

# Expression of c-Jun in human granulosa cells from patients participating in in vitro fertilization programs

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## Abstract

**Background:** The mitogen-activated protein kinase (MAPK) pathway is one of the major signaling pathways that transmit intracellular signals initiated by extracellular stimuli to the nucleus. The stress-activated protein kinase (SAPK)/c-Jun NH<sub>2</sub>-terminal kinase is a subfamily of MAP kinases implicated in cytokine and stress responses.

**Objective:** In this study, we have examined total and phosphorylated c-Jun in the mural and cumulus granulosa cells, and investigated also whether c-Jun can be responsible for the difference in the expression of apoptosis between mural and cumulus regions.

**Materials and Methods:** A total of 14 consecutive couples participating in IVF program were investigated. Aspirated follicular fluid was transferred into tissue culture dishes and oocyte-cumulus cells complexes were isolated. The cells were centrifuge and fixed with Bouin's solution and then were put on a glass slide. After fixation, the slides were stained by immunocytochemistry method.

The incidence of apoptotic granulosa cells was examined by a fluorescence microscope.

**Results:** The incidence of apoptotic granulosa cells was  $1.27 \pm 0.12$  in the mural region and  $0.38 \pm 0.07$  in the cumulus regions.

All mural and cumulus cells expressed total c-Jun in 7 patients while phosphorylated c-Jun was also expressed in all cells of the other 7 patients. There was no difference between apoptotic and nonapoptotic cells in the expression of total and phosphorylated c-Jun.

**Conclusion:** C-Jun may not be responsible for apoptotic effect on mural and cumulus cells.

**Key words:** Apoptosis, C-Jun, Granulosa cells, In vitro fertilization, Human.

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## Introduction

Programmed cell death (apoptosis) is an essential process in the regulation of cell numbers in various organs and tissues through a mechanism triggered by the activation of a molecular pathway that causes caspase mediated proteolytic cleavage of intracellular proteins (1-2). Consequently, cells dying by apoptosis undergo drastic and distinctive

morphological alterations such as cytoplasmic shrinkage, chromatin condensation, nuclear convolution and budding leading to the formation of apoptotic bodies (3). Apoptotic bodies are engulfed by macrophages or other adjacent cells and prevent extracellular release of the content of dying cells and thereby any subsequent local inflammatory reaction (4-6). The mitogen-activated protein kinase (MAPK) pathway is one of the major signaling pathways that transmit intracellular signals initiated by extracellular stimuli to the nucleus. The MAPK pathway regulates a variety of cellular functions, including cell proliferation, differentiation, and death (7-9).

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The MAPK pathway includes three distinct components: MAPKs, MAPK kinases (MAPKKs), and MAPKK kinases (MAPKKKs). MAPKKKs phosphorylate and activate MAPKKs, which in turn phosphorylate and activate MAPKs. When activated, MAPKs phosphorylate various proteins that include transcription factors, thereby regulating gene expression or other cellular functions. The family of mammalian MAPKs includes three subfamilies: extracellular signal-regulated kinase (Erk), p38MAPK and stress-activated protein kinase (SAPK)/c-Jun NH<sub>2</sub>-terminal kinase (JNK) (10-16).

The JNKs, are a subfamily of MAP kinases implicated in cytokine and stress responses (17). Inhibition of c-Jun either by a dominant negative mutant or by a neutralizing antibody led to reduced apoptosis upon nerve growth factor withdrawal in rat sympathetic neurons. Similarly, interference with c-Jun activity reduced apoptosis in human monoblastic leukemia cells (18-20). Persistent activation of JNK/SAPKs by dominant active MEKK-1 resulted in hyperphosphorylation and activation of c-Jun and increased apoptosis in pc12 cells (21, 22). The incidence of apoptotic bodies has been shown to be significantly prognostic in neoplasms such as non-Hodgkin's lymphoma, prostatic intraepithelial neoplasia, and prostatic cancer (23, 24).

A study showed higher significant incidence of apoptotic bodies in mural granulosa cell masses than in cumulus cell masses (25). Although they used from exogenous gonadotropin in their study, this phenomenon may reflect the differentiation of each region during follicular maturation (26, 27). Expression of c-Jun in the absence of c-fos may be implicated in programmed granulosa cell death in rat ovarian follicles (28). Phospho c-Jun was exclusively expressed in mitotic granulosa cells of follicles from transitional to antral stages in mouse ovarian follicles (29).

The proto-oncogenes, c-fos and c-Jun, are expressed during granulosa cells mitosis, in rat ovarian follicles (30). TNF receptor type 1 (TNFR1) increased c-Jun by activating stress-activated protein kinase/c-Jun-NH<sub>2</sub>-terminal kinase signaling via TNFR1 in mouse granulosa cells, and the induced c-Jun resulted in increased cell proliferation (31). In this study we have examined total and phosphorylated c-Jun in the mural and cumulus granulosa cells and investigated whether c-Jun can be responsible for the difference in the expression of apoptosis between mural granulosa and cumulus regions.

## Materials and methods

### Patients and follicle aspiration

A total of 14 consecutive couples with tubal infertility and a normal semen analysis were analyzed from Jun 2001 to October 2001 at Yamagata University Hospital, Yamagata, Japan. The mean age of the study group was 35 years (range, 28 to 41 years). For ovulation induction protocol, a GnRH analogue, buserelin acetate (Suprecur nasal; Hoechst, Tokyo, Japan) in along suppression protocol were started from mid-luteal phase. Human menopausal gonadotropin (150 to 300 IU/d, Humegon; Sankyo, Tokyo, Japan) with or without FSH (Fertinom P; Serono, Tokyo, Japan) was started on day 3 of the menstrual cycle. Human chorionic gonadotropin (10,000 IU; Mochida, Tokyo, Japan) was administered when one follicle achieved a mean diameter of 16 mm or more. Thirty-five hours after the administration of hCG, follicles were aspirated by transvaginal ultrasound retrieval (6.5 MHz; Mochida). All follicles with a mean diameter of 11 mm were aspirated using 20-mL syringes for suction.

### Cell fixation

Aspirated follicular fluid (FF) was transferred into tissue culture dishes (Falcon 3002; Becton Dickinson and company, Lincoln Park, NJ). Oocyte-cumulus cells complexes were isolated under a dissecting microscope (Olympus, Tokyo, Japan) and were put into an organ tissue culture dish (Falcon 3037; Becton Dickinson and Company) with human tubal fluid (HTF) medium (32). The mural granulosa cell masses were put into another dish with HTF medium. After oocytes were isolated mechanically from cumulus cell masses using 26-gauge needles, the cumulus cell masses were transferred into a centrifuge tube. Mural granulosa cells were also collected into another centrifuge tube, washed three times with PBS, then granulosa cells were transferred into a new centrifuge tube. Hyaluronidase solution (Sigma, St. Louis, MO, 0.1% w/v in HTF medium) was added into the tubes containing cumulus cell masses or mural granulosa cell masses (0.05% w/v of final concentration). Both kinds of cells masses were then pipetted thoroughly for 10 minutes, and then PBS was added to the centrifuge tube. The cells were centrifuged for 5 minutes with 1500 g. After second centrifuge, the cells were finally fixed with Bouin's solution for 25hr at room temperature. This fixation process was completed within 1hr after follicle aspiration to avoid post-aspiration cell death. After filtrating with nital

(Swiss Nylon Monofilament, NRK, Japan) and pipetting of the solution, the cells were put on a glass slide.

### Immunocytochemistry

After fixation, the slides were washed by PBS overnight, permeabilized for 2hr with 0.3% triton X-100 in PBS, washed three times with MilliQ and incubated with 3% BSA in PBS as a blocking solution for 30 min. They were washed with MilliQ once and then with PBS twice for 5 minutes.

The slides were incubated with c-Jun (#9162, Cell signaling technology, Beverly, MA) antibody at a dilution of 1:50 overnight in 4°C. Phospho-C-Jun (#9261, Cell signaling technology, Beverly, MA) antibody at a dilution of 1:1000 was also used in another procedure. For the negative control first antibodies were omitted. After washing in PBS, the slides were incubated with biotinylated goat anti rabbit IgA for 1hr as secondary antibody at a dilution of 1:300 (6). After washing in PBS, Streptavidin, Alexa 488 conjugate (S-11223; Eugene, Oregon USA) was used for antibody visualization (33).

The slides were washed by PBS three times and the nuclei of cells were stained with 0.5 µl of Hoechst 33258 (Wako, Osaka, Japan) with 0.5% w/v of DABCO (1,4-diazabicyclo-2,2,2-Octane, Sigma) in 90% glycerol:10% 0.2m Na<sub>2</sub>HPO<sub>3</sub>. Finally, the slides were cover-slipped, and then were observed using an Olympus Fluorescence microscope with a magnification of X200. A filter for the wavelength of 330-380 nm was used to search for Hoechst 33258 and a filter for the wavelength of 450-490 nm was employed for the Alexa green signal of the mural and cumulus cells. The microscope was equipped with a digital imaging camera.

### Quantification of apoptotic cells

The apoptotic changes recognized under light microscope are pyknosis of nuclei, decrease in cell volume, extraordinary surface convolutions, and the explosion of the cell into cytoplasmic fragments containing condensed chromatin. In this study apoptotic cells were defined as the cells containing fragments of condensed chromatin when examined by the fluorescence microscopy. One thousand granulosa cells were observed at random and the apoptotic cells were identified.

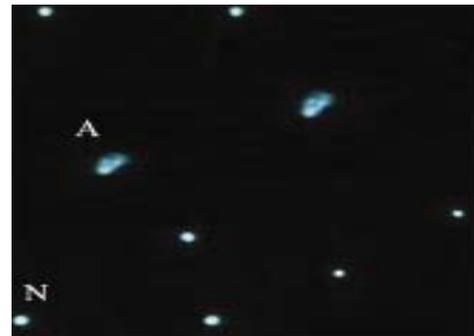
### Statistical analysis

All results expressed as means ± SEM values. Data were analyzed by sign rank test. Means were

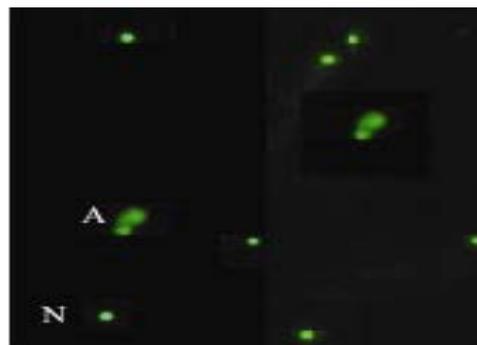
considered statistically significant at  $p < 0.05$ .

## Results

There was no difference in morphological features of apoptotic cells between mural and cumulus cells under fluorescence microscopy. For all patients (n=14), the incidence of apoptotic cells in mural granulosa cell masses was  $1.27 \pm 0.12$  and in the cumulus cells was  $0.38 \pm 0.07$ . All mural and cumulus cells expressed total c-Jun in 7 patients and phospholysed c-Jun was also expressed in all cells of the other 7 patients (figures 1, 2). In negative control without primary antibody (c-Jun and phospholysed c-Jun), none of the mural and cumulus cells were stained. There were no difference between apoptotic and nonapoptotic cells regarding the expression of total c-Jun and phosphorylated c-Jun.



**Figure 1.** Fluorescence micrograph ( $\times 100$ ) of normal cell (N) and apoptotic cell (A) stained with Hoechst 33258.



**Figure 2.** Fluorescence micrograph ( $\times 100$ ) of normal cell (N) and apoptotic cell (A) stained with phospholysed c-Jun.

## Discussion

These results provide the first evidence that mural and cumulus granulosa cells have total and phosphorylated c-jun.

In mammalian systems five distinguishable

MAPK modules have been identified so far. The extracellular signal-regulated kinase 1 and 2 (ERK1/2) cascade preferentially regulates cell growth and differentiation. JNK and p38 MAPK cascades mainly function in stress responses like inflammation and apoptosis (34).

In some cells such as rat sympathetic neurons, human monoblastic leukemia cells, 3T3 fibroblasts, pc12 cells, and granulosa cells in rat ovarian follicles c-Jun induces apoptosis and leads to cell death (22-26, 28).

Phospho c-Jun was exclusively expressed in mitotic granulosa cells of follicles from transitional to antral stages in mouse ovarian follicles (29-31).

In this study, we showed that in human mural and cumulus cells, c-Jun doesn't have any effect in apoptosis. But according to another study (Quinn *et al.* 1985), the higher incidence of apoptotic cells in mural regions is higher than that of cumulus cells (32). However, in our study we showed that there was no difference in expression of total and phosphorylated c-Jun between mural and cumulus cells. Both apoptotic and nonapoptotic granulosa cells revealed the expression of total and phosphorylated c-Jun.

In conclusion, these results presented that c-Jun may not be responsible for inducing apoptosis in mural and cumulus granulosa cells. Therefore the functions of c-Jun remain to be studied.

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