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NADPH oxidase-4 and MATER expressions in granulosa cells: relationships with ovarian aging

MATER and NOX in granulosa cells

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ABSTRACT

Aims - Relevant roles in follicular development and ovulation are played by maternal antigen that embryos require (MATER), product of a maternal effect gene, and by reactive oxygen species (ROS), indispensable for the induction of ovulatory genes. At the moment, the relationship between these two biological systems and their involvement in the ovarian aging have not been still clarified. The aim of the current experimental study was to analyse the age-related changes of the MATER and NOX proteins.

Materials and methods - MATER and ROS homeostasis was studied in granulosa cells (GCs) and cumulus cells (CCs) of infertile patients who undergone oocyte retrieval for in vitro fertilization cycles using Western blot and confocal immunofluorescence analysis. Samples were obtained from subjects with age ≥ 40 years (cases) and with age ≤ 37 years (controls).

Key findings - The expression pattern of MATER and NOX observed in GCs was not different from that observed in CCs. High levels of both proteins were detected in the control samples. A significant lower expression of both MATER and NOX4 was observed in the case *versus* control samples.

Significance - The expression of MATER and NOX4 proteins are closely related to the follicular development and ovulation with particular regard for ovarian aging.

Keywords: MATER; NOX4; granulosa cells; infertility.

Introduction

Maternal effect genes encode for proteins and transcripts that accumulate during oogenesis and are necessary to support the development of pre-implantation embryo. Over the past few years a number of maternal effect genes have been studied to understand the molecular mechanisms governing oocyte and early embryo development [1]. These factors play a role in folliculogenesis and oocyte development since they are involved in a coordinated development and communication between the oocyte and its surrounding somatic cells [2,3]. Specifically, during human folliculogenesis, maternal antigen that embryos require (MATER, or Nlrp5), a maternal effect gene, is expressed both in follicular cells and oocytes starting in late primary follicles and persisting for the entire maturation process and also after ovulation [4].

The passages from antral follicles to fertilization and early embryonic development may be particularly sensitive to exposures to environmental stressors. In recent years, there has been growing interest in the roles of reactive oxygen species (ROS) in female reproduction since endogenous ROS can induce oxidative stress or can play important roles as signalling molecules, for example, during ovulation [5]. Cumulus cells (CCs) that surround the oocytes, as well as follicular fluid, may protect the oocytes from the damaging effects of ROS [6]. Furthermore ROS augment oocyte aging, even more in relatively old oocytes, suggesting compromised antioxidant capacity in aged oocytes [7]. ROS are involved in signalling of growth factors, e.g. via the epidermal growth factor receptor, playing a crucial role in the ovulatory signalling cascade linked to the induction of ovulatory genes in rodents [8]. Thus, ROS appear indispensable for ovulation, at least in rodents. Parallels to the human situation are possible but remain to be proven [9].

NOX family members are the predominant contributors of ROS in many cellular systems [10]. NOX 2, 4 and 5 are the NOX isoforms expressed by human follicle. However, it is known that NOX 4 and NOX 5 isoforms are expressed in the human granulosa cells (GCs) [9]. NOX- derived ROS as second messenger molecule are involved in protein kinase C signalling pathway regulating oocyte maturation [11], and MATER protein interacts with such protein kinase in CCS under physiological conditions [12]. At the moment, the relationship between the two biological systems, i.e. MATER and ROS and their involvement in the ovarian aging have not been still clarified. Unfortunately, it is very difficult to study human oocyte, even if within a process of in vitro fertilization (IVF) program, the use of GCs and CCs can represent surrogate bioassays to study the biological processes involving oocyte. Based on these considerations, the aim of the current experimental study was to analyse the age-related changes of MATER and NOX proteins in human GCs and CCs.

Materials and Methods

Subjects

Human GCs and CCs were obtained from follicular aspirates of women undergoing IVF cycles. Samples were collected at the Unit of Obstetrics & Gynecology, IRCCS - ASMN of Reggio Emilia (Italy). An informed consent allowing the use of clinical data and biological samples for a not specified research purpose was signed by all infertile couples before treatment.

Patients scheduled for their first IVF cycle for infertility due to male factor were included in the study. Exclusion criteria were considered: gynaecological (endometriosis,

adenomyosis, fibroids, etc.), endocrinological (hyperprolactinemia, hypo- and hyperthyroidism, polycystic ovary syndrome, etc.) and metabolic (obesity, leanness, diabetes mellitus, dyslipidemia, etc.) diseases. Patients with previous ovarian surgery were also excluded. In order to avoid confounders and a potential overlapping of the findings, patients with an age of 38 and/or 39 years were excluded. Patients with an age equal or higher than 40 years were considered as cases, whereas patients with an age lower or equal to 37 years were considered as controls.

The same ovarian stimulation protocol was used for all patients. Briefly, to achieve ovarian suppression, all patients were treated with a gonadotropin releasing hormone (GnRH) agonist (Enantone 3.75, Takeda, Milan, Italy) starting during the luteal phase of the previous menstrual cycle. Fourteen to 20 days were needed for completed ovarian suppression as assessed by serum estradiol (E_2) concentrations ($E_2 < 50$ pg/mL) and by ovarian ultrasound (no follicles > 10 mm). When these suppression criteria were satisfied, recombinant follicle stimulating hormone (FSH) (Gonal F, Merck Serono, Rome, Italy) was started using an initial dose of 225 IU per day for at least the next 5 days. The ovarian response was then monitored daily by transvaginal ultrasound and serum E_2 assays. The FSH dosage was adjusted individually according to the ovarian response. When one or more follicles > 17 mm in diameter were obtained, 10,000 IU human chorionic gonadotropin (hCG), Gonasi HP, IBSA, Rome, Italy) were administered intramuscularly. Thirty-six hours after hCG administration, oocyte retrieval was performed by ultrasound-guided transvaginal aspiration (see data in Table I).

Experimental protocol

We focused our interest on the MATER and ROS proteins presence during aging in GCs and CCs. Among calcium independent - NOX isoforms and subunits, we investigated the expression of NOX4, of p22phox, a subunit shared by NOX1, 2, 3 and 4, and of p47phox, as regulating subunit only for NOX1, 2.

GCs and CCs were compared by western blot for the cited protein expression. Also immunofluorescence analysis of MATER and NOX were performed in order to assess the protein distribution into the follicle cell populations. Since no difference between GCs and CCs was observed, the study was carried out only with GCs.

Control samples and case samples of GCs were compared for the expression of a panel of proteins, including also antioxidant proteins such as SOD1 and SOD2.

To confirm the western blot results, immunofluorescence analysis was then performed.

GCs isolation

Pelleted follicular fluid samples of follicle >17 mm in diameter were isolated within one hour from the oocyte retrieval. Follicle cells from the same patient were layered onto a 50% Percoll gradient (Amersham Pharmacia Biotech., Freiburg, Germany) and the GC-enriched layer was isolated [13]. Such subpopulation was then fixed for immunofluorescence analysis or lysed for WB.

Immunofluorescence and confocal microscopy

For immunofluorescence analysis, samples were processed as previously described [14]. Isolated GCs and CCs were fixed in 4% paraformaldehyde in PBS and permeabilized with 1% Triton X-100 in PBS for 10 min.

After a treatment with 3% BSA in PBS for 30 min at room temperature, the cells were incubated with the primary antibodies diluted in PBS containing 3% BSA. Primary antibodies against NOX4 (rabbit anti-NOX4) and MATER (goat anti-Nalp5) were purchased from Santa Cruz Biotechnology (CA, USA) or the rabbit anti-Nalp5 from Abcam (Cambridge, UK).

Confocal imaging was performed by a Nikon A1 confocal laser scanning microscope. The confocal serial sections were processed with ImageJ software to obtain three-dimensional projections [15]. The image rendering was performed by Adobe Photoshop software (Adobe, San Jose, CA, USA).

Western blotting

Whole cell lysates from GCs and CCs and oocytes, as positive control, were processed as previously described [16]. Protein extracts were loaded onto SDS-polyacrylamide gel, blotted on Immobilon-P membranes (Millipore, Billerica, MA, USA), and processed by Western blot with the indicated antibody, detected by supersignal substrate chemiluminescence detection kit (Pierce, Rockford, IL, USA). Quantization of the signal was obtained by chemiluminescence detection on a Kodak Image Station 440CF and analysis with the Kodak 1D Image software (Rochester, NY, USA).

The primary antibodies used for experiments were: rabbit anti-p47phox (Cell Signalling Technology, Beverly, MA, USA), goat anti-matrin3, goat anti-Nalp5, rabbit anti-PARP, rabbit anti-SOD1, rabbit anti-p22phox, mouse anti- β actin (Santa Cruz Biotechnology, CA, USA), rabbit anti-Nox4 (Novus Biologicals, CO, USA), rabbit anti-Nalp5 from Abcam (Cambridge, UK) and sheep anti-SOD2 (Calbiochem, Darmstadt, Germany).

Matrin3, a nuclear matrix protein, was considered as internal loading control, instead of β actin or tubulin, in order to exclude red blood cells in this evaluation. Its expression is fairly constant in all groups.

Statistical analysis

In vitro experiments were performed in triplicate. For quantitative comparisons, values were reported as mean \pm standard deviation (SD) based on triplicate analysis for each sample. To test the significance of observed differences among the study groups, analysis of variance (ANOVA) test with post-hoc Bonferroni correction or Student's *t*-test were applied. A *P* value less than 0.05 was considered to be statistically significant.

Results

Cases (n. 20) and controls (n. 26) were significantly ($P<0.05$) different in terms of age (41.0 ± 1.5 vs. 33.1 ± 3.3 , respectively), clinical pregnancy (5% vs. 39%, respectively) and delivery (0% vs. 22%, respectively) rates.

The pre-study phase is shown in Figure 1. Western blot analysis of GCs and CCs, obtained from young patient, showed measurable levels of MATER in all the follicle cells (Figure 1A). Comparing again GCs and CCs for NADPH oxidase family expression, we checked the presence of NOX4 isoform and the p47phox subunit, NOX4 and p47phox were both present in CCs, while only NOX4 was prevalently expressed in GCs unlike p47phox (Figure 1B).

The expression of MATER and NOX4 was analysed in GCs and CCs also after stratification by age (Figure 1C). Specifically, the results were compared in three sub-groups characterised by increasing age (5 years difference). No difference between GCs and CCs was observed. Moreover, the protein expression gradually decreases with the increasing of the patient age achieving the greatest expressions in the youngest and the lowest in the oldest. The expression pattern of MATER and NOX4 observed in GCs was not different from that observed in CCs (Figure 1C).

Figure 2 shows the samples of GCs and CCs from a patient of the control group, marked for MATER and NOX4.

After seeing that the data obtained with the GCs have a close relationship with the findings in CCs, we decided to work exclusively with GCs due to their greater availability and their possible validity as indirect study of ovarian function.

MATER and NOX4 proteins were analysed in case and control samples (Figures 3A and 3B). The MATER expression was higher in the controls than in the cases samples, even if with an un-homogenous decrease among the patients. The MATER expression in the cases resulted less than the middle than in the controls ($P \leq 0.0001$ for both vs. control group) (Figure 3B). A huge expression decrease occurs also for NOX4 isoform ($P \leq 0.0001$) occurs (Figure 3B). On the other hand, p22phox expression was not statistically different (Figure 3B). Analysing the apoptotic process, an increase in the cleaved form of PARP (80 kDa) occurred in the cases ($P \leq 0.0001$).

The analysis of the expression of SOD1 and SOD2 enzymes demonstrated a protein expression significantly ($P \leq 0.0001$) lower in the case samples than in the control samples.

Figure 4 shows the immunofluorescence analysis for the NOX4 and MATER.

A dramatic decrease of the signal intensity for both the investigated markers was observed (Figure 4), thus the probability of co-localisation in parallel falls. The data in the graph confirm the findings from western blot analysis adding the statistical analysis evidence. The number of positive cells for MATER and NOX4 was statistically significant ($P < 0.0001$) different between case and control samples.

Discussion

Relevant roles in follicular development are played by MATER that is the product of a maternal effect gene. Also ROS homeostasis in the ovarian follicle appears indispensable for the induction of ovulatory genes. In this study we analysed GCs and we showed that MATER and NOX4, a source of ROS, appear to be factors that may be related to female

fertility. In young subjects indeed we find high levels of both proteins, however in the older patients we observed lower expression levels. It can therefore be assumed that these proteins are related to the correct follicular development and for the establishment of a pregnancy.

What roles physiological ROS levels may play is not well known, but evidence for the importance of ROS in many physiological events is mounting, also in the homeostasis of ovarian follicle [17]. In fact, follicular vascularity, intrafollicular oxygen content and mitochondrial activity are factors sustaining an optimal oocyte development. For example a positive correlation between ROS levels in follicular fluid and maturation parameters has been demonstrated. ROS were reported to improve in vitro maturation of bovine oocyte, and, also in humans FF, ROS levels were found higher in women who become pregnant in IVF than in nonpregnant patients [18]. Many NOX family members are the predominant contributors of ROS in many cellular systems.

The classical neutrophil NOX comprises a catalytic subunit gp91phox, which in conjunction with the p22phox subunit forms a membrane-bound heterodimer.

Additionally, a number of cytosolic regulatory subunits, such as p67phox and p47phox, are required for enzyme activation [19,20]. Recently, the isoforms NOX2 and NOX4, the homologues of subunit gp91phox, were shown to generate ROS in the ovary [11]. On the other hand, the impact of oxidative stress on oocyte maturation seems to be deleterious; a supraphysiological amount of ROS leading to oxidative stress is involved in the aetiology of defective embryo development [21]. Oocyte competence is deeply affected by aging and decreases rapidly after age 37 [22], probably due to a series of molecular alterations that drive the defects in chromatid separation [23]. Although the reason is not fully

understood, endocrine, paracrine, genetic, and metabolic factors are thought to be affecting the decrease in the quality of the follicular pool and oocytes.

Premature ovarian failure may offer a unique model for the study of the genetic mechanisms of ovarian aging [24], and MATER is a known ovarian autoantigen targeted in autoimmune syndromes of POF [25]. Our group demonstrated that MATER is expressed not only in the oocytes, but also in somatic follicle cells [4]. Furthermore, we found an interaction between MATER and Protein kinase C (PKC)- ϵ [12]. PKC- ϵ regulates various physiological functions and is characterized as a calcium-independent but phorbol ester/diacylglycerol-sensitive serine/threonine kinase (novel isoenzyme) [26]. Usually conventional PKC isoforms activates NOX enzymes *via* the direct phosphorylation of p47phox, a subunit of NOX1 and 2. On the other hand, NADPH oxidase-dependent generation of ROS, which in turn induces activation of PKC, particularly the prosurvival novel isoenzyme PKC ϵ , results in preconditioning against cell death [27,28]. Moreover, Kampfer *et al.* [9] showed that presence only of NOX 4 (calcium independent) and NOX 5 (with a calcium binding domain) in cultured human GCs, unlike other NOX forms. Therefore, in this study we decided to investigate the expression pattern of MATER and the calcium independent NOX isoform NOX4 in follicle cells of aged women with potential ROS unbalance.

Clinical data, shown in the result section and in Table I, clearly point out that, despite a similar embryo transfer number, the success in pregnancies (chemical or clinical) and then of deliveries, mainly only for young women (male sterility problem) occurs. Such result may be due to the low number of gametes with high quality from the women of the group older than 40 years (cases). It is known that, also in case of advancing age, high

proportion of oocytes result in developmentally incompetent embryos or embryos that fail to implant despite being chromosomally and morphologically normal (Meldrum et al 2016). Furthermore, this condition does not allow the easy recovery of CCs too.

However, GCs are easier to collect in order to be analysed.

The analysis of MATER, by Western blot and immunofluorescence approaches, demonstrates that GCs show an expression profile similar to the one of CCs even if with the highest expression in oocytes, of course, shown as a positive control. The redox state in follicular fluid could be due to the somatic cells. Therefore, we compared the NOX expression in GCs and CCs, observing a higher NOX4 presence in GCs, unlike for p47phox, a subunit regulating NOX1 and 2 (calcium-independent NOX isoforms) and p22phox, a subunit shared by several NADPH oxidases such as NOX1,2,3, and 4.

Therefore only ROS deriving from NOX4 isoform should to be affected during aging.

The comparison between GCs and CCs demonstrated also that, in both these follicle cells, aging induces a decrease in MATER and NOX4 expression, indicating a possible link among this maternal effect gene product, a defined and localized physiological ROS production and a natural occurring fertility decline. Indeed, that protein expression unbalance is reflected in the low success of pregnancies, linked to the oocyte quality.

It is likely that an optimal balance between oxygen available to the oocyte and antioxidants is critical to permit normal meiotic spindle formation and correct chromosome alignment; for example in a mouse model, correct chromosomal alignment was found in follicles with high oxygen and antioxidant levels [29]. Since the measure of follicle fluid content of ROS is not possible in human samples, we tested the expression in granulosa cells of other redox modulating enzymes. Indeed, with the weakening of the

antioxidant defence, aging occurs in GCs, accompanied by Cu/Zn superoxide dismutase, Mn superoxide dismutase, and catalase down-regulation [30]. As a result, the increase in oxidative damage associated with the weakening of antioxidant defence mechanisms causes aging of the ovaries [24]. For this reason, we tested also the presence of SOD proteins in the collected samples. The mitochondrial isoform (SOD2) but also the cytosolic one (SOD1) are less expressed in case samples, indicating that a minor antioxidant power occurs. This effect can be linked to a cell suffering situation as also shown by the increase of the cleaved form of PARP, protein dedicated to the control of DNA and the activation of repair mechanisms and involved in the apoptotic process.

Conclusions

This study demonstrated that in GCs aging induces a decrease in MATER and NOX4 expression, indicating a possible link among this maternal effect gene product, a defined physiological ROS presence and a natural occurring fertility decline.

Overall this findings can open the doors to the inclusion of MATER and NOX4 expression in granulosa cells in the diagnostic protocols of the centres of assisted reproduction in the search for a prognostic factor of infertility.

DECLARATION

Abbreviations:

Bovine serum albumin (BSA); cumulus cells (CCs), 1,4-diazabicyclo(2.2.2)octane (DABCO); 4',6-diamidino-2-phenylindole (DAPI); Ethylenediaminetetraacetic acid (EDTA); follicle fluid (FF); granulosa cells (GCs); immunofluorescence (IF); in vitro fertilization (IVF); Maternal Antigen that Embryos Require (MATER); phosphate

buffered saline (PBS); NADPH oxidase (NOX); oocyte (OO); reactive oxygen species (ROS); Tris-buffered saline (TBS); Triton-X-100 (TxTBS); western blot (WB).

Ethics approval and consent to participate

An informed consent allowing the use of clinical data and biological samples for a not specified research purpose was signed by all infertile couples before treatment and collected by the Unit of Obstetrics & Gynecology, IRCCS - ASMN of Reggio Emilia (Italy).

Availability of data and material

The dataset (clinical data) supporting the conclusions of this article is included within the article.

Consent for publication

Not applicable since the manuscript does not contain any individual person's data in any form.

Conflict of interest

The authors report no conflict of interest.

We declare that there was not a role of the funding body in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Authors' contributions

TM, PI of the group, design of the work, acquisition and interpretation of data for the work and drafting the manuscript; ER, as postdoc, IF data analysis; AN, biologist, granulosa and cumulus cells processing and acquisition of clinical data; FB, lab technician, acquisition of Western blot data; MZ, lab technician, statistical data analysis; FC, biologist, acquisition of clinical data; DM, biologist, acquisition of clinical data; SP, associate professor, interpretation of data and revising the work critically for important intellectual content; GBLS, full professor, revising the work critically for important intellectual content; ADP, full professor, final approval of the version to be published. All authors read and approved the final manuscript.

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Group	≤ 37	≥ 40
Age of patients	33.1 ± 3.3	41.0 ± 1.5
Age of menarche	11.9 ± 0.8	12.0 ± 0.7
duration of stimulation (days)	15.0 ± 3.5	14.5 ± 4.4
FSH IU/patient	2199.7 ± 1368.1	4907.6 ± 3186.1
E2 (pmol/L) the day of induction	1269.4 ± 761.5	1950.4 ± 782.3
follicle number $>16\text{mm}$	5.3 ± 2.9	4.9 ± 3.0
oocyte retrieved	5.8 ± 4.1	4.9 ± 4.0

Table I: clinical data of ovarian stimulation.

Legends to the figures

Figure 1. Western blot analysis of MATER and NOX expression by follicular cells. A) revealed with anti-MATER of total lysates of oocytes (OO), cumulus cells (CC) and granulosa cells (GC). B) Western blot analysis of total lysates of CC and GC samples revealed with anti-NOX4 and anti-p47phox. C) CC and GC samples obtained from patients with different age revealed with anti-NOX4 and anti-MATER. Actin detection was performed in order to show the amount of protein loaded in each lane. Presented data are representative of three independent experiments.

Figure 2. Immunofluorescence analysis of MATER and NOX expression in follicular cells. Representative immunofluorescence (IF) images of GC and CC samples labelled with DAPI (blue), NOX4 (green) and MATER (red). Bar=20 μ m. In the first column images show that NOX4 antibody labels in a cytoplasmic and perinuclear area of CCs and GCs, even with an higher intensity in GCs sample. MATER, shown in the second column is more uniformly expressed in GCs rather than in CCs. In the third column show that some cells co-express both MATER and NOX4.

Figure 3. Western blot analysis of GC samples obtained from cases and controls A) Three most representative samples of each group are here presented. Lysates were revealed for the indicated antibodies. Matrin (nuclear matrix protein) detection was performed in order to show the amount of protein loaded in each lane. B) The graph shows grey density values analysed for all samples. Statistical analysis is discussed in the text.

Figure 4. A) Immunofluorescence analysis of MATER and NOX expression in GC samples obtained from cases and controls . Representative immunofluorescence (IF) images of different group samples labelled with DAPI (blue), NOX4 (green) and MATER (red). Bar=20 μ m. Images show single staining for both the antibodies, and also the double staining to observe the overlap of the two signals (third column). Hence three cell areas for each group are here shown. All the images obtained from the entire cohort of patients have been considered for assemble the graph below. B) The data reported in the graph indicate the mean \pm the standard deviation of the percentage of positive cells for MATER, NOX4 and co-expression for NOX4 / MATER, obtained from at least 20 patients. Statistical analysis was carried out by One-way ANOVA test followed by Bonferroni post-test. *** $P < 0.0001$.

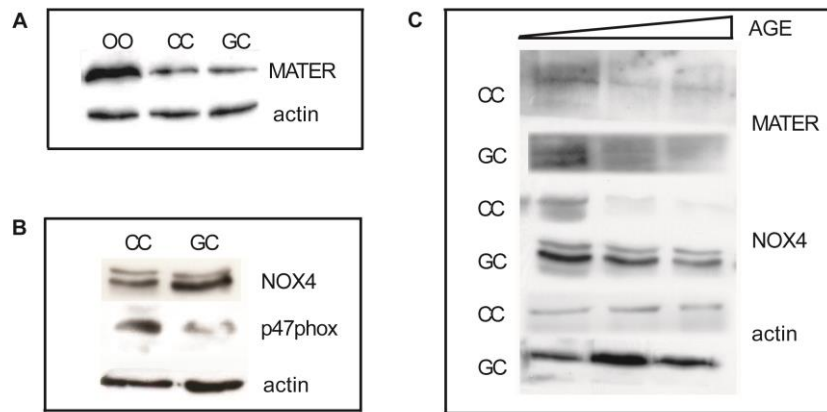


Figure 1

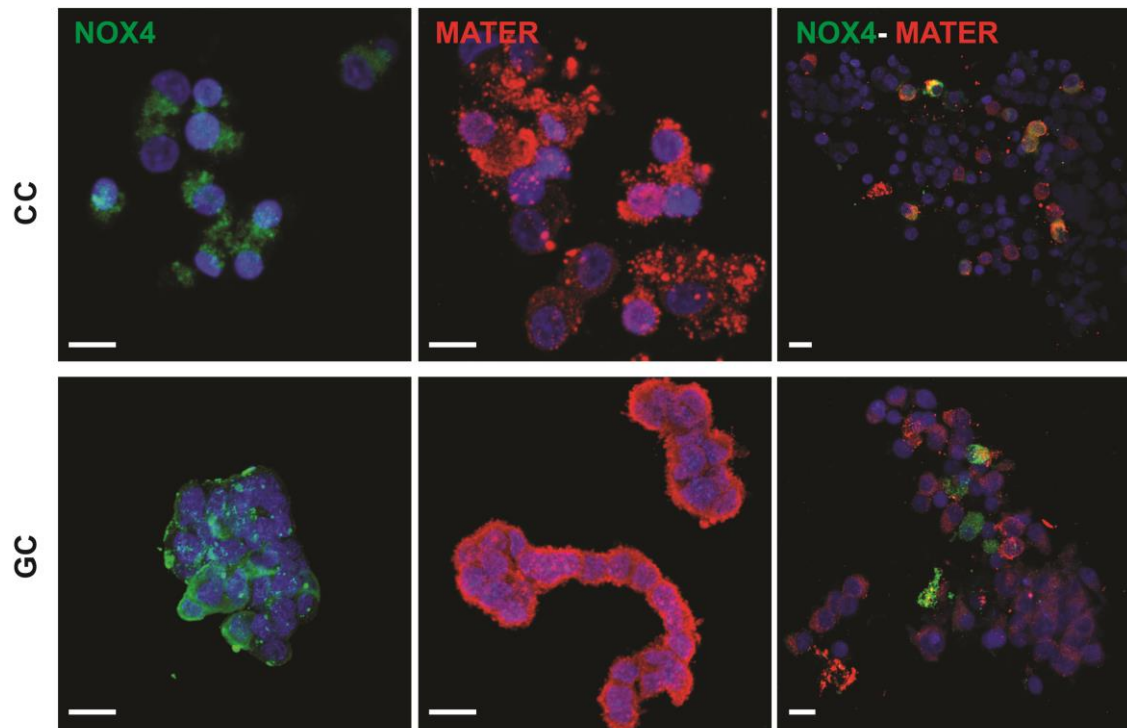


Figure 2

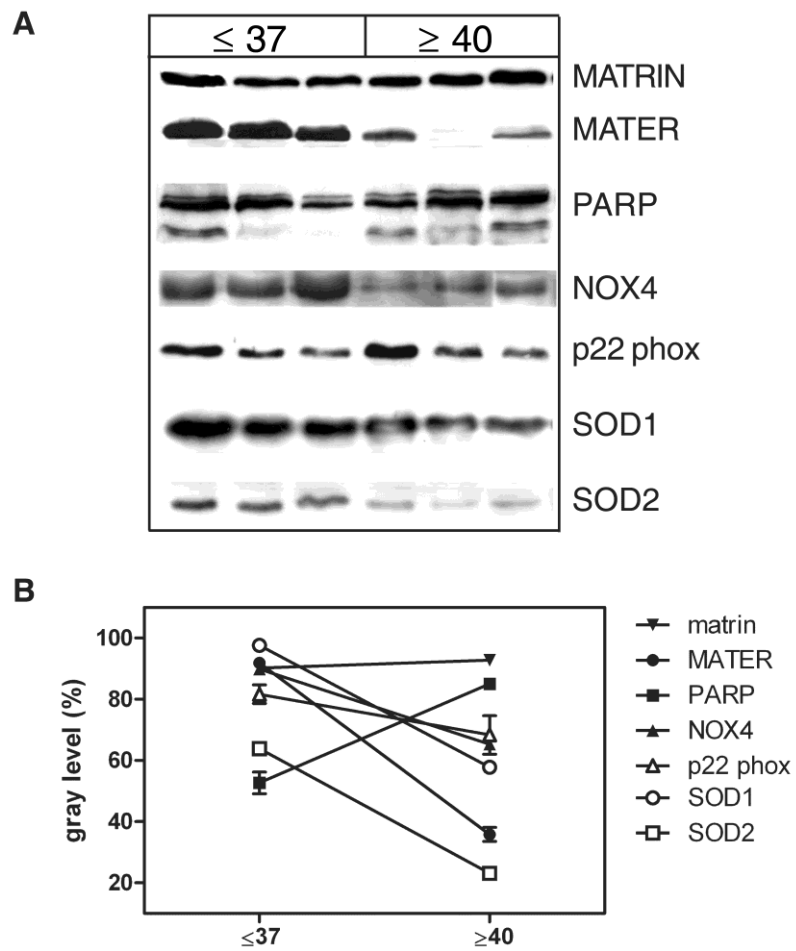
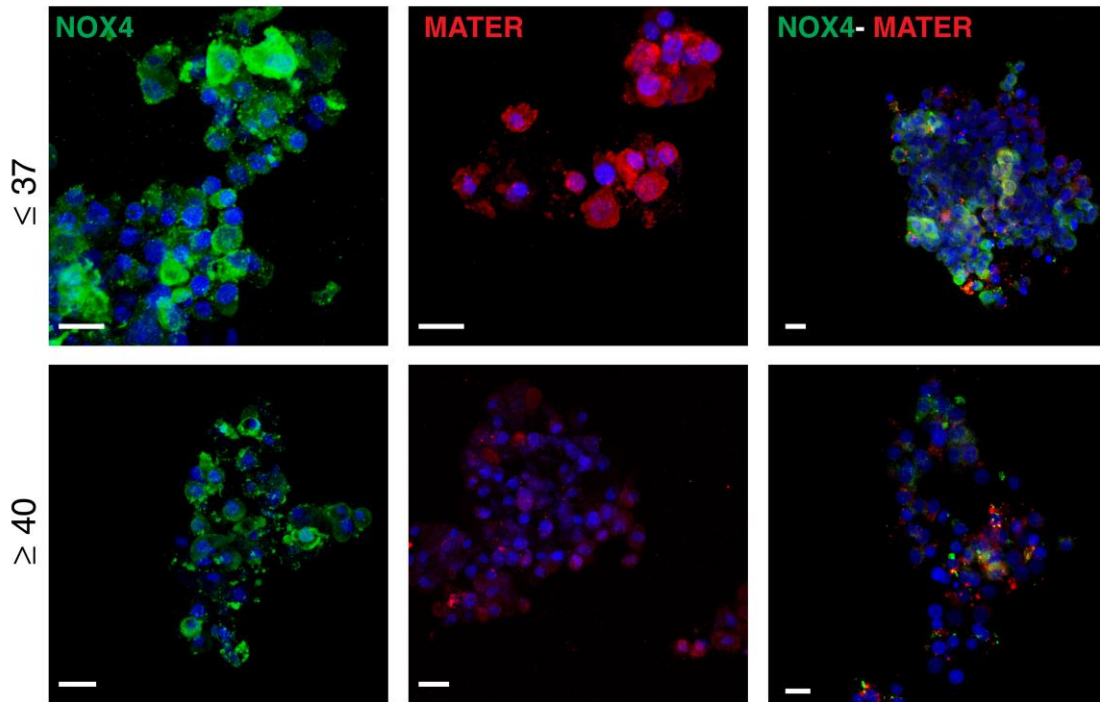


Figure 3

A



B

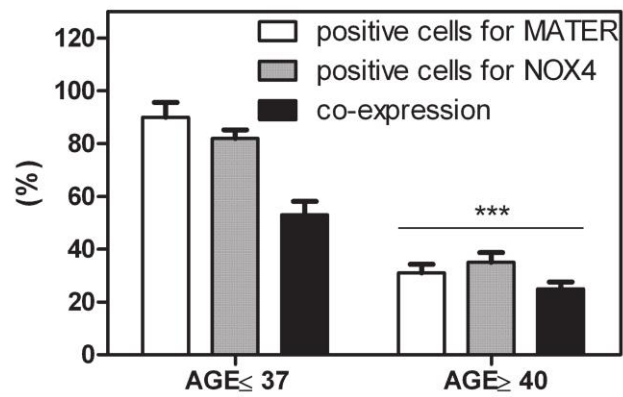


Figure 4