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Expression of c-MYC in Nuclear Speckles During Mouse Oocyte Growth and Preimplantation Development

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Abstract. Myelocytomatosis oncogene (*c-myc*) is a major transcriptional regulator that controls various biological processes, and its deregulated expression causes carcinogenesis. To investigate the involvement of *c-myc* in oogenesis and preimplantation development, the expression of c-MYC during these stages was examined by immunocytochemistry. A strong c-MYC signal was detected in the nucleus of growing and fully grown oocytes as well as in preimplantation embryos before the morula stage. The signal intensity decreased slightly at the morula stage, and no signal was detected in blastocysts. Close observation of the nucleus revealed that c-MYC was localized in small granules that appeared to be nuclear speckles controlling pre-mRNA splicing. Although the number of granules decreased during oocyte growth, their size increased. After fertilization, the granules of c-MYC disappeared from the pronuclei, and c-MYC was evenly distributed in the nucleoplasm at the 1-cell stage, but the granules reappeared at the 2-cell stage. These results suggest that *c-myc* is involved in oocyte growth and preimplantation development and that its role changes during these stages.

Key words: *c-myc*, Embryo, Nuclear speckles, Oocyte

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Myelocytomatosis oncogene (*c-myc*) was first identified as the cellular homolog of *v-myc*, the transforming element of avian myelocytomatosis leukemia virus MC29 [1]. It is ubiquitously expressed in normal tissues, but in some tumor cells, its expression is highly elevated or deregulated [2, 3]. While this overexpression is not sufficient for induction of oncogenic transformation, it contributes to the process [4, 5]. In normal cells, *c-myc* plays various important roles in cell survival, including transcriptional regulation, and the gene product, c-MYC protein contains both a leucine zipper and helix-loop-helix motifs, which allow it to bind to specific target sequences [6–8]; moreover, it forms a heterodimer with a protein called MAX, which is necessary for DNA binding, leading to the up- or downregulation of target gene transcription [9]. In addition, c-MYC is involved in DNA replication via a mechanism that does not involve transcriptional activation [10]. The suppression of *c-myc* expression by antisense RNA or specific antibodies inhibits DNA synthesis [11, 12]. Furthermore, *c-myc* is involved in various other cellular functions, such as chromatin modification, apoptosis, and differentiation [13].

C-MYC is an essential factor for normal embryonic development and differentiation. For example, suppression of c-MYC expression by antisense oligonucleotides reduces the developmental rate at the blastocyst stage *in vitro* [14], and a null mutation in *c-myc* results in embryonic lethality before day 10.5 *in vivo* [15]. However, the role of *c-myc* in oogenesis and early preimplantation development remains to be elucidated. Although *c-myc* RNA has been shown to be expressed during oocyte growth and preimplantation development, no data exist regarding the expression of c-MYC during these stages. In oocytes and early preimplantation embryos,

the expression patterns of certain proteins are different from those of the transcripts, since the translation of maternal mRNA is post-transcriptionally regulated in a complex manner [16, 17].

In the present study, we examined the expression of c-MYC during oogenesis and preimplantation development via immunocytochemistry. Our results indicate that c-MYC is expressed in growing and fully grown oocytes and embryos up to the 4-cell stage, but that expression decreases thereafter and is weak in blastocysts. We also found that c-MYC was localized in numerous small granules, which were identified as nuclear speckles.

Materials and Methods

Oocyte collection and culture

Growing oocytes (GOs) and fully-grown oocytes (FGOs) were collected from 12-day- and 8-week-old B6D2F1 female mice, respectively (Japan SLC, Shizuoka, Japan). The ovaries were removed from the mice and transferred to HEPES-buffered KSOM (synthetic oviductal medium enriched with potassium) [18] supplemented with 1 mg/ml bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) and 0.2 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich). The ovarian follicles were punctured using a needle to release the oocytes and their surrounding cumulus cells. The cumulus cells were removed gently using a narrow glass pipette.

In vitro fertilization and embryo culture

Metaphase II (MII)-stage oocytes were collected from 3-week-old female B6D2F1 mice that had been hormonally stimulated with 5 IU of equine chorionic gonadotropin (eCG; ASKA Pharmaceutical, Tokyo, Japan) and treated with 5 IU of human chorionic gonadotropin (hCG; ASKA Pharmaceutical) 48 h later. Superovu-

lated MII oocytes were collected 16 h after hCG injection and inseminated with spermatozoa obtained from male ICR mice (Japan SLC). Before insemination, the spermatozoa were cultured for 2 h in human tubal fluid (HTF) medium [19] supplemented with 10 mg/ml BSA for 2 h at 38°C under 5% CO₂ and 95% air to allow capacitation. Six hours after insemination, the fertilized oocytes were washed and cultured in KSOM medium. The embryos were collected for immunocytochemistry at the following time points after insemination: 1-cell (8 h), 2-cell (28 h), 4-cell (43 h), morula (60 h) and blastocyst stages (96 h).

Immunocytochemistry

The oocytes and embryos were fixed in 2.0% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min, washed with PBS containing 1 mg/ml BSA and permeabilized with 0.5% Triton X-100 for 15 min. The cells were then incubated overnight at 4°C in one of the following primary antibodies (diluted 1:100): anti-c-MYC (N-262; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-RNA polymerase II phosphorylated on serine 2 (H5; ab24758; Abcam, Cambridge, UK) or anti-RNA polymerase II phosphorylated on serine 5 (H14; MMS-134R; Covance, Princeton, NJ, USA). The antibodies were then probed with a 1:100 dilution of fluorescein isothiocyanate-conjugated anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) or Cy3.5-conjugated affinity-purified anti-mouse IgG (Rockland Immunochemicals, Gilbertsville, PA, USA) for 1 h at room temperature and mounted on a glass slide in Vectashield anti-bleaching solution (Vector Laboratories, Burlingame, CA, USA) containing 4',6-diamidino-2-phenylindole (DAPI; Dojindo Laboratories, Kumamoto, Japan). Fluorescence was detected by laser-scanning confocal microscopy (Carl Zeiss MicroImaging, Oberkochen, Germany).

Treatment with transcription inhibitor

To inhibit transcription, 5,6-dichlororibofuranosyl-benzimidazole (DRB; Sigma-Aldrich) was used. GOs were incubated in α -minimum essential medium (α -MEM) in the presence of 0.2 mM IBMX and 120 μ M DRB for 24 h at 38°C under 5% CO₂ and 95% air. After incubation, the oocytes were stained with anti-c-MYC antibodies.

Results

Expression of c-MYC during oocyte growth and preimplantation development

The expression of *c-myc* was examined in oocytes and preimplantation embryos by immunocytochemistry using anti-c-MYC antibodies (Fig. 1). A strong c-MYC signal was observed in the nuclei of GOs, FGOs and embryos before the morula stage. At the morula stage, the c-MYC signal decreased and was weakly detected in blastocysts.

Close investigation of the intranuclear distribution of c-MYC using magnified images revealed that it was concentrated as numerous small granules in GOs (Fig. 2A). In the FGOs, the size of the granules increased, while their number decreased (Fig. 2A and B). The reduced number of granules may be associated with a decrease in transcriptional activity during growth, since active transcription

Fig. 1. Expression of c-MYC during oogenesis and preimplantation development. Growing oocytes (GOs), fully grown oocytes (FGOs) and embryos at the 1-cell, 2-cell, 4-cell, morula and blastocyst stages were immunostained with anti-c-MYC antibodies. DNA was stained with 4',6-diamidino-2-phenylindole. Bar=30 μ m.

occurs in GOs but stops once they are fully grown [20, 21]. This hypothesis is supported by the observation that the number of granules was smaller in FGOs with a surrounded nucleolus (SN)-type nucleus, in which little to no transcription occurs, compared with those with a non-surrounded nucleolus (NSN)-type nucleus, in which active transcription still takes place (Fig. 2A and B) [20, 21]. After fertilization, c-MYC was evenly distributed in the pronuclei, and a few granules were observed, although the signal intensity in the nucleoplasm was greater than that during the other stages (Fig. 2A). Small granules reappeared at the 2-cell stage and were present thereafter until the morula stage.

Localization of c-MYC in nuclear speckles

In somatic cells, c-MYC has been shown to be localized in nuclear speckles containing various splicing factors [22, 23]. Furthermore, the distribution of speckles in somatic cells is very similar to that of the c-MYC granules in GOs and preimplantation embryos [24]. Therefore, the c-MYC granules observed in the nuclei of the oocytes and embryos most likely were also nuclear speckles. To address this issue, we immunostained FGOs with two different anti-phospho RNA polymerase II antibodies, H5 (phosphorylated on serine 2) and H14 (phosphorylated on serine 5), in accordance with data showing that nuclear speckles may be immunostained with H5 but not H14 [24–27]. Our results indicate that c-MYC was co-localized with RNA polymerase II phosphorylated on

Fig. 2. Intranuclear localization of c-MYC. (A) Growing oocytes (GOs), fully-grown oocytes (FGOs) with a surrounded nucleolus (SN)-type nucleus (FGO-SN) or non-surrounded nucleolus (NSN)-type nucleus (FGO-NSN) and embryos at the 1-cell, 2-cell, 4-cell and morula stages were immunostained with anti-c-MYC antibodies. DNA was stained with 4',6-diamidino-2-phenylindole. Bar=5 μ m. (B) Determination of the number of granules immunostained with anti-c-MYC antibodies. The number of granules was counted in GOs (n=12) and FGOs with a SN-type nucleus (n=21) or NSN-type nucleus (n=17). The data are presented as the mean $\pm$ SEM.

Fig. 3. Co-localization of c-MYC with phosphorylated RNA polymerase II. Fully grown oocytes were double-stained with anti-c-MYC and one of two anti-phosphorylated RNA polymerase II antibodies [RNA polymerase II phosphorylated on serine 2 (Ser2) or serine 5 (Ser5)]. Arrowheads indicate the c-MYC granules. Bar=5 μ m.

Fig. 4. Morphological changes in the c-MYC granules after inhibition of transcription with 5,6-dichlororibofuranosyl-benzimidazole (DRB). Growing oocytes were incubated in the presence or absence of 120 μ M DRB for 24 h and then stained with anti-c-MYC antibodies. DNA was stained with 4',6-diamidino-2-phenylindole. Bar=10 μ m.

serine 2 but not on serine 5 (Fig. 3), suggesting that c-MYC is localized in nuclear speckles during oocyte growth and preimplantation development.

To confirm that c-MYC is localized in nuclear speckles in oocytes, transcription was inhibited in GOs based on the observation that the number of nuclear speckles in somatic cells decreases,

while their size increases, when transcription is inhibited [27, 28]. In oocytes treated with DRB, a potent inhibitor of transcription, the number and size of c-MYC granules also decreased and increased, respectively (Fig. 4).

Discussion

In this study, we analyzed the expression pattern of c-MYC during oogenesis and preimplantation development and found that c-MYC was expressed in GOs, FGOs and embryos until the morula stage, but not in blastocysts (Fig. 1). Inspection of its intranuclear distribution revealed that c-MYC was concentrated in small granules, which we experimentally identified as nuclear speckles (Fig. 3 and 4). The distribution pattern in these granules changed before and after fertilization. Specifically, their number and size decreased and increased, respectively, during oocyte growth. The granules disappeared at the 1-cell stage and then reappeared at the 2-cell stage (Fig. 2). Thus, the distribution of c-MYC changes dynamically during oocyte growth and preimplantation development, suggesting its function during these stages.

In nuclear speckles, c-MYC may be involved in regulation of pre-mRNA splicing. Nuclear speckles are thought to control pre-mRNA splicing and mRNA export in mammalian cell nuclei because the splicing machinery for premature mRNAs, including small nuclear ribonucleoprotein particles (snRNPs), spliceosome subunits and other non-snRNP protein splicing factors such as SC-35, accumulate in these small nuclear organelles. Once transcription becomes active and pre-mRNAs are synthesized, the splicing machinery is assembled in speckles and transported toward peripheral chromatin [26, 27]. Therefore, c-MYC appears to be involved in pre-mRNA splicing. Our results support this hypothesis based on the changes in c-MYC distribution during transcription: the splicing machinery works in conjunction with transcription. Several small c-MYC granules were observed in the GOs, that appeared to cluster together to form a small number of large granules when the oocytes were fully grown (Fig. 2). In GOs, in which active transcription occurs to support a rapid increase in volume and accumulation of maternal mRNAs and other transcripts [29, 30], nuclear speckles localize near sites of transcription for efficient splicing of newly synthesized pre-mRNAs. In FGOs, however, nuclear speckles are not necessary; thus, they gather to form a few large speckles due to the lack of transcription [20, 21]. After fertilization, a few nuclear speckles were seen in the 1-cell embryos, in which transcription is still inert. At the 2-cell stage, however, the speckles reappeared when transcription of the zygotic genes began. The requirement for splicing machinery in the nuclear speckles and changes in the nuclear distribution of c-MYC during oocyte growth and preimplantation development suggest the involvement of c-MYC in pre-mRNA splicing.

C-MYC has been shown to function in cellular processes other than pre-mRNA splicing, including transcription, DNA replication and chromatin modification in the nucleoplasm. The concentration of c-MYC in the nucleoplasm was high at the 1-cell stage, but decreased during preimplantation development. At the blastocyst stage, no c-MYC was detected (Figs. 1 and 2). Preimplantation embryos are known to share certain characteristics with cancer

cells. For example, both cancer cells and preimplantation embryos lack cell-cycle checkpoints and thus proliferate at a fast rate. Furthermore, they can grow without serum *in vitro* [31, 32]. *C-Myc* may be involved in the formation of these characteristics in embryos since it contributes to the induction of oncogenic transformation in somatic cells [4, 5]. After fertilization, c-MYC may be present at a high concentration in the nucleoplasm of 1-cell embryos to establish these cancer cell-like characteristics. At the blastocyst stage, these characteristics may be lost due to the decrease in c-MYC.

In conclusion, we confirmed the expression of c-MYC in mouse oocytes and preimplantation embryos and found that its intranuclear distribution changes dynamically during oocyte growth and preimplantation development. This information will be useful for clarifying the functions of *c-myc* during these stages.

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