lek. wet. Jakub Kulus
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„Ekspresja wybranych genów związanych z sygnalizacją międzykomórkową oraz strukturą komórek ziarnistych pęcherzyka jajnikowego u świni domowej podczas krótkoterminowej pierwotnej hodowli in vitro”

Rozprawa na stopień doktora nauk weterynaryjnych
w dyscyplinie weterynaria

Promotor: dr hab. n. wet. Paweł Antosik prof. UMK

Katedra Diagnostyki i Nauk Klinicznych
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Spis treści

1. Wykaz publikacji stanowiących podstawę do nadania stopnia naukowego doktora

Publikacja I:

Jakub Kulus, Magdalena Kulus, Wiesława Kranc, Karol Jopek, Maciej Zdun, Małgorzata Józkowiak, Jędrzej M. Jaśkowski, Hanna Piotrowska-Kempisty, Dorota Bukowska, Paweł Antosik, Paul Mozdziak, Bartosz Kempisty

"Transcriptomic profile of new gene markers encoding proteins responsible for structure of porcine ovarian granulosa cells"

Biology, 2021, Vol. 10, nr 11, art. nr 1214, DOI: 10.3390/biology10111214

Punktacja MNiSW: 100; Impact Factor: 5,168; 5 – letni Impact Factor: 4,4;

Publikacja II:

Jakub Kulus, Wiesława Kranc, Magdalena Kulus, Piotr Dzięgiel, Dorota Bukowska, Paul Mozdziak, Bartosz Kempisty, Paweł Antosik

"Expression of genes regulating cell division in porcine follicular granulosa cells"

Cell Division, 2023, Vol. 18, p. 1–22. DOI: 10.1186/s13008-023-00094-7.

Punktacja MNiSW: 100; Impact Factor: 2,3; 5 – letni Impact Factor: 3,8;

Publikacja III:

Jakub Kulus, Wiesława Kranc, Magdalena Kulus, Dorota Bukowska, Hanna Piotrowska-Kempisty, Paul Mozdziak, Bartosz Kempisty, Paweł Antosik

"New Gene Markers of Exosomal Regulation Are Involved in Porcine Granulosa Cell Adhesion, Migration, and Proliferation"

International Journal of Molecular Sciences, 2023, Vol. 24, nr 14, art. nr. 11873, DOI: 10.3390/ijms241411873

Punktacja MNiSW: 140; Impact Factor: 5,6; 5 – letni Impact Factor: 6,2;

Łącznie współczynnik Impact Factor: 13,068 Łączna punktacja MNiSW: 340

Łącznie 5 - letni współczynnik Impact Factor: 14,4

2. Wykaz użytych skrótów

ABI3BP ang. *ABI family member 3 binding protein*

ABP białko wiążące aktynę (ang. *actin binding protein*)

ACTN1 aktynina (ang. *actinin alpha 1*)

ACTB β – aktyna (ang. *β – actin*)

ADAM23 ang. *ADAM metallopeptidase domain 23*

AKT kinaza białkowa B (ang. *protein kinase B*)

ANK2 ang. *ankyrin 2*

ANKRD1 ang. *ankyrin repeat domain 1*

ART techniki wspomaganego rozrodu (ang. *assisted reproductive technologies*)

CAV1 kaweolina 1 (ang. *caveolin 1*)

CCL2 ang. *chemokine (C-C motif) ligand 2*

CCNE2 cyklina E2 (ang. *cyclin E2*)

CCs komórki wzgórka jajonośnego (ang. *cumulus cells*)

CD44 antygen różnicowania 44 (ang. *cluster of differentiation 44*)

CD90 antygen różnicowania 90 (ang. *cluster of differentiation 90*)

CD105 antygen różnicowania 105 (ang. *cluster of differentiation 105*)

CD117 antygen różnicowania 117 (ang. *cluster of differentiation 117*)

CD166 antygen różnicowania 166 (ang. *cluster of differentiation 166*)

CDC20 ang. *cell division cycle 20*

CDC45 ang. *cell division cycle 45*

CDK1 kinaza zależna od cykliny 1 (ang. *cyclin-dependent kinase 1*)

cDNA komplementarny DNA (ang. *complementary DNA*)

CH homolog kalponiny (ang. *calponin homology*)

CHI3L1 ang. *chitinase-3-like protein 1*

CLIP4 ang. *CAP-Gly domain containing linker protein family member 4*

COC kompleks kumulus – oocyt (ang. *cumulus – oocyte complex*)

COL3A1 kolagen 3 podjednostka 1 (ang. *collagen type III alpha chain*)

COL14A1 kolagen 14 podjednostka 1 (ang. *collagen type XIV alpha chain*)

CNN1 kalponina (ang. *calponin 1*)

CNTLN centleina (ang. *centlein*)

CXCL10 ang. *C-X-C motif chemokine ligand 10*

DAPL1 ang. *death associated protein – like 1*

DAVID ang. *Database for Annotation, Visualization and Integrated Discovery*

DCN dekoryna (ang. *decorin*)

DMEM ang. *Dulbecco's Modified Eagle's Medium*

DNAJB1 ang. *DnaJ heat shock protein family (Hsp40) member B1*

ECM macierz zewnątrzkomórkowa (ang. *extracellular matrix*)

EDTA kwas wersenowy (ang. *ethylenediaminetetraacetic acid*)

EGFR receptor naskórkowego czynnika wzrostu (ang. *epidermal growth factor receptor*)

ERK ścieżka sygnalizacyjna ERK (ang. *ERK signaling pathway*)

ESPL1 separaza (ang. *extra spindle pole bodies like 1*)

EVs pęcherzyki zewnątrzkomórkowe (ang. *extracellular vesicles*)

FAK ścieżka sygnalizacyjna FAK (ang. *FAK signaling pathway*)

FBS płodowa surowica bydlęca (ang. *fetal bovine serum*)

FBXO5 ang. *F-Box Protein 5*

FCS płodowa surowica cielęca (ang. *fetal calf serum*)

FF płyn pęcherzykowy (ang. *follicular fluid*)

FI interakcja funkcjonalna (ang. *functional interaction*)

FMOD fibromodulina (ang. *fibromodulin*)

FN1 fibronektyna 1 (ang. *fibronectin 1*)

FRMD6 białko 6 zawierające domenę FERM (ang. *FERM domain containing 6*)

FSH hormon folikulotropowy (ang. *follicular stimulating hormone*)

FST folistatyna (ang. *follistatin)*

GCs komórki ziarniste (ang. *granulosa cells*)

GSN gelsolina (ang. *gelsolin*)

GJC połączenie szczelinowe typu neksus (ang. *gap junction connection*)

GO BP ontologia genów procesów biologicznych (ang. *gene ontology biological process*)

GO MF ontologia genów funkcji molekularnych (ang. *gene ontology molecular function*)

HSD3B1 ang. *hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 1*

HSD17B1 ang. *17β-Hydroxysteroid dehydrogenase 1*

ICAM międzykomórkowe cząsteczki adhezyjne (ang. *intercellular adhesion molecules)*

IGF1 insulinopodobny czynnik wzrostu IGF1 (*ang. insulin-like growth factor 1*)

IHH ang. *indian hedgehog signaling molecule*

IRS1 ang. *insulin receptor substrate 1*

ITGA2 podjednostka α-2 integryny (ang. *integrin subunit α-2*)

ITGA8 podjednostka α-8 integryny (ang. *integrin subunit α-8*)

ITGA11 podjednostka α-11 integryny (ang. *integrin subunit α-11*)

ITGB3 podjednostka β-3 integryny (ang. *integrin subunit β-3*)

KIF14 białko podobne do kinezyny 14 (ang. *kinesin family member 14*)

LAMB1 podjednostka β-1 lamininy (ang. *laminin subunit β-1*)

LH hormon luteinizujący (ang. *luteinizing hormone*)

LIPG lipaza śródbłonkowa (ang. *endothelial lipase, EL*)

LOX oksydaza lizylowa (ang. *lysyl oxidase*)

LRP1 białko związane z receptorem lipoprotein o niskiej gęstości 1 (ang. *LDL receptor related protein 1*)

MAL2 ang. *T-cell differentiation protein 2*

MAPK ścieżka sygnalizacyjna MAPK (ang. *MAPK signaling pathway*)

MGCs komórki ziarniste budujące ścianę pęcherzyka jajnikowego (ang. *mural granulosa cells*)

MXRA5 ang. *matrix remodeling associated 5*

NEBL ang. *nebulette*

NEK2 ang. *NIMA related kinase 2*

NEXN ang. *nexilin F-actin binding protein*

PAICS ang. *phosphoribosylaminoimidazole carboxylase and phosphoribosylaminoimidazolesuccinocarboxamide synthase*

PBS buforowana fosforanem sól fizjologiczna (ang. *phosphate-buffered saline*)

PCOLCE2 ang. *procollagen C-endopeptidase enhancer*

PCOS zespół policystycznych jajników (ang. *polycystic ovary syndrome*)

PI3K-AKT ścieżka sygnalizacyjna PI3K-AKT (ang. *PI3K-AKT signaling pathway*)

PLK2 ang. *polo like kinase 2*

POI przedwczesna niewydolność jajników (ang. *premature ovarian insufficiency*)

POSTN periostyna (ang. *periostin*)

RAP1 ścieżka sygnalizacyjna RAP1 (ang. *RAP1 signaling pathway*)

RGS2 ang. *regulator of G protein signaling 2*

RNA kwas rybonukleinowy (ang. *ribonucleic acid*)

RT-qPCR ilościowa łańcuchowa reakcja polimerazy z odwróconą transkrypcją (ang. *Reverse Transcription quantitative Polimerase Chain Reaction*)

SHAS2 syntaza hialuronianowa (ang. *hyaluronan synthase 2*)

SNX31 ang. *sorting nexin 31*

SPP1 ang. *secreted phosphoprotein 1*

STRING ang. *Search Tool for the Retrieval of Interacting Genes/Proteins*

TACC3 ang. *transforming acidic coiled-coil containing protein 3*

TAGLN transgelina (ang. *transgelin*)

TCs komórki osłonki pęcherzykowej (ang. *theca cells*)

TGFβ ścieżka sygnalizacyjna TGFβ (ang. *TGFβ signaling pathway*)

TMPO tymopoetyna (ang. *thymopoietin*)

TPM2 tropomiozyna 2 (ang. *tropomyosin 2, beta*)

TRIB2 ang. *tribbles pseudokinase 2*

TTK kinaza białkowa o podwójnej specyficzności (ang. *dual specificity protein kinase*)

VCAM-1 cząsteczka adhezyjna śródbłonka 1 (ang. *vascular cell adhesion molecule 1*)

3. Badania własne

3.1. Wprowadzenie

Komórki ziarniste (ang. *granulosa cells,* GCs), osadzone na błonie podstawnej (łac. *lamina basalis*), stanowią największą populację komórek budujących pęcherzyk jajnikowy [1]. Wśród nich najliczniej występują komórki tworzące ścianę pęcherzyka i wyścielające go od wewnątrz (ang. *mural granulosa cells,* MGCs). Kolejną grupę stanowią komórki ziarniste wzgórka jajonośnego (ang. *cumulus cells*, CCs), które tworzą charakterystyczne wyniesienie wewnątrz pęcherzyka jajnikowego [2]. W obrębie wzgórka jajonośnego (łac. *cumulus oophorus*), oprócz komórek ziarnistych, znajduje się zahamowany w metafazie II podziału mejotycznego oocyt II rzędu [3]. Komórki ziarniste, bezpośrednio otaczające komórkę jajową, tworzą charakterystyczny wieniec promienisty (łac. *corona radiata*). Oocyt od komórek ziarnistych wieńca promienistego oddzielony jest osłonką przejrzystą (łac. *zona pellucida*). Błona komórkowa oocytu oraz komórek ziarnistych jest silnie pofałdowana i tworzy liczne wypustki. Drążą one osłonkę przejrzystą, dzięki czemu komórki te kontaktują się i powstają połączenia szczelinowe typu neksus (ang. *gap junction connection,* GJC), które zbudowane są z białek błonowych – koneksyn [4]. Połączenia te występują także pomiędzy sąsiadującymi ze sobą komórkami ziarnistymi, umożliwiając swobodny przepływ jonów i niewielkich cząsteczek do 1,2 kDa [5]. Budowa taka wskazuje na istotną rolę w przepływie informacji międzykomórkowej, niezbędnej podczas wzrostu i dojrzewania pęcherzyka jajnikowego [6]. Z zewnątrz pęcherzyk jajnikowy otoczony jest łącznotkankową osłonką pęcherzykową (łac. *theca folliculi*), która dzieli się na warstwę wewnętrzną (łac. *tunica interna thecae folliculi*) oraz zewnętrzną (łac. *tunica externa thecae folliculi*). Warstwa wewnętrzna, przylegająca do błony podstawnej, charakteryzuje się występowaniem dużej liczby komórek (ang. *theca cells*) i włosowatych naczyń krwionośnych, zewnętrzna zaś jest uboższa w komórki i zawiera w przewadze włókna kolagenowe oraz większe naczynia krwionośne [7].

Podstawową rolą komórek ziarnistych jest ich udział w folikulogenezie oraz oogenezie [2]. Procesy te są ze sobą ściśle związane i zachodzą u zwierząt cyklicznie, w regularnie występujących po sobie fazach cyklu rujowego. Pod wpływem hormonów płciowych dochodzi do wzrostu i dojrzewania pęcherzyka jajnikowego oraz wykształcenia jego ostatecznej formy rozwojowej, zawierającej we wnętrzu jamę (pęcherzyk antralny). Jama ta wypełniona jest płynem pęcherzykowym (ang. *follicular fluid,* FF) i wraz z dalszym rozwojem pęcherzyka zwiększa swoją objętość [8]. Pęcherzyk jajnikowy odpowiada także za steroidogenezę, w którą

zaangażowane są komórki ziarniste wraz z komórkami warstwy wewnętrznej osłonki pęcherzykowej. Komórki osłonki pęcherzykowej rozpoczynają produkcję androgenów pod wpływem hormonu luteinizującego (ang. *luteinizing hormone,* LH) [9]. W kolejnym etapie zsyntetyzowane androgeny (androstendion) przedostają się przez błonę podstawną i docierają do komórek ziarnistych. Komórki te posiadają receptory dla hormonu folikulotropowego (ang. *follicle stimulating hormone*, FSH) i pod jego wpływem indukują aromatyzację androgenów do estrogenów (17β-estradiolu) [9]. Estrogeny wpływają korzystnie na unaczynienie osłonki wewnętrznej pęcherzyka jajnikowego, co zwiększa dostępność LH dla tych komórek. To z kolei prowadzi do zwiększonej syntezy androgenów w komórkach osłonki pęcherzyka i estrogenów w komórkach ziarnistych [7]. Podwyższony poziom estrogenów we krwi promuje proliferację komórek ziarnistych. Proliferacja tych komórek indukowana jest również poprzez 9 czynnik wzrostu i różnicowania (ang. *growth differentiation factor 9*, *GDF-9*) [10], wydzielany przez oocyt, co wskazuje na interakcje między GCs a komórką jajową. Wyżej opisane procesy prowadzą do powstania przedowulacyjnych pęcherzyków jajnikowych, z których mogą zostać uwolnione gotowe do zapłodnienia oocyty. W przypadku niedostatecznej produkcji estrogenów dochodzi do androgenizacji pęcherzyków jajnikowych, które ulegają atrezji, stopniowo zmniejszając tym samym pulę dostępnych do zapłodnienia komórek jajowych. Przedstawione wyżej różnice w stężeniu hormonów płciowych, związane z dojrzewaniem pęcherzyków jajnikowych, wpływają także na zmiany zachowania zwierząt, które są dobrze widoczne w okresie okołorujowym. Świnia domowa (łac. *Sus scrofa domestica*) należy do zwierząt poliestralnych z owulacją mnogą (poliowulacją), w trakcie której dochodzi do uwolnienia wielu kompleksów kumulus – oocyt (ang. *cumulus – oocyte complex,* COC).

Do prawidłowego funkcjonowania komórek zwierzęcych, w tym także komórek ziarnistych, wymagane jest odpowiednie mikrośrodowisko, w obrębie którego dochodzi do swobodnego przepływu informacji i wzajemnego komunikowania się. Macierz zewnątrzkomórkowa (ang. *extracellular matrix*, ECM) jest niezwykle ważną strukturą, obecną we wszystkich tkankach organizmu zwierzęcego [11]. Składa się z elementów o odmiennej strukturze chemicznej i pełni zróżnicowane funkcje [12]. Głównymi składnikami macierzy zewnątrzkomórkowej są dwie grupy cząsteczek. Pierwszą z nich stanowią białka włókniste, do których należą kolagen, elastyna, fibronektyna i laminina. Drugą grupą są proteoglikany. Cząsteczki te składają się z białkowego rdzenia, do którego przyłączone są glikozaminoglikany. Najczęściej występującym białkiem włóknistym jest kolagen, który wspiera rusztowanie tkankowe, ale także wpływa na adhezję, migrację i chemotaksję komórek

[13]. Proteoglikany z kolei wykazują właściwości wysoce hydrofilowe, powodując tworzenie hydrożelu, wypełniającego przestrzeń między białkami włóknistymi ECM. Ponadto proteoglikany są zaangażowane w wiele szlaków sygnalizacyjnych, związanych między innymi z receptorem insulinopodobnego czynnika wzrostu 1 (ang. *insulin-like growth factor 1*, *IGFIR*), receptorem naskórkowego czynnika wzrostu (ang. *epidermal growth factor receptor*, *EGFR*) oraz białkiem związanym z receptorem lipoprotein o niskiej gęstości 1 (ang. *LDL receptor related protein 1*, *LRP1*) [13]. Macierz zewnątrzkomórkowa stanowi unikalne mikrośrodowisko dla komórek, pełniąc dla nich nie tylko funkcje podporowe, ale także biorąc udział w szlakach transportu składników odżywczych, hormonów i sygnałów pozakomórkowych do komórek docelowych oraz służąc regulacji ekspresji genów i uwalnianiu cytokin [11,14]. Tak szeroki wachlarz pełnionych przez nią funkcji jest wynikiem zróżnicowanej struktury chemicznej [15].

W dotychczas przeprowadzonych przez innych autorów badaniach, ekspresja genów związanych z tworzeniem macierzy zewnątrzkomórkowej została wykazana w różnych obszarach pęcherzyka jajnikowego, takich jak: błona podstawna, osłonka przejrzysta, komórki ziarniste ściany i wzgórka jajonośnego oraz płyn pęcherzykowy [16]. Wykazano również, że profil ekspresji tych genów ulega zmianie w trakcie przebiegu procesu folikulogenezy [17], czego efektem jest zmienność konformacji łańcuchów lamininy w zależności od stopnia dojrzałości pęcherzyka jajnikowego [18]. Udział poszczególnych elementów składowych macierzy zewnątrzkomórkowej w folikulogenezie, potwierdzono również, wykazując ich wpływ na szlaki sygnalizacyjne związane z dojrzewaniem pęcherzyka jajnikowego [19]. Zaburzenia w funkcjonowaniu i strukturze ECM [20] mogą prowadzić do schorzeń w obrębie układu rozrodczego, w tym zespołu policystycznych jajników (ang. *polycystic ovary syndrome*, PCOS) [21–23], przedwczesnej niewydolności jajników (ang. *premature ovarian insufficient*, POI) [24,25] oraz nowotworów [26]. Do tej pory rola macierzy zewnątrzkomórkowej komórek ziarnistych na poziomie molekularnym została słabo poznana.

Wpływ na przebieg procesu folikulogenezy i oogenezy mają rozbudowane wzajemne interakcje występujące w komórkach ziarnistych, w które oprócz ECM zaangażowane są także kadheryny i integryny [27–31]. Kadheryny i integryny należą do białek transbłonowych, które biorą udział w sygnalizacji międzykomórkowej. Pierwsze z nich są odpowiedzialne za sygnalizację bezpośrednio między komórką a komórką [27], drugie zaś za sygnalizację między komórką a macierzą zewnątrzkomórkową [30]. Wśród kadheryn wyróżnia się kilka grup: N-kadheryna (kadheryna neuronalna), P-kadheryna (kadheryna łożyskowa), R-kadheryna

(kadheryna siatkówkowa), VE-kadheryna (kadheryna śródbłonka naczyniowego) oraz E-kadheryna (kadheryna nabłonkowa) występująca w nabłonkach i tym samym związana między innymi z funkcjonowaniem układu rozrodczego [27]. Ponad dwie dekady temu opisano ekspresję E-kadheryn w komórkach ziarnistych świń i powiązano ją z rozwojem pęcherzyków jajnikowych, rozumianym jako wpływ na utrzymanie integralności strukturalnej [32]. Ponadto wykazano dodatnią korelację między ekspresją E-kadheryny a pulą pierwotnych pęcherzyków w jajniku myszy, powstających podczas rozwoju embrionalnego [33]. Kadheryny nabłonkowe wpływają również na proces zapłodnienia poprzez wzmocnienie interakcji między plemnikiem a nabłonkiem jajowodu i komórką jajową, co zostało potwierdzone w modelu bydlęcym [34]. Drugą grupą białek transbłonowych są integryny, które należą do cząsteczek adhezyjnych zawierających w swojej budowie podjednostki α (ang. *integrin subunit α, ITGA*) i β (ang. *integrin subunit β, ITGB*) [35]. *ITGA* jest związana z tworzeniem ECM, zaś *ITGB* odgrywa jedną z kluczowych ról w regulacji wewnątrzkomórkowych kaskad sygnalizacyjnych (w tym FAK, AKT). Integryny, jako cząsteczki adhezyjne, wiążąc się z ECM, stale identyfikują skład macierzy zewnątrzkomórkowej, w odpowiedzi na co mogą zmieniać lub utrzymywać kształt komórki [36,37]. Podwyższona ekspresja genów kodujących integryny została już wcześniej opisana w komórkach ziarnistych świni i powiązano ją z procesami angiogenezy [38], morfogenezy [39], adhezji [40], a w przypadku *ITGB3* również apoptozy [41]. Z kolei obniżona ekspresja genów kodujących integryny negatywnie wpływa na adhezję komórek ziarnistych. Zaburzenia ekspresji integryn mogą prowadzić do zaburzeń takich jak PCOS u ludzi [42]. Stąd kadheryny i integryny, jako białka transbłonowe, wykazują istotną rolę w przebiegu folikulogenezy, oogenezy i owulacji.

Kolejnym istotnym elementem biorącym udział w sygnalizacji międzykomórkowej, choć także w podziale komórki, jest cytoszkielet [43]. Cytoszkielet jest strukturą dynamiczną, stale modyfikującą swój skład, w który wchodzą mikrofilamenty, mikrotubule i filamenty pośrednie [44]. Białkiem strukturalnym budującym mikrofilamenty jest aktyna, która jest najczęściej występującym białkiem w komórkach zwierzęcych. Funkcje pełnione przez mikrofilamenty są liczne i obejmują ich udział w ruchu i podziale komórek, sygnalizacji wewnątrzkomórkowowej [45] a także w endocytozie [46]. Mikrotubule składają się z białka tubuliny i są odpowiedzialne za transport różnych substancji [47] oraz tworzenie wrzeciona kariokinetycznego [48,49]. Z kolei filamenty pośrednie, składające się z białek takich jak wimentyna, keratyna i lamina, determinują kształt i stabilność komórek, ale także sygnalizację międzykomórkową [50]. Oprócz funkcji związanych ze stabilizacją środowiska komórkowego,

filamenty pośrednie wykazują aktywność w regulacji apoptozy, migracji i adhezji komórkowej oraz interakcjach z innymi składnikami cytoszkieletu [44]. Ponadto wykazano, że cytoszkielet wpływa na skład macierzy zewnątrzkomórkowej [51]. Geny kodujące białka związane z cytoszkieletem, w tym *TPM2* (ang. *tropomyosin 2, beta*), *VCAM-1* (ang. *vascular cell adhesion molecule 1*) [52,53] oraz *COL3A1* (ang. *collagen type III alpha chain*) [54,55], wpływają także na wystąpienie PCOS i POI. Zmiany w proporcjach poszczególnych składowych cytoszkieletu wykazano również w procesach nowotworowych [56]. Może wiązać się to z aktywnym udziałem cytoszkieletu w niekontrolowanym podziale komórek nowotworowych [57].

Uzupełnieniem omawianego zagadnienia, związanego z sygnalizacją międzykomórkową w obrębie komórek ziarnistych, są pęcherzyki zewnątrzkomórkowe (ang. *extracellular vesicles*, EVs). Cząsteczki te, o wielkości około 30 – 1000 nm, są obszarem intensywnych badań naukowych w ostatnich latach i przypisuje się im wiele ważnych dla aktywności komórek funkcji [58]. Pęcherzyki zewnątrzkomórkowe to cząsteczki otoczone błoną lipidową, które są zlokalizowane poza komórką. Wśród nich wyróżnia się małe pęcherzyki (ang. *small-EVs*) oraz duże pęcherzyki (ang. *large-EVs*). Do małych pęcherzyków zalicza się egzosomy, zaś do dużych mikropęcherzyki (ang. *microvesicles*, MVs) oraz ciałka apoptotyczne (ang. *apoptotic bodies*) [59]. Ponadto, niedawno opisano trzeci typ EVs, zwany migrasomami [59]. Pęcherzyki zewnątrzkomórkowe stanowią nośnik dla białek, lipidów, mRNA, mikroRNA, ncRNA, cirRNA będąc jednocześnie silnie zaangażowanymi w sygnalizację międzykomórkową [60]. EVs powstają w wyniku pączkowania lub wewnątrzkomórkowego transportu endocytarnego obejmującego fuzję ciał wielopęcherzykowych (ang. *multivesicular bodies*, MVBs) z błoną plazmatyczną [58]. Ważnym przekaźnikiem należącym do pęcherzyków zewnątrzkomórkowych, zaangażowanym bezpośrednio, jak i pośrednio w sygnalizację międzykomórkową, są egzosomy. Wpływają one na komórki docelowe poprzez bezpośredni kontakt z receptorami zewnątrzkomórkowymi lub po związaniu z błoną komórkową mogą podlegać endocytozie zależnej od klatryny [61]. Po fuzji z błoną komórkową egzosomy uwalniają przeniesiony ładunek wprost do cytozolu [62] lub wpływają na komórkę poprzez aktywację szlaków sygnalizacyjnych [63]. Te nanocząsteczki odgrywają ważną rolę w funkcjonowaniu układu rozrodczego [64–67] poprzez ich udział w adhezji komórek [68], migracji [59], proliferacji [69] i odpowiedzi na hipoksję [70]. Egzosomy w mikrośrodowisku komórek ziarnistych wpływają na skład macierzy zewnątrzkomórkowej poprzez udział w jej remodelingu, jednocześnie to właśnie ECM wpływa

na uwalnianie egzosomów z komórek [71]. Cytoszkielet jest również zaangażowany w uwalnianie EVs, gdyż poprzez polimeryzacje aktyny znajdującej się pod błoną cytoplazmatyczną, umożliwia pączkowanie i uwalnianie pęcherzyków na zewnątrz komórki [58]. Badanie wpływu egzosomów na skład ECM [72] oraz zaangażowania tych nanocząsteczek w proces angiogenezy [73,74] może być kluczowy dla zrozumienia podstaw molekularnych schorzeń jajnika [75].

Istotną, niedawno odkrytą, cechą komórek ziarnistych jest ich zdolność do różnicowania się w inne typy komórek [76,77]. Wykazano w nich ekspresję genów, określonych jako markery genetyczne, charakterystyczne dla mezenchymalnych multipotencjalnych komórek macierzystych, tj. *CD44*, *CD90*, *CD105*, *CD117*, *CD166* [78]. Potencjalnie stwarza to możliwości różnicowania komórek ziarnistych w komórki tkanki chrzęstnej, kostnej, nerwowej [79] oraz mięśniowej [80]. Istotny dla tej cechy komórek ziarnistych jest fakt, iż na różnicowanie się komórek ma wpływ ECM oraz egzosomy [68,81].

Wzajemne interakcje ECM, cytoszkieletu i pęcherzyków zewnątrzkomórkowych w komórkach ziarnistych wpływają na ich prawidłowe funkcjonowanie w zakresie sygnalizacji międzykomórkowej, adhezji, proliferacji, migracji i podziału [11,14,45,59,68,69,82–84]. Mechanizmy te, występujące w komórkach ziarnistych, nie są wystarczająco poznane na poziomie molekularnym. W tym celu konieczne jest zbadanie tych interakcji, co może dać podstawy do zrozumienia patofizjologii schorzeń występujących w obrębie jajnika. Dodatkowo wiedza ta może być wykorzystana w nowoczesnych technikach wspomaganego rozrodu (ang. *assisted reproductive technologies* – ART) a także medycynie regeneracyjnej opartej na komórkach macierzystych.

3.2. Cel badań

Celem badań było określenie profilu ekspresji wybranych genów odpowiedzialnych za sygnalizację międzykomórkową i tworzenie macierzy zewnątrzkomórkowej oraz cytoszkieletu w komórkach ziarnistych pęcherzyka jajnikowego świni, w trakcie ich krótkoterminowej pierwotnej hodowli *in vitro*.

Równolegle realizowane były następujące cele szczegółowe:

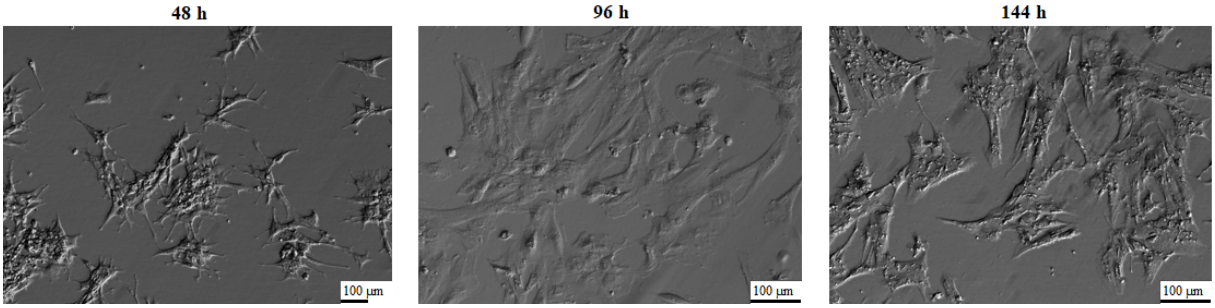
1. Badanie ekspresji wybranych genów biorących udział w steroidogenezie komórek ziarnistych jajnika świni w warunkach *in vitro*.
2. Typowanie ścieżek sygnalizacyjnych biorących udział w integracji środowiska zewnątrz i wewnątrzkomórkowego komórek ziarnistych jajnika świni w hodowli *in vitro*.
3. Wytypowanie genów, które mogą być uznane w przyszłości za nowe markery folikulogenezy u świni domowej.

3.3. Materiał i metody

Materiał do badań stanowiły jajniki pozyskane poubojowo od 120 dojrzałych płciowo loszek świni domowej, o średniej masie ciała 105 kg i wieku około 6 miesięcy. Zwierzęta poddane ubojowi w komercyjnej rzeźni utrzymywane były w zbliżonych warunkach hodowlanych. Zgodnie z obowiązującym prawem, zwierzęta przed ubojem poddane były badaniu klinicznemu, które nie wykazało zmian chorobowych. Po uboju, wyizolowane narządy rozrodcze były transportowane do laboratorium w roztworze soli fizjologicznej o temperaturze 38°C, w czasie do 30 minut. Zgodnie z obowiązującymi w Unii Europejskiej przepisami, badania przeprowadzane na materiale pozyskiwanym poubojowo nie wymagają zgody Lokalnej Komisji Etycznej ds. Doświadczeń na Zwierzętach.

Jajniki odizolowane od narządów rozrodczych umieszczono w roztworze PBS (ang. *phosphate-buffered saline*), uzupełnionym płodową surowicą bydlęcą (FBS; Sigma-Aldrich Co., St. Louis, MO, USA). Następnie za pomocą strzykawki o pojemności 5 ml i igły 20 G aspirowano płyn pęcherzykowy z pojedynczych pęcherzyków jajnikowych o średnicy powyżej 5 mm. Pozyskany płyn umieszczano w sterylnej szalce Petriego, a następnie izolowano i usuwano kompleksy kumulus-oocyt (COCs). Wyekstrahowany płyn pęcherzykowy filtrowano przez sterylne nylonowe sita komórkowe o średnicy oczek 40 µm (Biologix Group, Shandong, Chiny) w celu wyeliminowania resztek tkanek i większych agregatów komórek, w tym erytrocytów i komórek nabłonkowych. Otrzymaną zawiesinę wirowano w temperaturze pokojowej przez 10 minut, przy obrotach 200 × *g*, w celu uzyskania podziału na frakcje. Po odrzuceniu supernatantu, osad komórkowy zawieszano w roztworze kolagenazy typu I (Gibco, Thermo-Fischer Scientific, Waltham, MA, USA) 1 mg/1 ml DMEM i inkubowano przez 10 minut w łaźni wodnej o temperaturze 37°C, a następnie odwirowano (w takich samych warunkach jak podano wyżej). Uzyskany po odwirowaniu osad użyto do rozpoczęcia procedury hodowli pierwotnej *in vitro*. Do dalszych badań wykorzystano tylko próby o żywotności komórek wynoszącej powyżej 85%. Komórki wysiewano w liczbie 3×10^6 / butelkę hodowlaną (25 cm2, TPP, Trasadingen, Szwajcaria). Żywotność komórek i ich liczbę oceniano za pomocą automatycznego licznika komórek ADAM (NanoEnTek, Waltham, MA, USA). Do hodowli używano pożywkę hodowlaną składającą się z Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, Saint Louis, MO, USA), 2% płodowej surowicy cielęcej (FCS) (PAA, Linz, Austria), 10 mg/ml kwasu askorbinowego (Sigma-Aldrich, Saint Louis, MO, USA), 0,05 µM deksametazonu (Sigma-Aldrich, Saint Louis, MO, USA), 200 mM L-glutaminy (Invitrogen, Carlsbad, CA, USA), 10 mg/ml gentamycyny (Invitrogen, Carlsbad, CA, USA), 10 000

jednostek/ml penicyliny (Invitrogen, Carlsbad, CA, USA) i 10 000 µg/ml streptomycyny (Invitrogen, Carlsbad, CA, USA). Tak przygotowane butelki hodowlane wraz z komórkami utrzymywano w temperaturze 38,5 °C i 5% stężeniu CO2. Po osiągnięciu przez komórki ponad 80% konfluencji, odseparowywano je od podłoża za pomocą 0,05% trypsyny-EDTA (Invitrogen, Carlsbad, CA, USA), a następnie pasażowano. Morfologię GCs oceniano za pomocą odwróconego mikroskopu z kontrastem fazowym (Ryc. 1).



Rycina 1. Morfologia komórek ziarnistych podczas trwania hodowli *in vitro* w poszczególnych przedziałach czasowych (Kulus i wsp., 2021, *Biology*)

W celu przeprowadzenia doświadczenia wyznaczono następujące przedziały czasowe: 0 h – wartość referencyjna oraz 48 h, 96 h i 144 h. Hodowle *in vitro* zamykano w wyznaczonych przedziałach czasowych, a z pozyskanych komórek izolowano całkowity RNA, przy wykorzystaniu metody Chomczyńskiego – Sacchi, polegającej na degradowaniu błon komórkowych przy użyciu fenolu oraz tiocyjanianu guanidyny (TRI Reagent®, Sigma – Aldrich, St. Luis, USA). Po dodaniu chloroformu uzyskano podział roztworu na 3 fazy: wodną (zawierającą RNA), interfazę oraz fazę organiczną. Do wytrącenia RNA użyto izopropanolu. Stężenie wyizolowanego RNA zbadano przy użyciu spektrofotometru do pomiarów w kropli (Spektrofotometr NanoDrop, Thermo Scientific, Waltham, MA, USA), przy długości fali 260 nm, bazując na pomiarze gęstości optycznej (ang. *optical density*, OD). Do dalszych badań użyto 100 ng RNA, które poddano procesowi odwrotnej transkrypcji w celu uzyskania cDNA, zgodnie z załączonym przez producenta protokołem (Ambion® WT Expression Kit).

Kolejnym etapem badań było wykorzystanie metody mikromacierzy ekspresyjnych Affymetrix® Porcine Gene 1.1 ST Array Affymetrix (Affymetrix, Santa Clara, CA, USA), co umożliwiło zbadanie transkryptomu komórek ziarnistych w określonych w trakcie doświadczenia przedziałach czasowych. Dodatkowo, w oparciu o bazy danych genów i ich produktów białkowych (GeneAtlas System, Affymetrix GeneAtlas™ Operating Software), przeprowadzono analizę funkcjonalną. W celu skorygowania tła, normalizacji i podsumowania wyników zastosowano algorytm Robust Multiarray Averaging (RMA). Istotność statystyczną

zmian ekspresji analizowanych genów oceniono za pomocą moderowanej statystyki t z empirycznej metody Bayesa. Uzyskana wartość p została skorygowana o wielokrotne porównania przy użyciu współczynnika Benjaminiego i Hochberga. Istotnie zmienioną ekspresję genów ustalono w oparciu o wartość p wynoszącą <0,05 i krotność ekspresji wyższą niż 2. Listy genów ulegających zróżnicowanej ekspresji zostały wprowadzone do oprogramowania DAVID (Database for Annotation, Visualization and Integrated Discovery), gdzie uzyskano grupy ontologiczne genów GO MF (ang. *gene ontology molecular function*) [85]. Dane dotyczące ekspresji tych genów zostały poddane hierarchicznej procedurze klasteryzacji i przedstawione w postaci wykresu *heatmap* (mapy zmienności ekspresji genów). Szczegółowa analiza genów należących do wybranych terminów GO MF została przedstawiona w postaci wykresów przy użyciu biblioteki "GOplot" [86] i pakietu R "ClusterProfiler" [87]. W wybranych zestawach genów zbadano wzajemne relacje za pomocą pakietu GOplot. Pakiet GOplot został wykorzystany do obliczenia z-score (różnica w liczbie genów regulowanych w górę i w dół podzielona przez pierwiastek kwadratowy z liczby). Analiza z-score pozwoliła porównać wzbogacenie wybranych grup ontologicznych GO BP (ang. *gene ontology biological process*). Za pomocą oprogramowania STRING10 (Search Tool for the Retrieval of Interacting Genes, STRING Consortium, Lozanna, Szwajcaria) [88] zbadano interakcje między genami i kodowanymi przez nie białkami. Funkcjonalne interakcje (ang. *functional interaction* – FI) między genami należącymi do wybranych GO BP zostały zbadane za pomocą aplikacji REACTOME FIViz i oprogramowania Cytoscape 3.8.2 (San Diego, CA, USA).

W celu walidacji wyników otrzymanych z mikromacierzy ekspresyjnych, przeprowadzono ilościową łańcuchową reakcję polimerazy z odwrotną transkrypcją (RT-qPCR). Reakcję tą wykonano przy użyciu LightCycler real-time PCR detection system (Roche Diagnostics GmbH, Mannheim, Germany). Jako barwnik wykrywający użyto SYBR® Green I (Master Mix Qiagen GmbH, Hilden, Niemcy), a docelowe cDNA określono ilościowo przy użyciu metody względnej kwantyfikacji. Poziom ekspresji poszczególnych genów w każdej próbce obliczono względem genów referencyjnych PBGD (ang. *porphobilinogen deaminase*) i *ACTB* (ang. *β – actin*). Przedstawione analizy i wykresy zostały wykonane za pomocą Bioconductor (wersja 3.12) i przy użyciu języka programowania R (v4.1.2; R Core Team 2021).

3.4. Wyniki i dyskusja

W pierwszym etapie badań analizie poddano ekspresję genów związanych z tworzeniem macierzy zewnątrzkomórkowej oraz kodujących białka transbłonowe, takie jak integryny i kadheryny, w komórkach ziarnistych świni, które wspólnie odpowiadają za sygnalizację międzykomórkową. Metoda mikromacierzy ekspresyjnych pozwoliła na wyodrębnienie, z pozyskanego w trakcie hodowli *in vitro* materiału, 3380 genów o zróżnicowanej ekspresji. Zgodnie z tematyką pierwszego etapu badań, dalszym analizom poddano 81 genów, które przyporządkowano do siedmiu grup ontologicznych: "*extracellular matrix binding*", "*extracellular matrix structural constituent*", "*binding, bridging*", "cadherin binding", "*cell adhesion molecule binding*", "*collagen binding*" and "*cadherin binding involved in cell-cell adhesion*". Pośród tych 81 genów, 66 wykazało względem wartości referencyjnych podwyższony poziom ekspresji, natomiast 15 obniżony. Mapy cieplne (*heatmaps*) prezentujące profile transkryptomiczne genów dla poszczególnych grup ontologicznych wykazały wzrost ekspresji w trakcie prowadzonej hodowli *in vitro*. Przynależność do dwóch lub więcej grup ontologicznych wykazały 33 pośród 66 genów o podwyższonej ekspresji. Natomiast wśród 15 genów wykazujących obniżony poziom ekspresji, 5 wykazało przynależność do dwóch lub więcej grup ontologicznych. Do dalszych analiz wybrano 20 genów wykazujących największą zmienność w ekspresji, zarówno związaną z jej wzrostem (ang. *upregulation*), jak i spadkiem (ang. *downregulation*). Do dziesięciu genów wykazujących w komórkach ziarnistych najsilniej podwyższoną ekspresję należały *POSTN, ITGA2, FN1, LAMB1, ITGB3, CHI3L1, PCOLCE2, CAV1, DCN, COL14A1*. Natomiast do dziesięciu genów wykazujących najsilniej obniżoną ekspresję zaliczały się *SPP1, IRS1, CNTLN, TMPO, PAICS, ANK2, ADAM23, ABI3BP, DNAJB1, IGF1*. Wymienione wyżej geny i ich produkty białkowe odgrywają istotną rolę w licznych procesach i ścieżkach sygnalizacyjnych występujących w obrębie pęcherzyka jajnikowego. Udział tych białek jest kluczowy w tworzeniu macierzy zewnątrzkomórkowej, która dynamicznie zmieniając swoją strukturę, wpływa na aktywność procesów fizjologicznych oraz patologicznych w obrębie jajnika. Dekoryna (ang. *decorin*, *DCN*), której ekspresja była podwyższona w prowadzonych w ramach rozprawy doktorskiej badaniach, jest glikoproteiną wykazującą liczne funkcje w jajniku [89]. Jest zaangażowana w proces folikulogenezy oraz owulacji, co potwierdzają wyniki zróżnicowanej ekspresji tego genu w poszczególnych populacjach komórek ziarnistych uzyskane przez innych autorów [90]. Wykazano, że ekspresja *DCN* jest wyższa w MGCs tworzących od wewnątrz ścianę pęcherzyka jajnikowego i istotnych dla owulacji, niż w CCs [90]. Rolę dekoryny opisano także w steroidogenezie oraz proliferacji

komórek ziarnistych, dlatego jest uznawana za biomarker technik wspomaganego rozrodu [91,92]. Ekspresja *DCN* jest regulowana przez wiele ścieżek sygnałowych, między innymi PKA, PKC, ERK/MEK i PI3K [90], zaś sama reguluje aktywność ścieżki sygnałowej TGF-β [93]. Podobną rolę, związaną z udziałem w folikulogenezie i owulacji, pełnią dwa geny wykazujące podwyższoną ekspresję w badaniach prowadzonych w ramach rozprawy doktorskiej. Są to fibronektyna (ang. *fibronectin*, *FN1*) oraz periostyna (ang. *periostin*, *POSTN*), które ściśle związane są z funkcjonowaniem ECM. *FN1* bierze udział w ścieżce sygnalizacyjnej fibronektyna – integryna, ważnej dla funkcjonowania pęcherzyka jajnikowego [94]. Ponadto, regulując ścieżkę sygnałową FAK, wpływa na proces owulacji, luteinizacji oraz ekspansji komórek budujących kumulus [95]. Potwierdzono to poprzez inhibicję tej ścieżki sygnałowej, czego efektem było niewystąpienie owulacji [94]. Z kolei *POSTN* jest genem warunkującym remodeling tkanki włóknistej w obrębie pęcherzyka jajnikowego, co także jest kluczowe dla owulacji [96]. Oba geny poprzez wyżej opisaną aktywność wykazują związek z występowaniem zespołu policystycznych jajników [22,23]. Dodatkowo, istotną rolę w regulacji składu macierzy zewnątrzkomórkowej pełni kaweolina (ang. *caveolin*, *CAV-1*), która wykazała podwyższoną ekspresję w prowadzonych badaniach. Produkt białkowy tego genu odpowiada za formowanie egzosomów a także deponowanie poza obręb komórki produktów mających wpływ na skład i funkcjonowanie ECM [97]. Pośrednio może wpływać także na proces owulacji oraz luteinizacji komórek ziarnistych, co potwierdzono u bydła [98]. Dodatkowo wykazano, że obniżona ekspresja tego genu wpływa na ścieżkę sygnalizacyjną Notch2, powodując zmniejszenie ilości receptorów Lgr5 (*leucine-rich repeat containing G protein-coupled receptor 5*), co związane jest z występowaniem POI [24]. Integryny, jako białka transbłonowe, wykazują liczne interakcje z macierzą zewnątrzkomórkową, wpływając na jej skład i pośrednicząc w sygnalizacji międzykomórkowej [36]. Dodatkowo wykazano, że integryny, łącząc się z kadherynami, wpływają na ścieżkę sygnalizacyjną Rap1 zależną od GTP [99]. Ścieżka ta wykazuje istotną rolę w transporcie wewnątrz- i zewnątrzkomórkowym, będąc zaangażowana w endo- i egzocytarny transport białek [100]. Podwyższona ekspresja genów kodujących integryny *ITGA2* i *ITGB3* w badaniach przedstawionych w ramach rozprawy doktorskiej sugeruje ich bezpośrednią rolę w sygnalizacji międzykomórkowej, jak i pośrednią poprzez wpływ na uwalnianie pęcherzyków zewnątrzkomórkowych. Interakcje funkcjonalne pomiędzy poszczególnymi genami potwierdzono przy użyciu oprogramowania STRING. Genem wykazującym największą liczbę interakcji był gen *FN1*. Podkreśla to istotną rolę fibronektyny w sygnalizacji międzykomórkowej, polegającą na łączeniu się z glikoproteinami występującymi w ECM oraz integrynami. Dodatkowo, analiza interakcji funkcjonalnych

w prowadzonych badaniach wykazała korelację pomiędzy genami *DCN* i *IGF1* (ang. *insulin-like growth factor 1*) oraz *SPP1* (ang. *secreted phosphoprotein 1*) i *ITGB3*. Ekspresja wyżej wymienionych genów opisana była w komórkach ziarnistych w kontekście apoptozy [38,41,101] koniecznej do zajścia owulacji, co sugerować może ich rolę w tym procesie. W przeprowadzonych badaniach profil ekspresji genów uzyskany metodą mikromacierzy potwierdzono przy użyciu metody RT-qPCR. Podwyższony poziom ekspresji genów zaangażowanych w tworzenie macierzy zewnątrzkomórkowej wskazuje na jej istotną rolę w funkcjonowaniu komórek ziarnistych w warunkach *in vitro*. ECM, wspólnie z integrynami, ściśle uczestniczy w sygnalizacji międzykomórkowej a także wykazuje udział w procesach folikulogenezy, oogenezy i owulacji. Dodatkowo, poznanie ekspresji i interakcji genów związanych z ECM w odniesieniu do schorzeń w obrębie jajnika jest szczególnie istotne [21–23].

Drugi etap badań polegał na analizie ekspresji genów, których udział wykazano w tworzeniu cytoszkieletu i podziale komórkowym oraz interakcjach między środowiskiem zewnątrz- i wewnątrzkomórkowym w komórkach ziarnistych. Analizy ekspresji genów z wykorzystaniem mikromacierzy wykazały, że w 48 godzinie hodowli 828 genów charakteryzowało się podwyższoną ekspresją, a 610 obniżoną w odniesieniu do wartości referencyjnej, w 96 godzinie hodowli 1206 genów wykazało wzrost ekspresji, a 1104 jej spadek, natomiast po 144 godzinach hodowli 1025 genów zwiększyło poziom ekspresji, a 732 obniżyło. Geny wykazujące największą zmienność w ekspresji zostały przyporządkowane do 12 grup ontologicznych: "*actin cytoskeleton organization*", "*actin filament organization*", "*actin filament – based process*", "*cell – matrix adhesion*", "*cell – substrate adhesion*", "*chromosome segregation*", "*chromosome separation*", "*cytoskeleton organization*", "*DNA integrity checkpoint*", "*DNA replication initiation*", "*organelle fision*", "*organelle organization*". Na podstawie uzyskanych *heatmap* można zauważyć ogólną tendencję do wzrostu poziomu ekspresji genów podczas trwania hodowli, z wyjątkiem grup ontologicznych związanych z segregacją chromosomów oraz punktami kontrolnymi podziału komórki. Do dalszych analiz wybrano 20 genów, z których 10 charakteryzowało się podwyższoną ekspresją (*ITGA11, CNN1, CCl2, TPM2, ACTN1, VCAM-1, COL3A1, GSN, FRMD6, PLK2*) oraz 10 obniżoną (*KIF14, TACC3, ESPL1, CDC45, TTK, CDC20, CDK1, FBXO5, NEK2 – NIMA, CCNE2*). Cytoszkielet w głównej mierze składa się z białka aktyny, które przyjmując różne formy konformacyjne, wpływa na funkcjonowanie komórki, w tym na jej strukturę, transport wewnątrzkomórkowy i ruchliwość [43]. Białka wiążące aktynę (ang. *actin binding protein,*

ABP) wykazują wpływ na jej cząsteczkę poprzez udział w formowaniu nowych filamentów, ich polimeryzację i elongację [102–104]. W przeprowadzonym doświadczeniu do genów wykazujących podwyższoną ekspresję i kodujących białka wiążące aktynę, należały: kalponina (ang. *calponin 1*, *CNN1*), tropomiozyna (ang. *tropomyosin 2*, *TPM2*), aktynina (ang. *actinin alpha 1*, *ACTN1*) oraz gelsolina (ang. *gelsolin*, *GSN*). W badaniach prowadzonych w ramach rozprawy doktorskiej wykazano po raz pierwszy ekspresję genu *CNN1* w komórkach ziarnistych świni. Białko to wykazuje liczne interakcje, między innymi z *TPM2*, *ACTN1* i *GSN* [105]. Homolog kalponiny (ang. *calponin homology*, *CH*) jest jedną z najczęściej występujących domen w organizmie zwierzęcym, mającym wpływ na strukturę cytoszkieletu, aktywację ścieżek sygnałowych oraz metabolizm wapnia [106]. Domena ta dodatkowo wykazuje aktywność w ścieżce sygnałowej ERK, która umożliwia transdukcję sygnału między cytoszkieletem a macierzą zewnątrzkomórkową [106]. Potwierdza to występujące za pośrednictwem cytoszkieletu interakcje między środowiskiem wewnątrz- i zewnątrzkomórkowym. Tropomiozyna wpływa na skład cytoszkieletu, stabilizując jego wiązania [102]. W interakcje między komórką a środowiskiem zewnętrznym oraz pomiędzy poszczególnymi komórkami zaangażowana jest także aktynina. Opisano jej wpływ na aktywację ścieżki sygnałowej PI3K oraz współpracę z integrynami i międzykomórkowymi cząsteczkami adhezyjnymi (ang. *intercellular adhesion molecules*, *ICAM*) [107]. *ACTN1* pełni także rolę w takich procesach komórkowych jak cytokineza, adhezja i migracja [107]. Wpływ na filamenty aktynowe w komórce wykazuje również, zależna od stężenia wapnia gelsolina [107,108]. Mutacje genu GSN i zmienność w jego ekspresji wiążą się z występowaniem schorzeń w obrębie jajnika [109,110]. W prezentowanych badaniach ekspresja genu *ITGA11* (ang. *integrin subunit α-11*) uległa wzrostowi względem wartości referencyjnych. Integryny jako białka transbłonowe pełnią rolę przekaźników sygnału między cytoszkieletem a środowiskiem zewnętrznym, między innymi poprzez udział w ścieżce sygnałowej FAK [93]. Wykazano, że w poszczególnych fazach cyklu rujowego u świń ekspresja tych białek jest różna i zależy od stężenia progesteronu [111]. Genem istotnym dla procesu owulacji, wykazującym podwyższoną ekspresję w tym etapie badań był *CCl2* (ang. *chemokine C-C motif ligand 2*), którego ekspresja także zależy od stężenia progesteronu. Produkt białkowy genu *CCl2* jest cząsteczką prozapalną o charakterze chemotaktycznym, powodującym napływ białych krwinek do pęcherzyka jajnikowego [112]. Analogiczną rolę tego genu opisano także w aspekcie luteolizy u człowieka [113]. Dodatkowo, Ji i wsp. wykazali korelację między genem *CCl2* a genem *PTGS2*, który jest odpowiedzialny za syntezę prostaglandyny E2 [111]. Łącząc zatem dwa powyższe mechanizmy można stwierdzić, iż wystąpienie kontrolowanego procesu

zapalnego w mikrośrodowisku komórek ziarnistych tworzących ścianę pęcherzyka jajnikowego, jest kluczowe do zajścia owulacji. Istotną rolę w tworzeniu mikrośrodowiska komórek ziarnistych pełni kolagen. Podwyższoną ekspresję genów *COL14A1* (ang. *collagen type XIV alpha chain*), *COL3A1* (ang. *collagen type III alpha chain*) oraz *PCOLCE2* (ang. *procollagen C-endopeptidase enhancer*), regulujących jego syntezę, wykazano w badaniach prowadzonych w ramach rozprawy doktorskiej. Kolagen, łącząc się z laminą, stanowi główny składnik błony podstawnej komórek ziarnistych. Jego włókna charakteryzują się dużą zmiennością w swojej budowie, dzięki czemu dostosowuje się do funkcji pełnionej przez dany typ tkanki [114]. Kolagen jako główny składnik macierzy zewnątrzkomórkowej wpływa na jej funkcjonowanie, a także może odgrywać istotną rolę w patogenezie chorób jajnika [54,55]. Do genów odpowiedzialnych za regulację podziału komórki branych pod uwagę w prowadzonych badaniach należały: *CDK1* (ang. *cyclin-dependent kinase 1*), *CCNE2* (ang. *cyclin E2*), *CDC20* (ang. *cell division cycle 20*), *CDC45* (ang. *cell division cycle 45*), *NEK2 – NIMA* (ang. *NIMA related kinase 2*). Ścisłą interakcje wykazują cykliny i cyklino zależne kinazy, które wspólnie regulują możliwość zajścia po sobie faz podziału komórki: G1, S, G2, M [115]. Dodatkowo powyższe grupy genów wykazują wpływ na ścieżkę sygnałową MAPK i ERK [116], oddziałując tym samym na proliferację komórek ziarnistych. Rozbudowane interakcje genów odpowiedzialnych za podział komórki potwierdza także wykazana w niniejszej dysertacji funkcjonalna zależność między *CCNE2* a *NEK2* oraz wpływ genów *CDC20* oraz *CDC45* na ścieżkę sygnałową SMAD4 [117]. Przedstawiona przy użyciu oprogramowania STRING liczba 708 interakcji dla 148 genów związanych z włóknami filamentowymi oraz organizacją cytoszkieletu wskazuje na mnogość i różnorodność połączeń tam występujących, podkreślając poziom skomplikowania tej struktury.

W trzecim, uzupełniającym tematykę sygnalizacji międzykomórkowej etapie badań, skupiono się na procesach komórkowych, takich jak migracja, adhezja i proliferacja komórek ziarnistych. Badania te obejmowały określenie poziomu ekspresji genów związanych z wyżej wymienionymi procesami komórkowymi. Dodatkowo wykazano podwyższoną ekspresję genów należących do grupy ontologicznej „*vesicle – mediated transport*”, co świadczyć może o występowaniu tego rodzaju sygnalizacji międzykomórkowej w hodowanych *in vitro* komórkach ziarnistych. Co więcej, podwyższona ekspresja grup ontologicznych genów związanych z procesem angiogenezy i odpowiedzią komórek na hipoksję, wskazuje także na obecność egzosomów w mikrośrodowisku komórek ziarnistych, gdyż procesy te w dużej mierze oparte są o uwalnianie tego rodzaju pęcherzyków zewnątrzkomórkowych. Profil

transkrypcyjny hodowanych *in vitro* komórek ziarnistych określił, że w 48 godzinie hodowli 610 genów wykazało podwyższoną, a 827 obniżoną ekspresję względem godziny referencyjnej. W 96 godzinie 1104 geny prezentowały zwiększony poziom ekspresji, a 1206 obniżony, zaś w 144 godzinie hodowli *in vitro* 731 genów wykazało podwyższoną ekspresję, a 1025 obniżoną. Geny o zróżnicowanej ekspresji zostały przyporządkowane do 46 grup ontologicznych, z których 10 charakteryzowało się obniżoną a 36 podwyższoną ekspresją. Do grup ontologicznych o zwiększonym poziomie ekspresji należały między innymi: „*cell adhesion*", „*cell – cell adhesion*", „*cell – matrix adhesion*", „*cell migration*", „*integrin – mediated signaling pathway*", „*negative regulation of apoptotic process*", "*positive regulation of ERK1 and ERK2 cascade*", „*angiogenesis*", „*response to hypoxia*", „*wound healing*" oraz „*vesicle – mediated transport*", zaś o obniżonej ekspresji między innymi: „*cell cycle*" i „*cell division*". Największą ilość grup ontologicznych o podwyższonej ekspresji wykazano w 144 h doświadczenia, co wskazuje na tendencję do zwiększania poziomu ekspresji genów wraz z czasem trwania hodowli. Do dalszych analiz wybrano jedenaście genów o podwyższonej ekspresji (*LIPG, HSD3B1, CLIP4, LOX, ANKRD1, FMOD, SHAS2, TAGLN, ITGA8, MXRA5, NEXN*) oraz dziesięć o ekspresji obniżonej (*DAPL1, HSD17B1, SNX31, FST, NEBL, CXCL10, RGS2, MAL2, IHH, TRIB2*), a także związane z nimi grupy ontologiczne genów. Ścieżka sygnalizacyjna ERK, dla której w tym etapie badań określono zwiększony poziom ekspresji, jest elementem łączącym poprzednie etapy badań, gdyż wykazano funkcjonalny wpływ kalponiny i kaweoliny na jej funkcjonowanie. Gen *CAV-1* warunkuje transport wewnątrz – i zewnątrzkomórkowy, wpływając bezpośrednio na produkcję egzosomów [97]. Ponadto Karampoga i inni podają, że proces egzocytozy jest zależny od ECM [71]. Wskazuje to także na pośredni wpływ kaweoliny na uwalnianie egzosomów, gdyż gen ten uczestniczy w remodelingu macierzy zewnątrzkomórkowej. Co istotne, pęcherzyki zewnątrzkomórkowe, w tym egzosomy, wykazują zasadniczą rolę w adhezji [68] i migracji komórek [59] oraz gojeniu ran [118], czyli procesach, dla których ekspresja grup ontologicznych genów była podwyższona w prezentowanym etapie badań. Sugeruje to, że procesy zachodzące w komórkach ziarnistych zależne są, w pewnym stopniu, od uwalniania i aktywności pęcherzyków zewnątrzkomórkowych. Wśród genów wykazujących podwyższoną ekspresję w trzecim etapie badań były *MXRA5* (ang. *matrix remodeling associated 5*) oraz *FMOD* (ang. *fibromodulin*). Funkcja tych genów, polegająca na remodelingu macierzy zewnątrzkomórkowej, została już wcześniej opisana [13,119]. Kolejnym genem wykazującym wpływ na skład ECM jest, zależna od miedzi, oksydaza lizylowa (ang. *lysyl oxidase*, *LOX*) [120], dla której w niniejszych badaniach wykazano podwyższoną ekspresję. Pokazuje to, jak

duża liczba genów jest zaangażowana w regulację struktury ECM, a tym samym funkcjonowanie komórek ziarnistych. Gen *LOX* wykazuje także aktywność w ścieżkach sygnalizacyjnych takich jak MAPK, ERK, FAK [121], istotnych dla proliferacji i funkcjonowania GCs. Co więcej, gen ten pełni również istotną rolę w różnicowaniu komórek pluripotentnych do osteoblastów [121]. Warto dodać, że rola oksydazy lizylowej została opisana także w steroidogenezie u szczurów [122] oraz w zespole policystycznych jajników [123]. Na podstawie powyższych danych można stwierdzić, że gen *LOX* może w przyszłości stać się markerem dla procesów różnicowania się i steroidogenezy komórek ziarnistych w warunkach *in vitro*. W prezentowanych badaniach wykazano po raz pierwszy w komórkach ziarnistych świni ekspresję genu lipazy endotelialnej (ang. *endothelial lipase, LIPG, EL*). Gen ten odpowiada za metabolizm lipidów, w tym cholesterolu, i jest kluczowy dla mediowanej przez mitochondria steroidogenezy [124–126]. Podobnie jak przywołany wcześniej gen *LOX*, *LIPG* może zostać w przyszłości uznany jako marker steroidogenezy w komórkach ziarnistych hodowanych *in vitro*. Oprócz genów *LOX* i *LIPG*, istotnymi genami w aspekcie produkcji hormonów płciowych w komórkach ziarnistych są *HSD3B1 (ang. hydroxy-delta-5-steroid dehydrogenase)* i *HSD17B1* (ang. *17β-hydroxysteroid dehydrogenase 1*). Pierwszy z wyżej wymienionych genów wykazał w tym etapie badań podwyższoną ekspresję. Rolą *HSD3B1* jest konwersja pregnenolonu do progesteronu [127]. Natomiast gen *HSD17B1*, wykazujący obniżoną ekspresję w prowadzonych badaniach, odpowiada za przejście estronu w bardziej aktywną formę, czyli estradiol [128]. Ten przeciwstawny względem siebie kierunek ekspresji wyżej wymienionych genów wskazuje specyfikę steroidogenezy w komórkach ziarnistych. W warunkach *in vivo* podwyższona ekspresja genu *HSD17B1*, wraz z jednoczesnym obniżeniem ekspresji *HSD3B1*, prowadzi do zwiększonej produkcji estradiolu. Ma to miejsce w okresie okołoowulacyjnym i wpływa na aktywność płciową samicy i akceptację samca. Z kolei odwrotna sytuacja, gdzie ekspresja *HSD17B1* jest obniżona a ekspresja *HSD3B1* podwyższona, prowadzi do wzrostu stężenia progesteronu, co przyżyciowo ma miejsce w fazie lutealnej i w przypadku zapłodnienia odpowiada za utrzymanie ciąży. Wiedza ta może być wykorzystana w unowocześnieniu technik wspomaganego rozrodu, szczególnie prowadzonych *in vitro*. Dodatkowo, w prowadzonych badaniach podwyższoną ekspresję wykazał gen kodujący syntazę hialuronianową (ang. *hyaluronan synthase 2*, *SHAS2*). Produkt białkowy tego genu wpływa na migrację komórek i jest odpowiedzialny za ekspansję kumulusa oraz syntezę hialuronianu [129], czyli jednego z głównych składników ECM. Warto dodać, że migracja komórek zależy także od egzosomów, które w tym aspekcie wykazują mechanizm zarówno autokrynny, jak i parakrynny [59]. Nanocząsteczki te stymulują pozakomórkowe receptory

sygnalizacyjne oraz przemieszczając się w okolice błony komórkowej, są konieczne do zainicjowania procesu migracji [130]. Podwyższoną ekspresję w ostatnim etapie badań wykazała także grupa ontologiczna genów reprezentująca proces angiogenezy. Angiogeneza wpływa na folikulogenezę, owulację oraz powstanie ciałka żółtego, poprzez udział w tworzeniu odpowiedniego mikrośrodowiska naczyniowego jajnika. Proces powstawania nowych naczyń jest także ściśle uzależniony i regulowany przez egzosomy [74]. Co ciekawe, inhibicja procesu angiogenezy może spowalniać wyczerpywanie się puli pęcherzyków jajnikowych. Stąd kluczowe może okazać się wykorzystanie egzosomów jako cząsteczek terapeutycznych modulujących proces angiogenezy w schorzeniach jajnika.

3.5. Podsumowanie

Powyżej opisane badania były prowadzone przy zastosowaniu krótkoterminowej pierwotnej hodowli *in vitro* komórek ziarnistych jajnika świni domowej. Głównym celem rozprawy doktorskiej było określenie profilu transkryptomicznego genów regulujących wybrane procesy zachodzące w komórkach ziarnistych, utrzymywanych w warunkach *in vitro*. Badania były prowadzone w trzech etapach, z których rezultaty opublikowano w oryginalnych pracach naukowych.

W pierwszym etapie badań wykazano silnie podwyższoną ekspresję genów odpowiedzialnych za skład i funkcjonowanie macierzy zewnątrzkomórkowej. Struktura ta odpowiedzialna jest za proliferację komórek ziarnistych, ich rozwój oraz sygnalizację międzykomórkową. Dodatkowo, w tym etapie badań odnotowano podwyższoną ekspresję genów kodujących integryny i kadheryny, które jako białka transbłonowe stanowią dla ECM uzupełnienie sygnalizacji międzykomórkowej. Biorąc pod uwagę wpływ ECM na występowanie schorzeń w obrębie jajnika, określenie profilu ekspresji genów odpowiedzialnych za tworzenie mikrośrodowiska komórek ziarnistych może być wykorzystane w aspekcie terapii celowanej. Wytypowanie swoistych markerów genetycznych tych chorób stwarza możliwość wyciszania lub zmiany ich ekspresji.

W drugim etapie badań wykazano podwyższony poziom ekspresji grup ontologicznych genów warunkujących powstanie cytoszkieletu i jego udział w kontroli poszczególnych faz podziału komórki. Potwierdzono tym samym udział cytoszkieletu w proliferacji i sygnalizacji międzykomórkowej, poprzez jego wpływ na ścieżki sygnalizacyjne regulujące skład i funkcjonowanie ECM. Co ważne, geny kodujące białka transbłonowe biorące udział w sygnalizacji międzykomórkowej w trakcie prowadzonych badań także wykazały podwyższoną ekspresję. Dodatkowo, aktywny udział cytoszkieletu w folikulogenezie i oogenezie może mieć związek z występowaniem schorzeń układu rozrodczego takich jak PCOS i POI. Schorzenia te, jako wypadkowa funkcjonowania ECM i cytoszkieletu, wskazują na konieczność prowadzenia badań molekularnych mających na celu poznanie fundamentu ich patofizjologii.

Ostatni etap badań ukierunkowany był na analizę ekspresji genów zaangażowanych w procesy związane z migracją, proliferacją, adhezją komórek, angiogenezą, gojeniem ran i hipoksją. W oparciu o podwyższoną ekspresję grupy ontologicznej genów reprezentujących proces transportu pęcherzykowego oraz dostępne dane literaturowe stwierdzić można, że

istotną rolę w wyżej wymienionych procesach pełnią egzosomy. Wykazana po raz pierwszy w niniejszej rozprawie doktorskiej ekspresja genu *LIPG* w komórkach ziarnistych jajnika świni, wzbogaca wiedzę na temat steroidogenezy tych komórek. Natomiast wzajemne zestawienie poziomów ekspresji genów *HSD17B1* i *HSD3B1* może być wykorzystane w dalszych badaniach dotyczących funkcji komórek ziarnistych.

Przedstawione w powyższych badaniach wyniki wskazują na istotną rolę macierzy zewnątrzkomórkowej, cytoszkieletu, jak i pęcherzyków zewnątrzkomórkowych w sygnalizacji międzykomórkowej. Ta wzajemna współzależność jest konieczna do prawidłowego funkcjonowania komórek ziarnistych, ich podziału, proliferacji i migracji. Dokładne poznanie występujących ścieżek sygnałowych i transdukcji sygnału w mikrośrodowisku komórek ziarnistych może pozwolić na zrozumienie dialogu występującego pomiędzy środowiskiem zewnątrz- i wewnątrzkomórkowym. Wyniki przedstawione w niniejszej rozprawie doktorskiej mogą stanowić wkład do dalszych badań w tym kierunku.

3.6. Wnioski

1. Podwyższona ekspresja *CAV-1*, genu regulującego proces egzocytozy, świadczy zarówno o dynamicznych zmianach składu ECM, jak i intensywnej sygnalizacji międzykomórkowej w komórkach ziarnistych utrzymywanych w warunkach krótkotermonowej pierwotnej hodowli *in vitro*.
2. Wykazana po raz pierwszy w niniejszej pracy ekspresja genu *CNN-1* w komórkach ziarnistych świni, który odpowiedzialny jest za strukturę cytoszkieletu i transdukcję sygnału, może sugerować jego istotną rolę w tworzeniu i funkcjonowaniu mikrośrodowiska komórek ziarnistych hodowanych w warunkach *in vitro*.
3. Zmiany w profilu ekspresji genów *HSD17B1* oraz *HSD3B1* mogą oznaczać indukcję procesu steroidogenezy w komórkach ziarnistych utrzymywanych w warunkach krótkoterminowej pierwotnej hodowli *in vitro*.
4. Indukcja ścieżki sygnalizacyjnej ERK potwierdza jej istotną rolę w procesach formowania cytoszkieletu, macierzy zewnątrzkomórkowej oraz produkcji egzosomów w komórkach ziarnistych utrzymywanych w krótkoterminowej hodowli pierwotnej *in vitro.*
5. Opisana po raz pierwszy w rozprawie doktorskiej ekspresja genu *LIPG*, regulującego metabolizm lipidów w komórkach ziarnistych, może wskazywać na istotną rolę tego genu w procesie steroidogenezy pęcherzyka jajnikowego świni podczas folikulogenezy.

3.7. Piśmiennictwo

1. Amsterdam, A.; Rotmensch, S. Structure-Function Relationships during Granulosa Cell Differentiation. *Endocr. Rev.* **1987**, *8*, 309–337, doi:10.1210/EDRV-8-3-309.
2. Thibault, C.; Szöllösi, D.; Gérard, M. Mammalian Oocyte Maturation. *Reprod. Nutr. Dev.* **1987**, *27*, 865–896, doi:10.1051/RND:19870701.
3. Strączyńska, P.; Papis, K.; Morawiec, E.; Czerwiński, M.; Gajewski, Z.; Olejek, A.; Bednarska-Czerwińska, A. Signaling Mechanisms and Their Regulation during in Vivo or in Vitro Maturation of Mammalian Oocytes. *Reprod. Biol. Endocrinol.* **2022**, *20*, doi:10.1186/S12958-022-00906-5.
4. Buccione, R.; Schroeder, A.C.; Eppig, J.J. Interactions between Somatic Cells and Germ Cells Throughout Mammalian Oogenesis. *Biol. Reprod.* **1990**, *43*, 543–547, doi:10.1095/BIOLREPROD43.4.543.
5. Nicholson, B.J. Gap Junctions - From Cell to Molecule. *J. Cell Sci.* **2003**, *116*, 4479–4481, doi:10.1242/JCS.00821.
6. Carabatsos, M.J.; Sellitto, C.; Goodenough, D.A.; Albertini, D.F. Oocyte-Granulosa Cell Heterologous Gap Junctions Are Required for the Coordination of Nuclear and Cytoplasmic Meiotic Competence. *Dev. Biol.* **2000**, *226*, 167–179, doi:10.1006/DBIO.2000.9863.
7. Young, J.M.; McNeilly, A.S. Theca: The Forgotten Cell of the Ovarian Follicle. *Reproduction* **2010**, *140*, 489–504, doi:10.1530/REP-10-0094.
8. Gosden, R.G.; Hunter, R.H.F.; Telfer, E.; Torrance, C.; Brown, N. Physiological Factors Underlying the Formation of Ovarian Follicular Fluid. *J. Reprod. Fertil.* **1988**, *82*, 813–825, doi:10.1530/jrf.0.0820813.
9. Channing, C.P.; Hillensjo, T.; Schaerf, F.W. Hormonal Control of Oocyte Meiosis, Ovulation and Luteinization in Mammals. *Clin. Endocrinol. Metab.* **1978**, *7*, 601–624, doi:10.1016/S0300-595X(78)80011-5.
10. McGrath, S.A.; Esquela, A.F.; Lee, S.J. Oocyte-Specific Expression of Growth/Differentiation Factor-9. *Mol. Endocrinol.* **1995**, *9*, 131–136, doi:10.1210/MEND.9.1.7760846.
11. Walma, D.A.C.; Yamada, K.M. The Extracellular Matrix in Development. *Development* **2020**, *147*, doi:10.1242/dev.175596.
12. Hynes, R.O. The Extracellular Matrix: Not Just Pretty Fibrils. *Science (80-.).* **2009**, *326*, 1216–1219, doi:10.1126/science.1176009.
13. Frantz, C.; Stewart, K.M.; Weaver, V.M. The Extracellular Matrix at a Glance. *J. Cell Sci.* **2010**, *123*, 4195–4200, doi:10.1242/jcs.023820.
14. Mecham, R.P. Overview of Extracellular Matrix. *Curr. Protoc. Cell Biol.* **2012**, *57*, 10.1.1-10.1.16, doi:10.1002/0471143030.cb1001s57.
15. Mouw, J.K.; Ou, G.; Weaver, V.M. Extracellular Matrix Assembly: A Multiscale Deconstruction. *Nat. Rev. Mol. Cell Biol.* **2014**, *15*, 771–785, doi:10.1038/nrm3902.
16. Rodgers, R.J.; Irving-Rodgers, H.F.; Van Wezel, I.L. Extracellular Matrix in Ovarian Follicles. *Mol. Cell. Endocrinol.* **2000**, *163*, 73–79, doi:10.1016/S0303-7207(00)00219-7.
17. Hatzirodos, N.; Irving-Rodgers, H.F.; Hummitzsch, K.; Harland, M.L.; Morris, S.E.; Rodgers, R.J. Transcriptome Profiling of Granulosa Cells of Bovine Ovarian Follicles during Growth from Small to Large Antral Sizes. *BMC Genomics* **2014**, *15*, doi:10.1186/1471-2164-15-24.
18. Van Wezel, I.L.; Rodgers, H.F.; Rodgers, R.J. Differential Localization of Laminin Chains in Bovine Follicles. *J. Reprod. Fertil.* **1998**, *112*, 267–278, doi:10.1530/jrf.0.1120267.

19. Zhao, Y.; Feng, H.; Zhang, Y.; Zhang, J. V; Wang, X.; Liu, D. Current Understandings of Core Pathways for the Activation of Mammalian Primordial Follicles. *Cells* **2021**, 1–17, doi:doi.org/10.3390/cells10061491.
20. Kinnear, H.M.; Tomaszewski, C.E.; Chang, F.L.; Moravek, M.B.; Xu, M.; Padmanabhan, V.; Shikanov, A. The Ovarian Stroma as a New Frontier. *Reproduction* **2020**, *160*, R25–R39, doi:10.1530/REP-19-0501.
21. Xiang, S.; Xia, M. feng; Song, J. yan; Liu, D. qi; Lian, F. Effect of Electro-Acupuncture on Expression of IRS-1/PI3K/GLUT4 Pathway in Ovarian Granulosa Cells of Infertile Patients with Polycystic Ovary Syndrome-Insulin Resistance of Phlegm-Dampness Syndrome. *Chin. J. Integr. Med.* **2020**, *27*, doi:10.1007/s11655-020-3219-z.
22. Chen, X.; Huo, L.; Ren, L.; Li, Y.; Sun, Y.; Li, Y.; Zhang, P.; Chen, S.; Song, G.Y. Polycystic Ovary Syndrome Is Associated with Elevated Periostin Levels. *Exp. Clin. Endocrinol. Diabetes* **2019**, *127*, 571–577, doi:10.1055/a-0752-0061.
23. Patil, K.; Hinduja, I.; Mukherjee, S. Alteration in Angiogenic Potential of Granulosa-Lutein Cells and Follicular Fluid Contributes to Luteal Defects in Polycystic Ovary Syndrome. *Hum. Reprod.* **2021**, *36*, 1052–1064, doi:10.1093/humrep/deaa351.
24. Huang, K.; Dang, Y.; Zhang, P.; Shen, C.; Sui, X.; Xia, G.; Qin, Y.; Jiao, X.; Wang, C.; Huo, R.; et al. CAV1 Regulates Primordial Follicle Formation via the Notch2 Signalling Pathway and Is Associated with Premature Ovarian Insufficiency in Humans. *Hum. Reprod.* **2018**, *33*, 087–2095, doi:10.1093/humrep/dey299.
25. Henning, N.F.; LeDuc, R.D.; Even, K.A.; Laronda, M.M. Proteomic Analyses of Decellularized Porcine Ovaries Identified New Matrisome Proteins and Spatial Differences across and within Ovarian Compartments. *Sci. Rep.* **2019**, *9*, doi:10.1038/s41598-019-56454-3.
26. Cho, A.; Howell, V.M.; Colvin, E.K. The Extracellular Matrix in Epithelial Ovarian Cancer - A Piece of a Puzzle. *Front. Oncol.* **2015**, *5*, 245, doi:10.3389/fonc.2015.00245.
27. Piprek, R.P.; Kloc, M.; Mizia, P.; Kubiak, J.Z. The Central Role of Cadherins in Gonad Development, Reproduction, and Fertility. *Int. J. Mol. Sci.* **2020**, *21*, 1–21, doi:10.3390/ijms21218264.
28. Sueoka, K.; Kuji, N.; Shiokawa, S.; Tanaka, M.; Miyazaki, T.; Yoshimura, Y. Integrins and Reproductive Physiology: Expression and Modulation in Fertilization, Embryogenesis, and Implantation. *Fertil. Steril.* **1997**, *67*, 799–811, doi:10.1016/S0015-0282(97)81388-X.
29. Bronson, R.A.; Fusi, F.M. Integrins and Human Reproduction. *Mol. Hum. Reprod.* **1996**, *2*, 153–168, doi:10.1093/molehr/2.3.153.
30. Weber, G.F.; Bjerke, M.A.; DeSimone, D.W. Integrins and Cadherins Join Forces to Form Adhesive Networks. *J. Cell Sci.* **2011**, *124*, 1183–1193, doi:10.1242/jcs.064618.
31. Mui, K.L.; Chen, C.S.; Assoian, R.K. The Mechanical Regulation of Integrin-Cadherin Crosstalk Organizes Cells, Signaling and Forces. *J. Cell Sci.* **2016**, *129*, 1093–1100, doi:10.1242/jcs.183699.
32. Ryan, P.L.; Valentine, A.F.; Bagnell, C.A. Expression of Epithelial Cadherin in the Developing and Adult Pig Ovary. *Biol. Reprod.* **1996**, *55*, 1091–1097, doi:10.1095/biolreprod55.5.1091.
33. Yan, H.; Wen, J.; Zhang, T.; Zheng, W.; He, M.; Huang, K.; Guo, Q.; Chen, Q.; Yang, Y.; Deng, G.; et al. Oocyte-Derived E-Cadherin Acts as a Multiple Functional Factor Maintaining the Primordial Follicle Pool in Mice. *Cell Death Dis.* **2019**, *10*, 1–14, doi:10.1038/s41419-018-1208-3.
34. Caballero, J.N.; Gervasi, M.G.; Veiga, M.F.; Dalvit, G.C.; Perez-Martínez, S.; Cetica, P.D.; Vazquez-Levin, M.H. Epithelial Cadherin Is Present in Bovine Oviduct Epithelial Cells and Gametes, and Is Involved in Fertilization-Related Events. *Theriogenology*

2014, *81*, 1189–1206, doi:10.1016/j.theriogenology.2014.01.028.
35. Buck, C.A.; Horwitz, A.F. Integrin, a Transmembrane Glycoprotein Complex Mediating Cell-Substratum Adhesion. *J. Cell Sci. Suppl.* **1987**, *8*, 231–250, doi:10.1242/jcs.1987.supplement_8.13.
36. Clark, E.A.; Brugge, J.S. Integrins and Signal Transduction Pathways: The Road Taken. *Science (80-.).* **1995**, *268*, 233–239, doi:10.1126/science.7716514.
37. Giancotti, F.G.; Ruoslahti, E. Integrin Signaling. *Science (80-.).* **1999**, *285*, 1028–1032, doi:10.1126/science.285.5430.1028.
38. Chermuła, B.; Brązert, M.; Iżycki, D.; Ciesiółka, S.; Kranc, W.; Celichowski, P.; Ożegowska, K.; Nawrocki, M.J.; Jankowski, M.; Jeseta, M.; et al. New Gene Markers of Angiogenesis and Blood Vessels Development in Porcine Ovarian Granulosa Cells during Short-Term Primary Culture In Vitro. *Biomed Res. Int.* **2019**, *2019*, 6545210, doi:10.1155/2019/6545210.
39. Kulus, M.; Sujka-Kordowska, P.; Konwerska, A.; Celichowski, P.; Kranc, W.; Kulus, J.; Piotrowska-Kempisty, H.; Antosik, P.; Bukowska, D.; Iżycki, D.; et al. New Molecular Markers Involved in Regulation of Ovarian Granulosa Cell Morphogenesis, Development and Differentiation during Short-Term Primary in Vitro Culture—Transcriptomic and Histochemical Study Based on Ovaries and Individual Separated Follicles. *Int. J. Mol. Sci.* **2019**, *20*, doi:10.3390/ijms20163966.
40. Ozegowska, K.; Brazert, M.; Ciesiółka, S.; Nawrocki, M.J.; Kranc, W.; Celichowski, P.; Jankowski, M.; Bryja, A.; Jeseta, M.; Antosik, P.; et al. Genes Involved in the Processes of Cell Proliferation, Migration, Adhesion, and Tissue Development as New Potential Markers of Porcine Granulosa Cellular Processes In Vitro: A Microarray Approach. *DNA Cell Biol.* **2019**, *38*, 549–560, doi:10.1089/dna.2018.4467.
41. Kulus, M.; Kranc, W.; Sujka-Kordowska, P.; Mozdziak, P.; Jankowski, M.; Konwerska, A.; Kulus, J.; Bukowska, D.; Skowroński, M.; Piotrowska-Kempisty, H.; et al. The Processes of Cellular Growth, Aging, and Programmed Cell Death Are Involved in Lifespan of Ovarian Granulosa Cells during Short-Term IVC – Study Based on Animal Model. *Theriogenology* **2020**, *148*, 76–88, doi:10.1016/j.theriogenology.2020.02.044.
42. Hassani, F.; Oryan, S.; Eftekhari-Yazdi, P.; Bazrgar, M.; Moini, A.; Nasiri, N.; Sharifi-Zarchi, A. Downregulation of Extracellular Matrix and Cell Adhesion Molecules in Cumulus Cells of Infertile Polycystic Ovary Syndrome Women with and without Insulin Resistance. *Cell J.* **2019**, *21*, 35–42, doi:10.22074/cellj.2019.5576.
43. Fletcher, D.A.; Mullins, R.D. Cell Mechanics and the Cytoskeleton. *Nature* **2010**, *463*, 485, doi:10.1038/NATURE08908.
44. Hohmann, T.; Dehghani, F. The Cytoskeleton—A Complex Interacting Meshwork. *Cells 2019, Vol. 8, Page 362* **2019**, *8*, 362, doi:10.3390/CELLS8040362.
45. Jiang, X.; Qin, Y.; Kun, L.; Zhou, Y. The Significant Role of the Microfilament System in Tumors. *Front. Oncol.* **2021**, *11*, 333, doi:10.3389/FONC.2021.620390/BIBTEX.
46. Akamatsu, M.; Vasan, R.; Serwas, D.; Ferrin, M.; Rangamani, P.; Drubin, D.G. Principles of Self-Organization and Load Adaptation by the Actin Cytoskeleton during Clathrin-Mediated Endocytosis. *Elife* **2020**, *9*, doi:10.7554/ELIFE.49840.
47. Barlan, K.; Gelfand, V.I. Microtubule-Based Transport and the Distribution, Tethering, and Organization of Organelles. *Cold Spring Harb. Perspect. Biol.* **2017**, *9*, doi:10.1101/CSHPERSPECT.A025817.
48. Jones, M.C.; Zha, J.; Humphries, M.J. Connections between the Cell Cycle, Cell Adhesion and the Cytoskeleton. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **2019**, *374*, doi:10.1098/RSTB.2018.0227.
49. Forth, S.; Kapoor, T.M. The Mechanics of Microtubule Networks in Cell Division. *J. Cell Biol.* **2017**, *216*, 1525–1531, doi:10.1083/JCB.201612064.

50. Sanghvi-Shah, R.; Weber, G.F. Intermediate Filaments at the Junction of Mechanotransduction, Migration, and Development. *Front. Cell Dev. Biol.* **2017**, *5*, 81, doi:10.3389/FCELL.2017.00081/BIBTEX.
51. Geiger, B.; Bershadsky, A.; Pankov, R.; Yamada, K.M. Transmembrane Extracellular Matrix-Cytoskeleton Crosstalk. *Nat. Rev. Mol. Cell Biol.* **2001**, *2*, 793–805, doi:10.1038/35099066.
52. Kanmaz-Özer, M.; Vural, P.; Doru-Abbasolu, S.; Gedikbaşi, A.; Çil, E.; Karadağ, B.; Uysal, M. Polymorphisms of Vascular Cell Adhesion Molecule1 (VCAM1) in Polycystic Ovary Syndrome Determined by Quantitative Real-Time Polymerase Chain Reaction and Melting Curve Analysis. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **2012**, *160*, 174–178, doi:10.1016/J.EJOGRB.2011.11.013.
53. Li, L.; Mo, H.; Zhang, J.; Zhou, Y.; Peng, X.; Luo, X. The Role of Heat Shock Protein 90B1 in Patients with Polycystic Ovary Syndrome. *PLoS One* **2016**, *11*, e0152837, doi:10.1371/JOURNAL.PONE.0152837.
54. Kong, Y.; Yao, G.; He, J.; Yang, G.; Kong, D.; Sun, Y. LncRNA LNC-GULP1-2:1 Is Involved in Human Granulosa Cell Proliferation by Regulating COL3A1 Gene. *Fertil. Steril.* **2018**, *110*, e320, doi:10.1016/J.FERTNSTERT.2018.07.900.
55. Yao, G.; Kong, Y.; Yang, G.; Kong, D.; Xu, Y.; He, J.; Xu, Z.; Bai, Y.; Fan, H.; He, Q.; et al. Lnc-GULP1–2:1 Affects Granulosa Cell Proliferation by Regulating COL3A1 Expression and Localization. *J. Ovarian Res.* **2021**, *14*, 1–10, doi:10.1186/S13048-021-00769-1/FIGURES/4.
56. Ong, M.S.; Deng, S.; Halim, C.E.; Cai, W.; Tan, T.Z.; Huang, R.Y.J.; Sethi, G.; Hooi, S.C.; Kumar, A.P.; Yap, C.T. Cytoskeletal Proteins in Cancer and Intracellular Stress: A Therapeutic Perspective. *Cancers (Basel).* **2020**, *12*, doi:10.3390/CANCERS12010238.
57. Matthews, H.K.; Bertoli, C.; de Bruin, R.A.M. Cell Cycle Control in Cancer. *Nat. Rev. Mol. Cell Biol. 2021* **2021**, 1–15, doi:10.1038/s41580-021-00404-3.
58. Mathieu, M.; Martin-Jaular, L.; Lavieu, G.; Théry, C. Specificities of Secretion and Uptake of Exosomes and Other Extracellular Vesicles for Cell-to-Cell Communication. *Nat. Cell Biol.* **2019**, *21*, 9–17, doi:10.1038/S41556-018-0250-9.
59. Sung, B.H.; Parent, C.A.; Weaver, A.M. Extracellular Vesicles: Critical Players during Cell Migration. *Dev. Cell* **2021**, *56*, 1861–1874, doi:10.1016/J.DEVCEL.2021.03.020.
60. Cells, D.B.-; 2021, undefined Extracellular Vesicles in Organ Fibrosis: Mechanisms, Therapies, and Diagnostics. *mdpi.com* **2021**, *10*, doi:10.3390/cells10071596.
61. Prada, I.; Meldolesi, J. Binding and Fusion of Extracellular Vesicles to the Plasma Membrane of Their Cell Targets. *Int. J. Mol. Sci. 2016, Vol. 17, Page 1296* **2016**, *17*, 1296, doi:10.3390/IJMS17081296.
62. Mulcahy, L.A.; Pink, R.C.; Carter, D.R.F. Routes and Mechanisms of Extracellular Vesicle Uptake. *https://doi.org/10.3402/jev.v3.24641* **2014**, *3*, doi:10.3402/JEV.V3.24641.
63. Guan, S.; Li, Q.; Liu, P.; Xuan, X.; Du, Y. Experimental Immunology
Umbilical Cord Blood-Derived Dendritic Cells Loaded with BGC823 Tumor Antigens and DC-Derived Exosomes Stimulate Efficient Cytotoxic T-Lymphocyte Responses and Antitumor Immunity in Vitro and in Vivo. *Cent. Eur. J. of Immunology* **2014**, *39*, 142–151, doi:10.5114/CEJI.2014.43713.
64. Shen, Y.; You, Y.; Zhu, K.; Fang, C.; Chang, D.; Yu, X. Exosomes in the fIeld of Reproduction: A Scientometric Study and Visualization Analysis. *Front. Pharmacol.* **2022**, *13*, doi:10.3389/FPHAR.2022.1001652.
65. Muñoz, E.L.; Fuentes, F.B.; Felmer, R.N.; Yeste, M.; Arias, M.E. Extracellular Vesicles in Mammalian Reproduction: A Review. *Zygote* **2022**, *30*, 44–463, doi:10.1017/S0967199422000090.

66. de ávila, A.C.F.C.M.; Andrade, G.M.; Bridi, A.; Gimenes, L.U.; Meirelles, F.V.; Perecin, F.; da Silveira, J.C. Extracellular Vesicles and Its Advances in Female Reproduction. *Anim. Reprod.* **2020**, *16*, 31–38, doi:10.21451/1984-3143-AR2018-00101.
67. Llobat, L. Extracellular Vesicles and Domestic Animal Reproduction. *Res. Vet. Sci.* **2021**, *136*, 166–173, doi:10.1016/J.RVSC.2021.02.016.
68. Lan, Y.; Jin, Q.; Xie, H.; Yan, C.; Ye, Y.; Zhao, X.; Chen, Z.; Xie, Z. Exosomes Enhance Adhesion and Osteogenic Differentiation of Initial Bone Marrow Stem Cells on Titanium Surfaces. *Front. Cell Dev. Biol.* **2020**, *8*, 1216, doi:10.3389/FCELL.2020.583234/BIBTEX.
69. Miller, C.M.; Enninga, E.A.L.; Rizzo, S.A.; Phillipps, J.; Guerrero-Cazares, H.; Destephano, C.C.; Peterson, T.E.; Stalboerger, P.G.; Behfar, A.; Khan, Z. Platelet-Derived Exosomes Induce Cell Proliferation and Wound Healing in Human Endometrial Cells. *Regen. Med.* **2022**, *17*, 805–817, doi:10.2217/RME-2022-0095.
70. Jiang, H.; Zhao, H.; Zhang, M.; He, Y.; Li, X.; Xu, Y.; Liu, X. Hypoxia Induced Changes of Exosome Cargo and Subsequent Biological Effects. *Front. Immunol.* **2022**, *13*, 1140, doi:10.3389/FIMMU.2022.824188/BIBTEX.
71. Karampoga, A.; Tzaferi, K.; Koutsakis, C.; Kyriakopoulou, K.; Karamanos, N.K. Exosomes and the Extracellular Matrix: A Dynamic Interplay in Cancer Progression. *Int. J. Dev. Biol.* **2022**, *66*, 97–102, doi:10.1387/IJDB.210120NK.
72. Patel, N.J.; Ashraf, A.; Chung, E.J. Extracellular Vesicles as Regulators of the Extracellular Matrix. *Bioeng. 2023, Vol. 10, Page 136* **2023**, *10*, 136, doi:10.3390/BIOENGINEERING10020136.
73. Ribeiro, M.F.; Zhu, H.; Millard, R.W.; Fan, G.C. Exosomes Function in Pro- and Anti-Angiogenesis. *Curr. Angiogenes.* **2013**, *2*, 54, doi:10.2174/22115528113020020001.
74. Olejarz, W.; Kubiak-Tomaszewska, G.; Chrzanowska, A.; Lorenc, T. Exosomes in Angiogenesis and Anti-Angiogenic Therapy in Cancers. *Int. J. Mol. Sci.* **2020**, *21*, 1–25, doi:10.3390/IJMS21165840.
75. Xu, X.; Mu, L.; Li, L.; Liang, J.; Zhang, S.; Jia, L.; Yang, X.; Dai, Y.; Zhang, J.; Wang, Y.; et al. Imaging and Tracing the Pattern of Adult Ovarian Angiogenesis Implies a Strategy against Female Reproductive Aging. *Sci. Adv.* **2022**, *8*, 8683, doi:10.1126/SCIADV.ABI8683/SUPPL_FILE/SCIADV.ABI8683_MOVIES_S1_TO_S4.ZIP.
76. Kossowska-Tomaszczuk, K.; De Geyter, C. Cells with Stem Cell Characteristics in Somatic Compartments of the Ovary. *Biomed Res. Int.* **2013**, *2013*, doi:10.1155/2013/310859.
77. Oki, Y.; Ono, H.; Motohashi, T.; Sugiura, N.; Nobusue, H.; Kano, K. Dedifferentiated Follicular Granulosa Cells Derived from Pig Ovary Can Transdifferentiate into Osteoblasts. *Biochem. J.* **2012**, *447*, 239–248, doi:10.1042/BJ20120172.
78. Ai, A.; Tang, Z.; Liu, Y.; Yu, S.; Li, B.; Huang, H.; Wang, X.; Cao, Y.; Zhang, W. Characterization and Identification of Human Immortalized Granulosa Cells Derived from Ovarian Follicular Fluid. *Exp. Ther. Med.* **2019**, *18*, 2167–2177, doi:10.3892/ETM.2019.7802.
79. Józkowiak, M.; Hutchings, G.; Jankowski, M.; Kulcenty, K.; Mozdziak, P.; Kempisty, B.; Spaczyński, R.Z.; Piotrowska-Kempisty, H. The Stemness of Human Ovarian Granulosa Cells and the Role of Resveratrol in the Differentiation of MSCs-A Review Based on Cellular and Molecular Knowledge. *Cells* **2020**, *9*, doi:10.3390/cells9061418.
80. Brevini, T.A.L.; Pennarossa, G.; Rahman, M.M.; Paffoni, A.; Antonini, S.; Ragni, G.; DeEguileor, M.; Tettamanti, G.; Gandolfi, F. Morphological and Molecular Changes of Human Granulosa Cells Exposed to 5-Azacytidine and Addressed Toward Muscular Differentiation. *Stem Cell Rev. Reports* **2014**, *10*, 633–642, doi:10.1007/s12015-014-

9521-4.
81. Mushahary, D.; Spittler, A.; Kasper, C.; Weber, V.; Charwat, V. Isolation, Cultivation, and Characterization of Human Mesenchymal Stem Cells. *Cytom. Part A* **2018**, *93*, 19–31, doi:10.1002/CYTO.A.23242.
82. Kulus, J.; Kulus, M.; Kranc, W.; Jopek, K.; Zdun, M.; Józkowiak, M.; Jáskowski, J.M.; Piotrowska-Kempisty, H.; Bukowska, D.; Antosik, P.; et al. Transcriptomic Profile of New Gene Markers Encoding Proteins Responsible for Structure of Porcine Ovarian Granulosa Cells. *Biol. 2021, Vol. 10, Page 1214* **2021**, *10*, 1214, doi:10.3390/BIOLOGY10111214.
83. Bader, G.D.; Hogue, C.W.V. An Automated Method for Finding Molecular Complexes in Large Protein Interaction Networks. *BMC Bioinformatics* **2003**, *4*, 1–27, doi:10.1186/1471-2105-4-2/FIGURES/12.
84. Baruah, J.; Wary, K.K. Exosomes in the Regulation of Vascular Endothelial Cell Regeneration. *Front. Cell Dev. Biol.* **2020**, *7*, 353, doi:10.3389/FCELL.2019.00353/BIBTEX.
85. Fresno, C.; Fernandez, E.A. RDAVIDWebService: A Versatile R Interface to DAVID. *Bioinformatics* **2013**, *29*, 2810–2811, doi:10.1093/bioinformatics/btt487.
86. Walter, W.; Sánchez-Cabo, F.; Ricote, M. GOplot: An R Package for Visually Combining Expression Data with Functional Analysis. *Bioinformatics* **2015**, *31*, 2912–2914, doi:10.1093/bioinformatics/btv300.
87. Yu, G.; Wang, L.G.; Han, Y.; He, Q.Y. ClusterProfiler: An R Package for Comparing Biological Themes among Gene Clusters. *Omi. A J. Integr. Biol.* **2012**, *16*, 284–287, doi:10.1089/omi.2011.0118.
88. von Mering, C.; Jensen, L.J.; Snel, B.; Hooper, S.D.; Krupp, M.; Foglierini, M.; Jouffre, N.; Huynen, M.A.; Bork, P. STRING: Known and Predicted Protein-Protein Associations, Integrated and Transferred across Organisms. *Nucleic Acids Res.* **2005**, *33*, D433-7, doi:10.1093/nar/gki005.
89. Peng, J.Y.; Gao, K.X.; Xin, H.Y.; Han, P.; Zhu, G.Q.; Cao, B.Y. Molecular Cloning, Expression Analysis, and Function of Decorin in Goat Ovarian Granulosa Cells. *Domest. Anim. Endocrinol.* **2016**, *57*, 108–116, doi:10.1016/j.domaniend.2016.05.006.
90. Kedem, A.; Ulanenko-Shenkar, K.; Yung, Y.; Yerushalmi, G.M.; Maman, E.; Hourvitz, A. Elucidating Decorin's Role in the Preovulatory Follicle. *J. Ovarian Res.* **2020**, *13*, doi:10.1186/s13048-020-0612-3.
91. Messini, C.I.; Vasilaki, A.; Korona, E.; Anifandis, G.; Georgoulias, P.; Dafopoulos, K.; Garas, A.; Daponte, A.; Messinis, I.E. Effect of Resistin on Estradiol and Progesterone Secretion from Human Luteinized Granulosa Cells in Culture. *Syst. Biol. Reprod. Med.* **2019**, *65*, 350–356, doi:10.1080/19396368.2019.1615151.
92. Sawada, Y.; Sato, T.; Saito, C.; Ozawa, F.; Ozaki, Y.; Sugiura-Ogasawara, M. Clinical Utility of Decorin in Follicular Fluid as a Biomarker of Oocyte Potential. *Reprod. Biol.* **2018**, *18*, 33–39, doi:10.1016/j.repbio.2017.12.001.
93. Kulus, J.; Kulus, M.; Kranc, W.; Jopek, K.; Zdun, M.; Józkowiak, M.; Edrzej, J.; Ja´skowski, M.J.; Piotrowska-Kempisty, H.; Bukowska, D.; et al. Transcriptomic Profile of New Gene Markers Encoding Proteins Responsible for Structure of Porcine Ovarian Granulosa Cells. *Biol. 2021, Vol. 10, Page 1214* **2021**, *10*, 1214, doi:10.3390/BIOLOGY10111214.
94. Kitasaka, H.; Kawai, T.; Hoque, S.A.M.; Umehara, T.; Fujita, Y.; Shimada, M. Inductions of Granulosa Cell Luteinization and Cumulus Expansion Are Dependent on the Fibronectin-Integrin Pathway during Ovulation Process in Mice. *PLoS One* **2018**, *13*, e0192458, doi:10.1371/journal.pone.0192458.
95. Asem, E.K.; Carnegie, J.A.; Tsang, B.K. Fibronectin Production by Chicken Granulosa

Cells in Vitro: Effect of Follicular Development. *Acta Endocrinol. (Copenh).* **1992**, *127*, 466–470, doi:10.1530/acta.0.1270466.
96. Lussier, J.G.; Diouf, M.N.; Lévesque, V.; Sirois, J.; Ndiaye, K. Gene Expression Profiling of Upregulated MRNAs in Granulosa Cells of Bovine Ovulatory Follicles Following Stimulation with HCG. *Reprod. Biol. Endocrinol.* **2017**, *15*, doi:10.1186/s12958-017-0306-x.
97. Albacete-Albacete, L.; Navarro-Lérida, I.; López, J.A.; Martín-Padura, I.; Astudillo, A.M.; Ferrarini, A.; Van-Der-Heyden, M.; Balsinde, J.; Orend, G.; Vázquez, J.; et al. ECM Deposition Is Driven by Caveolin-1–Dependent Regulation of Exosomal Biogenesis and Cargo Sorting. *J. Cell Biol.* **2020**, *219*, doi:10.1083/JCB.202006178.
98. Diouf, M.N.; Lefebvre, R.; Silversides, D.W.; Sirois, J.; Lussier, J.G. Induction of Alpha-Caveolin-1 (ACAV1) Expression in Bovine Granulosa Cells in Response to an Ovulatory Dose of Human Chorionic Gonadotropin. *Mol. Reprod. Dev.* **2006**, *73*, 1353–1360, doi:10.1002/mrd.20513.
99. Retta, S.F.; Balzac, F.; Avolio, M. Rap1: A Turnabout for the Crosstalk between Cadherins and Integrins. *Eur. J. Cell Biol.* **2006**, *85*, 283–293, doi:10.1016/j.ejcb.2005.09.007.
100. Balzac, F.; Avolio, M.; Degani, S.; Kaverina, I.; Torti, M.; Silengo, L.; Small, J.V.; Retta, S.F. E-Cadherin Endocytosis Regulates the Activity of Rap1: A Traffic Light GTPase at the Crossroads between Cadherin and Integrin Function. *J. Cell Sci.* **2005**, *118*, 4765–4783, doi:10.1242/jcs.02584.
101. Velazquez, M.A.; Zaraza, J.; Oropeza, A.; Webb, R.; Niemann, H. The Role of IGF1 in the in Vivo Production of Bovine Embryos from Superovulated Donors. *Reproduction* **2009**, *137*, 161–180, doi:10.1530/REP-08-0362.
102. Pollard, T.D. Actin and Actin-Binding Proteins. *Cold Spring Harb. Perspect. Biol.* **2016**, *8*, doi:10.1101/CSHPERSPECT.A018226.
103. Lappalainen, P. Actin-Binding Proteins: The Long Road to Understanding the Dynamic Landscape of Cellular Actin Networks. *Mol. Biol. Cell* **2016**, *27*, 2519–2522, doi:10.1091/MBC.E15-10-0728/ASSET/IMAGES/LARGE/2519FIG1.JPEG.
104. Harris, A.R.; Jreij, P.; Belardi, B.; Joffe, A.M.; Bausch, A.R.; Fletcher, D.A. Biased Localization of Actin Binding Proteins by Actin Filament Conformation. *Nat. Commun. 2020 111* **2020**, *11*, 1–13, doi:10.1038/s41467-020-19768-9.
105. Liu, R.; Jin, J.P. Calponin Isoforms CNN1, CNN2 and CNN3: Regulators for Actin Cytoskeleton Functions in Smooth Muscle and Non-Muscle Cells. *Gene* **2016**, *585*, 143–153, doi:10.1016/J.GENE.2016.02.040.
106. Yin, L.M.; Schnoor, M.; Jun, C.D. Structural Characteristics, Binding Partners and Related Diseases of the Calponin Homology (CH) Domain. *Front. Cell Dev. Biol.* **2020**, *8*, doi:10.3389/FCELL.2020.00342.
107. Yang, X.; Pang, Y.; Zhang, J.; Shi, J.; Zhang, X.; Zhang, G.; Yang, S.; Wang, J.; Hu, K.; Wang, J.; et al. High Expression Levels of ACTN1 and ACTN3 Indicate Unfavorable Prognosis in Acute Myeloid Leukemia. *J. Cancer* **2019**, *10*, 4286, doi:10.7150/JCA.31766.
108. Zhou, Y.; Deng, X.; Ma, X.; Zang, N.; Li, H.; Li, G.; Li, D.; Li, C.; Huang, W.; He, M. Cellular Transcriptomics: Gelsolin Negatively Regulates the Expression of Apoptosis-Associated Genes and Inhibits Apoptosis in Hepatocarcinoma Cells. *Int. J. Clin. Exp. Pathol.* **2015**, *8*, 13871.
109. Jiang, Y.; Jiao, B.; Liao, X.; Xiao, X.; Liu, X.; Shen, L. Analyses Mutations in GSN, CST3, TTR, and ITM2B Genes in Chinese Patients With Alzheimer's Disease. *Front. Aging Neurosci.* **2020**, *12*, 292, doi:10.3389/FNAGI.2020.581524/BIBTEX.
110. Asare-Werehene, M.; Nakka, K.; Reunov, A.; Chiu, C.T.; Lee, W.T.; Abedini, M.R.;

Wang, P.W.; Shieh, D. Bin; Dilworth, F.J.; Carmona, E.; et al. The Exosome-Mediated Autocrine and Paracrine Actions of Plasma Gelsolin in Ovarian Cancer Chemoresistance. *Oncogene 2019 397* **2019**, *39*, 1600–1616, doi:10.1038/s41388-019-1087-9.

111. Ji, H.; Long, V.; Briody, V.; Chien, E.K. Progesterone Modulates Integrin A2 (ITGA2) and A11 (ITGA11) in the Pregnant Cervix. *Reprod. Sci.* **2011**, *18*, 156–163, doi:10.1177/1933719110382305.
112. Chan Jin Park, A.; Lin, P.-C.; Zhou, S.; Duffy, D.M.; Lydon, J.P.; Ko Correspondence, C.J. Progesterone Receptor Serves the Ovary as a Trigger of Ovulation and a Terminator of Inflammation In Brief. *CellReports* **2020**, *31*, 107496, doi:10.1016/j.celrep.2020.03.060.
113. Nio-Kobayashi, J.; Kudo, M.; Sakuragi, N.; Kimura, S.; Iwanaga, T.; Colin Duncan, W. Regulated C-C Motif Ligand 2 (CCL2) in Luteal Cells Contributes to Macrophage Infiltration into the Human Corpus Luteum during Luteolysis. *Mol. Hum. Reprod.* **2015**, *21*, 645–654, doi:10.1093/MOLEHR/GAV028.
114. Rodgers, R.J.; Irving-Rodgers, H.F.; Russell, D.L. Extracellular Matrix of the Developing Ovarian Follicle. *Reproduction* **2003**, *126*, 415–424, doi:10.1530/rep.0.1260415.
115. Li, J.; Qian, W.P.; Sun, Q.Y. Cyclins Regulating Oocyte Meiotic Cell Cycle Progression†. *Biol. Reprod.* **2019**, *101*, 878–881, doi:10.1093/BIOLRE/IOZ143.
116. Pan, B.; Liu, C.; Zhan, X.; Li, J. Protegrin-1 Regulates Porcine Granulosa Cell Proliferation via the EGFR-ERK1/2/P38 Signaling Pathway in Vitro. *Front. Physiol.* **2021**, *12*, 733, doi:10.3389/FPHYS.2021.673777/BIBTEX.
117. Zhang, L.; Du, X.; Wei, S.; Li, D.; Li, Q. A Comprehensive Transcriptomic View on the Role of SMAD4 Gene by RNAi-Mediated Knockdown in Porcine Follicular Granulosa Cells. *Reproduction* **2016**, *152*, 81–89, doi:10.1530/REP-16-0034.
118. Hettich, B.F.; Ben-Yehuda Greenwald, M.; Werner, S.; Leroux, J.C. Exosomes for Wound Healing: Purification Optimization and Identification of Bioactive Components. *Adv. Sci.* **2020**, *7*, 2002596, doi:10.1002/ADVS.202002596.
119. Peng, S. qing; Zhu, X. ren; Zhao, M. zhi; Zhang, Y. fan; Wang, A. ran; Chen, M. bin; Ye, Z. yu Identification of Matrix-Remodeling Associated 5 as a Possible Molecular Oncotarget of Pancreatic Cancer. *Cell Death Dis. 2023 142* **2023**, *14*, 1–14, doi:10.1038/s41419-023-05684-5.
120. Cai, L.; Xiong, X.; Kong, X.; Xie, J. The Role of the Lysyl Oxidases in Tissue Repair and Remodeling: A Concise Review. *Tissue Eng. Regen. Med.* **2017**, *14*, 15, doi:10.1007/S13770-016-0007-0.
121. Laczko, R.; Csiszar, K. Lysyl Oxidase (LOX): Functional Contributions to Signaling Pathways. *Biomol. 2020, Vol. 10, Page 1093* **2020**, *10*, 1093, doi:10.3390/BIOM10081093.
122. Harlow, C.R.; Rae, M.; Davidson, L.; Trackman, P.C.; Hillier, S.G. Lysyl Oxidase Gene Expression and Enzyme Activity in the Rat Ovary: Regulation by Follicle-Stimulating Hormone, Androgen, and Transforming Growth Factor-β Superfamily Members in Vitro. *Endocrinology* **2003**, *144*, 154–162, doi:10.1210/EN.2002-220652.
123. Zhang, C.; Ma, J.; Wang, W.; Sun, Y.; Sun, K. Lysyl Oxidase Blockade Ameliorates Anovulation in Polycystic Ovary Syndrome. *Hum. Reprod.* **2018**, *33*, 2096–2106, doi:10.1093/HUMREP/DEY292.
124. Yu, J.E.; Han, S.Y.; Wolfson, B.; Zhou, Q. The Role of Endothelial Lipase in Lipid Metabolism, Inflammation, and Cancer. *Histol. Histopathol.* **2018**, *33*, 1, doi:10.14670/HH-11-905.
125. Miller, W.L.; Bose, H.S. Early Steps in Steroidogenesis: Intracellular Cholesterol

Trafficking: Thematic Review Series: Genetics of Human Lipid Diseases. *J. Lipid Res.* **2011**, *52*, 2111, doi:10.1194/JLR.R016675.
126. Bassi, G.; Sidhu, S.K.; Mishra, S. The Expanding Role of Mitochondria, Autophagy and Lipophagy in Steroidogenesis. *Cells* **2021**, *10*, doi:10.3390/CELLS10081851.
127. Mourikes, V.E.; Santacruz Márquez, R.; Deviney, A.; Neff, A.M.; Laws, M.J.; Flaws, J.A. Imidacloprid and Its Bioactive Metabolite, Desnitro-Imidacloprid, Differentially Affect Ovarian Antral Follicle Growth, Morphology, and Hormone Synthesis In Vitro. *Toxics 2023, Vol. 11, Page 349* **2023**, *11*, 349, doi:10.3390/TOXICS11040349.
128. Yuan, X.; Zhou, X.; Qiao, X.; Wu, Q.; Yao, Z.; Jiang, Y.; Zhang, H.; Zhang, Z.; Wang, X.; Li, J. FoxA2 and P53 Regulate the Transcription of HSD17B1 in Ovarian Granulosa Cells of Pigs. *Reprod. Domest. Anim.* **2021**, *56*, 74–82, doi:10.1111/RDA.13850.
129. Yung, Y.; Ophir, L.; Yerushalmi, G.M.; Baum, M.; Hourvitz, A.; Maman, E. HAS2-AS1 Is a Novel LH/HCG Target Gene Regulating HAS2 Expression and Enhancing Cumulus Cells Migration. *J. Ovarian Res.* **2019**, *12*, 1–7, doi:10.1186/S13048-019-0495-3/TABLES/1.
130. Sung, B.H.; Ketova, T.; Hoshino, D.; Zijlstra, A.; Weaver, A.M. Directional Cell Movement through Tissues Is Controlled by Exosome Secretion. *Nat. Commun. 2015 61* **2015**, *6*, 1–14, doi:10.1038/ncomms8164.

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Transcriptomic Profile of New Gene Markers Encoding Proteins Responsible for Structure of Porcine Ovarian Granulosa Cells

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Simple Summary: The extracellular matrix (ECM) is involved in many physiological processes that occur in the ovary and affect reproduction in animals and humans. The ECM has been shown to significantly affect folliculogenesis, ovulation, and corpus luteum formation. This is mainly due to the involvement of ECM in intercellular signaling. In the present study, we report the gene expression profile of porcine granulosa cells during their primary in vitro culture. The genes presented are related to ECM formation but also to cadherins and integrins that influence intercellular dialogue. During the study, it was shown that most of the genes were upregulated. A detailed understanding of the expression of genes such as POSTN, CHI3L1, CAV-1, IRS1, DCN in in vitro culture of granulosa cells may provide a basis for further studies on the molecular mechanisms occurring within the ovary. Knowledge of ECM-related gene expression within granulosa cells can also be used to study the recently discovered stemness of these cells. Moreover, the presented data may serve for the development of assisted reproduction techniques, which, especially in vitro, are becoming increasingly common.

Abstract: The extracellular matrix (ECM) in granulosa cells is functionally very important, and it is involved in many processes related to ovarian follicle growth and ovulation. The aim of this study was to describe the expression profile of genes within granulosa cells that are associated with extracellular matrix formation, intercellular signaling, and cell–cell fusion. The material for this study was ovaries of sexually mature pigs obtained from a commercial slaughterhouse. Laboratory-derived granulosa cells (GCs) from ovarian follicles were cultured in a primary in vitro culture model. The extracted genetic material (0, 48, 96, and 144 h) were subjected to microarray expression analysis. Among 81 genes, 66 showed increased expression and only 15 showed decreased expression were assigned to 7 gene ontology groups "extracellular matrix binding", "extracellular matrix structural constituent", "binding, bridging", "cadherin binding", "cell adhesion molecule binding", "collagen binding" and "cadherin binding involved in cell-cell adhesion". The 10 genes with the highest expression (POSTN, ITGA2, FN1, LAMB1, ITGB3, CHI3L1, PCOLCE2, CAV1, DCN, COL14A1) and 10 of the most down-regulated (SPP1, IRS1, CNTLN, TMPO, PAICS, ANK2, ADAM23, ABI3BP, DNAJB1, IGF1) were selected for further analysis. The results were validated by RT-qPCR. The

current results may serve as preliminary data for further analyses using in vitro granulosa cell cultures in assisted reproduction technologies, studies of pathological processes in the ovary as well as in the use of the stemness potential of GCs.

Keywords: porcine granulosa cells; extracellular matrix; gene expression profile; folliculogenesis; reproductive physiology

1. Introduction

The extracellular matrix (ECM) is an extremely important structure present in all tissues of the animal organism. It exhibits various functions and is composed of many substances of different origins [1,2]. The major components of the extracellular matrix include two groups of molecules. The first is the fibrous proteins, which include collagens, elastin, fibronectin, and laminin. The most common of these proteins is collagen, which provides a support for tissue scaffolding but also affects cell adhesion, cell migration, and chemotaxis [3]. The second group are proteoglycans, which consist of a protein core to which glycosaminoglycans are attached. These molecules are highly hydrophilic, causing the formation of a hydrogel that fills the space between the fibrous proteins of the ECM. Additionally, proteoglycans are involved in many signaling pathways related to insulin-like growth factor 1 receptor (IGFIR), epidermal growth factor receptor (EGFR), low-density lipoprotein-receptor-related protein 1 (LRP1) [3]. The fact that an extracellular matrix is composed of a variety of components results in a vast array of functions [4]. It is a unique microenvironment for cells (including the delivery of osmotic forces), a mechanical support or pathway for the passage of nutrients, hormones and extracellular signals to the target cells as well as regulation of gene expression and cytokine release [1,5]. The complicated ultrastructure of the matrix provides the filter material, and the ability to bind growth factors required for local action. The ECM also influences cell behavior by participating in the migration, anchoring, division, and death of cells. Taking into account all these functions, it can be stated that it enables cells to specialize in a specific way by creating their microenvironment [6]. The role of the extracellular matrix in the development of cancer has also been demonstrated, including those associated with epithelial cells in the ovary [7]. The role of the ECM has also been described in the pathogenesis of other diseases related to the reproductive system, including PCOS (polycystic ovary syndrome) [8–10] and POI (premature ovarian insufficiency) [11,12]. The extensively described involvement of extracellular matrix components in signaling pathways within the ovary highlights the role of the ECM in folliculogenesis [13] and in pathological processes [14] which altogether may provide valuable therapeutic insights. Until now, little is known about the molecular-level processes involved in the ECM and its role in the maturation and growth of mammalian ovarian follicles, and this role seems to be significant [15]. An example is the presence of different laminin chains in ovarian follicles depending on their developmental stage, which is associated with antrum formation [16]. In addition, the expression of genes related to ECM remodeling was also found to change during follicle maturation [17]. ECM in ovarian follicles is expressed in various compartments, such as basal lamina, follicular fluid, zona pellucida, granulosa membrane, or cumulus [6]. The basal lamina is a flat sheet of extracellular matrix, which is specialized in separating the epithelial cells lying on it from the lower layers. Its structure is of a lattice type consisting of a network of type IV collagen and laminine. Entaxins/nidogen are attached to this network, stabilizing the structure. In various proportions fibronectin, heparan sulfate, and others are also connected [6,18–20]. Furthermore, these components may be present in different conforms, composed of various chains, thus affecting the uniqueness of a specific basal lamina, which in turn affects its functionality [6,21].

It is important to consider the role of cell signaling on reproductive efficiency, including via the ECM, cadherins, and integrins [22–26]. Cadherins and integrins are among the

transmembrane proteins that are closely involved in intercellular signaling. The former are responsible for cell-to-cell signaling and their action is closely related to catenins (α, β and p120). The second are responsible for signaling between the cell and the ECM. The action of both groups of proteins is through their effect on F-actin [26]. Several groups are distinguished among the cadherins: N-cadherin (neural cadherin), P-cadherin (placental cadherin), R-cadherin (retinal cadherin), VE-cadherin (vascular endothelial cadherin), and the E-cadherin (epithelial cadherin) found in the epithelium of various tissues and have been implicated in the function of the reproductive system [22]. More than two decades ago, the expression of these transmembrane proteins in porcine GCs was described and linked to the development of ovarian follicles by maintaining the structural integrity of the follicle through E-cadherin [27]. Furthermore, a positive correlation between e-cadherin expression and the pool of primary follicles in the mouse ovary formed during embryonic development was demonstrated due to effects on cell–cell junction integrity and NOVOX expression [28] which may affect the occurrence of POI. These proteins also affect the fertilization process by allowing the interaction between sperm and oviduct epithelium and oocyte which was confirmed in a bovine model [29]. Intensive research in recent years on granulosa cells has further demonstrated their stemness potential. To date, human-derived granulosa cells have demonstrated the ability to differentiate into muscle, cartilage, bone, and even nerve tissue [30–33]. Porcine granulosa cells have shown the ability to differentiate into bone tissue [34,35]. These data suggest the possibility of using these cells in the treatment of degenerative diseases and also in regenerative medicine. Further studies conducted on porcine GCs, considered as an animal model for humans, may provide important information in the context of using these cells in therapy. Granulosa cells play an important role in the processes of folliculogenesis and oogenesis [36,37]. One of the most important functions of GCs is their participation in steroidogenesis as they perform aromatization of androgens to estrogens, which are secreted into the follicular fluid. In addition, the proliferation of GCs leads to the growth and development of the entire ovarian follicle [38]. The aim of this study was to determine the expression profile of genes responsible for processes related to cadherin and collagen binding and structuralization of ECM in porcine granulosa cells cultured in vitro. The present study compared the expression levels of selected ECM-related genes in vivo (0 h) and during in vitro culture. Cells isolated from the natural microenvironment (which for GCs is the ovarian follicle) and subjected to in vitro culture show an altered gene expression profile. Therefore, the mechanisms governing expression profile dynamics may be the basis for further research, especially in the field of assisted reproduction techniques based on in vitro culture. Additionally, characterization of extracellular matrix-related genes within them may elucidate the molecular basis of GCs function, which is important to elucidate the pathogenesis of ovarian-related diseases. The results obtained in this study, show significant upregulation of expression levels of genes related to both ECM formation and function, confirm the occurrence of these processes in vitro during GCs culture, which is associated with the development of these cells. The expression patterns observed in the current study, together with the positive effects of ECM on the efficiency of cell differentiation reported in the literature [39,40], may provide a valuable background for further research in studies on the pluripotency of GCs [41,42].

2. Materials and Methods

2.1. Animals

A total of 40 crossbred Landrace gilts with a median age of 170 days and weight of 98 kg were used in this study. All animals were housed under identical conditions. The animals in the study reached sexual maturity as pigs become sexually mature at 4–6 months of age.

2.2. Collection of Porcine Ovarian Granulosa Cells

Ovaries (n = 80) were recovered at slaughter and transported to the laboratory at 38 °C in 0.9% NaCl within 30 min. In the laboratory, the ovaries of each animal were placed in PBS supplemented with fetal bovine serum (FBS; Sigma-Aldrich Co., St. Louis, MO,

USA). Thereafter, single preovulatory large follicles, with a diameter estimated greater than 5 mm (n = 300), were opened into a sterile Petri dish by puncturing using a 5 ml syringe and 20 G needle, and the cumulus-oocyte complexes (COCs) and follicular fluid (FF) were recovered. The transcriptomic profile of mural GCs, which constitute a significant majority among the GCs population was analyzed. The follicular fluid was used to isolate GCs, whereas the COCs were discarded. The extracted follicular fluid after discarding COCs was filtered through sterile nylon cell strainers with a mesh diameter of 40 µm (Biologix Group, Shandong, China) to eliminate tissue debris and larger cell aggregates (including blood cells) or epithelium. The resulting suspension was centrifuged at room temperature for 10 min, 200 rpm, to obtain individual cell fractions. The GCs pellet was then resuspended in collagenase type I solution (Gibco, Thermo-Fischer Scientific, Waltham, MA, USA) 1 mg/1 mL DMEM and incubated 10 min in a 37 °C water bath and centrifuged (under the same conditions). The obtained cell pellet was resuspended in culture medium to establish in vitro culture under the conditions described below. Granulosa cells collected from ovarian follicles were pooled to homogenize the sample.

2.3. In Vitro Primary Culture of Porcine Granulosa Cells

A primary in vitro culture model was used in this study with four time intervals for each biological repeat. For microarray expressions, cultures were maintained in two biological replicates for each time interval. For validation by RT-qPCR, cultures were maintained in a triplicate biological sample model for each time interval. Primary cultures were established from GCs in four bottles with 3×10^6 cells per dish (25 cm^2 cell culture flask, TPP, Trasadingen, Switzerland). The number of cells and their viability were assessed using the ADAM Automatic Cell Counter (NanoEnTek, Waltham, MA, USA). From the cell suspension, a 20 µL sample for number and viability analysis was stained with propium iodide and examined in a fluorescence analyzer on disposable microchips. By staining the cell nuclei, the counter is able to distinguish single cells in aggregates. Only those samples with viability above 85% were used for further studies. Cells in culture were kept until culture termination when the material was collected at 0 h, 48 h, 96 h, 144 h. The culture medium was changed every 72 h.

Culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, Saint Louis, MO, USA), 2% fetal calf serum (FCS) (PAA, Linz, Austria), 10 mg/mL ascorbic acid (Sigma-Aldrich, Saint Louis, MO, USA), 0.05 µM dexamethasone (Sigma-Aldrich, Saint Louis, MO, USA), 200 mM L-glutamine (Invitrogen, Carlsbad, CA, USA), 10 mg/mL gentamycin (Invitrogen, Carlsbad, CA, USA), 10,000 units/mL penicillin and 10,000 µg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were cultivated at 38.5 °C under aerobic conditions (5% CO_2). Once the adherent cells were more than 80% confluent, they were detached with 0.05% trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) for 3 min. and then passaged. The morphology of the GCs was evaluated using an inverted phase-contrast microscope, and the results of these observations are presented in Figure 1. GCs underwent significant morphological changes. When the cells were seeded into culture bottles, the shape of the cells was close to spherical, where the cells formed a suspension in the medium. After 24 h of culture, the cells became adherent to the medium, and after 48 h, the cells assumed a star-like shape. At subsequent time intervals, the GCs became wider, more fibroblast-like. The strong adherence to the dish surface, shape change, and flattening of the cells is related to the secretion of extracellular matrix components, which correlates with the increased expression of ECM-related genes during the study.

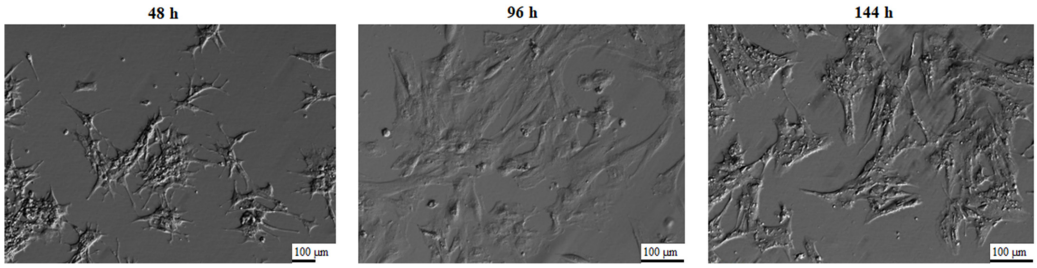

Figure 1. Porcine granulosa cells cultures used for the experiment, presenting changes in their morphology, following 48 h, 96 h, and 144 h of in vitro culture (magnification ×100 (48 h), magnification ×200 (96 h, 144 h)).

2.4. Microarray Expression Analysis and Statistics

The Affymetrix procedure was previously described by Trejter et al. [43] and used in studies involving porcine oviduct epithelial cells (OECs) [44–46] as well as oocytes [47–49]. Briefly, cDNA was subjected to Total RNA (100 ng) (Ambion® WT Expression Kit, Thermo Fisher Scientific Inc., Wilmington, DE, USA). Obtained cDNA was biotin labeled and fragmented by Affymetrix GeneChip® WT Terminal Labeling and Hybridization (Affymetrix, Santa Clara, CA, USA). Biotin-labeled fragments of cDNA (5.5 µg) were hybridized to Affymetrix® Porcine Gene 1.1 ST Array Strip (Affymetrix, Santa Clara, CA, USA) (48 °C/20 h). Then, microarrays were washed and stained according to the technical protocol using Affymetrix GeneAtlas Fluidics Station (Affymetrix, Santa Clara, CA, USA). Subsequently, the array strips were scanned by the Imaging Station of the GeneAtlas System (Affymetrix, Santa Clara, CA, USA). The preliminary analysis of the scanned chips was performed using Affymetrix GeneAtlasTM Operating Software. The quality of gene expression data was checked according to quality control criteria provided by the software. Obtained CEL files were imported into the downstream data analysis software. All of the presented analyses and graphs were performed by Bioconductor and R programming language. Each CEL file was merged with a description file. In order to correct background, normalize and summarize results, we used Robust Multiarray Averaging (RMA) algorithm.

Statistical significance of analyzed genes was performed by moderated t-statistics from the empirical Bayes method. Obtained *p*-value was corrected for multiple comparisons using Benjamini and Hochberg's false discovery rate. The selection of significantly changed gene expression was based on a *p*-value under 0.05 and expression fold higher than 2. Differentially expressed genes were subjected to the selection of genes involved in cadherin and collagen binding and structuralization of extracellular matrix components. Differentially expressed gene lists were uploaded to DAVID software (Database for Annotation, Visualization and Integrated Discovery), where "extracellular matrix binding", "extracellular matrix structural constituent", "binding, bridging", "cadherin binding", "cell adhesion molecule binding", "collagen binding" and "cadherin binding involved in cell-cell adhesion" GO MF terms were obtained. Expression data of these genes were subjected to a hierarchical clusterization procedure and presented as a heatmap graph. Detailed analysis of genes belonging to selected GO MF terms was presented as plots using "GOplot" library [50] and "ClusterProfiler" R package [51]. In the chosen gene sets, we investigated their mutual relations using the GOplot package. Moreover, the GOplot package was used to calculate the z-score (difference in the number of up- and down-regulated genes divided by the square root of the count). The z-score analysis allowed us to compare the enrichment of the selected GO BP terms.

Using STRING10 (Search Tool for the Retrieval of Interacting Genes, STRING Consortium, Lausanne, Switzerland) software, interactions between genes and the proteins they encode have been investigated. The STRING database includes information on protein/gene interactions, including experimental data, computational prediction methods,

and public text collections. The STRING database engine supplied the molecular network of interactions formed between the chosen genes. Search criteria are provided based on gene/protein co-occurrence in scientific texts (textmining), coexpression, and experimentally observed interactions.

The functional interaction between genes that belong to the chosen GO BP terms was investigated by the REACTOME FIViz application to the Cytoscape 3.8.2 software (San Diego, CA, USA). The Reactome FIViz app is designed to find pathways and network patterns related to cancer and other types of diseases. This app accesses the pathways stored in the Reactome database, allowing to do pathway enrichment analysis for a set of genes, visualize hit pathways using manually laid-out pathway diagrams directly in Cytoscape, and investigate functional relationships among genes in hit pathways. The app can also access the Reactome Functional Interaction (FI) network, a highly reliable, manually curated pathway-based protein functional interaction network covering over 60% of human proteins.

2.5. Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

Total RNA was isolated from GCs in 0 h and after 48 h, 96 h, and 144 h in vitro culture using an RNeasy mini column from Qiagen GmbH (Qiagen GmbH, Hilden, Germany). The RNA samples were resuspended in 20 µl of RNase-free water and stored in liquid nitrogen. RNA samples were treated with DNase I and reverse-transcribed (RT) into cDNA. RT-qPCR was conducted in a LightCycler real-time PCR detection system (Roche Diagnostics GmbH, Mannheim, Germany) using SYBR® Green I (Master Mix Qiagen GmbH, Hilden, Germany) as a detection dye, and target cDNA was quantified using the relative quantification method. The relative abundance of analyzed transcripts in each sample was standardized to the internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For amplification, 2 µL of cDNA solution was added to 18 µL of QuantiTect® SYBR® Green PCR (Master Mix Qiagen GmbH, Hilden, Germany) and primers (Table 1). One RNA sample of each preparation was processed without the RT reaction to provide a negative control for subsequent PCR.

Table 1. Oligonucleotide sequences of primers used for RT-qPCR analysis.

Gene		Primer Sequence (5′-3′)	Product Size (bp)
IRS1	F	CCTAGCACCAACAGGACTCA	239
	R	GAAGAGATGAAACCGCCGTC	
TMPO	F	GCTCAGTGGAAAGTCAGCAG	241
	R	CCTGTCAATTTGCTGCCACT	
PAICS	F	AGTCATGCTACACAGGCCAT	235
	R	TTACCATCTGCAGCCCTTCA	
ANK2	F	TTGTAACGGAGGAGGTCACC	222
	R	AACGCAGGTAGTTCATCCCA	
ADAM23	F	AGCAGCTCAATACCAGGGTT	235
	R	TTCACACCAACTCCCCTTGT	
IGF1	F	TTCTACTTGGCCCTGTGCTT	222
	R	CTCCAGCCTCCTCAGATCAC	
CNTLN	F	ACCTCAACCCATAAAGCCCA	186
	R	TGTGGCAAAAGGAAGCTGTC	
DNAJB1	F	AGGACCATACCCGTTGTGTT	167
	R	AGGACGGTTCTTGAGGTCTG	
POSTN	F	ATTGACCGTGTCCTCACACA	212
	R	GCCACTTTGTCTCCCATGAT	

Table 1. *Cont.*

Gene		Primer Sequence (5′-3′)	Product Size (bp)
ITGA2	F	CATGCCAGATCCCTTCATCT	153
	R	CGCTTAAGGCTTGGAAACTG	
FN1	F	TGAGCCTGAAGAGACCTGCT	113
	R	CAGCTCCAATGCAGGTACAG	
LAMB1	F	CTTCACCACCTTGGACCACT	216
	R	AGCTGTGGCTCATAGCGAAT	
ITGB3	F	GGCTTCAAAGACAGCCTCAC	175
	R	AGTCCTTTTCCGAGCACTCA	
CHI3L1	F	GGATGCAAGTTCCGACAGAT	202
	R	GAGGATCCCTTTCTCCTTGG	
DCN	F	CTCTCTGGCCAACACTCCTC	155
	R	GCGGGCAGAAGTCATTAGAG	
PCOLCE2	F	TGTAAACGGACTGGGACTCC	184
	R	CGATGACCTTGGCACTCATG	
COL14A1	F	AGTTCCAGCCCAGCAATACT	229
	R	ATCGTCCAGTACAGCCAACA	

To quantify the specific genes expressed in the GCs, the expression levels of specific mRNAs in each sample were calculated relative to PBGD and ACTB. To ensure the integrity of these results, the additional housekeeping gene 18S was used as an internal standard to demonstrate that PBGD and ACTB mRNAs were not differentially regulated in GC groups. The gene for 18S rRNA expression has been identified as an appropriate housekeeping gene for use in quantitative PCR studies. Expression of PBGD, ACTB, and 18S mRNA was measured in cDNA samples from isolated GCs. The statistical significance of the analyzed genes was performed using moderated t-statistics from the empirical Bayes method. The obtained *p*-value was corrected for multiple comparisons using Benjamini and Hochberg's false discovery rate.

3. Results

The Affymetrix® Porcine Gene 1.1 ST Array Strip (Affymetrix, Santa Clara, CA, USA) for the microarray gene expression analysis of porcine granulosa cells allows the study of the gene expression of 27,558 transcripts at 0, 48, 96, and 144 h of in vitro granulosa cell culture. Genes with more than 2-fold changes and corrected *p*-values less than 0.05 were selected for downstream analysis. A total of 3380 differentially expressed genes (DEGs) were identified according to the above criteria. The list of DEGs was uploaded to the GEO database (ID:GSE134361). Microarray gene expression analysis with subjecting the list of DEGs to DAVID software, which showed that the genes can be assigned to 1901 GO BP (Gene Ontology Biological Process), 162 GO MF (Gene Ontology Molecular Function), and 182 GO CC (Gene Ontology Cellular Component) terms. Gene Ontology is the knowledge database of gene function and provides the basis for computational analyses used in molecular biology and genetics. This paper focused on the genes involved in cadherin and collagen binding and structuralization of extracellular matrix components. The DAVID software indicated the following GO MF terms, which cover the above processes: "extracellular matrix binding", "extracellular matrix structural constituent", "binding, bridging", "cadherin binding", "cell adhesion molecule binding", "collagen binding" and "cadherin binding involved in cell-cell adhesion". The 81 genes involved in those processes were clustered using hierarchical clustering and presented as heatmaps (Figure 2). Interestingly, 66 genes were upregulated, which is the greater part of the list of genes used for hierarchical clustering. Only 15 genes, in contrast, were downregulated. Some of the downregulated genes form small clusters in "cadherin binding" and "cadherin binding involved in cell-cell adhesion" GO MF terms. The direction of expression change (upregulation or downreg-

ulation) was maintained in granulosa cell culture in subsequent points of analysis (after 48, 96, and 144 h of in vitro culture). The mean value of the fold change ratio of each gene between 48 h, 96 h, and 144 h was calculated. For further analysis we choose the 10 most significantly upregulated and downregulated genes and their symbols, fold changes, and corrected *p*-values are shown in Table 2.

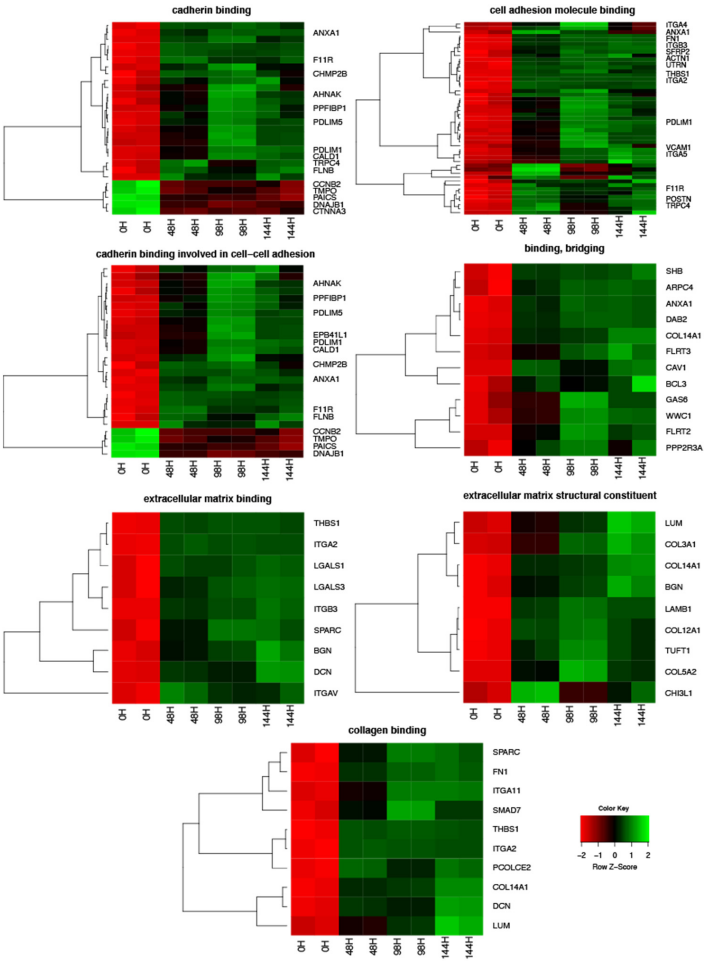

Figure 2. Heatmaps presenting differentially expressed genes involved in "extracellular matrix binding", "extracellular matrix structural constituent", "binding, bridging", "cadherin binding", "cell adhesion molecule binding", "collagen binding" and "cadherin binding involved in cell-cell adhesion" based on GO MF terms. Each row on the *Y*-axis represents a single transcript. The red color indicates downregulated genes while the green indicates upregulated.

Table 2. The 10 most significantly upregulated and 10 most significantly downregulated genes involved in cadherin and collagen binding and structuralization of extracellular matrix components.

Gene Symbol	Gene Name	Fold Change	Adj. p. val.
POSTN	periostin, osteoblast specific factor	95.2	3.5×10^{7}
ITGA2	Integrin, alpha 2 (CD49B, alpha 2 subunit of VLA -2 receptor)	42.7	3.5×10^{7}
FN1	Fibronectin 1	35.4	3.6×10^{7}
LAMB1	Laminin, beta 1	33.3	3.5×10^{7}
ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	29.9	3.5×10^{7}
CHI3L1	Chitinase 3-like 1 (cartilage glycoprotein-39)	29.6	1.4×10^{5}
PCOLCE2	Procollagen C-endopeptidase enhancer 2	26.2	4.6×10^{7}
CAV1	Caveolin 1, caveolae protein, 22kDa	22.3	4.3×10^{7}
DCN	decorin	21.6	4.4×10^{7}
COL14A1	Collagen, type XIV, alpha 1	16.0	5.3×10^{7}
SPP1	Secreted phosphoprotein 1	−2.5	1.4×10^{2}
IRS1	Insulin receptor substrate 1	−2.6	1.9×10^{4}
CNTLN	Centlein, centrosmal protein	−2.6	5.1×10^{4}
TMPO	thymopoietin	−2.7	1.3×10^{4}
PAICS	Phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazole succinocarboxamide synthetase	−2.9	8.0×10^{5}
ANK2	Ankyrin 2	−4.3	2.1×10^{5}
ADAM23	ADAM metalloptidase domain 23	−4.9	1.0×10^{5}
ABI3BP	ABI family, member 3 (NESH) binding protein	−5.3	2.1×10^{5}
DNAJB1	DnaJ (Hsp40) homolog, subfamily B, member 1	−6.4	2.3×10^{6}
IGF1	Insulin-like growth factor 1 (somatomedin C)	−8.3	1.0×10^{4}

Z-scores, reveal whether the molecular function is more likely to be decreased (negative value) or increased (positive value). The z-scores were presented as segments of inner circles in Figure 3. As can be seen from the figure, the expression of most genes was increased (green dots) in all ontological groups. The z-scores had positive values, indicating that the GO MF terms were upregulated. The "cell adhesion molecule binding" term contained the highest number of genes. The expression pattern did not change at any of the analyzed time points. Considering all information, the subsequent analysis was based only on 48H/0H comparison.

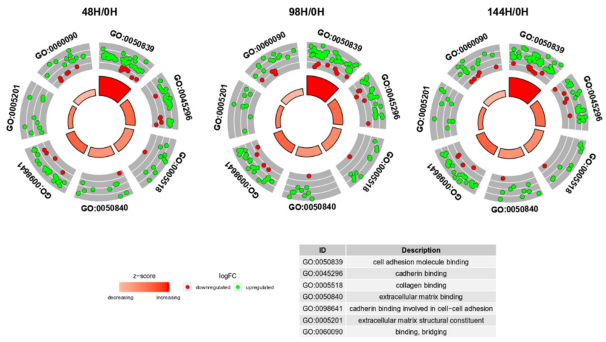

Figure 3. The circular scatter plots of differentially expressed genes involved in "extracellular matrix binding", "extracellular matrix structural constituent", "binding, bridging", "cadherin binding", "cell adhesion molecule binding", "collagen binding" and "cadherin binding involved in cell-cell adhesion" GO MF terms. Each dot represents a single gene. The z-scores were presented as segments of inner circles.

Subsequently, the interaction between the seven selected ontological groups of the 81 genes was evaluated. One of the most visually appealing ways of presenting such interaction is by using a dendrogram (Figure 4). Clusters contain functionally related genes based on their expression pattern. The middle circle represents a logarithm of fold change (logFC) of differentially expressed genes assigned to the studied GO terms. The GO terms are shown as the outer ring. The genes whose expression is downregulated form a small cluster (blue part of the middle circle—as can be seen for the gene IGF1), which is consistent with the previous observations. Clusters of the same color over the entire width of the outer circle represent genes that are unique for a specific GO term (as can be seen for the gene ANK2). Clusters of different colors on the cross-section of the outer circle show sets of genes that are likely to be functionally related (as can be seen for the genes DNAJB1, COL14A1, ITGA2). The dendrogram showed that 25 of the 81 genes belong simultaneously to three of the selected GO MF terms: "cadherin binding", "cell adhesion molecule binding" and "cadherin binding involved in cell-cell adhesion".

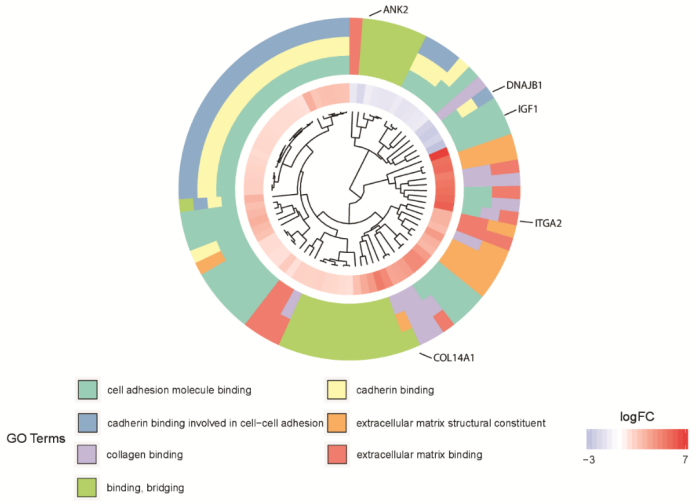

Figure 4. The dendrogram of the 81 differentially expressed genes involved in "extracellular matrix binding", "extracellular matrix structural constituent", "binding, bridging", "cadherin binding", "cell adhesion molecule binding", "collagen binding" and "cadherin binding involved in cell-cell adhesion" GO MF terms. The DEGs were clustered based on their logFC values.

In the gene ontology database, single genes may belong to many ontological terms. In our study, among 15 down-regulated genes, 5 of them belonged to two or more ontology groups. Of the 66 up-regulated genes, 33 showed belonging to more than one GO. Therefore, plots with visualization of fold change values and the relationship between genes and selected GO MF terms were employed to depict the data (Figure 5A,B). The relationship was also presented as a heatmap (Figure 6). Genes were selected on the basis of the logFC [2.5] (absolute value, i.e., >2.5 and <−2.5).

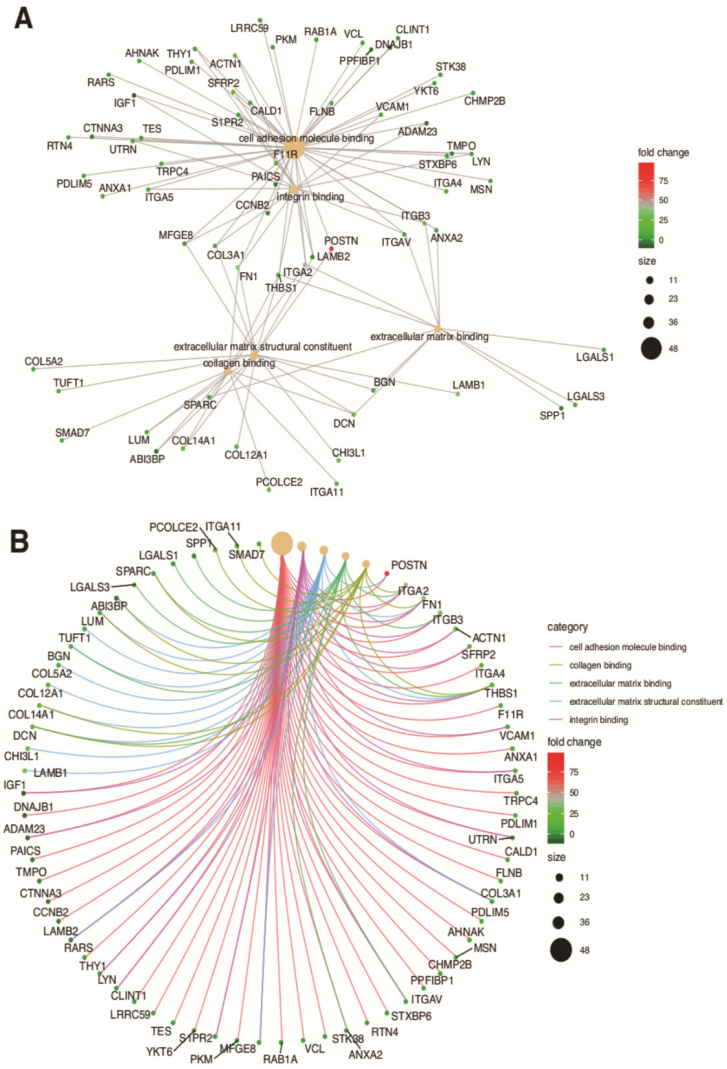

Figure 5. Analysis of enriched gene ontological groups involved in (**A**) cell adhesion molecule binding, collagen binding, extracellular matrix binding, extracellular matrix structural constituent, integrin binding. The network plots presenting the linkages of genes and GO MF terms; (**B**) cadherin and collagen binding and structuralization of extracellular matrix components. The network plots presenting the linkages of genes and GO MF terms.

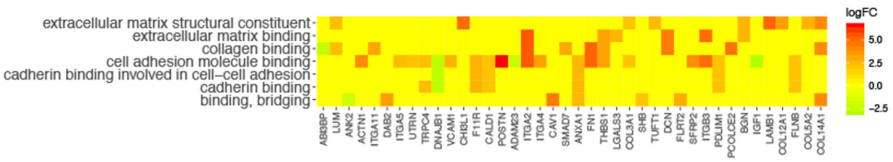

Figure 6. Heatmap presenting the relationship between genes and selected GO MF terms. Yellow color of tiles indicates the absence of logFC values.

A STRING interaction network was used to demonstrate the interaction between the 10 most up-regulated and the 10 most down-regulated genes analyzed, which can predict the relationship between the protein products of the genes described (Figure 7). In the last stage, the functional interactions between chosen genes with REACTOME FIViz app to Cytoscape 3.8.2 software were evaluated. The results were shown in Figure 8.

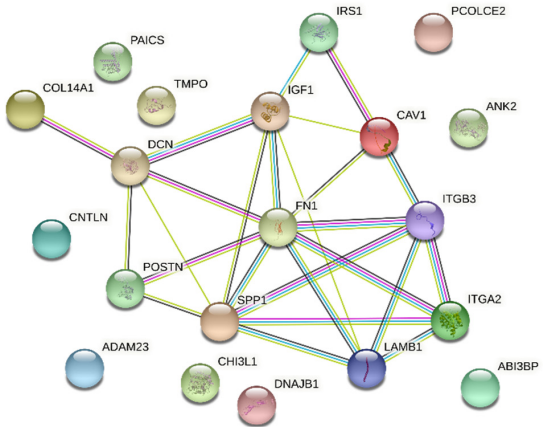

Figure 7. STRING-generated interaction network among 20 chosen differently expressed genes belonging to the "extracellular matrix binding", "extracellular matrix structural constituent", "binding, bridging", "cadherin binding", "cell adhesion molecule binding", "collagen binding" and "cadherin binding involved in cell-cell adhesion" GO MF terms. The strength of the interaction score was reflected by the intensity of the edges.

Results from microarray expression were confirmed by quantitative RT-qPCR on porcine granulosa cells. These data sets were collected, compared, and presented as bar graphs (Figures 9 and 10). All 20 selected genes were validated. For 3 downregulated genes via microarray analysis, the reaction conditions could not be established. Non-specific products were formed in the reaction. Only 7 downregulated genes have been presented in the graph (Figure 9). Moreover, the expression pattern for one gene (IGF1) was not confirmed. The expression direction of upregulated genes in all cases was confirmed (Figure 10). The direction of expression change was different on all days of culture intervals. The RT-qPCR result can be more representative because this method has greater quantitative precision, as opposed to the whole transcriptome analysis provided by microarrays.

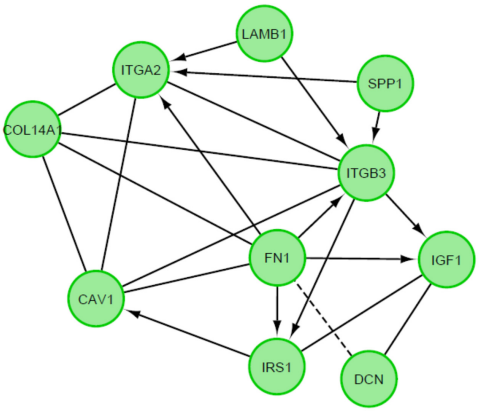

Figure 8. Functional interaction (FI) between 20 chosen differently expressed genes. In above figure "->" stands for activating/catalyzing, "-" for FIs extracted from complexes or inputs, and "—" for predicted FIs.

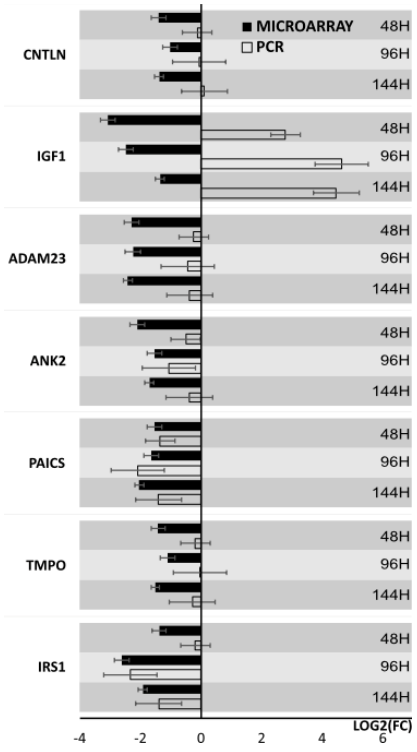

Figure 9. Bar graph showing validation results of microarrays of downregulated genes obtained by RT-qPCR.

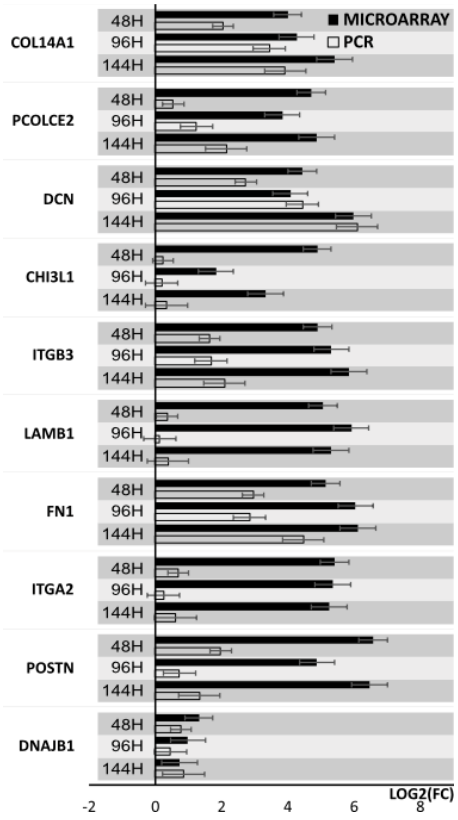

Figure 10. Bar graph showing validation results of microarrays of upregulated genes obtained by RT-qPCR.

4. Discussion

The contact of the cell with the external environment, including the ECM, is crucial for the continuity of the communication, the reception of stimuli, and the occurrence of basic processes such as migration, proliferation, differentiation, morphogenesis, as well as providing tissue homeostasis [25]. The high complexity of ECM composition, its modification in different tissues, and its activity prove that it is not only an extracellular spatial skeleton of tissues. The processes of cell–cell but also cell–ECM adhesion depend on many integrated networks of protein interactions as well as signaling pathways [52]. Considering the wide range of functions performed by the ECM, as well as its significance in cell function in vitro, it is very important to determine the changes in gene expression relative to in vivo conditions. Due to the relatively different function of cumulus cells (located in the closest proximity to the oocyte) and mural GCs, the gene expression profile between these subpopulations may differ. Thus, the changes that occur during in vitro culture can be significantly different, and knowledge of this transcriptomic profile will allow for further studies on GCs cultured in vitro. Further research, including in the field of assisted human and animal reproduction techniques, determination of mechanisms of ovarian pathology, or stemness, GCs use in regenerative medicine, may rely on the transcriptomic data. In this study, among 81 genes with statistically significant differential expression,

10 the most upregulated POSTN, ITGA2, FN1, LAMB1, ITGB3, CHI3L1, PCOLCE2, CAV1, DCN, COL14A1, and 10 the most down-regulated SPP1, IRS1, CNTLN, TMPO, PAICS, ANK2, ADAM23, ABI3BP, DNAJB1, IGF1 were selected for further analysis.

It has been shown that ECM components have a direct effect on biological processes within the ovary such as folliculogenesis, ovulation and steroidogenesis. Glycoproteins, which play an important role in ovulation are involved in signaling with other ECM components including proteins, integrins and growth factors, deserve special attention. These include, among others: DCN (decorin), FN1 (fibronectin) and POSTN (periostin). These genes showed a significant increase in expression during in vitro culture of GCs compared to the reference value. The description of increased expression of DCN in mural granulosa cells (MGCs) than cumulus granulosa cells (CGCs) may indicate involvement in ovulation. The effect of LH/hCG on the upregulation of this gene expression was also shown to be mediated through PKA, PKC, ERK/MEK, and PI3K pathways [53]. The role of DCN is to participate in cell proliferation, cell cycle regulation and apoptosis [54] and its expression has been described in human, goat, pig, and bovine granulosa cells [19,53–55]. Additionally, it may also be associated with cellular aging [56], folliculogenesis and ovulation [53]. Therefore, its role as a marker used in assisted reproduction biotechnologies is described [57]. On the other hand, the increased eCG-mediated DCN expression in goat granulosa may be disrupted by the tissue inhibitor of metalloproteinase 3 (TIMP3) present [58]. A role for DCN in steroidogenesis has also been suggested and demonstrated by resistin in vitro. Resistin is a protein for which one of the receptors is decorin, found in ovarian granulosa cells. It has been shown that the application of resistin can influence the regulation of steroidogenesis through Akt, MAPK, Stat-3, PPARγ, and NF-kB signaling pathways, leading to a decrease in estradiol and progesterone levels [59]. The DCN gene is associated with the TGF-β signaling pathway (Figure A1). This pathway indirectly affects cellular processes related to the cell cycle and apoptosis.

In turn, integrins are bound by various ECM components, including fibronectin (Figure A2). The type of this signaling interaction is referred to as input-output and involves the fibronectin receptor [60]. The fibronectin signaling pathway shows further association with MAPK/ERK, involved in the cell cycle (Figure A3). The FAK (focal adhesion kinase, Figure A4) signaling pathway engages FN1, an important component of the ECM, thereby mediating processes in the ovary, particularly ovulation, luteinization, and cumulus cell expansion [61]. Inhibition of the FAK pathway results in the arrest of ovulation, demonstrating the crucial role of the ECM in this process [62]. Fibronectin was shown to be involved in ovulation and luteinization of GCs, as it enhanced progesterone production in vitro, compared to collagen, which promoted estrogen synthesis [63]. Additionally, an increase in the expression of this gene in GCs of beef cattle after super-stimulation was demonstrated [64].

It is worth considering that the expression of this gene in the follicular fluid of lambs is lower than in sheep [65] indicating that the expression may depend on various factors related to the physiological status of the animal. It has been described that under the stimulation of hCG in bovine granulosa cells the expression of the POSTN gene increases and its role in fibrous tissue remodeling may induce ovulation [66]. This gene has also been shown to influence cell adhesion, migration, proliferation, differentiation, and survival [67]. Confirmation of the role of this gene in the aforementioned processes may be provided by the relationship between elevated POSTN levels and PCOS in humans [9]. The role of FN1 in PCOS is associated with effects on vasculature development within the ovary, which significantly affects folliculogenesis and corpus luteum formation [10].

Another glycoprotein showing roles in ECM remodeling is CHI3L1 (chitinase-3-like protein 1 CHI3L1) gene, which also showed up-regulation during conducted culture. Its high expression level is an indicator of poor prognosis for ovarian cancer patients [68] and can be used as a goal for targeted therapy [69]. Summarizing the described glycoproteins and their role in ovarian processes, it should be pointed out that they are mainly responsible for ovulation. Although additionally their role in steroidogenesis has been demonstrated.

It is also important that the above ECM elements are associated with the occurrence of PCOS. These data and our results show that not only the glycoproteins themselves but also their association with integrins and their participation in signaling pathways are important in the assessment of their role in this disorder. Demonstration of the expression levels of these genes in the context of their interactions may be a basis for studies related to the therapy of, among others, PCOS and their strong upregulation within GCs and association with ovulation may be used in reproductive biotechnologies based on in vitro studies. This suggests that further research is needed in this direction, but gives hope that glycoproteins may be a target for therapy and use in assisted reproductive techniques.

Not only the ECM glycoproteins mentioned above are involved in these processes within the ovary. CAV1 (caveolin-1) protein is strictly responsible for the composition of the extracellular matrix. It is involved in the formation of exosomes and sorting of protein components of the extracellular matrix as well as their deposition [70]. Thus, it leads to changes in ECM composition and, consequently, its function. CAV1 gene expression in bovine granulosa cells and its role in the process of ovulation and luteinization have been demonstrated [71]. Given the role of the CAV-1 gene in extracellular matrix formation, it has been shown that in vitro culture its expression is affected by anethole. It was observed that anethole, as a natural substance with antioxidant properties, influences the maturation of goat ovarian follicles [72]. It has also been shown that downregulation of CAV-1 gene expression in female ovaries pathway affects the Notch2 signaling and causes a decrease in Leucine-rich repeat containing G protein-coupled receptor 5 (Lgr5) leading to POI (premature ovarian insufficiency) [11]. Lgr5 protein affects cell proliferation, differentiation and is also a biomarker of adult stem cells [73]. Moreover, the IRS1 gene (insulin receptor substrate 1) protein, which showed downregulated expression in our study compared with the reference value, is associated with the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway and also PI3K/AKT (Figure A5) [74,75]. IRS1 affects cell metabolism regulation, survival, and apoptosis [76]. The association of its overexpression with GCs proliferation was demonstrated in PCOS (polycystic ovary syndrome) study, where it activated the MAPK/ERK pathway and promoted the disorder [77]. The MAPK/ERK pathway is also associated with ovulation, as it is completely inhibited in mice with MAPK1/3 deletion in GCs [78].

A group of widely distributed and important proteins are integrins, adhesion molecules, as transmembrane proteins are composed of α and β components [79]. ITGA is associated with ECM formation, ITGB plays one of the key roles in regulating intracellular signaling cascades (including FAK, AKT—Figures A4 and A5). With reference to carcinogenesis, in particular ovarian cancer, a role for ITGA2 in spheroid formation is described, thus promoting metastasis in the peritoneal cavity [80–82]. The PI3K/AKT pathway is also closely related to the FOXO family proteins (Figure A6), a member of the transcription factor class. The upregulated ITGA2 gene affects AKT phosphorylation [83], making it closely related to the FAK signaling pathways. Integrins bind to focal adhesion components to identify modifications in the ECM to keep the shape or reshape the cell [84,85]. Cadherin–integrin interactions play an important role, involving among others the Rap1 pathway [60], associated with GTP (Figure A7). Rap1 is involved in the regulation of integrin activity downstream of cadherins [86] and maintains cadherin connections [87]. ITGA2, ITGB3 genes play an important role in many signaling pathways. The upregulation of these genes has been previously described in porcine granulosa cells, and it was associated with their angiogenesis [55], development, morphogenesis [88], adhesion, tissue development [89], and in the case of ITGB3 and FN1 also with the process of apoptosis [56]. These three genes belong to ontology groups closely related to cell adhesion, formation, and binding of extracellular matrix components confirming the role of the ECM in the processes previously described related to granulosa cell proliferation [55,88–90]. Decreased expression of the genes mentioned above affects ECM and cell adhesion within the ovary, affecting the possibility of diseases within the ovary, including PCOS (polycystic ovary syndrome) in humans. It is important to consider the role of ECM in folliculogenesis and

ovulation [91]. Furthermore, in the current study, the down-regulated gene ABI3BP (ABI family member 3 binding protein), encoding an extracellular matrix protein that binds to integrins has been demonstrated. Studies on MSCs have shown that their activity is essential for osteogenic and adipogenic differentiation [92]. Through association with the fibronectin III domain, it promotes cell attachment and with the ECM [93].

One of the main components of lamina basalis is laminin, which binds to collagen. Increased expression of the laminin subunit beta 1 (LAMB1) gene, which belonged to the ontology group (GO: ECM structural constituent) is illustrated in Figure 1. The LAMB-1 gene has been described in ovarian follicles at different developmental stages and also in the corpus luteum in mice [18]. In addition, laminin protein can influence the shape and proliferation of GCs and also indirectly affect estradiol secretion in the antral follicles [63]. Thus highlighting the role of laminin, a basic component of lamina basalis in human folliculogenesis [94].

Another important component of the lamina basalis is collagen, which during the development of the ovarian follicle may change its conformation, amount, and proportion of isoforms [19]. In the present study, increased expression of PCOLCE2 (procollagen C-endopeptidase enhancer 2) and COL14A1 (collagen type XIV alpha chain) genes, which among others belong to the "collagen binding" ontology group was observed. Thus, up-regulation of the aforementioned genes indicates active modeling of ECM composition in primary in vitro culture of porcine GCs. Other ongoing in vitro studies have demonstrated increased expression of collagens (*COL1A2*, *COL3A1*, *COL5A2*, *COL12A1*, *COL15A1*, *COL6A3*) in the theca interna of the bovine ovarian follicle, leading to their consideration as cellular markers of this structure [95].

IGF1 (insulin-like growth factor 1) is one of the described regulators of folliculogenesis and oogenesis and is also involved in cell proliferation. Expression of this gene was the most strongly reduced during in vitro culture of porcine GCs relative to the reference value. The number of its receptors in the ovary changes depending on the presence of the corpus luteum and is therefore dependent on steroid hormones [96]. In turn, down-regulation of IGF1 is associated with apoptotic processes [97], and it has additionally been shown to increase apoptotic gene regulation through activation of PI3K/AKT (Figure A4) in bovine granulosa cells [98]. Another gene with reduced expression is SPP1 (secreted phosphoprotein 1), which was shown in bovine GCs where its expression level in the largest follicles (>10.7 mm) was lower than in smaller follicles (>7.8 mm) [99]. This demonstrates that the expression profile of follicle stage-specific genes is altered and is associated with development or atresia. This gene is otherwise known as osteopontin (OPN) and is associated with immune processes, particularly during ovarian follicular atresia [100]. It also plays an important role in wound healing processes, as it is responsible for cell adhesion to the ECM through the integrin-binding sequence [101].

The down-regulated PAICS gene (phosphoribosylaminoimidazole carboxylase and phosphoribosylaminoimidazolesuccinocarboxamide synthase) encodes an enzyme that catalyzes steps 6 and 7 of the de novo purine biosynthesis pathway [102] and was indicated in the corpus luteum in cattle. Its expression level was shown to be higher in the ovary than in skeletal muscle, indicating that these tissues require high levels of purines for the normal proliferation of GCs [103].

Both physical and functional interactions between particular genes are confirmed by STRING interaction (Figure 7). Interactions are mainly formed by genes closely related to extracellular matrix formation. These connections occur mostly between upregulated genes, although they also occur between downregulated genes IGF1, IRS1, SPP1. The gene showing the highest number of interactions was FN1, which by binding to genes encoding extracellular matrix glycoproteins confirms its participation in transmitting intercellular signaling. In addition, the strongly expressed interaction of FN1 to integrins ITGA2 and ITGB3 (and between) confirms the activity of these genes and their influence on the numerous signaling pathways described above. Also noteworthy is the connection between the DCN and IGF1 genes and between SPP1 and ITGB3. These genes, among

others, are associated with apoptosis processes and may have a strong influence on changes within granulosa cells leading to ovulation, which may confirm their involvement in this process. Other genes that do not show direct interaction on STRING interaction may show it indirectly, therefore, their function in ovarian processes cannot be excluded.

5. Conclusions

The incompletely understood molecular basis of processes within the ovarian follicle leading to ovulation and luteinization requires precise determination of the gene expression profile within, among others, granulosa cells. Therefore, the results can be broadly applied not only to understand the physiology of the ovary but also to study pathological processes and assisted reproduction techniques. In addition, compared to in vivo studies, in vitro methods are less expensive, easier to perform, and do not require the approval of ethical committees if the material used in the study comes from commercially slaughtered animals. The expression profile of genes in porcine granulosa cells cultured in vitro, associated with cadherin and collagen binding and structuralization of extracellular matrix (ECM) components were carefully evaluated, and the culture of GCs in vitro showed increased expression of genes highly responsible for extracellular matrix (ECM) formation. ECM is involved in the regulation of many processes within the ovarian follicle, including its development, maturation, ovulation, and corpus luteum formation. The pig has been used as a model in human medicine, so the results of this study may be valuable in determining pathological mechanisms within the ovary, including finding targets for clinical therapy in humans. Of special attention is the description of the expression levels of genes such as POSTN, CHI3L1, CAV-1, IRS1, DCN, which according to literature data are associated with diseases such as POI, PCOS, and ovarian cancer. The current and previously demonstrated increased expression of POSTN, FN1, ITGB3, ITGA2, LAMB1, DCN genes in granulosa cells, associated with the processes of growth, proliferation, maturation and differentiation, may become potential new molecular markers of proliferation of these cells in vitro what can be valuable in assisted animal reproduction techniques. Additionally, the described expression profile of genes related to extracellular matrix structuralization, which plays an important role in stem cell differentiation and regeneration processes, may provide important knowledge in this aspect. Signaling pathways and interactions associated with the described genes demonstrate the multidirectional role of the ECM in both spatial structure formation, intercellular signaling describing even more fully the role of selected genes.

Author Contributions: Conceptualization, B.K. and P.A.; methodology, W.K. and H.P.-K., M.J.; software, K.J. and M.Z.; validation, K.J., M.Z. and D.B.; formal analysis, J.K., M.K. and K.J.; investigation, J.K., M.K. and W.K.; resources, D.B., P.A. and J.M.J.; data curation, P.M., H.P-K. and D.B.; writing—original draft preparation, J.K. and M.K.; writing—review and editing, P.M. and W.K.; visualization, K.J. and M.J.; supervision, B.K., J.M.J. and P.M.; project administration, B.K.; funding acquisition, P.A. and D.B. All authors have read and agreed to the published version of the manuscript.

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Appendix A

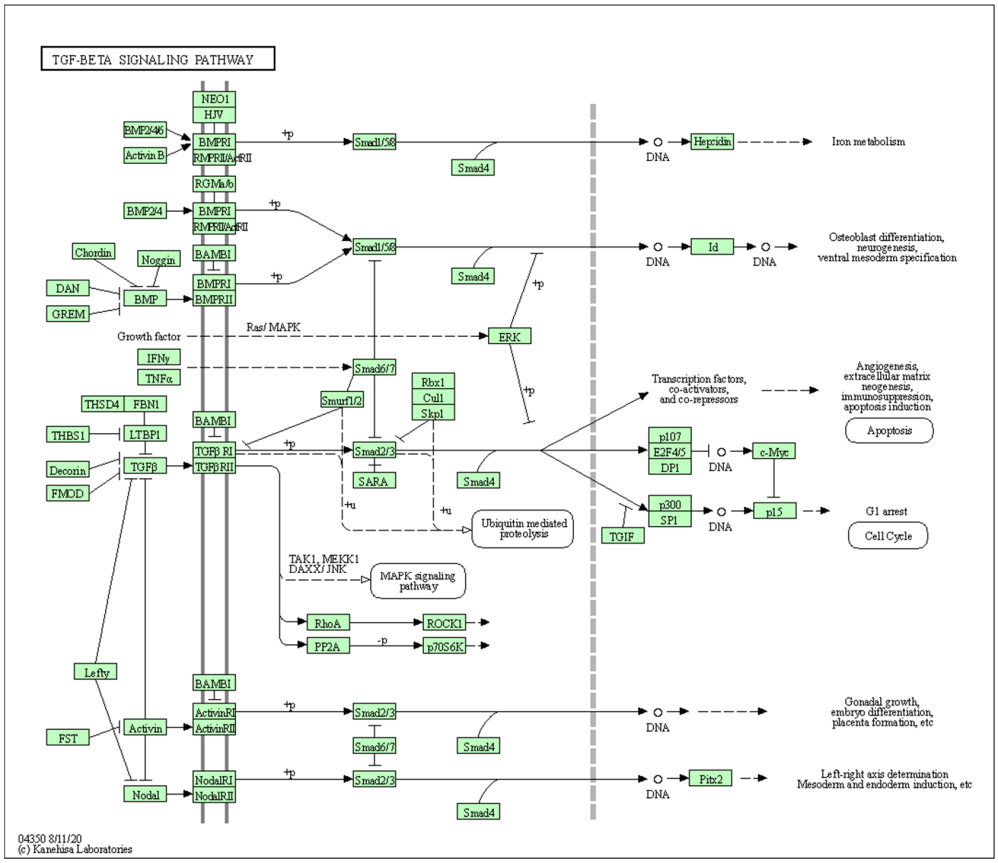

Figure A1. TGF-β signaling pathway, KEGG pathway. KEGG, Kyoto Encyclopedia of Genes and Genomes. Copyright permission obtained.

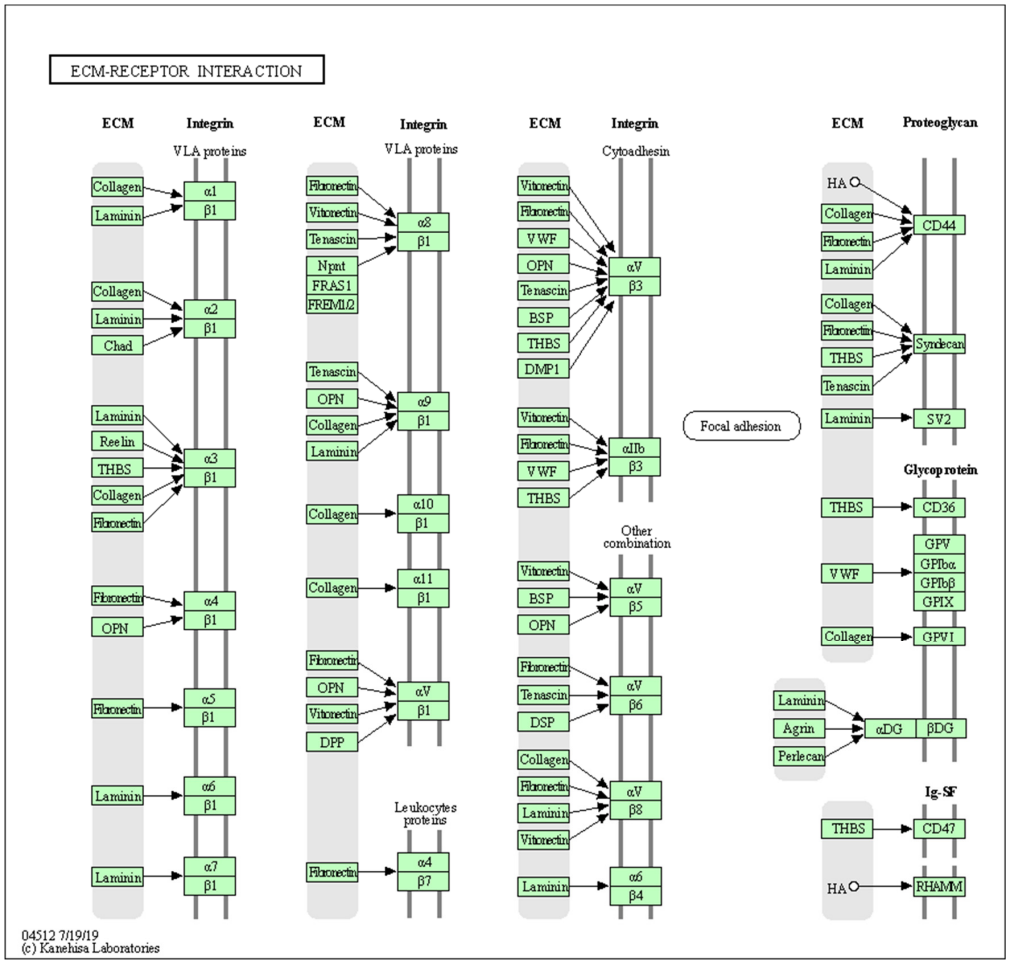

Figure A2. ECM-receptor interaction signaling pathway, KEGG pathway. KEGG, Kyoto Encyclopedia of Genes and Genomes. Copyright permission obtained.

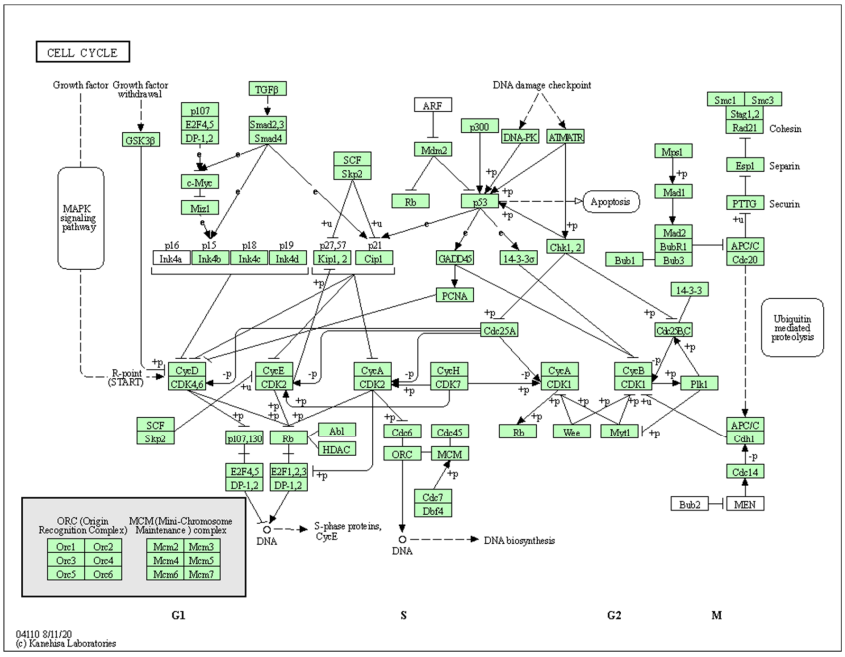

Figure A3. Cell cycle signaling pathway, KEGG pathway. KEGG, Kyoto Encyclopedia of Genes and Genomes. Copyright permission obtained.

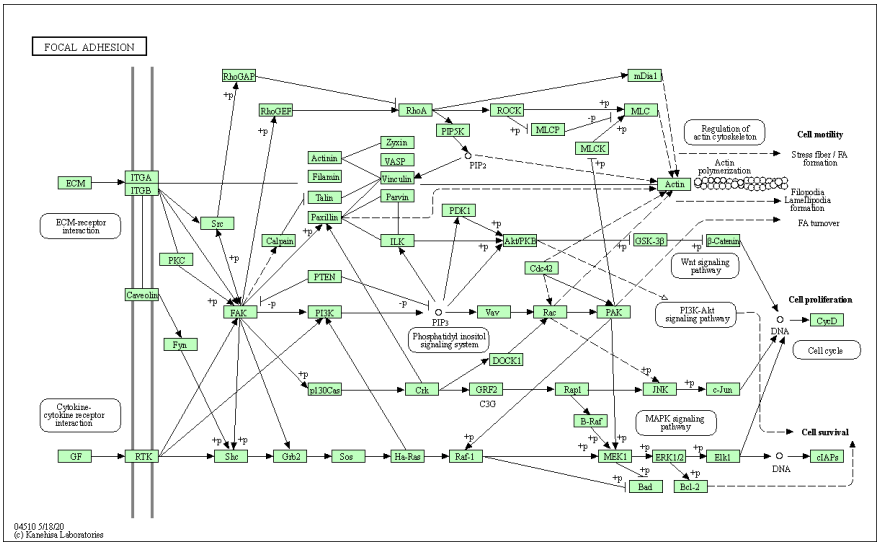

Figure A4. Focal adhesion signaling pathway, KEGG pathway. KEGG, Kyoto Encyclopedia of Genes and Genomes. Copyright permission obtained.

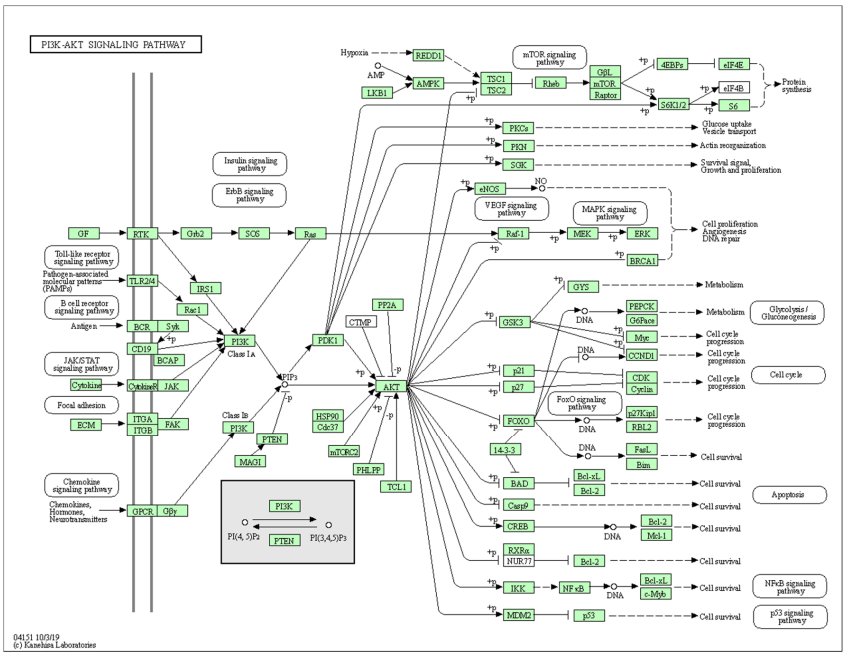

Figure A5. PI3K-AKT signaling pathway, KEGG pathway. KEGG, Kyoto Encyclopedia of Genes and Genomes. Copyright permission obtained.

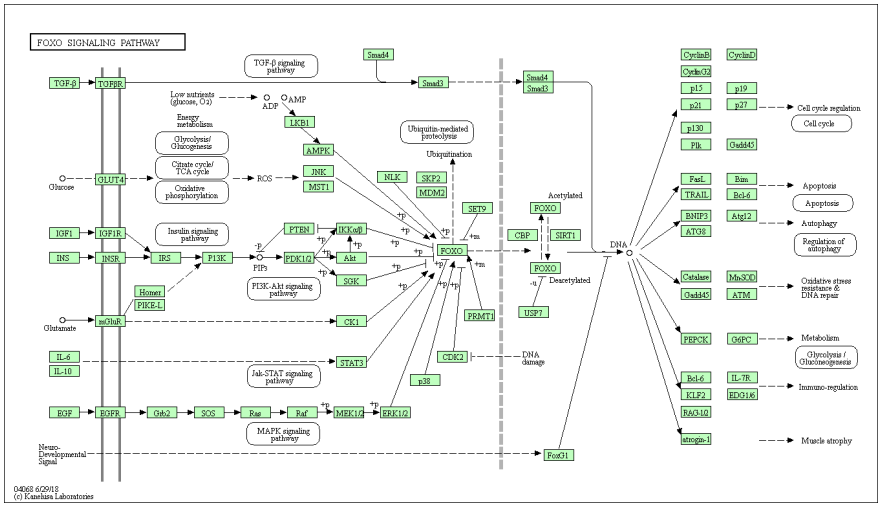

Figure A6. FOXO signaling pathway, KEGG pathway. KEGG, Kyoto Encyclopedia of Genes and Genomes. Copyright permission obtained.

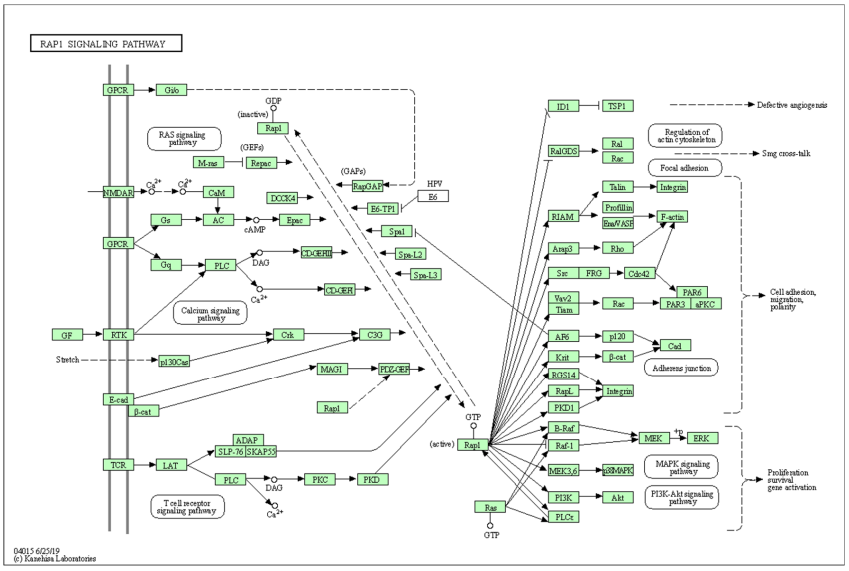

Figure A7. RAP1 signaling pathway, KEGG pathway. KEGG, Kyoto Encyclopedia of Genes and Genomes. Copyright permission obtained.

References

1. Walma, D.A.C.; Yamada, K.M. The extracellular matrix in development. *Development* **2020**, *147*, dev175596. [CrossRef] [PubMed]
2. Hynes, R.O. The extracellular matrix: Not just pretty fibrils. *Science* **2009**, *326*, 1216–1219. [CrossRef] [PubMed]
3. Frantz, C.; Stewart, K.M.; Weaver, V.M. The extracellular matrix at a glance. *J. Cell Sci.* **2010**, *123*, 4195–4200. [CrossRef] [PubMed]
4. Mouw, J.K.; Ou, G.; Weaver, V.M. Extracellular matrix assembly: A multiscale deconstruction. *Nat. Rev. Mol. Cell Biol.* **2014**, *15*, 771–785. [CrossRef]
5. Mecham, R.P. Overview of Extracellular Matrix. *Curr. Protoc. Cell Biol.* **2012**, *57*, 10.1.1–10.1.16. [CrossRef]
6. Rodgers, R.J.; Irving-Rodgers, H.F.; Van Wezel, I.L. Extracellular matrix in ovarian follicles. *Mol. Cell Endocrinol.* **2000**, *163*, 73–79. [CrossRef]
7. Cho, A.; Howell, V.M.; Colvin, E.K. The extracellular matrix in epithelial ovarian cancer—A piece of a puzzle. *Front. Oncol.* **2015**, *5*, 245. [CrossRef]
8. Xiang, S.; Xia, M.-f.; Song, J.-y.; Liu, D.-q.; Lian, F. Effect of Electro-acupuncture on Expression of IRS-1/PI3K/GLUT4 Pathway in Ovarian Granulosa Cells of Infertile Patients with Polycystic Ovary Syndrome-Insulin Resistance of Phlegm-Dampness Syndrome. *Chin. J. Integr. Med.* **2020**, *27*, 330–335. [CrossRef]
9. Chen, X.; Huo, L.; Ren, L.; Li, Y.; Sun, Y.; Li, Y.; Zhang, P.; Chen, S.; Song, G.Y. Polycystic Ovary Syndrome is Associated with Elevated Periostin Levels. *Exp. Clin. Endocrinol. Diabetes* **2019**, *127*, 571–577. [CrossRef]
10. Patil, K.; Hinduja, I.; Mukherjee, S. Alteration in angiogenic potential of granulosa-lutein cells and follicular fluid contributes to luteal defects in polycystic ovary syndrome. *Hum. Reprod.* **2021**, *36*, 1052–1064. [CrossRef]
11. Huang, K.; Dang, Y.; Zhang, P.; Shen, C.; Sui, X.; Xia, G.; Qin, Y.; Jiao, X.; Wang, C.; Huo, R.; et al. CAV1 regulates primordial follicle formation via the Notch2 signalling pathway and is associated with premature ovarian insufficiency in humans. *Hum. Reprod.* **2018**, *33*, 087–2095. [CrossRef]
12. Henning, N.F.; LeDuc, R.D.; Even, K.A.; Laronda, M.M. Proteomic analyses of decellularized porcine ovaries identified new matrisome proteins and spatial differences across and within ovarian compartments. *Sci. Rep.* **2019**, *9*, 20001. [CrossRef] [PubMed]
13. Zhao, Y.; Feng, H.; Zhang, Y.; Zhang, J.V.; Wang, X.; Liu, D. Current Understandings of Core Pathways for the Activation of Mammalian Primordial Follicles. *Cells* **2021**, *10*, 1491. [CrossRef] [PubMed]
14. Kinnear, H.M.; Tomaszewski, C.E.; Chang, F.L.; Moravek, M.B.; Xu, M.; Padmanabhan, V.; Shikanov, A. The ovarian stroma as a new frontier. *Reproduction* **2020**, *160*, R25–R39. [CrossRef] [PubMed]
15. Woodruff, T.K.; Shea, L.D. The Role of the Extracellular Matrix in Ovarian Follicle Development. *Reprod. Sci.* **2007**, *14*, 6–10. [CrossRef] [PubMed]
16. Van Wezel, I.L.; Rodgers, H.F.; Rodgers, R.J. Differential localization of laminin chains in bovine follicles. *J. Reprod. Fertil.* **1998**, *112*, 267–278. [CrossRef]

17. Hatzirodos, N.; Irving-Rodgers, H.F.; Hummitzsch, K.; Harland, M.L.; Morris, S.E.; Rodgers, R.J. Transcriptome profiling of granulosa cells of bovine ovarian follicles during growth from small to large antral sizes. *BMC Genom.* **2014**, *15*, 24. [CrossRef] [PubMed]
18. Irving-Rodgers, H.F.; Hummitzsch, K.; Murdiyarso, L.S.; Bonner, W.M.; Sado, Y.; Ninomiya, Y.; Couchman, J.R.; Sorokin, L.M.; Rodgers, R.J. Dynamics of extracellular matrix in ovarian follicles and corpora lutea of mice. *Cell Tissue Res.* **2010**, *339*, 613–624. [CrossRef]
19. Rodgers, R.J.; Irving-Rodgers, H.F.; Russell, D.L. Extracellular matrix of the developing ovarian follicle. *Reproduction* **2003**, *126*, 415–424. [CrossRef]
20. Irving-Rodgers, H.F.; Rodgers, R.J. Extracellular matrix of the developing ovarian follicle. *Semin. Reprod. Med.* **2006**, *24*, 195–203. [CrossRef]
21. Berkholtz, C.B.; Shea, L.D.; Woodruff, T.K. Extracellular matrix functions in follicle maturation. *Semin. Reprod. Med.* **2006**, *24*, 262–269. [CrossRef] [PubMed]
22. Piprek, R.P.; Kloc, M.; Mizia, P.; Kubiak, J.Z. The central role of cadherins in gonad development, reproduction, and fertility. *Int. J. Mol. Sci.* **2020**, *21*, 8264. [CrossRef] [PubMed]
23. Sueoka, K.; Kuji, N.; Shiokawa, S.; Tanaka, M.; Miyazaki, T.; Yoshimura, Y. Integrins and reproductive physiology: Expression and modulation in fertilization, embryogenesis, and implantation. *Fertil. Steril.* **1997**, *67*, 799–811. [CrossRef]
24. Bronson, R.A.; Fusi, F.M. Integrins and human reproduction. *Mol. Hum. Reprod.* **1996**, *2*, 153–168. [CrossRef] [PubMed]
25. Weber, G.F.; Bjerke, M.A.; DeSimone, D.W. Integrins and cadherins join forces to form adhesive networks. *J. Cell Sci.* **2011**, *124*, 1183–1193. [CrossRef]
26. Mui, K.L.; Chen, C.S.; Assoian, R.K. The mechanical regulation of integrin-cadherin crosstalk organizes cells, signaling and forces. *J. Cell Sci.* **2016**, *129*, 1093–1100. [CrossRef]
27. Ryan, P.L.; Valentine, A.F.; Bagnell, C.A. Expression of epithelial cadherin in the developing and adult pig ovary. *Biol. Reprod.* **1996**, *55*, 1091–1097. [CrossRef]
28. Yan, H.; Wen, J.; Zhang, T.; Zheng, W.; He, M.; Huang, K.; Guo, Q.; Chen, Q.; Yang, Y.; Deng, G.; et al. Oocyte-derived E-cadherin acts as a multiple functional factor maintaining the primordial follicle pool in mice. *Cell Death Dis.* **2019**, *10*, 160. [CrossRef]
29. Caballero, J.N.; Gervasi, M.G.; Veiga, M.F.; Dalvit, G.C.; Perez-Martínez, S.; Cetica, P.D.; Vazquez-Levin, M.H. Epithelial cadherin is present in bovine oviduct epithelial cells and gametes, and is involved in fertilization-related events. *Theriogenology* **2014**, *81*, 1189–1206. [CrossRef]
30. Kossowska-Tomaszczuk, K.; De Geyter, C. Cells with stem cell characteristics in somatic compartments of the ovary. *Biomed. Res. Int.* **2013**, *2013*, 310859. [CrossRef]
31. Kossowska-Tomaszczuk, K.; De Geyter, C.; De Geyter, M.; Martin, I.; Holzgreve, W.; Scherberich, A.; Zhang, H. The Multipotency of Luteinizing Granulosa Cells Collected from Mature Ovarian Follicles. *Stem Cells* **2009**, *27*, 210–219. [CrossRef] [PubMed]
32. Brevini, T.A.L.; Pennarossa, G.; Rahman, M.M.; Paffoni, A.; Antonini, S.; Ragni, G.; DeEguileor, M.; Tettamanti, G.; Gandolfi, F. Morphological and Molecular Changes of Human Granulosa Cells Exposed to 5-Azacytidine and Addressed Toward Muscular Differentiation. *Stem Cell Rev. Rep.* **2014**, *10*, 633–642. [CrossRef]
33. Józkowiak, M.; Hutchings, G.; Jankowski, M.; Kulcenty, K.; Mozdziak, P.; Kempisty, B.; Spaczyński, R.Z.; Piotrowska-Kempisty, H. The Stemness of Human Ovarian Granulosa Cells and the Role of Resveratrol in the Differentiation of MSCs-A Review Based on Cellular and Molecular Knowledge. *Cells* **2020**, *9*, 1418. [CrossRef] [PubMed]
34. Mattioli, M.; Gloria, A.; Turriani, M.; Berardinelli, P.; Russo, V.; Nardinocchi, D.; Curini, V.; Baratta, M.; Martignani, E.; Barboni, B. Osteo-regenerative potential of ovarian granulosa cells: An in vitro and in vivo study. *Theriogenology* **2012**, *77*, 1425–1437. [CrossRef]
35. Oki, Y.; Ono, H.; Motohashi, T.; Sugiura, N.; Nobusue, H.; Kano, K. Dedifferentiated follicular granulosa cells derived from pig ovary can transdifferentiate into osteoblasts. *Biochem. J.* **2012**, *447*, 239–248. [CrossRef]
36. Rybska, M.; Knap, S.; Jankowski, M.; Jeseta, M.; Bukowska, D.; Antosik, P.; Nowicki, M.; Zabel, M.; Kempisty, B.; Jaśkowski, J.M. Characteristic of factors influencing the proper course of folliculogenesis in mammals. *Med. J. Cell Biol.* **2018**, *6*, 33–38. [CrossRef]
37. Chamier-Gliszczyńska, A.; Kałuzna, S.; Stefańska, K.; Celichowski, P.; Antosik, P.; Bukowska, D.; Bruska, M.; Zakova, J.; Machatkova, M.; Jeseta, M.; et al. Analysis of expression of genes responsible for regulation of cellular proliferation and migration-Microarray approach based on porcine oocyte model. *Med. J. Cell Biol.* **2019**, *7*, 48–57. [CrossRef]
38. Nguyen, T.; Lee, S.; Hatzirodos, N.; Hummitzsch, K.; Sullivan, T.R.; Rodgers, R.J.; Irving-Rodgers, H.F. Spatial differences within the membrana granulosa in the expression of focimatrix and steroidogenic capacity. *Mol. Cell Endocrinol.* **2012**, *363*, 62–73. [CrossRef]
39. Mushahary, D.; Spittler, A.; Kasper, C.; Weber, V.; Charwat, V. Isolation, cultivation, and characterization of human mesenchymal stem cells. *Cytom. Part A* **2018**, *93*, 19–31. [CrossRef]
40. Hendijani, F. Explant culture: An advantageous method for isolation of mesenchymal stem cells from human tissues. *Cell Prolif.* **2017**, *50*, e12334. [CrossRef]
41. Stefańska, K.; Sibiak, R.; Hutchings, G.; Dompe, C.; Moncrieff, L.; Janowicz, K.; Jeseta, M.; Kempisty, B.; Machatkova, M.; Mozdziak, P. Evidence for existence of molecular stemness markers in porcine ovarian follicular granulosa cells. *Med. J. Cell Biol.* **2019**, *7*, 183–188. [CrossRef]
42. Moncrieff, L.; Mozdziak, P.; Jeseta, M.; Machatkova, M.; Kranc, W.; Kempisty, B. Ovarian follicular cells—Living in the shadow of stemness cellular competence. *Med. J. Cell Biol.* **2019**, *7*, 134–140. [CrossRef]
43. Trejter, M.; Hochol, A.; Tyczewska, M.; Ziolkowska, A.; Jopek, K.; Szyszka, M.; Malendowicz, L.K.; Rucinski, M. Sex-related gene expression profiles in the adrenal cortex in the mature rat: Microarray analysis with emphasis on genes involved in steroidogenesis. *Int. J. Mol. Med.* **2015**, *35*, 702–714. [CrossRef] [PubMed]

44. Chamier-Gliszczyńska, A.; Brązert, M.; Sujka-Kordowska, P.; Popis, M.; Ożegowska, K.; Stefańska, K.; Kocherova, I.; Celichowski, P.; Kulus, M.; Bukowska, D.; et al. Genes involved in angiogenesis and circulatory system development are differentially expressed in porcine epithelial oviductal cells during long-term primary in vitro culture—A transcriptomic study. *Med. J. Cell Biol.* **2018**, *6*, 163–173. [CrossRef]
45. Nawrocki, M.J.; Celichowski, P.; Jankowski, M.; Kranc, W.; Bryja, A.; Borys-Wójcik, S.; Jeseta, M.; Antosik, P.; Bukowska, D.; Bruska, M.; et al. Ontology groups representing angiogenesis and blood vessels development are highly up-regulated during porcine oviductal epithelial cells long-term real-time proliferation—A primary cell culture approach. *Med. J. Cell Biol.* **2018**, *6*, 186–194. [CrossRef]
46. Stefańska, K.; Chamier-Gliszczyńska, A.; Jankowski, M.; Celichowski, P.; Kulus, M.; Rojewska, M.; Antosik, P.; Bukowska, D.; Bruska, M.; Nowicki, M.; et al. Epithelium morphogenesis and oviduct development are regulated by significant increase of expression of genes after long-term in vitro primary culture—A microarray assays. *Med. J. Cell Biol.* **2018**, *6*, 195–204. [CrossRef]
47. Kranc, W.; Brązert, M.; Ożegowska, K.; Budna-Tukan, J.; Celichowski, P.; Jankowski, M.; Bryja, A.; Nawrocki, M.J.; Popis, M.; Jeseta, M.; et al. Response to abiotic and organic substances stimulation belongs to ontologic groups significantly up-regulated in porcine immature oocytes. *Med. J. Cell Biol.* **2018**, *6*, 91–100. [CrossRef]
48. Borys-Wójcik, S.; Kocherova, I.; Celichowski, P.; Popis, M.; Jeseta, M.; Bukowska, D.; Antosik, P.; Nowicki, M.; Kempisty, B. Protein oligomerization is the biochemical process highly up-regulated in porcine oocytes before in vitro maturation (IVM). *Med. J. Cell Biol.* **2018**, *6*, 155–162. [CrossRef]
49. Budna, J.; Celichowski, P.; Bryja, A.; Jeseta, M.; Jankowski, M.; Bukowska, D.; Antosik, P.; Nowicki, A.; Brüssow, K.P.; Bruska, M.; et al. Expression Changes in Fatty acid Metabolic Processrelated Genes in Porcine Oocytes During in Vitro Maturation. *Med. J. Cell Biol.* **2018**, *6*, 48–54. [CrossRef]
50. Walter, W.; Sánchez-Cabo, F.; Ricote, M. GOplot: An R package for visually combining expression data with functional analysis. *Bioinformatics* **2015**, *31*, 2912–2914. [CrossRef]
51. Yu, G.; Wang, L.G.; Han, Y.; He, Q.Y. ClusterProfiler: An R package for comparing biological themes among gene clusters. *OMICS J. Integr. Biol.* **2012**, *16*, 284–287. [CrossRef] [PubMed]
52. Onodera, T.; Sakai, T.; Hsu, J.C.F.; Matsumoto, K.; Chiorini, J.A.; Yamada, K.M. Btbd7 regulates epithelial cell dynamics and branching morphogenesis. *Science* **2010**, *329*, 562–565. [CrossRef] [PubMed]
53. Kedem, A.; Ulanenko-Shenkar, K.; Yung, Y.; Yerushalmi, G.M.; Maman, E.; Hourvitz, A. Elucidating Decorin's role in the preovulatory follicle. *J. Ovarian Res.* **2020**, *13*, 15. [CrossRef]
54. Peng, J.Y.; Gao, K.X.; Xin, H.Y.; Han, P.; Zhu, G.Q.; Cao, B.Y. Molecular cloning, expression analysis, and function of decorin in goat ovarian granulosa cells. *Domest. Anim. Endocrinol.* **2016**, *57*, 108–116. [CrossRef]
55. Chermuła, B.; Brazert, M.; Izycki, D.; Ciesiółka, S.; Kranc, W.; Celichowski, P.; Ozegowska, K.; Nawrocki, M.J.; Jankowski, M.; Jeseta, M.; et al. New Gene Markers of Angiogenesis and Blood Vessels Development in Porcine Ovarian Granulosa Cells during Short-Term Primary Culture in Vitro. *Biomed. Res. Int.* **2019**, *2019*, 6545210. [CrossRef]
56. Kulus, M.; Kranc, W.; Sujka-Kordowska, P.; Mozdziak, P.; Jankowski, M.; Konwerska, A.; Kulus, J.; Bukowska, D.; Skowroński, M.; Piotrowska-Kempisty, H.; et al. The processes of cellular growth, aging, and programmed cell death are involved in lifespan of ovarian granulosa cells during short-term IVC—Study based on animal model. *Theriogenology* **2020**, *148*, 76–88. [CrossRef]
57. Sawada, Y.; Sato, T.; Saito, C.; Ozawa, F.; Ozaki, Y.; Sugiura-Ogasawara, M. Clinical utility of decorin in follicular fluid as a biomarker of oocyte potential. *Reprod. Biol.* **2018**, *18*, 33–39. [CrossRef] [PubMed]
58. Peng, J.; Xin, H.; Han, P.; Gao, K.; Gao, T.; Lei, Y.; Ji, S.; An, X.; Cao, B. Expression and regulative function of tissue inhibitor of metalloproteinase 3 in the goat ovary and its role in cultured granulosa cells. *Mol. Cell Endocrinol.* **2015**, *412*, 104–115. [CrossRef]
59. Messini, C.I.; Vasilaki, A.; Korona, E.; Anifandis, G.; Georgoulias, P.; Dafopoulos, K.; Garas, A.; Daponte, A.; Messinis, I.E. Effect of resistin on estradiol and progesterone secretion from human luteinized granulosa cells in culture. *Syst. Biol. Reprod. Med.* **2019**, *65*, 350–356. [CrossRef]
60. Balzac, F.; Avolio, M.; Degani, S.; Kaverina, I.; Torti, M.; Silengo, L.; Small, J.V.; Retta, S.F. E-cadherin endocytosis regulates the activity of Rap1: A traffic light GTPase at the crossroads between cadherin and integrin function. *J. Cell Sci.* **2005**, *118*, 4765–4783. [CrossRef]
61. Asem, E.K.; Carnegie, J.A.; Tsang, B.K. Fibronectin production by chicken granulosa cells in vitro: Effect of follicular development. *Eur. J. Endocrinol.* **1992**, *127*, 466–470. [CrossRef] [PubMed]
62. Kitasaka, H.; Kawai, T.; Hoque, S.A.M.; Umehara, T.; Fujita, Y.; Shimada, M. Inductions of granulosa cell luteinization and cumulus expansion are dependent on the fibronectin-integrin pathway during ovulation process in mice. *PLoS ONE* **2018**, *13*, e0192458. [CrossRef] [PubMed]
63. Huet, C.; Pisselet, C.; Mandon-Pépin, B.; Monget, P.; Monniaux, D. Extracellular matrix regulates ovine granulosa cell survival, proliferation and steroidogenesis: Relationships between cell shape and function. *J. Endocrinol.* **2001**, *169*, 347–360. [CrossRef]
64. Dias, F.C.F.; Khan, M.I.R.; Sirard, M.A.; Adams, G.P.; Singh, J. Differential gene expression of granulosa cells after ovarian superstimulation in beef cattle. *Reproduction* **2013**, *146*, 181–191. [CrossRef]
65. Wu, Y.; Lin, J.; Han, B.; Wang, L.; Chen, Y.; Liu, M.; Huang, J. Proteomic profiling of follicle fluids after superstimulation in one-month-old lambs. *Reprod. Domest. Anim.* **2018**, *53*, 186–194. [CrossRef] [PubMed]
66. Lussier, J.G.; Diouf, M.N.; Lévesque, V.; Sirois, J.; Ndiaye, K. Gene expression profiling of upregulated mRNAs in granulosa cells of bovine ovulatory follicles following stimulation with hCG. *Reprod. Biol. Endocrinol.* **2017**, *15*, 88. [CrossRef] [PubMed]

67. Kudo, A. Periostin in fibrillogenesis for tissue regeneration: Periostin actions inside and outside the cell. *Cell Mol. Life Sci.* **2011**, *68*, 3201–3207. [CrossRef] [PubMed]
68. Lin, H.-W.; Chiang, Y.-C.; Sun, N.-Y.; Chen, Y.-L.; Chang, C.-F.; Tai, Y.-J.; Chen, C.-A.; Cheng, W.-F. CHI3L1 results in poor outcome of ovarian cancer by promoting properties of stem-like cells. *Endocr.-Relat. Cancer* **2019**, *26*, 73–88. [CrossRef]
69. Zhao, T.; Su, Z.; Li, Y.; Zhang, X.; You, Q. Chitinase-3 like-protein-1 function and its role in diseases. *Signal Transduct. Target. Ther.* **2020**, *5*, 201. [CrossRef]
70. Albacete-Albacete, L.; Navarro-Lérida, I.; López, J.A.; Martín-Padura, I.; Astudillo, A.M.; Ferrarini, A.; Van-Der-Heyden, M.; Balsinde, J.; Orend, G.; Vázquez, J.; et al. ECM deposition is driven by caveolin-1–dependent regulation of exosomal biogenesis and cargo sorting. *J. Cell Biol.* **2020**, *219*, e202006178. [CrossRef]
71. Diouf, M.N.; Lefebvre, R.; Silversides, D.W.; Sirois, J.; Lussier, J.G. Induction of alpha-caveolin-1 (αCAV1) expression in bovine granulosa cells in response to an ovulatory dose of human chorionic gonadotropin. *Mol. Reprod. Dev.* **2006**, *73*, 1353–1360. [CrossRef]
72. Sá, N.A.R.; Araújo, V.R.; Correia, H.H.V.; Ferreira, A.C.A.; Guerreiro, D.D.; Sampaio, A.M.; Escobar, E.; Santos, F.W.; Moura, A.A.; Lôbo, C.H.; et al. Anethole improves the in vitro development of isolated caprine secondary follicles. *Theriogenology* **2017**, *89*, 226–234. [CrossRef] [PubMed]
73. Xu, L.; Lin, W.; Wen, L.; Li, G. Lgr5 in cancer biology: Functional identification of Lgr5 in cancer progression and potential opportunities for novel therapy. *Stem Cell Res. Ther.* **2019**, *10*, 219. [CrossRef] [PubMed]
74. Kayampilly, P.P.; Menon, K.M.J. Dihydrotestosterone inhibits insulin-stimulated cyclin D2 messenger ribonucleic acid expression in rat ovarian granulosa cells by reducing the phosphorylation of insulin receptor substrate-1. *Endocrinology* **2006**, *147*, 464–471. [CrossRef]
75. Corbould, A.; Kim, Y.B.; Youngren, J.F.; Pender, C.; Kahn, B.B.; Lee, A.; Dunaif, A. Insulin resistance in the skeletal muscle of women with PCOS involves intrinsic and acquired defects in insulin signaling. *Am. J. Physiol. Endocrinol. Metab.* **2005**, *288*, E1047–E1054. [CrossRef] [PubMed]
76. Niu, P.X.; Huang, Z.; Li, C.C.; Fan, B.; Li, K.; Liu, B.; Yu, M.; Zhao, S.H. Cloning, chromosomal localization, SNP detection and association analysis of the porcine IRS-1 gene. *Mol. Biol. Rep.* **2009**, *36*, 2087–2092. [CrossRef]
77. Cai, G.; Ma, X.; Chen, B.; Huang, Y.; Liu, S.; Yang, H.; Zou, W. MicroRNA-145 Negatively Regulates Cell Proliferation Through Targeting IRS1 in Isolated Ovarian Granulosa Cells from Patients with Polycystic Ovary Syndrome. *Reprod. Sci.* **2017**, *24*, 902–910. [CrossRef]
78. Fan, H.Y.; Liu, Z.; Shimada, M.; Sterneck, E.; Johnson, P.F.; Hedrick, S.M.; Richards, J.S. MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility. *Science* **2009**, *324*, 938–941. [CrossRef]
79. Buck, C.A.; Horwitz, A.F. Integrin, a transmembrane glycoprotein complex mediating cell-substratum adhesion. *J. Cell Sci. Suppl.* **1987**, *1987*, 231–250. [CrossRef]
80. Parise, L.V.; Lee, J.W.; Juliano, R.L. New aspects of integrin signaling in cancer. *Semin. Cancer Biol.* **2000**, *10*, 407–414. [CrossRef]
81. Hood, J.D.; Cheresh, D.A. Role of integrins in cell invasion and migration. *Nat. Rev. Cancer* **2002**, *2*, 91–100. [CrossRef]
82. Shield, K.; Riley, C.; Quinn, M.A.; Rice, G.E.; Ackland, M.L.; Ahmed, N. α2β1 integrin affects metastatic potential of ovarian carcinoma spheroids by supporting disaggregation and proteolysis. *J. Carcinog.* **2007**, *6*, 11. [CrossRef] [PubMed]
83. Ma, L.; Sun, Y.; Li, D.; Li, H.; Jin, X.; Ren, D. Overexpressed ITGA2 contributes to paclitaxel resistance by ovarian cancer cells through the activation of the AKT/FoxO1 pathway. *Aging* **2020**, *12*, 5336–5351. [CrossRef] [PubMed]
84. Clark, E.A.; Brugge, J.S. Integrins and signal transduction pathways: The road taken. *Science* **1995**, *268*, 233–239. [CrossRef]
85. Giancotti, F.G.; Ruoslahti, E. Integrin signaling. *Science* **1999**, *285*, 1028–1032. [CrossRef]
86. Retta, S.F.; Balzac, F.; Avolio, M. Rap1: A turnabout for the crosstalk between cadherins and integrins. *Eur. J. Cell Biol.* **2006**, *85*, 283–293. [CrossRef]
87. Watanabe, T.; Sato, K.; Kaibuchi, K. Cadherin-mediated intercellular adhesion and signaling cascades involving small GTPases. *Cold Spring Harb. Perspect. Biol.* **2009**, *1*, a003020. [CrossRef] [PubMed]
88. Kulus, M.; Sujka-Kordowska, P.; Konwerska, A.; Celichowski, P.; Kranc, W.; Kulus, J.; Piotrowska-Kempisty, H.; Antosik, P.; Bukowska, D.; Iżycki, D.; et al. New molecular markers involved in regulation of ovarian granulosa cell morphogenesis, development and differentiation during short-term primary in vitro culture-transcriptomic and histochemical study based on ovaries and individual separated follicles. *Int. J. Mol. Sci.* **2019**, *20*, 3966. [CrossRef]
89. Ozegowska, K.; Brazert, M.; Ciesiółka, S.; Nawrocki, M.J.; Kranc, W.; Celichowski, P.; Jankowski, M.; Bryja, A.; Jeseta, M.; Antosik, P.; et al. Genes Involved in the Processes of Cell Proliferation, Migration, Adhesion, and Tissue Development as New Potential Markers of Porcine Granulosa Cellular Processes In Vitro: A Microarray Approach. *DNA Cell Biol.* **2019**, *38*, 549–560. [CrossRef]
90. Antosik, P.; Jeseta, M.; Kranc, W.; Chachuła, A.; Bryja, A.; Budna, J.; Ciesiołka, S.; Wojtanowicz-Markiewicz, K.; Bukowska, D.; Brussow, K.P.; et al. Expression of integrins and GDF9 mRNAs is associated with Ovarian follicle size and donor puberty status in pigs. *Med. Weter.* **2016**, *72*, 750–754. [CrossRef]
91. Hassani, F.; Oryan, S.; Eftekhari-Yazdi, P.; Bazrgar, M.; Moini, A.; Nasiri, N.; Sharifi-Zarchi, A. Downregulation of extracellular matrix and cell adhesion molecules in cumulus cells of infertile polycystic ovary syndrome women with and without insulin resistance. *Cell J.* **2019**, *21*, 35–42. [CrossRef]
92. Hodgkinson, C.P.; Naidoo, V.; Patti, K.G.; Gomez, J.A.; Schmeckpeper, J.; Zhang, Z.; Davis, B.; Pratt, R.E.; Mirotsou, M.; Dzau, V.J. Abi3bp is a multifunctional autocrine/paracrine factor that regulates mesenchymal stem cell biology. *Stem Cells* **2013**, *31*, 1669–1682. [CrossRef]
93. Manabe, R.; Tsutsui, K.; Yamada, T.; Kimura, M.; Nakano, I.; Shimono, C.; Sanzen, N.; Furutani, Y.; Fukuda, T.; Oguri, Y.; et al. Transcriptome-based systematic identification of extracellular matrix proteins. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 12849–12854. [CrossRef]

94. Hao, J.; Tuck, A.R.; Prakash, C.R.; Damdimopoulos, A.; Sjödin, M.O.D.; Lindberg, J.; Niklasson, B.; Pettersson, K.; Hovatta, O.; Damdimopoulou, P. Culture of human ovarian tissue in xeno-free conditions using laminin components of the human ovarian extracellular matrix. *J. Assist. Reprod. Genet.* **2020**, *37*, 2137–2150. [CrossRef] [PubMed]
95. Hatzirodos, N.; Hummitzsch, K.; Irving-Rodgers, H.F.; Rodgers, R.J. Transcriptome comparisons identify new cell markers for theca interna and granulosa cells from small and large antral ovarian follicles. *PLoS ONE* **2015**, *10*, e0119800. [CrossRef] [PubMed]
96. Gallelli, M.F.; Bianchi, C.; Lombardo, D.; Rey, F.; Rodriguez, F.M.; Castillo, V.A.; Miragaya, M. Leptin and IGF1 receptors in alpaca (Vicugna pacos) ovaries. *Anim. Reprod. Sci.* **2019**, *200*, 96–104. [CrossRef]
97. Velazquez, M.A.; Zaraza, J.; Oropeza, A.; Webb, R.; Niemann, H. The role of IGF1 in the in vivo production of bovine embryos from superovulated donors. *Reproduction* **2009**, *137*, 161–180. [CrossRef] [PubMed]
98. Mani, A.M.; Fenwick, M.A.; Cheng, Z.; Sharma, M.K.; Singh, D.; Wathes, D.C. IGF1 induces up-regulation of steroidogenic and apoptotic regulatory genes via activation of phosphatidylinositol-dependent kinase/AKT in bovine granulosa cells. *Reproduction* **2010**, *139*, 139–151. [CrossRef] [PubMed]
99. Hayashi, K.G.; Ushizawa, K.; Hosoe, M.; Takahashi, T. Differential genome-wide gene expression profiling of bovine largest and second-largest follicles: Identification of genes associated with growth of dominant follicles. *Reprod. Biol. Endocrinol.* **2010**, *8*, 11. [CrossRef]
100. Skinner, M.K.; Schmidt, M.; Savenkova, M.I.; Sadler-Riggleman, I.; Nilsson, E.E. Regulation of granulosa and theca cell transcriptomes during ovarian antral follicle development. *Mol. Reprod. Dev.* **2008**, *75*, 1457–1472. [CrossRef] [PubMed]
101. Lamort, A.S.; Giopanou, I.; Psallidas, I.; Stathopoulos, G.T. Osteopontin as a Link between Inflammation and Cancer: The Thorax in the Spotlight. *Cells* **2019**, *8*, 815. [CrossRef] [PubMed]
102. Zalkin, H.; Dixon, J.E. De novo purine nucleotide biosynthesis. *Prog. Nucleic Acid Res. Mol. Biol.* **1992**, *42*, 259–287. [CrossRef] [PubMed]
103. Bønsdorff, T.; Gautier, M.; Farstad, W.; Rønningen, K.; Lingaas, F.; Olsaker, I. Mapping of the bovine genes of the *de novo* AMP synthesis pathway. *Anim. Genet.* **2004**, *35*, 438–444. [CrossRef] [PubMed]

4.2 Publikacja II

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Cell Division

RESEARCH Open Access

Expression of genes regulating cell division in porcine follicular granulosa cells

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Abstract

Background Cell cycle regulation influences the proliferation of granulosa cells and affects many processes related to ovarian folliclular growth and ovulation. Abnormal regulation of the cell cycle can lead to many diseases within the ovary. The aim of this study was to describe the expression profile of genes within granulosa cells, which are related to the formation of the cytoskeleton, organization of cell organelles inside the cell, and regulation of cell division. Established in vitro primary cultures from porcine ovarian follicle granulosa cells were maintained for 48, 96, 144 h and evaluated via microarray expression analysis.

Results Analyzed genes were assigned to 12 gene ontology groups "actin cytoskeleton organization", "actin filament organization", "actin filament—based process", "cell—matrix adhesion", "cell—substrate adhesion", "chromosome segregation", "chromosome separation", "cytoskeleton organization", "DNA integrity checkpoint", "DNA replication initiation", "organelle fision", "organelle organization". Among the genes with significantly changed expression, those whose role in processes within the ovary are selected for consideration. Genes with increased expression include (ITGA11, CNN1, CCl2, TPM2, ACTN1, VCAM-1, COL3A1, GSN, FRMD6, PLK2). Genes with reduced expression inlcude (KIF14, TACC3, ESPL1, CDC45, TTK, CDC20, CDK1, FBXO5, NEK2—NIMA, CCNE2). For the results obtained by microarray expressions, quantitative validation by RT-qPCR was performed.

Conclusions The results indicated expression profile of genes, which can be considered as new molecular markers of cellular processes involved in signaling, cell structure organization. The expression profile of selected genes brings new insight into regulation of physiological processes in porcine follicular granulosa cells during primary in vitro culture.

Keywords Follicular granulosa cells, Cellular signaling, Cytoskeleton organization, Cell cycle, Gene expression profile, Transcriptomics

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Background

The ability of animal cells to divide, transmit signals, move, and perform metabolic activity depends on the cytoskeleton. In addition, the cytoskeleton is responsible for cell shape, durability, and resistance to compression [1]. Depending on the environmental conditions, the cell, has the ability to adjust its shape, whether it is in vitro or in vivo [2]. The cytoskeleton is a dynamic structure, modifying its composition continuously. Components that build the cytoskeleton include microfilaments, microtubules, and intermediate filaments. The structural protein that builds microfilaments is actin, which is the most common protein in cells. The functions performed by microfilaments are numerous and include cell movement, intracellular signaling, and cell division [3], but also has been shown to be significantly involved in endocytosis [4]. Microtubules are made of the protein tubulin, are responsible for the transport of substances [5], and form the karyokinetic spindle responsible for the spread of chromosomes to the daughter cells [6]. Polyglutamination of tubulin also affecting the shape of the nucleus, according to recent data [7]. Intermediate filaments made of proteins such as vimentin, keratin and lamin are responsible for the majority of cell shape determination and cell stability but also for cell signaling [8]. In addition to functions related to stabilization of the cell environment, intermediate filaments also show activity in apoptosis, migration, adhesion, and interactions with other cytoskeletal components [2]. A functionally active animal cell requires the correct interactions of cytoskeletal components. Changes in the ratio of individual components of the cytoskeleton are described in diseases including cancer [9].

Granulosa cells (GCs) are the largest population of cells that make up the ovarian follicle. They have been shown to be intimately involved in the processes of folliculogenesis and oogenesis [10, 11]. Granulosa cells are responsible for steroidogenesis and their dialogue with an oocyte leads to its competence for fertilization [12]. The cytoskeleton involved in the cell division [13] influences the proliferation of granulosa cells in the ovarian follicle. The cytoskeleton has been shown to influence steroidogenesis in rat granulosa cells [14] through cholesterol transport [15, 16] and influence on localization of cell organelles [5, 17], including mitochondria in the cytoplasm [18]. A very important role of the cytoskeleton has been demonstrated in meiotic division of the oocyte [19, 20]. This results in an asymmetric division and the formation of a haploid oocyte and two polar bodies allowing the oocyte to retain the maternal components necessary for the initial development of the embryo. Critical steps in the above division are positioning of the nucleus, formation and migration of spindles, segregation of chromosomes, and extrusion of polar bodies, in which actin filaments are involved [19]. The processes involved in cell division, which is part of the cell cycle, are important for cell proliferation. The transition between the different phases G1, S, G2, M in the cell cycle is regulated by many genes and signaling pathways [21]. Cell division includes both cytoplasm and cell nucleus division, which must be controlled by checkpoints [22]. Abnormalities occurring during cell division can result in the formation of defective cells and consequently cell death. A number of genes responsible for cell division have been shown to be expressed, which if not properly expressed can cause the processes of tumorigenesis [23].

Additionally, intracellular and extracellular signaling is very important in processes related to animal reproduction and its efficiency [24]. The cytoskeleton and cell adhesion molecules (integrins, cadherins), catenins but also extracellular matrix (ECM) are involved in cell signaling [25, 26]. The orientation of the division spindle depends on extracellular matrix proteins confirming the interplay between the ECM and the cytoskeleton in cell division [27]. The main transmembrane proteins involved in the cell division are the integrins, which bind to cytoskeletal actin filaments [28]. A major role for these transmembrane proteins in reproduction in animals has been demonstrated [29]. Additionally, the recently described ability of granulosa cells to differentiate into other cell lineages gives these cells additional value [30, 31]. Small Rho GTPases affect cytoskeleton composition while showing effects on mesenchymal cell differentiation into adipose or muscle cells [32, 33]. Also the recently described effect of actin remodeling on the ability of mesenchymal stem cells to differentiate requires further research [34].

The aim of this study was to evaluate the expression profile of genes related to the formation of cytoskeleton, which is involved in cell division, spatial organization of cell organelles, metabolic activity and intercellular signaling in porcine granulosa cells. Describing the expression of individual genes related to intercellular signaling in granulosa cells may contribute to understanding the molecular basis of these cellular processes. Given the ability to co-culture granulosa cells along with oocytes, these data may be used in future studies to improve the efficiency of in vitro assisted reproductive techniques.

Results

The porcine granulosa cells were collected at 48, 96, and 144-h of cultivation and compared to the 0-h of the experiment as a control group. The general profile of the transcriptome changes is shown in Fig. 1, where dots represent the mean gene expression. Concerning the cut-off criteria for differentially expressed genes

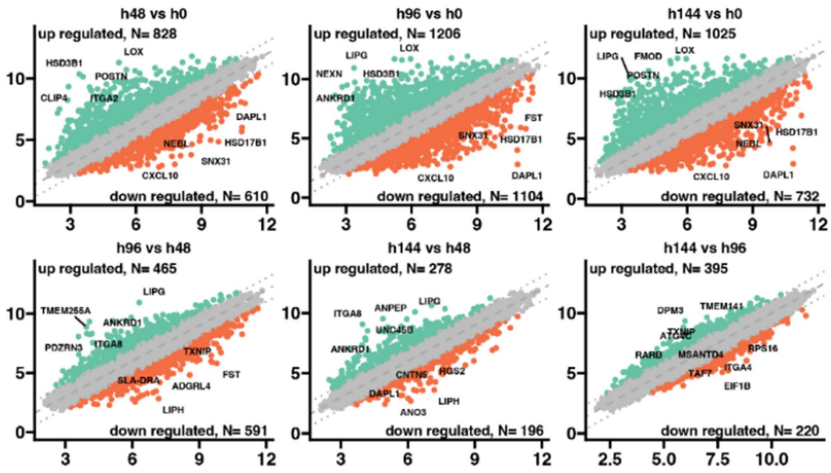


Fig. 1 Distribution of differentially expressed genes visualized as scattered plots. Each dot represents the mean expression (two biological replicates) of an individual gene obtained from a normalized microarray study. The gray dotted lined (cut-off values) were established according to the following parameters: |fold change| = 2 and p value = 0.05. Genes above the cut-off lines were considered as differentially expressed genes and are shown as orange (down-regulated) and turquoise (up-regulated) dots. The total numbers of up- and down-regulated genes are given in the top right and top left corners, respectively. The symbols of the five most differentially expressed genes from each compression are marked on the plots

(|fold change| > 2, and p value < 0.05), the scattered plots indicate that on 48-h of the experiment 828 genes were upregulated, while 610 were downregulated in comparison to 0 h of cells cultivation. At 96-h, it was revealed that 1206 activated and 1104 activated genes were, while at the end of the experiment at 144-h, it was indicated that 1025 upregulated and 732 downregulated genes in comparison to 0-h of the experiment. It was found that the most changes have been indicated during the 96-h of the experiment compared to the control. Meanwhile, the lowest number of deregulated genes were revealed between 48-h and controls. Commonly expressed genes were also identified across all analyzed groups. The expression of LOX, and HS3B1 were upregulated, and the expression of HSD17B1, SNX31, DAPL1, and CXCL10 were inhibited in all experimental groups compared to the control. Expression changes were also analyzed between experimental groups. It has been indicated that at 96- and 144-h groups ANKRD1, and ITGA8 genes are upregulated compared to 48-h of the experiment.

Next, the differentially expressed genes were classified by hierarchic clustering and visualized it as a heatmaps (Fig. 2, 3 and 4). The figures show both the expression values and expression fold changes between compared groups, according to the clustered ontological groups. It has been revealed that at the cluster related to actin filament and cytoskeleton organization (Fig. 2), the most downregulated gene across all analyzed groups was NEK2, and KIF14. Nevertheless, the expression of CAV1, TPM2, ACTN1, and CCL2 has been mostly upregulated throughout the experiment. Moreover, analysis of the cell–matrix and cell-substrate adhesion processes (Fig. 3), shows that only the KIF14 was downregulated across all analyzed groups. For the most upregulated genes, we include POSTN, FN1, ITGB8, LAMB1, ACTN1, ITGA2, and COL3A4. Furthermore, the differentially expressed genes arranged in chromosome segregation and DNA replication (Fig. 4) revealed that the expression of CCNE2 was mostly inhibited, while the expression of PLK2, and CDKN1A has been activated. Furthermore, the examination of genes of organelle fission and organization clusters (Fig. 5) indicates, that CDC20, NEK2, FBXO5, and MASTL were downregulated, while expression of PDE3A, TPM2, CCL2, DCN, CAV1, CLU, PTK7, and ETS1 has been mostly upregulated across all analyzed groups compared to control.

Furthermore, STRING and Metascape were employed as effective online platforms to perform functional analysis of protein–protein interactions and combine it with functional enrichment interactome analysis, gene annotation, and membership search [35, 36]. For the STRING analysis, four lists containing GO BP terms were used for

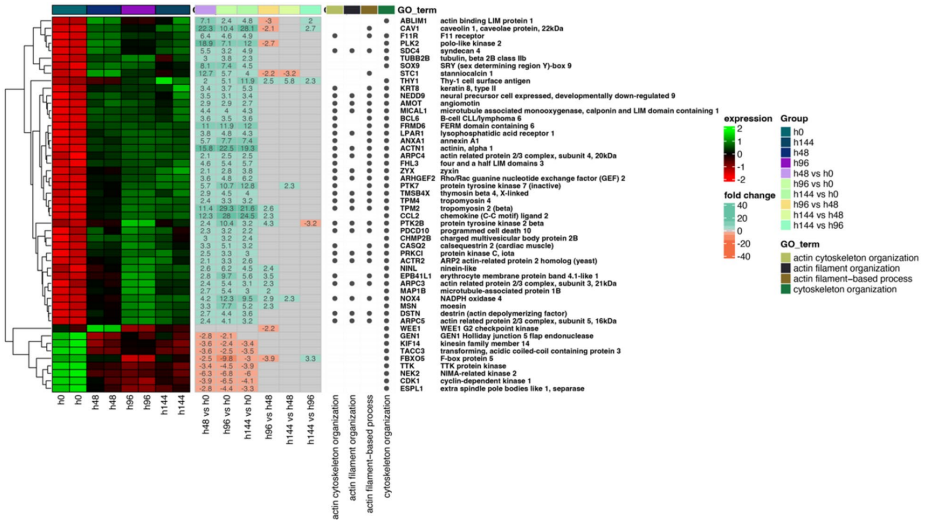


Fig. 2 Heatmap of expressed genes related to actin filament and cytoskeleton organization. The heatmap shows the expressed genes (left side), expression fold changes (center), and GO term ontological groups with gene names (right side) of all analyzed groups. The legends on the right side illustrate the colors used for the visualization

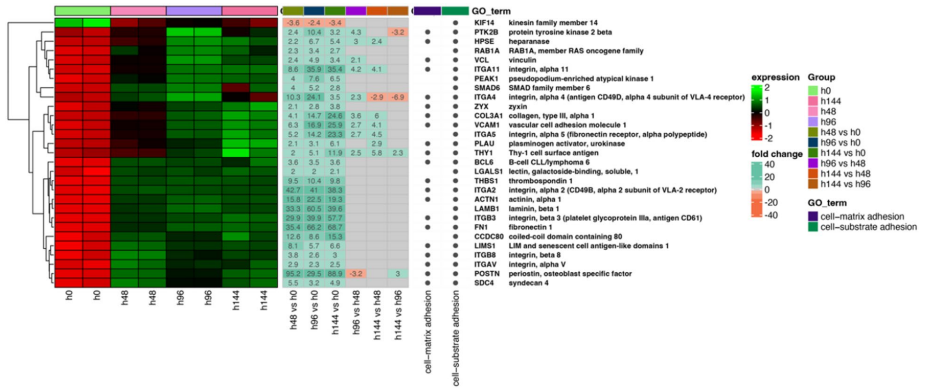


Fig. 3 Heatmap of expressed genes related to cell–matrix and cell-substrate adhesion, according to the GO term. The heatmap shows the expressed genes (left side), expression fold changes (center), and GO term ontological groups with gene names (right side) of all analyzed groups. The legends on the right side illustrate the colors used for the visualization

the differentially expressed genes (according to received heatmaps). The STRING analysis of proteins related to actin filament and cytoskeleton organization indicates that for all 148 nodes, 708 edges have been revealed (Fig. 6). The line thickness indicates the strength of data support from the sources of text mining and experiments with a cutoff value of medium confidence (0.522). The

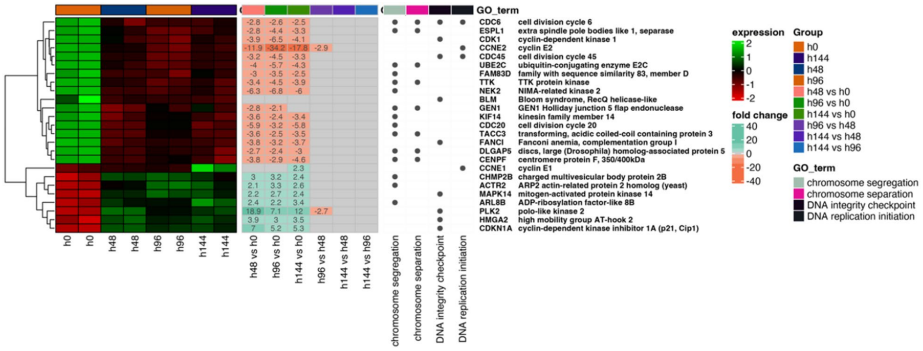


Fig. 4 Heatmap of expressed genes related to chromosome segregation and DNA replication, according to the GO term. The heatmap shows the expressed genes (left side), expression fold changes (center), and GO term ontological groups with gene names (right side) of all analyzed groups. The legends on the right side illustrate the colors used for the visualization

protein–protein interaction (PPI) enrichment p-value was < 10–16.

Moreover, all statistically enriched GO terms were identified, from which the most enriched were five processes: actin filament-based process (GO:0030029, log10(P) = − 29.6), cell-substrate adhesion (GO:0031589, log10(P) = − 26.7), positive regulation of cell migration (GO:0030335, log10(P) = − 21.6), response to wounding (GO:0009611, log10(P) = − 21.1), and regulation of cell cycle process (GO:0010564, log10(P) = − 20.4) (Fig. 7A). A subset of representative terms was selected from the entire cluster, converted them into a network layout (Fig. 7B, C), and applied the MCODE algorithm on this network to identify neighborhoods where proteins are densely connected (Fig. 7D).

The protein–protein interaction of molecules related to cell–matrix and cell-substrate adhesion, according to the GO BP terms, revealed 66 nodes with a number of edges ranged 155 (Fig. 8). The medium confidence (0.55) cutoff value and the PPI enrichment p-value were < 10–16. The 20 GO terms from which the actin filament-based process (GO:0030029, log10(P) = − 43.5) was the most significant (Fig. 9A) were identified. The network layout and MCODE algorithm on this network let us identify two neighborhoods that are densely connected among analyzed proteins (Fig. 9B–D).

The analysis of protein–protein interactions related to chromosome segregation and DNA replication performed by STRING analysis disclosed 34 nodes with 138 edges, with a cutoff value of medium confidence (0.698), and the PPI enrichment p-value was < 10–16 (Fig. 10).

The Metascape analysis indicates that the most enriched GO processes were cell-substrate adhesion (GO:0031589, log10(P) = − 45.7), regulation of cell-substrate adhesion (GO:0010810, log10(P) = − 23), and PID integrin1 pathway (M18, log10(P) = − 21.2) (Fig. 11A). Moreover, the selected subset of representative terms from the full cluster and converted them into a network layout revealed 20 different biological processes, according to the node colors, while the MCODE algorithm defined only one neighborhood (Fig. 11B–D).

The analysis of protein interactions belonging to organelle fission and organization indicates 117 nodes with 389 edges, with a cutoff value of medium confidence (0.485), and the PPI enrichment p-value was < 10–16 (Fig. 12).

Next, the analysis of the function of selected genes revealed that actin cytoskeleton organization (GO:0030036, log10(P) = − 22.6), and regulation of cell cycle process (GO:0010564, log10(P) = − 21.5) (Fig. 13A). The network layout and MCODE algorithm on this network let us identify six neighborhoods that are densely connected among analyzed proteins (Fig. 13B–D).

In conclusion, among all analyzed groups, we observed commonly activated biological processes like actin filament-based process (GO:0030029) and regulation of cell cycle process (GO:0010564).

Results from microarray expression were confirmed by quantitative RT-qPCR. These data sets were collected, compared, and presented as a bar graph (Fig. 14). 6 selected genes were validated. The RT-qPCR result can be more representative because this method has greater quantitative precision, as opposed to the whole transcriptome analysis provided by microarrays.

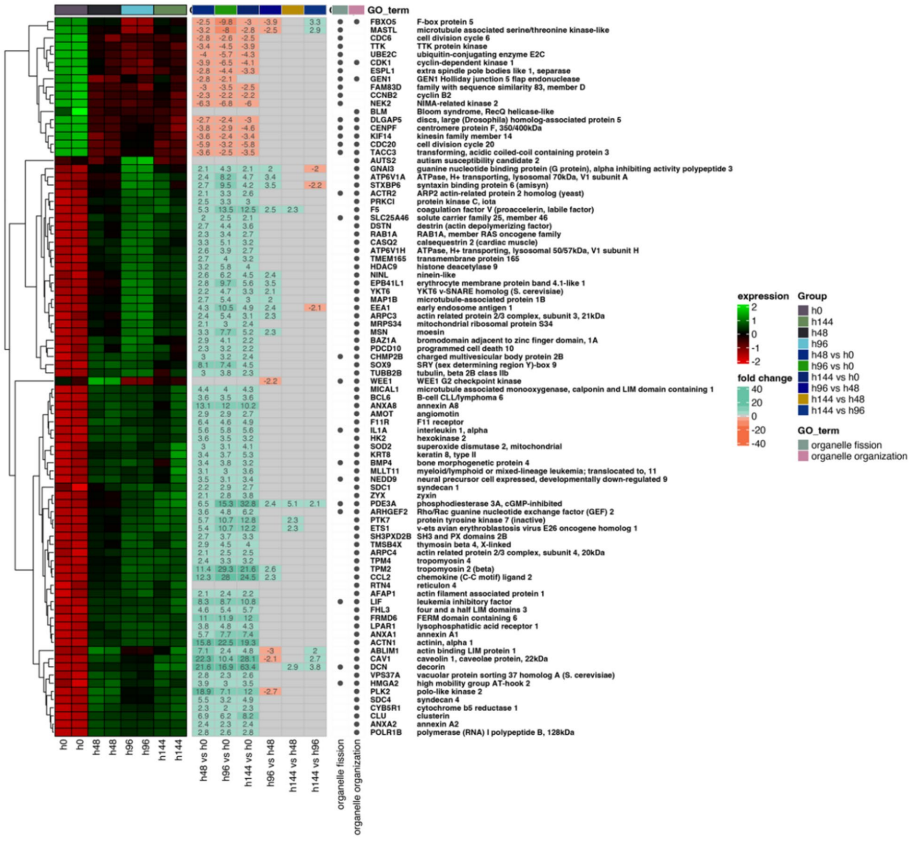


Fig. 5 Heatmap of expressed genes related to organelle fission and organization, according to the GO term. The heatmap shows the expressed genes (left side), expression fold changes (center), and GO term ontological groups with gene names (right side) of all analyzed groups. The legends on the right side illustrate the colors used for the visualization

Discussion

As a dynamically changing structure, the cytoskeleton influences cellular metabolism by adjusting individual components depending on signals received from the external environment. Intercellular signaling based on chemical signals is well established, but signaling based on physical signals (including through the cytoskeleton) requires further study [1]. Knowledge of the expression of genes related to the internal environment of the cell extends the knowledge needed to understand the molecular mechanisms. Taking into account the involvement of cytoskeleton in processes related to cell differentiation and division [32, 33], the transcriptome results can be used to conduct further studies related to stemness potential of GCs [37, 38], among others from proteomic and metabolomic aspects. Demonstration of the expression of particular genes connected with cell-to-cell and cell-to-environment signaling relevant in the context of reproduction is valuable for conducting research on assisted reproduction techniques conducted in vitro. The 10 upregulated genes (ITGA11, CNN1, CCl2, TPM2, ACTN1, VCAM-1, COL3A1, GSN, FRMD6, PLK2) and

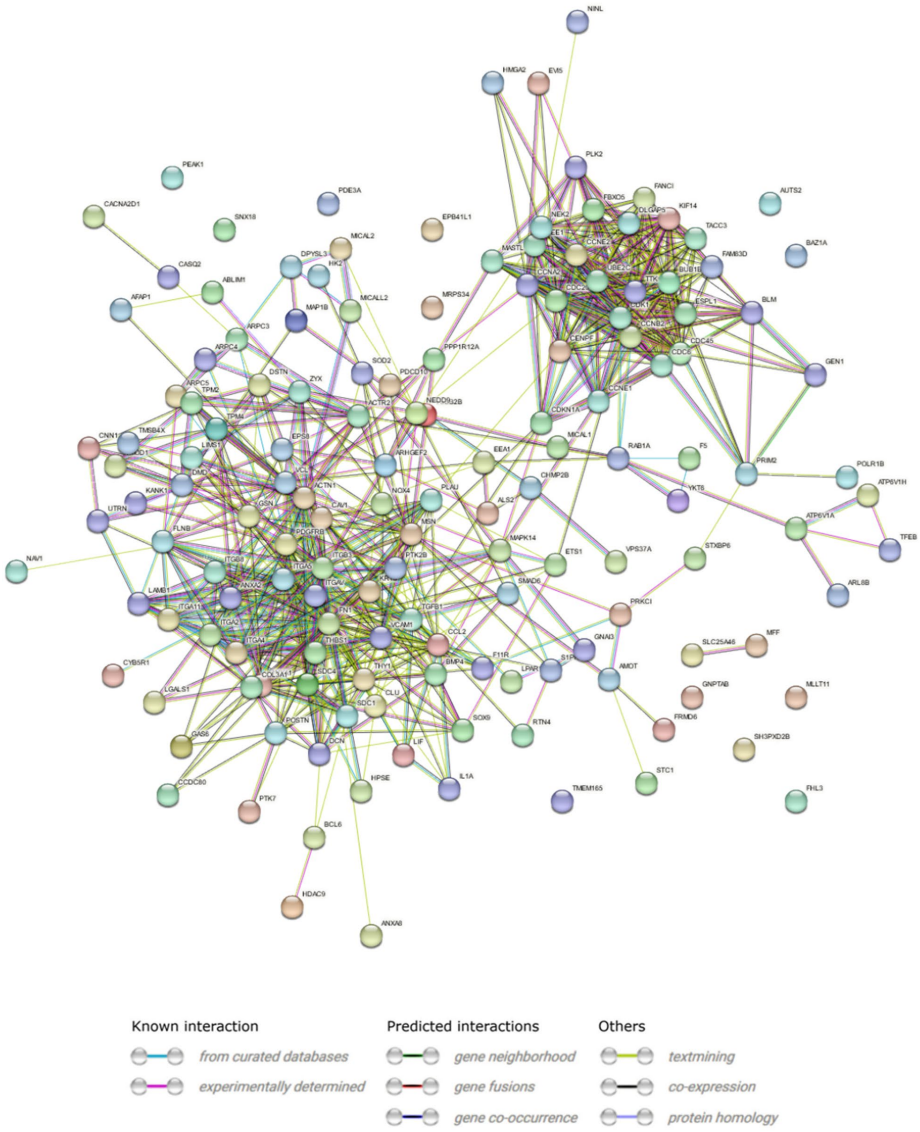


Fig. 6 STRING-generated interaction occurrence between differently expressed genes. The intensity of the edges reflects the strength of interaction score. Proteins are shown as nodes and the color of each link defined the type of evidence available for the interaction between two proteins

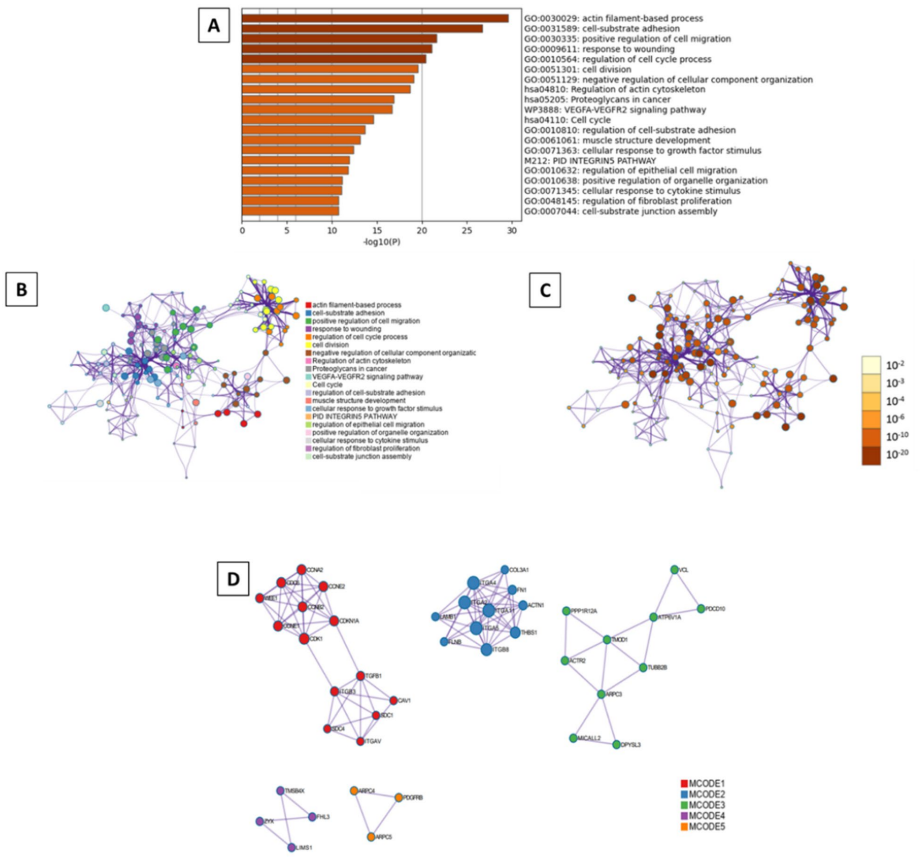


Fig. 7 Metascape functional analysis of transcriptome profiles based on differently expressed genes. **A** Heatmap of Gene Ontology (GO) enriched terms colored by p-values. **B** Clustered network of GO enriched terms where color represent its cluster identity. A circle node represents each term, the size of node is proportional to the number of input genes fall under that term, and its color represent its cluster identity. **C** Clustered network of GO enriched terms colored by p-value, where terms containing more genes tend to have a more significant p-value. **D** Protein–protein interaction (PPI) network clustered to five most significant MCODE components form the PPI network. A circle node represents each term, the size of node is proportional to the number of input genes fall under that term, and its color represent its cluster identity

10 downregulated genes (KIF14, TACC3, ESPL1, CDC45, TTK, CDC20, CDK1, FBXO5, NEK2—NIMA, CCNE2) were selected for further evaluation.

The main protein building block of the cytoskeleton is actin, which can assume different conformations in the cell [39]. Spontaneous polymerization of actin molecules builds the cytoskeleton. Depending on its conformation, actin is involved in various processes within the cell, including cellular structure, motility and intracellular transport [39]. Actin-binding proteins play an important role in actin remodeling in the cytoskeleton regulating almost every aspect of actin filament formation, including maintaining a large pool of actin monomers available for polymerization, initiating the formation of new filaments, promoting elongation [39–41]. The actin-binding proteins whose expression was

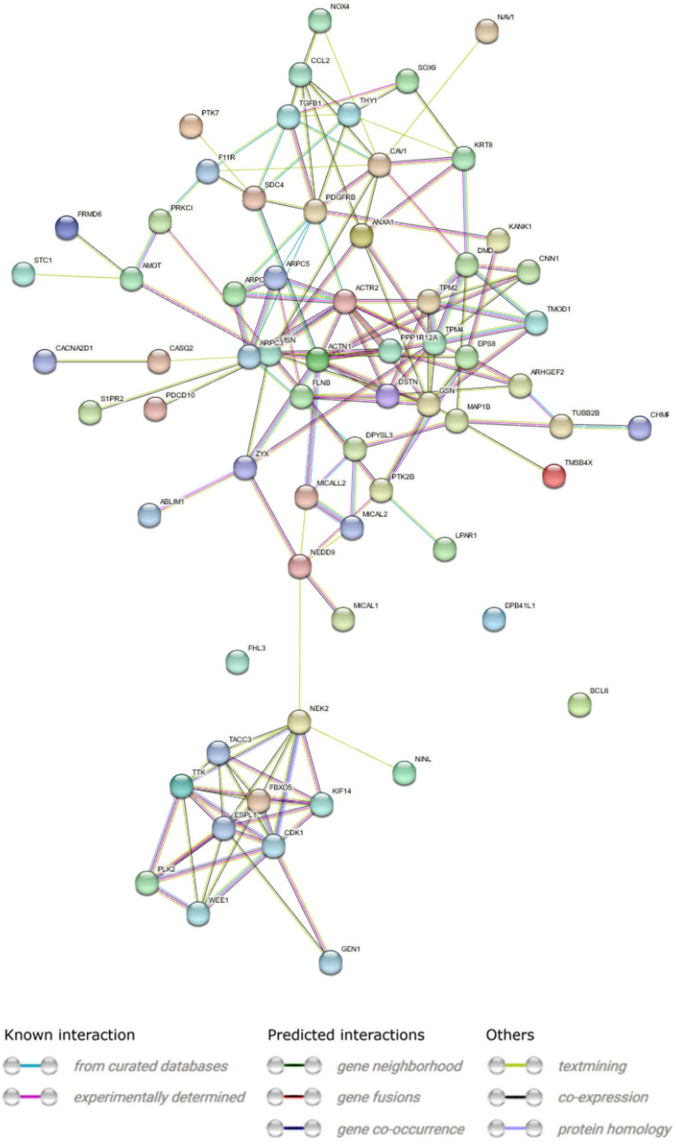


Fig. 8 STRING-generated interaction occurrence between differently expressed genes. The intensity of the edges reflects the strength of interaction score. Proteins are shown as nodes and the color of each link defined the type of evidence available for the interaction between two proteins

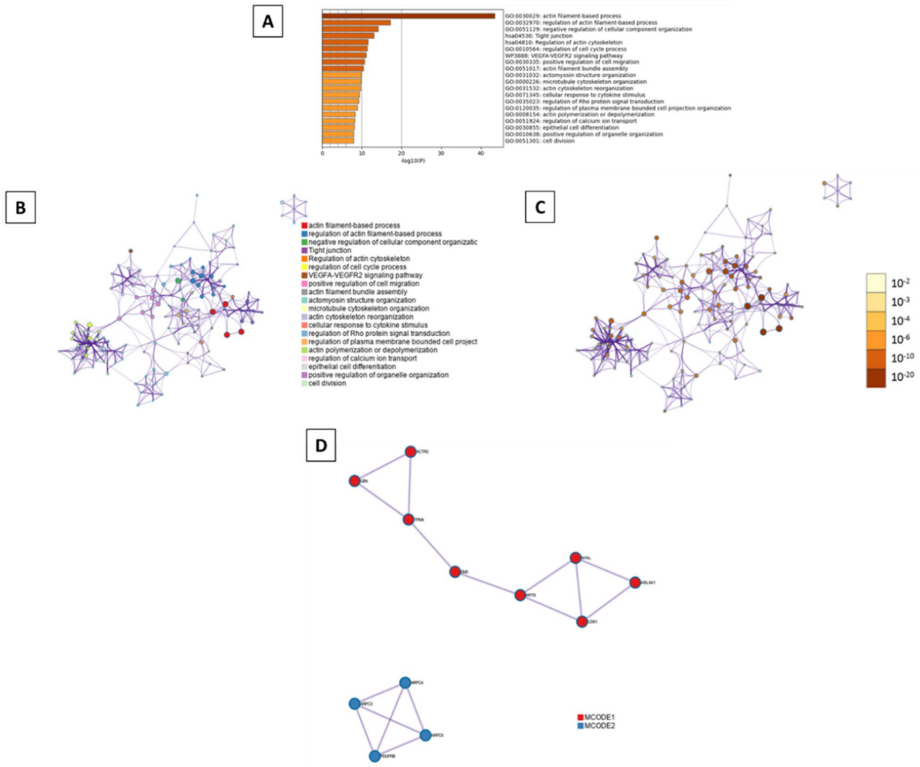


Fig. 9 Metascape functional analysis of transcriptome profiles based on differently expressed genes. **A** Heatmap of Gene Ontology (GO) enriched terms colored by p-values. **B** Clustered network of GO enriched terms where color represent its cluster identity. A circle node represents each term, the size of node is proportional to the number of input genes fall under that term, and its color represent its cluster identity. **C** Clustered network of GO enriched terms colored by p-value, where terms containing more genes tend to have a more significant p-value. **D** Protein–protein interaction (PPI) network clustered to five most significant MCODE components form the PPI network

determined in our study are: CNN1 (calponin 1), TPM2 (tropomyosin 2, beta), ACTN (actinin, alpha 1), GSN (gelsolin). The CH (calponin homology) domain is one of the most common in animal cells, being responsible for actin cytoskeleton organization, activation of signaling pathways and calcium metabolism. The following three domains are distinguished: CH1—CH2 occurring in tandem and CH3 [42]. Point mutations present in diseases have been shown to affect the affinity of the CH1-CH2 domains for F-actin [43]. A large role has recently been attached to the role of the CH domain in cancer, involving the Rho/ROCK1 signaling pathway [44]. To date, specific expression of CNN1 in muscle tissue has been demonstrated, linking this protein to muscle contraction [45]. The role of CH domain is not limited to actin binding and cytoskeleton remodeling, it has been shown that CH domain of calponin binds to ERK (extracellular regulated kinase) leading to signal transduction between cytoskeleton and extracellular matrix [42]. CNN1 gene expression was first demonstrated in porcine granulosa cells in the present study. Highlighting the importance of calponin, it has been shown to bind to many proteins found in the cytoskeleton, including tropomyosin, actinin, and gelsolin [45]. The cytoskeleton protein TPM2 binding to actin filaments protects them from degenerative action of cofilin, maintaining a stable cytoskeleton

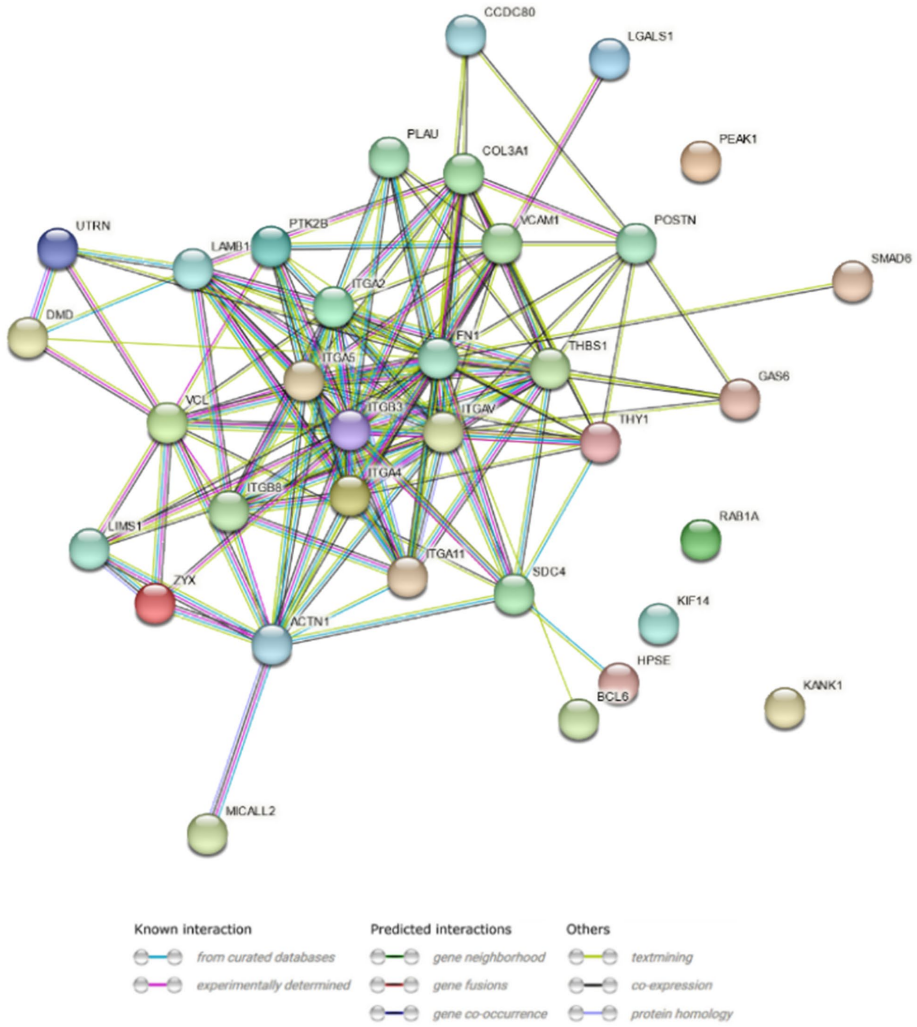


Fig. 10 STRING-generated interaction occurrence between differently expressed genes. The intensity of the edges reflects the strength of interaction score. Proteins are shown as nodes and the color of each link defined the type of evidence available for the interaction between two proteins

structure [39]. Elevated expression of this gene was previously shown in the granulosa of pre-ovulatory follicles in mice [46]. Additionally, tropomyosin beta has been shown to be down-regulated in granulosa cells of women with PCOS (Polycystic ovary syndrome) [47]. Actinin (ACTN1) belongs to actin filament cross-linking proteins

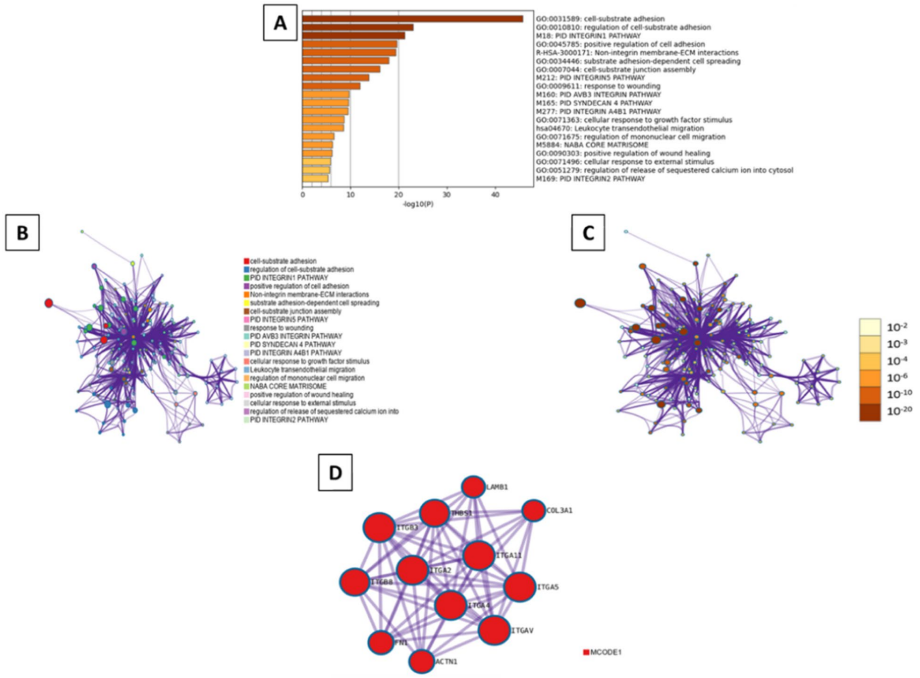
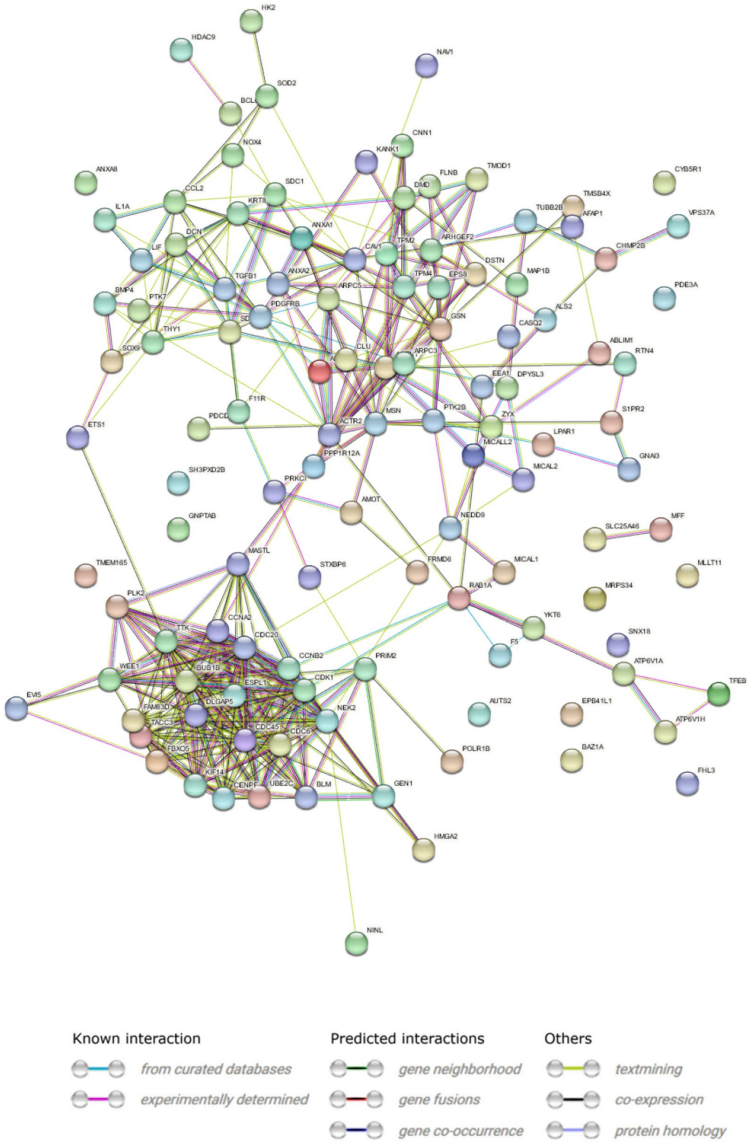


Fig. 11 Metascape functional analysis of transcriptome profiles based on differently expressed genes. **A** Heatmap of Gene Ontology (GO) enriched terms colored by p-values. **B** Clustered network of GO enriched terms where color represent its cluster identity. A circle node represents each term, the size of node is proportional to the number of input genes fall under that term, and its color represent its cluster identity. **C** Clustered network of GO enriched terms colored by p-value, where terms containing more genes tend to have a more significant p-value. **D** Protein–protein interaction (PPI) network clustered to five most significant MCODE components form the PPI network

[2]. This protein indirectly affects cytokinesis, cell adhesion and migration without affecting actin assembly. Actinins affect cell–matrix signaling through activation of PI3K and also cell-to-cell signaling through integrins and intercellular adhesion molecules (ICAM) [48]. To date, expression of ACTN1 and TPM2 genes has been demonstrated in human granulosa cells [49]. A cytoskeletal protein showing a major influence on the composition of actin filaments is gelsolin (GSN) [39]. Gelsolin is composed of two to six domains, and the activity of breaking the connections between actin molecules is dependent on calcium concentration [50]. Mutations in this gene have been shown to cause multiple diseases [51]. It has been described that gelsolin is also associated with ovarian disease in humans [52–54], and GSN has been identified as a marker for these disease processes [53]. It has been described that increased GSN gene expression is associated with suppression of apoptotic processes, whereas downregulation is associated with increased apoptosis and this protein is also recognized as a therapeutic target [50]. Differentiation of this gene expression in mice between mural granulosa cells and cumulus cells was also demonstrated [55]. As transmembrane proteins, integrins are responsible for transmitting signals between cells by influencing the composition of the cytoskeleton through interactions with GTPases [56]. The signaling occurs through the connection between the actin cytoskeleton and the extracellular matrix [57]. It has been shown that ITGA11 expression in rat uterine endometrium is dependent on progesterone, but also on miR-126a-3p [58, 59], which by affecting its expression may be involved in embryo implantation and pregnancy maintenance. The importance of progesterone has been confirmed in studies on pregnant sows [60] and expression of the ITGA11 gene in the porcine ovary during different phases of the sexual cycle [61]. A marked increase



Known interaction
from curated databases
experimentally determined

Predicted interactions
gene neighborhood
gene fusions
gene co-occurrence

Others
textmining
co-expression
protein homology

Fig. 12 STRING-generated interaction occurrence between differently expressed genes. The intensity of the edges reflects the strength of interaction score. Proteins are shown as nodes and the color of each link defined the type of evidence available for the interaction between two proteins

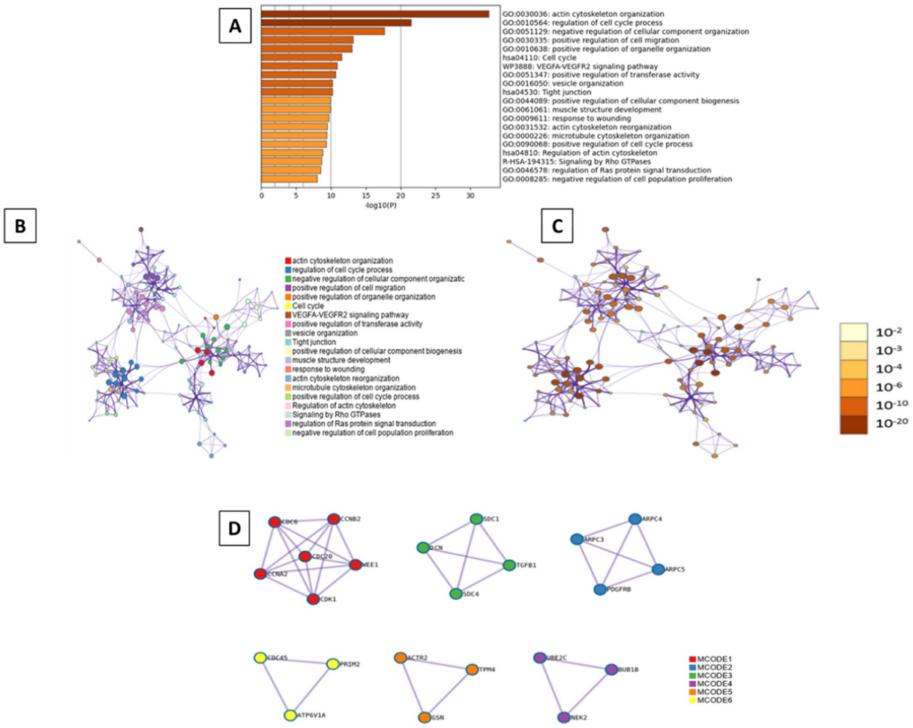


Fig. 13 Metascape functional analysis of transcriptome profiles based on differently expressed genes. **A** Heatmap of Gene Ontology (GO) enriched terms colored by p-values. **B** Clustered network of GO enriched terms where color represent its cluster identity. A circle node represents each term, the size of node is proportional to the number of input genes fall under that term, and its color represent its cluster identity. **C** Clustered network of GO enriched terms colored by p-value, where terms containing more genes tend to have a more significant p-value. **D** Protein–protein interaction (PPI) network clustered to five most significant MCODE components form the PPI network

in ITGA11 gene expression during differentiation of murine satellite cells (MSCs) into muscle cells has been described [62], which may be important in the context of the described differentiation potential of granulosa cells. ITG, GSN, ACTN genes have been shown to be involved in regulation of actin cytoskeleton. ITG affects signaling pathways, including FAK, thus regulating the activity of Rho GTPases, including Rac. This in turn leads to stabilization of actin filaments by gelsolin (GSN). An important element regulated by Rho proteins is ROCK (Rho-kinase), which by regulating ACTN transcription affects actin filament polymerization. The mechanism stabilizes the cytoskeleton while regulating intercellular signaling (Fig. 15).

CCl2 (chemokine C–C motif ligand 2) as a chemotactic, proinflammatory substance causes the influx of white blood cells within the ovarian follicle, which is involved in the regulation of ovulation. CCl2 expression has been shown to be dependent on progesterone receptors (Pgr), which influence the expression of the Ptgs2 gene involved in PGE2 synthesis leading to a physiologically controlled inflammatory response necessary for ovulation to occur [63]. Expression of the CCl2 gene in bovine granulosa cells is also described, demonstrating its role in the gaining of competence by the oocyte [64]. In addition, it has been described that CCl2 in human granulosa shows a role in luteolysis by affecting macrophage infiltration into the corpus luteum [65]. CCl2 in combination with BMP15 affect apoptosis of porcine cumulus granulosa

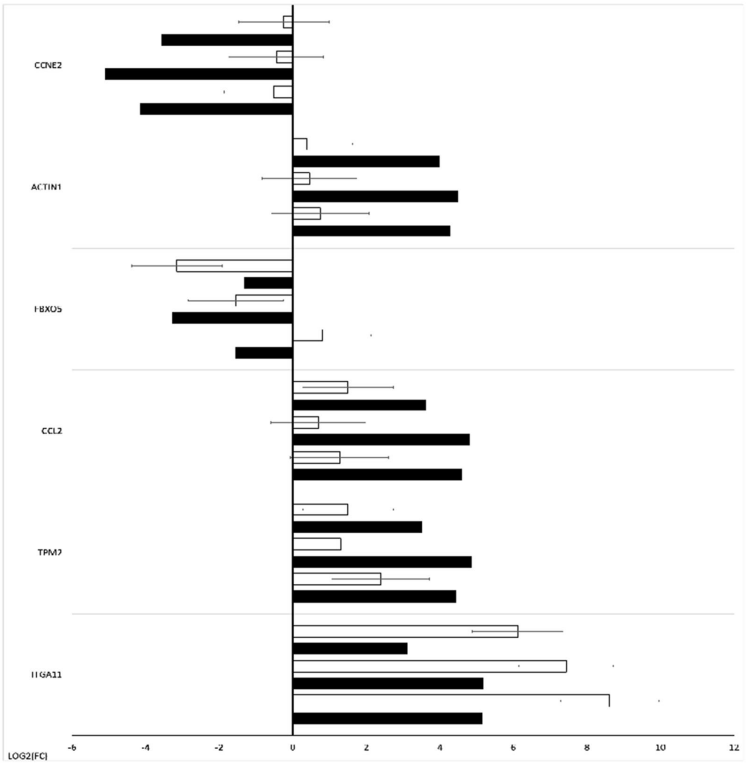


Fig. 14 Bar graph showing the microarray validation results obtained by RT-qPCR. Black bar indicates results of microarray expressions, white bar indicates results of RT-qPCR

cells has also been described [66]. VCAM-1 is identified as a molecule involved in cell adhesion, regulates inflammation-related vascular adhesion and transendothelial migration of leukocytes such as macrophages and T lymphocytes [67] suggesting the involvement of VCAM-1 in luteolysis,. Additionally, VCAM-1 has been shown to be associated in cancer [68], autoimmune diseases [69]. VCAM-1 has been suggested to be upregulated in PCOS (Polycytic ovary syndrome) [70], where it has been shown that this gene correlates with androgen production in theca cells of mouse ovaries, [71]. Ovarian processes such as oogenesis, folliculogenesis and ovulation require close cooperation between the cytoskeleton and the extracellular matrix (ECM) which is encoded by genes described in porcine granulosa cells [72]. It is worth noting the role of COL3A1, an ECM component protein in folliculogenesis, whose expression has been demonstrated within the ovary in both cattle and pigs, mono- and polyovulatory animals [73]. COL3A1 expression in PCOS [74] as well as POI [75] suggest that Lnc-GULP1-2:1 (long non-coding RNA) could be used to alter COL3A1 expression, treating it as a therapeutic target. Additionally, COL3A1 has been shown to be a biomarker for ovarian cancers [76] and also its elevated expression reduces the effects of anticancer drugs in vitro [77]. The control of mitotic and meiotic cell division is carried out by polo-like kinases (PLKs). One of them is PLK2, which also affects cell shape and cell death [78]. It was also shown that PLK2 activity in rat granulosa cells is influenced by hormonal induction of both LH and hCG [79]. Increased expression of PLK2 shown in the above work affects cell cycle arrest in granulosa cells, which is necessary for their luteinization in the perovulatory period. The FRMD-6 protein belongs to the FERM superfamily of proteins and has been shown to

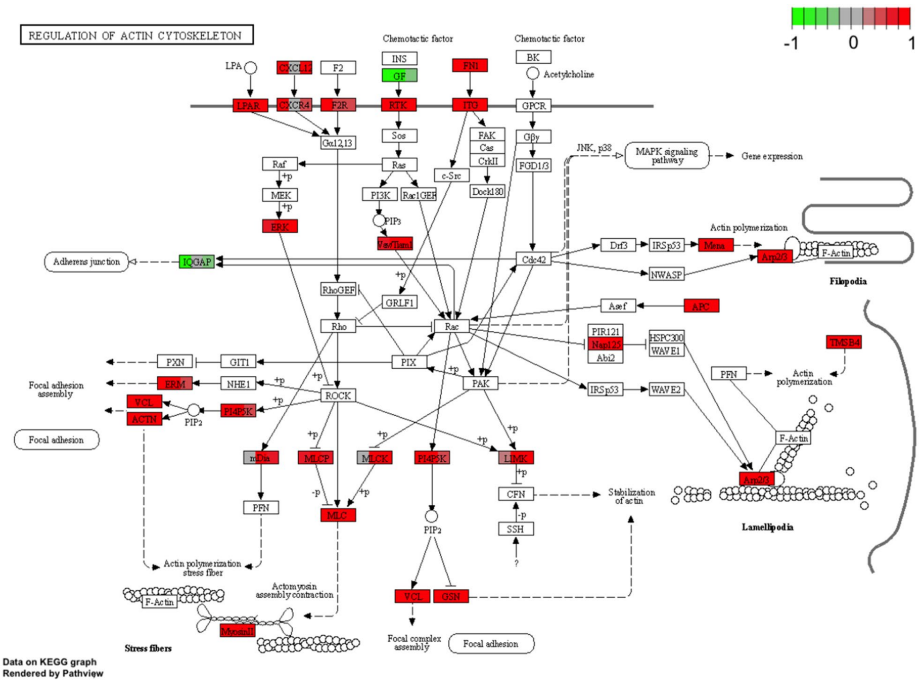


Fig. 15 Regulation of actin cytoskeleton pathway, KEGG pathway. KEGG, Kyoto Encyclopedia of Genes and Genomes. Copyright permission obtained

be involved in the regulation of the Hippo signaling pathway [80]. This pathway has been shown to significantly affect ovarian follicle development and ovulation in cattle [81, 82]. Additionally, the Hippo signaling pathway is dependent on actin remodeling [83]. Cell proliferation in animal organisms depends on cell division, which must occur in an orderly manner and regulated by multiple mechanisms. The cell cycle is divided into individual phases associated with cell division and growth, consisting of G1, S, G2, M. Only the proper transition between the different stages of cell division allows proper karyo and cytokinesis, which is further controlled by checkpoints. Many genes have been described whose protein products are responsible for normal cell division and are also involved in controlling the succession of the different phases of oogenesis [21]. If abnormalities occur during mitosis or meiosis, this can lead to cell dysfunction, cell death and uncontrolled cell division leading to cancer [84, 85]. Many cancers within the ovary have been described that are associated with abnormal cell division within the ovary [86, 87]. In relation to this type of disorders, knowledge of gene transcription associated with cell division is valuable because it may explain the basis of many diseases and also highlight potential therapeutic targets. All of the down-regulated expression genes are cell cycle related and have been described to be involved in cell division. Interestingly, a similar direction of expression of genes responsible for cell division was obtained in in vitro experiments on human [88] and porcine granulosa cells [89]. These genes affecting cell proliferation within the ovary affect granulosa cell function [90, 91], while at the same time may cause many diseases within the ovary [86, 87]. A particularly important gene associated with cell division is CDK1, which binds to CCNs cyclins and is responsible for regulation of cell cycle events, including transition between G1, S, G2, M phases [21]. Both CDKs and CCNs have been shown to influence granulosa cell proliferation by affecting the MAPK and ERK pathways [92]. Deletion of the CDK1 gene causes early embryonic death in mice [21], and its expression in pig granulosa

cells is modulated by the RSPO2 gene involved in the WNT signaling pathway [93]. Regulation of pig granulosa cell proliferation is associated with the CCNE2 gene [89] but also by numerous miRNAs [94]. Recent studies support a large role for cyclins in granulosa cell growth, which was confirmed by targeted suppressive effects of miRNAs on CCND2 [95]. There are many interactions between genes responsible for cell division such as the functional interaction between the CCNE2 and NEK2 genes [89]. Although NEK2 has previously been shown to interact with many other genes while demonstrating its involvement in cancer treatment within the ovary [96]. Continued research on treatment resistance caused by NEK2 confirms its important role in this regard and identifies this gene as a therapeutic target [97]. However, the activity of NEK2 as a kinase in granulosa cells derived from healthy porcine ovarian follicles was blocked [91]. It has been shown that the FBXO5 gene may be a prognostic biomarker in breast cancers, thus providing a potential therapeutic target [98]. In addition, the demonstrated interaction between FBXO5 (Emi1) and CDC20 confirms their role in regulating cell division through APC (anaphase promoting complex) inhibition [99]. The TGF-β/SMAD signaling pathway (Fig. 16) has a very important role in regulating ovarian function, where SMAD4 plays a large role within SMAD [100]. It was shown that silencing of SMAD4 caused an increase in many key cell cycle markers, including CDK1 and also cyclins (CNNA2, CNNB1 and CNNB2) confirming its role in granulosa cell proliferation. Additionally, SMAD4 silencing resulted in upregulation of the expression of other important cell cycle checkpoints, including CDC20 and CDC45 [100].

CDC20 has also been shown to play an important role in meiosis occurring in the oocyte, where detected mutations within this gene lead to infertility in women [101]. Interestingly, exogenous administration of gonadotropins has been shown to downregulate expression in human granulosa cells of both the CDC20 and CDC45 genes [102]. Genes closely involved in cell cycle regulation are TTK, ESPL1, TACC3, KIF14. They have been shown to be expressed in ovarian, uterine, breast, lung and colorectal cancers [84, 85, 103–106]. The available literature shows that these genes are potential therapeutic targets in cancer and determine resistance to treatment [84, 85, 103–106]. These data emphasize the necessity of understanding the expression of genes responsible for cell cycle regulation and constituting checkpoints in cells associated with the reproductive system of animals, including granulosa cells.

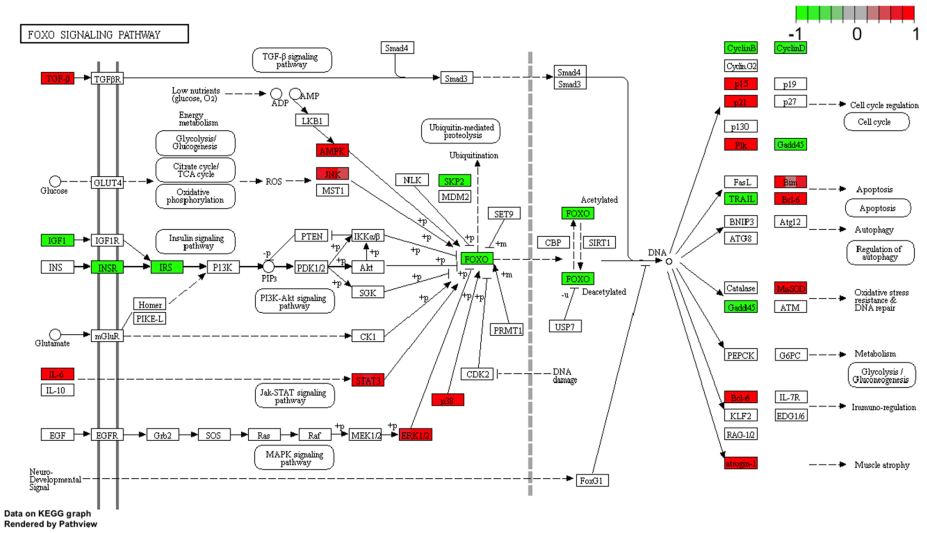


Fig. 16 FOXO signaling pathway, KEGG pathway. KEGG, Kyoto Encyclopedia of Genes and Genomes. Copyright permission obtained

Conclusions

Successful animal and human reproduction is largely dependent on interactions that occur between granulosa cells and the oocyte. Knowledge of the mechanisms and interactions occurring between the cytoskeleton and the external environment of the cells that make up the ovarian follicle provides an opportunity to understand the basis of diseases occurring within the ovary. The cytoskeleton has been shown to influence the composition of the extracellular matrix [26]. Many glycoproteins found in the ECM of granulosa cells have been described to influence the occurrence of diseases within the ovary, including PCOS and POI [107–109]. Interestingly, several genes encoding cytoskeleton-related proteins have also been described in relation to PCOS, including: TPM2, VCAM-1 [47, 70], and in the case of COL3A1 also in the aspect of POI [74, 75]. Given some ability to modulate cytoskeleton composition with actin-binding proteins [41, 110], it should be possible to influence ECM composition. Such an approach seems reasonable as a possibility to apply targeted therapy. Equally important in the context of reproductive disorders are the cell mechanisms involved in cell division, both within granulosa cells and within the oocyte [64, 90, 91]. Noteworthy are the cell division checkpoints, which, if not working properly, may impede cell replication. The TTK, ESPL1, TACC3, KIF14 genes first described in porcine granulosa cells provide new information about their cell cycle regulation. The results may provide a basis for further research on their use as a therapeutic target.

Methods

Animals

A total of 40 crossbred Landrace gilts with a median age of 170 days and weight of 98 kg were used in this study. All animals were housed under identical conditions. The animals in the study reached sexual maturity at 4–6 months of age and were in the follicular phase of sexual cycle.

Collection of porcine ovarian granulosa cells

Ovaries (n = 80) were recovered at slaughter and transported to the laboratory at 38 °C in 0.9% NaCl within 30 min of harvest. In the laboratory, the ovaries of each animal were placed in PBS supplemented with fetal bovine serum (FBS; Sigma-Aldrich Co., St. Louis, MO, USA). Thereafter, single preovulatory large follicles, with a diameter estimated greater than 5 mm (n = 300), were opened into a sterile Petri dish by puncturing using a 5 ml syringe and 20 G needle, and the cumulus-oocyte complexes (COCs) and follicular fluid (FF) were recovered. The transcriptomic profile of mural GCs, which constitute a significant majority among the GCs population was analyzed. The follicular fluid was used to isolate GCs, whereas the COCs were discarded. The extracted follicular fluid after discarding COCs was filtered through sterile nylon cell strainers with a mesh diameter of 40 µm (Biologix Group, Shandong, China) to eliminate tissue debris and larger cell aggregates (including blood cells) or epithelium. The resulting suspension was centrifuged at room temperature for 10 min, 200 rpm, to obtain individual cell fractions. The GCs pellet was then resuspended in collagenase type I solution (Gibco, Thermo-Fischer Scientific, Waltham, MA, USA) 1 mg/1 mL DMEM and incubated 10 min in a 37 °C water bath and centrifuged (under the same conditions). The cell pellet was resuspended in culture medium to establish in vitro culture under the conditions described below. Granulosa cells collected from ovarian follicles were pooled to homogenize the sample.

In vitro primary culture of porcine granulosa cells

A primary in vitro culture model was used in this study with four time intervals. For microarray expressions, cultures were maintained in two biological replicates for each time interval. For validation by RT-qPCR, cultures were maintained in a triplicate biological sample model for each time interval. Primary cultures were established from GCs in four bottles with 3×10^6 cells per dish (25 cm^2 cell culture flask, TPP, Trasadingen, Switzerland). The number of cells and their viability were assessed using the ADAM Automatic Cell Counter (NanoEnTek, Waltham, MA, USA). From the cell suspension, a 20 µL sample for number and viability analysis was stained with propium iodide and examined in a fluorescence analyzer on disposable microchips. By staining the cell nuclei, the counter is able to distinguish single cells in aggregates. Only those samples with viability above 85% were used for further studies. Cells in culture were kept until culture termination when the material was collected at 0 h, 48 h, 96 h, 144 h. The culture medium was changed every 72 h.

Culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, Saint Louis, MO, USA), 2% fetal calf serum (FCS) (PAA, Linz, Austria), 10 mg/mL ascorbic acid (Sigma-Aldrich, Saint Louis, MO, USA), 0.05 µM dexamethasone (Sigma-Aldrich, Saint Louis, MO, USA), 200 mM L-glutamine (Invitrogen, Carlsbad, CA, USA), 10 mg/mL gentamycin (Invitrogen, Carlsbad, CA, USA), 10,000 units/mL penicillin and 10,000 µg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). Cells were cultivated at 38.5 °C under aerobic conditions (5% CO2). Once the adherent cells were more than 80% confluent, they were detached with

0.05% trypsin–EDTA (Invitrogen, Carlsbad, CA, USA) for 3 min. and then passaged. When the cells were seeded into culture bottles, the shape of the cells was close to spherical, where the cells formed a suspension in the medium. After 24 h of culture, the cells became adherent to the medium, and after 48 h, the cells assumed a star-like shape. At subsequent time intervals, the GCs became wider, more fibroblast-like. The strong adherence to the dish surface, shape change, and flattening of the cells is related to the secretion of extracellular matrix components, which correlates with the increased expression of ECM-related genes during the study.

Microarray expression analysis and statistics

The Affymetrix procedure was previously described by Trejter et al. [111] and used in studies involving porcine oviduct epithelial cells (OECs) [112–114] as well as oocytes [115–117]. Briefly cDNA was subjected from Total RNA (100 ng) (Ambion® WT Expression Kit). Obtained cDNA was biotin labeled and fragmentated by Affymetrix GeneChip® WT Terminal Labeling and Hybridization (Affymetrix). Biotin-labeled fragments of cDNA (5.5 µg) were hybridized to Affymetrix® Porcine Gene 1.1 ST Array Strip (45 °C/20 h). Then, microarrays were washed and stained according to the technical protocol using Affymetrix GeneAtlas Fluidics Station. Subsequently the array strips were scanned by Imaging Station of GeneAtlas System. The preliminary analysis of the scanned chips was performed using Affymetrix GeneAtlasTM Operating Software. The quality of gene expression data was checked according to quality control criteria provided by the software. Obtained CEL files were imported into downstream data analysis software. All of presented analyses and graphs were performed by Bioconductor and R programming language (v4.1.2; R Core Team 2021). Each CEL file was merged with a description file. A Robust Multiarray Averaging (RMA) algorithm was used to correct background.

To show the total number of up- and down-regulated genes, the principal component analysis (PCA) of filtered data set was performed and visualized using "factoextra" library [118]. Differentially expressed genes (DEGs) from each comparison were visualized hierarchic clustering of differentially expressed genes as a heatmap using "ComplexHeatmap" library [119]. The established cut-off criteria for DEGs were based on the differences in the absolute value from the expression fold change greater than 2. Functional protein partners among all input gene list were identifies using the Search Tool for the Retrieval of Interacting Genes (STRING) (version 11.5) analysis web portal (https://string-db.org/) and by Metascape [35, 36]. The score of minimum required interaction was medium confidence (0.4). While the PPI network contains more than three nodes, the Detection (MCODE) algorithm has been used to revealed clusters directly related to genes within PPI [120]. Next, according to the p-value in the generated network, MCODE created and assigned a unique colour.

Table 1 Oligonucleotide sequences of primers used for RT-qPCR analysis

Gene	Primer sequence (5′-3′)		Product size (bp)
CCNE2	F	GATGGTGCTTGCAGTGAAGA	216
	R	CGATGGCTAGAATGCACAGA	
FBX05	F	AAGCCTCAAAGCCTGCATTC	221
	R	TCACCTTCGAAGCACAGTCT	
ITGA11	F	GAGGCTCCACAGGAAAGTCT	151
	R	CTTCTCATCGCTGTCACTGC	
CCl2	F	TCTCCAGTCACCTGCTGCTA	185
	R	TCCAGGTGGCTTATGGAGTC	
TPM2	F	AGTTTCCCCAAGTCTCTGCA	184
	R	TCCGTCCCTTTCAGCTTCTT	
ACTIN1	F	GGCAAGATGAGAGTGCACAA	172
	R	AGATGTCCTGGATGGCAAAG	

Real-time quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was isolated from GCs in 0 h and after 48 h, 96 and 144 h in vitro culture using an RNeasy mini column from Qiagen GmbH (Hilden, Germany). The RNA samples were resuspended in 20 µl of RNase-free water and stored in liquid nitrogen. RNA samples were treated with DNase I and reverse-transcribed (RT) into cDNA. RT-qPCR was conducted in a LightCycler real-time PCR detection system (Roche Diagnostics GmbH, Mannheim, Germany) using SYBR® Green I as a detection dye, and target cDNA was quantified using the relative quantification method. The relative abundance of analyzed transcripts in each sample was standardized to the internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For amplification, 2 µl of cDNA solution was added to 18 µl of QuantiTect® SYBR® Green PCR (Master Mix Qiagen GmbH, Hilden, Germany) and primers (Table 1). One RNA sample of each preparation was processed without the RT-reaction to provide a negative control for subsequent PCR.

To quantify the specific genes expressed in the GCs, the expression levels of specific mRNAs in each sample were calculated relative to PBGD and ACTB. To ensure the integrity of these results, the additional housekeeping gene, 18S, was used as an internal standard to demonstrate that PBGD and ACTB mRNAs were not

differentially regulated in GC groups. The gene for 18S rRNA expression has been identified as an appropriate housekeeping gene for use in quantitative PCR studies. Expression of PBGD, ACTB, and 18S mRNA was measured in cDNA samples from isolated GCs. The statistical significance of the analyzed genes was performed using moderated t-statistics from the empirical Bayes method. The obtained p-value was corrected for multiple comparisons using the Benjamini and Hochberg's false discovery rate.

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Not applicable

Author contributions
Conception BK and PA; Design of the work, WK and PD; Creation of new software used in the work, MK; Acquisition, analysis, JK, MK and WK; Interpretation of data, PM, DB; Writing—original draft preparation, JK and WK; Writing—review and editing, PM and MK; Funding acquisition, PA and PM All authors read and approved the final manuscript.

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Availability of data and materials
Not applicable.

Declarations

Ethics approval and consent to participate
Animals were not involved in the study. The material used in the study was collected post-mortem at the slaughterhouse, and is treated as waste. Such an action within the European Union does not require ethics committee approval.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References

1. Fletcher DA, Mullins RD. Cell mechanics and the cytoskeleton. Nature. 2010;463(7280):485.
2. Hohmann T, Dehghani F. The cytoskeleton—a complex interacting meshwork. Cells. 2019;8(4):362.
3. Jiang X, Qin Y, Kun L, Zhou Y. The significant role of the microfilament system in tumors. Front Oncol. 2021;17(11):333.
4. Akamatsu M, Vasan R, Serwas D, Ferrin M, Rangamani P, Drubin DG. Principles of self-organization and load adaptation by the actin cytoskeleton during clathrin-mediated endocytosis. Elife. 2020;1:9.
5. Barlan K, Gelfand VI. Microtubule-based transport and the distribution, tethering, and organization of organelles. Cold Spring Harb Perspect Biol. 2017. https://doi.org/10.1101/cshperspect.a025817.
6. Forth S, Kapoor TM. The mechanics of microtubule networks in cell division. J Cell Biol. 2017;216(6):1525–31. https://doi.org/10.1083/jcb.201612064.
7. Wang L, Paudyal SC, Kang Y, Owa M, Liang F-X, Spektor A, et al. Regulators of tubulin polyglutamylation control nuclear shape and cilium disassembly by balancing microtubule and actin assembly. Cell Res. 2021. https://doi.org/10.1038/s41422-021-00584-9.
8. Sanghvi-Shah R, Weber GF. Intermediate filaments at the junction of mechanotransduction, migration, and development. Front Cell Dev Biol. 2017;5(SEP):81.
9. Ong MS, Deng S, Halim CE, Cai W, Tan TZ, Huang RYJ, et al. Cytoskeletal proteins in cancer and intracellular stress: a therapeutic perspective. Cancers (Basel). 2020. https://doi.org/10.3390/cancers12010238.
10. Chamier-Gliszczyńska A, Kałuzna S, Stefańska K, Celichowski P, Antosik P, Bukowska D, et al. Analysis of expression of genes responsible for regulation of cellular proliferation and migration-Microarray approach based on porcine oocyte model. Med J Cell Biol. 2019;7(2):48–57.
11. Rybska M, Knap S, Jankowski M, Jeseta M, Bukowska D, Antosik P, et al. Characteristic of factors influencing the proper course of folliculogenesis in mammals. Med J Cell Biol. 2018. https://doi.org/10.2478/acb-2018-0006.
12. Irving-Rodgers HF, Rodgers RJ. Extracellular matrix of the developing ovarian follicle. Semin Reprod Med. 2006;24(4):195–203.
13. Jones MC, Zha J, Humphries MJ. Connections between the cell cycle, cell adhesion and the cytoskeleton. Philos Trans R Soc B. 2019. https://doi.org/10.1098/rstb.2018.0227.
14. Carnegie JA, Tsang BK. The cytoskeleton and rat granulosa cell steroidogenesis: possible involvement of microtubules and microfilaments. Biol Reprod. 1988;38(1):100–8.
15. El Zowalaty AE, Li R, Zheng Y, Lydon JP, DeMayo FJ, Ye X. Deletion of RhoA in progesterone receptor-expressing cells leads to luteal insufficiency and infertility in female mice. Endocrinology. 2017;158(7):2168–78.
16. Shen WJ, Zaidi SK, Patel S, Cortez Y, Ueno M, Azhar R, et al. Ablation of vimentin results in defective steroidogenesis. Endocrinology. 2012;153(7):3249–57.
17. Nekrasova OE, Mendez MG, Chernoivanenko IS, Tyurin-Kuzmin PA, Kuczmarski ER, Gelfand VI, et al. Vimentin intermediate filaments modulate the motility of mitochondria. Mol Biol Cell. 2011;22(13):2282–9. https://doi.org/10.1091/mbc.e10-09-0766.
18. Miller WL. Steroid hormone synthesis in mitochondria. Mol Cell Endocrinol. 2013;379(1–2):62–73.
19. Duan X, Sun SC. Actin cytoskeleton dynamics in mammalian oocyte meiosis. Biol Reprod. 2019;100(1):15–24.
20. Pereira GR, Lorenzo PL, Carneiro GF, Ball BA, Pegoraro LMC, Pimentel CA, et al. Influence of equine growth hormone, insulin-like growth factor-I and its interaction with gonadotropins on in vitro maturation and cytoskeleton morphology in equine oocytes. Animal. 2013;7(9):1493–9.
21. Li J, Qian WP, Sun QY. Cyclins regulating oocyte meiotic cell cycle progression†. Biol Reprod. 2019;101(5):878–81.
22. Barnum KJ, O'Connell MJ. Cell cycle regulation by checkpoints. Methods Mol Bio. 2014;1170:29.
23. Matthews HK, Bertoli C, de Bruin RAM. Cell cycle control in cancer. Nat Rev Mol Cell Biol. 2021. https://doi.org/10.1038/s41580-021-00404-3.
24. Bowen JA, Hunt JS. The role of integrins in reproduction. Proc Soc Exp Biol Med. 2000;223(4):331–43.
25. Weber GF, Bjerke MA, DeSimone DW. Integrins and cadherins join forces to form adhesive networks. J Cell Sci. 2011;124(8):1183–93.
26. Geiger B, Bershadsky A, Pankov R, Yamada KM. Transmembrane extracellular matrix-cytoskeleton crosstalk. Nat Rev Mol Cell Biol. 2001;2(11):793–805.
27. Théry M, Racine V, Pépin A, Piel M, Chen Y, Sibarita JB, et al. The extracellular matrix guides the orientation of the cell division axis. Nat Cell Biol. 2005;7(10):947–53.
28. D'Occhio MJ, Campanile G, Zicarelli L, Visintin JA, Baruselli PS. Adhesion molecules in gamete transport, fertilization, early embryonic development, and implantation—role in establishing a pregnancy in cattle: a review. Mol Reprod Dev. 2020;87(2):206–22.
29. Merc V, Frolikova M, Komrskova K. Role of integrins in sperm activation and fertilization. Int J Mol Sci. 2021;22(21):11809.
30. Kossowska-Tomaszczuk K, De Geyter C. Cells with stem cell characteristics in somatic compartments of the ovary. Biomed Res Int. 2013. https://doi.org/10.1155/2013/310859.
31. Józkowiak M, Hutchings G, Jankowski M, Kulcenty K, Mozdziak P, Kempisty B, et al. The stemness of human ovarian granulosa cells and the role of resveratrol in the differentiation of MSCs-A review based on cellular

and molecular knowledge. Cells. 2020. https://doi.org/10.3390/cells9061418.
32. Sordella R, Jiang W, Chen GC, Curto M, Settleman J. Modulation of Rho GTPase signaling regulates a switch between adipogenesis and myogenesis. Cell. 2003;113(2):147–58.
33. McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Dev Cell. 2004;6(4):483–95.
34. Khan AU, Qu R, Fan T, Ouyang J, Dai J. A glance on the role of actin in osteogenic and adipogenic differentiation of mesenchymal stem cells. Stem Cell Res Ther. 2020;11(1):1–14.
35. Szklarczyk D, Gable AL, Lyon D, Junge A, Wyder S, Huerta-Cepas J, et al. STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic Acids Res. 2019;47(D1):D607–13.
36. Zhou Y, Zhou B, Pache L, Chang M, Khodabakhshi AH, Tanaseichuk O, et al. Metascape provides a biologist-oriented resource for the analysis of systems-level datasets. Nat Commun. 2019. https://doi.org/10.1038/s41467-019-09234-6.
37. Moncrieff L, Mozdziak P, Jeseta M, Machatkova M, Kranc W, Kempisty B. Ovarian follicular cells—living in the shadow of stemness cellular competence. Med J Cell Biol. 2019;7(3):134–40.
38. Stefańska K, Sibiak R, Hutchings G, Dompe C, Moncrieff L, Janowicz K, et al. Evidence for existence of molecular stemness markers in porcine ovarian follicular granulosa cells. Med J Cell Biol. 2019;7(4):183–8.
39. Pollard TD. Actin and Actin-Binding Proteins. Cold Spring Harb Perspect Biol. 2016. https://doi.org/10.1101/cshperspect.a018226.
40. Lappalainen P. Actin-binding proteins: the long road to understanding the dynamic landscape of cellular actin networks. Mol Biol Cell. 2016;27(16):2519–22. https://doi.org/10.1091/mbc.e15-10-0728.
41. Harris AR, Jreij P, Belardi B, Joffe AM, Bausch AR, Fletcher DA. Biased localization of actin binding proteins by actin filament conformation. Nat Commun. 2020;11(1):1–13.
42. Yin LM, Schnoor M, Jun CD. Structural characteristics, binding partners and related diseases of the calponin homology (CH) domain. Front Cell Dev Biol. 2020. https://doi.org/10.3389/fcell.2020.00342.
43. Harris AR, Belardi B, Jreij P, Wei K, Shams H, Bausch A, et al. Steric regulation of tandem calponin homology domain actin-binding affinity. Mol Biol Cell. 2019;30(26):3112.
44. Wang Z, Li TE, Chen M, Pan JJ, Shen KW. miR-106b-5p contributes to the lung metastasis of breast cancer via targeting CNN1 and regulating Rho/ROCK1 pathway. Aging (Albany NY). 2020;12(2):1867–87.
45. Liu R, Jin JP. Calponin isoforms CNN1, CNN2 and CNN3: Regulators for actin cytoskeleton functions in smooth muscle and non-muscle cells. Gene. 2016;585(1):143–53.
46. Nagaraja AK, Middlebrook BS, Rajanahally S, Myers M, Li Q, Matzuk MM, et al. Defective gonadotropin-dependent ovarian folliculogenesis and granulosa cell gene expression in inhibin-deficient mice. Endocrinology. 2010;151(10):4994.
47. Li L, Mo H, Zhang J, Zhou Y, Peng X, Luo X. The role of heat shock protein 90b1 in patients with polycystic ovary syndrome. PLoS One. 2016;11(4):e0152837. https://doi.org/10.1371/journal.pone.0152837.
48. Yang X, Pang Y, Zhang J, Shi J, Zhang X, Zhang G, et al. High expression levels of ACTN1 and ACTN3 indicate unfavorable prognosis in acute myeloid leukemia. J Cancer. 2019;10(18):4286.
49. Sasson R, Rimon E, Dantes A, Cohen T, Shinder V, Land-Bracha A, et al. Gonadotrophin-induced gene regulation in human granulosa cells obtained from IVF patients. Modulation of steroidogenic genes, cytoskeletal genes and genes coding for apoptotic signalling and protein kinases. Mol Hum Reprod. 2004;10(5):299–311.
50. Zhou Y, Deng X, Ma X, Zang N, Li H, Li G, et al. Cellular transcriptomics: gelsolin negatively regulates the expression of apoptosis-associated genes and inhibits apoptosis in hepatocarcinoma cells. Int J Clin Exp Pathol. 2015;8(11):13871.
51. Jiang Y, Jiao B, Liao X, Xiao X, Liu X, Shen L. Analyses mutations in GSN, CST3, TTR, and ITM2B genes in Chinese patients with Alzheimer's disease. Front Aging Neurosci. 2020;10(12):292.
52. Asare-Werehene M, Nakka K, Reunov A, Chiu CT, Lee WT, Abedini MR, et al. The exosome-mediated autocrine and paracrine actions of plasma gelsolin in ovarian cancer chemoresistance. Oncogene. 2019;39(7):1600–16.
53. Zhang W, Ou X, Wu X. Proteomics profiling of plasma exosomes in epithelial ovarian cancer: a potential role in the coagulation cascade, diagnosis and prognosis. Int J Oncol. 2019;54(5):1719–33. https://doi.org/10.3892/ijo.2019.4742/abstract.
54. Schiewek J, Schumacher U, Lange T, Joosse SA, Wikman H, Pantel K, et al. Clinical relevance of cytoskeleton associated proteins for ovarian cancer. J Cancer Res Clin Oncol. 2018;144(11):2195–205. https://doi.org/10.1007/s00432-018-2710-9.
55. Wigglesworth K, Lee KB, Emori C, Sugiura K, Eppig JJ. Transcriptomic diversification of developing cumulus and mural granulosa cells in mouse ovarian follicles. Biol Reprod. 2015;92(1):23–4.
56. Delon I, Brown NH. Integrins and the actin cytoskeleton. Curr Opin Cell Biol. 2007;19(1):43–50.
57. Martino F, Perestrelo AR, Vinarský V, Pagliari S, Forte G. Cellular mechanotransduction: From tension to function. Front Physiol. 2018;9(JUL):824.
58. Ji H, Long V, Briody V, Chien EK. Progesterone modulates integrin α2 (ITGA2) and α11 (ITGA11) in the pregnant cervix. Reprod Sci. 2011;18(2):156–63.
59. Li Z, Jia J, Gou J, Tong A, Liu X, Zhao X, et al. Mmu-miR-126a-3p plays a role in murine embryo implantation by regulating Itga11. Reprod Biomed Online. 2015;31(3):384–93.
60. Samborski A, Graf A, Krebs S, Kessler B, Bauersachs S. Deep sequencing of the porcine endometrial transcriptome on day 14 of pregnancy. Biol Reprod. 2013;88(4):1–13.
61. Yang S, Zhou X, Pei Y, Wang H, He K, Zhao A. Identification of differentially expressed genes in porcine ovaries at proestrus and estrus stages using RNA-seq technique. Biomed Res Int. 2018. https://doi.org/10.1155/2018/9150723.
62. Grassot V, Da Silva A, Saliba J, Maftah A, Dupuy F, Petit JM. Highlights of glycosylation and adhesion related genes involved in myogenesis. BMC Genomics. 2014;15(1):1–17. https://doi.org/10.1186/1471-2164-15-621.
63. Chan JP, Lin P-C, Zhou S, Duffy DM, Lydon JP, Ko CJ. Progesterone receptor serves the ovary as a trigger of ovulation and a terminator of inflammation in brief. Cell Reports. 2020;31:107496. https://doi.org/10.1016/j.celrep.2020.03.060.
64. Nivet AL, Vigneault C, Blondin P, Sirard MA. Changes in granulosa cells' gene expression associated with increased oocyte competence in bovine. Reproduction. 2013;145(6):555–65.
65. Nio-Kobayashi J, Kudo M, Sakuragi N, Kimura S, Iwanaga T, Colin DW. Regulated C-C motif ligand 2 (CCL2) in luteal cells contributes to macrophage infiltration into the human corpus luteum during luteolysis. Mol Hum Reprod. 2015;21(8):645–54.
66. Zhai B, Liu H, Li X, Dai L, Gao Y, Li C, et al. BMP15 prevents cumulus cell apoptosis through CCL2 and FBN1 in porcine ovaries. Cell Physiol Biochem. 2013;32(2):264–78.
67. Kong DH, Kim YK, Kim MR, Jang JH, Lee S. Emerging roles of vascular cell adhesion molecule-1 (VCAM-1) in immunological disorders and cancer. Int J Mol Sci. 2018. https://doi.org/10.3390/ijms19041057.
68. Zhang D, Bi J, Liang Q, Wang S, Zhang L, Han F, et al. VCAM1 promotes tumor cell invasion and metastasis by inducing EMT and transendothelial migration in colorectal cancer. Front Oncol. 2020;10:1066.
69. Sommaggio R, Máñez R, Costa C. TNF, pig CD86, and VCAM-1 identified as potential targets for intervention in xenotransplantation of pig chondrocytes. Cell Trans. 2009;18(12):1381–93.
70. Kanmaz-Özer M, Vural P, Doru-Abbasolu S, Gedikbaşi A, Çil E, Karadağ B, et al. Polymorphisms of vascular cell adhesion molecule1 (VCAM1) in polycystic ovary syndrome determined by quantitative real-time polymerase chain reaction and melting curve analysis. Eur J Obstet Gynecol Reprod Biol. 2012;160(2):174–8.
71. Candelaria NR, Padmanabhan A, Stossi F, Cecilia Ljungberg M, Shelly KE, Pew BK, et al. VCAM1 is induced in ovarian theca and stromal cells in a mouse model of androgen excess. Endocrinology. 2019;160(6):1377–93.
72. Kulus J, Kulus M, Kranc W, Jopek K, Zdun M, Józkowiak M, et al. Transcriptomic profile of new gene markers encoding proteins responsible for structure of porcine ovarian granulosa cells. Biol. 2021;10(11):1214.
73. Parkes WS, Amargant F, Zhou LT, Villanueva CE, Duncan FE, Pritchard MT. Hyaluronan and collagen are prominent extracellular matrix components in bovine and porcine ovaries. Genes (Basel). 2021;12(8):1186.
74. Yao G, Kong Y, Yang G, Kong D, Xu Y, He J, et al. Lnc-GULP1–2:1 affects granulosa cell proliferation by regulating COL3A1 expression and

localization. J Ovarian Res. 2021;14(1):1–10. https://doi.org/10.1186/s13048-021-00769-1.
75. Kong Y, Yao G, He J, Yang G, Kong D, Sun Y. lncRNA LNC-GULP1-2:1 is involved in human granulosa cell proliferation by regulating COL3A1 gene. Fertil Steril. 2018;110(4):e320.
76. Engqvist H, Parris TZ, Kovács A, Nemes S, Werner Rönnerman E, De Lara S, et al. Immunohistochemical validation of COL3A1, GPR158 and PITHD1 as prognostic biomarkers in early-stage ovarian carcinomas. BMC Cancer. 2019;19(1):1–12. https://doi.org/10.1186/s12885-019-6084-4.
77. Januchowski R, Świerczewska M, Sterzyńska K, Wojtowicz K, Nowicki M, Zabel M. Increased expression of several collagen genes is associated with drug resistance in ovarian cancer cell lines. J Cancer. 2016;7(10):1295–310.
78. Ma S, Charron J, Erikson RL. Role of Plk2 (Snk) in mouse development and cell proliferation. Mol Cell Biol. 2003;23(19):6936–43. https://doi.org/10.1128/MCB.23.19.6936-6943.2003.
79. Li F, Jo M, Curry TE, Liu J. Hormonal induction of polo-like kinases (Plks) and impact of Plk2 on cell cycle progression in the rat ovary. PLoS ONE. 2012. https://doi.org/10.1371/annotation/1a9779fe-f0ab-4937-a3ce-1bc7fb0268df.
80. Chen D, Yu W, Aitken L, Gunn-Moore F, Amadoro G. Willin/FRMD6: a multi-functional neuronal protein associated with Alzheimer's disease. Cells. 2021;10(11):3024.
81. Dos Santos EC, Lalonde-Larue A, Antoniazzi AQ, Barreta MH, Price CA, Dias Gonçalves PB, et al. YAP signaling in preovulatory granulosa cells is critical for the functioning of the EGF network during ovulation. Mol Cell Endocrinol. 2022;541:111524.
82. Plewes MR, Hou X, Zhang P, Liang A, Hua G, Wood JR, et al. Yes-associated protein 1 is required for proliferation and function of bovine granulosa cells in vitro. Biol Reprod. 2019;101(5):1001.
83. Seo J, Kim J. Regulation of Hippo signaling by actin remodeling. BMB Rep. 2018;51(3):151.
84. Yang Q, Yu B, Sun J. TTK, CDC25A, and ESPL1 as prognostic biomarkers for endometrial cancer. Biomed Res Int. 2020. https://doi.org/10.1155/2020/4625123.
85. Jiang F, Kuang B, Que Y, Lin Z, Yuan L, Xiao W, et al. The clinical significance of transforming acidic coiled-coil protein 3 expression in non-small cell lung cancer. Oncol Rep. 2016;35(1):436–46.
86. Liu Y, Zhu K, Guan X, Xie S, Wang Y, Tong Y, et al. TTK is a potential therapeutic target for cisplatin-resistant ovarian cancer. J Ovarian Res. 2021;14(1):128.
87. Gui T, Yao C, Jia B, Shen K. Identification and analysis of genes associated with epithelial ovarian cancer by integrated bioinformatics methods. PLoS ONE. 2021. https://doi.org/10.1371/journal.pone.0253136.
88. Wissing ML, Kristensen SG, Andersen CY, Mikkelsen AL, Høst T, Borup R, et al. Identification of new ovulation-related genes in humans by comparing the transcriptome of granulosa cells before and after ovulation triggering in the same controlled ovarian stimulation cycle. Hum Reprod. 2014;29(5):997–1010.
89. Kulus M, Kranc W, Sujka-Kordowska P, Celichowski P, Konwerska A, Jankowski M, et al. Transcriptomic analysis of expression of genes regulating cell cycle progression in porcine ovarian granulosa cells during short-term in vitro primary culture. Histochem Cell Biol. 2020;153(6):397–412. https://doi.org/10.1007/s00418-020-01860-2.
90. Du X, Li Q, Yang L, Zeng Q, Wang S, Li Q. Transcriptomic data analyses reveal that sow fertility-related lincRNA NORFA Is essential for the normal states and functions of granulosa cells. Front Cell Dev Biol. 2021;23(9):227.
91. Yang F, Liu Q, Chen Y, Ye H, Wang H, Zeng S. Integrative proteomic and phosphoproteomic analyses of granulosa cells during follicular atresia in porcine. Front Cell Dev Biol. 2021;15(8):1722.
92. Pan B, Liu C, Zhan X, Li J. Protegrin-1 regulates porcine granulosa cell proliferation via the EGFR-ERK1/2/p38 signaling pathway in vitro. Front Physiol. 2021;21(12):733.
93. Zhou X, He Y, Li N, Bai G, Pan X, Zhang Z, et al. DNA methylation mediated RSPO2 to promote follicular development in mammals. Cell Death Dis. 2021;12(7):1–13.
94. Donadeu FX, Schauer SN, Sontakke SD. Involvement of miRNAs in ovarian follicular and luteal development. J Endocrinol. 2012;215(3):323–34.
95. Wang L, Chen Y, Wu S, Tang J, Chen G, Li F. miR-135a suppresses granulosa cell growth by targeting Tgfbr1 and Ccnd2 during Folliculogenesis in mice. Cells. 2021;10(8):2104.
96. Liu X, Gao Y, Lu Y, Zhang J, Li L, Yin F. Upregulation of NEK2 is associated with drug resistance in ovarian cancer. Oncol Rep. 2014;31(2):745–54. https://doi.org/10.3892/or.2013.2910/abstract.
97. Kokuryo T, Yokoyama Y, Yamaguchi J, Tsunoda N, Ebata T, Nagino M. NEK2 is an effective target for cancer therapy with potential to induce regression of multiple human malignancies. Anticancer Res. 2019;39(5):2251–8.
98. Liu Y, Pan B, Qu W, Cao Y, Li J, Zhao H. Systematic analysis of the expression and prognosis relevance of FBXO family reveals the significance of FBXO1 in human breast cancer. Cancer Cell Int. 2021;21(1):1–25. https://doi.org/10.1186/s12935-021-01833-y.
99. Reimann JDR, Freed E, Hsu JY, Kramer ER, Peters JM, Jackson PK. Emi1 is a mitotic regulator that interacts with Cdc20 and inhibits the anaphase promoting complex. Cell. 2001;105(5):645–55.
100. Zhang L, Du X, Wei S, Li D, Li Q. A comprehensive transcriptomic view on the role of SMAD4 gene by RNAi-mediated knockdown in porcine follicular granulosa cells. Reproduction. 2016;152(1):81–9.
101. Zhao L, Xue S, Yao Z, Shi J, Chen B, Wu L, et al. Biallelic mutations in CDC20 cause female infertility characterized by abnormalities in oocyte maturation and early embryonic development. Protein Cell. 2020;11(12):921–7. https://doi.org/10.1007/s13238-020-00756-0.
102. Lu CL, Yan ZQ, Song XL, Xu YY, Zheng XY, Li R, et al. Effect of exogenous gonadotropin on the transcriptome of human granulosa cells and follicular fluid hormone profiles. Reprod Biol Endocrinol. 2019. https://doi.org/10.1186/s12958-019-0489-4.
103. Liu Y, Zhu K, Guan X, Xie S, Wang Y, Tong Y, et al. TTK is a potential therapeutic target for cisplatin-resistant ovarian cancer. J Ovarian Res. 2021;14(1):128.
104. Gui T, Yao C, Jia B, Shen K. Identification and analysis of genes associated with epithelial ovarian cancer by integrated bioinformatics methods. PLoS ONE. 2021. https://doi.org/10.1371/journal.pone.0253136.
105. Huo Q, Chen S, Li Z, Wang J, Li J, Xie N. Inhibiting of TACC3 promotes cell proliferation, cell invasion and the EMT pathway in breast cancer. Front Genet. 2021;3(12):649.
106. Hou PF, Jiang T, Chen F, Shi PC, Li HQ, Bai J, et al. KIF4A facilitates cell proliferation via induction of p21-mediated cell cycle progression and promotes metastasis in colorectal cancer. Cell Death Dis. 2018;9(5):1–15.
107. Chen X, Huo L, Ren L, Li Y, Sun Y, Li Y, et al. Polycystic ovary syndrome is associated with elevated periostin levels. Exp Clin Endocrinol Diabetes. 2019;127(9):571–7.
108. Hassani F, Oryan S, Eftekhari-Yazdi P, Bazrgar M, Moini A, Nasiri N, et al. Downregulation of extracellular matrix and cell adhesion molecules in cumulus cells of infertile polycystic ovary syndrome women with and without insulin resistance. Cell J. 2019;21(1):35–42.
109. Henning NF, LeDuc RD, Even KA, Laronda MM. Proteomic analyses of decellularized porcine ovaries identified new matrisome proteins and spatial differences across and within ovarian compartments. Sci Rep. 2019. https://doi.org/10.1038/s41598-019-56454-3.
110. Palani S, Ghosh S, Ivorra-Molla E, Clarke S, Suchenko A, Balasubramanian MK, et al. Calponin-homology domain mediated bending of membrane-associated actin filaments. Elife. 2021. https://doi.org/10.7554/eLife.61078.
111. Trejter M, Hochol A, Tyczewska M, Ziolkowska A, Jopek K, Szyszka M, et al. Sex-related gene expression profiles in the adrenal cortex in the mature rat: microarray analysis with emphasis on genes involved in steroidogenesis. Int J Mol Med. 2015;35(3):702–14.
112. Chamier-Gliszczyńska A, Brązert M, Sujka-Kordowska P, Popis M, Ożegowska K, Stefańska K, et al. Genes involved in angiogenesis and circulatory system development are differentially expressed in porcine epithelial oviductal cells during long-term primary in vitro culture—a transcriptomic study. Med J Cell Biol. 2018;6(4):163–73.
113. Nawrocki MJ, Celichowski P, Jankowski M, Kranc W, Bryja A, Borys-Wójcik S, et al. Ontology groups representing angiogenesis and blood vessels development are highly up-regulated during porcine oviductal epithelial cells long-term real-time proliferation—a primary cell culture approach. Med J Cell Biol. 2018;6(4):186–94.

114. Stefańska K, Chamier-Gliszczyńska A, Jankowski M, Celichowski P, Kulus M, Rojewska M, et al. Epithelium morphogenesis and oviduct development are regulated by significant increase of expression of genes after long-term in vitro primary culture—a microarray assays. Med J Cell Biol. 2018;6(4):195–204.
115. Kranc W, Brązert M, Ożegowska K, Budna-Tukan J, Celichowski P, Jankowski M, et al. Response to abiotic and organic substances stimulation belongs to ontologic groups significantly up-regulated in porcine immature oocytes. Med J Cell Biol. 2018. https://doi.org/10.2478/acb-2018-0015.
116. Borys-Wójcik S, Kocherova I, Celichowski P, Popis M, Jeseta M, Bukowska D, et al. Protein oligomerization is the biochemical process highly up-regulated in porcine oocytes before in vitro maturation (IVM). Med J Cell Biol. 2018;6(4):155–62.
117. Budna J, Celichowski P, Bryja A, Jeseta M, Jankowski M, Bukowska D, et al. Expression changes in fatty acid metabolic processrelated genes in porcine oocytes during in vitro maturation. Med J Cell Biol. 2018;6(2):48–54.
118. Extract and Visualize the Results of Multivariate Data Analyses [R package factoextra version 1.0.7] | Semantic Scholar. https://www.semanticscholar.org/paper/Extract-and-Visualize-the-Results-of-Multivariate-Kassambara-Mundt/5cb503e3db8609405d9f286fadc2a8bb867e5b6e. Accessed 5 Mar 2023.
119. Golkar-Narenji A, Antosik P, Nolin S, Rucinski M, Jopek K, Zok A, et al. Gene ontology groups and signaling pathways regulating the process of avian satellite cell differentiation. Genes (Basel). 2022;13(2):242.
120. Bader GD, Hogue CWV. An automated method for finding molecular complexes in large protein interaction networks. BMC Bioinformatics. 2003;4(1):1–27. https://doi.org/10.1186/1471-2105-4-2.

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4.3 Publikacja III

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New Gene Markers of Exosomal Regulation Are Involved in Porcine Granulosa Cell Adhesion, Migration, and Proliferation

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Abstract: Exosomal regulation is intimately involved in key cellular processes, such as migration, proliferation, and adhesion. By participating in the regulation of basic mechanisms, extracellular vesicles are important in intercellular signaling and the functioning of the mammalian reproductive system. The complexity of intercellular interactions in the ovarian follicle is also based on multilevel intercellular signaling, including the mechanisms involving cadherins, integrins, and the extracellular matrix. The processes in the ovary leading to the formation of a fertilization-ready oocyte are extremely complex at the molecular level and depend on the oocyte's ongoing relationship with granulosa cells. An analysis of gene expression from material obtained from a primary in vitro culture of porcine granulosa cells was employed using microarray technology. Genes with the highest expression (LIPG, HSD3B1, CLIP4, LOX, ANKRD1, FMOD, SHAS2, TAGLN, ITGA8, MXRA5, and NEXN) and the lowest expression levels (DAPL1, HSD17B1, SNX31, FST, NEBL, CXCL10, RGS2, MAL2, IHH, and TRIB2) were selected for further analysis. The gene expression results obtained from the microarrays were validated using quantitative RT-qPCR. Exosomes may play important roles regarding intercellular signaling between granulosa cells. Therefore, exosomes may have significant applications in regenerative medicine, targeted therapy, and assisted reproduction technologies.

Keywords: porcine granulosa cells; cellular signaling; extracellular vesicles; cell adhesion; cell migration and proliferation; transcriptomics; extracellular matrix

1. Introduction

In oocyte maturation during oogenesis, granulosa cells (GCs) are necessary and surround the oocyte, interacting with it in numerous ways [1]. Within the granulosa cells found in the ovarian follicle, there are mural granulosa cells (mGCs), which occur at the periphery of the ovarian follicle and are closely associated with steroidogenesis and ovulation [2]. The second group of granulosa cells—namely, cumulus cells (CCs)—are those that are in direct

contact with and surround the oocyte, forming close multiple intercellular connections with the oocyte [2]. These connections are of the gap junction type (nexus type), allowing, among other things, ion exchange [3]. Granulosa cells are responsible for the maturation of the oocyte, although they are also responsible for meiotic arrest through the regulation of cAMP levels. [4]. The adequate pool of these cells in the ovarian follicle depends on their division and proliferation. The proliferation of granulosa cells depends on a number of factors that are involved in the activation of signaling pathways, e.g., EGFR, PDGF, VEGF, TGF-β, MAPK, FAK/AKT, and ERK. Some of these factors have been well-known for years [5], but with active research, newer ones are being described, such as Procr (Protein C receptor) [6], Protegrin-1 [7], and the KAT2B gene [8]. The current direction of research should focus on a multifaceted view of cell signaling and its effects on the proliferation and migration of GCs. Moreover, properly functioning granulosa cells require efficient intercellular signaling, which involves the integrins, cadherins, and the extracellular matrix (ECM) that constitute the microenvironment for them [9]. The present study shows the upregulation of ontology groups of genes related to the effects of integrins on cell adhesion and the activation of signaling pathways. Integrins affect signaling dictated by Rho GTPase, which is involved in cytokinesis and cell migration [10]. In addition, the cytoskeleton, which is a dynamic structure in terms of composition and structure, also significantly affects intercellular signaling [11]. The GTPase RhoA is involved in the process of cytoskeleton change [12].

The upregulation of the vesicle-mediated transport ontology group demonstrated in this article suggests an important role for extracellular vesicles (EVs) in the activity of granulosa cells, especially in intercellular signaling. EVs are structures with a lipid membrane released outside the cell. They provide a carrier for proteins, RNAs, mRNAs, and microRNAs while being heavily involved in cell signaling. They are formed by budding or intracellular endocytic trafficking [13]. The cytoskeleton is also involved in EV secretion, which requires polymerization of actin located under the cytoplasmic membrane, allowing budding and the release of vesicles outside the cell [13]. Exosomes, belonging to extracellular vesicles, take direct and indirect roles in intercellular signaling and have been shown to play an important role in the functioning of the reproductive system [14–17]. These nanoparticles, through their involvement in the regulation of cell morphology, can promote cell adhesion [18]. In addition, exosomes transporting protein molecules released from cells have been suggested to promote cell migration, as described in inflammatory processes [19]. Recent reports have also indicated that exosomes are actively involved in the processes of proliferation [20] and the response to hypoxia [21]. Exosomes affect the composition of the ECM (through its remodeling), but, at the same time, the ECM affects the release of exosomes from the cell [22]. The ECM's and exosomes' formation and composition are important for the signaling pathways that take place in them, the passage of nutrients and hormones, and the initiation of many cellular mechanisms (migration and cell division) [23,24].

The success of the cell cycle requires interaction in multiple fields, both in the intracellular and extracellular environment. For this purpose, it is necessary to maintain proper interactions between the cytoskeleton and the extracellular matrix while varying cell adhesion and proliferation [25]. Microtubules are involved in the formation of the karyokinetic spindle [26], while intermediate filaments show an important role in cell adhesion and interaction with other components of the cytoskeleton [27]. The link between the cell cycle and cell adhesion has been confirmed through integrin receptors, which, by connecting the cell to the ECM, lead to the activation of a cell cycle signaling pathway progression, particularly the G1/S phase transition [28].

The interaction of the ECM, the cytoskeleton, and the release of EVs during the cell cycle affects the proper functioning of cells in terms of cell signaling, adhesion, proliferation, migration, and division [9–11,19,20,23–25,29]. These interactions on a molecular basis within GCs are not very well understood. Therefore, the goal of the current study was to investigate the expression profile of genes involved in the regulation of cell adhesion, mi-

gration, proliferation, and wound healing in porcine granulosa cells, as these are processes associated with exosomes' formation and composition.

2. Results

The porcine granulosa cells were collected at four time points, representing different stages of a short-term in vitro culture: 0 h (serving as an ex vivo reference); 48 h (representing the initial in-vitro-associated changes in culture); 96 h (an assumed "point of loss" of most of the cell's physiological properties); and 144 h (the end point of the short-term culture). Obtaining information on the level and direction of gene expression in culture provides important new data regarding dynamic changes in the cell population. The transcriptomic profile of gene expression was compared to the control group (0 h). The general profile of the transcriptome changes is shown in Figure 1, where dots represent the mean gene expression. With respect to the assumed cut-off criteria for differentially expressed genes (|fold change| = 2, and p value < 0.05), we demonstrated 610 upregulated and 827 downregulated genes in the 48 h vs. 0 h comparison, 1104 upregulated and 1206 downregulated genes in the 96 h vs. 0 h comparison, and 731 upregulated and 1025 downregulated genes in the 144 vs. 0 h comparison. In the 48 h vs. 0 h comparison, the genes with the highest fold change of expression included: LOX, POSTN, ITGA2, HSD3B1, and CLIP4. In the 96 h vs. 0 h comparison, the most downregulated genes were DAPL1 and HSD17B1, with overexpression of LOX, LIPG, and ANKRD1 genes. In the 144 h vs. 0 h comparison, we observed decreased expression of DAPL1 and HSD17B1 and increased expression of POSTN, HSD3B1, and LIPG genes.

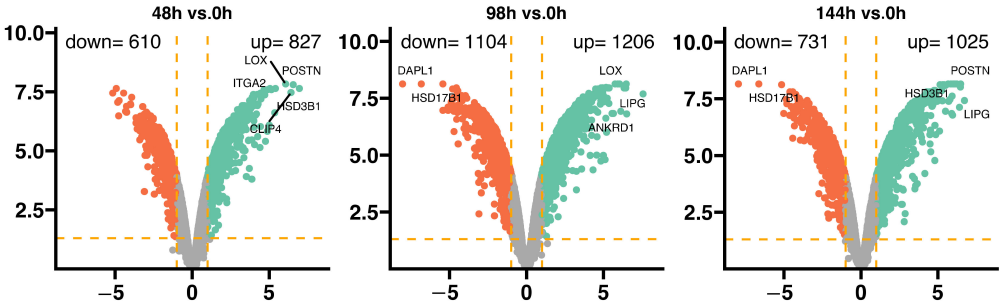

Figure 1. General expression profiles visualized as volcano plots. Each dot represents the mean expression (two biological replicates) of an individual gene obtained from a normalized microarray study. The orange dotted lines (cut-off values) were established according to the following parameters: |fold change| = 2 and p value = 0.05. Genes above the cut-off lines were considered as differentially expressed genes and are shown as red (downregulated) and green (upregulated) dots. The total numbers of upregulated and downregulated genes are given in the top right and top left corners, respectively. The symbols of the five most differentially expressed genes from each comparison are marked on the plots.

A principal component analysis (PCA) was performed to show similarities and differences in the analyzed transcriptomic profiles of the studied groups (Figure 2). PCA analysis showed a very strong separation of the studied groups, where the first component (Dim1) explained 88.8% of the differences between the groups. The 0 h and 48 h groups were considerably separated from the others, while the 96 h and 144 h groups were distinctly separate. The Venn diagram illustrates that many genes overlapped between the compared experimental conditions, and 548 genes were upregulated and 462 were downregulated in comparison to the control group, regardless of the duration of the cultivation.

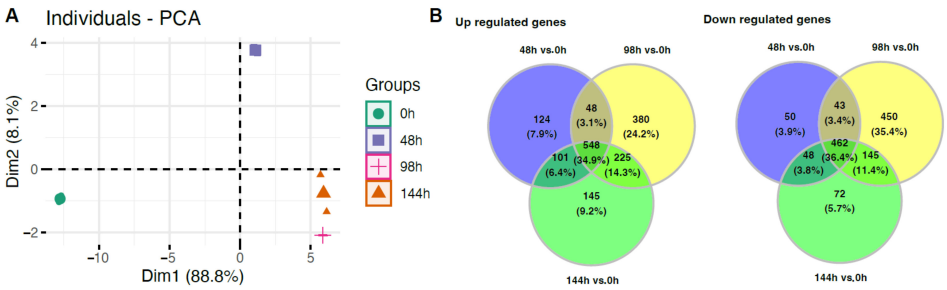

Figure 2. (**A**) Principal component analysis (PCA) plot of the first two components of the filtered microarray data set. (**B**) Venn diagrams indicating common upregulated and downregulated genes in all analyzed groups.

The fold change values of the top ten upregulated genes in the 48 h vs. 0 h comparison (Figure 3) ranged from 124.46 to 29.91. The fold change values of the top ten downregulated genes in the 48 h vs. 0 h comparison ranged from −16.08 to −35.75. The ten genes with enhanced expression in the 48 h vs. 0 h comparison were: hydroxy-delta-5-steroid dehydrogenase 3-beta and steroid delta-isomerase 1 (HSD3B1); periostin (POSTN); CAP-GLY domain containing linker protein family member 4 (CLIP4); Lysol oxidase (LOX); integrin alpha 2 (ITGA2); serpin protease inhibitor clade B (ovalbumin) member 2 (SERPINB2); fibronectin 1 (FN1); laminin beta 1 (LAMB1); hyaluronian synthase 2 (SHAS2); and integrin beta 3 (ITGB3). The ten downregulated genes in the axis cells compared to the controls were: phosphodiesterase 7B (PDE7B); synaptotagmin X (SYT10); Rh family B glycoprotein (RHBG); Indian hedgehog (IHH); mal T-cell differentiation protein 2 (MAL2); nebulette (NEBL); chemokine (C-X-C motif) ligand 10 (CXCL10); death associated protein-like 1 (DAPL1); sorting nexin 31 (SNX31); and hydroxysteroid (17-beta) dehydrogenase 1 (HSD17B1).

The fold change values of the top ten upregulated genes in the 96 h vs. 0 h comparison (Figure 4) ranged from 190.61 to 50.19. The fold change values of the top ten downregulated genes in the 96 h vs. 0 h comparison ranged from −28.00 to −265.08. The ten genes with enhanced expression in the 96 h vs. 0 h comparison were: Lipase (LIPG); ankyrin repeat domain 1 (ANKRD1); lysyl oxidase (LOX); nexin (NEXN); hydroxy-delta-5−steroid dehydrogenase 3-beta and steroid delta-isomerase 1 (HSD3B1); hyaluronian synthase 2 (SHAS2); fibronectin 1 (FN1); laminin beta 1 (LAMB1); transgelin (TAGLN); and matrix-remodelling associated 5 (MXRA5). The ten downregulated genes in the 96 h vs. 0 h comparison were: Tribbles pseudokinase 2 (TRIB2); pyruvate dehydrogenase kinase isozyme 4 (PDK4); regulator of G-protein signaling 2 (RGS2); thioredoxin interacting protein (TXNIP); cyclin E2 (CCNE2); chemokine (C-X-C motif) ligand 10 (CXCL10); follistatin (FST); sortin nexin 31 (SNX31); hydroxysteroid (17-beta) dehydrogenase 1 (HSD17B1); and death associated protein-like 1 (DAPL1).

The fold change values of the top ten upregulated genes in the 144 h vs. 0 h comparison (Figure 5) ranged from 105.90 to 59.54. The fold change values of the top ten downregulated genes in the 144 h vs. 0 h comparison ranged from −21.64 to −247.18. The ten genes with overexpression in the 144 h vs. 0 h comparison were: Lipase (LIPG); periostin (POSTN); hydroxy-delta-5-steroid dehydrogenase 3−beta and steroid delta-isomerase 1 (HSD3B1); fibromodulin (FMOD); lysyl oxidase (LOX); fibronectin 1 (FN1); decorin (DCN); hyaluronian synthase 2 (SHAS2); CAP-GLY domain containing linker protein family member 4 (CLIP4); and integrin alpha 8 (ITGA8).

48h vs. 0h

Gene symbol	Gene name	Fold change
HSD3B1	hydroxy–delta–5–steroid dehydrogenase, 3 beta– and steroid delta–isomerase 1	124.46
POSTN	periostin, osteoblast specific factor	95.23
CLIP4	CAP–GLY domain containing linker protein family, member 4	85.59
LOX	lysyl oxidase	67.34
ITGA2	integrin, alpha 2 (CD49B, alpha 2 subunit of VLA–2 receptor)	42.68
SERPINB2	serpin peptidase inhibitor, clade B (ovalbumin), member 2	41.25
FN1	fibronectin 1	35.38
LAMB1	laminin, beta 1	33.30
SHAS2	hyaluronan synthase 2	32.19
ITGB3	integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	29.91
PDE7B	phosphodiesterase 7B	−16.08
SYT10	synaptotagmin X	−19.17
RHBG	Rh family, B glycoprotein	−19.28
IHH	indian hedgehog	−20.09
MAL2	mal, T–cell differentiation protein 2	−21.75
NEBL	nebulette	−26.72
CXCL10	chemokine (C–X–C motif) ligand 10	−27.26
DAPL1	death associated protein–like 1	−28.69
SNX31	sorting nexin 31	−30.30
HSD17B1	hydroxysteroid (17–beta) dehydrogenase 1	−35.75

Figure 3. List of the top 20 genes with the highest (10 genes) and lowest (10) expression fold change between 48 h and 0 h of the cells' cultivation.

98h vs. 0h

Gene symbol	Gene name	Fold change
LIPG	lipase, endothelial	190.61
ANKRD1	ankyrin repeat domain 1 (cardiac muscle)	91.18
LOX	lysyl oxidase	88.95
NEXN	nexilin (F actin binding protein)	87.60
HSD3B1	hydroxy–delta–5–steroid dehydrogenase, 3 beta– and steroid delta–isomerase 1	80.52
SHAS2	hyaluronan synthase 2	69.65
FN1	fibronectin 1	66.17
LAMB1	laminin, beta 1	60.55
TAGLN	transgelin	53.77
MXRA5	matrix–remodelling associated 5	50.19
TRIB2	tribbles pseudokinase 2	−28.00
PDK4	pyruvate dehydrogenase kinase, isozyme 4	−29.19
RGS2	regulator of G–protein signaling 2	−29.73
TXNIP	thioredoxin interacting protein	−34.10
CCNE2	cyclin E2	−34.23
CXCL10	chemokine (C–X–C motif) ligand 10	−36.04
FST	follistatin	−43.04
SNX31	sorting nexin 31	−43.14
HSD17B1	hydroxysteroid (17–beta) dehydrogenase 1	−113.74
DAPL1	death associated protein–like 1	−265.08

Figure 4. List of the top 20 genes with the highest (10 genes) and lowest (10) expression fold change between 96 h and 0 h of the cells' cultivation.

144h vs. 0h

Gene symbol	Gene name	Fold change
LIPG	lipase, endothelial	105.90
POSTN	periostin, osteoblast specific factor	88.95
HSD3B1	hydroxy–delta–5–steroid dehydrogenase, 3 beta– and steroid delta–isomerase 1	88.08
FMOD	fibromodulin	84.85
LOX	lysyl oxidase	75.58
FN1	fibronectin 1	68.74
DCN	decorin	63.38
SHAS2	hyaluronan synthase 2	62.22
CLIP4	CAP–GLY domain containing linker protein family, member 4	60.71
ITGA8	integrin, alpha 8	59.54
TGFBR3	transforming growth factor, beta receptor III	–21.64
ITM2A	integral membrane protein 2A	–23.29
MAL2	mal, T–cell differentiation protein 2	–24.61
KCNN2	potassium channel, calcium activated intermediate/small conductance subfamily N alpha, member 2	–24.76
RGS2	regulator of G–protein signaling 2	–28.29
NEBL	nebulette	–28.79
CXCL10	chemokine (C–X–C motif) ligand 10	–32.28
SNX31	sorting nexin 31	–35.74
HSD17B1	hydroxysteroid (17–beta) dehydrogenase 1	–99.00
DAPL1	death associated protein–like 1	–247.18

Figure 5. List of the top 20 genes with the highest (10 genes) and lowest (10) expression fold change between 144 h and 0 h of the cells' cultivation.

The ten downregulated genes in the 144 h vs. 0 h comparison were: Transforming growth factor beta receptor III (TGFBR3); integral membrane protein 2A (ITM2A); mal T-cell differentiation protein 2 (MAL2); potassium channel, calcium activated intermediate/small conductance subfamily N alpha member 2 (KCNN2); regulator of G−protein signaling 2 (RGS2); nebulette (NEBL); chemokine (C-X-C motif) ligand 10 (CXCL10); sorting nexin 21 (SNX31); hydroxysteroid (17-beta) dehydrogenase 1 (HSD17B1); and death associated protein-like 1 (DAPL1).

In conclusion, commonly overexpressed genes for all the analyzed groups were: HSD3B1, LOX, FN1, and SHAS2. Meanwhile, inhibited expression was observed in all groups for CXCL10, DAPL1, and SNX31 in comparison to the control.

Further analysis of the enrichment in the relevant ontological groups was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) bioinformatics tool with the GO BP Direct database (Figure 6).

The analysis revealed 46 ontological groups. For all analyzed groups, some similarities in patterns in the gene expression profile were revealed between groups in comparison to the control. The downregulated genes were responsible for inhibition processes, such as cell division, mitotic cell cycle, and mitotic sister chromatid segregation. Meanwhile, upregulated genes comparable in all three groups were angiogenesis, cell adhesion, cell-cell adhesion, cell-matrix adhesion, cellular response to transforming growth factor beta stimulus, collagen fibril organization, endodermal cell differentiation, heart development, integrin-mediated signaling pathway, negative regulation of apoptotic process, positive regulation of angiogenesis, positive regulation of cell migration, response to hypoxia, and wound healing.

The relevant GO ontological groups with adjusted *p*-values below 0.05 and N per group > 2 are presented as a bubble in Figure 6. The analysis of the expression patterns in the 48 h group in comparison to the control revealed a total of twenty-two upregulated and seven downregulated GO BP terms. Meanwhile, in the 96 h group, we showed that twenty GO BP terms were activated and six terms were inhibited. The highest number of activated GO BP terms (29 terms) was observed at 144 h, with six inhibited terms.

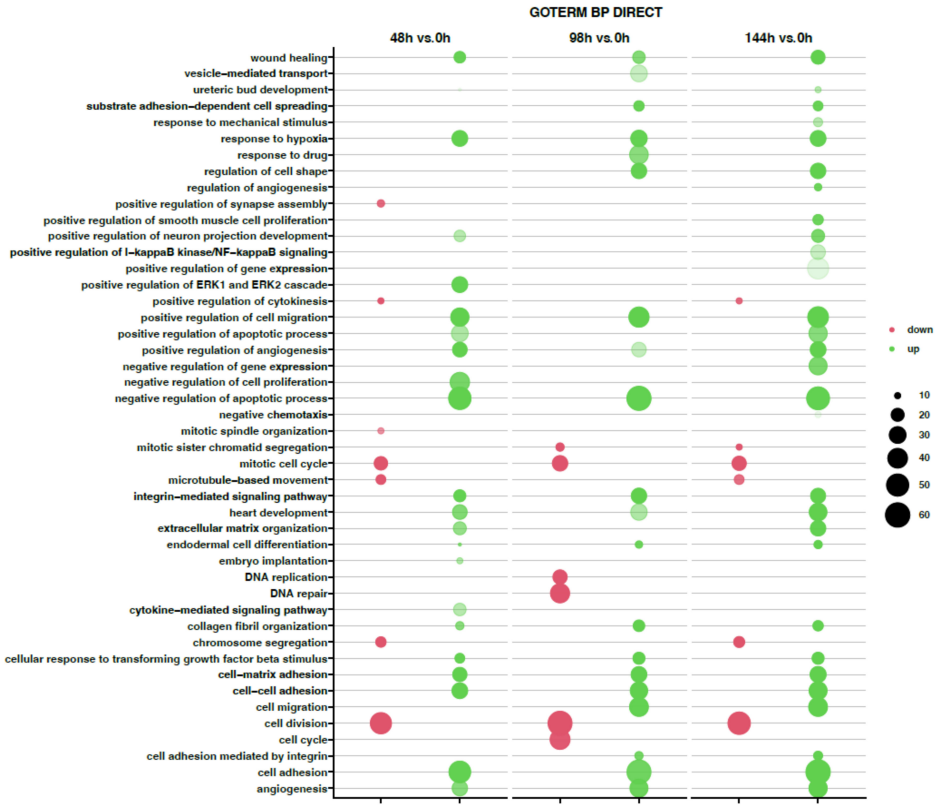

Figure 6. Bubble plot of overrepresented gene sets in DAVID GO PB DIRECT annotations database obtained from comparisons in gene expression profiles between 48 h, 96 h, and 144 h vs. control (0 h). The graph shows only the GO groups above the established cut-off criteria (p with correction < 0.05, a minimal number of genes per group >2). The size of each bubble reflects the number of differentially expressed genes assigned to the GO BP terms. The intensity of the bubble's transparency displays a *p*-value (more transparent indicates closer to the $p = 0.05$ cut-off value). The green bubbles indicate overexpressed genes, and the red bubbles indicate downregulated genes.

Hierarchic clustering of differentially expressed genes in all analyzed groups has been shown as a heatmap and presented in Figure 7. Genes belonging to the first seven most significantly enriched ontological groups (lowest adjusted *p*-value) are shown as dark squares. Expression values are scaled by rows and presented as colors and ranges. As observed, the expression of all analyzed genes decreased according to the time of the experiments. In accordance with previous results, most genes, regardless of the time of the experiment, were assigned to the cell division and cell cycle GO terms.

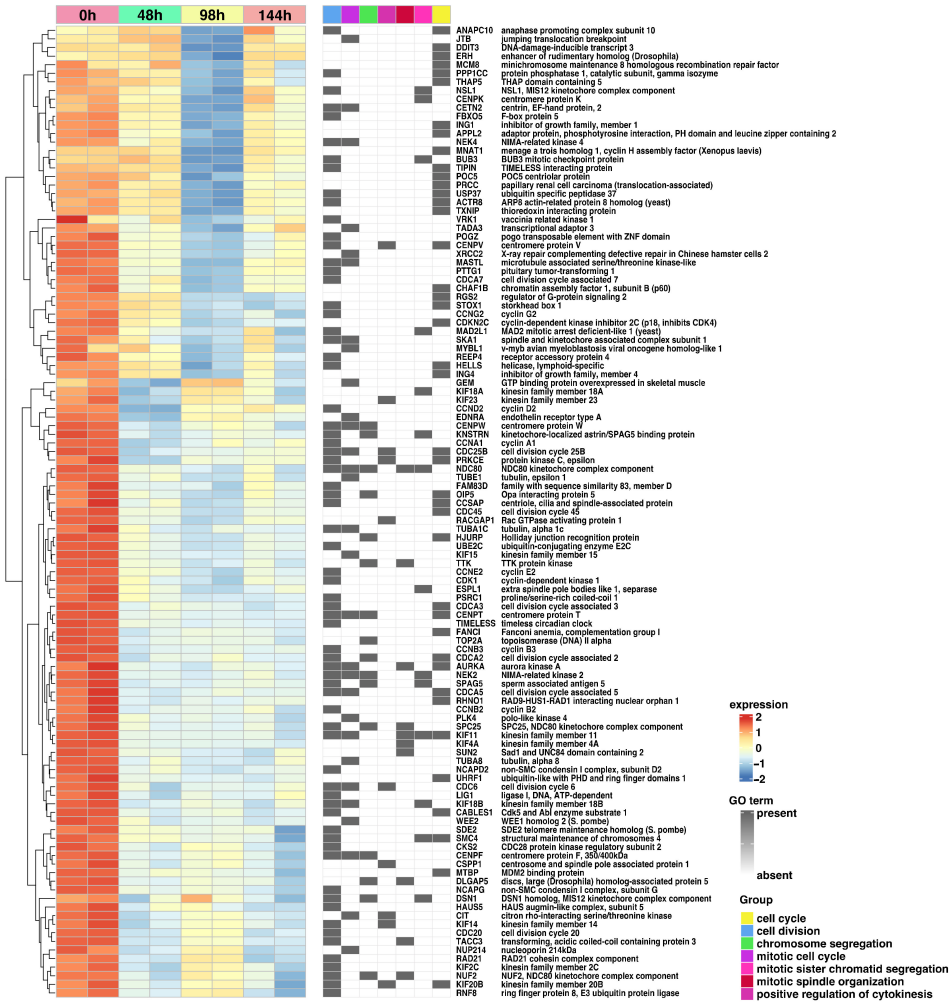

Figure 7. Heatmap with hierarchic clustering of differentially expressed genes in all analyzed groups. Genes belonging to the first seven most significantly enriched ontological groups (lowest adjusted *p*-value) are shown as dark squares. Expression values are scaled by rows and presented as colors and range from red (high expression) to yellow (moderate) to blue (low expression).

Next, powerful bioinformatic tools, such as the Gene Set Enrichment Analysis (GSEA), were used to confirm the obtained results. The GSEA was performed for the 48/0 h, 96/0 h, and 144/0 h experimental groups. The normalized expression level data from the microarray were uploaded to the software and allowed us to generate the list of significantly represented terms from the Hallmark database software version 4.1.2 (BioConductor software, Boston, MA, USA).

The strongest enriched term in the comparison between 48 h, 144 h, and 0 h referred to "wound healing." Meanwhile, the strongest enriched term in the comparison between 96 h and 0 h referred to "gastrulation." This means that the expression of those terms was

higher in the analyzed groups in comparison to controls. Detailed results of this analysis are presented in Figures 8–10. Despite a different methodological approach, the GSEA analysis presented relatively similar groups, as shown in the analysis of ontological groups by DAVID. This group's enriched terms strictly related to the cell cycle pathway, such as wound healing, extracellular matrix organization, and cell-matrix adhesion.

Quantitative RT-qPCR was used to validate the results from the microarray expression. Results for 11 selected genes are presented as a bar graph (Figure 11). The differences in gene expression shown in Figure 11 are due to the greater sensitivity of RT-qPCR than microarray expression methods.

The analyses focused on cellular processes, such as the migration, adhesion, and proliferation of granulosa cells. Extracellular vesicles, mainly exosomes, which represent a form of intercellular signaling based on exocytary release, have been shown to play an important role in these processes. Interestingly, increased expression of genes belonging to the "vesicle—mediated transport" ontological group was demonstrated, indicating an important role for this type of intercellular signaling in cultured granulosa cells.

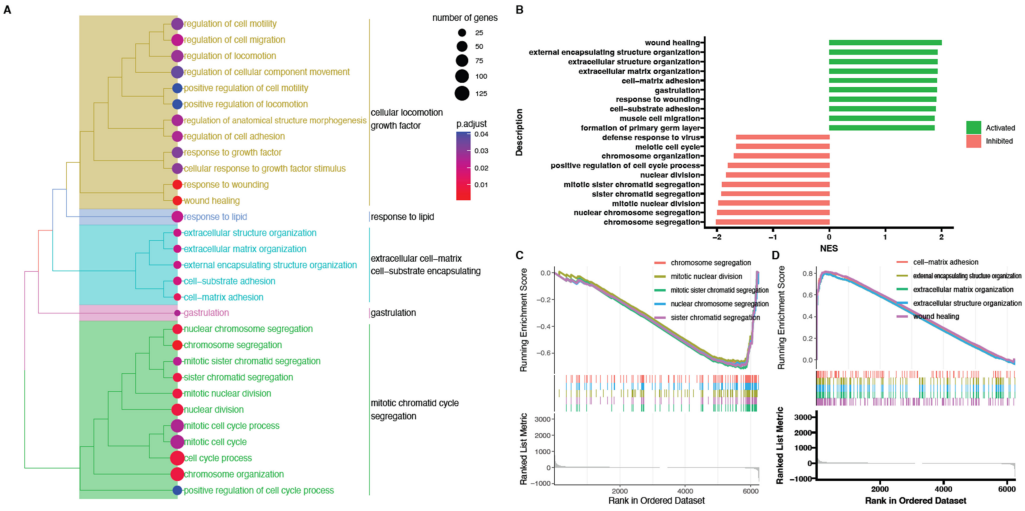

Figure 8. Gene set enrichment analysis (GSEA) cells in 48 h cultivation compared to control (0 h). (**A**) Clusterization of enriched gene sets into common functional clusters. Each cluster is marked with a different color. (**B**) Bar plot with the ten most activated and inhibited gene terms according to the normalized enrichment score (NES) values. (**C**) Detailed enrichment plots for the five most inhibited gene sets showing the profile of the running ES score and positions of genes on the rank-ordered list. (**D**) Detailed enrichment plots for the five most activated gene sets showing the profile of the running ES score and positions of genes on the rank-ordered list.

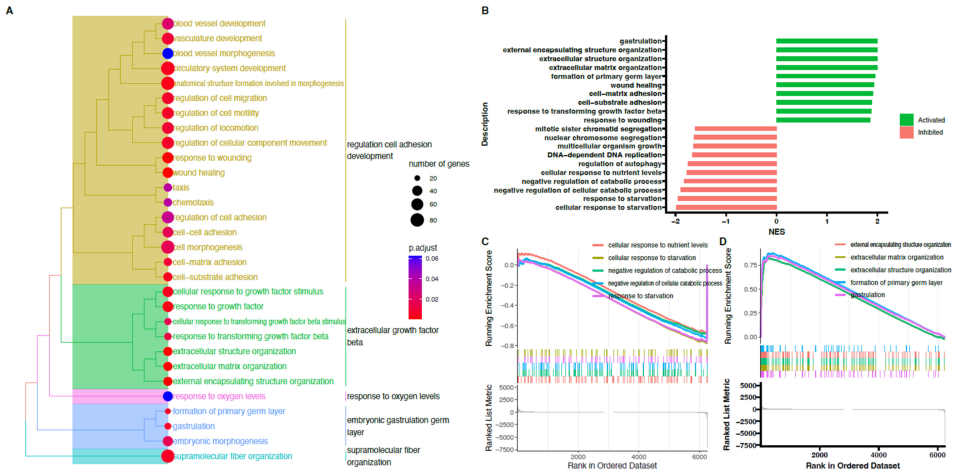

Figure 9. Gene set enrichment analysis (GSEA) cells in 96 h cultivation compared to control (0 h). (**A**) Clusterization of enriched gene sets into common functional clusters. Each cluster is marked with a different color. (**B**) Bar plot with the ten most activated and inhibited gene terms according to the normalized enrichment score (NES) values. (**C**) Detailed enrichment plots for the five most inhibited gene sets showing the profile of the running ES score and positions of genes on the rank-ordered list. (**D**) Detailed enrichment plots for the five most activated gene sets showing the profile of the running ES score and positions of genes on the rank-ordered list.

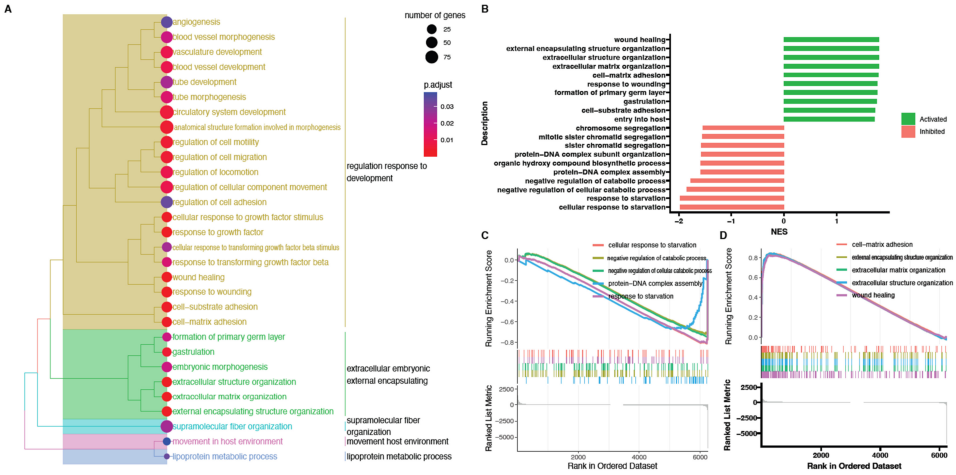

Figure 10. Gene set enrichment analysis (GSEA) cells in 144 h cultivation compared to control (0 h). (**A**) Clusterization of enriched gene sets into common functional clusters. Each cluster is marked with a different color. (**B**) Bar plot with the ten most activated and inhibited gene terms according to the normalized enrichment score (NES) values. (**C**) Detailed enrichment plots for the five most inhibited gene sets showing the profile of the running ES score and positions of genes on the rank-ordered list. (**D**) Detailed enrichment plots for the five most activated gene sets showing the profile of the running ES score and positions of genes on the rank-ordered list.

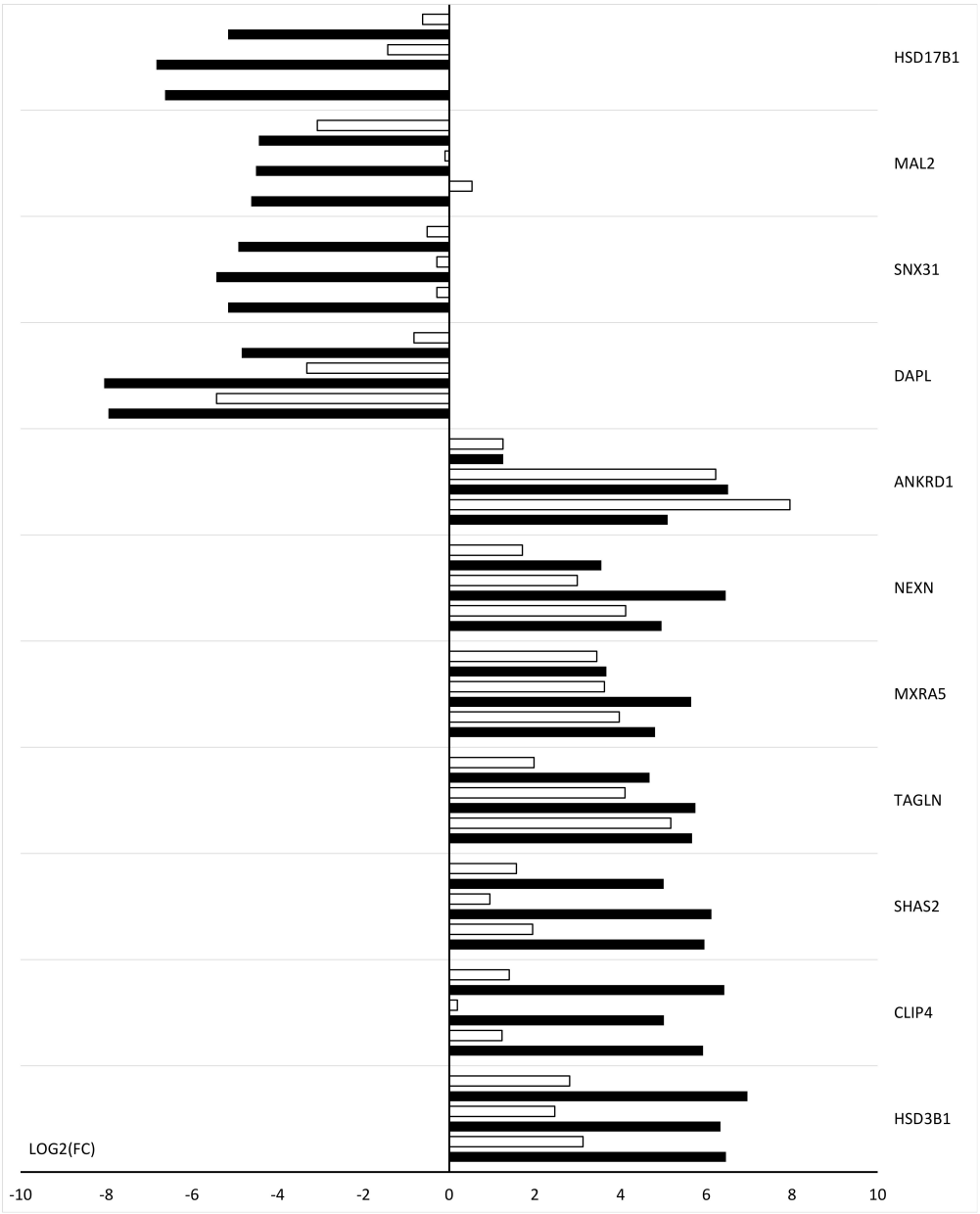

Figure 11. Bar graph showing the microarray validation results obtained by RT-qPCR. The black bar indicates the results of microarray expressions; the white bar indicates the results of RT-qPCR.

3. Discussion

The granulosa cells' function in steroidogenesis, folliculogenesis, and oogenesis requires proper intercellular signaling (both physical and chemical). The ECM, cytoskeleton, transmembrane proteins, and multiple signaling pathways are involved in this signaling. It is worth noting that extracellular vesicles are also important in this context, with their role in cell adhesion, cell-to-cell adhesion, migration, and proliferation [9–11,19,20,23–25,29]. The demonstrated elevated expression of genes mainly related to the processes of migration, proliferation, and granulosa cell adhesion clearly suggests that exosomes are important in these processes. Exosomal influence on the molecular level is not completely understood. A thorough understanding of these mechanisms and the messengers involved can be used in assisted reproductive techniques (ART) and in the treatment of ovarian disorders, such as PCOS (polycystic ovary syndrome) and POI (premature ovarian insufficiency). In addition, given the elevated expression of genes included in the wound healing ontology group in granulosa cells and the previously demonstrated potential for stemness [30,31], as well as the important role of exosomes in tissue regeneration [32], further research linking these aspects is needed. In this study, eleven genes with increased expression (LIPG, HSD3B1, CLIP4, LOX, ANKRD1, FMOD, SHAS2, TAGLN, ITGA8, MXRA5, and NEXN), ten with decreased expression (DAPL1, HSD17B1, SNX31, FST, NEBL, CXCL10, RGS2, MAL2, IHH, and TRIB2), and selected ontology groups were chosen for further analysis.

Cells are the basic building blocks of living organisms, which require numerous interactions among themselves and between the cell and the extracellular environment to function properly. For this purpose, cells exhibit adhesion, which is carried out by various components that build the cell, including cadherins, integrins (cell adhesion molecules—CAM), and the cytoskeleton. Proper communication requires continuous changes in cell adhesion, thereby remodeling the structures involved. These connections show varying degrees of complexity depending on the tissue the cell type builds [33]. An important element in the aspect of intercellular signaling and also in granulosa cells is the extracellular matrix. The combination of the ECM with integrins (transmembrane proteins) allows the transmission of signals. In addition to elevated expression of the ECM-associated genes ITGA2 and ITGB3 in porcine GCs [9], the present study also showed significant upregulation of ITGA8 gene expression. This integrin (ITGA8) has so far been described in bovine cumulus cells, where it is responsible for integrin-mediated cell adhesion [34]. Expression of this gene is also significantly modified by progesterone, which has been shown in the oviduct [35] and may be equally important in the ovary. In reference to previous results [9,34] and the elevated expression of the integrin-mediated signaling pathway ontology group in our research, the role of integrins in cell signaling within the ovarian follicle is highlighted.

It is noteworthy that in the context of intercellular signaling [36] and cell adhesion [18], an important role has recently been demonstrated for extracellular vesicles, including exosomes. The ECM plays an important role in the transport of extracellular vesicles, which, depending on the degree of stress (resulting from its composition), affects the diffusion of EVs [37]. Exosomes affect target cells through direct contact with extracellular receptors, or, after binding to the cell membrane, can be uptaken by clathrin-dependent endocytosis [38]. After fusion with the cell membrane, exosomes release the transferred type of molecule directly into the cytosol [39] or influence the recipient cell through the activation of signaling pathways [40]. A potential second pathway based on caveolin has also been demonstrated [41]. However, elevated expression of the CAV1 gene has been shown to negatively affect exosome uptake [42]. This is related to the effect of CAV1, which inhibits the ERK1/2 signaling pathway; this signaling pathway showed elevated expression in our research [42]. Interestingly, exosomes are involved in regulating the composition of the extracellular matrix [43,44], thereby affecting the cellular processes mediated by the ECM. The action of exosomes is not only limited to constituting the structural components of the ECM, but also stimulates cells to release enzymes responsible for ECM remodeling (matrix metalloproteases-MMPs) [43]. It is worth adding that the

upregulation of the CAV-1 gene, which is responsible for ECM remodeling by participating in exosome formation [45], was presented in our previous studies [9]. As nanoparticles with biological activity, exosomes have a wide range of functions in the mammalian tissues. Due to their characteristics, they are being carefully studied for use as drug transporters and molecular markers of diseases [46].

ECM composition and cell adhesion are influenced by the Matrix-remodeling associated (MXRA) protein family [47] and also by fibromodulin (FMOD) [34], which are the genes that showed increased expression (Figures 4 and 5). The composition of the ECM influences the microenvironment of cells and thus is also associated with pathological conditions. Elevated MXRA5 gene expression has been demonstrated in pancreatic cancer [48]. The protein encoded by the LOX gene, Cu-dependent lysyl oxidase (LOX), also has an important effect on ECM remodeling and was upregulated. This protein is involved in a number of signaling pathways, e.g., EGFR, PDGF, VEGF, TGF-β, MAPK, and FAK/AKT [49]. LOX has been shown to interact with the cytoskeleton, and its expression in the nucleus has been demonstrated, suggesting activity in cell division [50,51]. In addition, LOX expression is regulated by integrin–collagen fusion, confirming its role in mechanotransduction [52]. LOX showed a multiplicity of functions in granulosa cells, including effects on signaling pathways MAPK, ERK, and FAK [49], which are important for the function of GCs [9]. And, given the involvement of the LOX gene in the differentiation of pluripotent cells into osteoblasts [53] in relation to the stemness potential of GCs [30,31], this gene requires further careful study. LOX also shows a role in steroidogenesis within rat ovaries [54] and in PCOS [55]. However, it may be an important marker for processes related to reproduction and cell differentiation in GCs. Expression of the LIPG (EL endothelial lipase) gene has not been described in pig granulosa cells. This lipase regulates lipoproteins' metabolism [56], which, as sources of cholesterol [57], are important for mitochondria-mediated steroidogenesis [58]. Exosomes are also involved in the metabolism of lipids, including cholesterol [59]. LIPG expression is affected by IL-1β [60] similarly to LOX [49], although it is also affected by sex hormones [61]. This indicates that the genes of interest may be crucial for steroidogenesis in porcine granulosa cells. Upregulated 3β-hydroxysteroid dehydrogenase 1 (HSD3B1) plays an important role in steroidogenesis [62]; its expression is dependent on imidacloprid [62] and estrogen [63].

Another ontological group showing increased expression is the repression of apoptosis. The negative regulation of apoptosis in the cancer cells described is associated with increased expression of the ANKRD1 gene. The expression of ANKRD1 was upregulated. The process of natural cell death is also regulated by exosomes through their effect on TNF related apoptosis inducing ligand (TRAIL) [64]. The ANKRD1 gene shows a positive effect on the differentiation of hMSCs into adipocytes and a negative effect on osteoblastocytes [65]. In addition, this gene regulates cell sensitivity to cisplatin, thereby affecting ER stress-induced apoptosis (caused by hypoxia) [66]. TAGLN (transgelin), like the ANKRD1 gene, showed upregulation and is associated with the differentiation of hMSCs into osteoblasts and adipocytes [67]. In view of the potential of GCs to differentiate into other cell types, they may provide a basis for further research in this direction.

Genes belonging to the response to hypoxia ontology group showed significant upregulation in our study. This process in the aspect of the reproductive system, especially the ovarian microenvironment, is very important [68]. It is responsible for maintaining the proper oxygen concentration necessary for folliculogenesis and ovulation [69]. The response to conditions of reduced oxygen concentration involves the release of hypoxia-inducible factors (HIFs) [68]. The reduced oxygen concentration condition also affects the exosomes that are released [21], particularly in the case of exosomes secreted in the tumor microenvironment (TME) [70]. Additionally, changes in exosomes have been described in the context of hypoxia-maintained human umbilical vein endothelial cells (HUVECs), which were later used in regenerative medicine [71]. Hypoxia is linked to the process of angiogenesis as an element very important for the formation of the corpus luteum [72]. The genes encoding proteins involved in the process of angiogenesis showed upregulation.

Angiogenesis within the ovarian follicle is very important, and its improper regulation can be associated with various disorders, such as PCOS and POI [73]. Each ovarian follicle undergoing folliculogenesis manifests a temporary, individual vascularization pattern. It has been shown that inhibition of angiogenesis within the ovary slows the depletion of the ovarian follicle pool and can be used to treat POI [74]. Exosomes have been shown to promote angiogenesis through the suppression of HIK-1 expression [75] as well as by microRNA-92a-3p [76].

Another ontological group showing increased expression is wound healing. This process strictly depends on the blood supply to the tissues undergoing healing. The healing process is also closely related to exosomes [77–79], which significantly influence its course (probably also by influencing angiogenesis [75,76]), and they are important for their application in clinical practice [80]. The involvement of previously described ANKRD1 and LOX genes, whose expression was upregulated, was demonstrated to display an important role in wound healing. ANKRD1 affects the interaction of fibroblasts with collagen fibers [81]. LOX, on the other hand, is involved in ECM remodeling during new tissue reconstruction [82].

Cell migration is very important for many processes related to development, embryogenesis, immune response, and wound healing, among others. The current study demonstrated increased expression of genes belonging to the cell migration ontology group. SHAS2 (swine hyaluronic acid synthase 2) was upregulated in the present study; it is mainly responsible for the cumulus expansion process (one of the LH-mediated ovulatory processes), and inhibition of its expression leads to reduced migration of granulosa cells [83]. This gene is also responsible for the synthesis of hyaluronyan, the main component of the ECM [84]. In addition, hyaluronic acid is an important component affecting exosomes with regard to their bone-regenerative capacity [85]. SHAS2 has been described in pig CCs [86]. Additionally, cell viability and migration depend on the expression of the CLIP4 gene (upregulated (Figures 3 and 5)), whose knockdown causes a significant decrease in cell viability [87]. EVs, including exosomes, also have an impact on migration [88,89]. They play a key role in migration, conducting it in an autocrine and paracrine way [19]. Exosomes stimulate extracellular signaling receptors, and their deposition near the cell membrane is required to initiate the migration process [90]. In addition, interactions between exosomes and the ECM via integrins, among other things, show the importance of connecting the cell to the extracellular environment [91].

Downregulation of the HSD17B1 (hydroxysteroid 17-beta dehydrogenase 1) gene results in a decrease in estrogen because the enzyme is responsible for the last step of steroidogenesis in porcine granulosa cells. This process is further regulated positively by the p53 protein and negatively by FoxA2 [92]. Changes in sex hormone levels in relation to PCOS have also been shown to be caused by changes in HSD17B1 gene expression in follicular fluid (FF) exosomes [93]. A negative effect of dioxin on the expression of the HSD17B1 gene has been described, thereby causing the inhibition of steroidogenesis [94]. In addition, this gene has been identified as a marker of steroidogenesis in ovine granulosa cells, thus affecting fecundity in this species [95]. A downregulation of FST (follistatin) was revealed in the current study, which could positively affect porcine GCs' proliferation and estrogen secretion [96]. FST also affects the TGF-β signaling pathway, which is closely responsible for ovarian follicle development [97] and the survival rate of follicles [98]. Expression of the CXCL10 (C-X-C motif chemokine ligand 10) gene exhibited reduced expression, but it has no effect on steroidogenesis within luteinized ovarian granulosa cells [99]. However, CXCL10 has been shown to affect the production of COL1A1 and COL1A2, which, as a component of the ECM, can affect fibrosis within the ovary, leading to POI [99].

4. Materials and Methods

4.1. Animals

Ovaries were collected post-slaughter from 40 sexually mature gilts. Animals slaughtered in a commercial slaughterhouse were kept under similar breeding conditions on registered farms. At slaughter, the animals had reached an average weight of 98 kg and an age of about 6 months (+/−10 days).

4.2. Collection of Porcine Ovarian Granulosa Cells

The research material was transported to the laboratory at 38 °C in 0.9% NaCl within 30 min of harvesting. In the laboratory, ovaries isolated from the reproductive organs were placed in PBS (phosphate-buffered saline) solution supplemented with fetal bovine serum (FBS; Sigma-Aldrich Co., St. Louis, MO, USA). Follicular fluid (FF) was then aspirated from individual pre-ovulatory ovarian follicles larger than 5 mm in diameter using a 5 mL syringe and a 20 G needle. The fluid thus extracted was deposited into a sterile Petri dish, and then cumulus–oocyte complexes (COCs) were recovered for rejection. The extracted vesicular fluid after COCs rejection was filtered through sterile nylon cell screens with a mesh diameter of 40 µm (Biologix Group, Shandong, China) to eliminate tissue debris and larger cell aggregates, including erythrocytes and epithelial cells. The resulting suspension was centrifuged at room temperature for 10 min at 200× *g* to divide the solution into fractions. After discarding the supernatant, the GCs pellet was then suspended in collagenase type I solution (Gibco, Thermo-Fischer Scientific, Waltham, MA, USA) and 1 mg/1 mL DMEM and incubated for 10 min in a 37 °C water bath, followed by centrifugation (under the same conditions as stated above). Granulosa cells were taken from different ovarian follicles to homogenize the sample, and the pellet obtained after centrifugation was used to establish the primary culture.

4.3. In Vitro Primary Culture of Porcine Granulosa Cells

A primary in vitro culture model was used in this study with four time intervals. For microarray expressions, cultures were maintained in two biological replicates for each time interval. For validation by RT-qPCR, cultures were maintained in a triplicate biological sample model for each time interval. Primary cultures were established from GCs in four bottles. Cells were seeded at 3×10^6/culture bottle (25 cm^2, TPP, Trasadingen, Switzerland). The number of cells and their viability were assessed using an ADAM automatic cell counter (NanoEnTek, Waltham, MA, USA). Only samples with a cell viability above 85% were used for further studies. The culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, Saint Louis, MO, USA), 2% fetal calf serum (FCS) (PAA, Linz, Austria), 10 mg/mL ascorbic acid (Sigma-Aldrich, Saint Louis, MO, USA), 0.05 µM dexamethasone (Sigma-Aldrich, Saint Louis, MO, USA), 200 mM L-glutamine (Invitrogen, Carlsbad, CA, USA), 10 mg/mL gentamicin (Invitrogen, Carlsbad, CA, USA), 10,000 units/mL penicillin (Invitrogen, Carlsbad, CA, USA), and 10,000 µg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). The culture bottles prepared in this way, together with the cells, were maintained at 38.5 °C and 5% CO2. After the cells reached more than 80% confluence, they were detached from the medium with 0.05% trypsin-EDTA (Invitrogen, Carlsbad, CA, USA) and then passaged. Cells in culture were kept until culture termination, and the material was collected at 0 h, 48 h, 96 h, and 144 h. The culture medium was changed every 72 h.

4.4. Microarray Expression Analysis and Statistics

The total RNA from porcine granulosa cells was isolated using TRI Reagent (Sigma, St Louis, MO, USA), and an RNeasyMinElute cleanup Kit (Qiagen, Hilden, Germany). The RNA for transcriptome study was collected from two independent replicates for each experimental variant: (1) control—0 h, (2) 48 h, (3) 96 h, and (4) 144 h. Each replicate contained pooled RNA from three independent experiments. The microarray study was performed according to the previously described protocol [100,101].

First, the total RNA (100 ng) from each sample was submitted to a two-step cDNA synthesis reaction, biotin labeling, and fragmentation according to the manufacturer's instructions (GeneChip® WT Plus Reagent Kit, Affymetrix, Santa Clara, CA, USA). Then, the biotin-labeled fragments of cDNA were hybridized to the Affymetrix® PorGene 1.1 ST Array Strip (45 °C/20 h). Next, the microarrays were stained by the Affymetrix GeneAtlas Fluidics Station of GeneAtlas System. The microarrays were scanned by the Imaging Station of the GeneAtlas System (Affymetrix, Santa Clara, CA, USA). The Affymetrix GeneAtlas Operating System was performed for the analysis of the obtained results. The quality of the gene expression data was confirmed using the software's quality control criteria.

All analyses were performed by BioConductor software with the relevant Bioconductor libraries through the statistical R programming language (v4.1.2; R Core Team 2021). For the normalization, background correction, and calculation of the expression values of the analyzed genes, the robust multiarray average (RMA) normalization algorithm implement in the "Affy" library was applied [102]. To show the total number of upregulated and downregulated genes, the principal component analysis (PCA) of the filtered data set was performed and visualized using the "factoextra" library [103]. Next, the DAVID (Database for Annotation, Visualization, and Integrated Discovery) bioinformatics tool was used for functional annotation and clusterization of differentially expressed genes (DEGs) [102,104]. The established cut-off criteria for DEGs was based on the differences in the absolute value from the expression fold change greater than 2. Furthermore, the expressed genes were assigned to relevant GO terms, with the subsequent selection of significantly enriched GO terms using the GO BP DIRECT database. The *p*-values of selected GO terms were corrected using the Benjamini–Hochberg correction [105]. DEGs from each comparison were visualized through hierarchic clustering of differentially expressed genes as a heatmap using the "ComplexHeatmap" library [106]. Genes belonging to the first seven most significantly enriched ontological groups (lowest adjusted *p*-value) were shown on the figures with the expression values of analyzed genes.

GSEA was carried out using the "clusterProfiler" Bioconductor library [107]. The aim of the analysis was to identify the level of depletion or enrichment in GO terms through the calculation of the normalized enrichment score (NES) with the relevant *p*-value. Normalized fold change values from all of the genes were log2 transformed, sorted, and used as an argument for the "gseGO" function. Gene set enrichment was performed with reference to the "biological process" GO category, assuming that the minimum size of each geneSet for analyzing = 50 and *p*-value cut-off = 0.05. Then, hierarchical clustering of enriched terms based on pairwise similarities calculation with Jaccard's similarity index was performed. The result of the analysis qualified individual GO terms to clusters based on their functional similarity. The obtained clusters were presented as a tree plot. The ten ontology groups with the highest enrichment score (the highest NES value) and the ten groups with the most depleted enrichment score (the lowest NES value) were visualized as a bar chart. Enrichment plots for five of the most enriched and depleted GO terms were also presented.

4.5. Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

Total RNA was isolated from GCs at 0 h and after 48 h, 96 h, and 144 h in vitro culture using an RNeasy mini column from Qiagen GmbH (Hilden, Germany). The RNA samples were resuspended in 20 µL of RNase-free water and stored in liquid nitrogen. RNA samples were treated with DNase I and reverse-transcribed (RT) into cDNA. RT-qPCR was conducted in a LightCycler real-time PCR detection system (Roche Diagnostics GmbH, Mannheim, Germany) using SYBR® Green I as a detection dye, and the target cDNA was quantified using the relative quantification method. The relative abundance of analyzed transcripts in each sample was standardized to the internal standard glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For amplification, 2 µL of cDNA solution was added to 18 µL of QuantiTect® SYBR® Green PCR (Master Mix Qiagen GmbH, Hilden, Germany) and primers (Table 1). One RNA sample of each preparation was processed without the RT

reaction to provide a negative control for subsequent PCR. Eleven randomly selected genes were chosen to validate the microarray results.

Table 1. Oligonucleotide sequences of primers used for RT-qPCR analysis.

Gene		Primer Sequence (5′-3′)	Product Size (bp)
HSD17B1	F	GTGTCAGAGGCTTGCTAGGG	200
	R	CAGCACAATCTCAAGGCTGA	
MAL2	F	ATCCTCGTCATGGAAAGGTG	202
	R	TGCCACTCATTCATGGTTGT	
SNX31	F	AGGTGACCTTCCTTGGGACT	222
	R	CCGGAACTTCAATCTGCATT	
DAPL	F	CCTGCTCTGGAGAAGGTCAC	151
	R	GGGCCTAAGGAAAGTTTTGG	
ANKRD1	F	CTGCTTGAGGTGGGGAAGTA	178
	R	GTGTCTCACTGTCTGGGGAA	
NEXN	F	GAAGCAAGGAGAAGCATGGC	151
	R	CCTCCTCTGTTCGTCGTCTT	
MXRA5	F	TGCTGGCACTGTTTTCTCAC	212
	R	TCGGAGAGGATTCATGAGGC	
TAGLN	F	TTAAAGGCCGCTGAGGACTA	233
	R	ATGACATGCTTTCCCTCCTG	
SHAS2	F	ATCGCGGCCTATCAAGAAGA	204
	R	GCCCTTTTCGTGGAAGTTGT	
CLIP4	F	CCCTTAGAAATGGCCGATGC	162
	R	ATCTCCCAACTTCAGGCCAA	
HSD3B1	F	TCCACACCAGCAGCATAGAG	245
	R	CATGTGGGCAAAGATGAATG	

To quantify the specific genes expressed in the GCs, the expression levels of specific mRNAs in each sample were calculated relative to PBGD and ACTB. To ensure the integrity of these results, the additional housekeeping gene, 18S, was used as an internal standard to demonstrate that PBGD and ACTB mRNAs were not differentially regulated in GC groups. The gene for 18S rRNA expression has been identified as an appropriate housekeeping gene for use in quantitative PCR studies. The expression of PBGD, ACTB, and 18S mRNA was measured in cDNA samples from isolated GCs. The statistical significance of the analyzed genes was performed using moderated t-statistics from the empirical Bayes method. The *p*-value was corrected for multiple comparisons using Benjamini and Hochberg's false discovery rate.

5. Conclusions

There appears to be an association between the expression of genes involved in cell adhesion, proliferation, migration, division, and intercellular signaling and EV production and composition in granulosa cells. The literature suggests that the ECM and cytoskeleton are also involved in these signaling pathways of granulosa cells. The exosomes in the microenvironment of granulosa cells affect the composition of the ECM, which is a key element of the ovulation process. ECM is also crucial in the aspect of reproductive disorders, such as PCOS and POI. Therefore, these studies can be used to identify genetic markers of processes, largely based on EVs, that can be used in assisted reproductive techniques (ART), reproductive tract disorders, and regenerative medicine.

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References

1. Cecconi, S.; Ciccarelli, C.; Barberi, M.; Macchiarelli, G.; Canipari, R. Granulosa Cell-Oocyte Interactions. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **2004**, *115* (Suppl 1), S19–S22. [CrossRef]
2. Turathum, B.; Gao, E.M.; Chian, R.C. The Function of Cumulus Cells in Oocyte Growth and Maturation and in Subsequent Ovulation and Fertilization. *Cells* **2021**, *10*, 2292. [CrossRef]
3. Kidder, G.M.; Mhawi, A.A. Gap Junctions and Ovarian Folliculogenesis. *Reproduction* **2002**, *123*, 613–620. [CrossRef] [PubMed]
4. Strączyńska, P.; Papis, K.; Morawiec, E.; Czerwiński, M.; Gajewski, Z.; Olejek, A.; Bednarska-Czerwińska, A. Signaling Mechanisms and Their Regulation during in Vivo or in Vitro Maturation of Mammalian Oocytes. *Reprod. Biol. Endocrinol.* **2022**, *20*, 37. [CrossRef] [PubMed]
5. Robker, R.L.; Richards, J.S. Hormone-Induced Proliferation and Differentiation of Granulosa Cells: A Coordinated Balance of the Cell Cycle Regulators Cyclin D2 and P27Kip1. *Mol. Endocrinol.* **1998**, *12*, 924–940. [CrossRef] [PubMed]
6. Wang, J.; Chu, K.; Wang, Y.; Li, J.; Fu, J.; Zeng, Y.A.; Li, W. Procr-Expressing Granulosa Cells Are Highly Proliferative and Are Important for Follicle Development. *iScience* **2021**, *24*, 102065. [CrossRef] [PubMed]
7. Pan, B.; Liu, C.; Zhan, X.; Li, J. Protegrin-1 Regulates Porcine Granulosa Cell Proliferation via the EGFR-ERK1/2/P38 Signaling Pathway in Vitro. *Front. Physiol.* **2021**, *12*, 733. [CrossRef] [PubMed]
8. Kranc, W.; Brązert, M.; Budna, J.; Celichowski, P.; Bryja, A.; Nawrocki, M.J.; Ożegowska, K.; Jankowski, M.; Chermuła, B.; Dyszkiewicz-Konwińska, M.; et al. Genes Responsible for Proliferation, Differentiation, and Junction Adhesion Are Significantly up-Regulated in Human Ovarian Granulosa Cells during a Long-Term Primary in Vitro Culture. *Histochem. Cell Biol.* **2019**, *151*, 125–143. [CrossRef] [PubMed]
9. Kulus, J.; Kulus, M.; Kranc, W.; Jopek, K.; Zdun, M.; Józkowiak, M.; Jáskowski, J.M.; Piotrowska-Kempisty, H.; Bukowska, D.; Antosik, P.; et al. Transcriptomic Profile of New Gene Markers Encoding Proteins Responsible for Structure of Porcine Ovarian Granulosa Cells. *Biology* **2021**, *10*, 1214. [CrossRef]
10. Bader, G.D.; Hogue, C.W.V. An Automated Method for Finding Molecular Complexes in Large Protein Interaction Networks. *BMC Bioinform.* **2003**, *4*, 2. [CrossRef]
11. Jiang, X.; Qin, Y.; Kun, L.; Zhou, Y. The Significant Role of the Microfilament System in Tumors. *Front. Oncol.* **2021**, *11*, 333. [CrossRef]
12. McBeath, R.; Pirone, D.M.; Nelson, C.M.; Bhadriraju, K.; Chen, C.S. Cell Shape, Cytoskeletal Tension, and RhoA Regulate Stem Cell Lineage Commitment. *Dev. Cell* **2004**, *6*, 483–495. [CrossRef]
13. Mathieu, M.; Martin-Jaular, L.; Lavieu, G.; Théry, C. Specificities of Secretion and Uptake of Exosomes and Other Extracellular Vesicles for Cell-to-Cell Communication. *Nat. Cell Biol.* **2019**, *21*, 9–17. [CrossRef]
14. Shen, Y.; You, Y.; Zhu, K.; Fang, C.; Chang, D.; Yu, X. Exosomes in the fIeld of Reproduction: A Scientometric Study and Visualization Analysis. *Front. Pharmacol.* **2022**, *13*, 1001652. [CrossRef] [PubMed]
15. Muñoz, E.L.; Fuentes, F.B.; Felmer, R.N.; Yeste, M.; Arias, M.E. Extracellular Vesicles in Mammalian Reproduction: A Review. *Zygote* **2022**, *30*, 440–463. [CrossRef]
16. de ávila, A.C.F.C.M.; Andrade, G.M.; Bridi, A.; Gimenes, L.U.; Meirelles, F.V.; Perecin, F.; da Silveira, J.C. Extracellular Vesicles and Its Advances in Female Reproduction. *Anim. Reprod.* **2020**, *16*, 31–38. [CrossRef] [PubMed]
17. Llobat, L. Extracellular Vesicles and Domestic Animal Reproduction. *Res. Vet. Sci.* **2021**, *136*, 166–173. [CrossRef] [PubMed]
18. Lan, Y.; Jin, Q.; Xie, H.; Yan, C.; Ye, Y.; Zhao, X.; Chen, Z.; Xie, Z. Exosomes Enhance Adhesion and Osteogenic Differentiation of Initial Bone Marrow Stem Cells on Titanium Surfaces. *Front. Cell Dev. Biol.* **2020**, *8*, 1216. [CrossRef]
19. Sung, B.H.; Parent, C.A.; Weaver, A.M. Extracellular Vesicles: Critical Players during Cell Migration. *Dev. Cell* **2021**, *56*, 1861–1874. [CrossRef]
20. Miller, C.M.; Enninga, E.A.L.; Rizzo, S.A.; Phillipps, J.; Guerrero-Cazares, H.; Destephano, C.C.; Peterson, T.E.; Stalboerger, P.G.; Behfar, A.; Khan, Z. Platelet-Derived Exosomes Induce Cell Proliferation and Wound Healing in Human Endometrial Cells. *Regen. Med.* **2022**, *17*, 805–817. [CrossRef]

21. Jiang, H.; Zhao, H.; Zhang, M.; He, Y.; Li, X.; Xu, Y.; Liu, X. Hypoxia Induced Changes of Exosome Cargo and Subsequent Biological Effects. *Front. Immunol.* **2022**, *13*, 1140. [CrossRef]
22. Karampoga, A.; Tzaferi, K.; Koutsakis, C.; Kyriakopoulou, K.; Karamanos, N.K. Exosomes and the Extracellular Matrix: A Dynamic Interplay in Cancer Progression. *Int. J. Dev. Biol.* **2022**, *66*, 97–102. [CrossRef] [PubMed]
23. Walma, D.A.C.; Yamada, K.M. The Extracellular Matrix in Development. *Development* **2020**, *147*, 175596. [CrossRef]
24. Mecham, R.P. Overview of Extracellular Matrix. *Curr. Protoc. Cell Biol.* **2012**, *57*, 10.1.1–10.1.16. [CrossRef]
25. Jones, M.C.; Zha, J.; Humphries, M.J. Connections between the Cell Cycle, Cell Adhesion and the Cytoskeleton. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **2019**, *374*, 20180227. [CrossRef] [PubMed]
26. Forth, S.; Kapoor, T.M. The Mechanics of Microtubule Networks in Cell Division. *J. Cell Biol.* **2017**, *216*, 1525–1531. [CrossRef]
27. Sanghvi-Shah, R.; Weber, G.F. Intermediate Filaments at the Junction of Mechanotransduction, Migration, and Development. *Front. Cell Dev. Biol.* **2017**, *5*, 81. [CrossRef]
28. Kamranvar, S.A.; Rani, B.; Johansson, S. Cell Cycle Regulation by Integrin-Mediated Adhesion. *Cells* **2022**, *11*, 2521. [CrossRef]
29. Baruah, J.; Wary, K.K. Exosomes in the Regulation of Vascular Endothelial Cell Regeneration. *Front. Cell Dev. Biol.* **2020**, *7*, 353. [CrossRef] [PubMed]
30. Moncrieff, L.; Mozdziak, P.; Jeseta, M.; Machatkova, M.; Kranc, W.; Kempisty, B. Ovarian Follicular Cells—Living in the Shadow of Stemness Cellular Competence. *Med. J. Cell Biol.* **2019**, *7*, 134–140. [CrossRef]
31. Stefańska, K.; Sibiak, R.; Hutchings, G.; Dompe, C.; Moncrieff, L.; Janowicz, K.; Jeseta, M.; Kempisty, B.; Machatkova, M.; Mozdziak, P. Evidence for Existence of Molecular Stemness Markers in Porcine Ovarian Follicular Granulosa Cells. *Med. J. Cell Biol.* **2019**, *7*, 183–188. [CrossRef]
32. Hade, M.D.; Suire, C.N.; Suo, Z. Mesenchymal Stem Cell-Derived Exosomes: Applications in Regenerative Medicine. *Cells* **2021**, *10*, 1959. [CrossRef] [PubMed]
33. Sergé, A. The Molecular Architecture of Cell Adhesion: Dynamic Remodeling Revealed by Videonanoscopy. *Front. Cell Dev. Biol.* **2016**, *4*, 36. [CrossRef] [PubMed]
34. Martínez-Moro, Á.; González-Brusi, L.; Lamas-Toranzo, I.; O'callaghan, E.; Esteve-Codina, A.; Lonergan, P.; Bermejo-Álvarez, P. RNA-Sequencing Reveals Genes Linked with Oocyte Developmental Potential in Bovine Cumulus Cells. *Mol. Reprod. Dev.* **2022**, *89*, 399–412. [CrossRef]
35. Dinh, D.T.; Breen, J.; Akison, L.K.; DeMayo, F.J.; Brown, H.M.; Robker, R.L.; Russell, D.L. Tissue-Specific Progesterone Receptor-Chromatin Binding and the Regulation of Progesterone-Dependent Gene Expression. *Sci. Rep.* **2019**, *9*, 11966. [CrossRef]
36. Gurung, S.; Perocheau, D.; Touramanidou, L.; Baruteau, J. The Exosome Journey: From Biogenesis to Uptake and Intracellular Signalling. *Cell Commun. Signal.* **2021**, *19*, 47. [CrossRef]
37. Lenzini, S.; Bargi, R.; Chung, G.; Shin, J.W. Matrix Mechanics and Water Permeation Regulate Extracellular Vesicle Transport. *Nat. Nanotechnol.* **2020**, *15*, 217–223. [CrossRef]
38. Prada, I.; Meldolesi, J. Binding and Fusion of Extracellular Vesicles to the Plasma Membrane of Their Cell Targets. *Int. J. Mol. Sci.* **2016**, *17*, 1296. [CrossRef]
39. Mulcahy, L.A.; Pink, R.C.; Carter, D.R.F. Routes and Mechanisms of Extracellular Vesicle Uptake. *J. Extracell. Vesicles* **2014**, *3*, 1–14. [CrossRef]
40. Guan, S.; Li, Q.; Liu, P.; Xuan, X.; Du, Y. Experimental Immunology Umbilical Cord Blood-Derived Dendritic Cells Loaded with BGC823 Tumor Antigens and DC-Derived Exosomes Stimulate Efficient Cytotoxic T-Lymphocyte Responses and Antitumor Immunity in Vitro and in Vivo. *Cent. Eur. J. Immunol.* **2014**, *39*, 142–151. [CrossRef]
41. Kiss, A.L.; Botos, E. Endocytosis via Caveolae: Alternative Pathway with Distinct Cellular Compartments to Avoid Lysosomal Degradation? *J. Cell. Mol. Med.* **2009**, *13*, 1228–1237. [CrossRef]
42. Svensson, K.J.; Christianson, H.C.; Wittrup, A.; Bourseau-Guilmain, E.; Lindqvist, E.; Svensson, L.M.; Mörgelin, M.; Belting, M. Exosome Uptake Depends on ERK1/2-Heat Shock Protein 27 Signaling and Lipid Raft-Mediated Endocytosis Negatively Regulated by Caveolin-1. *J. Biol. Chem.* **2013**, *288*, 17713. [CrossRef] [PubMed]
43. Patel, N.J.; Ashraf, A.; Chung, E.J. Extracellular Vesicles as Regulators of the Extracellular Matrix. *Bioengineering* **2023**, *10*, 136. [CrossRef] [PubMed]
44. Brigstock, D.R. Extracellular Vesicles in Organ Fibrosis: Mechanisms, Therapies, and Diagnostics. *Cells* **2021**, *10*, 1596. [CrossRef]
45. Albacete-Albacete, L.; Navarro-Lérida, I.; López, J.A.; Martín-Padura, I.; Astudillo, A.M.; Ferrarini, A.; Van-Der-Heyden, M.; Balsinde, J.; Orend, G.; Vázquez, J.; et al. ECM Deposition Is Driven by Caveolin-1–Dependent Regulation of Exosomal Biogenesis and Cargo Sorting. *J. Cell Biol.* **2020**, *219*, e202006178. [CrossRef] [PubMed]
46. Zhang, Y.; Liu, Y.; Liu, H.; Tang, W.H. Exosomes: Biogenesis, Biologic Function and Clinical Potential. *Cell Biosci.* **2019**, *9*, 19. [CrossRef]
47. Walker, M.G.; Volkmuth, W. Cell Adhesion and Matrix Remodeling Genes Identified by Co-Expression Analysis. *Gene Funct. Dis.* **2002**, *3*, 109–112. [CrossRef]
48. Peng, S.; Zhu, X.; Zhao, M.; Zhang, Y.; Wang, A.; Chen, M.; Ye, Z. Identification of Matrix-Remodeling Associated 5 as a Possible Molecular Oncotarget of Pancreatic Cancer. *Cell Death Dis.* **2023**, *14*, 157. [CrossRef]
49. Laczko, R.; Csiszar, K. Lysyl Oxidase (LOX): Functional Contributions to Signaling Pathways. *Biomolecules* **2020**, *10*, 1093. [CrossRef]

50. Oleggini, R.; Biology, A.D.D.-B. Lysyl Oxidase Regulates MMTV Promoter: Indirect Evidence of Histone H1 Involvement. *Biochem. Cell Biol.* **2011**, *89*, 522–532. [CrossRef]
51. Iturbide, A.; de Herreros, A.G.; Peiró, S. A New Role for LOX and LOXL 2 Proteins in Transcription Regulation. *Wiley Online Libr.* **2015**, *282*, 1768–1773. [CrossRef]
52. Gao, A.E.; Sullivan, K.E.; Black, L.D. Lysyl Oxidase Expression in Cardiac Fibroblasts Is Regulated by A2β1 Integrin Interactions with the Cellular Microenvironment. *Biochem. Biophys. Res. Commun.* **2016**, *475*, 70–75. [CrossRef]
53. Alcudia, J.F.; Martinez-Gonzalez, J.; Guadall, A.; Gonzalez-Diez, M.; Badimon, L.; Rodriguez, C. Lysyl Oxidase and Endothelial Dysfunction: Mechanisms of Lysyl Oxidase down-Regulation by pro-Inflammatory Cytokines. *Front. Biosci.* **2008**, *13*, 2721–2727. [CrossRef]
54. Harlow, C.R.; Rae, M.; Davidson, L.; Trackman, P.C.; Hillier, S.G. Lysyl Oxidase Gene Expression and Enzyme Activity in the Rat Ovary: Regulation by Follicle-Stimulating Hormone, Androgen, and Transforming Growth Factor-β Superfamily Members in Vitro. *Endocrinology* **2003**, *144*, 154–162. [CrossRef] [PubMed]
55. Zhang, C.; Ma, J.; Wang, W.; Sun, Y.; Sun, K. Lysyl Oxidase Blockade Ameliorates Anovulation in Polycystic Ovary Syndrome. *Hum. Reprod.* **2018**, *33*, 2096–2106. [CrossRef] [PubMed]
56. Yu, J.E.; Han, S.Y.; Wolfson, B.; Zhou, Q. The Role of Endothelial Lipase in Lipid Metabolism, Inflammation, and Cancer. *Histol. Histopathol.* **2018**, *33*, 1–10. [CrossRef] [PubMed]
57. Miller, W.L.; Bose, H.S. Early Steps in Steroidogenesis: Intracellular Cholesterol Trafficking: Thematic Review Series: Genetics of Human Lipid Diseases. *J. Lipid Res.* **2011**, *52*, 2111. [CrossRef] [PubMed]
58. Bassi, G.; Sidhu, S.K.; Mishra, S. The Expanding Role of Mitochondria, Autophagy and Lipophagy in Steroidogenesis. *Cells* **2021**, *10*, 1851. [CrossRef]
59. Wang, W.; Zhu, N.; Yan, T.; Shi, Y.N.; Chen, J.; Zhang, C.J.; Xie, X.J.; Liao, D.F.; Qin, L. The Crosstalk: Exosomes and Lipid Metabolism. *Cell Commun. Signal.* **2020**, *18*, 119. [CrossRef]
60. Mikłosz, A.; Łukaszuk, B.; Chabowski, A.; Górski, J. Treadmill Running Changes Endothelial Lipase Expression: Insights from Gene and Protein Analysis in Various Striated Muscle Tissues and Serum. *Biomolecules* **2021**, *11*, 906. [CrossRef]
61. Wang, C.; Niimi, M.; Kitajima, S.; Matsuhisa, F.; Yan, H.; Dong, S.; Liang, J.; Fan, J. Sex Hormones Affect Endothelial Lipase-Mediated Lipid Metabolism and Atherosclerosis. *Lipids Health Dis.* **2019**, *18*, 226. [CrossRef] [PubMed]
62. Mourikes, V.E.; Santacruz Márquez, R.; Deviney, A.; Neff, A.M.; Laws, M.J.; Flaws, J.A. Imidacloprid and Its Bioactive Metabolite, Desnitro-Imidacloprid, Differentially Affect Ovarian Antral Follicle Growth, Morphology, and Hormone Synthesis In Vitro. *Toxics* **2023**, *11*, 349. [CrossRef] [PubMed]
63. Samardzija Nenadov, D.; Pogrmic-Majkic, K.; Fa, S.; Stanic, B.; Tubic, A.; Andric, N. Environmental Mixture with Estrogenic Activity Increases Hsd3b1 Expression through Estrogen Receptors in Immature Rat Granulosa Cells. *J. Appl. Toxicol.* **2018**, *38*, 879–887. [CrossRef] [PubMed]
64. Munich, S.; Sobo-Vujanovic, A.; Buchser, W.J.; Beer-Stolz, D.; Vujanovic, N.L. Dendritic Cell Exosomes Directly Kill Tumor Cells and Activate Natural Killer Cells via TNF Superfamily Ligands. *Oncoimmunology* **2012**, *1*, 1074–1083. [CrossRef]
65. Yi, X.; Wu, P.; Fan, Y.; Gong, Y.; Liu, J.; Xiong, J.; Xu, X. Identification of Candidate Genes Simultaneously Shared by Adipogenesis and Osteoblastogenesis from Human Mesenchymal Stem Cells. *Folia Histochem. Cytobiol.* **2022**, *60*, 179–190. [CrossRef] [PubMed]
66. Lei, Y.; Henderson, B.R.; Emmanuel, C.; Harnett, P.R.; Defazio, A. Inhibition of ANKRD1 Sensitizes Human Ovarian Cancer Cells to Endoplasmic Reticulum Stress-Induced Apoptosis. *Oncogene* **2015**, *34*, 485–495. [CrossRef]
67. Elsafadi, M.; Manikandan, M.; Dawud, R.A.; Alajez, N.M.; Hamam, R.; Alfayez, M.; Kassem, M.; Aldahmash, A.; Mahmood, A. Transgelin Is a TGFβ-Inducible Gene That Regulates Osteoblastic and Adipogenic Differentiation of Human Skeletal Stem Cells through Actin Cytoskeleston Organization. *Cell Death Dis.* **2016**, *7*, e2321. [CrossRef]
68. Lim, M.; Thompson, J.G.; Dunning, K.R. HYPOXIA AND REPRODUCTIVE HEALTH: Hypoxia and Ovarian Function: Follicle Development, Ovulation, Oocyte Maturation. *Reproduction* **2021**, *161*, F33–F40. [CrossRef]
69. Baddela, V.S.; Sharma, A.; Michaelis, M.; Vanselow, J. HIF1 Driven Transcriptional Activity Regulates Steroidogenesis and Proliferation of Bovine Granulosa Cells. *Sci. Rep.* **2020**, *10*, 3906. [CrossRef]
70. He, G.; Peng, X.; Wei, S.; Yang, S.; Li, X.; Huang, M.; Tang, S.; Jin, H.; Liu, J.; Zhang, S.; et al. Exosomes in the Hypoxic TME: From Release, Uptake and Biofunctions to Clinical Applications. *Mol. Cancer* **2022**, *21*, 19. [CrossRef]
71. Li, L.; Mu, J.; Zhang, Y.; Zhang, C.; Ma, T.; Chen, L.; Huang, T.; Wu, J.; Cao, J.; Feng, S.; et al. Stimulation by Exosomes from Hypoxia Preconditioned Human Umbilical Vein Endothelial Cells Facilitates Mesenchymal Stem Cells Angiogenic Function for Spinal Cord Repair. *ACS Nano* **2022**, *16*, 10811–10823. [CrossRef] [PubMed]
72. Nishimura, R.; Okuda, K. Multiple Roles of Hypoxia in Ovarian Function: Roles of Hypoxia-Inducible Factor-Related and-Unrelated Signals during the Luteal Phase. *Reprod. Fertil. Dev.* **2015**, *28*, 1479–1486. [CrossRef] [PubMed]
73. Dompe, C.; Kulus, M.; Stefańska, K.; Kranc, W.; Chermuła, B.; Bryl, R.; Pieńkowski, W.; Nawrocki, M.J.; Petitte, J.N.; Stelmach, B.; et al. Human Granulosa Cells—Stemness Properties, Molecular Cross-Talk and Follicular Angiogenesis. *Cells* **2021**, *10*, 1396. [CrossRef] [PubMed]
74. Xu, X.; Mu, L.; Li, L.; Liang, J.; Zhang, S.; Jia, L.; Yang, X.; Dai, Y.; Zhang, J.; Wang, Y.; et al. Imaging and Tracing the Pattern of Adult Ovarian Angiogenesis Implies a Strategy against Female Reproductive Aging. *Sci. Adv.* **2022**, *8*, 8683. [CrossRef] [PubMed]
75. Olejarz, W.; Kubiak-Tomaszewska, G.; Chrzanowska, A.; Lorenc, T. Exosomes in Angiogenesis and Anti-Angiogenic Therapy in Cancers. *Int. J. Mol. Sci.* **2020**, *21*, 5840. [CrossRef]

76. Chen, S.; Chen, X.; Luo, Q.; Liu, X.; Wang, X.; Cui, Z.; He, A.; He, S.; Jiang, Z.; Wu, N.; et al. Retinoblastoma Cell-Derived Exosomes Promote Angiogenesis of Human Vesicle Endothelial Cells through MicroRNA-92a-3p. *Cell Death Dis.* **2021**, *12*, 695. [CrossRef]
77. Hettich, B.F.; Ben-Yehuda Greenwald, M.; Werner, S.; Leroux, J.C. Exosomes for Wound Healing: Purification Optimization and Identification of Bioactive Components. *Adv. Sci.* **2020**, *7*, 2002596. [CrossRef]
78. Zhou, C.; Zhang, B.; Yang, Y.; Jiang, Q.; Li, T.; Gong, J.; Tang, H.; Zhang, Q. Stem Cell-Derived Exosomes: Emerging Therapeutic Opportunities for Wound Healing. *Stem Cell Res. Ther.* **2023**, *14*, 107. [CrossRef]
79. Bo, Y.; Yang, L.; Liu, B.; Tian, G.; Li, C.; Zhang, L.; Yan, Y. Exosomes from Human Induced Pluripotent Stem Cells-Derived Keratinocytes Accelerate Burn Wound Healing through MiR-762 Mediated Promotion of Keratinocytes and Endothelial Cells Migration. *J. Nanobiotechnol.* **2022**, *20*, 291. [CrossRef]
80. Li, D.; Wu, N. Mechanism and Application of Exosomes in the Wound Healing Process in Diabetes Mellitus. *Diabetes Res. Clin. Pract.* **2022**, *187*, 109882. [CrossRef]
81. Samaras, S.E.; Almodóvar-García, K.; Wu, N.; Yu, F.; Davidson, J.M. Global Deletion of Ankrd1 Results in a Wound-Healing Phenotype Associated with Dermal Fibroblast Dysfunction. *Am. J. Pathol.* **2015**, *185*, 96. [CrossRef]
82. Cai, L.; Xiong, X.; Kong, X.; Xie, J. The Role of the Lysyl Oxidases in Tissue Repair and Remodeling: A Concise Review. *Tissue Eng. Regen. Med.* **2017**, *14*, 15. [CrossRef] [PubMed]
83. Yung, Y.; Ophir, L.; Yerushalmi, G.M.; Baum, M.; Hourvitz, A.; Maman, E. HAS2-AS1 Is a Novel LH/HCG Target Gene Regulating HAS2 Expression and Enhancing Cumulus Cells Migration. *J. Ovarian Res.* **2019**, *12*, 21. [CrossRef]
84. Tan, J.X.; Wang, X.Y.; Li, H.Y.; Su, X.L.; Wang, L.; Ran, L.; Zheng, K.; Ren, G.S. HYAL1 Overexpression Is Correlated with the Malignant Behavior of Human Breast Cancer. *Int. J. Cancer* **2011**, *128*, 1303–1315. [CrossRef]
85. Deng, H.; Wang, J.; An, R. Hyaluronic Acid-Based Hydrogels: As an Exosome Delivery System in Bone Regeneration. *Front. Pharmacol.* **2023**, *14*, 1131001. [CrossRef]
86. Kimura, N.; Totsukawa, K.; Sato, E. Significance of Mammalian Cumulus-Oocyte Complex Matrix in Oocyte Meiotic Maturation: Review of the Synthetic Control and Possible Roles of Hyaluronan (HA) and HA-Binding Protein. *J. Mamm. Ova Res.* **2006**, *23*, 36–51. [CrossRef]
87. Ahn, J.; Han, K.S.; Heo, J.H.; Bang, D.; Kang, Y.H.; Jin, H.A.; Hong, S.J.; Lee, J.H.; Ham, W.S. FOXC2 and CLIP4: A Potential Biomarker for Synchronous Metastasis of ≤7-Cm Clear Cell Renal Cell Carcinomas. *Oncotarget* **2016**, *7*, 51423. [CrossRef] [PubMed]
88. Liu, Y.; Su, C.Y.; Yan, Y.Y.; Wang, J.; Li, J.J.; Fu, J.J.; Wang, Y.Q.; Zhang, J.Y. Exosomes of A549 Cells Induced Migration, Invasion, and EMT of BEAS-2B Cells Related to Let-7c-5p and MiR-181b-5p. *Front. Endocrinol.* **2022**, *13*, 1395. [CrossRef]
89. Ma, S.; Song, L.; Bai, Y.; Wang, S.; Wang, J.; Zhang, H.; Wang, F.; He, Y.; Tian, C.; Qin, G. Improved Intracellular Delivery of Exosomes by Surface Modification with Fluorinated Peptide Dendrimers for Promoting Angiogenesis and Migration of HUVECs. *RSC Adv.* **2023**, *13*, 11269–11277. [CrossRef]
90. Sung, B.H.; Ketova, T.; Hoshino, D.; Zijlstra, A.; Weaver, A.M. Directional Cell Movement through Tissues Is Controlled by Exosome Secretion. *Nat. Commun.* **2015**, *6*, 7164. [CrossRef]
91. Jimenez, L.; Yu, H.; McKenzie, A.J.; Franklin, J.L.; Patton, J.G.; Liu, Q.; Weaver, A.M. Quantitative Proteomic Analysis of Small and Large Extracellular Vesicles (EVs) Reveals Enrichment of Adhesion Proteins in Small EVs. *J. Proteome Res.* **2019**, *18*, 947–959. [CrossRef] [PubMed]
92. Yuan, X.; Zhou, X.; Qiao, X.; Wu, Q.; Yao, Z.; Jiang, Y.; Zhang, H.; Zhang, Z.; Wang, X.; Li, J. FoxA2 and P53 Regulate the Transcription of HSD17B1 in Ovarian Granulosa Cells of Pigs. *Reprod. Domest. Anim.* **2021**, *56*, 74–82. [CrossRef] [PubMed]
93. Yu, L.; Liu, M.; Wang, Z.; Liu, T.; Liu, S.; Wang, B.; Pan, B.; Dong, X.; Guo, W. Correlation between Steroid Levels in Follicular Fluid and Hormone Synthesis Related Substances in Its Exosomes and Embryo Quality in Patients with Polycystic Ovary Syndrome. *Reprod. Biol. Endocrinol.* **2021**, *19*, 74. [CrossRef] [PubMed]
94. Karman, B.N.; Basavarajappa, M.S.; Hannon, P.; Flaws, J.A. Dioxin Exposure Reduces the Steroidogenic Capacity of Mouse Antral Follicles Mainly at the Level of HSD17B1 without Altering Atresia. *Toxicol. Appl. Pharmacol.* **2012**, *264*, 1–12. [CrossRef]
95. Wang, C.; Zhao, Y.; Yuan, Z.Y.; Wu, Y.; Zhao, Z.; Wu, C.; Hou, J.; Zhang, M. Genome-Wide Identification of MRNAs, LncRNAs, and Proteins, and Their Relationship with Sheep Fecundity. *Front. Genet.* **2022**, *12*, 2667. [CrossRef]
96. Zhou, Q.Y.; Wan, M.C.; Wei, Q.P.; Song, Q.L.; Xiong, L.G.; Huo, J.H.; Huang, J.N. Expression, Regulation, and Functional Characterization of FST Gene in Porcine Granulosa Cells. *Anim. Biotechnol.* **2016**, *27*, 295–302. [CrossRef]
97. Patton, B.K.; Madadi, S.; Pangas, S.A. Control of Ovarian Follicle Development by TGF-β Family Signaling. *Curr. Opin. Endocr. Metab. Res.* **2021**, *18*, 102–110. [CrossRef]
98. Bai, X.; Wang, S. Signaling Pathway Intervention in Premature Ovarian Failure. *Front. Med.* **2022**, *9*, 3545. [CrossRef]
99. Wang, C.; Sun, Y. Induction of Collagen I by CXCL10 in Ovarian Theca–Stroma Cells via the JNK Pathway. *Front. Endocrinol.* **2022**, *13*, 823740. [CrossRef]
100. Budna, J.; Chachuła, A.; Kaźmierczak, D.; Rybska, M.; Ciesiółka, S.; Bryja, A.; Kranc, W.; Borys, S.; Zok, A.; Bukowska, D.; et al. Morphogenesis-Related Gene-Expression Profile in Porcine Oocytes before and after in Vitro Maturation. *Zygote* **2017**, *25*, 331–340. [CrossRef]

101. Stelcer, E.; Komarowska, H.; Jopek, K.; Żok, A.; Iżycki, D.; Malińska, A.; Szczepaniak, B.; Komekbai, Z.; Karczewski, M.; Wierzbicki, T.; et al. Biological Response of Adrenal Carcinoma and Melanoma Cells to Mitotane Treatment. *Oncol. Lett.* **2022**, *23*, 120. [CrossRef]
102. Dennis, G.; Sherman, B.T.; Hosack, D.A.; Yang, J.; Gao, W.; Lane, H.C.; Lempicki, R.A. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol.* **2003**, *4*, P3. [CrossRef] [PubMed]
103. Extract and Visualize the Results of Multivariate Data Analyses [R Package Factoextra Version 1.0.7] | Semantic Scholar. Available online: https://www.semanticscholar.org/paper/Extract-and-Visualize-the-Results-of-Multivariate-Kassambara-Mundt/5cb503e3db8609405d9f286fadc2a8bb867e5b6e (accessed on 5 March 2023).
104. Golkar-Narenji, A.; Antosik, P.; Nolin, S.; Rucinski, M.; Jopek, K.; Zok, A.; Sobolewski, J.; Jankowski, M.; Zdun, M.; Bukowska, D.; et al. Gene Ontology Groups and Signaling Pathways Regulating the Process of Avian Satellite Cell Differentiation. *Genes* **2022**, *13*, 242. [CrossRef] [PubMed]
105. Benjamini, Y.; Cohen, R. Weighted False Discovery Rate Controlling Procedures for Clinical Trials. *Biostatistics* **2017**, *18*, 91–104. [CrossRef] [PubMed]
106. Gu, Z.; Eils, R.; Schlesner, M. Complex Heatmaps Reveal Patterns and Correlations in Multidimensional Genomic Data. *Bioinformatics* **2016**, *32*, 2847–2849. [CrossRef] [PubMed]
107. Yu, G.; Wang, L.G.; Han, Y.; He, Q.Y. ClusterProfiler: An R Package for Comparing Biological Themes among Gene Clusters. *Omics* **2012**, *16*, 284–287. [CrossRef]

5. Streszczenie

Macierz zewnątrzkomórkowa (ang. *extracellular matrix, ECM*) jest niezwykle ważną strukturą obecną we wszystkich tkankach organizmu zwierzęcego. Jest ona zaangażowana w wiele procesów fizjologicznych, także tych zachodzących w jajniku. Wykazano, że ECM znacząco wpływa na folikulogenezę, owulację i tworzenie ciałka żółtego. Cytoszkielet, podobnie jak ECM, jest strukturą dynamiczną, stale modyfikującą swój skład. Poprzez zaangażowanie w podział komórek wpływa na proliferację komórek ziarnistych w pęcherzyku jajnikowym. Cytoszkielet, wraz z białkami transbłonowymi (integrynami i kadherynami) a także macierzą zewnątrzkomórkową, jest ściśle zaangażowany w sygnalizację komórkową. W ostatnim czasie wiele uwagi poświęca się roli pęcherzyków zewnątrzkomórkowych (ang. *extracellular vesicles, EVs*) w sygnalizacji międzykomórkowej. Wśród tych nanocząsteczek wyróżnia się egzosomy, które będąc nośnikami białek, lipidów czy cząsteczek DNA lub RNA, są ściśle zaangażowane w regulację procesów komórkowych takich jak: adhezja, proliferacja i migracja. Dokładne poznanie interakcji pomiędzy ECM, cytoszkieletem oraz EVs w mikrośrodowisku pęcherzyka jajnikowego pozwoli na lepsze zrozumienie molekularnych podstaw procesów fizjologicznych jak i patologicznych w obrębie jajnika.

W badaniach obejmujących rozprawę doktorską wykorzystano komórki ziarniste jajnika świni domowej, które stanowią najliczniejszą populację komórek tworzących pęcherzyk jajnikowy. Wykazano, że są one ściśle zaangażowane w procesy folikulogenezy i oogenezy, a także są odpowiedzialne za steroidogenezę. Dodatkowo, poprzez ich nieustanny dialog z komórką jajową aktywnie uczestniczą w nabywaniu przez nią kompetencji do zapłodnienia. Metodyka badawcza rozprawy doktorskiej została oparta na prowadzeniu pierwotnej hodowli *in vitro* komórek ziarnistych jajnika świni domowej oraz określeniu profilu ekspresji genów regulujących tworzenie macierzy zewnątrzkomórkowej, cytoszkieletu oraz uczestniczących w podziale komórki i sygnalizacji międzykomórkowej, szczególnie w oparciu o pęcherzyki zewnątrzkomórkowe. Wykorzystanie metody mikromacierzy ekspresyjnych pozwoliło na określenie profilu transkryptomicznego komórek z poszczególnych przedziałów czasowych (0 h, 48 h, 96 h i 144 h), a walidację pozyskanych wyników przeprowadzono z zastosowaniem procedury RT-qPCR.

Opublikowane wyniki pierwszego etapu badań wykazały w świńskich komórkach ziarnistych hodowanych *in vitro* zwiększoną ekspresję genów kodujących kadheryny i kolagen oraz zaangażowanych w powstanie macierzy zewnątrzkomórkowej. Wyniki drugiego etapu badań przedstawiają profil ekspresji genów, które można uznać za nowe markery molekularne

procesów komórkowych zaangażowanych w organizację cytoszkieletu i jego udział w sygnalizacji międzykomórkowej. Trzeci etap prowadzonych badań przedstawia analizę ekspresji genów zaangażowanych w adhezję komórkową, proliferację, migrację oraz produkcję pęcherzyków zewnątrzkomórkowych. Przedstawiony profil ekspresji wybranych genów wnosi nowe spojrzenie na regulacje procesów fizjologicznych, szczególnie sygnalizację międzykomórkową w komórkach ziarnistych pęcherzyka jajnikowego świni. Badania te mogą być zatem wykorzystane w technikach wspomaganego rozrodu (ang. *assisted reproductive technologies, ART*) prowadzonych *in vitro* oraz dostarczać nowych danych dotyczących patofizjologii zaburzeń w obrębie jajnika.

6. Summary

The extracellular matrix (ECM) is an extremely important structure present in all tissues of the animal body. It is involved in many physiological processes, including those occurring in the ovary. The ECM has been shown to significantly affect folliculogenesis, ovulation and corpus luteum formation. The cytoskeleton, like the ECM, is a dynamic structure, constantly modifying its composition. Through its involvement in cell division, it influences the proliferation of granulosa cells in the ovarian follicle. The cytoskeleton, along with trans-membrane proteins (integrins and cadherins) and also the extracellular matrix, is closely involved in cell signaling. Recently, the role of extracellular vesicles (EVs) in intercellular signaling has focused much attention. Among these nanoparticles are exosomes, which, being carriers of proteins, lipids or DNA or RNA molecules, are intimately involved in the regulation of cellular processes such as adhesion, proliferation and migration. A thorough understanding of the interactions between the ECM, cytoskeleton and EVs in the microenvironment of the ovarian follicle will allow us to better understand the molecular basis of physiological as well as pathological processes of the ovary.

In the research performed in the dissertation, were used granulosa cells of the domestic pig ovary, which constitute the most abundant population of cells forming the ovarian follicle. They have been shown to be intimately involved in the processes of folliculogenesis and oogenesis, and are also responsible for steroidogenesis. In addition, through their constant dialogue with the ovum, they actively participate in the oocyte acquisition of competence for fertilization. The research methodology of the dissertation was based on conducting primary *in vitro* culture of granulosa cells of the domestic pig ovary and determining the expression profile of genes regulating the formation of the extracellular matrix, cytoskeleton and participating in cell division and intercellular signaling, specifically based on extracellular vesicles. The use of the expression microarray method allowed the determination of the transcriptomic profile of cells from specific time intervals (0 h, 48 h, 96 h and 144 h), and the validation of the obtained results was carried out using the RT-qPCR procedure.

The published results of the first stage of the study showed increased expression of genes encoding cadherins and collagen, as well as those involved in extracellular matrix (ECM) formation, in porcine granulosa cells cultured *in vitro*. The results of the second stage of the study present the expression profile of genes that can be considered new molecular markers of cellular processes involved in the organization of the cytoskeleton and its participation in intercellular signaling. The third stage of the conducted research presents the expression

analysis of genes involved in cell adhesion, proliferation, migration and production of extracellular vesicles (EVs). The presented expression profile of selected genes brings new insights into the regulation of physiological processes, especially intercellular signaling in granulosa cells of the porcine ovarian follicle. This research can therefore be used in assisted reproduction techniques (ART) conducted *in vitro* and provide new data on the pathophysiology of ovarian disorders.