Zygote **25** (October), pp. 583–591. © Cambridge University Press 2017
doi:10.1017/S0967199417000429 First Published Online 08 August 2017

Relationship between apoptosis and survival molecules in human cumulus cells as markers of oocyte competence

Liana Bosco[1], *Roberto Chiarelli*[2], *Maria Carmela Roccheri*[2], *Domenica Matranga*[3] *and Giovanni Ruvolo*[4]

Università degli Studi di Palermo, STEBICEF; Università degli Studi di Palermo, Scienze per la Promozione della Salute e Materno Infantile 'G.D' Alessandro'; and Centro di Biologia della Riproduzione, Palermo, Italy

Date submitted: 06.04.2017. Date revised: 06.04.2017. Date accepted: 28.06.2017

Summary

To select from a single patient the best oocytes able to reach the blastocyst stage, we searched for valuable markers for oocytes competence. We evaluated the DNA fragmentation index (DFI) and the level of some survival molecules, such as AKT, pAKT and pERK1/2, in individual cumulus cell–oocyte complexes (COC). The study included normo-responder women. The average age of the patients was 34.3. DFI in cumulus cells was evaluated using the terminal deoxynucleotidyl transferase (TdT) dUTP nick-end labelling (TUNEL) assay *in situ*. AKT, pAKT and pERK1/2 were measured by immunological assay and densitometric analysis of fluorescent signals using NIS-Elements BR 3.10 image software. Statistical analysis was performed using STATA SE/14.1. The study focused on 53 patients involved after informed consent. Out of 255 MII oocytes, 197 were fertilized and the derived embryos had the following evolution: 117 completed the development to blastocyst and were transferred to uterus; 57 were vitrified at the blastocyst stage; and 23 were arrested during *in vitro* culture at different stages of cleavage. We found a significant statistical difference between the DFI of cumulus cells of the arrested embryos and the transferred blastocysts ($P = 0.004$), confirming that DFI could be considered as a valuable marker of oocyte competence. In addition, the pAKT/DFI ratio was higher in cumulus cells of oocytes able to produce blastocysts, indicating that DFI is significantly lower when pAKT is higher ($P = 0.043$). This study demonstrates for the first time that the relationship between apoptosis and survival molecules can be used as a marker to select the best oocytes.

Keywords: Apoptosis, Human cumulus cells, Molecular markers, Oocyte competence, Survival molecules

Introduction

About 15% of couples have fertility issues during their reproductive years (World Health Organization, 1983). *In vitro* fertilization (IVF) has the highest success rates of pregnancy and live-birth outcomes among treatments available for infertile couples, even if the more conventional methods of embryo selection are still associated with a relatively low success rate, with a clinical pregnancy rate (PR) of 30% per transfer (Andersen *et al*., 2008). The assessment of oocyte and embryo competence in IVF treatment is critical to determine which one embryo to be transferred is the most viable. To date, the evaluation of embryo competence uses morpho-kinetics information (Herrero & Meseguer, 2013). The transfer of two or three embryos is a common practice that aims to increase pregnancy probability but can increase the risk of multiple gestations, resulting in preterm birth and its complications, such as cerebral palsy and infant death (Strömberg *et al*., 2002). In the field of assisted

[1]All correspondence to: Liana Bosco. Università degli Studi di Palermo, Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche (STEBICEF), Viale delle Scienze, Ed.16, 90128 Palermo, Italy. Tel: +39 09123897411. Fax: +39 0916577210. E-mail: liana.bosco@unipa.it
[2]Università degli Studi di Palermo, Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche (STEBICEF), Viale delle Scienze, Ed.16, 90128 Palermo, Italy.
[3]Università degli Studi di Palermo, Scienze per la Promozione della Salute e Materno Infantile 'G.D'Alessandro', Via del Vespro, 133, 90127 Palermo, Italy.
[4]Centro di Biologia della Riproduzione, via V. Villareale, 54, 90141 Palermo, Italy.

reproduction, the selection of embryos with a high implantation potential remains one of the major goals, so that only one embryo needs to be transferred. One of the most significant research goals is still the development of non-invasive, objective and accurate testing to assess oocyte and embryo viability. Some countries have ethical and legal limitations that restrict research on embryos. During the assisted reproduction treatment (ART) cycle, the selection of a pool of oocytes with the highest potential to develop into a viable blastocyst and subsequently a healthy baby is one of the major goals of gamete competence research.

The fertility potential of individual oocytes has been evaluated with non-invasive approaches in ART cycles, without compromising oocyte integrity (Varras *et al*., 2012). The easy availability of cumulus and granulosa cells gives the opportunity to perform studies, as these cells are always discarded before intracytoplasmic sperm injection (ICSI) procedures. A very important role is played by cumulus cells through gap junctions, which establish a physical connection between cumulus cells and the oocyte. As a consequence of this close molecular dialogue, cumulus cells may reflect oocyte quality, when considering cumulus DNA fragmentation or apoptosis as a marker of competence (Bosco *et al*., 2015). Physiologically, apoptosis occurs in the male and female germ lines during maturation of gametes, to eliminate germinal cells that carry genetic or chromosomal abnormalities through macrophage activity (Bosco *et al*., 2005; Ruvolo *et al*., 2013a,b).

The negative effect of apoptosis of granulosa cells is supported by several data on IVF outcomes (Nakahara *et al*., 1997; Oosterhuis *et al*., 1998; Jancar *et al*., 2007). This hypothesis has been sustained by studies (Oosterhuis *et al*., 1998) carried out on granulosa cells from women who have become pregnant after IVF and embryo transfer (IVF-ET). A higher incidence of apoptotic cells in the granulosa is associated with a higher incidence of empty follicles, fewer oocytes retrieved, low rate of fertilization and poor quality of oocytes and embryos (Nakahara *et al*., 1997, 1998).

Specific metabolic pathways activated by gonadotropins and by a number of growth factors support the survival of somatic cells of follicles to reach ovulation and prevent apoptosis (Quirk *et al*., 2004). Clinical outcomes in ICSI cycles have been improved by specific ovarian stimulation treatment, reducing apoptotic rate in cumulus cells and by supporting oocyte competence (Ruvolo *et al*., 2007).

Several studies have been published on gene expression profiles of cumulus and granulosa cells that could provide an insight into cellular health and viability, as well as into oocyte competence, also expressed in terms of chromosomal normalcy (Pacella *et al*., 2012).

The normal physiology of multicellular organisms relies on the regulation of cell survival. Cell death suppression induction is a key process in the development and homeostasis of almost all organisms, so perturbing the death or survival mechanisms that may lead to a large number of disease processes (Datta *et al*., 1997).

The central role of some molecules that support the survival pathway, exerting an anti-apoptotic action, has been confirmed by several studies. Specifically, it has been shown that pERK1/2 and pAKT are certainly involved in the control of apoptosis.

Recent studies have indicated that ERK1/2 (also known as MAPK3/1) is an essential mediator by which luteinizing hormone (LH) dictates the remarkable changes in follicular cell fate during ovulation and luteinization.

Promotion of cell death by ERK activation may result from the suppression of survival signalling pathways. Many growth and survival factors are able to activate the AKT pathway that plays a key role in the regulation of cell survival (Amaravadi & Thompson, 2005). It is still unclear how ERK1/2 inhibits AKT. The coexistence of ERK1/2 and AKT has been reported in a multimolecular complex containing at least ERK1/2, AKT, ribosomal S6 kinase 1, and phosphoinositide-dependent kinase 1 (Sinha *et al*., 2004).

Although ERK has generally been considered as a survival signalling pathway, there is now clear evidence indicating that the ERK pathway mediates apoptosis induced by different stimuli and in different tissues.

AKT has a key role in mediating growth factor–induced survival and suppressing the apoptotic death of various cell types induced by a variety of stimuli, including growth factor withdrawal, cell-cycle discordance, loss of cell adhesion and DNA damage (Kauffmann-Zeh *et al*.,1997; Sinha *et al*., 2004).

pAKT, the active form of AKT, regulates apoptosis by a direct mechanism linked to events resulting in the phosphorylation and inactivation of pro-apoptotic factors, such as Bad and pro-caspase-9 (Thompson & Thompson, 2004). The regulation of expression and phosphorylation of AKT in each stage of follicle development is required for the proliferation of granulosa cells (Goto *et al*., 2007). Although it is known that AKT regulates cell survival in many cell types, in human cumulus cells this role is not completely understood.

The aim of this study was to investigate the relationship between apoptosis of cumulus cells and AKT, pAKT and pERK1/2 molecules, by comparing embryos developed to blastocyst stage with embryos arrested during *in vitro* culture. To this purpose, the DFI of individual cumulus cell–oocyte complexes (COC), associated with the level of markers of oocyte

https://doi.org/10.1017/S0967199417000429 Published online by Cambridge University Press

competence (AKT, pAKT and pERK1/2), in patients undergoing ICSI procedures was studied.

Materials and methods

This prospective observational study was performed from September 2012 until September 2014 on 53 patients that were included in a programme of blastocyst embryo transfer at the Centro di Biologia della Riproduzione, Palermo, Italy (ethical approval on 27 June 2012 by Centro di Biologia della Riproduzione). Patients signed an informed consent form, which included both the agreement for the study participation and for the possible use of any discarded cumulus cells for apoptosis rate assessment.

The patients were normo-responder patients with a normal basic level of FSH (<12 IU/ml), <28 body mass index (kg/m^2) (average 22 ± 3.18), <38 years old [average 34.3 ± standard deviation (SD) 3.5] and treated with a GnRH agonist (Buserelin, Suprefact, Sanofi-Aventis, Italy, 0.2 ml/day). The patients were excluded in the case of azoospermia or severe oligoasthenospermic patients (motile sperm count less than 0.5 million/ml).

The patients were stimulated with r-hFSH (150IU). The stimulation therapy continued with a fixed daily dose of 225 IU of r-hFSH (Gonal-F; Serono Pharma, Rome, Italy). Follicular growth was monitored daily using ultrasound and serum estradiol E2 levels starting on day 6 of stimulation, modifying the dose of r-hFSH as a consequence. At day 8 randomization was carried out, only if patients presented a value of serous E2 <180 pg/ml, and if the ovarian condition showed at least six follicles with diameters ranging between 7–10 mm and no follicle with a diameter >12 mm. The ovulatory dose of 10,000 IU of hCG (Gonasi; AMSA SRL, Rome, Italy) was administered when at least three or four follicles presented a diameter ≥18 mm. Only metaphase II (MII) oocytes were fertilized by ICSI. Sequential media (VitroLife, IVF, G1 and G2 media, Sweden) were used for blastocyst culture. Only 5–6-day-old blastocysts were transferred or vitrified (Kitazato, Spain) while embryos arrested at different cleavage stages were discarded after 8 days of culture.

Apoptosis in cumulus cells was examined using the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. The level of pAKT protein, was assessed by *in situ* immunofluorescence assay, as described below.

Cumulus cell preparation

After hyaluronidase treatment, about 800 cumulus cells were obtained from individual COC. These cells were collected in HEPES-buffered medium and centrifuged twice for 7 min at 800 rpm and then fixed in 3.7% paraformaldehyde for 1 h. Cumulus cells were centrifuged for 7 min at 2000 rpm, the supernatant was removed and phosphate-buffered saline (PBS) was added. The cytospin method was used to mount cells on polylysine-coated glass slides to start *in situ* immunocytochemistry experiments and the TUNEL assay.

Fluorescent *in situ* TUNEL assay

As described by Ruvolo *et al*. (2007), and using the limited amount of cumulus cells available from a single COC (400 cells/sample were analyzed), we applied *in situ* methods for DNA fragmentation rate. Cumulus cells were washed in PBS and permeabilized on ice in 0.1% Triton X-100 and 0.1% sodium citrate in PBS, then washed three times in PBS at room temperature. Cumulus cells were then incubated for 60 min at 37°C in a humidified chamber in 50 µl of a mixture containing 5 µl of nucleotide mix, 1 µl of TdT enzyme, and 45 µl of equilibration buffer (DeadEndFluorometric TUNEL System, Promega Italia SRL, Milano, Italy).

An additional slide was incubated with the same mixture without the TdT enzyme (negative control), while another slide was treated briefly with a DNase buffer solution containing 10 unit/ml of DNase I (positive control). The reaction was blocked with saline sodium citrate (SSC), then washed three times in PBS. Cumulus cells were stained with propidium iodide (1 µg/ml) and observed under an Olympus BX 50 microscope equipped with a reflected light fluorescence attachment (Olympus), and a ×20/0.40 objective.

Immunofluorescence *in situ* assay in cumulus cells

This type of immunological assay was preferred in order to obtain specific results using a limited amounts of cumulus cells from a single COC. Specifically, 100 cells/sample per each immune assay were analyzed. The specificity of antibodies was tested in granulosa cells.

The cells were washed in PBS and permeabilized for 10 min at 4°C in a solution of 0.1% Triton X-100 and 0.1% sodium citrate in PBS. Cells were washed three times in PBS and incubated overnight at 4°C with anti-pAKT polyclonal antibody (sc-7985-R, 1:50, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-AKT antibody (9272, 1:25, Cell Signaling, Danvers, MA, USA) and anti-pERK1/2 antibody (9101, 1:50, Cell Signaling, Danvers, MA, USA) in a solution containing 3% BSA in PBS. The primary antibody was omitted in the negative controls. After three rinses with PBS, secondary antibodies, specifically anti-rabbit IgG (whole molecule)–FITC (F0382, 1:50, Sigma) for

https://doi.org/10.1017/S0967199417000429 Published online by Cambridge University Press

anti-pAKT and anti-AKT or anti-mouse IgG (whole molecule) F(ab)$_2$ fragment-Cy3 (C2181, 1:50, Sigma) for anti-pERK1/2, were added to the samples for 1 h. Cumulus cells were stained for 10 min with Hoechst 33342 (Invitrogen), mounted in 10 μl DABCO solution (de-ionized H_2O, 1 M Tris–HCl pH 8, 2 mM DABCO, glycerol) and observed under an Olympus BX 50 microscope equipped with a reflected light fluorescent attachment (Olympus), and a ×20/0.40 objective. Densitometric analysis of fluorescent signals was carried out using NIS-Elements BR 3.10 image analyzer software (Nikon) as reported by Choi *et al*. (2012). Numerical values represent intensity.

Electrophoretic analysis and protein gel blot in granulosa cells

Western blot analysis was performed on granulosa cells because these were more numerous than cumulus cells and belonged to the same cell type. We did not carry out any additional purification, because the very low number of leukocytes (1–4%) usually present within the granulosa cells is not enough to interfere with the western blot results (Van Deerlin *et al*., 1997). Granulosa cells were washed in PBS, pelleted by centrifugation and lysed in a lysis buffer as reported by Chiarelli *et al*. (2011). The lysates were centrifuged at 1000 rpm for 1 min at 4°C. Protein concentrations were estimated by the Bradford assay (Bio-Rad, Hercules, CA, USA). Electrophoretic analysis and immunoblots were performed according to Chiarelli (Chiarelli *et al*., 2014; Librizzi *et al*., 2015) with minor modifications. Lysates of cells were submitted to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) with subsequent protein transfer onto Hybond-ECL nitrocellulose membranes (GE Healthcare, Piscataway, NJ, USA) with a Novablot semidry apparatus (Amersham Pharmacia Biotech, Piscataway, NJ, USA) (Chiarelli *et al*., 2014).

Specifically, the antibodies adopted were: anti-pAKT antibody (sc-7985-R, 1:750, Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-AKT antibody (9272, 1:750, Cell Signaling, Danvers, MA, USA); anti-pERK1/2 antibody (9101, 1:750, Cell Signaling, Danvers, MA, USA). Anti-actin antibody (A5060, 1:500, Sigma-Aldrich, Saint Louis, Missouri, USA) was used as the loading control.

The secondary antibody was a horseradish peroxidase-conjugated anti-rabbit IgG antibody (W4011, Promega, Madison, WI, USA, 1:25,000), for anti-pAKT and anti-AKT or anti-mouse IgG antibody (W402b, Promega, Madison, WI, USA, 1:25,000) for anti-pERK1/2. Protein bands were visualized using a Molecular imager VersaDoc MP Imaging Systems (Bio-Rad) and the ImmunStar Western C.

ImageJ software was used for densitometric analysis of bands and the normalization was carried out with respect to the values related to the anti-actin antibody reaction.

Data normalization

As each patient had a personal clinical history and an individual response to hormonal stimulation, and as the selection of oocytes to fertilize concerned the individual patient, the data were analyzed at patient level. In order to evaluate the quality of individual COC compared with the total of the analyzed complexes within the same patient, we analyzed data from cumulus cells following the formula for statistical normalization, in which the minimum and the maximum were chosen within the same patient. With respect to TUNEL data (DFI), assuming that a low percentage of apoptosis in cumulus cells coincides with a better quality of the corresponding oocyte, normalized data for each patient ranged between 0 (the maximum DFI of the cumulus cells) and 1 (the minimum). Conversely, the molecules of the survival pathways, AKT, pAKT and pERK1/2, among the COCs within the same patient were normalized ranging between 0 (the minimum value of the densitometry analysis) and 1 (the maximum).

Statistical analysis

All continuous variables (DFI, level of molecules and ratio between molecules and DFI) were positively skewed so that they were expressed as median value and interquartile range (Q_1 is the 25th and Q_3 is 75th percentiles). Statistical significance of the difference between level of molecules and DFI of oocytes resulting in blastocysts transferred versus arrested was tested through the repeated measures analysis of variance (ANOVA) test of log-transformed variables. In order to take into account the correlation structure existing for oocytes observed within the same patient, the association between level of molecules and DFI was obtained through a Generalized Estimating Equations (GEE) model with Gaussian distribution and log link. The most appropriate model was chosen according to the Quasi-likelihood under Independence Criterion (QIC). Exponentiated coefficients were given to estimate the variation in one molecule that was expected for one unit increase in another molecule. With three molecules, there were three multiple comparisons, so the *P*-value for statistical significance after Bonferroni's correction for multiplicity was $P = 0.05/3 = 0.017$. Statistical analysis was performed using STATA SE/14.1 software.

This is an observational study, feasible and ethically acceptable as it uses cumulus cells/granulosa that are usually discarded after decumulation of the COC,

https://doi.org/10.1017/S0967199417000429 Published online by Cambridge University Press

Table 1 Statistical significance of the differences between transferred and arrested embryos for DFI and molecular performances

	Arrested ($n = 23$) median (Q_1–Q_3)	Transferred (blastocyst stage) ($n = 117$) median (Q_1–Q_3)	P-value[a]
DFI	1.96 (1.32–2.39)	1.34 (1.00–2.33)	0.004
AKT	1.33 (1.15–1.71)	1.17 (1.00–1.59)	0.101
pAKT	1.37 (1.12–1.71)	1.25 (1.02–1.64)	0.416
pERK1/2	1.29 (1.10–1.62)	1.22 (1.00–1.61)	0.392
AKT/DFI	0.57 (0.50–1.25)	0.91 (0.53–1.16)	0.158
pAKT/DFI	0.58 (0.42–1.03)	0.99 (0.54–1.37)	0.043
pERK1/2/DFI	0.71 (0.47–1.02)	0.99 (0.52–1.36)	0.066

[a]Repeated measures ANOVA test of log-transformed data; $P < 0.05$ for statistical significance.

prior to performing ICSI. All activities were performed after the patient informed consent signature was obtained. There was no experimental therapeutic intervention on the patients. The Internal Review Board (IRB) considered this research ethically acceptable (27 June 2012).

Results

Study design and size

This prospective observational study was performed on 53 patients. In total, 255 MII oocytes were analyzed, of which 197 were fertilized and the derived embryos had the following evolutions: 117 transferred blastocysts, 57 vitrified and 23 arrested. In total, 58 oocytes were not analyzed because of failed fertilization, or immature condition [germinal vesicle (GV) or metaphase I (MI)].

Cumulus cell investigation

The analyses on cumulus cells of DFI were performed by TUNEL assay and the levels of AKT, pAKT and pERK1/2 were evaluated by immunofluorescence assay (the specificity of all antibodies was tested by immunoblot, as described in Materials and methods).

The DFI of cumulus cells was significantly different between arrested and transferred embryos that reached the blastocyst stage (1.96 vs. 1.34, $P = 0.004$), confirming that cumulus cell DFI could be considered as a marker of oocyte competence. It also could be considered as a predictive tool to define the higher probability of the resulting embryo to develop to the blastocyst stage. Likewise, we found statistically significant differences between cumulus cells and oocytes based on pAKT/DFI ratio results of transferred and arrested embryos ($P = 0.043$).

Conversely, the analysis of the levels of individual molecules (AKT, pAKT, pERK1/2) did not reach statistical significance in any case (Table 1).

Representative samples showing DNA fragmentation in cumulus cells from transferred (A1) or an arrested (A4) embryo that expressed green fluorescent chromatin are shown in Fig. 1(*A*). The total nuclei stained with propidium iodide in A2 and A5 and the merge in A3 and A6 are shown. Collected data showed a relatively high DFI in cumulus cells of arrested embryo compared with transferred embryo.

Further analysis by immunofluorescence *in situ* with pAKT antibody is shown in Fig. 1(*B*), showing representative fields of samples of cumulus cells of a transferred (Fig. 1*B1*) and an arrested (Fig. 1*B4*) embryo. The total nuclei stained with Hoechst in Fig. 1(*B2*) and Fig. 1(*B5*) and the merged image in Fig. 1(*B3*) and Fig. 1(*B6*) are shown. Collected data showed a relatively low level of pAKT in cumulus cells of arrested embryo compared with transferred embryo.

In order to evaluate specificity of pAKT antibody utilized for the immunofluorescence we performed protein gel blot analysis with samples of granulosa/cumulus cells of arrested embryos (lines 1, 4, 5) and transferred embryos (line 2, 3), normalized with actin antibody and quantified using densitometry (Fig. 1*C*).

Wider implication of the findings

We found that in the cumulus cells of the oocytes able to produce blastocysts, the pAKT/DFI ratio was higher than in cumulus cells of arrested embryos, indicating that DNA fragmentation is lower when the pAKT level is higher.

The univariate associations of AKT, pAKT and pERK1/2 molecules in the cumulus cells of all samples showed a significant direct correlation within pairs of molecules. In detail, an increase of one unit in AKT was associated with +30% increase ($P < 0.001$) in pAKT; an increase of one unit in AKT was associated with +26% increase ($P < 0.001$) in pERK1/2; an increase of one unit in pAKT was associated with +9% increase ($P = 0.001$) in pERK1/2 (data not shown in the table).

https://doi.org/10.1017/S0967199417000429 Published online by Cambridge University Press

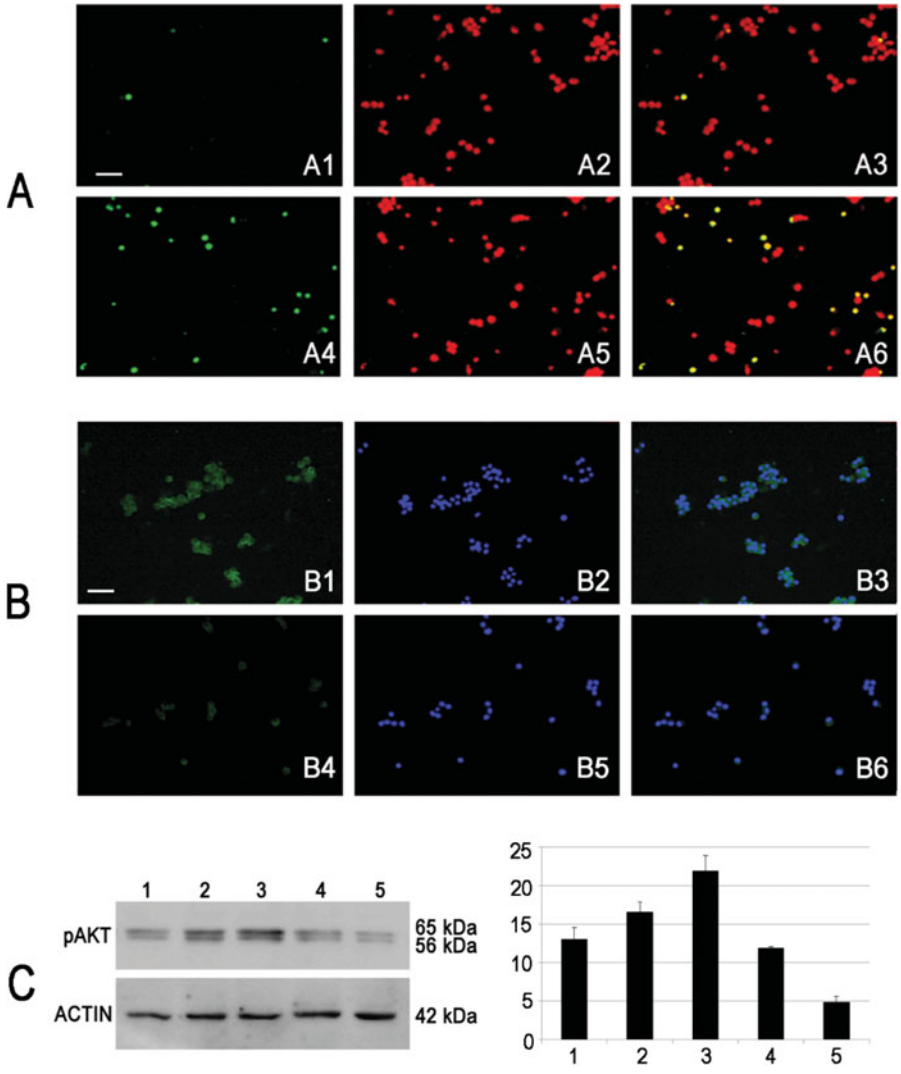

Figure 1 Cumulus cell detection. The images of representative fields show cumulus cells observed under a fluorescence microscope (*A*, *B*) and immunoblot detection against pAKT (*C*). Cumulus cells derived from an oocyte that was fertilized and the related embryo was transferred (*A1–A3*; *B1–B3*). Cumulus cells derived from an oocytes that was fertilized but the related embryo was arrested (*A4–A6*; *B4–B6*). Bar 30 μM. (*A*) DNA fragmentation detection by TUNEL assay (*A1*, *A4*), nuclei stained with propidium iodide (*A2*, *A5*), merge of green and red (*A3*, *A6*). (*B*) Detection by immunofluorescence of pAKT protein (*B1*, *B4*), nuclei stained with Hoechst (*B2*, *B5*), merge of green and blue (*B3*, *B6*). Bar 30 μM. (*C*) Immunoblot of total lysates of granulosa/cumulus cells derived from oocytes that were fertilized and the related embryos were arrested (lines 1, 4, and 5) and transferred (lines 2, and 3), reacted with anti-pAKT antibody. The same samples were incubated with anti-actin antibody. The histogram shows results obtained from the analysis of pAKT band intensities normalized by comparison with actin and reported as arbitrary units.

Discussion

The continuous cross-talk between the oocyte and the somatic follicular cells is essential for follicular development and oocyte maturation (Hutt & Albertini, 2007), suggesting that the progression of the follicle through different developmental stages is supported by factors exclusively or predominantly expressed by the oocyte (Soyal *et al.*, 2000; Suzumori *et al.*, 2002; Rajkovic *et al.*, 2004). The surrounding somatic cells provide most of the substrates for energy metabolism and biosynthesis to the oocyte. In particular, oocyte maturation and competence acquisition are essentially regulated by the cumulus cells (Larsen *et al.*, 1986; Ashkenazi *et al.*, 2005).

The biological significance of lower levels of DFI, in cumulus cells, could be related to the ability to maintain the physiological function for a longer time supporting the synchronization of nuclear and cytoplasmic maturation in the oocytes that is one of

https://doi.org/10.1017/S0967199417000429 Published online by Cambridge University Press

the main properties of oocyte competence. The lower apoptosis rate in cumulus cells could be evidence of the activation of survival pathways (Ruvolo *et al*., 2007, 2013b), probably involved in better oocyte quality that could be associated with a significant reduction in the number of immature oocytes collected after ovarian stimulation and increase in the clinical outcome (Ruvolo *et al*., 2007).

The central role of EGF and the EGF-like factors in supporting the survival pathway through the action of epidermal growth factor receptor (EGFR) has been demonstrated by Cameron *et al*. (1996).

LH seems to have a pivotal role in regulating and activating specific proteins involved in the surviving pathways. Several studies have demonstrated that LH increases the cAMP level, stimulating adenylyl cyclase, activating kinase A and down-streaming the ERK1 and ERK2 signalling cascade (Ashkenazi *et al*., 2005). The consequence seems to be the down-regulation of genes related to follicular development induced by ERK1/2 with the up-regulation of the ovulation-related genes (Richards, 2005).

ERK and AKT are able to regulate the apoptotic pathway, as demonstrated in several papers. The action of activated ERK (pERK) seems to be different in different tissues. In human cervical carcinoma HeLa cells, pERK plays an active role in mediating cisplatin-induced apoptosis of HeLa cells and works upstream of caspase activation to initiate the apoptotic signal, through cytochrome *c* release, and dependent activation of caspase-9 through Apaf-1 (Wang *et al*., 2000). In contrast, other studies have suggested that a survival signal activation is delivered by the ERK pathway (Hayakawa *et al*., 1999; Persons *et al*., 1999). Conversely, pAKT seems to always have a survival effect, inhibiting the apoptotic pathway (Zhang *et al*., 2014).

Our data seem to demonstrate that pAKT has an anti-apoptotic effect also on the somatic cells of human follicles. For the first time in cumulus cells, we have shown a relationship between pAKT and the oocyte competence. Increased levels of pAKT were found in cumulus cells from oocytes that produce embryos that are able to reach blastocyst stage compared with embryos arrested during *in vitro* culture. The amount of pAKT seems to be associated with a reduced apoptosis rate in the cumulus cells. As shown, an increase of one unit of AKT is associated with remarkable increases in pAKT and pERK1/2; while an increase in pAKT is associated with a small increase in pERK1/2. These data show a significant direct correlation within pairs of molecules, suggesting that these molecules act as survival factors.

The present study is not aimed at finding a correlation with the PR as it was designed as a prospective observational analysis and two or three blastocysts for each patient were transferred. Conversely, our results strongly suggest that DFI in the cumulus cells could be considered as a molecular marker of oocyte competence, to be evaluated as a prognostic pattern of blastocyst formation. We also demonstrated that the relationship between pAKT and apoptosis is inversely correlated with the cumulus cells, comparing embryos developed until blastocyst stage and embryos arrested during *in vitro* culture. This result could help to reduce the number of oocytes to be fertilized and blastocysts to be transferred, with the important consequence of lowering the risk of multiple pregnancies.

Acknowledgements

The authors are grateful to the host institution: Department STEBICEF of Palermo University and to Chiara Martino for proofreading. Funding to pay this study was provided by a grant for fertility innovation: GFI 2012–24.

Competing interests

Authors declare that they have no competing interests.

References

Amaravadi, R. & Thompson, C.B. (2005). The survival kinases Akt and Pim as potential pharmacological targets. *J. Clin. Invest.* **115**, 2618–24.

Andersen, A.N., Goossens, V., Ferraretti, A.P., Bhattacharya, S., Felberbaum, R., de Mouzon, J. & Nygren, K.G. (2008). Assisted reproductive technology in Europe, 2004: results generated from European registers by ESHRE. *Hum. Reprod.* **23**, 756–71.

Ashkenazi, H., Cao, X., Motola, S., Popliker, M., Conti, M. & Tsafriri, A. (2005). Epidermal growth factor family members: endogenous mediators of the ovulatory response. *Endocrinology* **146**, 77–84.

Bosco, L., Ruvolo, G., Morici, G., Manno, M., Cittadini, E. & Roccheri, M.C. (2005). Apoptosis in human unfertilized oocytes after intracytoplasmic sperm injection. *Fertil. Steril.* **84**, 1417–23.

Bosco, L., Ruvolo, G., Chiarelli, R., Agnello, M. & Roccheri, M.C. (2015). Selection of the best oocytes for intracytoplasmic sperm injection (ICSI) using apoptotic analysis of cumulus cells. *J. Biol. Res.* **88**, 31–32.

Cameron, M.R., Foster, J.S., Bukovsky, A. & Wimalasena, J. (1996). Activation of mitogen-activated protein kinases by gonadotropins and cyclic adenosine 5′-monophosphates in porcine granulosa cells. *Biol. Reprod.* **55**, 111–9.

Chiarelli, R., Agnello, M. & Roccheri, M.C. (2011). Sea urchin embryos as a model system for studying autophagy induced by cadmium stress. *Autophagy* **7**, 1028–34.

Chiarelli, R., Agnello, M., Bosco, L. & Roccheri, M.C. (2014). Sea urchin embryos cadmium exposed as an experimental

model system for studying the relationship between autophagy and apoptosis. *Mar. Environ. Res.* **93**, 47–55.

Choi, S.K., Galán, M., Kassan, M., Partyka, M., Trebak, M. & Matrougui, K. (2012). Poly(ADP-ribose) polymerase 1 inhibition improves coronary arteriole function in type 2 diabetes mellitus. *Hypertension* **59**, 1060–8.

Datta, S.R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y. & Greenberg, M.E. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91**, 231–41.

Goto, M., Iwase, A., Ando, H., Kurotsuchi, S., Harata, T. & Kikkawa, F. (2007). PTEN and Akt expression during growth of human ovarian follicles. *J. Assist. Reprod. Genet.* **24**, 541–6.

Hayakawa, J., Ohmichi, M., Kurachi, H., Ikegami, H., Kimura, A., Matsuoka, T., Jikihara, H., Mercola, D. & Murata, Y. (1999). Inhibition of extracellular signal-regulated protein kinase or c-Jun N-terminal protein kinase cascade, differentially activated by cisplatin, sensitizes human ovarian cancer cell line. *J. Biol. Chem.* **274**, 31648–54.

Herrero, J. & Meseguer, M. (2013). Selection of high potential embryos using time-lapse imaging: the era of morphokinetics. *Fertil. Steril.* **99**, 1030–4.

Hutt, K. J. & Albertini, D. F. (2007). An oocentric view of folliculogenesis and embryogenesis. *Reprod. Biomed. Online* **14**, 758–64.

Jancar, N., Kopitar, A.N., Ihan, A., Virant Klun, I. & Bokal, E.V. (2007). Effect of apoptosis and reactive oxygen species production in human granulosa cells on oocyte fertilization and blastocyst development. *J. Assist. Reprod. Genet.* **24**, 91–7.

Kauffmann-Zeh, A., Rodriguez-Viciana, P., Ulrich, E., Gilbert, C., Coffer, P., Downward, J. & Evan, G. (1997). Suppression of *c*-Myc-induced apoptosis by Ras signalling through PI(3)K and PKB. *Nature* **385**, 544–8.

Larsen, W.J., Wert, S.E. & Brunner, G.D. (1986). A dramatic loss of cumulus cell gap junctions is correlated with germinal vesicle breakdown in rat oocytes. *Dev. Biol.* **113**, 517–21.

Librizzi, M., Chiarelli, R., Bosco, L., Sansook, S., Gascon, J.M., Spencer, J., Caradonna, F. & Luparello, C. (2015). The histone deacetylase inhibitor JAHA down-regulates pERK and global DNA methylation in MDA-MB231 breast cancer cells. *Materials* **8**, 7041–7.

Nakahara, K., Saito, H., Saito, T., Ito, M., Ohta, N., Takahashi, T. & Hiroi, M. (1997). The incidence of apoptotic bodies in membrana granulosa can predict prognosis of ova from patients participating in in vitro fertilization programs. *Fertil. Steril.* **68**, 312–7.

Nakahara, K., Saito, H., Saito, T., Ito, M., Ohta, N., Takahashi, T. & Hiroi, M. (1998). Ovarian fecundity in patients with endometriosis can be estimated by the incidence of apoptotic bodies. *Fertil. Steril.* **69**, 931–5.

Oosterhuis, G.J., Michgelsen, H.W., Lambalk, C.B., Schoemaker, J. & Vermes, I. (1998). Apoptotic cell death in human granulosa-lutein cells: a possible indicator of in vitro fertilization outcome. *Fertil. Steril.* **70**, 747–9.

Pacella, L., Zander-Fox, D.L., Armstrong, D.T. & Lane, M. (2012). Women with reduced ovarian reserve or advanced maternal age have an altered follicular environment. *Fertil. Steril.* **98**, 986–94.

Persons, D.L., Yazlovitskaya, E.M., Cui, W. & Pelling, J.C. (1999). Cisplatin-induced activation of mitogen-activated protein kinases in ovarian carcinoma cells: inhibition of extracellular signal-regulated kinase activity increases sensitivity to cisplatin. *Clin. Cancer Res.* **5**, 1007–14.

Quirk, S.M., Cowan, R.G. & Harman, R.M. (2004). Progesterone receptor and the cell cycle modulate apoptosis in granulosa cells. *Endocrinology* **145**, 5033–43.

Rajkovic, A., Pangas, S.A., Ballow, D., Suzumori, N. & Matzuk, M.M. (2004). NOBOX deficiency disrupts early folliculogenesis and oocyte-specific gene expression. *Science* **305**, 1157–9.

Richards, J.S. (2005). Ovulation: new factors that prepare the oocyte for fertilization. *Mol. Cell. Endocrinol.* **234**, 75–9.

Ruvolo, G., Bosco, L., Pane, A., Morici, G., Cittadini, E. & Roccheri, M.C. (2007). Lower apoptosis rate in human cumulus cells after administration of recombinant luteinizing hormone to women undergoing ovarian stimulation for in vitro fertilization procedures. *Fertil. Steril.* **87**, 542–6.

Ruvolo, G., Roccheri, M.C., Brucculeri, A.M., Longobardi, S., Cittadini, E. & Bosco, L. (2013a). Lower sperm DNA fragmentation after r-FSH administration in functional hypogonadotropic hypogonadism. *J. Assist. Reprod. Genet.* **30**, 497–503.

Ruvolo, G., Fattouh, R.R., Bosco, L., Brucculeri, A.M. & Cittadini, E. (2013b). New molecular markers for the evaluation of gamete quality. *J. Assist. Reprod. Genet.* **30**, 207–12.

Sinha, D., Bannergee, S., Schwartz, J.H., Lieberthal, W. & Levine, J.S. (2004). Inhibition of ligand-independent ERK1/2 activity in kidney proximal tubular cells deprived of soluble survival factors up-regulates Akt and prevents apoptosis. *J. Biol. Chem.* **279**, 10962–72.

Soyal, S.M., Amleh, A. & Dean, J. (2000). FIGalpha, a germ cell-specific transcription factor required for ovarian follicle formation. *Development* **127**, 4645–54.

Strömberg, B., Dahlquist, G., Ericson, A., Finnström, O., Köster, M. & Stjernqvist, K. (2002). Neurological sequelae in children born after in-vitro fertilisation: a population-based study. *Lancet* **359**, 461–5.

Suzumori, N., Yan, C., Matzuk, M.M. & Rajkovic, A. (2002). Nobox is a homeobox-encoding gene preferentially expressed in primordial and growing oocytes. *Mech. Dev.* **111**, 137–41.

Thompson, J.E. & Thompson, C.B. (2004). Putting the rap on Akt. *J. Clin. Oncol.* **22**, 4217–26.

Van Deerlin, P.G., Cekleniak, N., Coutifaris, C., Boyd, J. & Strauss, J.F. 3rd. (1997). Evidence for the oligoclonal origin of the granulosa cell population of the mature human follicle. *J. Clin. Endocrinol. Metab.* **82**, 3019–24.

Varras, M., Polonifi, K., Mantzourani, M., Stefanidis, K., Papadopoulos, Z., Akrivis, C. & Antsaklis, A. (2012). Expression of antiapoptosis gene survivin in luteinized ovarian granulosa cells of women undergoing IVF or ICSI and embryo transfer: clinical correlations. *Reprod. Biol. Endocrinol.* **10**, 74.

Wang, X., Martindale, J.L. & Holbrook, N.J. (2000). Requirement for ERK activation in cisplatin-induced apoptosis. *J. Biol. Chem.* **275**, 39435–43.

World Health Organization. *Report of the Meeting on the Prevention of Infertility at the Primary Health Care Level*. 1983, WHO, Geneva, WHO/MCH/1984.4.

Zhang, C., Zhu, J., Zhang, J., Li, H., Zhao, Z., Liao, Y., Wang, X., Su, J., Sang, S., Yuan, X. & Liu, Q. (2014). Neuroprotective and anti-apoptotic effects of valproic acid on adult rat cerebral cortex through ERK and Akt signaling pathway at acute phase of traumatic brain injury. *Brain Res*. **1555**, 1–9.

https://doi.org/10.1017/S0967199417000429 Published online by Cambridge University Press