność inwazyjnych komórek nowotworowych, częściową ich plastyczność i uczestnictwo w mimikrze naczyniopodobnej. Najważniejsze wnioski płynące z tej publikacji:

- Wykazałem różnicę w poziomach ekspresji alfa-katuliny w różnych liniach komórkowych TNBC, gdzie dwie linie komórkowe mające najbardziej agresywny fenotyp (MDA-MB-231 i HCC1806) okazały się mieć dużo wyższy poziom ekspresji *CTNNAL1* w porównaniu do nieinwazyjnej linii MCF-7.
- Wykazałem, że w ludzkich tkankach z nowotworów piersi wzrastający poziom alfa-katuliny koreluje ze wzrastającym stopniem zaawansowania choroby nowotworowej.
- W modelu sferoidowym 3D wyciszenie alfa-katuliny w liniach komórkowych MDA-MB-231 i HCC1806 prowadzi do znaczącego spadku potencjału inwazyjności i migracji.
- Scharakteryzowałem nowy system reporterowi, wktórym ekspresja GFP zachodzi bezpośrednio z promotora alfa-katuliny. Okazało się, że w liniach komórkowych TNBC ok. 40% komórek eksprymuje wysoko alfa katulinę. Ponadto w modelu 3D komórki najwyżej eksprymujące katulinę lokalizują się na brzegu inwazyjnym sfery a ekspresja GFP pokrywa się z ekspresją markera EMT wimentyny.
- Analiza RNAseq komórek najbardziej inwazyjnych (GFP+) porównanych do komórek negatywnych (GFP-) pokazała 1856 genów o podwyższonej ekspresji i 1617 o obniżonej ekspresji. Najbardziej zróżnicowanymi genami były te należące do kategorii ruchliwości komórkowej, inwazyjności, migracji komórek śródbłonka naczyń, z dużą ilością genów zaangażowanych w proces tworzenia naczyń

krwionośnych. W kategorii regulatorów transkrypcyjnych o najwyższej ekspresji były VEGF, HGF i Myc.

- Porównując listę genów o podwyższonej ekspresji z genami o obniżonej ekspresji z grupy komórek z wyciszoną katuliną otrzymałem swoistą listę genów zależnych od alfa-katuliny, gdzie pojawiły się geny specyficzne dla angiogenezy - *PECAM1, ESM1, CD146, CDH5, ENG, ICAM4, PTN, FGF1, ANKRD1, GATA2, KDR, NOS3, SERPINE1, TIE1*. Świadczy to o korelacji ekspresji alfa-katuliny z genami odpowiedzialnymi za migrację komórkową, a także możliwą modulację potencjału adhezyjnego komórek nowotworowych i ich interakcję z unaczynieniem.
- W liście genów o podwyższonej ekspresji w moich wynikach wyjątkowo ciekawym wydał się CD146 (MCAM), który znany jest jako marker śródbłonka, ale także jako receptor promujący angiogenezę śródnowotworową i migrację komórkową [42]. Barwienia immunohistochemiczne wykazały, że plastyczne komórki inwazyjne, wysoce eksprymujące alfa-katulinę również eksprymują MCAM tworząc jednocześnie struktury naczyniopodobne (proces mimikry naczyniopodobnej).
- Wiedząc, że poziom ekspresji alfa-katuliny wpływa na plastyczność i inwazyjność komórek nowotworowych, sprawdziłem czy w przypadku braku ekspresji katuliny zmienia się profil markerów CSC. Zauważyłem spadek poziomu ekspresji markera macierzystości CD44 specyficznego dla komórek raka piersi w przypadku wyciszenia genu alfa-katuliny. Ponadto komórki z wyciszoną katuliną nie były w stanie odtwarzać sfer z kolejnymi pasażami. Oznacza to, iż katulina jest zaangażowana w funkcje adhezyjne komórek nowotworowych wpływając tym samym na ich potencjał macierzystości.

Podsumowując, alfa-katulina jest bardzo istotnym białkiem w procesie nowotworzenia. Poziom jej ekspresji wpływa na właściwości adhezyjne i migracyjne komórek nowotworowych. Zwiększona plastyczność komórek raka piersi może prowadzić do zwiększonej macierzystości i możliwości przeprowadzenia procesu mimikry naczyniopodobnej, która pomaga komórkom guza pierwotnego przejść EMT i osiągając dystalne organy zapoczątkować przerzutowanie. Alfa-katulina mogłaby wydawać się świetnym celem terapii przeciwnowotworowej, jednakże jej ważne funkcje w trakcie rozwoju zarodkowego, homeostazie organizmu i szeregu procesów fizjologicznych wykluczają ją jako bezpośredni target terapeutyczny

Dlatego kluczowym będzie lepsze poznanie jej biologii i białek oddziaływujących a także jej udziału w procesie tworzenia struktur naczyniopodobnych i procesie przerzutowania, co może potencjalnie doprowadzić do scharakteryzowania nowych celów dla celowanej terapii przeciwnowotworowej.

Oświadczenia współautorów

Warszawa 21.11.22
(miejscowość, data)

Agnieszka Kobielak

(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **„Emerging Roles of the α-Catenin Family Member α-Catulin in Development, Homeostasis and Cancer Progression"** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

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A. Kobielak

(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa 21.11.22
(miejscowość, data)

Agnieszka Kobielak

(imię i nazwisko)

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Jako współautor pracy pt. **„Catulin reporter marks a heterogeneous population of invasive breast cancer cells with some demonstrating plasticity and participating in vascular mimicry"** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: Koordynowanie badań zawartych w publikacji, pomysły na eksperymenty, redagowanie manuskryptu, finansowanie publikacji.

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obejmował on: analiza większości wyników, napisanie tekstu publikacji, stworzenie wszystkich figur, pomysł na większość eksperymentów.

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A. Kobielak
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 15.11.2022 r.
(miejscowość, data)

Tomasz Pieczonka
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **„Emerging Roles of the α-Catenin Family Member α-Catulin in Development, Homeostasis and Cancer Progression"** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi: częściowy przegląd literaturowy i przygotowanie jednego z podrozdziałów.

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(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Mateusza Gielata
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Tomasz Pieczonka
(podpis oświadczającego)

*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

Warszawa, 23.11.2022
(miejscowość, data)

Kamila Karpińska

(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **„Emerging Roles of the α-Catenin Family Member α-Catulin in Development, Homeostasis and Cancer Progression"** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi:

.....przygotowanie rozdziału czwartego p.t. "α-Catulin and Its Function during Development".....
.....
.....

Mój udział procentowy w przygotowaniu publikacji określam jako…10%….. %.

Wkład Mateusza Gielata w powstawanie publikacji określam jako…70%….. %,
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Kamila Karpińska
(podpis oświadczającego)

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Warszawa, 23.11.2022
(miejscowość, data)

Kamila Karpińska
(imię i nazwisko)

OŚWIADCZENIE

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.....
.....

Mój udział procentowy w przygotowaniu publikacji określam jako…10….. %.

Wkład Mateusza Gielata w powstawanie publikacji określam jako…70….. %,
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(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

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Kamila Karpińska
.....
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*w szczególności udziału w przygotowaniu koncepcji, metodyki, wykonaniu badań, interpretacji wyników

WARSZAWA, 24.11.2022
(miejscowość, data)

Łukasz Boryń
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **„Catulin reporter marks a heterogeneous population of invasive breast cancer cells with some demonstrating plasticity and participating in vascular mimicry"** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowił:

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(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji*)

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24.11.2022r.
(miejscowość, data)

Aleksandra Gwiazdowska
(imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. **„Catulin reporter marks a heterogeneous population of invasive breast cancer cells with some demonstrating plasticity and participating in vascular mimicry"** oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji stanowi opieka nad doświadczeniami i pomoc w eksperymentach na doświadczeniach

Mój udział procentowy w przygotowaniu publikacji określam jako 10 %

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obejmował on: analiza większości wyników, napisanie tekstu publikacji, stworzenie wszystkich figur, pomysł na większość eksperymentów.

(merytoryczny opis wkładu kandydata do stopnia w powstanie publikacji)*

Jednocześnie wyrażam zgodę na wykorzystanie w/w pracy jako część rozprawy doktorskiej mgr Mateusza Gielata
(imię i nazwisko kandydata do stopnia)

Aleksandra Gwiazdowska
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Lp.	Opis bibliograficzny	Impact Factor	MEiN
Artykuły			
1.	**Gielata M**, Karpińska K, Płeczonka T, et al. Emerging Roles of the α-Catenin Family Member α-Catulin in Development, Homeostasis and Cancer Progression. International Journal of Molecular Sciences. 2022;23(19):11962. [Rodzaj publikacji: praca poglądowa]	6,208	140
2.	**Gielata M**, Karpińska K, Gwiazdowska A, Boryń Ł, Kobielak A. Catulin reporter marks a heterogeneous population of invasive breast cancer cells with some demonstrating plasticity and participating in vascular mimicry. Sci Reports. 2022;12(1):12673. [Rodzaj publikacji: praca oryginalna]	4,997	140
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Piśmiennictwo

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