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„Analiza profilu ekspresji wybranych genów regulujących procesy wzrostu, różnicowania i apoptozy w komórkach ziarnistych jajnika świni podczas ich krótkoterminowej pierwotnej hodowli in vitro”

Rozprawa na stopień doktora nauk rolniczych

w dyscyplinie weterynaria

Promotor: dr hab. n. wet. Paweł Antosik

Katedra Chirurgii Weterynaryjnej

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Spis treści

1. Wykaz publikacji stanowiących podstawę do nadania stopnia naukowego doktora

Publikacja I:

Kulus Magdalena, Sujka-Kordowska Patrycja, Konwerska Aneta, Celichowski Piotr, Kranc Wiesława, Kulus Jakub, Piotrowska-Kempisty Hanna, Antosik Paweł, Bukowska Dorota, Iżycki Dariusz, Bruska Małgorzata, Zabel Maciej, Nowicki Michał, Kempisty Bartosz

"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"

International Journal of Molecular Sciences, 2019, Vol. 20, nr 16, art. nr 3966, DOI: 10.3390/ijms20163966

Punktacja MNiSW: 140; Impact Factor: 4,556

Publikacja II:

Kulus Magdalena, Kranc Wiesława, Sujka-Kordowska Patrycja, Mozdziak Paul, Jankowski Maurycy, Konwerska Aneta, Kulus Jakub, Bukowska Dorota, Skowroński Mariusz Tomasz, Piotrowska-Kempisty Hanna, Nowicki Michał, Kempisty Bartosz, Antosik Paweł

"*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, Vol. 148, s. 76-88, DOI: 10.1016/j.theriogenology.2020.02.044

Punktacja MNiSW: 140; Impact Factor: 2,094

Publikacja III:

Kulus Magdalena, Kranc Wiesława, Sujka-Kordowska Patrycja, Celichowski Piotr, Konwerska Aneta, Jankowski Maurycy, Jeseta Michal, Skowroński Mariusz Tomasz, Piotrowska-Kempisty Hanna, Bukowska Dorota, Zabel Maciej, Bruska Małgorzata, Mozdziak Paul, Kempisty Bartosz, Antosik Paweł

"Transcriptomic analysis of expression of genes regulating cell cycle progression in porcine ovarian granulosa cells during short-term in vitro primary culture"

Histochemistry and Cell Biology, 2020, Vol. 153, nr 6, s. 397-412, DOI: 10.1007/s00418-020-01860-2

Punktacja MNiSW: 100; Impact Factor: 3,418

Łączna punktacja MNiSW: 380 Łącznie współczynnik Impact Factor: 10,068

2. Wykaz użytych skrótów

ACTB β – aktyna (ang. *β – actin*)

ANXA1 aneksyna A1 (ang. *annexin A1*)

CABLES1 ang. *Cdk5 and Abl enzyme substrate 1*

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 endoglina (ang. *endoglin*)

CD117 receptor c-kit (ang. *c-kit ligand*)

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

cDNA komplementarny DNA (ang. *complementary DNA*)

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

CSRNP3 ang. *cysteine and serine rich nuclear protein 3*

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

DAB1 ang. *DAB adaptor protein 1*

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*

EDTA kwas wersenowy (ang. *ethylenediaminetetraacetic acid*)

EMP1 ang. *epithelial membrane protein 1*

ESR1 receptor estrogenowy typu 1 (ang. *estrogen receptor 1*)

ETS1 ang. *ETS proto-oncogene 1, transcription factor*

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

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

FF płyn pęcherzykowy (ang. *follicular fluid*)

FGFR2 receptor 2 czynnika wzrostu fibroblastów (ang. *fibroblast growth factor receptor 2*)

FI interakcja funkcjonalna (ang. *functional interaction*)

FMOD fibromodulina (ang. *fibromodulin*)

FN1 fibronektyna 1 (ang. *fibronectin 1*)

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

GCs komórki ziarniste (ang. *granulosa cells*)

GDF-9 czynnik wzrostu i różnicowania 9 (ang. *growth differentiation factor 9*)

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

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

H&E hematoksylina i eozyna

H1FOO histon H1 specyficzny dla oocytów (ang. *oocyte-specific H1 histone*)

IFIT3 ang. *interferon induced protein with tetratricopeptide repeats 3*

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

IGFBP5 ang. *insulin like growth factor binding protein 5*

IHH ang. *indian hedgehog signaling molecule*

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

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

ITM2A ang. *integral membrane protein 2A*

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

LH hormon luteinizujący (ang. *luteinizing hormone*)

LIF ang. *interleukin 6 family cytokine*

MCOLN3 mukolipina 3 (ang. *mucolipin 3*)

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

NCAPD2 ang. *non-SMC condensin I complex subunit D2*

NEBL ang. *nebulette*

NEK2 ang. *NIMA related kinase 2*

NUP210 nukleoporyna 210 (ang. *nucleoporin 210*)

OD gęstość optyczna (ang. *optical density*)

P4 progesteron

PBGD deaminaza porfobilinogenu (ang. *porphobilinogen deaminase*)

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

PDE3A fosfodiestraza 3A (ang. *phosphodiesterase 3A*)

PDK4 ang. *pyruvate dehydrogenase kinase 4*

PDPN podoplanina (ang. *podoplanin*)

PLK2 ang. *polo like kinase 2*

POSTN periostyna (ang. *periostin*)

PPARD ang. *peroxisome proliferator activated receptor δ*

PPAT ang. *phosphoribosyl pyrophosphate amidotransferase*

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*)

SCUBE1 ang. *signal peptide, CUB domain and EGF like domain containing 1*

SERPINB2 inhibitor aktywatora plazminogenu-2 (ang. *serpin family B member 2*)

SFRP2 ang. *secreted frizzled – related protein 2*

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

TAGLN transgelina (ang. *transgelin*)

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

TGFB1 transformujący czynnik wzrostu typu β-1 (ang. *transforming growth factor β-1*)

TGFBR3 ang. *transforming growth factor β receptor 3*

THBS1 trombospondyna 1 (ang. *thrombospondin 1*)

TMOD1 tropomodulina 1 (ang. *tropomodulin 1*)

TNF – α czynnik martwicy nowotworów (ang. *tumor necrosis factor α*)

TNFSF10 ang. *TNF superfamily member 10*

TXNIP ang. *thioredoxin interacting protein*

3. Badania własne

3.1. Wprowadzenie

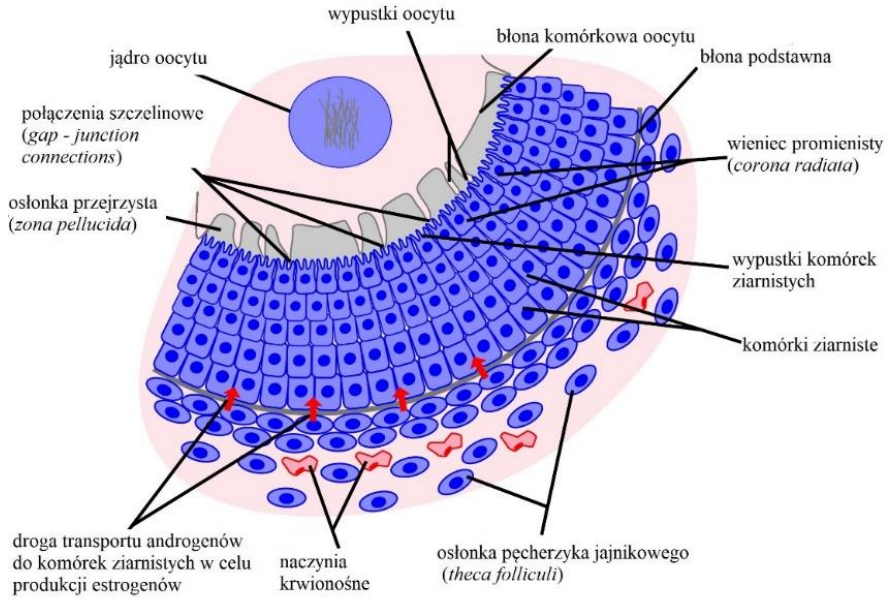
Folikulogeneza, czyli rozwój pęcherzyka jajnikowego, skutkuje licznymi zmianami zarówno pod względem morfologii pęcherzyka jajnikowego, jak i syntezy hormonów płciowych (Channing i Tsafriri 1977). Prowadzi to do cyklicznych zmian w narządach układu rozrodczego oraz w zachowaniu się zwierząt, nazywanych rują (Schnurrbusch i Erices 1979). Oogeneza (rozwój oocytu) jest ściśle związana z folikulogenezą, a rozbudowane wzajemne interakcje komórek budujących pęcherzyk jajnikowy, w tym szczególnie komórek ziarnistych (ang. *granulosa cells,* GCs), prowadzą do rozwoju komórki jajowej przygotowywanej do jej uwolnienia w procesie owulacji (Channing i wsp. 1978). Przebieg opisanych wyżej mechanizmów jest dobrze poznany, jednak ich podłoże molekularne wymaga dalszych badań.

Dojrzały, przedowulacyjny pęcherzyk jajnikowy jest zbudowany z kilku populacji komórek. Najliczniejszą grupę stanowią osadzone na błonie podstawnej (łac. *lamina basalis*) komórki ziarniste budujące wewnętrzną warstwę ściany pęcherzyka (ang. *mural granulosa cells,* MGCs). Z kolei komórki ziarniste, tworzące wzgórek jajonośny (łac. *cumulus oophorus*), określa się mianem komórek wzgórka jajonośnego (ang. *cumulus cells,* CCs). W obrębie wzgórka znajduje się oocyt II rzędu, zahamowany w metafazie II podziału mejotycznego. Warstwa komórek bezpośrednio otaczająca oocyt przyjmuje postać wieńca promienistego (łac. *corona radiata*) (Clement 1987). Wokół pęcherzyka jajnikowego tworzy się łącznotkankowa osłonka pęcherzykowa, którą budują komórki osłonki pęcherzykowej (ang. *theca cells,* TCs). Wyróżnia się jej warstwę wewnętrzną (łac. *tunica interna thecae folliculi*), bogatą w komórki i naczynia krwionośne oraz warstwę zewnętrzną (łac. *tunica externa thecae folliculi*), zawierającą niewiele komórek, nieliczne miocyty gładkie oraz włókna kolagenowe (Magoffin 2005). Podczas wzrostu pęcherzyka jajnikowego pomiędzy komórkami ziarnistymi tworzą się przestrzenie, z czasem łączące się z sobą w jedną jamę, którą wypełnia płyn pęcherzykowy (ang. *follicular fluid,* FF). Jedną z głównych funkcji komórek ziarnistych jest synteza estrogenów (żeńskich hormonów steroidowych), które wydzielane są do płynu pęcherzykowego (Truman i wsp. 2017). Produkcja tych hormonów odbywa się w ścisłej współpracy tych komórek z komórkami wewnętrznej warstwy osłonki pęcherzyka, które w wyniku oddziaływania hormonu luteinizującego (LH, ang. *luteinizing hormone*) rozpoczynają produkcję androgenów. Wytworzony w tym procesie androstendion przemieszcza się przez błonę podstawną do komórek ziarnistych (Ryc. 1), które poprzez sygnały hormonalne, pochodzące z przysadki, w tym hormon folikulotropowy

(FSH, ang. *follicular stimulating hormone*), przeprowadzają aromatyzację tych substratów do estrogenów (głównie do 17β-estradiolu) (Magoffin 2005; Nguyen i wsp. 2012). W wyniku dodatniego sprzężenia zwrotnego, syntetyzowane estrogeny powodują zwiększoną waskularyzację osłonki wewnętrznej (zwiększenie dostępności LH, FSH), mitozę i proliferację komórek ziarnistych (zwiększenie liczby receptorów dla FSH), co prowadzi do zintensyfikowania procesu aromatyzacji androgenów. Konsekwencją opisanego wyżej sprzężenia jest powstanie przedowulacyjnych pęcherzyków jajnikowych, natomiast pęcherzyki produkujące niewielką ilość estrogenów ulegają androgenizacji, co prowadzi do ich atrezji. Dodatkowo sam oocyt stymuluje proliferację komórek ziarnistych poprzez wydzielanie czynnika GDF–9 (ang. *growth differentiation factor 9*) (Su i wsp. 2009). Niezwykle ważną rolę pełnią komórki ziarniste budujące wieniec promienisty, gdyż pozostają one w bezpośrednim kontakcie z oocytem dzięki licznym mikrokosmkom (Piotrowska i wsp. 2013). Między oocytem a komórkami ziarnistymi wytwarza się osłonka przejrzysta (łac. *zona pellucida*) zbudowana z glikoprotein (Sinowatz i wsp. 1995). Jest ona penetrowana zarówno przez wypustki komórek wieńca promienistego, jak i samego oocytu. W miejscach ich kontaktu wytwarzają się połączenia szczelinowe typu neksus (ang. *gap junction connection,* GJC), zbudowane z białek błonowych – koneksyn (Aasen i wsp. 2019). Dzięki tym połączeniom zapewniona jest bezpośrednia wymiana jonów, małych cząsteczek czy wtórnych przekaźników (o masie do 1,2 kDa), która odbywa się na zasadzie przenikania zgodnie z gradientem stężeń (Kidder i Mhawi 2002; Wang i wsp. 2009; Sugimura i wsp. 2017). Połączenia typu neksus występują nie tylko pomiędzy komórkami wieńca promienistego a oocytem, ale także pomiędzy poszczególnymi komórkami ziarnistymi, co umożliwia pośredniczenie w „dialogu" z oocytem (Kempisty i wsp. 2013, 2014). Prowadzone w ostatnich latach badania wskazują, iż komórki ziarniste mają duży wpływ na dojrzewanie jądrowe i cytoplazmatyczne oocytu. Potwierdzeniem tego jest zahamowanie tych procesów w przypadku zbyt wczesnej denudacji (odizolowania oocytu od komórek CCs), co negatywnie wpływa na kompetencję rozwojową komórki jajowej (Tanghe i wsp. 2002; Appeltant i wsp. 2015). W przypadku świni domowej (łac. *Sus scrofa domestica*) dochodzi do owulacji mnogiej (poliowulacji) dojrzałych pęcherzyków jajnikowych. Następuje uwolnienie oocytu wraz z otaczającymi go komórkami ziarnistymi wieńca promienistego – CCs, które razem nazywane są kompleksem kumulus – oocyt (ang. *cumulus – oocyte complex,* COC), podczas gdy komórki ziarniste ściany pęcherzyka (MGCs) pozostają w jego wnętrzu i pełnią funkcję steroidogenną. Jednocześnie komórki budujące warstwę wewnętrzną osłonki pęcherzykowej wnikają w ścianę zapadniętego pęcherzyka i przekształcają się w komórki

paraluteinowe. Bardzo istotną rolę odgrywają komórki warstwy ziarnistej, które przekształcają się w komórki luteinowe odpowiedzialne za syntezę hormonów steroidowych (głównie progesteronu – P4). Hormon ten stymuluje aktywność błony śluzowej macicy, a w rezultacie podtrzymuje ciążę w przypadku zapłodnienia (Maekawa i wsp. 2016).

Najnowsze techniki badawcze, w tym metody biologii molekularnej, stwarzają możliwości dokładnego poznania mechanizmów regulujących przebieg folikulogenezy i oogenezy. Nowe informacje mogą okazać się istotne dla zwiększenia skuteczności technik wspomaganego rozrodu stosowanych u ludzi i zwierząt. Dokładne poznanie mechanizmów regulujących kompetencję rozwojową oocytów może również w znaczący sposób przybliżyć nas do wyjaśnienia na poziomie molekularnym procesów patologicznych, prowadzących do niepłodności.



Ryc. 1. Schemat budowy pęcherzyka jajnikowego, na podstawie publikacji w *Histochem. Cell Biol*., (Kulus i wsp. 2020a)

Ostatnie doniesienia literaturowe wskazują na bardzo istotny aspekt dotyczący komórek ziarnistych (GCs), określający ich znaczny potencjał macierzystości. Potencjał ten został ostatnio wykazany w komórkach ziarnistych pochodzących z pęcherzyków jajnikowych, które stanowią istotną pulę komórek ostatecznie niezróżnicowanych (Kossowska-Tomaszczuk i wsp. 2010; Varras i wsp. 2012). Stwierdzono to w trakcie badań nad ludzkimi komórkami ziarnistymi, które podczas pierwotnej hodowli *in vitro* wykazały ekspresję genów uznanych za molekularne markery multipotencjalnych mezenchymalnych komórek macierzystych,

tj. *CD44*, *CD90*, *CD105*, *CD117*, *CD166* (Atlasi i wsp. 2008; Kossowska-Tomaszczuk i Geyter 2013). Po zastosowaniu odpowiednich mediów zaobserwowano różnicowanie się komórek ziarnistych w komórki tkanki mięśniowej (Brevini i wsp. 2014), chrzęstnej, kostnej oraz nerwowej (Kossowska-Tomaszczuk i wsp. 2009). W odniesieniu do komórek świni także wykazano znaczną plastyczność GCs, która przejawiała się różnicowaniem ich w komórki o potencjale osteogennym (Mattioli i wsp. 2012). Wykazana zdolność komórek ziarnistych do kierunkowego różnicowania się, po przeprowadzeniu dalszych badań w tym zakresie, może zostać wykorzystana w medycynie regeneracyjnej, w tym leczeniu chorób degeneracyjnych i chronicznych.

3.2. Cele badań

Głównym celem przeprowadzonych badań było określenie profilu ekspresji wybranych genów regulujących procesy wzrostu, różnicowania i apoptozy w jajnikowych komórkach ziarnistych świni, podczas pierwotnej hodowli *in vitro*.

Cel główny został zrealizowany poprzez następujące cele szczegółowe:

1. Izolację oraz ustalenie warunków krótkoterminowej hodowli *in vitro* jajnikowych komórek ziarnistych świni domowej;
2. Analizę profilu ekspresji genów przy wykorzystaniu metody mikromacierzy (typu Affymetrix);
3. Walidację uzyskanych wyników ekspresji wybranych genów za pomocą techniki RT-qPCR.

3.3. Materiał i metody

Materiał do badań stanowiły komórki ziarniste, izolowane z jajników pobranych poubojowo od świń. Samice świń, 126 loszek, były utrzymywane w zbliżonych warunkach hodowlanych, pochodziły z zarejestrowanych gospodarstw. Zwierzęta te osiągnęły wiek 170 dni oraz masę ciała ok. 98 kg, były także dojrzałe płciowo. Skierowane do uboju zwierzęta zostały wcześniej zbadane i zaklasyfikowane jako klinicznie zdrowe. Wyizolowane narządy układu rozrodczego były następnie transportowane do laboratorium w czasie nie dłuższym niż 30 min., zanurzone w roztworze 0,9% NaCl o temperaturze 38°C.

W laboratorium jajniki przenoszono do roztworu PBS (buforowana fosforanem sól fizjologiczna, ang. *phosphate-buffered saline*) wzbogaconego 2% płodową surowicą bydlęcą (ang. *fetal bovine serum*, FBS; Sigma – Aldrich Co., St. Louis, MO, USA). Komórki ziarniste były pozyskiwane na drodze aspiracji płynu pęcherzykowego poprzez punkcję pęcherzyków o średnicy > 5 mm za pomocą igły 20-G i strzykawki 5 ml do sterylnych płytek Petriego. Pozyskany płyn pęcherzykowy, wraz z zawieszonymi w nim komórkami, był następnie wirowany (200 × *g*, 10 min., w temperaturze pokojowej), a uzyskany osad komórkowy wykorzystano do założenia hodowli pierwotnej. Komórki wysiewano do butelek hodowlanych z zakrętką wentylacyjną (25 $cm^{2,}$ TPP 90025, BioNovo) w liczbie 3×10^6 / butelkę. Zliczanie komórek wykonano za pomocą automatycznych urządzeń do pomiaru ilości i oceny żywotności komórek (Vi-Cell XR Cell Viability Analyzer, Beckman Coulter, IN, USA; ADAM MC Automated Cell Counter, NanoEntek, Waltham, MA, USA). Komórki ziarniste hodowano w pożywce Dulbecco's Modified Eagle's Medium (DMEM, Sigma – Aldrich Co., St. Louis, MO, USA), która została wzbogacona o: 2% płodową surowicę cielęcą (ang. *fetal calf serum*, FCS; PAA, Linz, Austria), 10 mg/ml kwasu askorbinowego (Sigma – Aldrich, USA), 0,05 μM deksametazonu (Sigma – Aldrich, USA), 4 mM l-glutaminy (ang. *l-glutamine*), 10 mg/ml gentamycyny (Invitrogen, USA), 10,000 U/ml penicyliny (Invitrogen, USA) oraz 10,000 μg/ml streptomycyny (Invitrogen, USA) o temperaturze 38,5°C i w warunkach 5% stężenia CO_2. Medium hodowlane było wymieniane co 72 h. Pasaż komórek wykonywano w momencie osiągnięcia przez komórki 80% konfluencji (pokrycie 80% powierzchni naczynia hodowlanego), używając do ich separacji od powierzchni dna naczynia roztworu 0,05% trypsyny – EDTA (Invitrogen, USA).

Przeprowadzone badania podzielone zostały na następujące etapy:

- → izolacja komórek ziarnistych z pęcherzyków jajnikowych świń;
- → pierwotna hodowla *in vitro;*
- → izolacja RNA metodą Chomczyńskiego – Sacchi;
- → wykonanie mikromacierzy ekspresyjnych Affymetrix® Porcine Gene 1.1 ST Array Afffymetrix (Affymetrix, Santa Clara, CA, USA);
- → ilościowa łańcuchowa reakcja polimerazy z odwróconą transkrypcją (RT-qPCR);
- → badanie histologiczne, barwienie preparatów hematoksyliną i eozyną (H&E).

W badaniach wybrano model krótkoterminowej pierwotnej hodowli *in vitro*, podczas której zastosowano przedziały czasowe umożliwiające zarejestrowanie zmian w ekspresji genów. Wyznaczono 4 przedziały czasowe: 1) 0 h – wartość referencyjna; 2) 48 h – początkowe zmiany w hodowli; 3) 96 h – zakładany „punkt utraty" właściwości fizjologicznych komórki oraz 4) 144 h – punkt końcowy krótkoterminowej hodowli *in vitro*. Po upływie wskazanych godzin hodowli z komórek ziarnistych izolowano RNA, który poddawano dalszym analizom.

Izolację całkowitego RNA wykonano z wykorzystaniem metody Chomczyńskiego – Sacchi. Błony komórkowe zdegradowano fenolem oraz tiocyjanianem guanidyny (TRI Reagent®, Sigma – Aldrich, St. Luis, USA), dodając chloroform uzyskano 3 fazy roztworu (wodna – wraz z badanym RNA, interfaza oraz organiczna), natomiast wytrącenie RNA nastąpiło po użyciu izopropanolu. Jakość i stężenie wyizolowanego RNA zostały zbadane poprzez pomiar gęstości optycznej (OD, ang. *optical density*) przy długości fali 260 nm z wykorzystaniem spektrofotometru do pomiarów w kropli (Spektrofotometr NanoDrop, Thermo Scientific, Waltham, MA, USA). W kolejnych etapach badań molekularnych wykorzystano 100 ng RNA w procesie odwrotnej transkrypcji celem uzyskania cDNA, zgodnie z protokołem załączonym przez producenta (Ambion® WT Expression Kit).

Technika mikromacierzy ekspresyjnych Affymetrix® została wykorzystana w celu pełnego profilowania transkryptomu komórek ziarnistych w poszczególnych przedziałach czasowych. Dzięki wykorzystaniu baz danych dotyczących genów (GeneAtlas System, Affymetrix GeneAtlas™ Operating Software) i ich produktów białkowych, w tym także danych literaturowych, wykonano analizę funkcjonalną mikromacierzy. Algorytm Robust Multiarray Averaging (RMA) został wykorzystany w celu normalizacji wyników, które następnie poddano annotacji wg wskazań producenta. Statystyczna istotność różnic w poziomie ekspresji

badanych genów była analizowana metodą Bayesa z poprawkami Benjaminiego – Holdberga. Selekcję genów o istotnie zmienionej ekspresji oparto na wartości p wynoszącej <0,05 i średniej zmianie wartości ekspresji >2. Wybrane w ten sposób geny dodano do bazy DAVID (Database for Annotation, Visualization and Integrated Discovery), gdzie otrzymano wyselekcjonowane grupy ontologiczne genów z bazy danych Gene Ontology Biological Process (GO BP). Poszczególne grupy ontologiczne obejmują geny odpowiedzialne za różne procesy biologiczne. Otrzymane wyniki ekspresji zostały poddane hierarchicznej procedurze klasteryzacji i przedstawione jako „mapy cieplne" (ang. *heat maps*) wybranych genów wchodzących w skład grup ontologicznych. Ilościowa łańcuchowa reakcja polimerazy z odwrotną transkrypcją (RT-qPCR) została przeprowadzona przy użyciu LightCycler real-time PCR detection system (Roche Diagnostics GmbH, Mannheim, Germany) i wykorzystana w celu walidacji wyników otrzymanych podczas analizy mikromacierzy ekspresyjnych. W reakcji qPCR użyto QuantiTect® SYBR® Green PCR (Master Mix Qiagen GmbH, Hilden, Germany) a także genów referencyjnych: *PBGD* (ang. *porphobilinogen deaminase*) i *ACTB* (ang. *β – actin*). Bazując na uzyskanych wynikach, wygenerowano sieć interakcji między poszczególnymi genami (STRING network (von Mering i wsp. 2005)) oraz oceniono ich powiązania funkcjonalne (REACTOME FIViz app to Cytoscape 3.6.0 software). Wszystkie prezentowane analizy i wykresy zostały opracowane przy użyciu pakietu Bioconductor (wersja 3.12) oraz oprogramowania statystycznego R (wersja 4.0.4). Analiza statystyczna uzyskanych mikromacierzy była wykonywana pakietem limma (wersja 3.46.0) (Ritchie i wsp. 2015), natomiast annotacja do grup ontologicznych pakietem RDAVIDWebService (wersja 3.12) (Fresno i Fernandez 2013). W celu dalszej analizy wybranych zestawów genów i oceny ich wzajemnych relacji zastosowano pakiet GOplot (wersja 1.0.2.) (Walter i wsp. 2015).

W celu analizy prawidłowej budowy histologicznej pozyskanych jajników i poszczególnych pęcherzyków jajnikowych tkanki były utrwalane przez 48 godzin w płynie Bouin'a, odwodnione w szeregu alkoholi oraz zatopione w parafinie. Za pomocą półautomatycznego obrotowego mikrotomu (Leica RM 2145, Leica Microsystems, Nussloch, Niemcy) bloczki parafinowe zostały pocięte na skrawki o grubości 4 μm, które następnie rutynowo zabarwiono przy użyciu hematoksyliny i eozyny. Mikroskop świetlny Olympus BX61VS został wykorzystany w celu oglądania uzyskanych preparatów i dokumentacji fotograficznej.

3.4. Wyniki i dyskusja

Pierwszy etap badań polegał na analizie ekspresji wybranych genów w komórkach ziarnistych, związanych z pełnieniem przez te komórki ściśle określonych funkcji. Selekcja grup ontologicznych genów, w oparciu o metodę mikromacierzy ekspresyjnych, pozwoliła wyróżnić trzy grupy genów odpowiedzialnych odpowiednio za komórkową morfogenezę („*cell morphogenesis*", GO:0000902), rozwój komórek („*cell development*", GO:0048468) oraz różnicowanie się komórek („*cell differentiation*", GO:0030154). Geny należące do tych grup charakteryzowały się podobnym wzorem ekspresji w analizowanych przedziałach czasowych, z tendencją do wzrostu ekspresji badanych genów wraz z upływem czasu doświadczenia. W wyniku przeprowadzonego badania wyodrębniono 265 genów (należących do trzech wspomnianych grup ontologicznych), wśród których wybrano 10 o najbardziej podwyższonej ekspresji (ang. *up – regulation*) oraz 10 o najbardziej obniżonej ekspresji (ang. *down – regulation*). W trakcie prowadzonej pierwotnej hodowli *in vitro* komórek ziarnistych podwyższoną ekspresję wykazały geny: *POSTN*, *FN1*, *ITGB3*, *TAGLN*, *LAMB1*, *ITGA2*, *IGFBP5*, *CAV1*, *PDPN*, *TMOD1*. Natomiast obniżona ekspresja została odnotowana w przypadku genów: *DAPL1*, *CXCL10*, *NEBL*, *ITM2A*, *TGFBR3*, *IHH*, *MCOLN3*, *SCUBE1*, *DAB1*, *IGF1*. Wśród 20 analizowanych genów aż 19 wykazało przynależność do grupy ontologicznej różnicowania się komórek („*cell differentiation*"). Do pozostałych dwóch grup ontologicznych przypisano 7 genów (grupa „*cell morphogenesis*") oraz 9 genów (grupa „*cell development*"). W przypadku 6 genów wykazano przynależność do wszystkich opisywanych grup ontologicznych. Uzyskane metodą mikromacierzy ekspresyjnych wyniki zostały poddane walidacji ilościową metodą RT-qPCR. Potwierdziło to omawiany wyżej charakter ekspresji genów poddanych analizie, a istotne statystycznie różnice w ich ekspresji wynikają z większej czułości metody RT-qPCR. Genem o najwyższym poziomie ekspresji podczas krótkoterminowej pierwotnej hodowli *in vitro* komórek ziarnistych był *POSTN* (ang. *periostin*), którego białkowy produkt jest odpowiedzialny nie tylko za procesy komórkowej adhezji, ale także pełni istotną rolę w procesie regeneracji tkanki kostnej (Rios i wsp. 2005). Wprawdzie do tej pory nie wykazano jego roli w przebiegu folikulogenezy, niemniej jednak ostatnie badania wykazały podwyższoną ekspresję *POSTN* w hodowli *in vitro* świńskich komórek ziarnistych (Ożegowska i wsp. 2019). Najsilniej obniżony poziom ekspresji wykazał gen *DAPL1* (ang. *death associated protein – like 1*), należący do grupy „*cell differentiation*". Produkt białkowy tego genu jest silnie związany z procesami apoptozy, a podwyższony poziom jego ekspresji wykazano w badaniach nad zapaleniem macicy krów mlecznych (Salilew-

Wondim i wsp. 2016). Znaczące obniżanie ekspresji omawianego genu w trakcie hodowli, w stosunku do wartości referencyjnej, może wskazywać na zmianę właściwości komórek ziarnistych podczas ich hodowli *in vitro*. Kolejnym genem, którego ekspresja poddana była analizie, jest *IGF1* (ang. *insulin like growth factor 1*), którego powiązanie jest szeroko opisane w kontekście folikulogenezy. Dowiedziono, iż gen ten pośrednio, jako jeden z czynników, uczestniczy w regulacji owulacji mnogiej u zwierząt (Sirotkin i wsp. 2017), co potwierdzono w naszym doświadczeniu na komórkach ziarnistych świń, w którym wykazano jego podwyższoną ekspresję w początkowym etapie eksperymentu (wartość referencyjna – 0 godz.). W odniesieniu do wyników przedstawiających zależność poszczególnych genów (STRING network) należy zwrócić uwagę na ważne interakcje genu *FN1* (ang. *fibronectin 1*). Zostały one potwierdzone jego wpływem na aktywację ekspresji genów: *ITGA2* (ang. *integrin subunit α-2*), *ITGB3* (ang. *integrin subunit β-3*), *IGF1*, co wskazuje na interakcję funkcjonalną pomiędzy analizowanymi genami (Functional Interaction, FI). Przeprowadzone badania pozwoliły na wskazanie genów w jajnikowych komórkach ziarnistych świń, kodujących białka biorące udział w procesach związanych z morfogenezą, ich różnicowaniem i rozwojem. Analiza ta pozwoliła dodatkowo wyróżnić potencjalne markery genetyczne procesu folikulogenezy oraz owulacji, które w przyszłości mogą być istotne w odniesieniu do zaawansowanych biotechnik wspomaganego rozrodu u zwierząt.

Dalszy etap prowadzonych badań umożliwił określenie ekspresji genów wykazujących aktywność w procesach związanych ze wzrostem komórek, ich starzeniem się oraz programowaną śmiercią komórki. Analiza wykonana w oparciu o mikromacierze ekspresyjne pozwoliła wyróżnić siedem grup ontologicznych: proces apoptozy („*apoptotic process*", GO:0006915), starzenie się komórek („*aging*", GO:0007568), wzrost komórek („*cell growth*", GO:0016049), regulacja śmierci komórkowej („*regulation of cell death*", GO:0010941), dodatnia regulacja śmierci komórkowej („*positive regulation of cell death*", GO:0010942), ujemna regulacja śmierci komórkowej („*negative regulation of cell death*", GO:0060548), śmierć komórkowa („*cell death*", GO:0008219). W badaniu tym wykazano tendencję do zwiększania poziomu ekspresji analizowanych genów podczas krótkoterminowej hodowli *in vitro*. W trakcie analizy 182 genów, wykazujących zróżnicowaną ekspresję, wybrano 10 genów o najwyższym i 10 genów o najniższym poziomie ekspresji. Do genów o wzrastającej ekspresji należały: *POSTN*, *FMOD*, *FN1*, *DCN*, *ITGB3*, *SERPINB2*, *SFRP2*, *IGFBP5*, *EMP1*, *CCL2*, natomiast największy spadek ekspresji zanotowano dla: *DAPL1*, *TGFBR3*, *IHH*, *ESR1*, *PPARD*, *PDK4*, *CSRNP3*, *IFIT3*, *TXNIP*, *TNFSF10*. Większość genów poddanych analizie

wykazywała przynależność do kilku grup ontologicznych. Najmniej licznie reprezentowana była grupa ontologiczna „*aging*", do której przynależały geny *CCL2* (ang. *chemokine (C-C motif) ligand 2*), *IGFBP5* (ang. *insulin like growth factor binding protein 5*) oraz *DCN* (ang. *decorin*), z czego dwa ostatnie należały tylko do tej grupy ontologicznej. Genami wykazującymi przynależność tylko do jednej grupy, „*cell growth*" były *POSTN, FMOD* (ang. *fibromodulin*), *FN1, ITGB3*, które jednocześnie ulegały najwyższej ekspresji w trakcie prowadzonego doświadczenia oraz gen *TGFBR3* (ang. *transforming growth factor β receptor 3*) wykazujący najbardziej obniżony poziom ekspresji. Wyniki otrzymane metodą mikromacierzy ekspresyjnych poddano walidacji ilościową metodą RT-qPCR. Analiza ta umożliwiła ilościowe określenie poziomu ekspresji wybranych genów. Badanie profilu ekspresji pozwoliło na wyodrębnienie dwóch genów, które, podobnie jak w poprzednim etapie badań, wykazały silny wzrost (*POSTN*) oraz znaczący spadek ekspresji (*DAPL1*). Gen *POSTN* wykazuje także podwyższoną ekspresję w tkankach, w których odbywają się procesy regeneracji i angiogenezy (Rios i wsp. 2005). Ostatnie wyniki wskazują także, że gen ten może być związany z procesami nowotworowymi w obrębie jajnika (Tang i wsp. 2018; Mariani i wsp. 2019). Gen *DAPL1*, wykazujący najbardziej obniżoną ekspresję, reprezentował grupy ontologiczne „*cell death*" oraz „*apoptotic process*", co dowodzi, że jest ściśle związany z procesami programowanej śmierci komórki. Kolejnym genem związanym z procesem starzenia się oraz atrezją pęcherzyków jajnikowych (Hayashi i wsp. 2010) był gen *IGFBP5*, wykazujący podwyższony poziom ekspresji. Gen ten przynależał tylko do jednej grupy ontologicznej „*aging*". Podobnie jak w poprzednim etapie badań, genem wykazującym największą ilość powiązań z innymi genami (STRING network) jest *FN1*. Wykazano, że nawiązuje on bezpośrednie interakcje z 7 innymi genami, przy czym najsilniejsza dotyczy genu *ITGB3*, który jest z nim ściśle związany pod względem funkcjonalnym (Functional Interaction, FI). Powyższa analiza pozwoliła wyróżnić geny należące do grup związanych zarówno z procesami wzrostu, starzenia się, jak i programowanej śmierci komórki. Natomiast na szczególną uwagę zasługują geny powiązane z apoptozą i atrezją pęcherzyków jajnikowych, co wskazuje na ich rolę jako specyficznych markerów genetycznych związanych z tymi procesami.

W ostatnim, podsumowującym, etapie badań skupiono się na procesach związanych z regulacją cyklu komórkowego. Wykonana w tym celu analiza transkryptomu komórek ziarnistych jajnika świni pozwoliła wyodrębnić geny należące do siedmiu wyróżnionych poniżej grup ontologicznych. Grupa ontologiczna „*cell cycle*" (GO:0007049) reprezentuje geny

funkcjonalnie odpowiedzialne za procesy biochemiczne i morfologiczne zachodzące w obrębie dzielących się komórek, natomiast geny należące do grupy „*cell cycle process*" (GO:0022402) są zaangażowane w replikację i segregację chromosomów. Geny odpowiedzialne za podział komórki należą z kolei do grupy ontologicznej „*cell division*" (GO:0051301). Przejście komórki pomiędzy poszczególnymi fazami cyklu komórkowego regulowane jest przez geny z grup ontologicznych „*cell cycle phase transition*" (GO:0044770), w tym pomiędzy fazą G2/M cyklu komórkowego („*cell cycle G2/M phase transition*", GO:0044839) oraz pomiędzy fazą G1/S („*cell cycle G1/S phase transition*", GO:0044843). Istotnym etapem cyklu komórkowego jest punkt kontrolny (grupa ontologiczna „*cell cycle checkpoint*", GO:0000075), który reguluje prawidłowe następowanie po sobie jego poszczególnych faz. Wśród analizowanych grup ontologicznych zaobserwowano tendencję do spadku poziomu ekspresji w poszczególnych przedziałach czasowych hodowli pierwotnej komórek ziarnistych. Spośród 133 genów w obrębie wymienionych grup do dalszej analizy wybrano 10 genów z najbardziej podwyższoną (*SFRP2*, *PDE3A*, *PDPN*, *FGFR2*, *ETS1*, *PLK2*, *LIF*, *THBS1*, *ANXA1*, *TGFB1*) oraz 10 z najbardziej obniżoną ekspresją (*RGS2*, *NUP210*, *CCNE2*, *PPAT*, *H1FOO*, *CABLES1*, *NEK2*, *NCAPD2*, *TXNIP*, *IGF1*). W obrębie opisywanych genów większość z nich wykazywała przynależność jednocześnie do kilku grup ontologicznych, a grupa „*cell cycle G2/M phase transition*" była reprezentowana tylko przez jeden gen – *NEK2* (ang. *NIMA related kinase 2*). Najliczniejszą grupą ontologiczną była „*cell cycle*", do której przynależność wykazały wszystkie geny (za wyjątkiem *SFRP2*, ang. *secreted frizzled – related protein 2*). Walidację zmian ekspresji wybranych 20 genów wykonano za pomocą ilościowej metody RT-qPCR. Najwyższy poziom ekspresji wykazał gen *SFRP2*, który przynależał tylko do grupy ontologicznej „*cell division*". Gen ten należy do rodziny antagonistów ścieżki sygnałowej WNT. Ścieżka ta odpowiedzialna jest między innymi za procesy związane z rozwojem, proliferacją oraz różnicowaniem komórek ziarnistych, a także steroidogenezą (Zamberlam i wsp. 2019). Ekspresja tego genu była opisywana wcześniej w jajnikowych komórkach ziarnistych myszy (Hernandez-Gonzalez i wsp. 2006) i ludzi (Ekart i wsp. 2013). Najniższy poziom ekspresji odnotowano w przypadku genu *RGS2* (ang. *regulator of G protein signaling 2*), który opisano jako marker genetyczny procesu luteinizacji komórek ziarnistych (Kranc i wsp. 2015) oraz *CCNE2* (ang. *cyclin E2*), związanego z proliferacją bydlęcych komórek ziarnistych (Shimizu i wsp. 2013) a także będącego kluczowym regulatorem cyklu komórkowego mysich komórek ziarnistych (Meinsohn i wsp. 2018). Dzięki modelowi powiązań opisywanych genów (STRING network) wykazano silną korelację między genami *TGFB1* (ang. *transforming growth factor β-1*) a *THBS1* (ang. *thrombospondin 1*), czego

wynikiem jest interakcja funkcjonalna (FI) między innymi w zakresie atrezji pęcherzyków jajnikowych (Terenina i wsp. 2017). Wyniki ostatniego etapu badań pozwoliły na wskazanie genów, których produkty białkowe zaangażowane są w procesy regulacji cyklu komórkowego jajnikowych komórek ziarnistych świń. Dodatkowo ekspresja części analizowanych genów została opisana po raz pierwszy w obrębie komórek ziarnistych jajnika świni. Ponadto uzyskane wyniki wskazują na nowe markery molekularne regulujące proces atrezji pęcherzyków jajnikowych świń.

Jak wcześniej opisano, jajniki świni, stanowiące materiał do badań, były oceniane pod względem klinicznym (morfologia jajnika), a ewentualne występujące zmiany w ich obrębie dyskwalifikowały je jako materiał badawczy. Dodatkowo, w celu potwierdzenia prawidłowej budowy gonad oraz charakterystyki morfologicznej pęcherzyków jajnikowych, wykonano badanie histologiczne.

3.5. Podsumowanie

W omawianych powyżej badaniach wykorzystano model komórek ziarnistych jajników świń w krótkoterminowej hodowli pierwotnej *in vitro*. Uzyskane wyniki badań zostały przedstawione w formie trzech oryginalnych publikacji naukowych. Głównym celem prowadzonych badań było określenie kierunku zmian i poziomu ekspresji wybranych genów.

Zdecydowana większość genów zidentyfikowanych w pierwszym etapie badań (publikacja w Int J Mol Sci., 2019; (Kulus i wsp. 2019)) wykazała przynależność do grupy ontologicznej związanej z różnicowaniem się komórek („*cell differentiation*"). Grupa ta jest odpowiedzialna za procesy nabywania określonych cech strukturalnych i funkcjonalnych, podczas których komórki rozpoczynają określony kierunek rozwoju. Podwyższona ekspresja genów z tej grupy ontologicznej wskazuje na potencjał macierzystości komórek ziarnistych hodowanych w warunkach *in vitro*, co potwierdzają liczne dane literaturowe (Kossowska-Tomaszczuk i wsp. 2009b; Mattioli i wsp. 2012; Varras i wsp. 2012; Dzafic i wsp. 2013, 2014; Kossowska-Tomaszczuk i Geyter 2013).

Warto podkreślić, że wykazanie ekspresji genów związanych z procesami atrezji pęcherzyków jajnikowych (publikacja w Theriogenology, 2020, (Kulus i wsp. 2020b)) może przyczynić się do dogłębnego poznania procesów molekularnych sterujących tym zjawiskiem. Zdecydowanie podwyższona ekspresja genu *IGFBP5* (pomiędzy 48 a 144 godziną hodowli), należącego wyłącznie do grupy ontologicznej „*aging*", który został wcześniej opisany jako marker atrezji pęcherzyków jajnikowych (Hayashi i wsp. 2010), potwierdza rozpoczęcie zjawiska starzenia się komórek w hodowli pierwotnej *in vitro*, szczególnie w porównaniu do wartości referencyjnej (0 h, *in vivo*), gdzie ekspresja była silnie obniżona. Atrezja pęcherzyków jajnikowych rozpoczyna się apoptozą komórek ziarnistych, która może być wywołana poprzez niedostateczne stężenie czynników przeżywalności, np. gonadotropin i czynników wzrostu lub poprzez związanie specyficznego ligandu, np. czynnika martwicy nowotworów (ang. *tumor necrosis factor α*, TNF – α) (Guthrie i Garrett 2001). Zbyt wczesna atrezja może stanowić podłoże zaburzeń rozrodczych samic, co może wpływać na pogorszenie wskaźników rozrodu.

W badaniach określono także poziomy ekspresji genów związanych z regulacją cyklu komórkowego podczas pierwotnej hodowli świńskich komórek ziarnistych (wyniki opublikowano w Histochem. Cell Biol., 2020, (Kulus i wsp. 2020a)). Spośród analizowanych genów na uwagę zasługuje gen *SFRP2*, którego poziom ekspresji w trakcie prowadzonej

hodowli był podwyższony w stosunku do wartości referencyjnej. Gen ten należy do rodziny antagonistów ścieżki WNT, co wskazuje na jego udział w procesach atrezji pęcherzyków jajnikowych. Wykazana podwyższona ekspresja genu *THBS1* wraz z ko-ekspresją z genem *IGF1* wiąże się z procesem atrezji pęcherzyków jajnikowych, co potwierdzono także w badaniach innych autorów (Terenina i wsp. 2017).

Dzięki szczegółowej charakterystyce profilu ekspresji genów w komórkach ziarnistych jajnika świni, możliwe jest poszerzenie stanu aktualnej wiedzy dotyczącej procesów folikulogenezy, co może wpłynąć na poprawę skuteczności technik wspomaganego rozrodu u tego gatunku. Warto podkreślić, że komórki ziarniste wyizolowane z pęcherzyków jajnikowych i hodowane *in vitro* mogą wykazywać zupełnie nowe, niepoznane dotychczas właściwości. Z uwagi na duże podobieństwo filogenetyczne świni domowej do człowieka, gatunek ten stanowi wartościowy model doświadczalny w badaniach biomedycznych.

3.6. Wnioski

1. Sugeruje się, że procesy wzrostu, różnicowania i apoptozy w jajnikowych komórkach ziarnistych świń są regulowane poprzez ekspresję specyficznych genów, wśród których najważniejszą rolę pełnią: *FMOD, POSTN, DAPL1, FN1* czy *SFRP2*.
2. Metoda profilowania ekspresji genów przy wykorzystaniu mikromacierzy pozwoliła na wskazanie nowych molekularnych markerów procesów zachodzących w komórkach ziarnistych podczas krótkoterminowej hodowli pierwotnej *in vitro.*
3. Komórki ziarniste hodowane *in vitro* wykazały ekspresję dużej liczby genów należących do grupy ontologicznej „*cell differentiation*", co może świadczyć o ich wysokim potencjale macierzystości, a gen *POSTN* może zostać uznany za nowy molekularny marker tego procesu.
4. Komórki ziarniste jajnika świni podczas krótkoterminowej hodowli *in vitro* wykazują ekspresję genów regulujących proces atrezji pęcherzykowej (m.in. *IGFBP5, SFRP2, DAPL1, THBS1* wraz z ko-ekspresją z *IGF1*). Geny te mogą być uznane za nieopisane dotychczas markery tego procesu.
5. Zmiany poziomu ekspresji genów zaangażowanych w ważne procesy fizjologiczne komórek ziarnistych mogą zostać wykorzystane jako nowe markery dla poprawy efektywności biotechnik stosowanych w rozrodzie zwierząt.

3.7. Piśmiennictwo

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4. Pełne treści artykułów naukowych stanowiących cykl prac rozprawy doktorskiej

4.1. Publikacja I

International Journal of
Molecular Sciences

MDPI

Article

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

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Abstract: Nowadays, science has a lot of knowledge about the physiology of ovarian processes, especially folliculogenesis, hormone production and ovulation. However, the molecular basis for these processes remains largely undiscovered. The cell layer surrounding the growing oocyte—granulosa cells—are characterized by high physiological capabilities (e.g., proliferation, differentiation) and potential for growth in primary cultures, which predisposes them for analysis in the context of possible application of their cultures in advanced methods of assisted reproduction. In this study, we have used standard molecular approaches to analyze markers of these processes in primarily in vitro cultured porcine granulosa, subjected to conditions usually applied to cultures of similar cells. The material for our research came from commercially slaughtered pigs. The cells were obtained by enzymatic digestion of tissues and in vitro culture in appropriate conditions. The obtained genetic material (RNA) was collected at specific time intervals (0 h—before culture; reference, 48, 98, 144 h) and then analyzed using expression microarrays. Genes that showed a fold change greater than |2| and an adjusted *p* value lower than 0.05 were described as differentially expressed. Three groups of genes: "Cell morphogenesis", "cell differentiation" and "cell development" were analyzed. From 265 differently expressed genes that belong to chosen ontology groups we have selected *DAPL1*, *CXCL10*, *NEBL*, *IHH*, *TGFBR3*, *SCUBE1*, *DAB1*, *ITM2A*, *MCOLN3*, *IGF1* which are most downregulated and *PDPN*, *CAV1*, *TMOD1*, *TAGLN*, *IGFBP5*, *ITGB3*, *LAMB1*, *FN1*, *ITGA2*, *POSTN* genes whose expression is upregulated through the time of culture, on which we focused in downstream analysis. The results were also validated using RT-qPCR. The aim of our work was to conduct primary in vitro culture of granulosa cells, as well as to analyze the expression of gene groups in relation to the proliferation of follicular granulosa cells in the model of primary culture in real time. This knowledge should provide us with a molecular insight into the processes occurring during the in vitro cultures of porcine

granulosa cells, serving as a basic molecular entry on the extent of the loss of their physiological properties, as well as gain of new, culture-specific traits.

Keywords: pig; granulosa; cells morphogenesis

1. Introduction

The female reproductive system is an extremely rich environment with many different cell types. The granulosa layer of the ovarian follicle plays a key role in the nutrition of the oocyte. The growing oocyte undergoes several biochemical and morphological changes. This process occurs simultaneously with folliculogenesis, starting during early fetal development [1]. Ovaries in pigs form on the 26th day of pregnancy and follicles appear after about 40 days [2]. The oocyte is initially surrounded by one layer of granulosa cells located on the basal membrane. They protect the oocyte and ensure the supply of nutrients [3]. During the formation phase of the antral follicle we distinguish granulosa cells (GCs) and theca cells (TCs). An important aspect is also the participation of granulosa cells in the formation cumulus cell layer. It has also been shown [4] that granulosa, theca, basement membrane and capillaries are involved in the transfer of small metabolites to oocytes.

Our previous experience has shown that porcine granulosa cells can be cultured in vitro in short—term culture [5]. It was proved that these cells show logarithmic growth in vitro and significant expression of connexin 43 and cyclin—dependent kinase 4 mRNAs. Also, through detailed analyses, we have shown the movement of these proteins between the nucleus and cytoplasm in the cultured cells. This indicates the likely contribution of these molecules to nuclear—cytoplasmic shuttling of signals during cumulus—oocyte communication. Granulosa cells can be a great source of potential knowledge about complex processes occurring in cells of significant physiological interaction devoid of their usual environment, the molecular mechanisms of which still remain undiscovered. An extremely important aspect is the communication between oocytes and granulosa cells, which, among other things, play a role in the synthesis and expression of many hormones responsible for the development of gametes, but also for the processes associated with the physiology of reproduction, which adds the possibility that granulosa cultures could someday be used in advanced assisted reproduction techniques [6].

In our current research we have used microarray assays, together with RT-qPCR validation, to analyze the expression patterns of genes involved in the morphogenesis, differentiation and cellular development. The “cell differentiation” ontological group, contains genes that participate in processes during which undifferentiated cells acquire specialization, both morphological and functional, occupying a specific location in the mature body. On the other hand, the process whose specific outcome is the progress of the cell in time (but does not include the stages associated with the assignment of the cell to a specific fate) are regulated by genes belonging to “cellular development” gene ontology (GO). Then, the “cell morphogenesis” ontology group describes the genes involved in the development process, in which the size or shape of the cell is generated and organized. The aim of our work was to conduct primary in vitro culture of granulosa cells, to analyze the expression of gene groups in relation to the proliferation of follicular granulosa cells in the model of primary culture in real time. This knowledge should provide us with a molecular insight into the processes occurring during the in vitro cultures of porcine granulosa cells, serving as a basic molecular entry on the extent of the loss of their physiological properties, as well as gain of new, culture-specific traits.

The presented studies indicate not only the possibility of GCs proliferation in primary culture conditions in vitro, but also the expression of genes involved in differentiation-related processes, indicates a new potential of GCs devout of their physiological environment. Several authors conducted similar analyzes on human GCs indicating the potential of these cells to differentiate into other cell types in primary in vitro culture. Kossowka-Tomaszczuk et al. and Kranc et al. indicate such

properties of human GCs maintained in long-term primary culture. The presented studies relate to porcine cells, suggesting that short-term culture also possibly induces differentiative potential of GCs. The presented research should be treated as the first stage basic entry, identifying individual genes involved in the processes of differentiation and proliferation. The results aim to serve as a basis for further proteomic studies, results of which can be compiled with those presented in this manuscript and possibly extrapolated to become meaningful in clinical situations. Confirmation of the stem-like potential of GCs and the possibility of their differentiation towards other types of cells may be used primarily in regenerative and reconstructive medicine, as well as approaches associated with assisted reproduction techniques. The main aim of the study was to identify the genes responsible for the proliferation and differentiation of GCs in short-term primary in vitro cultures.

2. Results

Whole transcriptome profiling by Affymetrix microarray allowed us to analyze the granulosa gene expression changes at 48, 96 and 144 h of in vitro culture. With the use of Affymetrix® Porcine Gene 1.1 ST Array Strip we examined expression of 27,558 transcripts. Genes with fold change higher than abs (2) and with a corrected p-value lower than 0.05 were considered as differentially expressed. This set of genes consists of 3380 different transcripts, complete list of which can be found in the GEO database (ID: GSE134361).

Up and down regulated gene sets were subjected to the Database for Annotation, Visualization and Integrated Discovery (DAVID) search separately and only ones with an adj. *p* Value lower than 0.05 were selected. The DAVID software analysis showed that the differently expressed genes belonged to 344 GO BP Terms. In this paper we focused on the chosen "cell development", "cell differentiation" and "cell morphogenesis" GO BP terms. These sets of genes were subjected to a hierarchical clusterization procedure and presented as heatmaps (Figure 1). There were 265 genes of differential expression in the gene ontologies of interest, from which 114 genes were downregulated and 151 were upregulated compared to the reference point. The gene symbols, fold changes in expression, Entrez gene IDs and corrected *p*-values of that genes are shown in Table 1.

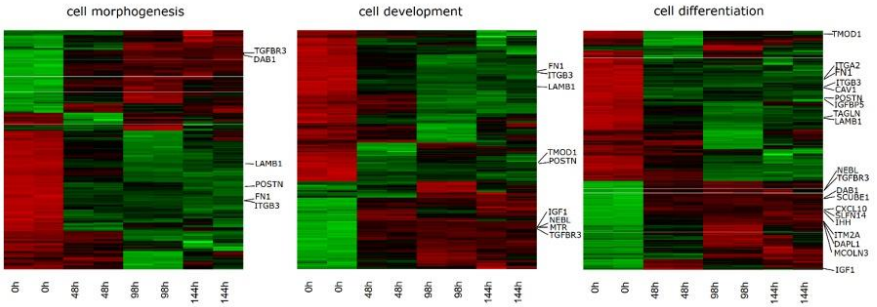

Figure 1. Heat map representation of differentially expressed genes belonging to the chosen "cell development", "cell differentiation" and "cell morphogenesis" gene ontology (GO) BP terms. Arbitrary signal intensity acquired from microarray analysis is represented by colors (green, higher; red, lower expression). Log2 signal intensity values for any single gene were resized to Row z-score scale (from −2, the lowest expression to +2, the highest expression for a single gene). Due to the large number of genes on the heatmap, only the genes chosen for analysis in the manuscript were marked, with their names indicated.

To further investigate the changes within the chosen GO BP terms, we measured the enrichment levels of each selected GO BPs. The enrichment levels were expressed as z-scores and presented as circular visualizations (Figure 2). As can be seen in the Figure, all three ontological groups

contain a larger proportion of genes of increased expression (green color of the inner ring), with "cell differentiation" and "cell development" containing the biggest number of genes.

Table 1. Fold changes, adjusted *p* values and ENTREZ gene ID of the 20 chosen differentially expressed genes belonging to the "cell morphogenesis", "cell development" and "cell differentiation gene ontology (GO) terms.

Gene	FC 48 h/0 h	FC 96 h/0 h	FC 14 h4/0 h	*p* Value 48 h/0 h	*p* Value 96 h/0 h	*p* Value 144 h/0 h	Entrez ID
CXCL10	0.036689	0.027744	0.030979	3.47×10^{6}	1.07×10^{6}	1.53×10^{6}	3627
DAB1	0.112175	0.096043	0.092698	3.33×10^{6}	1.07×10^{6}	1.26×10^{6}	1600
DAPL1	0.03486	0.003772	0.004046	1.00×10^{6}	9.14×10^{8}	9.57×10^{8}	92196
IGF1	0.119841	0.180781	0.391369	9.96×10^{5}	0.000172	0.006284	3479
IHH	0.049771	0.0538	0.047738	2.16×10^{6}	1.06×10^{6}	1.09×10^{6}	3549
ITM2A	0.11303	0.043537	0.042942	7.74×10^{6}	7.05×10^{7}	8.20×10^{7}	9452
MCOLN3	0.114236	0.052489	0.068606	9.09×10^{6}	1.06×10^{6}	1.98×10^{6}	55283
NEBL	0.037428	0.037303	0.034729	9.56×10^{7}	3.68×10^{7}	4.09×10^{7}	10529
SCUBE1	0.084451	0.063451	0.087118	4.12×10^{6}	1.09×10^{6}	2.53×10^{6}	80274
TGFBR3	0.065878	0.045288	0.04622	4.56×10^{7}	1.51×10^{7}	1.50×10^{7}	7049
CAV1	22.31557	10.4332	28.12126	4.28×10^{7}	4.59×10^{7}	1.50×10^{7}	857
FN1	35.37896	66.1651	68.74185	3.59×10^{7}	9.92×10^{8}	9.57×10^{8}	2335
IGFBP5	29.89599	20.97384	31.2743	4.42×10^{7}	3.09×10^{7}	2.28×10^{7}	3488
ITGA2	42.67896	41.0013	38.26346	3.52×10^{7}	1.21×10^{7}	1.26×10^{7}	3673
ITGB3	29.91255	39.85199	57.68212	3.52×10^{7}	1.21×10^{7}	9.57×10^{8}	3690
LAMB1	33.30078	60.54652	39.6371	3.52×10^{7}	9.14×10^{8}	9.57×10^{8}	3912
HPDPN	18.98888	18.65575	22.39775	2.14×10^{5}	1.11×10^{5}	1.07×10^{5}	10630
POSTN	95.22897	29.49005	88.94606	3.52×10^{7}	1.80×10^{7}	9.57×10^{8}	10631
TAGLN	25.56666	53.77139	50.96687	3.52×10^{7}	9.14×10^{8}	9.57×10^{8}	6876
TMOD1	23.71613	5.946509	9.468941	1.00×10^{6}	6.63×10^{6}	2.71×10^{6}	7111

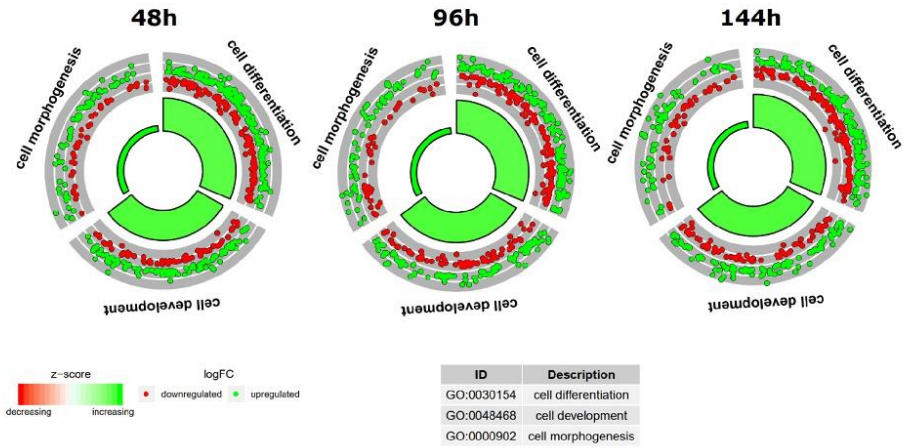

Figure 2. The circular visualization of the results of gene-annotation enrichment analysis. The outer circle shows a scatter plot for each term of the logFC of the assigned genes. Red circles display up-regulation and blue ones down-regulation. The inner circle is the representation of the z-score. The size and the color of the bar correspond to the value of z-score.

To better understand the interaction between chosen GO BP terms we performed the hierarchical clusterization of the gene expression profiles. The resulting dendrogram was combined with fold changes (FC) of studied gene expression and gene assignment to studied terms. The results were presented in Figure 3. Most of the genes of altered expression that belong to "cell morphogenesis" GO are also present in the other gene ontologies of interest. Unique genes of differential expression that

are not a part of other gene ontologies, appear mostly in the "cell development" group, with minor fractions characteristic for "cell morphogenesis". "Cell differentiation" did not contain any differentially expressed genes unique only for that group.

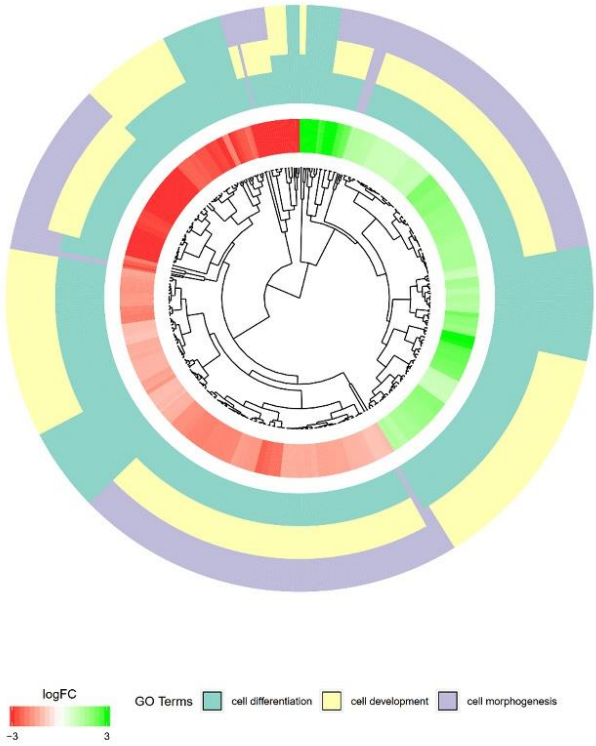

Figure 3. The representation of hierarchical clusterization, fold change and assignment of differently expressed genes that belongs to chosen "cell development", "cell differentiation" and "cell morphogenesis" GO BP terms. Genes are grouped together based on their expression patterns, with the clusterization pattern represented by dendrogram inside the circle. The middle ring represents the logarithm of gene expression fold change of studied genes. The outer ring represents the terms assigned to the genes.

Among 265 differently expressed genes that belongs to GO BP terms of interest we have chosen 10 with most upregulated and 10 with most downregulated expression levels, on which we focused during downstream analysis.

In the gene ontology database, genes that form one particular GO group can also belong to other different GO term categories. For this reason, we explore the gene intersections between the selected GO BP terms. The relation between those GO BP terms was presented as a circle plot (Figure 4) as well as the heatmap (Figure 5). As can be seen on the figures, most (11/20) genes of the most altered expression is characteristic uniquely for the "cell differentiation" group, with the remaining seven being members of all three GOs of interest.

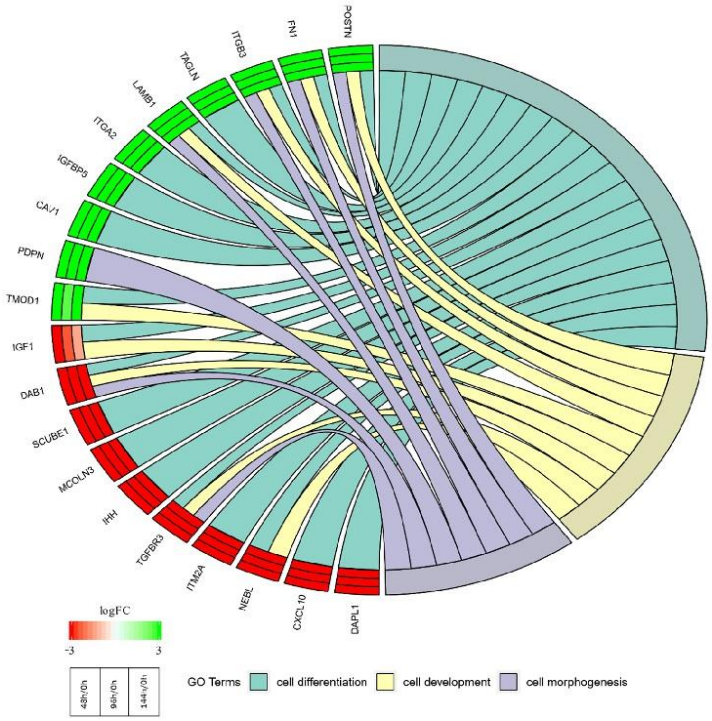

Figure 4. The representation of the mutual relationship between 10 most upregulated and 10 most downregulated genes that belongs to "cell development", "cell differentiation" and "cell morphogenesis" GO BP terms. The ribbons indicate which gene belongs to which categories. The genes were sorted by logFC from most to least changed gene, with the most upregulated genes presented topmost and the most downregulated genes located at the bottom of the figure. The tips of the ribbons present the direction of the change (upregulation-green; downregulation-red), with each of their parts showing change in particular culture period (innermost—48 h/0 h; middle—96 h/0 h; outermost—144 h/0 h).

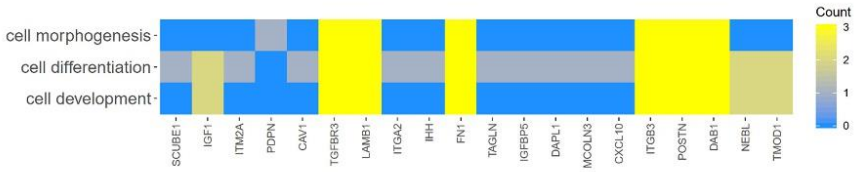

Figure 5. Heatmap showing the gene occurrence between 10 most upregulated and 10 most downregulated genes that belongs to "cell development", "cell differentiation" and "cell morphogenesis" GO BP terms.

A STRING interaction network was generated among chosen differentially expressed genes belonging to each of the selected GO BP terms [7]. Using such a prediction method provided us with a molecular interaction network formed between protein products of studied genes (Figure 6). According to the STRING database, the biggest amount of confirmed interactions can be observed between *POSTN*, *FN1*, *ITGB3*, *ITGA2* and *DAB1* genes. Some interactions between *IGF1*, *IGFBP5*, *CAV1* and *LAMB1* genes were also observed.

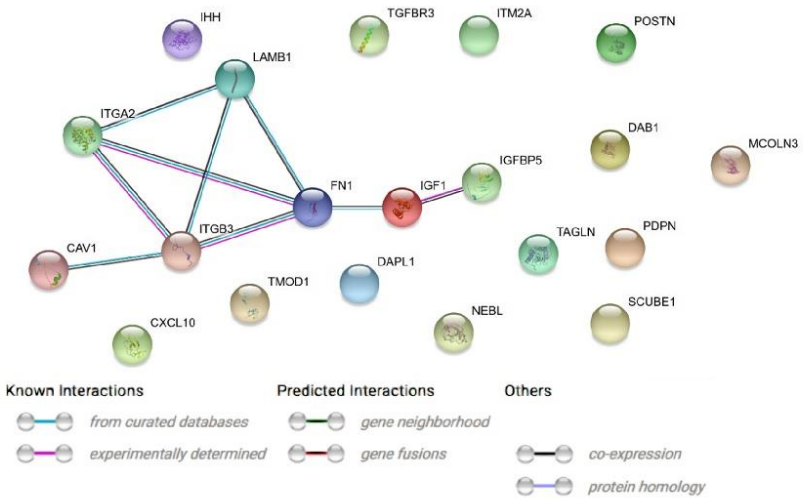

Figure 6. STRING-generated interaction network among differentially expressed genes belonging to the between 10 most upregulated and 10 most downregulated genes that belongs to "cell development", "cell differentiation" and "cell morphogenesis" GO BP terms. The intensity of the edges reflects the strength of interaction score.

Finally, we have investigated the functional interactions between chosen genes with the REACTOME FIViz app to Cytoscape 3.6.0 software. The results are shown in Figure 7.

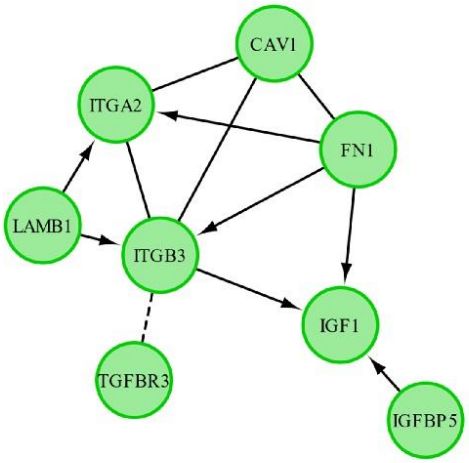

Figure 7. Functional interaction (FI) between 10 most upregulated and 10 most downregulated genes that belong to "cell development", "cell differentiation" and "cell morphogenesis" GO BP terms. In the figure "→" stands for activating/catalyzing, "—" stands for FIs extracted from complexes or inputs, and "—" stands for predicted FIs.

The results of this figure present interactions between eight out of 20 genes of interest. *FN1* catalyses the expression of three genes (*ITGA2*, *ITGB3*, *IGF1*), *LAMB1* catalyses the expression of two genes (*ITGA*, *ITGB3*). Finally, the expression of IGF1 is catalysed by three genes (*IGFBP5*, *FN1*, *ITGB3*).

The results of the microarray analysis were validated with the RT-qPCR methods. The obtained values were compared between both approaches and are presented as a bar graph (Figure 8).

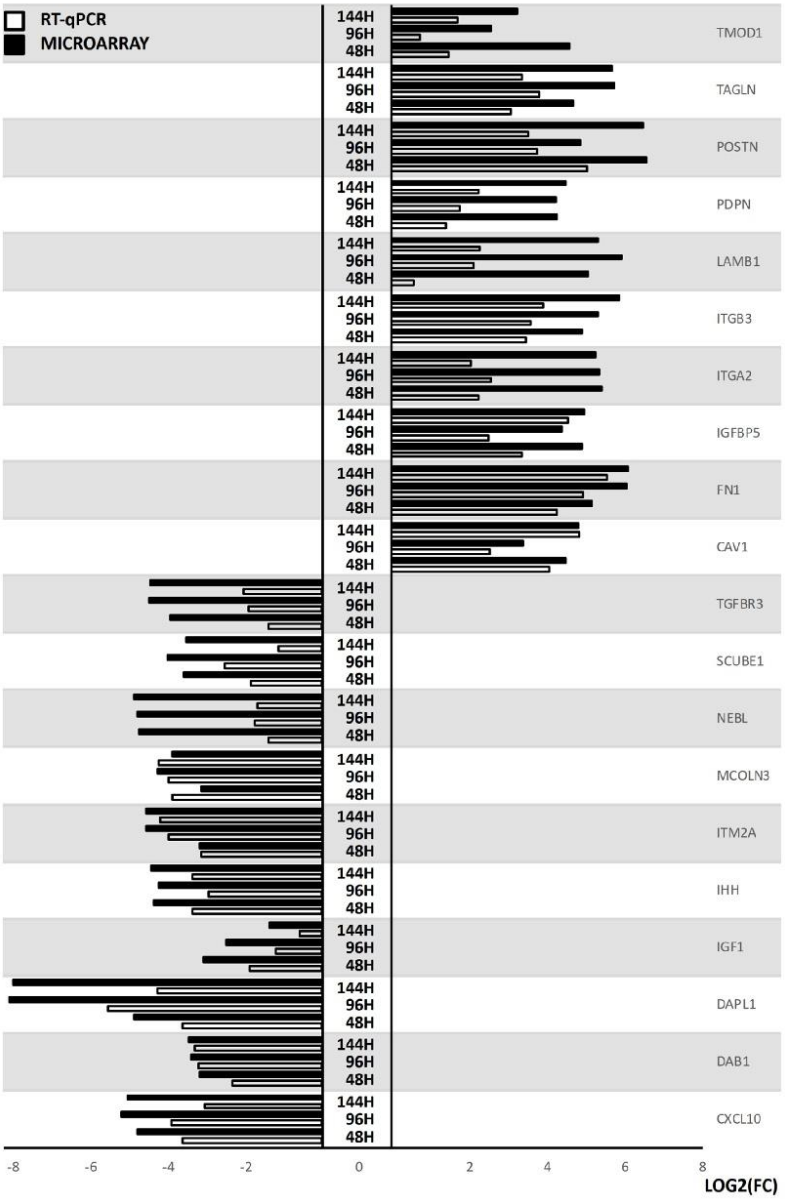

Figure 8. The results of RT-qPCR validation of the analysed genes, presented in the form of bar graph. All of the changes in expression were deemed significant at $p < 0.05$ after adjustment for multiple comparisons.

Overall, the direction changes in the expression of genes of interest were validated in every example. However, their scale often varies highly between the methods. This fact is explainable, as these methods are different in sensitivity and specificity, which further supports the need for quantitative validation of microarray results.

Histochemical studies revealed the proper morphology of collected ovaries stained with H and E. Ovarian follicles in every stage of development were observed (Figure 9).

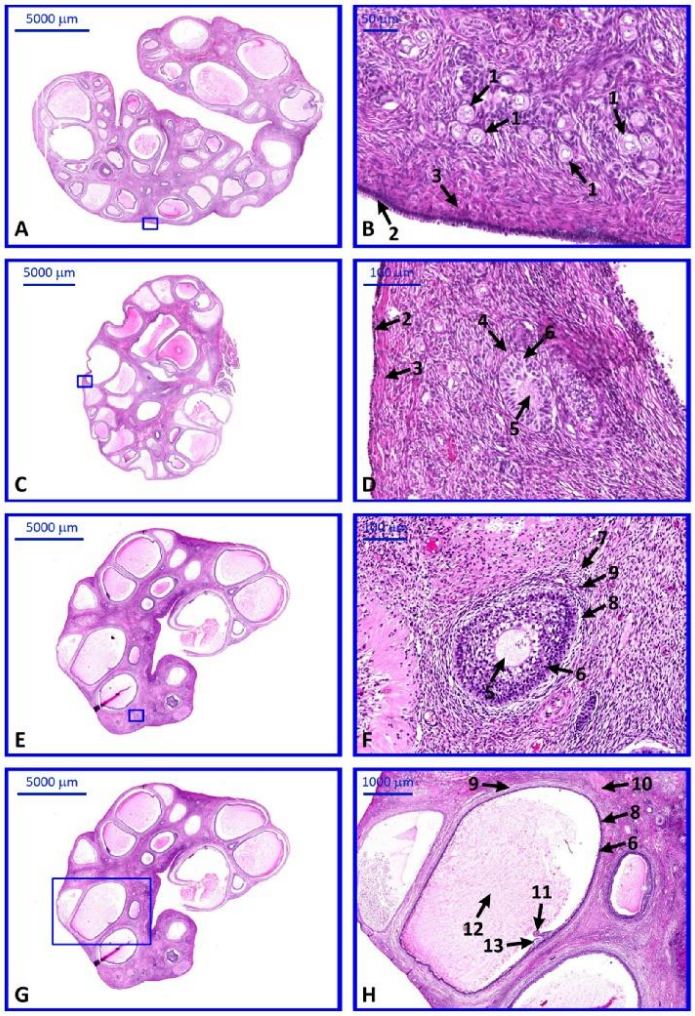

Figure 9. H and E stained ovaries (**A**,**C**,**E**,**G**) with follicles in particular stages of development (**B**,**D**,**F**,**H**). **B**,**D**,**F**,**H** represent selected areas of **A**,**C**,**E**,**G** observed in higher magnification (with blue square indicating the magnified region). Arrows: 1—primordial follicles, 2—germinal epithelium, 3—tunica albuginea, 4—primary follicle, 5—oocyte, 6—granulosa cells, 7—secondary follicle, 8—theca interna, 9—theca externa, 10—mature follicle, 11—oocyte with corona radiata, 12—antrum, 13—cumulus oophorus.

Individual isolated follicles varied in size. Large (>5 mm), medium (3–5 mm) and small (<3 mm) follicles were observed (Figure 10).

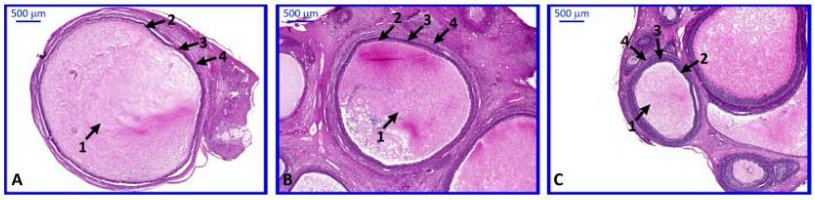

Figure 10. Individual isolated follicles stained with routine H and E method. Follicles varied in size; we can observe large (>5 mm), medium (3–5 mm) and small follicles (<3 mm). Medium and small follicles were collected in groups containing more than one follicle. (**A**)—large follicle, (**B**)—medium follicle, (**C**)—small follicle. Arrows: 1—antrum, 2—granulose cells, 3—theca interna, 4—theca externa.

3. Discussion

Granulosa cells are known to be closely related to mammalian oocytes. They surround them from the very beginning, and still in fetal life, support their development, maturation, and formation of ovarian follicles [8]. They are associated with the functioning of the reproductive system, as well as the maintenance of pregnancy through participation in the synthesis of steroid hormones [9]. After ovulation, when the follicle ruptures, the granulosa cells fill the formed gap and transform into lutein cells, whose main purpose is to produce progesterone and maintain pregnancy. The resulting structure is called the corpus luteum. The physiology of these processes is well known, but the deep molecular mechanisms and gene interactions of granulosa cells are still insufficiently described [10]. Extremely dynamic changes in the area of the mammalian mature ovary require adequate supply of blood vessels. Particularly dynamic processes related to angiogenesis occur in the corpus luteum. In a short time, a network of blood vessels is created, allowing cells with hormonal activity to obtain oxygen, nutrients, as well as hormone precursors necessary for the synthesis and release of progesterone in the early stages of pregnancy [11]. In modern human medicine and advanced procedures of assisted reproduction techniques in farm animals, understanding of all the mechanisms influencing the increase of reproductive efficiency seems to be of key importance. In addition, recent studies prove that ovarian granulosa cells can gain and/or exhibit programmed stem cell properties during in vitro culture [12–14].

Using modern research methods, we have the opportunity to learn more about the molecular basis of functioning of such microenvironments as ovarian follicles. Pigs, as animals physiologically similar to humans, are a commonly used models in medical research. Only through detailed analysis will it be possible to get deeper into the background of some clinical diseases and perhaps to get closer to the paths of their solution. Research on cells of the reproductive system can lead to new strategies for increasing the reproductive potential of animals and treating infertility in humans.

In our present research, we have measured the gene expression changes in three ontological groups ("cell morphogenesis", "cell differentiation", "cell development"), using the microarray approach. Our experiments were based on the successful primary short-term culture of granulosa cells (GCs) obtained from ovaries of pigs killed in a slaughterhouse. The obtained cells were subjected to short term in vitro culture, up to 144 h. RNA material was isolated from the cells before the primary culture (0 hour) and then after 48, 96 and 144 h of its course. We analyzed 20 genes that showed the biggest changes in expression. From 265 differentially expressed genes belonging to the GOs of interest, we have selected *DAPL1*, *CXCL10*, *NEBL*, *IHH*, *TGFBR3*, *SCUBE1*, *DAB1*, *ITM2A*, *MCOLN3*, *IGF1*, showing the biggest upregulation, as well as *PDPN*, *CAV1*, *TMOD1*, *TAGLN*, *IGFBP5*, *ITGB3*, *LAMB1*, *FN1*, *ITGA2*, *POSTN* genes whose expression was downregulated through the time of culture. All of the abovementioned genes were focused on during downstream analysis. Six of these genes belonged to all three groups (*TGFBR3*, *DAB1*, *ITGB3*, *LAMB1*, *FN1*, *POSTN*), and three others to two

groups (*NEBL*, *IGF1*, *TMOD1*—cell differentiation and cell development). In all genes belonging to the three ontological groups of interest, a similar, uniform pattern of expression was observed. The 0-h expression analysis, used as a reference point, exhibited the lowest levels of expression. A significant increase in expression was described in the subsequent culture hours (48, 98, 144 h), which concerned almost all genes from the three groups. The value of 0 h, the starting point of cultures, was assumed as a point of reference in this study. This method served to fulfil the aim of comparing the gene expression patters in different timepoints of in vitro cultures. Hence, it was assumed that 0h relatively well reflects the physiological gene expression of granulosa cells.

The most down-regulated gene was *DAPL1* (death associated protein—like 1), belonging to an ontological group of "cell differentiation". Its role is described in the early stages of epithelial differentiation, but also in the processes of apoptosis. Significant increases in *DAPL1* gene expression was observed in the study on uterine inflammation in dairy cows, analyzing levels of its transcripts in endometrium [15]. The downregulation of this gene's expression in subsequent cultures stages suggests a change in the properties of granulosa cells during primary in vitro culture. A similar pattern of expression is exhibited by the 9 genes described below. The next gene, *CXCL10* (*C-X-C* motif chemokine ligand 10) also belonging to the "cell differentiation" group is also known as interferon–gamma inducible protein-10 (IP-10). The *CXCL10* gene was described in pigs by Liu and Xiong, where it was proven to be highly expressed in muscle and weakly expressed in fat and kidneys [16]. The *CXCL10* gene codes a cytokine associated with inflammatory processes, such as activation of immune cells, differentiation, but also apoptosis or cell growth regulation. Another gene that shows significant down-regulation in our in vitro studies of cultured GCs in relation to the reference point is *NEBL*. NEBL (nebulette) is a protein that binds to actin and plays an important role in Z-disk assembly. Mainly found in the heart muscle, where it combines sarcomeric actin with desmin fibers in sarcomers [17]. We observed a significant decrease in the expression of this gene in GCs' primary culture. *Indian hedgehog protein* (*IHH*) is an important gene encoding an intracellular signaling protein that plays a role in cell development. A special role is attributed to *IHH* in the process of ossification and mesenchymal cell proliferation [18]. This gene belongs to the "cell differentiation" group and was definitely upregulated. *TGFBR3* (*transforming growth factor beta receptor 3*) is an important signal point for cell differentiation and development, also in such an important process as the formation of coronary vessels in the heart [19]. *TGFBR3* contributes to the response to the FSH hormone signals. The decrease in these gene's expression in subsequent days of culture suggests a change in properties of granulosa cells. Hence, our future studies could Focus on the secretive properties of GCs in in vitro culture conditions. Furthermore, *TGFBR3* also plays a role in the epithelial to mesenchymal transition. It was detected, among others, in the porcine uterus [20]. *SCUBE1* (*signal peptide CUB domain and EGF like domain containing 1*) is closely related to endothelial cells (differentiation) and vascular system. It occurs in highly vascularized tissues. Its expression also manifests a strong connection with the cell surface [21]. This gene, belonging to "cell differentiation" GO, showed down-regulation during our culture. Subsequently, *DAB1* (*DAB protein adaptor 1*) is a gene encoding adaptive protein necessary for intracellular Reelin signal transmission. This in turn controls the migration and differentiation of postmitotic neurons in the brain development process [22]. We have determined that this gene belongs to all three ontological groups, which reflects its role in morphogenesis, differentiation and cellular development. We observed a significant downregulation of *DAB1*, which probably indicates the loss of physiological properties of the aforementioned processes in in vitro cultured porcine granulosa cells. It is possible that this gene may become a marker of neuronal differentiation of granulosa cells, which requires further research. Considering the aim of our research, the expression of the abovementioned four genes is particularly important. Their high expression at 0h suggests that granulosa cells exhibit mesenchymal stem cell like potential in physiological conditions. The next gene, the *ITM2A* (*integral membrane of 2A protein*) encodes a porcine protein that is highly homologous to the integrated 2A (ITM2A) membrane protein of humans and mice. This gene is described in fat and spleen, in the lungs, as well as in the muscles, liver, small intestine, large intestine and kidneys [23]. In our studies, we have

shown a significantly reduced expression of this gene, which only belongs to the "cell differentiation" gene ontology. Another gene of decreased expression from the "cell differentiation" GO is *MCOLN3* (mucolipin 3), which was found in various pig tissues, also in the ovary [24]. The important role of *MCOLN3* is described in the regulation of Ca^{2+} homeostasis in the endosomal pathway, as well as in melanosomal transduction and hair cell maturation, as demonstrated by mouse studies [25]. The last down-regulated gene was *IGF1* (*insulin like growth factor 1*), belonging to the "cell differentiation" and "cell development" groups. This gene was analyzed in studies on ovarian follicle development in cattle and pigs in order to compare single and multiovulatory animals. These experiments showed that IGF1 was one of the factors limiting or promoting multiple ovulation [26]. Therefore, it can be assumed that high expression in 0h of this gene in the in vitro culture of granulosa cells may testify to the ability of these cells to regulate the ovulation processes in physiological condition. *IGFBP5* (*insulin like growth factor binding protein 5*), which has shown up–regulation, is not correlated with the IGF1 gene. The presence of these genes' expression was shown in the ovary and other organs of pigs [24]. The only gene that belongs exclusively to the "cell morphogenesis" group is *PDPN*, which may predispose it to becoming a marker of in vitro GC culture morphogenesis. *PDPN* (*podoplanin*) is a protein–coding gene that is quite widespread in porcine lungs, ovaries and other tissues. In our research it was up-regulated during the in vitro culture of GCs. The gene was also described in studies of pancreatic cancer, where fibroblasts expressed increased expression of *PDPN*. Podoplanin, a transmembrane glycoprotein, is selectively expressed by lymphatic endothelial cells [27]. It is possible that its presence in culture is related to cell differentiation in the vascular direction, because it was strongly associated with neoplastic angiogenesis. The *TMOD1* gene (*tropomodulin 1*) was found to belong to two ontological groups ("cell differentiation" and "cell development"). The protein coded by this gene plays an important role in the physiology of muscles, especially skeletal muscles, and its expression was mainly noted in the psoas major. An increase in the expression of these gene in subsequent stages of the culture suggests that granulosa cells gain new properties when cultured in vitro. The *TAGLN* (*transgelin*) gene is associated with the differentiation of epithelium in human intestine cell line cultures [28]. In GC, in vitro primary culture, this gene was up–regulated and belonged to the "cell differentiation" group. *FN1* (*fibronectin 1*) represents all three ontological groups, and its expression was recorded to be upregulated. Significant upregulation of the *FN1* gene was observed in an experiment on the primary culture of pig buccal mucosa cells in vitro. It was observed that increased expression of this gene, among other factors, was accompanied by cellular proliferation [29]. Fibronectin is a protein commonly found in extracellular matrix of blood vessels but also in dissolved form in blood [30]. Increased expression suggests that fibronectin is also present in the GC extracellular matrix. It activates ITGB3 (integrin subunit beta 3) proteins, which also showed upregulation in our experiment and belong to the same three ontological groups. By analyzing the gene correlation (STRING) we also observed the co-expression of proteins coded by these two genes. *ITGB3* also shows co–expression with downregulated *IGF1* gene and two of the analyzed upregulated genes: *ITGA2* and *LAMB1*. The *LAMB1* gene (*laminin subunit beta 1*) mediates the migration and organization of cells to tissues during embryonic development through interaction with other extracellular matrix components. It also plays a role in embryo implantation, as indicated by a mice study [31]. Laminin protein plays a role in the regulation of tissue proliferation, differentiation and repair [32]. In our study, this gene showed upregulation and belonged to all three analyzed ontological groups. Notable expression of this gene suggests that in vitro cultured GCs might gain new properties, including the potential to differentiate into other cell types. Another gene that plays a role in the early stages of embryogenesis [33] is *ITGA2* (*integrin subunit alpha 2*). Its expression was recorded in porcine kidneys, ovaries and other tissues. The correlation with *FN1* and *ITGB3* genes was also found for the *CAV1* gene (caveolin 1). This gene belongs only to the "cell differentiation" group and, like the two genes mentioned above, it was upregulated. The proteins encoded by these genes can form interactive complexes participate in cell differentiation during angiogenesis [34]. The *FN1* and *POSTN* genes are strongly correlated, showing co-expression and belonging to all three ontological groups. The *POSTN* gene (*periostin*) is responsible for cellular adhesion and bone regeneration [35].

In our research, this gene indicated the strongest up–regulation. Using STRING-generated interaction network, we have observed that 11 of the 20 analyzed genes did not show direct interactions with other genes. However, indirect interactions cannot be excluded, either through genes not detected in this study or those that have not shown significant changes in expression.

Analyzing the above results, it should be remembered that the change in the expression of individual genes may result not only from changes in nucleotide sequences but also from epigenetic changes occurring during the change of environment from physiological to ex vivo. Many factors, including age and obesity, have an effect on reproduction and epigenetics of germ cells [36]. Studies conducted on human cumulus cells suggest that the quality of oocytes and implantation of embryos may depend on whether the woman is overweight or not. Much less mature oocytes and inferior embryos were obtained from obese women than from women without obesity [37].

To summarize, our research showed expression of several genes of porcine ovarian granulosa cells, which manifested during short-term primary in vitro culture. Three ontological groups of genes were distinguished: "Cell morphogenesis", "cell development" and "cell differentiation". The observed gene expression data may indicate advanced molecular processes connected with proliferation of granulosa cells in in vitro culture. The vast majority (19 out of 20) of the described genes belonged to the "cell differentiation" group, while the "cell morphogenesis" group had the least representatives (7 genes). Some of these genes play a role in angiogenesis, which occurs abundantly in the ovary. Significant expression of genes from the described groups may reflect the ability of GCs to rapidly multiply and differentiate in the corpus luteum. However, it can also suggest the appearance of novel differentiation properties achieved during the course of in vitro culture. Our research is intended to provide further clarification to the molecular bases of processes occurring during the short-term in vitro culture of granulosa cells. The results partly support the thesis that some cells devoid of their physiological environment lose their original properties, instead gaining potential for differentiation into other cell types. However, it needs to be noted that this manuscript describes an entry level study. While there is a possibility that the results can be extrapolated into knowledge applicable in clinical situations, they need further validation on the protein level, followed by additional studies conducted in in vivo or simulated clinical conditions. Hence, these outcomes could serve as an entry-level molecular reference for further studies of stem-like properties of ovarian granulosa cells, as well as their potential application in the processes of assisted reproduction of animals and humans.

4. Materials and Methods

4.1. Animals

A total of 43 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. Pigs acquire sexual maturity between 4-6 months of age, and therefore the material obtained came from young, sexually mature, gilts ready for reproduction. All of the specimen were slaughtered in the anestrous stage of the estrous cycle. As the ovaries were obtained from animals of relatively equal age, the collected samples exhibited high homogeneity. The experiment was approved by Poznan University of Life Sciences Bioethical Committee (Resolution 32/2012, approved 1 June 2012).

4.2. Collection of Porcine Ovaries and In Vitro Culture of Granulosa Cells

Ovaries (n = 86) and reproductive tracts were recovered at slaughter and transported to the laboratory at 38 °C in 0.9% NaCl within 30 min. 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 an estimated diameter greater than 5 mm (n = 300), were opened into a sterile Petri dish by puncturing with a 5 mL syringe and 20-G needle. The cumulus-oocyte complexes (COCs) and follicular fluid (FF) were recovered. The follicular fluid was the used to isolate GCs, whereas the COCs were discarded. The method employed, as well as the choice of material

was chosen to most closely reflect the usual approaches used in assisted reproduction techniques, as this study could serve as a reference for future research aiming to improve the processes of in vitro fertilization and assisted reproduction of animals and possibly humans.

The culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich), 2% fetal calf serum (FCS; increased concentration required to maintain adequate cell viability in culture) (PAA, Linz, Austria), 10 mg/mL ascorbic acid (Sigma-Aldrich), 0.05 µM dexamethasone (Sigma-Aldrich), 200 mM L-glutamine (Invitrogen, Carlsbad, CA, USA), 10 mg/ml gentamycin (Invitrogen), 10,000 units/mL penicillin and 10,000 µg/mL streptomycin (Invitrogen). The 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) for 3 min and counted using a Z2 counter or cell viability analyzer (Vi-Cell XR 2.03; both Beckman Coulter, Brea, CA, USA). For our experiments, 3×10^6 cells per dish were used for the culture, with the number achieved by adequate pooling of the material obtained from the punctured follicles into samples of comparable cell number. The cell samples were collected in 4 time intervals, representing different stages of short-term in vitro culture: 0 h—serving as an ex vivo reference; 48 h—representing initial in vitro associated changes in culture; 96 h—an assumed "point of loss" of most of the cell's physiological properties; 144 h—the end point of short-term culture.

4.3. Histological Analysis

For the histochemical study ovaries and single follicles from 3 animals were collected and immediately fixed in Bouin's solution for 48 h. Due to technical limitations, follicles smaller than 5 mm were pooled into groups usually containing from 3 to 5 follicles. Subsequently, tissues were dehydrated and embedded in paraffin blocks. Then, they were cut into 4 µm thick sections with a semi-automatic rotary microtome (Leica RM 2145, Leica Microsystems, Nussloch, Germany). Both ovaries and single follicles were stained with a routine hematoxylin and eosin (H and E) staining method, following the steps of: Deparafinization and rehydration, H and E staining, and dehydration. Histological sections were observed and evaluated under light microscope. Selected pictures were taken with high-resolution scanning technique and Olympus BX61VS microscope scanner (Olympus, Tokyo, Japan).

4.4. Microarray Expression Analysis and Statistics

The Affymetrix procedure was previously described by Trejter et al. [38] and was used in our previous works regarding porcine oviductal cells [39–41] and porcine oocytes [42–44]. The cDNA was reverse transcribed from the total RNA (100 ng) (Ambion® WT Expression Kit). Obtained cDNA was biotin labeled and fragmented using Affymetrix GeneChip® WT Terminal Labeling and Hybridization Kit (Affymetrix, Santa Clara, CA, USA). Biotin-labeled fragments of cDNA (5.5 µg) were hybridized to Affymetrix® Porcine Gene 1.1 ST Array Strip (48 °C/20 h). Then, the microarrays were washed and stained according to the technical protocol of the Affymetrix GeneAtlas Fluidics Station. Subsequently, the array strips were scanned by the Imaging Station of GeneAtlas System. The preliminary analysis of the scanned chips was performed using Affymetrix GeneAtlas™ 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 using Bioconductor and the R programming language. Each CEL file was merged with a description file. In order to correct background, normalize and summarize results, we have used the robust multiarray averaging (RMA) algorithm.

Statistical significance of analyzed genes was performed by 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. The selection of genes of significantly changed expression was based on a *p*-value beneath 0.05 and expression fold-change higher than 2. Differentially expressed gene lists (separate for up and downregulated genes) were uploaded into DAVID software (Database for Annotation, Visualization and Integrated Discovery) [45], where differentially expressed

genes belonging to "cell development", "cell differentiation" and "cell morphogenesis" were obtained. Expression data of these genes was subjected to hierarchical clusterization procedure and presented as a heatmap graph.

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

Total RNA was isolated from GCs before and after in vitro culture using 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. The 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. Target cDNA was quantified using the relative quantification method. The relative abundance of the analyzed transcripts in each sample was standardized to the internal standards. For amplification, 1 µL of cDNA solution was added to 9 µL of QuantiTect® SYBR® Green PCR (Master Mix Qiagen GmbH, Hilden, Germany) and primers (Table 2). One RNA sample of each preparation was processed without the RT-reaction to provide a negative control for subsequent PCR.

Table 2. Oligonucleotide sequences of primers used for RT-qPCR analysis.

Gene	Gene ID	Primer Sequence (5′–3′)	Product Size (bp)
CXCL10	494019	CTCCTGAAAGGCCCATCATA GCACATGGGATAGAGGAGGA	193
DAB1	100037307	TACGTTTGTGGGAAGGAAGG CTTCCTTCTTTTGGCTGGTG	202
DAPL1	100157537	CCTGCTCTGGAGAAGGTCAC GGGCCTAAGGAAAGTTTTGG	151
IGF1	47523587	TTCTACTTGGCCCTGTGCTT CTCCAGCCTCCTCAGATCAC	222
IHH	397174	CTCCACTGCCCTCTCAGAAC AGCTCGCAGCTGTGTCACTA	182
ITM2A	595131	TCTCGTAGGCCTTTCCTTCA AGGCAGGAAGTAGGGCTCTC	163
MCOLN3	100625693	TCCGAGTGCCTTTTCTCACT CGGATATAAACGTGCGGAGT	238
NEBL	100522395	CAAACCCTTCAAGGCTACCA CTGAGAACACGCTTCCATCA	177
SCUBE1	100524621	AATCCAATGAAGCCAACAGC AGGGCCTTGATCAGCTTCTT	160
TGFBR3	397512	TTTGTTTTAGCTGGGGGTTG TGGCCACAGGGATTTTTAAG	177
CAV1	404693	TAGGTCAGCAGCCTCCCTAA CTGGTGAGAGGCAGGAAAAG	243
FN1	397620	TGAGCCTGAAGAGACCTGCT CAGCTCCAATGCAGGTACAG	113
IGFBP5	397182	TGGGGGTTTGTTTCTCTGAC TTCTGGCAGGTAGAGCAGGT	181
ITGA2	397483	CATGCCAGATCCCTTCATCT CGCTTAAGGCTTGGAAACTG	153
ITGB3	397063	GGCTTCAAAGACAGCCTCAC AGTCCTTTTCCGAGCACTCA	175
LAMB1	396707	CTTCACCACCTTGGACCACT AGCTGTGGCTCATAGCGAAT	216
PDPN	100738269	AGCAGATGCTGTGTCCCTCT TATGGAACCTGGGCTGGTAG	201
POSTN	100152401	ATTGACCGTGTCCTCACACA GCCACTTTGTCTCCCATGAT	212
TAGLN	397021	TTAAAGGCCGCTGAGGACTA ATGACATGCTTTCCCTCCTG	233
TMOD1	100316850	AGCCCTAACGGAAGAAGAGC CCTTTGCTTGCTTTTCCAAG	170

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 rRNA*, was used as an internal standard to demonstrate that *PBGD* and *ACTB* mRNAs were not differentially regulated in GC groups. *18S rRNA* has been identified as an appropriate housekeeping gene for use in quantitative PCR studies. Again, the statistical significance of analyzed genes was performed by 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.

Author Contributions: M.K.—formal analysis, investigation, writing—original draft; P.S.-K.—data curation, investigation, methodology; A.K.—data curation, investigation, methodology; P.C.—formal analysis, software, visualisation; W.K.—investigation, validation; J.K.—formal analysis, investigation; H.P.-K.—data curation, validation; P.A.—funding acquisition, resources; D.B.—funding acquisition, resources; D.I.—data curation, funding acquisition; M.B.—funding acquisition, supervision; M.Z.—funding acquisition, supervision; M.N.—funding acquisition, project administration, supervision; B.K.—conceptualisation, funding acquisition, project administration, supervision, writing—review and editing.

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4.2. Publikacja II

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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

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ABSTRACT

The oogenesis and folliculogenesis are closely linked and occur simultaneously in the growing ovarian follicles. Biochemical and morphological changes in oocytes (OC) and surrounding granulosa cells (GCs) are highly complex and depend on many factors, including intercellular communication. GCs are cells with many functions, often crucial for the proper viability of the oocyte and subsequent positive fertilization. The purpose of this study was to analyze gene expression in porcine GCs, to define differentially expressed genes belongs to the "cell growth", "aging", "positive regulation of cell death", "apoptotic process", "regulation of cell death", "cell death" and "negative regulation of cell death" ontology groups during the short – term primary *in vitro* culture. Microarrays were employed to study the transcriptome contained in the total RNA of the cultured GCs. Ovaries were obtained after slaughter, from 40 gilts of swine aged 170 days. The cells were obtained through puncture of the ovaries, collection of follicular fluid, removal of the cumulus - oocyte complexes and centrifugation. The cells were then cultured *in vitro*. The RNA material was obtained before the culture was established (0h) and then after 48h, 96h and 144h of its course. From 182 differently expressed genes belonging to the these ontology groups, we have selected POSTN, FN1, FMOD, ITGB3, DCN, SERPINB2, SFRP2, IGFBP5, EMP1, and CCL2 which were upregulated, as well as DAPL1, ESR1, IHH, TGFBR3, PPARD, PDK4, TXNIP, IFIT3, CSRNP3, and TNFSF10 genes whose expression was downregulated during the time of *in vitro* culture of the GCs. The significance of the differential gene expression is to provide new information on the molecular aspects of *in vitro* granulosa cell culture.

1. Introduction

The processes taking place in the mammalian ovary of are extremely complicated and begin during the fetal life [1]. Ovaries in pigs embryos are formed on the 26th day of pregnancy and follicles appear after about 40 days [2]. The growing oocyte (oogenesis) in the ovarian follicle undergoes a number of biochemical and

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morphological changes, and the subsequent process is the formation of follicles (folliculogenesis) [3]. Both processes are closely related, with the molecular mechanisms of cell interaction being poorly understood.

Granulosa cells are somatic cells of many functions. Together with oocytes, they form the ovarian follicle, the basic functional structure of mammalian ovaries, and they are hormonally active (conversion of androgens to estrogens), co-form corpus luteum (production of progesterone), and communicate with the oocyte [4]. The whole population of granulosa cells can be divided into two types: mural GCs, which adhere to the follicle wall, and cumulus cells (*cumulus oophorus*), which closely surround the oocyte. The layer of cumulus GCs that directly surrounds the oocyte is the *corona radiata* [5]. This division is also related to the function performed by these two types of GCs. Mural GCs are mainly responsible for steroidogenesis, and cumulus GCs maintain communicative contact with the oocyte through close intercellular connections [6]. There are microvilli, penetrating zona pellucida, between oocyte and GCs. At this point there are gap junctions (nexus type) [7,8] providing exchange of ions and others, which ensures mutual correlation.

The oocyte secretes the growth and differentiation factor (GDF − 9) and bone morphogenic protein − 15 (BMP − 15), which is responsible for the proliferation and differentiation of granulosa cells [9]. Additionally, produced estrogens and progesterone affect granulosa cells, which show expression of receptor genes [10].

Both oocyte and follicle development processes depend on sex hormones, especially follicular stimulating hormone (FSH) and luteinizing hormone (LH). Receptors of these hormones are present in GCs during the formation of follicles. Therefore, it is an important factor preventing atresia of ovarian follicles [6].

After the LH surge, granulosa cumulus cells have the ability to spread within the follicle. Their growth is accompanied by the characteristic process of extracellular matrix formation (rich in long chains of hyaluronic oligosaccharides), called cumulus expansion or mucification. The active components of this matrix are produced by cumulus GCs, which receive signals from mural GCs and OCs, as well as via the blood vessels reaching the ovarian follicle. The functioning of this matrix is crucial for ovulation, oocyte migration through the fallopian tube and fertilization [11]. Among other factors, oocytes are responsible for cumulus expansion through secretion of cumulus expansion enabling factors (CEEFs), which most likely include GDF-9 and BMP-15 [12].

Methodologies of collecting and cultivating GCs in primary culture have revealed many properties of GCs devout of their usual physiological environment. GCs have the properties of *in vitro* differentiation towards other somatic cells, giving a wide range of regenerative possibilities, e.g. showing osteogenic potential in the bone formation process [13]. The development of modern techniques of assessing molecular aspects of many intracellular processes allows us to get closer to their foundations. Microarrays prove to be an important tool for global evaluation of transcriptome of a selected tissue [14]. Results obtained during the research provide information on gene expression in GC population in primary culture. Increased knowledge of the molecular basis of ovarian follicle maturation, growth and competence may provide a basis for improving *in vitro* fertilization procedures, thereby increasing porcine embryo production, which may also be reflected in human reproduction research and possible treatment of infertility.

The objective of this study was to analyze gene expression in porcine GCs, to define differentially expressed genes belongs to the "cell growth", "aging", "positive regulation of cell death", "apoptotic process", "regulation of cell death", "cell death" and "negative regulation of cell death" ontology groups during the short − term primary *in vitro* culture. Subjecting GCs to conditions that could be applied to human GCs, obtained from the remnant IVF material, should help to understand the exact molecular processes occurring during their prolonged culture. This knowledge could be further applied in using the cultured GCs in assisted reproduction research, as well as the study of their plasticity in the context of regenerative and reconstructive medicine.

2. Material and methods

2.1. Animals

A total of 43 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. Pigs acquire sexual maturity between 4 and 6 months of age, and therefore the material obtained came from young, sexually mature, gilts ready for reproduction. As the ovaries were obtained from animals of relatively equal age, the collected samples exhibited high homogeneity. All experimental procedures involving animals were approved by Poznan University of Life Sciences Bioethical Committee (Resolution 32/2012, approved 1/6/2012).

2.2. Collection of porcine ovaries and in vitro culture of granulosa cells

Ovaries (n = 86) and reproductive tracts were recovered at slaughter and transported to the laboratory at 38 °C in 0.9% NaCl within 30 min. The ovaries of each animal were placed in PBS supplemented with fetal bovine serum (FBS; Sigma-Aldrich Co., St. Louis, MO, USA). After that, single preovulatory large follicles, with an estimated diameter greater than 5 mm (n = 300), were opened into a sterile Petri dish by puncturing with a 5 ml syringe and 20-G needle. The cumulus-oocyte complexes (COCs) and follicular fluid (FF) were recovered. The follicular fluid was then used to isolate GCs, whereas the COCs were discarded. The method employed, as well as the choice of material, was chosen to most closely reflect the usual approaches used in assisted reproduction techniques, as this study could serve as a reference for future research aiming to improve the processes of *in vitro* fertilization and assisted reproduction of animals and possibly humans.

The culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, USA), 2% fetal calf serum (FCS; increased concentration required to maintain adequate cell viability in culture) (PAA, Linz, Austria), 10 mg/ml ascorbic acid (Sigma-Aldrich, USA), 0.05 μM dexamethasone (Sigma-Aldrich, USA), 200 mM L-glutamine (Invitrogen, USA), 10 mg/ml gentamycin (Invitrogen, USA), 10,000 units/ml penicillin and 10,000 μg/ml streptomycin (Invitrogen, USA). The cells were cultured at 38.5 °C under aerobic conditions (5% CO_2). Once the adherent cells were more than 80% confluent (expanded to 80% of the flask's surface), they were detached with 0.05% trypsin-EDTA (Invitrogen, USA) for 3 min and counted using a Z2 counter or cell viability analyzer (Vi-Cell XR 2.03; both Beckman Coulter, USA). For our experiments, 3×10^6 cells were seeded per 25 cm^2 culture flask, with the number achieved by adequate pooling of the material obtained from the punctured follicles into samples of comparable cell numbers. The cell samples were collected in 4 time intervals, representing different stages of short-term *in vitro* culture: 0h-serving as an *ex vivo* reference; 48h-representing initial *in vitro* associated changes in culture; 96h-an assumed "point of loss" of most of the cell's physiological properties; 144h-the endpoint of short-term culture. The medium was changed every 72h.

2.3. Histological analysis

In the experiment, the morphology and histological structure of the collected ovaries and follicles were also assessed. For this purpose, five ovaries were collected. Subsequently, separate follicles were collected from 2 of those ovaries and fixed in Bouin's solution for 48 h. Subsequently, the obtained samples were embedded in paraffin and cut into four μm thick sections with a semi-automatic rotary microtome (Leica RM 2145, Leica Microsystems, Nussloch, Germany). The sections were collected from the mid-part of gonads and follicles. Then, the preparations were stained using routine hematoxylin and eosin (H&E) staining. Histological sections were evaluated using a light microscope, with selected pictures taken with the use of high-resolution scanning technique and Olympus BX61VS microscope scanner (Olympus).

2.4. Microarray expression analysis and statistics

The Affymetrix procedure was previously described by Trejter et al. [15] and was used in our previous works regarding porcine oviductal cells [16–18] and porcine oocytes [3,19,20]. The cDNA was reverse transcribed from the total RNA obtained from the isolated follicular GCs (100 ng) (Ambion® WT Expression Kit). Obtained cDNA was biotin-labeled and fragmented using Affymetrix GeneChip® WT Terminal Labeling and Hybridization Kit (Affymetrix, Santa Clara, CA, USA). Biotin-labeled fragments of cDNA (5.5 μg) were hybridized to Affymetrix® Porcine Gene 1.1 ST Array Strip (48 °C/20 h). Then, the microarrays were washed and stained according to the technical protocol of the Affymetrix GeneAtlas Fluidics Station. Subsequently, the array strips were scanned by the Imaging Station of the GeneAtlas System. The preliminary analysis of the scanned chips was performed using Affymetrix GeneAtlas™ 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 using Bioconductor and the R programming language. Each CEL file was merged with a description file. In order to correct background, normalize, and summarize results, we have used the Robust Multiarray Averaging (RMA) algorithm.

Statistical significance of analyzed genes was performed by 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. The selection of genes of significantly changed expression was based on a p-value beneath 0.05, and expression fold-change higher than 2. Differentially expressed gene lists (separate for up and downregulated genes) were uploaded to the DAVID software (Database for Annotation, Visualization, and Integrated Discovery) [21]. Expression data of these genes were subjected to hierarchical clusterization procedure and presented as a heatmap graph. Among generated Gene Ontology (GO) terms we focused on "aging", "apoptotic process", "cell death", "cell growth", "negative regulation of cell death", "positive regulation of cell death" and "regulation of cell death" Gene Ontology Biological Process (GO BP) terms.

Subsequently, sets of differentially expressed genes from the selected GO BP terms were applied to STRING software (Search Tool for the Retrieval of Interacting Genes/Proteins) for interaction prediction. STRING is a vast database that contains information on protein/gene interactions, including experimental data, computational prediction methods, and public text collections.

To further investigate the chosen gene sets, we investigated their mutual relations using the GOplot package [22].

Finally, the functional interaction between genes belonging to the chosen GO BP terms was investigated using the REACTOME FIViz application to the Cytoscape 3.6.0 software. The ReactomeFIViz 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 perform a 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 before and after IVC using 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. The 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

Table 1
Sequences of primers used in the RT-qPCR validation of the microarray results.

Gene	Gene ID	Primer sequence (5′-3′)	Product size (bp)
CXCL10	494019	CTCCTGAAAGGCCCATCATA GCACATGGGATAGAGGAGGA	193
DAB1	100037307	TACGTTTGTGGGAAGGAAGG CTTCCTTCTTTTGGCTGGTG	202
DAPL1	100157537	CCTGCTCTGGAGAAGGTCAC GGGCCTAAGGAAAGTTTTGG	151
IGF1	47523587	TTCTACTTGGCCCTGTGCTT CTCCAGCCTCCTCAGATCAC	222
IHH	397174	CTCCACTGCCCTCTCAGAAC AGCTCGCAGCTGTGTCACTA	182
ITM2A	595131	TCTCGTAGGCCTTTCCTTCA AGGCAGGAAGTAGGGCTCTC	163
MCOLN3	100625693	TCCGAGTGCCTTTTCTCACT CGGATATAAACGTGCGGAGT	238
NEBL	100522395	CAAACCCTTCAAGGCTACCA CTGAGAACACGCTTCCATCA	177
SCUBE1	100524621	AATCCAATGAAGCCAACAGC AGGGCCTTGATCAGCTTCTT	160
TGFBR3	397512	TTTGTTTTAGCTGGGGGTTG TGGCCACAGGGATTTTTAAG	177
CAV1	404693	TAGGTCAGCAGCCTCCCTAA CTGGTGAGAGGCAGGAAAAG	243
FN1	397620	TGAGCCTGAAGAGACCTGCT CAGCTCCAATGCAGGTACAG	113
IGFBP5	397182	TGGGGGTTTGTTTCTCTGAC TTCTGGCAGGTAGAGCAGGT	181
ITGA2	397483	CATGCCAGATCCCTTCATCT CGCTTAAGGCTTGGAAACTG	153
ITGB3	397063	GGCTTCAAAGACAGCCTCAC AGTCCTTTTCCGAGCACTCA	175
LAMB1	396707	CTTCACCACCTTGGACCACT AGCTGTGGCTCATAGCGAAT	216
PDPN	100738269	AGCAGATGCTGTGTCCCTCT TATGGAACCTGGGCTGGTAG	201
POSTN	100152401	ATTGACCGTGTCCTCACACA GCCACTTTGTCTCCCATGAT	212
TAGLN	397021	TTAAAGGCCGCTGAGGACTA ATGACATGCTTTCCCTCCTG	233
TMOD1	100316850	AGCCCTAACGGAAGAAGAGC CCTTTGCTTGCTTTTCCAAG	170

dye. Target cDNA was quantified using the relative quantification method. The relative abundance of the analyzed transcripts in each sample was standardized to the internal standards. For amplification, 1 μl of cDNA solution was added to 9 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 rRNA*, was used as an internal standard to demonstrate that *PBGD* and *ACTB* mRNAs were not differentially regulated in GC groups. *18S rRNA* has been identified as an appropriate housekeeping gene for use in quantitative PCR studies. Again, the statistical significance of analyzed genes was performed by 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.

3. Results

Whole transcriptome profiling by Affymetrix microarray revealed the granulosa gene expression changes at 48, 96, and 144 h of *in vitro* culture. With the use of Affymetrix® Porcine Gene 1.1 ST Array Strip, we examined the expression of 27558 transcripts. Genes with a fold change higher than abs (2) and with a corrected p-value lower than 0.05 were considered as differentially expressed. This set of genes consists of 3380 different transcripts, a complete list of which can be found in the GEO database (ID: GSE134361).

Up and down-regulated gene sets were subjected to the DAVID search separately and only ones with adj. A P-value lower than 0.05 were selected. The DAVID software analysis showed that the differently expressed genes belonged to 344 GO BP Terms. In this paper, we focused on the chosen "cell development", "cell differentiation" and "cell morphogenesis" GO BP terms. In this paper, we focused on „aging", „apoptotic process", „cell death", „cell growth", „negative regulation of cell death", „positive regulation of cell death" and „regulation of cell death" GO BP terms. These sets of genes were subjected to hierarchical clusterization procedure and presented as heatmaps (Fig. 1). The gene symbols, fold changes in expression, Entrez gene IDs, and corrected p-values of that genes were shown in Table 2.

To further investigate the changes within the chosen GO BP terms, we measured the enrichment levels of each selected GO BPs. The enrichment levels were expressed as z-score and presented as circular visualization (Fig. 2).

Chosen GO BP terms contain 182 differently expressed genes. Most of these genes are present in several groups at the same time. To better understand the interaction between chosen GO BP terms, we performed the hierarchical clusterization of the gene expression profiles. The resulting dendrogram was combined with fold changes (FC) of studied gene expression and gene assignment to studied terms. The results were presented in Fig. 3.

Subsequently, we calculated the mean value of the fold change ratio of each gene between 48h, 96h, and 144h. Based on that criteria, we choose ten most downregulated and ten most upregulated genes to further analysis.

In the Gene Ontology database, it is possible for genes to belong to more than one gene ontology. For this reason, we explore the gene intersections between the selected GO BP terms. The relation between those GO BP terms was presented as a circle plot (Fig. 4) as well as a heatmap (Fig. 5). Among the 20 genes chosen for this study, only 7 GO BP terms contain at least one gene from the chosen set (as seen on Fig. 1).

STRING interaction network was generated among chosen differentially expressed genes belonging to each of the selected GO BP terms [23]. Using such a prediction method provided us with a molecular interaction network formed between protein products of studied genes (Fig. 6). Finally, we have investigated the functional interactions between chosen genes with the REACTOME FIViz app to Cytoscape 3.6.0 software. The results were shown in (Fig. 7).

To confirm the correct morphology of the studies ovaries and follicles, a histological examination was performed. The results detailed the morphological structure of collected ovaries and follicles, revealing follicles at all stages of development. The assessment of the degree of follicular maturity is possible due to the analysis of their morphological characteristics. Primordial follicles are characterized by the presence of an oocyte and one layer of flattened follicular cells. In the primary follicles, granulosa cells are formed, arranged in one or many layers. In turn, in the secondary follicles, the spaces between granulosa cells are formed, connecting in the mature follicle and forming an antrum. Granulosa cells are arranged on the antrum's periphery (Fig. 8).

Mature follicles vary in size. Small follicles are characterized by a diameter of less than 3 mm. In medium follicles, the diameter is between 3 and 5 mm, and large follicles exceed the 5 mm in size (Fig. 9).

Finally, the microarray results were quantitatively validated using the RT-qPCR method. The results were compiled, compared, and presented as a bar graph (Fig. 10). RT-qPCR confirmed all microarray results.

4. Discussion

Ten of the most up-regulated and 10 of the most down-regulated genes belonging to these ontology groups were selected for analysis. Specifically, *POSTN, FN1, FMOD, ITGB3, DCN, SERPINB2, SFRP2, IGFBP5, EMP1*, and *CCL2* which are upregulated, as well as *DAPL1, ESR1, IHH, TGFBR3, PPARD, PDK4, TXNIP, IFIT3, CSRNP3*, and *TNFSF10* whose expression is downregulated through the time of *in vitro* GCs culture. The most upregulated genes were *POSTN, FMOD, FN*1, and *ITGB3*, with all of them representing only the „cell growth" ontological group.

Periostin (*POSTN*) is an adhesion glycoprotein present mainly in tissues rich in collagen (connective tissue), which exhibited a strong up-regulation of this gene in porcine GCs *in vitro*. Increased expression of this gene has been observed in many different tissues, including placenta, heart valves, ligaments, but also in tissues exposed to stress, where tissue repair and increased angiogenesis were present [24]. It was also shown that *POSTN* was involved in the interactions between macrophages and ovarian cancer cells [25], as well as is associated with ovarian cancer with intestinal metastases [26]. Recently published studies have also described a strong up-regulation of the *POSTN* gene in pig GC cultures. To date, a connection between POSTN and folliculogenesis has not yet been revealed [27]. It is, therefore, possible that POSTN's upregulation may be an important new marker associated with cell growth processes. The *FN1* gene shows a significant correlation (especially co-expression) with the *POSTN* gene. Fibronectin (FN1) is a protein commonly found in the extracellular matrix of blood vessels, as well as in dissolved form in the blood. It also has an important role in the structure of the ovary, as it is a component that builds the basement membrane of the ovarian follicle. In studies conducted on the primary culture of cells from swine buccal cheeks, it was observed that an increase in *FN1* gene expression correlates with increased cell

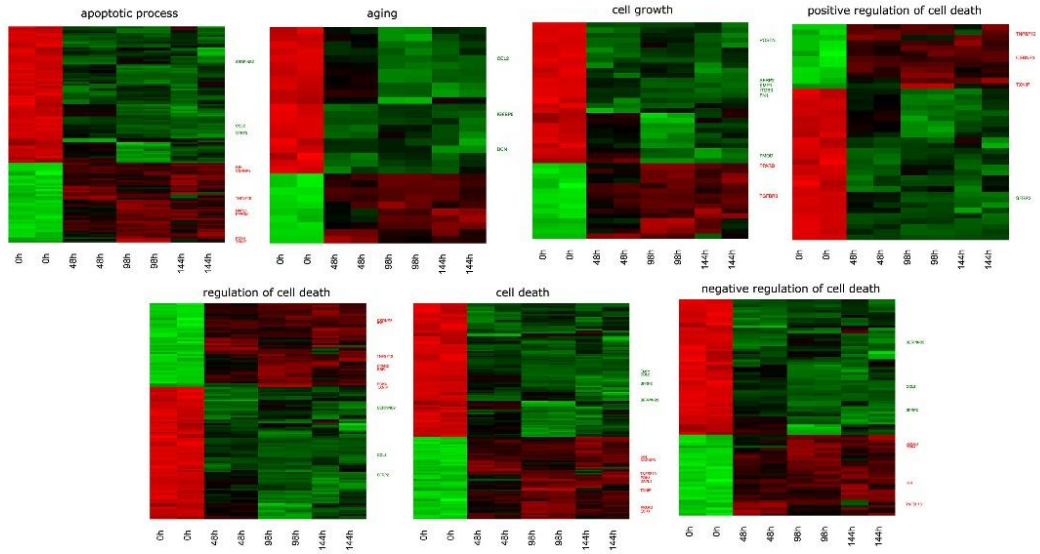

Fig. 1. Heat map representation of differentially expressed genes belonging to the „aging", „apoptotic process", „cell death", „cell growth", „negative regulation of cell death", „positive regulation of cell death" and „regulation of cell death" GO BP terms. Arbitrary signal intensity acquired from microarray analysis is represented by colors (green, higher; red, lower expression). Log2 signal intensity values for any single gene were resized to Row Z-Score scale (from −2, the lowest expression to +2, the highest expression for single gene). Only the location of described genes was marked on the diagram. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

proliferation [28]. Ożegowska et al. showed an increased expression of this gene in porcine GCs' *in vitro* culture, suggesting that it may participate in the regulation of cell proliferation *in vitro* [27]. Another gene, strongly up-regulated and correlated with *FN1*, is *FMOD* (fibromodulin), also belonging exclusively to the „cell growth" ontological group. Tormin and others conducted research on various subpopulations of human mesenchymal stem cells (MSC), where they demonstrated that FMOD is one of the molecules of cell surface, which enables identification of subpopulations of MSC with limited potential for differentiation [29]. *In vivo*, fibromodulin promotes angiogenesis in wound healing [30]. Direct interaction with the *FN1* gene is shown by the integrin subunit β-3 gene (*ITGB3*), which is also known as *CD61*. This gene is involved in the construction of transmembrane glycoproteins, which participate in many physiological processes. A link with the expression of this gene was demonstrated in cell lines infected with classical swine fever virus (CSFV) strains, which most probably was associated with strong adhesion of infected cells [31,32]. Ożegowska et al., for the first time, showed a significant up-regulation of the *ITGB3* gene

Table 2
Gene symbols, expression change ratio, Entrez gene IDs and corrected p values of studied genes.

Gene Symbol	Fold Ratio 0H/48H	Fold Ratio 0H/96H	Fold Ratio 0H/144H	P Val 0H/48H	P Val 0H/98H	P Val 0H/144H	Entrez Gene ID
DAPL1	−28.6861	−265.078	−247.176	1.00E-06	9.14E-08	9.57E-08	92196
ESR1	−8.09079	−41.7063	−18.1511	2.17E-05	7.04E-07	2.71E-06	2099
IHH	−20.0921	−18.5874	−20.9478	2.16E-06	1.06E-06	1.09E-06	3549
TGFBR3	−15.1795	−22.081	−21.6357	4.56E-07	1.51E-07	1.50E-07	7049
PPARD	−6.3915	−26.4032	−16.2152	8.24E-06	3.18E-07	7.27E-07	5467
PDK4	−2.41018	−29.1875	−15.9739	0.001618	8.79E-07	2.67E-06	5166
TXNIP	−2.70209	−34.0998	−4.53	0.00158	1.12E-06	0.000125	10628
IFIT3	−7.6518	−14.1799	−5.2724	9.85E-05	1.26E-05	0.000231	3437
CSRNP3	−8.1292	−6.9678	−8.53974	5.01E-06	3.21E-06	2.65E-06	80034
TNFSF10	−8.58694	−7.69706	−4.4102	0.002277	0.001798	0.012257	8743
CCL2	12.31755	28.04021	24.47926	1.00E-06	1.62E-07	2.27E-07	6347
EMP1	13.61155	28.38631	28.07128	2.79E-06	4.44E-07	5.38E-07	2012
IGFBP5	29.89599	20.97384	31.2743	4.42E-07	3.09E-07	2.28E-07	3488
SFRP2	15.34471	30.79202	38.31993	9.81E-07	1.80E-07	1.50E-07	6423
SERPINB2	41.24699	8.918724	44.89473	2.67E-06	1.68E-05	1.34E-06	5055
DCN	21.58632	16.85223	63.38248	4.36E-07	2.52E-07	9.57E-08	1634
ITGB3	29.91255	39.85199	57.68212	3.52E-07	1.21E-07	9.57E-08	3690
FMOD	7.111499	46.80442	84.85236	9.96E-05	1.61E-06	1.09E-06	2331
FN1	35.37896	66.1651	68.74185	3.59E-07	9.92E-08	9.57E-08	2335
POSTN	95.22897	29.49005	88.94606	3.52E-07	1.80E-07	9.57E-08	10631

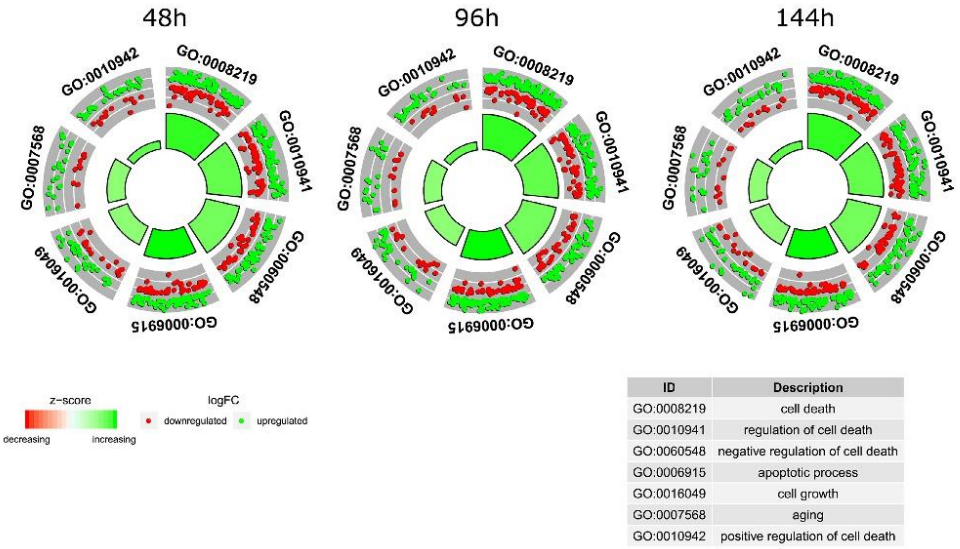

Fig. 2. The Circular visualization of the results of gene-annotation enrichment analysis. The outer circle shows a scatter plot for each term of the logFC of the assigned genes. Green circles display up-regulation and red ones down-regulation. The inner circle is the representation of Z-score. The size and the color of the bar correspond to the value of z-score. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

in pig GCs' primary culture [27]. The authors suggest that ITGB gene may be a new marker for cellular adhesion, proliferation and ovarian tissue development processes. In our current research, we have observed the up-regulation of this gene, an exclusive member of the "cell growth" group. Another gene that has shown a significantly increased expression in our *in vitro* studies of cultured GCs is decorin (*DCN*). Its strong expression also correlated with interactions with equally up-regulated genes (*FMOD, FN1, POSTN*), mainly as co-expression. The decorin gene belongs to the ontological group „aging". Decorin's expression in porcine GCs was described by Chermuła et al. [4], where it was associated with angiogenesis in the ovary, with its activity supposedly depending on the microenvironment. The next gene representing the „aging" ontological group is the *IGFBP5* (insulin-like growth factor binding protein 5) gene, which also showed high expression. It is an important component of the insulin-like growth factor (IGF) system. Studies on rat ovaries have shown that this gene is expressed by granulosa cells, mainly in atretic follicles, and in smaller amounts by interstitial tissue [33]. Therefore, its significant expression in the „aging" group may be a potential marker of aging of the follicle or granulosa cell *in vitro* culture. The authors of the aforementioned studies suggest that the *IGFBP5* gene may be an autocrine/paracrine regulator, especially of preantral ovarian follicle atresia. Studies conducted by Hayashi et al. based on bovine ovarian follicles showed significant expression of *IGFBP5* by GCs and theca cells of atretic follicles [34]. Recently, up-regulation of this gene has also been noted in the study of *in vitro* cultured porcine GCs [27]. Two more significantly upregulated genes were: *CCL2* (C–C motif chemokine ligand 2) and *SFRP2* (secreted frizzled – related protein 2). Both of them belong to several ontological groups (*CCL2* to 5 groups; *SFRP2* to 6 groups). The expression of the *CCL2* gene in GCs has been recorded in studies on bovine ovarian follicles [34]. It was suggested that this gene is involved in immune processes, but also in the development of follicles and atresia. These results are in line with our research because one of the groups to which this gene belongs is „aging" (related to atresia), followed by "cell death", "regulation of cell death", "negative regulation of cell death" and "apoptotic process". Increased regulation of these two genes was also observed in primary porcine GC culture in ovarian angiogenesis studies [4]. The *SERPINB2* gene (serpin family B member 2) showed significant up – regulation in our study and is found in 4 ontological groups („cell death", „regulation of cell death", „negative regulation of cell death" and „apoptotic process"). It correlates with the *POSTN* gene, demonstrating co-expression. This gene shows expression during pregnancy and in the immune response, and its role is to regulate fibrinolysis [35]. The last up – regulated gene we describe is the epithelial protein 1 membrane (EMP1). It belongs to two ontological groups: „cell death" and „cell growth". So far we have not found a link between this gene and GCs, its expression was only observed in ovarian cancer, where its down – regulation was correlated with the progression of the disease [36].

The most considerable reduced regulation of expression was found in the *DAPL1* gene (death-associated protein-like 1), belonging to two ontological groups: „cell death" and „apoptotic process". The close association of DAPL1 gene with ovarian cells, in particular GCs, has not yet been reported in the literature. However, a significant increase in the expression of the *DAPL1* gene has been observed in studies on uterine inflammation in dairy cows that analyzed transcripts in the endometrium [37]. Significantly downregulation was also detected for the *TGFBR3* (transforming growth factor-beta receptor 3) gene, which belongs to the „cell growth" group. Together with FSH, the transforming growth factor β (TGF-β) superfamily has an essential role in the apoptosis process, which leads to follicular atresia [38]. TGFBR3 gene is a vital signal point for cell differentiation and development, as well as for organogenesis, including the

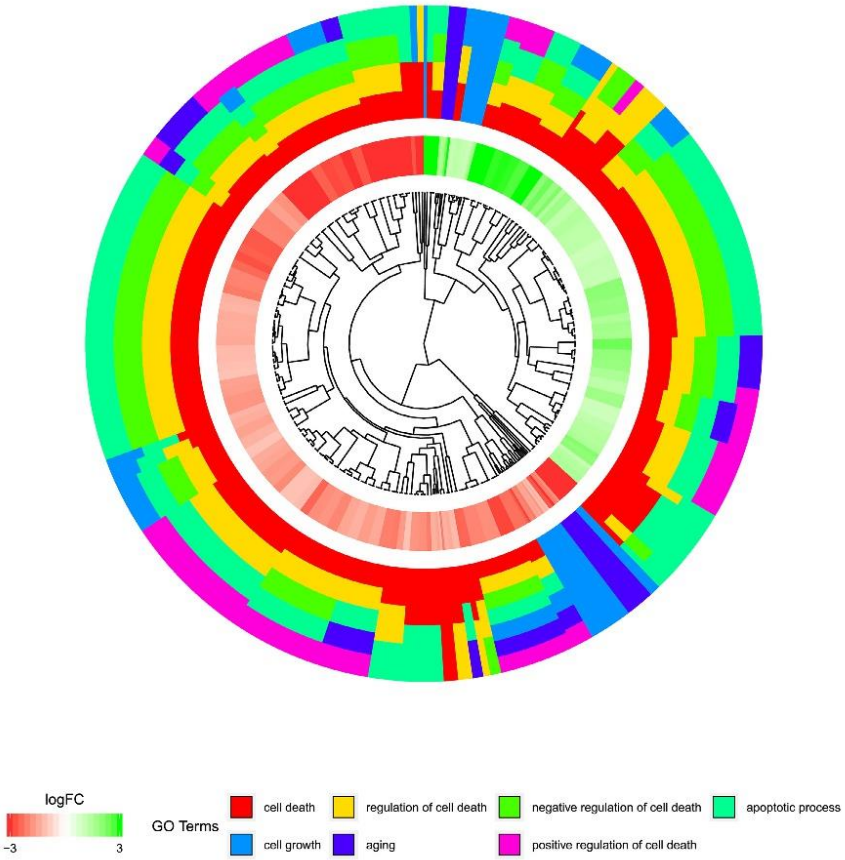

Fig. 3. The Representation of hierarchical clusterization, fold change and assignment of differently expressed genes that belongs „aging", „apoptotic process", „cell death", „cell growth", „negative regulation of cell death", „positive regulation of cell death" and „regulation of cell death" GO BP terms. Genes are grouped together based on their expression patterns and the clusterization pattern is represent by dendrogram inside the circle. The middle ring represents the logarithm of gene expression fold change of studied genes. The outer ring represents the terms assigned to the genes.

formation of coronary vessels in the heart [39]. The RGFBR gene that also showed a substantial decrease in expression, belonging to the „cell death" and „regulation of cell death" groups, was *ESR1* (estrogen receptor 1, also known as estrogen receptor α). The alpha estrogen receptor, similarly to its beta variant, is present in granulosa cells of preovulatory follicles, as well as in early corpora lutea confirming the regulation of ovarian function by gonadal hormones [40,41]. The remaining down-regulated genes of interest (*IHH, PPARD, PDK4, CSRNP3, IFIT3, TXNIP, TNFSF10*) belonged to several ontological groups, with all of them being a part of „cell death", „regulation of cell death" and „apoptotic process".

Besides, *IHH* (Indian hedgehog signaling molecule) and *PDK4* (pyruvate dehydrogenase kinase 4) were also expressed in „negative regulation of cell death", while *CSRNP3* (cysteine and serine-rich nuclear protein 3) and *TXNIP* (thioredoxin interacting protein) was also part of the „positive regulation of cell death". Previous mouse studies have shown that progesterone affects the expression of the *IHH* gene [42]. In our experiment, we observed a decrease in the expression of this gene, which can be attributed to a lack of stimulation with progesterone. Physiologically, *IHH* is a gene encoding intracellular protein that contributes to cell development. A unique role is assigned to IHH in the process of ossification and proliferation of mesenchymal cells [43]. In the available literature, there are reports linking the *PDK4* gene overexpression during the development of ovarian cancer. In such cases, this gene has been found to confer apoptosis resistance on cancer cells, leading to the regulation of cell stem-like traits [44]. The *CSRNP3* gene in our research shows a correlation with the *TNFSF10* gene (tumor necrosis factor superfamily member 10). There are reports that *TNFSF10* is associated with apoptosis in granulosa cells during vesicular atresia in porcine ovaries [45,46] confirming confirms that both genes in our study belong to ontological groups associated with apoptosis and cell death, which may also indicate the marker potential of these genes. The expression of the IFIT3 gene (interferon-induced protein with tetratricopeptide repeats 3),

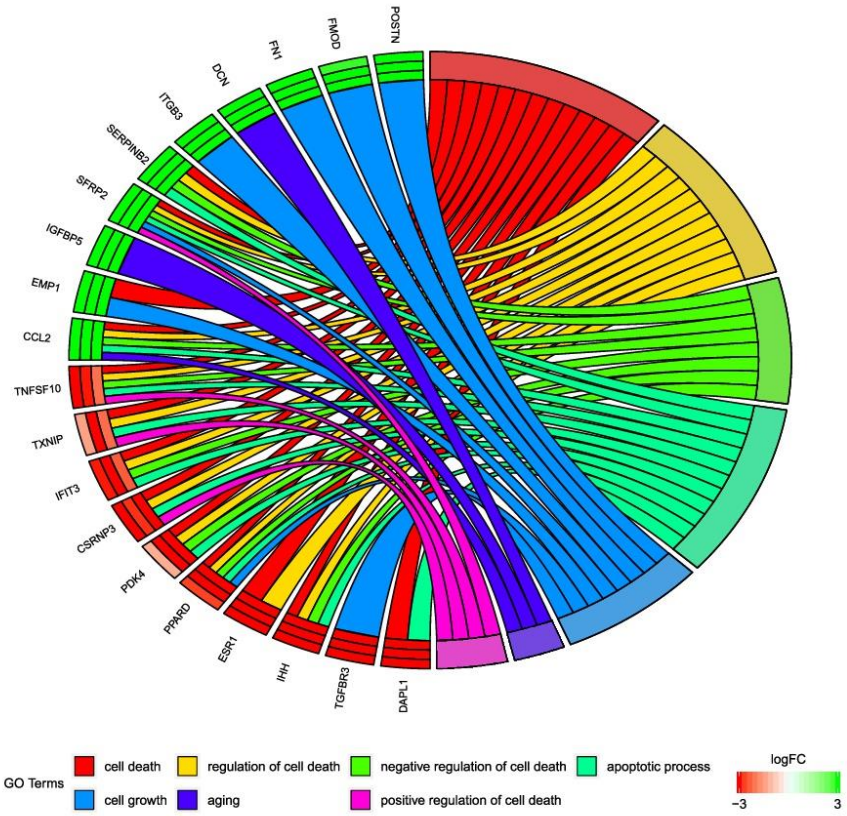

Fig. 4. The representation of the mutual relationship between 20 chosen genes that belongs to „aging", „apoptotic process", „cell death", „cell growth", „negative regulation of cell death", „positive regulation of cell death" and „regulation of cell death" GO BP terms. The ribbons indicate which gene belongs to which categories. The colors of 3 inner bars near each gene correspond to logFC after 48h, 96h and 144h respectively. The genes were sorted by logFC. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

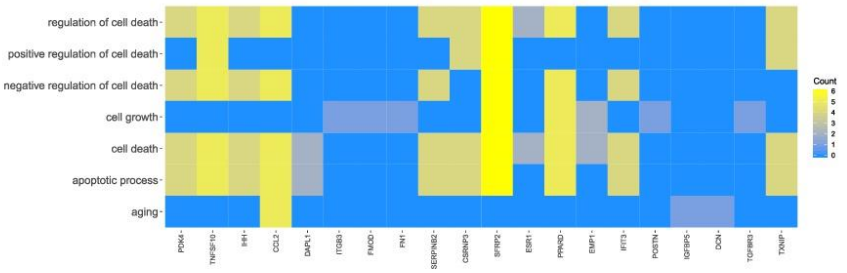

Fig. 5. Heatmap showing the gene occurrence between 20 chosen genes that „aging", „apoptotic process", „cell death", „cell growth", „negative regulation of cell death", „positive regulation of cell death" and „regulation of cell death" GO BP terms. Yellow color indicates the gene occurrence in indicated GO BP Term. The intensity of color correlates with number of GO BP Terms that selected gene belongs to. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

responsible for immune processes, has been reported in pluripotent human stem cells (hPSCs) research. Significantly lower expression of this gene was detected in the hPSCs line in comparison with to somatic cells [47]. This gene is a negative regulator of cell proliferation. In our experiment, we also noted down-regulation of the *IFIT3* gene in ontological groups

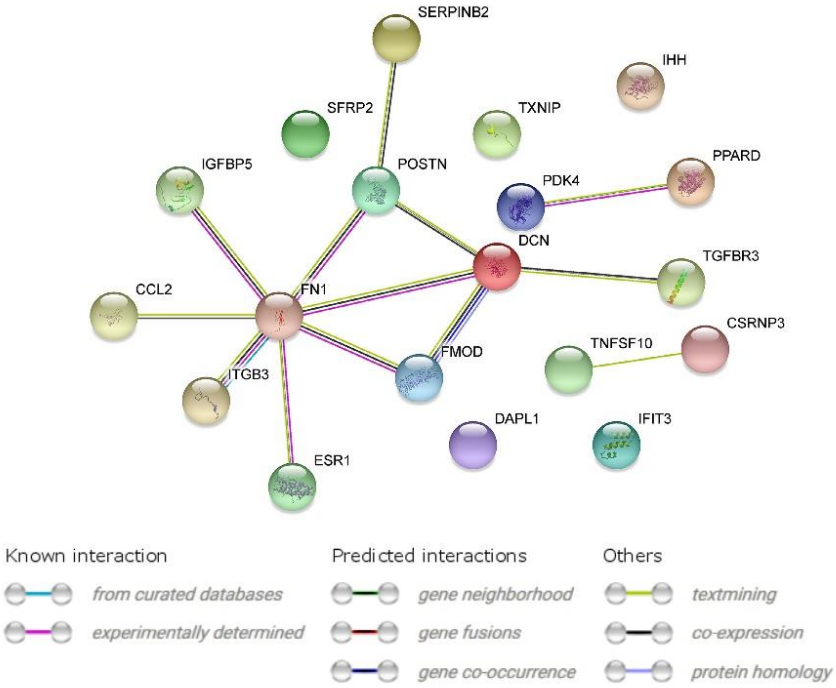

Fig. 6. STRING-generated interaction network among 20 chosen genes belonging to the „aging", „apoptotic process", „cell death", „cell growth", „negative regulation of cell death", „positive regulation of cell death" and „regulation of cell death" GO BP terms. The intensity of the edges reflects the strength of int**eraction score.**

corresponding to apoptosis. The downregulation of the *TXNIP* gene (thioredoxin interacting protein), responsible for glucose metabolism, was recorded in our studies. Earlier, a decrease in the expression of this gene (IVM) in bovine cumulus cells was observed [48]. GCs are responsible for supplying lactate and pyruvate to the oocyte, as OC is not able to metabolize glucose on its own.

Other studies indicated decreased expression of *TXNIP* during IVM of swine oocytes after stimulation with estrogens [49]. The last gene described in our study is *PPARD* (peroxisome proliferator-activated receptor delta), characterized by downregulation in five ontological groups (associated with apoptosis and death, as well as in the „cell growth" group). This gene is closely related to fat metabolism, and its expression is widely recorded. Its expression was noted in studies on porcine oviductal epithelial cells during long – term primary culture [50] and on porcine ovarian GCs during short – term primary *in vitro* culture [4]. New studies also provide information that the regulation of *PPARD* expression in ovarian follicles is dependent on LH and FSH. FSH increases the expression of all isoforms, while LH only affects alpha and gamma. The last two isoforms were also dependent on steroid hormones (such as progesterone and estradiol) [51].

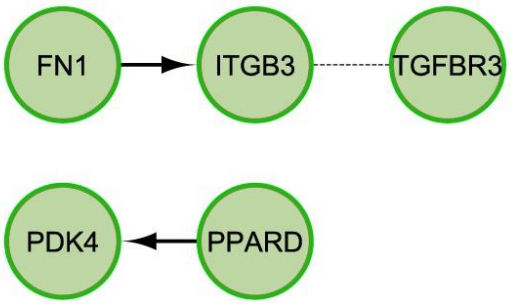

Fig. 7. Functional interaction (FI) between 20 chosen genes that belongs to „aging", „apoptotic process", „cell death", „cell growth", „negative regulation of cell death", „positive regulation of cell death" and „regulation of cell death" GO BP terms. In following figure „->„ stands for activating/catalyzing, „-|" for inhibition, „-" for FIs extracted from complexes or inputs, and „—" for predicted FIs.

To summarize, apart from few available studies on some of the analyzed genes referencing folliculogenesis, notable changes in their expression indicate their potential role as markers of essential life processes of porcine granulosa cells in primary *in vitro* culture. This research might serve as a molecular entry helping to fully understand the processes driving the GCs in non-physiological conditions, becoming the basis of further research on GC application in assisted reproduction techniques. Furthermore, the results shed further light on the potential plasticity of GCs *in vitro*, with possible application of this properties in regenerative and reconstructive medicine.

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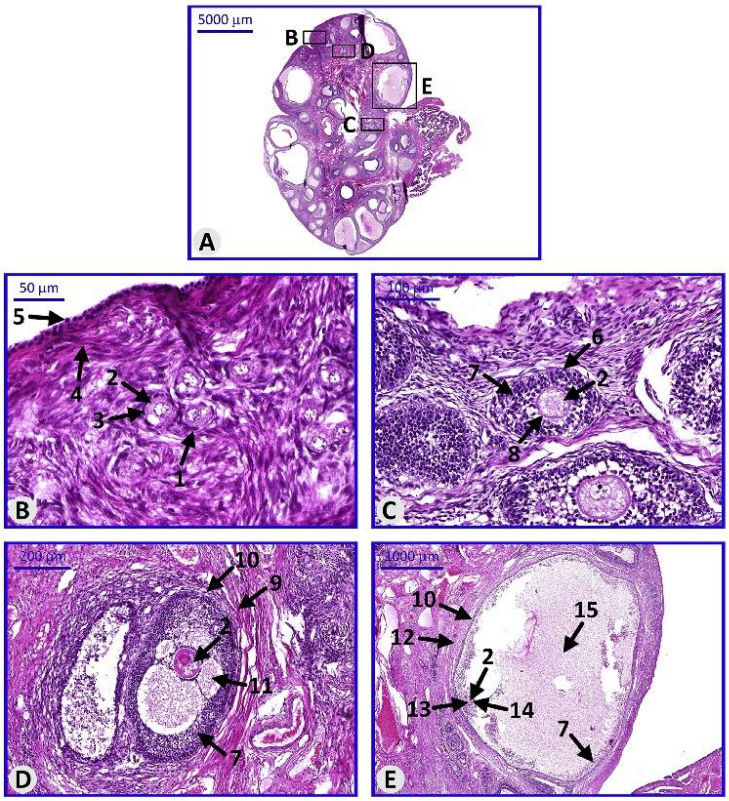

Fig. 8. Microphotograph representing the histological structure of crossbred Landrace gilt ovaries (H&E staining). Primordial, primary, secondary and mature follicles are present. A-ovary, B-E – selected area of A, observed in higher magnification, representing follicles in particular stages of development. Arrows: 1 – primordial follicles, 2 – oocyte, 3 – follicular cells, 4 – tunica albuginea, 5 – germinal epithelium, 6 – primary multilaminar follicle, 7 – granulosa cells, 8 – zona pellucida, 9 – secondary follicle, 10 – theca interna and theca externa, 11 – forming antrum, 12 – Graafian follicle, 13 – cumulus oophorus, 14 – corona radiata, 15 – antrum.

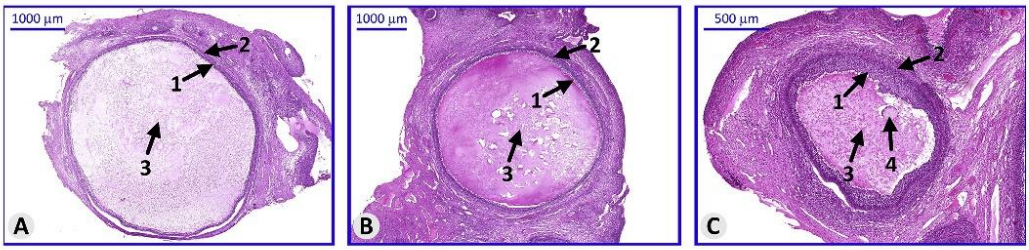

Fig. 9. Isolated mature follicles varying in size (H&E staining). A – large follicle (>5 mm), B – medium follicle (3–5 mm), C – small follicle (<3 mm). Arrows: 1 – granulosa cells, 2 – theca interna and theca externa, 3 – antrum.

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Author contributions

M.K.-Formal Analysis, Investigation, Writing-original draft; W.K.-Investigation, Validation, Writing part of the manuscript draft; P.S-K.-Data Curation, Investigation, Methodology; P.M.-Formal Analysis, Software; M.J.-Formal Analysis, Investigation; A.K.- Data Curation, Investigation, Methodology; J.K.-Formal Analysis, Software; D.B.- Funding Acquisition, Resources; M.S. - Writing-

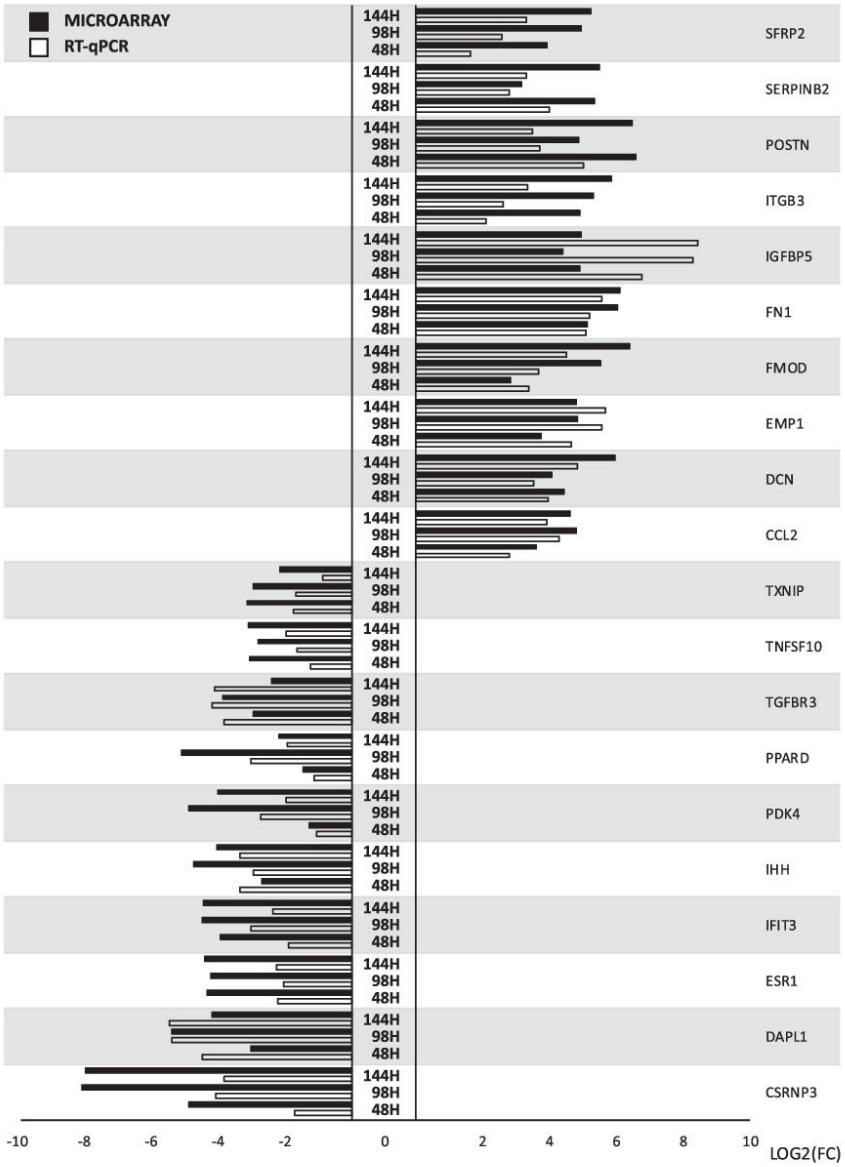

Fig. 10. The results of RT-qPCR validation of the analyzed genes, presented in a form of bar graph.

review and editing; H.P-K.- Data Curation, Validation; M.N.- Funding Acquisition, Project Administration, Supervision; B.K.- Conceptualisation, Funding Acquisition, Project Administration, Supervision, Writing-review and editing; P.A.-Funding Acquisition, Resources; Project administration.

Declaration of competing interest

None.

CRediT authorship contribution statement

Magdalena Kulus: Formal analysis, Investigation, Writing - original draft. **Wiesława Kranc:** Investigation, Validation, Writing - original draft. **Patrycja Sujka-Kordowska:** Data curation, Investigation, Methodology. **Paul Mozdziak:** Formal analysis, Software. **Maurycy Jankowski:** Formal analysis, Investigation. **Aneta Konwerska:** Data curation, Investigation, Methodology. **Jakub Kulus:** Formal analysis, Software. **Dorota Bukowska:** Funding acquisition, Resources. **Mariusz Skowroński:** Writing - review & editing. **Hanna Piotrowska-Kempisty:** Data curation, Validation. **Michał Nowicki:** Funding acquisition, Project administration, Supervision. **Bartosz Kempisty:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing. **Paweł Antosik:** Funding acquisition, Resources, Project administration.

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4.3. Publikacja III

Histochemistry and Cell Biology
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ORIGINAL PAPER

Transcriptomic analysis of expression of genes regulating cell cycle progression in porcine ovarian granulosa cells during short-term in vitro primary culture

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Abstract
The primary function of ovarian granulosa cells (GCs) is the support of oocytes during maturation and development. Molecular analyses of granulosa cell-associated processes, leading to improvement of understanding of the cell cycle events during the formation of ovarian follicles (folliculogenesis), may be key to improve the in vitro fertilization procedures. Primary in vitro culture of porcine GCs was employed to examine the changes in the transcriptomic profile of genes belonging to "cell cycle", "cell division", "cell cycle process", "cell cycle phase transition", "cell cycle G1/S phase transition", "cell cycle G2/M phase transition" and "cell cycle checkpoint" ontology groups. During the analysis, microarrays were employed to study the transcriptome of GCs, analyzing the total RNA of cells from specific periods of in vitro cultures. This research was based on material obtained from 40 landrace gilts of similar weight, age and the same living conditions. RNA was isolated at specific timeframes: before the culture was established (0 h) and after 48 h, 96 h and 144 h in vitro. Out of 133 differentially expressed genes, we chose the 10 most up-regulated (*SFRP2*, *PDPN*, *PDE3A*, *FGFR2*, *PLK2*, *THBS1*, *ETS1*, *LIF*, *ANXA1*, *TGFB1*) and the 10 most downregulated (*IGF1*, *NCAPD2*, *CABLES1*, *H1FOO*, *NEK2*, *PPAT*, *TXNIP*, *NUP210*, *RGS2* and *CCNE2*). Some of these genes known to play key roles in the regulation of correct cell cycle passage (up-regulated SFRP2, PDE3A, PLK2, LIF and down-regulated CCNE2, TXNIP, NEK2). The data obtained provide a potential reference for studies on the process of mammalian folliculogenesis, as well as suggests possible new genetic markers for cell cycle progress in in vitro cultured porcine granulosa cells.

Keywords Pig · Ovarian follicle · Granulosa cells · Primary culture · Microarray

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Introduction

During the life cycle, cells go through distinct successive stages, which have been divided into phases. The order in which they occur, as well as their correctness are determined primarily by expression of a particular set of genes. Abnormalities in the passage of individual phases lead to the formation of abnormal cells, including those that give rise to a variety of cancers (Bertoli et al. 2013). Specific "checkpoints", verify progression at each stage of the cell cycle, controlling the DNA integrity. These mechanisms are able to detect damaged DNA and/or replication errors, excluding these fragments from further activity or arresting the cycle, which prevents the formation of abnormal cells (Bertoli et al. 2013). The importance of these checkpoints is evidenced by significant percentage of mutations within their regulatory proteins leading to the onset of oncogenesis. Cells in the G1 phase of the cell cycle need process a number of information governing the transition to the next phase-S-replication. Errors in the processes of growth, proliferation and stress management in the G1 phase were found to lead to the formation of cancers (Massagué 2004). Through an in-depth analysis of molecular processes of these critical cell cycle stages, it may be possible to identify particular causes of oncogenesis and provide a basic reference for the potential development of targeted therapies.

In a mature ovarian follicle, two walls of ovarian granulosa cells surround the fluid filled cavity and the developing oocyte (Budna et al. 2017; Rybska et al. 2018a). The female gamete is surrounded directly by a layer of cumulus cells (CCs), with the oocyte adjacent portion referred to as corona radiata. The second layer, lining the inside of the follicle, are the mural granulosa cells (GCs), surrounded from the outside by a basement membrane. Finally, the outermost cells building the ovarian follicles are theca cells (TCs). All types of ovarian granulosa play a very important physiological role in the follicular function. TCs produce androgenic substrate, necessary for the production of estrogen (Gilchrist et al. 2004; Magoffin 2005). CCs belonging to corona radiata adhere to the zona pellucida, a glycoprotein capsule penetrated by microvilli from both the oocyte and the cumulus cells. This process facilitates communication between these cells, conducted via ions and molecules exchanged using gap-junction nexus connections (Kempisty et al. 2014). Finally, GCs play a role in the formation of the corpus luteum after ovulation, consequently participating in progesterone production (Chermuła et al. 2019).

Furthermore, mammalian ovaries are a rich source of cells that can be used for molecular research. The granulosa cells (GCs) of the ovarian follicle are an interesting example, being able to proliferate in primary in vitro cultures despite the lack of their usual physiological environment, with recent findings indicating many possibilities for their application in molecular studies (Kranc et al. 2015, 2016). Molecular analysis of the basic granulosa cell-associated processes, as well as understanding of their cell cycle events during the formation of ovarian follicles (folliculogenesis) may be a key factor to improve the in vitro fertilization procedures, providing us with new molecular markers indicating the correctness of the folliculogenesis process, as well as oocyte development and maturation (Kranc et al. 2018; Rybska et al. 2018b).

Additionally, ovarian granulosa cells show significant stem-like potential. Literature data indicates that female GCs expressed molecular markers that are characteristic for mesenchymal stem cells (e.g. CD29, CD44, CD105, CD90) or pluripotent stem cells (Oct4, Nanog, Sox2, Tert) (Kossowska-Tomaszczuk et al. 2009, 2010). Additionally, other studies have shown that GCs may differentiate into osteoblasts (Mattioli et al. 2012) and chondrocytes (Varras et al. 2012). Thanks to the characteristics shown by these cells and relatively simple methods of obtaining them, they may become a model for developing stem cell therapies, with potential application in fields such as regenerative medicine. However, it is first necessary to fully describe the molecular basis of their functioning in in vitro culture, including the genes responsible for the cell cycle, which will allow for their further in vivo study and potential applications in clinical situations.

The objective of the current study was to study transcriptomic profiling of in vitro cultured porcine GCs. The focus was placed on transcriptomic profile alterations of genes belonging to "cell cycle", "cell division", "cell cycle process", "cell cycle phase transition", "cell cycle G1/S phase transition", "cell cycle G2/M phase transition" and "cell cycle checkpoint" ontology group. The knowledge gained should help to improve the understanding of GC functioning in in vitro conditions, as well as provide a basic molecular reference for further in vivo studies that could possibly lead to application of ovarian granulosa in clinical situations, as well as improve and optimized the currently used IVF techniques.

Materials and methods

Animals

Samples were obtained from 40 crossbred landrace gilts, all kept in the same conditions (feed, breeding, housing). These pigs had a mean weight of 98 kg and age of 170 days. The experiment was approved by the Poznan University of Medical Sciences Bioethical Committee (Resolution 32/2012, approved 1st of June 2012).

Collection of porcine ovaries and in vitro culture of granulosa cells

The reproductive organs were transported to the laboratory under appropriate temperature conditions (38 °C) in 0.9% NaCl within 30 min of slaughter. Then, the ovaries (80 in total) of the individual animals were isolated and placed in PBS supplemented with fetal bovine serum (FBS; Sigma-Aldrich Co., St. Louis, MO, USA). Large pre-ovulatory follicles with diameters above 5 mm ($n = 300$) were selected. Individual follicles were punctured with a sterile 20-G needle and aspirated with a 5 ml syringe. The procedure was performed in a Petri dish, recovering cumulus-oocyte complexes (COCs) and follicular fluid (FF). Subsequently, GCs were obtained while COCs were discarded.

The obtained cells were suspended in the culture medium and counted using ADAM Cell Counter and Viability Analyzer (Bulldog Bio, Portsmouth, NH, USA) and seeded on specific culture vessels. In this study, a medium of the following composition was used: Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, USA), 2% fetal calf serum (FCS) (PAA, Linz, Austria), 10 mg/ml ascorbic acid (Sigma-Aldrich, USA), 0.05 µM dexamethasone (Sigma-Aldrich, USA), 4 mM L-glutamine (Invitrogen, USA), 10 mg/ml gentamycin (Invitrogen, USA), 10,000 units/ml penicillin and 10,000 µg/ml streptomycin (Invitrogen, USA).

Stable conditions of 38.5 °C and 5% CO_2 were maintained during the culture of the obtained cells. At the indicated time intervals of the culture: 0 h, 24 h, 48 h and 96 h, GCs separated from the bottom of the culture dish using 0.05% trypsin–EDTA (Invitrogen, USA) for 3 min. To count the cells in the samples, an ADAM Cell Counter and Viability Analyzer (Bulldog Bio, Portsmouth, NH, USA) were used.

Microarray expression analysis and statistics

The Affymetrix procedure was previously described by Trejter et al. (2015). cDNA was reverse transcribed from the Total RNA of each sample (100 ng) (Ambion® WT Expression Kit). Obtained cDNA was biotin labeled and fragmentated using Affymetrix GeneChip® WT Terminal Labeling and Hybridization. Biotin-labeled fragments of cDNA (5.5 µg) were hybridized to the Affymetrix® Porcine Gene 1.1 ST Array Strip (48 °C/20 h). Then, the microarrays were washed and stained according to the technical protocol of the Affymetrix GeneAtlas Fluidics Station. Subsequently, the array strips were scanned by the Imaging Station of the GeneAtlas System. The preliminary analysis of the scanned chips was performed using the Affymetrix GeneAtlas™ 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 compiled using Bioconductor and R programming language. Each CEL file was merged with a description file. To correct background, normalize and summarize results, a Robust Multiarray Averaging (RMA) algorithm was employed.

Statistical significance of the analyzed genes was conducted using moderated *t*-statistics from the empirical Bayes method. Obtained *p* value was corrected for multiple comparisons using the Benjamini and Hochberg's false discovery rate. The selection of significantly changed gene expression was based on a *p* value beneath 0.05 and expression fold higher than 2. Differentially expressed genes were subjected to the selection of genes associated with cell cycle progression. Differentially expressed gene lists (separate for up and down regulated groups) were uploaded to the DAVID software (Database for Annotation, Visualization and Integrated Discovery), with enriched Gene Ontology terms extracted. Among the Enriched Gene Ontology terms, we have chosen those containing at least 5 genes and exhibiting a Benjamini method calculated *p* value lower than 0.05. Among the enriched Gene Ontology terms, we have chosen "cell cycle checkpoint", "cell cycle G1/S phase transition", "cell cycle G2/M phase transition", "cell cycle phase transition", "cell cycle process", "cell cycle" and "cell division" Gene Ontology Biological Process (GO BP) terms. Expression data of genes within the selected GO BP terms were subjected to hierarchical clusterization procedure and presented as heatmaps.

To further analyze the chosen gene sets, we investigated their mutual relations using the GOplot package (Walter et al. 2015). Moreover, the GOplot package was used to calculate the *z*-score (the number of up-regulated genes minus the number of down-regulated genes divided by the square root of the count). *z*-Score analysis allowed us to compare the enrichment of the selected GO BP terms.

Moreover, the interactions between proteins coded by selected genes and the genes itself were investigated using the STRING10 software (Search Tool for the Retrieval of Interacting Genes). STRING database contains information on protein/gene interactions, including experimental data, computational prediction methods and public text collections. STRING database engine provided us with a molecular interaction network formed between the genes of interest. The search criteria are based on co-occurrences of genes/proteins in scientific texts (textmining), co-expression and experimentally observed interactions.

Finally, the functional interactions between the genes belonging to the chosen GO BP terms were investigated using the REACTOME FIViz application to the Cytoscape 3.6.0 software. The ReactomeFIViz 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 perform 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. It 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.

RT-qPCR validation

Total RNA was isolated from GCs after 0 h, 24 h, 48 h and 96 h of in vitro culture using the Chomczyński and Sacchi method (Chomczynski and Sacchi 1987; Borys-Wójcik et al. 2018; Chamier-Gliszczyńska et al. 2018). The RNA samples were resuspended in 20 µl of RNase-free water and stored in liquid nitrogen. The 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. Target cDNA was quantified using the relative quantification method. The relative abundance of the analyzed transcripts in each sample was standardized to the internal standards. For amplification, 1 µl of cDNA solution was added to 9 µl of QuantiTect® SYBR® Green PCR (Master Mix Qiagen GmbH, Hilden, Germany) and primers (Table 1). Each experiment was performed in 3 biological and three technical replicates.

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, an additional housekeeping gene, 18S rRNA, was used as an internal standard to demonstrate that PBGD and ACTB mRNAs were not differentially regulated in GC groups. 18S rRNA has been identified as an appropriate housekeeping gene for use in quantitative PCR studies. Again, 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.

Table 1 Oligonucleotide sequences of primers used to conduct the RT-qPCR reactions

Gene	Gene ID	Primer sequence (5′–3′)
CCNE2	9134	**F**: GATGGTGCTTGCAGTGAAGA
		R: CGATGGCTAGAATGCACAGA
RGS2	5997	**F**: CTAAGGCGGTCCAATCACAT
		R: GCCCTCAAAAGACAGCAGAC
NUP210	23225	**F**: GCAACTGAAGCACCTGAACA
		R: ATGGCACCAAAGACCTTGAC
TXNIP	10628	**F**: CAAGCCAGCCAACTCAAGAG
		R: TTCGAGCAGAGACAGACACC
PPAT	5471	**F**: ACCGTGAAGTCTTACCTGGA
		R: TCGAAGATACAGAAAGCCATTGG
NEK2	4751	**F**: TGGGAAGATCAGAGAAGGCA
		R: TGGAGTCCTGCAGCTTTTCT
H1FOO	132243	**F**: CAGTCACCTCCCAGAACCAC
		R: TGTTCCCCATCTTCGTTTTGG
CABLES1	91768	**F**: CGTCGTCTCATCTCCCAGAG
		R: CATTCCTGGTGTCGTGCTG
NCAPD2	9918	**F**: CATTTCAGGCTGCCTTTCGA
		R: CTGGGAGTGGCGGGATAC
IGF1	3479	**F**: TTCTACTTGGCCCTGTGCTT
		R: CTCCAGCCTCCTCAGATCAC
TGFB1	7040	**F**: AAGCGGCAACCAAATCTATG
		R: CACGTGCTGCTCCACTTTTA
ANXA1	301	**F**: GGCCTTGGAACTGATGAAGA
		R: CCTCAGATCGGTCACCCTTA
LIF	3976	**F**: GAGGGAACCCAGAGTCTTCC
		R: TAGCACTGCTGGATGTCAGG
ETS1	2113	**F**: CATTGAGCGAGGTGAAGACA
		R: TCTGCCTTTGCTTTCCAAGT
THBS1	7057	**F**: CAAAGAGTTGGCCAGTGAGC
		R: ATGATGGGGCAGGACACTTT
PLK2	10769	**F**: CTTCGGGTACCAGCTCTCAG
		R: TAGGCAGATCTCCACCATCC
FGFR2	2263	**F**: GATGCCATCTCGTCCGGA
		R: TGGACAGCGGAACTTGACA
PDE3A	5139	**F**: ATGAGGCACCTTCATCCAGT
		R: TTCACTAGAGAACCCGGTCG
PDPN	10630	**F**: AATGTGGAAGGTGCCAGTTC
		R: TTCGTGGGGTCACTGTGTAA
SFRP2	6423	**F**: GGAAGAGGGACACTCATGGA
		R: TGATTGGAAAGGGAGCATTC

F forward primer, *R* reverse primer

Histological examination

Histological examination was performed on ovaries and separated follicles. For this purpose, 3 whole ovaries were collected, with a dozen follicles isolated from 2 ovaries. Immediately after collection, the organs were fixed in Bouin's solution for 48 h. Subsequently, ovaries and follicles were embedded in paraffin and then cut into 4 µm thick sections with a semi-automatic rotary microtome (Leica RM 2145, Leica Microsystems, Nussloch, Germany). Then, the sections were stained with routine hematoxylin and eosin (H&E) staining method, following the protocol of deparaffinization and rehydration, H&E staining and dehydration. Finally, histological sections were evaluated under light microscope and selected pictures were taken with the use of

high-resolution scanning technique and Olympus BX61VS microscope scanner (Olympus, Tokyo, Japan).

Results

Whole transcriptome profiling with Affymetrix microarrays allowed to analyze the granulosa gene expression changes at 48, 96 and 144 h of in vitro culture, with 0 h sample serving as an entry point reference. With the use of Affymetrix® Porcine Gene 1.1 ST Array Strip, the expression of 27,558 transcripts was examined. Genes with fold change higher than abs (2) and with corrected *p* value lower than 0.05 were considered as differentially expressed. This set of genes consists of 3380 different transcripts, the complete list of which can be found in the GEO database (ID: GSE134361).

Up and down-regulated gene sets were subjected to the Database for Annotation, Visualization and Integrated Discovery (DAVID) search separately and only ones with an adj. *p* value lower than 0.05 were selected. The DAVID software analysis showed that the differently expressed genes belonged to 344 GO BP Terms. In this paper we focused on "cell cycle checkpoint", "cell cycle G1/S phase transition", "cell cycle G2/M phase transition", "cell cycle phase transition", "cell cycle process", "cell cycle" and "cell division" GO BP terms. These sets of genes were subjected to a hierarchical clusterization procedure and presented as heatmaps (Fig. 1). The gene symbols, fold changes in expression, Entrez gene IDs and corrected *p* values of that genes are shown in Table 2.

To further investigate the changes within the chosen GO BP terms, we measured the enrichment levels of each selected GO BPs. The enrichment levels were expressed as *z*-scores and presented as circular visualizations (Fig. 2).

Chosen GO BP terms contained 133 differently expressed genes. Therefore, we have calculated the mean value of fold change ratio of each gene between 48, 96 and 144 h. Based on that criteria, we choose 10 most downregulated and 10 most upregulated genes for further analysis.

In the gene ontology database, genes that form one particular GO group can also belong to other different GO term categories. For this reason, we explore the gene intersections between the selected GO BP terms. The relation between those GO BP terms was presented as a circle plot (Fig. 3) as well as the heatmap (Fig. 4). Among the 20 chosen genes only 7 GO BP terms contain at least one gene from the chosen set.

A STRING interaction network was generated among chosen differentially expressed genes belonging to each of the selected GO BP terms. Using such a prediction method provided us with a molecular interaction network formed between the protein products of studied genes (Fig. 5).

Finally, the functional interactions between chosen genes were examined with the REACTOME FIViz app to Cytoscape 3.6.0 software. The results are shown in Fig. 6.

The results of the microarray analysis were validated with the RT-qPCR methods. The obtained values were compared between both approaches and presented as a bar graph (Fig. 7).

As can be seen in Fig. 7, the direction of changes of gene expression was confirmed in all of the analyzed examples. While the scale of the changes often varies, it is understandable due to different specificity, selectivity and sensitivity of both of the methods used, with RT-qPCR tending to be much more quantitatively accurate.

Histological analysis was performed to confirm that the ovaries taken to isolate granulosa cells show the proper structure, further proofing the identity of the analyzed cells. In addition, histological image analysis enabled the observation of GCs at various stages of follicular maturation, with particular focus on mature follicles.

Histological analysis revealed the proper structure of the collected ovaries. The organs are surrounded by tunica albuginea and the germinal epithelium. Follicles are present in the ovaries at all stages of development: numerous oocyte containing primordial follicles and one layer of flattened cells; primary follicles in which the oocyte is surrounded by 1 to many layers of granulosa cells; secondary follicles in which follicular fluid-filled space (antrum) appears between granulosa cells; and mature follicles, with an oocyte located at one of the follicle's poles and a large antrum surrounded by granulosa cells (Fig. 8).

Collected separated follicles varied in size. According to the diameter of follicles, they can be classified into 3 different groups: large follicles (> 5 mm), medium (3–5 mm) and small follicles (< 3 mm) (Fig. 9).

Granulosa cells, formed during the transformation of flat cells present in primary follicles, are observed at all other stages of follicular maturation. In the primary follicles, these cells form one to several layers surrounding the oocyte (Fig. 8d-7). In the next stage, their number increases, while a cavity (antrum) filled with fluid formed at the same time (Fig. 8f-7). In a mature follicle, granulosa cells accumulate on one of the poles of follicle, forming the cumulus oophorus and corona radiata around the oocyte, as well as surrounding a fully formed follicular antrum (Fig. 8h-7, 13, 14; Fig. 9a–c-2).

Discussion

Ovarian granulosa cells (GCs) play several roles in the female ovary (Jankowski et al. 2018). By contributing to the construction of the ovarian follicle wall, these cells enable forming of the fluid-filled cavity, which provides the oocyte with an environment for development. A direct link to the

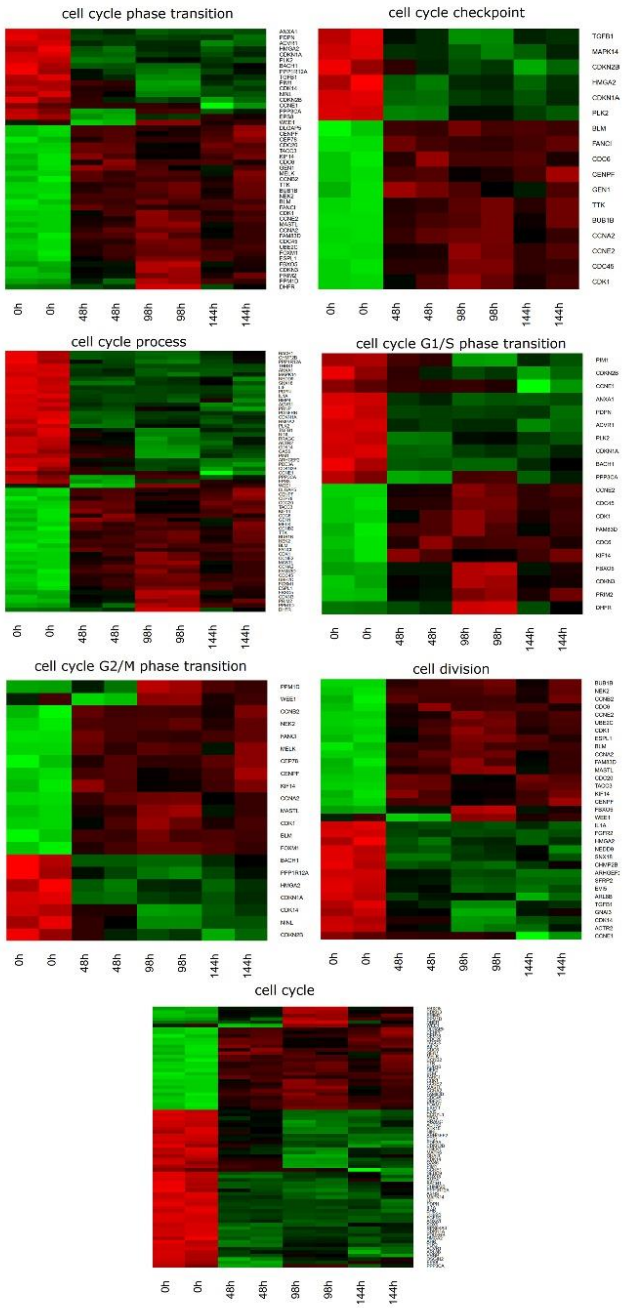


Fig. 1 Heat map representation of differentially expressed genes belonging to the "cell cycle checkpoint", "cell cycle G1/S phase transition", "cell cycle G2/M phase transition", "cell cycle phase transition", "cell cycle process", "cell cycle" and "cell division" GO BP terms. Arbitrary signal intensity acquired from microarray analysis is represented by colors (green, higher; red, lower expression). log2 signal intensity values for any single gene were resized to Row *z*-score scale (from −2, the lowest expression to +2, the highest expression for single gene)

Table 2 Fold rations and *p* values of differentially expressed genes of interest analysed in this study

Gene	Gene ID	Fold ratio 0H/48H	Fold ratio 0H/96H	Fold ratio 0H/144H	*p* value 0H/48H	*p* value 0H/96H	*p* value 0H/144H
CCNE2	9134	−1.192E+01	−3.423E+01	−1.777E+01	9.34E−06	9.04E−07	2.79E−06
RGS2	5997	−3.630E+00	−2.973E+01	−2.829E+01	1.15E−04	5.23E−07	6.54E−07
NUP210	23225	−6.899E+00	−2.075E+01	−1.881E+01	8.07E−04	3.78E−05	6.28E−05
TXNIP	10628	−2.702E+00	−3.410E+01	−4.530E+00	1.58E−03	1.12E−06	1.25E−04
PPAT	5471	−4.889E+00	−1.596E+01	−9.949E+00	5.19E−05	1.56E−06	5.23E−06
NEK2	4751	−6.253E+00	−6.790E+00	−6.013E+00	4.01E−05	1.64E−05	3.12E−05
H1FOO	132243	−5.181E+00	−5.013E+00	−6.962E+00	5.30E−06	2.60E−06	1.41E−06
CABLES1	91768	−5.840E+00	−4.871E+00	−6.439E+00	1.41E−05	1.21E−05	7.03E−06
NCAPD2	9918	−4.661E+00	−6.661E+00	−5.229E+00	1.93E−05	3.24E−06	8.81E−06
IGF1	3479	−8.344E+00	−5.532E+00	−2.555E+00	9.96E−05	1.72E−04	6.28E−03
TGFB1	7040	4.842E+00	1.036E+01	5.224E+00	3.48E−05	2.35E−06	1.86E−05
ANXA1	301	5.681E+00	7.665E+00	7.361E+00	5.56E−06	1.17E−06	1.63E−06
LIF	3976	8.345E+00	8.656E+00	1.079E+01	2.91E−05	1.36E−05	1.08E−05
ETS1	2113	5.431E+00	1.067E+01	1.223E+01	6.20E−06	6.41E−07	5.91E−07
THBS1	7057	9.549E+00	1.038E+01	9.767E+00	9.81E−07	3.32E−07	4.37E−07
PLK2	10769	1.894E+01	7.062E+00	1.203E+01	1.08E−06	3.21E−06	1.33E−06
FGFR2	2263	1.403E+01	1.521E+01	1.731E+01	1.48E−06	5.90E−07	5.70E−07
PDE3A	5139	6.473E+00	1.527E+01	3.278E+01	1.19E−05	1.02E−06	4.29E−07
PDPN	10630	1.899E+01	1.866E+01	2.240E+01	2.14E−05	1.11E−05	1.07E−05
SFRP2	6423	1.534E+01	3.079E+01	3.832E+01	9.81E−07	1.80E−07	1.50E−07

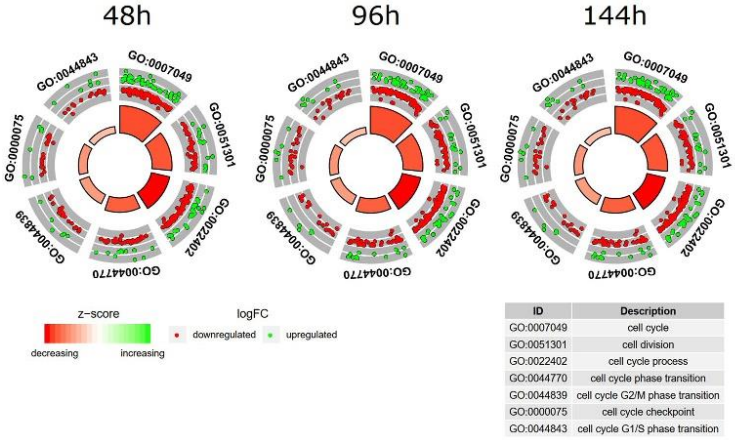
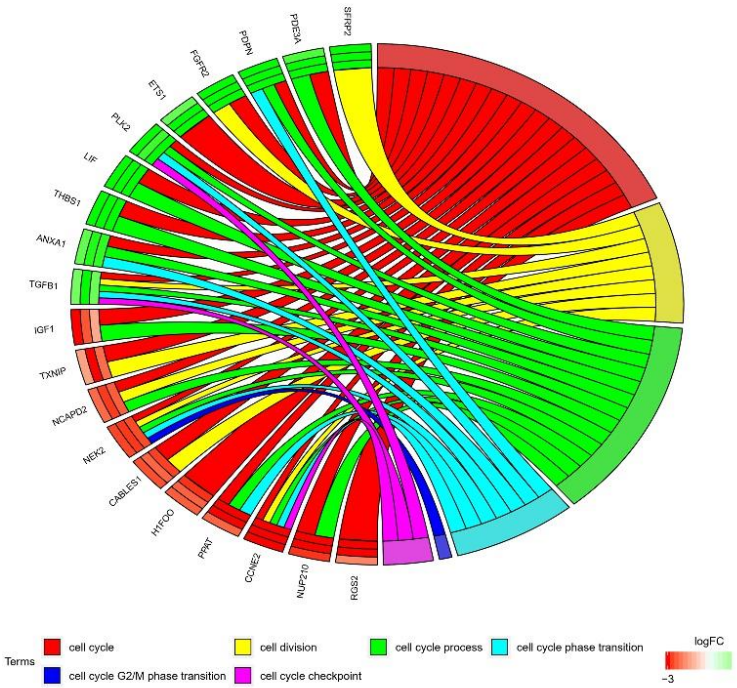


Fig. 2 The circular visualization of the results of gene-annotation enrichment analysis. The outer circle shows a scatter plot for each term of the logFC of the assigned genes. Green circles display up-regulation and red ones down-regulation. The inner circle is the representation of *z*-score. The size and the color of the bar correspond to the value of *z*-score

oocyte is established by the CCs, namely the corona radiata, through penetration of the zona pellucida using microvilli. GCs provide the oocyte with a supply of ions, nutrients and signal molecules through gap junctions (Kempisty et al. 2013). Another activity of GCs their participation in the synthesis and secretion of steroid sex hormones. After ovulation, GCs together with theca cells fill the interior of the follicle and form a corpus luteum, producing progesterone, the key hormone responsible for maintaining pregnancy. A schematic of the ovarian wall, outlining the features necessary for the distinct function of the granulosa cells is presented on Fig. 10. A broad range of GC properties indicates their great plasticity, which reinforces the belief that the molecular background of GCs processes in in vitro culture should be thoroughly studied. They are also cells of great differentiative potential towards lineages of largely different physiological characteristics e.g. osteoblasts (Mattioli et al. 2012). GCs are a rich source of cells used for research



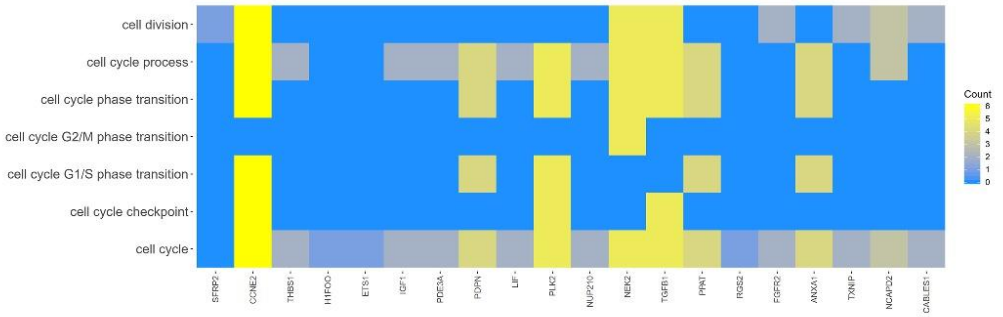


Fig. 3 The representation of the mutual relationship between 20 chosen genes that belongs to "cell cycle checkpoint", "cell cycle G1/S phase transition", "cell cycle G2/M phase transition", "cell cycle phase transition", "cell cycle process", "cell cycle" and "cell division" GO BP terms. The ribbons indicate which gene belongs to which categories. The colors of 3 inner bars near each gene corresponds to logFC after 48 h, 96 h and 144 h, respectively. The genes were sorted by logFC

Fig. 4 Heatmap showing the gene occurrence between 20 chosen genes that belongs "cell cycle checkpoint", "cell cycle G1/S phase transition", "cell cycle G2/M phase transition", "cell cycle phase transition", "cell cycle process", "cell cycle" and "cell division" GO BP terms. Yellow color indicates the gene occurrence in indicated GO BP term. The intensity of colour correlates with number of GO BP Terms that selected gene belongs to

on molecular backgrounds of the ovarian processes, which are the basis for mammalian reproduction. Granulosa can be divided into two types: mural GCs (building the wall of the follicle) and cumulus GCs (cumulus cells, CCs), which directly surround oocytes and form the *cumulus oophorus* (Rybska et al. 2018c).

The progression of the cell cycle is a sequence of successive phases in which cells perform planned actions necessary

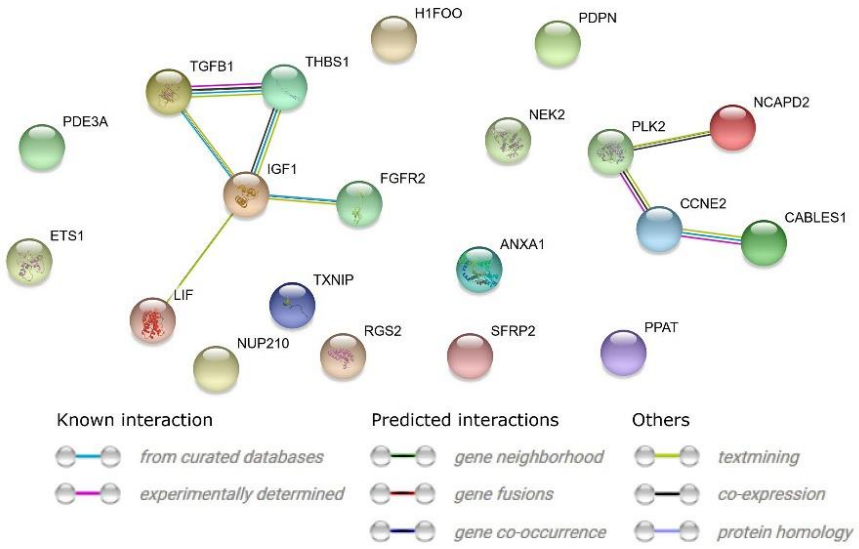


Fig. 5 STRING-generated interaction network among 20 chosen genes belonging to the "cell cycle checkpoint", "cell cycle G1/S phase transition", "cell cycle G2/M phase transition", "cell cycle phase transition", "cell cycle process", "cell cycle" and "cell division" GO BP terms. The intensity of the edges reflects the strength of interaction score

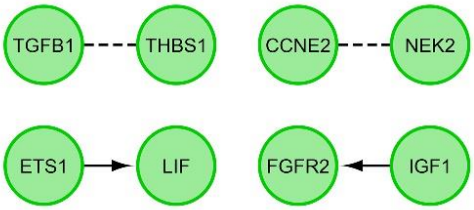


Fig. 6 Functional interaction (FI) between 20 chosen genes that belongs to "cell cycle checkpoint", "cell cycle G1/S phase transition", "cell cycle G2/M phase transition", "cell cycle phase transition", "cell cycle process", "cell cycle" and "cell division" GO BP terms. In the following figure "−>" stands for activating/catalyzing, "−|" for inhibition, "–" for FIs extracted from complexes or inputs, and "—" for predicted FIs

for proliferation. The checkpoint system is designed to prevent the cell from entering the next phase until the successful completion of events from the previous. Proliferation of most cells is regulated mainly in G1 phase, with some examples of its occurrence in phase G2. For example, oocytes (OC) can be retained in this phase for up to several decades until they are given the hormonal signals for further division. This is related to the oocyte development process called oogenesis. During this process, during nuclear and cytoplasmic maturation, proteins and mRNAs necessary for further development are accumulated (Budna et al. 2018).

The current study focused on measuring the level of porcine granulosa cell gene expression at the start of their primary culture (0 h) and after 48 h, 96 h and 144 h of its course. We have focused on genes belonging to seven ontological groups: "cell cycle", "cell division", "cell cycle process", "cell cycle phase transition", "cell cycle G1/S phase transition", "cell cycle G2/M phase transition" and "cell cycle checkpoint". Out of 133 differentially expressed genes from these GOs, we chose the 10 most up-regulated (*SFRP2*, *PDPN*, *PDE3A*, *FGFR2*, *PLK2*, *THBS1*, *ETS1*, *LIF*, *ANXA1*, *TGFB1*) and the 10 most downregulated (*IGF1*, *NCAPD2*, *CABLES1*, *H1FOO*, *NEK2*, *PPAT*, *TXNIP*, *NUP210*, *RGS2* and *CCNE2*). Almost all of the genes were members of the "cell cycle" ontological group, with the exception of the most upregulated *SFRP2*. The "cell cycle" ontological group includes the genes responsible for the progression of all biochemical and morphological events taking place in replicating cells. "Cell division" is defined as the process in which cell components are divided and partitioned to produce more cells. It is not synonymous with the term 'cytokinesis', which does not include the process of nuclear division. The "cell cycle process", on the other hand, refers to a process that ensures precise and complete genome replication and chromosome segregation. "Cell cycle phase

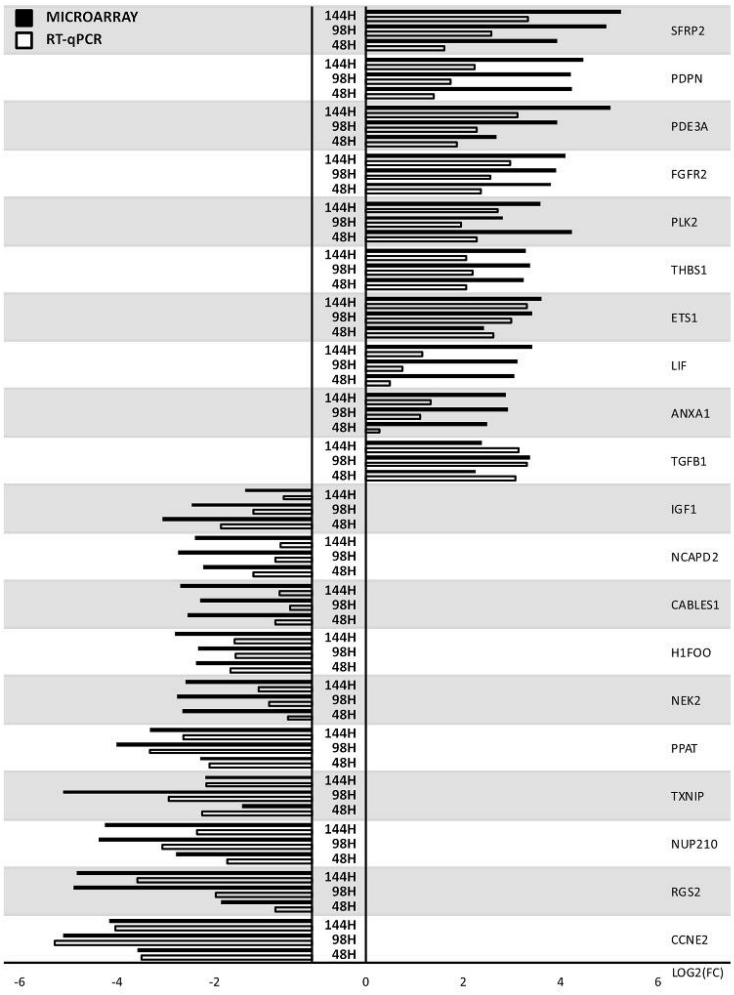


Fig. 7 The results of RT-qPCR validation of microarray results presented in the form of a bar graph

transition" references the process of cellular transition between the phases of the cell cycle. Genes participating in cell transition from G1 to S and from G2 to M are contained in the "cell cycle G1/S phase transition" and "cell cycle G2/M phase transition" GOs, respectively. The "cell cycle checkpoint" ontological group consists of genes controlling the cycle progression by monitoring the integrity of the cell after each of its phases. Their actions include detection of damage or lack of damage, often leading to downstream signal transduction.

The most up-regulated gene was *SFRP2* (*secreted frizzled-related protein 2*), which exclusively belongs to the "cell division" ontological group. It is a member of the secreted frizzled-related protein (SFRP) family, the antagonists of the WNT signaling pathways (Zamberlam et al. 2019). The WNT signaling affects the development of follicles, proliferation and differentiation of granulosa cells. This gene is commonly expressed in ovarian follicle granulosa (Hernandez-Gonzalez et al. 2006; Ekart et al. 2013). Its expression may predispose this gene to become a marker of cell division in GC in vitro culture. Another gene expressing significant up-regulation in "cell cycle", "cell cycle process" and "cell cycle phase transition ontology groups was *PDPN* (*podoplanin*), encoding a type I integral membrane

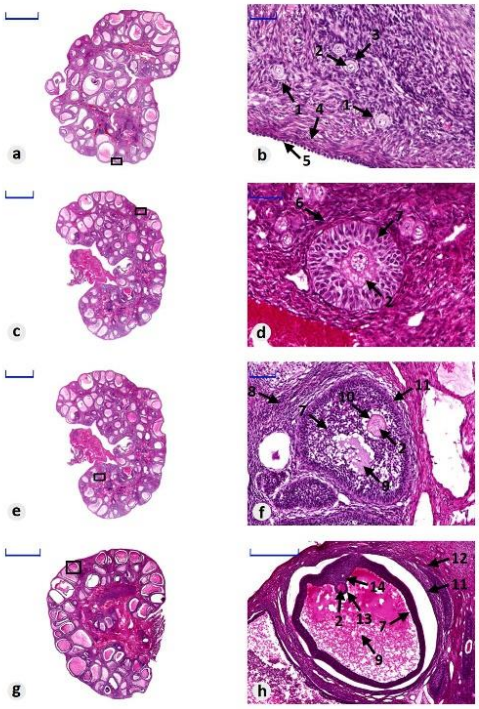


Fig. 8 Mid-part histological sections of crossbred Landrace gilts ovaries, stained with H&E, representing their structure and follicles in all stages of development. **a**, **c**, **e**, **g** Whole ovaries (scale bars: 5000 µm), **b**, **d**, **f**, **h** selected areas of **a**, **c**, **e**, **g** observed in higher magnification (scale bars: B, D-50 µm, F-100 µm, H-500 µm). Arrows: 1—primordial follicles, 2—oocyte, 3—follicular cells, 4—tunica albuginea, 5—germinal epithelium, 6—primary follicle, 7—granulosa cells, 8—secondary follicle, 9—antrum, 10—zona pellucida, 11—theca interna and theca externa, 12—mature follicle, 13—corona radiata, 14—cumulus oophorus

glycoprotein. Previous publications indicate its involvement in cancer-associated angiogenesis (Shindo et al. 2013). Increased expression of this gene was also demonstrated in porcine GCs (Chermuła et al. 2019). We have also observed up-regulation of *PDE3A* (*phosphodiesterase 3A*), which belongs to the "cell cycle" and "cell cycle process" GOs. Studies on in vitro matured porcine oocytes showed that PDE3A is the main PDE degrader of cAMP in oocytes. Specific inhibition of cAMP degradation by PDE3 prevents the resumption of oocyte meiosis (Sasseville et al. 2006). It also has an important role during the ovulatory gonadotropin surge (Sasseville et al. 2007). The *FGFR2* gene (*fibroblast growth factor receptor 2*), belonging to the "cell cycle" and "cell division" GOs, showed significant up-regulation during primary in vitro culture of the GCs. FGFR is a protein activated by IGF1. Its relationships with the reproductive system were indicated, among others, in the porcine umbilical cord during pregnancy (Chrusciel et al. 2011), swine endometrium (Wollenhaupt et al. 2004, 2005), swine fallopian tubes (Wollenhaupt et al. 2004) and porcine GCs (Chermuła et al. 2019). Expression of *PLK2* gene (*polo-like kinase 2*) was recorded in bovine GCs and theca cells and was recognized as a possible new genetic marker (Hatzirodos et al. 2015). Studies on rat ovaries have shown that *PLK2* is strongly involved in cell cycle processes. Excessive expression of this gene was shown to retain GCs in the G0/G1 phase (Li et al. 2012). The *PLK2* gene plays a role in normal cell division, with its expression also observed in swine buccal mucosa cells, indicating it as a marker of processes associated with that tissue (Dyszkiewicz-Konwińska et al. 2018). In our research, this gene has been shown to belong to the "cell cycle", "cell cycle process", "cell cycle phase transition" and "cell cycle checkpoint" ontology groups. This data indicates that PLK2 gene is a candidate marker of the cell cycle driving processes, with particular participation in checkpoint control. Another up-regulated gene is *THBS1* (*thrombospondin 1*), the increased expression of which was recorded during follicular atresia in swine GCs (Terenina et al. 2017). *ETS1* (*ETS proto-oncogene 1*) is a transcription factor

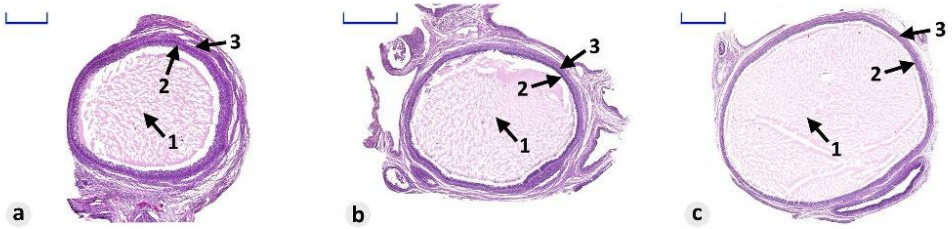


Fig. 9 Microphotograph representing separated mature follicles classified into 3 groups according to their size (H&E staining). **a** Small follicle (<3 mm; scale bar—500 µm), **b** medium follicle (3–5 mm; scale bar—1000 µm), **c** large follicles (> 5 mm; scale bar—1000 µm). Arrows: 1—antrum, 2—granulosa cells, 3—theca interna and theca externa

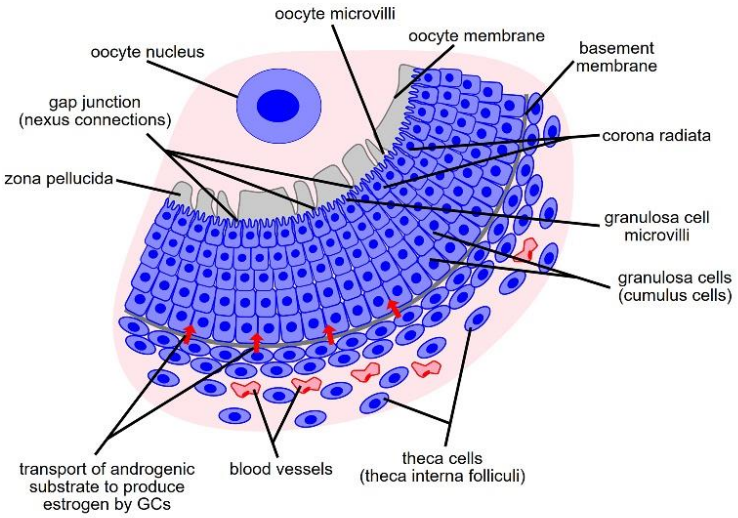


Fig. 10 A diagram of a cross-section of the ovarian follicle, outlining its layout and features enabling functions of the distinct granulosa types

involved in the regulation of extracellular matrix reconstruction (Garrett-Sinha 2013). Up-regulation of this gene has been observed in various human cancers, as well as the culture of porcine buccal mucosa cells (Dyszkiewicz-Konwińska et al. 2018). *LIF* (*leukaemia inhibitory factor interleukin 6 family cytokine*) plays an important role in normal cell cycle development, especially through its participation in checkpoint control. This gene was studied using porcine induced pluripotent stem cells (iPSC) and in vitro matured metaphase II oocytes (Yuan et al. 2014). Other studies analyzed the effect of porcine leukemia recombinant inhibitory factor (pLIF) on in vitro maturation of oocytes (Dang-Nguyen et al. 2014). A significant increase in the rate of maturation of oocytes in cumulus-oocyte complexes cultured with the addition of pLIF was observed, with lack of effect with the same protein was added after the removal of cumulus cells (Dang-Nguyen et al. 2014). LIF may play a role in the maturation of oocytes through regulation of the cell cycle predisposing LIF to become a genetic marker of GC in vitro culture. *ANXA1* (*annexin A1*) gene, encoding a membrane phospholipid-binding protein also exhibited upregulation along the time of culture and has a role in inhibiting phospholipase A2, as well as an anti-inflammatory effect. In humans, this gene has been shown to be associated with various types of cancer (Pessolano et al. 2018). Apoptosis of in vitro cultured swine kidney cells was observed after the addition of TNF-alpha, associated with the transfer of *ANXA1* to or around the nucleus (Ishido 2005). *TGFB1* (*transforming growth factor beta 1*) is a gene involved in many cellular processes, including tissue repair, inflammatory cell chemoattraction and angiogenesis, also known as a mediator of fibroblast–myofibroblast differentiation (Dyszkiewicz-Konwińska et al. 2018). In our study, it showed up-regulation in the "cell cycle", "cell cycle process", "cell cycle phase transition", "cell cycle checkpoint" and "cell division" ontology groups. The most down-regulated gene was *CCNE2* (*cyclin E2*), which, as shown in studies on bovine GCs, is associated with proliferation of granulosa cells in particular stages of folliculogenesis (Shimizu et al. 2013). This gene is one of the key regulators of the cell cycle, with its expression recorded in mouse GCs (Meinsohn et al. 2018). The *CCNE2* gene belongs to the same ontological groups as the *TGFB1* gene described above. The regulator of G protein signaling 2 is a protein encoded by the *RGS2* gene, a known marker of GC luteinization (Kranc et al. 2015). In our research, we noted a significant down-regulation of this gene in the "cell cycle" GO. *NUP210* (*nucleoporin 210*) is a gene encoding one of the building blocks of nuclear pores, allowing the transport of molecules between the nucleus and cytoplasm. Recently, the role of this gene as a critical regulator of the muscle and neuron differentiation process has also been described (Gomez-Cavazos and Hetzer 2015). To the best of our knowledge, the expression of this gene in the cells of the porcine ovary has not yet been described. However, its expression in granulosa cells and bovine theca cells has been noted (Hatzirodos et al. 2015). In our study, *NUP210* showed downregulation in the "cell cycle" and "cell cycle process" groups. The *TXNIP* gene (*thioredoxin interacting protein*), belonging to the "cell cycle" and "cell division" groups, was downregulated in our research. The expression of this gene was observed in bovine cumulus cells (Salhab et al. 2013), porcine oocytes

(Ożegowska et al. 2018), CCs (Borys et al. 2018) and porcine oviductal epithelial cells (Kulus et al. 2019a, b). It is responsible for regulating the response to oxidative stress but also contributes to glucose metabolism and lactate production. It was shown that this gene is important in the meiotic maturation process of mouse oocytes (Lee et al. 2013). Analysis of gene expression during GC short-term primary in vitro culture, also revealed a downregulation of the *PPAT* gene (*phosphoribosyl pyrophosphate amidotransferase*), which encodes a member of the family of phosphorus-pyramid transfer proteins. PPAT in cattle was closely related to the *PAICS* gene, with its location mapped to BTA6 (Bønsdorff et al. 2004). Among the analyzed genes, the only representative of the "cell cycle G2/M phase transition" ontological group was the *NEK2* (*NIMA related kinase 2*) gene. This gene also belonged to other GOs, with its downregulation observed during the culture. Earlier studies indicate its active participation in the start and progression of metaphase II in in vitro maturated porcine oocytes (Fujioka et al. 2000) suggesting potential uses of this gene as a marker of G2/M cycle phase transition in in vitro cumulus–oocyte complexes. The *H1FOO* gene (*oocyte-specific H1 histone*), belonging to only one ontological group ("cell cycle"), showed downregulation in our studies. The expression of this gene is restricted to the ovary. Sheng and co-authors studied the localization of *H1FOO* in pig ovaries at different stages of postpartum (Sheng et al. 2015). The studies showed its differential presence in oocytes depending on the stage of follicle development. In GCs, weak *H1FOO* expression was observed in primordial follicles, with a moderate increase in early growing follicles, developing antral follicles and antral follicles only after 72dpp and 95dpp, respectively (Sheng et al. 2015). These studies suggest that GCs may influence oocyte development through the *H1FOO* signalling. In the current study, GCs were cultured without oocytes, which could possibly explain the downregulation of this gene. Such a close link between this gene and the ovary, as the only place of its expression and function, may predispose it as an in vitro genetic marker of ovarian cells. *CABLES1* (*Cdk5 and Abl enzyme–substrate 1*) is a gene closely related to the regulation of the cell cycle (Lee et al. 2007). Its downregulation has been recorded in "cell cycle" and "cell division" groups. The loss of *CABLES1* expression was associated with the presence of ovarian cancer in humans, with excessive expression of this gene leading to cancer cell apoptosis (Sakamoto et al. 2008). These findings prove the function of this gene as a suppressor of ovarian cancer in humans. In our studies, *NCAPD2* (*non SMC condensin I complex subunit D2*) showed downregulation, in "cell cycle", "cell cycle process" and "cell division" GOs. This gene plays a role in the condensation of chromatin preceding cell division (Martin et al. 2016). Its expression was recorded in the ovary during studies on the resistance of human ovarian cancer cells to cisplatin (Solár and Sytkowski 2011). It was also found that mutations within this gene may have a significant effect on oncogenic processes in the mouse ovary (Emmanuel et al. 2011). The last downregulated gene was *IGF1* (*insulin-like growth factor 1*). It is associated with such important processes as cell growth, prevention of apoptosis and cell proliferation. It has been proven that it interacts with the FSH and LH hormones, influencing the proliferation of GCs (Filus and Zdrojewicz 2014). It is also believed that it increases the influence of gonadotropins on ovarian steroidogenesis (Kranc et al. 2017). Experiments on bovine and porcine ovaries have shown that IGF1 was one of the factors limiting or promoting multiple ovulation (Sirotkin et al. 2017).

Mutual correlations of the analyzed genes were analyzed using STRING-generated interaction network. Most genes (11) did not show a direct correlation with others. However, it is impossible to exclude indirect mutual regulation.

Genes whose expression in GCs was deemed to be of importance for the processes of cell cycle progression were revealed in the gene analysis. Several of these genes were earlier associated with the reproductive system, with some described for the first time in this context. The comparison of the results of this study with the research mentioned above may be the basis for the consolidation of some data on the expression of the genes of interest in the given context. However, the presence of potential new gene markers may provide a new point of reference for subsequent research. In conclusion, significant changes in the expression of the described genes and their functional participation in cell cycle processes indicate their potential role as markers of primary porcine granulosa cell in vitro cultures.

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Author contributions MK conducting of the experiments, choice of models, experimental design, preparation of part of the medical methodology, writing of manuscript parts—original draft preparation, conceptualization; WK writing of manuscript parts, preparation of RNA isolation protocol, validation; PS-K data curation, investigation, methodology; PC data analysis, figure preparation, and writing part of result manuscript parts; AK histochemical reaction and assays; MJa data analysis, and language corrections; MJe data curation, funding acquisition; MTS adjustment of graphic material, revision of the manuscript; HP-K software; experimental design and models analysis; DB funding acquisition, resources; MZ editorial assistance, histological evaluation and assays; MB funding acquisition, supervision; PM funding acquisition, supervision; BK Project supervision, Project design, revision of methodology, editorial supervision, senior author, and major assistance; PA Project supervision, Project design. All authors approved the final article.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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5. Streszczenie

Komórki ziarniste, budujące pęcherzyk jajnikowy, są ściśle zaangażowane w procesy folikulogenezy i oogenezy, a poprzez wzajemne oddziaływania z oocytem i pozostałymi komórkami pęcherzyka jajnikowego oraz osłonki pęcherzykowej odgrywają zasadniczą rolę w fizjologii jajnika i całego układu rozrodczego. Procesy zachodzące w gonadzie żeńskiej są ogólnie dobrze poznane, jednak ich molekularne podłoże wymaga dokładniejszych badań. Mnogość interakcji pomiędzy komórkami jajnika czyni te procesy wysoce skomplikowanymi oraz zależnymi od swoistego „dialogu" międzykomórkowego. W ostatnich latach odkryto, iż komórki ziarniste wykazują zdolność do wielokierunkowego różnicowania się, co jest wyrazem ich potencjału macierzystości. Wiedza ta stwarza nowe możliwości wykorzystania tych komórek w medycynie weterynaryjnej, ale także ludzkiej. Dokładna analiza mechanizmów regulacji tych procesów jest niezbędna do dalszych etapów badań. Ponadto, uzyskane dane stanowić mogą uzupełnienie podstawowej wiedzy dotyczącej procesów zachodzących w jajniku. W niniejszej pracy dokonano analizy profilu ekspresji wybranych genów regulujących procesy wzrostu, różnicowania i apoptozy w komórkach ziarnistych świń podczas ich krótkoterminowej pierwotnej hodowli *in vitro*. Dzięki zastosowaniu metody mikromacierzy ekspresyjnych analizie poddano transkryptom komórek z poszczególnych przedziałów czasowych (0 h, 48 h, 96 h oraz 144 h) i wybrano geny o największym zróżnicowaniu poziomu ich ekspresji. W dalszej kolejności wyodrębniono geny pochodzące z grup ontologicznych związanych z rozwojem, różnicowaniem się i morfogenezą komórek, a także geny biorące udział we wzroście, starzeniu się i apoptozie oraz geny regulujące cykl komórkowy. W opublikowanych wynikach pierwszego etapu badań wykazano, że spośród 20 analizowanych genów aż 19 należy do grupy ontologicznej „*cell differentiation*", których ekspresja w komórkach ziarnistych może potwierdzać ich zdolności do różnicowania się w inne typy komórek. Dodatkowo, wyróżniono geny, których rola w folikulogenezie nie została do tej pory określona, chociaż wcześniejsze badania wskazują na ich ekspresję w świńskich komórkach ziarnistych. Mogą one zatem kandydować do stania się markerami genetycznymi ważnych procesów zachodzących w obrębie komórek ziarnistych w warunkach hodowli *in vitro*. Należy zwrócić uwagę, że przedstawione wyniki wskazują także kilka genów, które wykazały związek z procesami apoptozy i atrezji pęcherzyka jajnikowego, a wyraźna ekspresja genów z grup ontologicznych odpowiedzialnych za zaprogramowaną śmierć komórki potwierdza ich wpływ na przebieg tych procesów. Ostatni etap badań

poświęcony został analizie poziomu ekspresji genów z grup ontologicznych związanych z regulacją cyklu komórkowego.

Podsumowując, uzyskane dane ilustrujące aktywność molekularnego podłoża wyżej wymienionych procesów stanowią uzupełnienie dotychczasowej wiedzy o mechanizmach regulacyjnych i ścieżkach sygnałowych w obrębie pęcherzyka jajnikowego. Dodatkowo, niedawno odkryte macierzyste właściwości komórek ziarnistych, częściowo sugerowane danymi uzyskanymi w niniejszej pracy, mogą wskazać nowe kierunki badań i możliwości wykorzystania tych komórek w nowoczesnej medycynie regeneracyjnej.

6. Summary

Granulosa cells, forming the ovarian follicle, are closely involved in the folliculogenesis and oogenesis processes. By interacting with the oocyte and other ovarian follicle cells, they are essential for the physiology of the ovary and the entire reproductive system. The processes taking place in the female gonad are generally well known, but their molecular background requires more detailed research. The multiple interactions between ovarian cells make these processes highly sophisticated and dependent on a specific intercellular "dialogue". In recent years, it has been discovered that granulosa cells show stem-like potential and can differentiate towards other cell lineages. This knowledge presents new possibilities of using these cells in veterinary, as well as in human medicine. A thorough analysis of the regulation mechanisms of these processes is necessary for further stages of research. Furthermore, the data obtained may complement the basic knowledge of the processes taking place in the ovary. In this paper, expression profile analysis of selected genes regulating growth, differentiation, and apoptotic processes in porcine granulosa cells during their short-term primary *in vitro* culture was performed. Thanks to the use of expressive microarrays, transcripts of cells from particular time intervals (0 h, 48 h, 96 h and 144 h) were analyzed, and genes with the highest differential expression levels were selected. Then, we have further extracted genes coming from ontological groups related to cell development, differentiation and morphogenesis, as well as genes participating in growth, aging and apoptosis, and cell cycle regulation. The published results of the first stage of the study showed that, out of the 20 analyzed genes, as many as 19 belong to the "*cell differentiation*" ontological group, expression of which in granulosa cells may confirm their ability to differentiate into other cell types. Additionally, genes with no apparent role in folliculogenesis have also been identified, although previous studies indicate their expression in porcine granulosa cells. Therefore, these genes may be candidates for genetic markers of critical processes occurring within granulosa cells under *in vitro* culture conditions. It should be noted that the presented results also indicate several genes that have shown a connection with apoptosis and follicular atresia, and the significant expression of genes from ontological groups responsible for programmed cell death confirms their influence on the course of these processes. The last stage of the study was devoted to the analysis of the level of expression of genes from ontological groups associated with cell cycle regulation.

To conclude, the obtained data referring to the molecular background of the processes mentioned above may complement the existing knowledge about the regulatory mechanisms and signal pathways within the ovarian follicle. Additionally, recently discovered stem-like

properties of granulosa cells, partially confirmed in this paper, may indicate new research directions and possibilities of their use in modern regenerative medicine.