

# Immunocytochemical Study of Mouse Oocytes Suggests Conserved Keratin Organisation in Tetrapod Oogenesis

Venera P. Nikolova, Stefka M. Delimitreva, Ralitsa S. Zhivkova, Irina V. Chakarova, Valentina P. Hadzhinesheva, Maya D. Markova

Department of Biology, Medical Faculty, Medical University of Sofia, Bulgaria; E-mail: veneran@yahoo.com

**Abstract:** Cytokeratins were localised in oocytes of laboratory mice at different maturation stages and the results were compared to data about other mammals and nonmammalian vertebrates. In prophase I (germinal vesicle stage), keratins were localised in cortical and perinuclear position in compliance with published reports for amphibians, reptiles and mammals. In oocytes at the stage of metaphase I and mature metaphase II, the immunofluorescent reaction in the cortex was no longer as obvious as in previous stages, suggesting relaxation of keratin cytoskeleton. While for mammals there is controversy in the literature among reports related to oogenesis of different species, our finding is fully consistent with results obtained for amphibians (*Xenopus*) and, for the first time, suggests uniform cytokeratin distribution pattern during tetrapod oogenesis. This evolutionary conserved localisation and reorganisation from germinal vesicle stage to metaphase II suggests important, though still largely unknown, functions of the oocyte keratin cytoskeleton.

**Key words:** oogenesis, mammalian oocytes, cytokeratin, immunofluorescence

## Introduction

Cytokeratins are known to be present in oocytes of different vertebrates but few studies have systematically addressed their organisation at consecutive stages of oogenesis. The preliminary and fragmentary nature of available studies, especially on mammals, has not allowed to summarise data across vertebrate classes. Keratins have been found by biochemical methods in eggs of teleost fishes *Cyprinus carpio* (see MENCARELLI, COTELLI 1997) and *Danio rerio* (see CHUA, LIM 2000) and immunocytochemically localised in oocytes of the amphibians *Xenopus laevis* (see TORPEY *et al.* 1992; GARD *et al.* 1997), the reptiles *Podarcis sicula* (MAURIZII *et al.* 1997) and various mammals (GALLICANO *et al.* 1994; KABASHIMA *et al.* 2010). In *Xenopus*, which has been studied in most detail, keratin cytoskeleton has been shown to assemble in the oocyte cortex and around the germinal vesicle (GV) of early oocytes, to become interconnected and polarised at later stages and to disassemble by the end of oocyte maturation (GARD *et al.* 1997; CLARKE, ALLAN 2003; KLOC *et al.* 2005). In the lizard *Podarcis*, it first appears in growing oocytes as a thin cortical layer and becomes thicker by the end of previtellogenic oocyte growth; no data have been reported for later stages (MAURIZII *et al.* 1997). Studies on mammals so far have been typically limited to one stage of oogenesis, such as mature eggs (GALLICANO *et al.* 1994), and have generated results that are not consistent with those for other vertebrates. The most systematic work, done on hamster oocytes (KABASHIMA *et al.* 2010), describes delicate keratin network in the GV periphery and large cortical aggregates in prophase-arrested oocytes. During oocyte maturation, cortical aggregates break into small granules while the filament network extends over the ooplasm. The authors characterised the

observed keratin reorganisation as increasing complexity, which is the exact opposite of the changes in *Xenopus* oogenesis. We detected cytokeratins in mouse oocytes in a preceding study (NIKOLOVA *et al.* 2011) and showed them to be a component of extraction-resistant cytoskeletal structures. In the present work, we performed an immunolocalisation of a set of cytokeratins in mouse oocytes at different maturation stages in order to compare mammalian keratin reorganisation with the pattern described in oogenesis of other vertebrates.

## Materials and Methods

Mouse oocytes were obtained following the protocol used in our earlier study (NIKOLOVA *et al.* 2011). Prepubertal laboratory BALB/c females were stimulated with 5 IU of Menogon (follicle-stimulating hormone) and, 48 hours later, with 5 IU of Pregnyl (luteinizing hormone). After additional 16-18 hours, mature oocytes at metaphase II stage were isolated from the oviducts and immature oocytes at GV and metaphase I stages were retrieved from unovulated follicles.

Immunofluorescent localisation was performed on whole-mounts of oocytes and cumulus-oocyte complexes. Cells were fixed in 2% paraformaldehyde and the immunofluorescent localisation was performed as previously described (DELIMITREVA *et al.* 2006). Cytokeratins were labelled by monoclonal antibody clone PCK-26 (Sigma-Aldrich) recognising type II cytokeratins 1, 5, 6 and 8, diluted 1:300. In control samples, this primary antibody was omitted. After washing, oocytes were incubated with FITC-conjugated secondary antibody (Sigma-Aldrich) diluted 1:300 and washed again. Chromatin was visualised using Hoechst 33258 (Sigma-Aldrich). The reaction was then observed by epifluorescence using fluorescent microscope Axioskop 20 (Zeiss).

## Results

In immature (GV stage) oocytes, cytokeratins were observed in two regions: the cytoplasmic cortex underlying the cell membrane and the prophase nucleus. Initially, staining of the nuclear region was confined to the periphery. However, after formation of the dense spherical heterochromatin nucleolar rim, i.e. karyosphere (DE LA FUENTE *et al.* 2004), bright reaction associated with this structure was detected but not spreading inside the nucleolus (Fig. 1).

The observed distribution of cytokeratins changed after germinal vesicle breakdown. In oocytes at metaphase I, there was a bright staining in the cap-

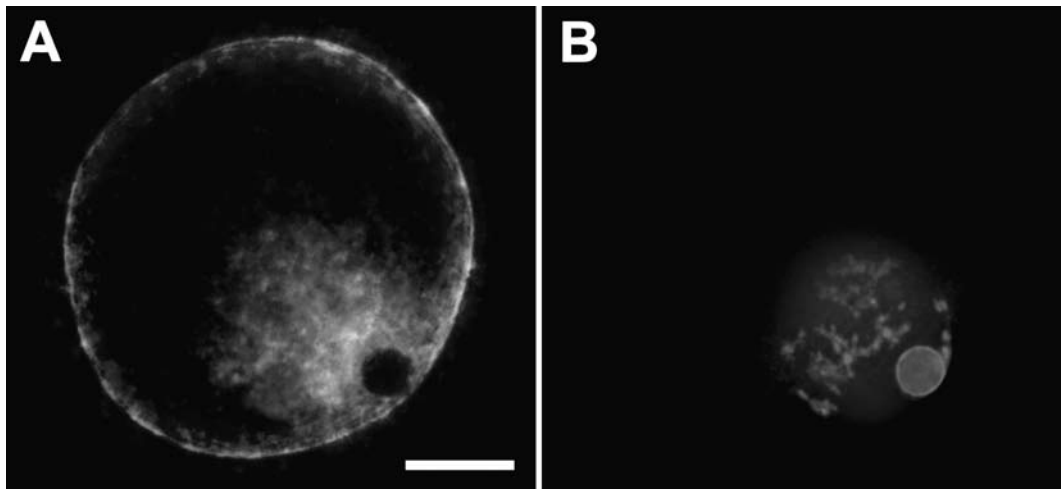
shaped cortical region overlying the meiotic spindle. The intensity of fluorescence in the rest of the cortex was diminished. The spindle itself was also stained, and the metaphase chromosomes produced strong reaction (Fig. 2). The same staining pattern was observed in mature metaphase II oocytes.

No reaction was observed in negative controls.

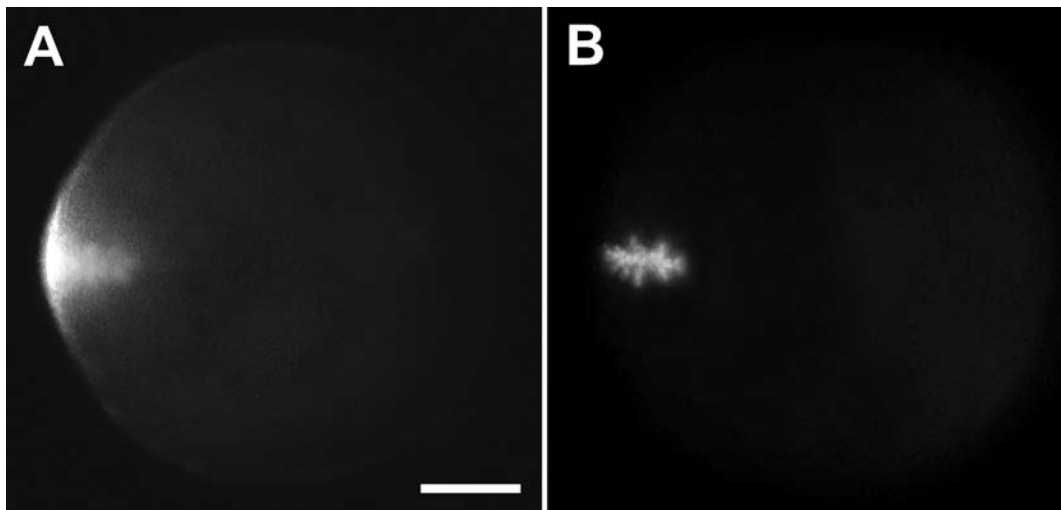
## Discussion

The aim of the present study was not only to characterise the intracellular distribution of cytokeratins at different stages of mouse oogenesis but also to compare the findings to data reported for other vertebrates in order to obtain insights about the evolution and function of keratin cytoskeleton in oocytes. Such phylogenetic approach often helps to evaluate the biological significance of cellular structures (MARINOVA *et al.* 1989). At present, oocyte cytokeratins have been studied immunocytochemically in tetrapods from three classes – amphibians, reptiles and mammals. For all of them, keratin structures have been localised to the cortex of immature oocytes in prophase I, and for *Xenopus* and mammals, reaction has been observed also at the nuclear periphery (GARD *et al.* 1997; MAURIZII *et al.* 1997; KABASHIMA *et al.* 2010). Therefore, our results and published reports of other authors suggest a conserved pattern of keratin distribution in early (GV stage) oocytes of different tetrapods.

For later stages of oogenesis, however, such consensus of data does not exist. These stages are excluded altogether from the study on lizards (MAURIZII *et al.* 1997), presumably because the immunolocalisation of proteins of yolk mass in late-stage (i.e. vitellogenic) oocytes of nonmammalian amniotes poses great technical difficulties. In *Xenopus*, the keratin cytoskeleton has been reported to disintegrate during oocyte maturation (GARD *et al.* 1997; CLARKE, ALLAN 2003; KLOC *et al.* 2005). For mammals, the authors of a detailed study on hamster (KABASHIMA *et al.* 2010) describe fragmentation of cytokeratins in the cortex after GV breakdown and interpret these data as increasing complexity of the keratin network with a possible role in maintaining cell integrity under physical stress after ovulation. This poses the question whether there is a major difference in the intracellular distribution of cytokeratins between amphibians and mammals. Our results show decreased reaction for keratins in mouse cortical cytoplasm after transition to metaphase I. This is consistent with the disassembly observed by several authors in maturing frog oocytes (GARD *et al.* 1997; CLARKE, ALLAN 2003; KLOC *et al.* 2005) and opposite to the increas-



**Fig. 1.** Fluorescent image of a GV stage oocyte stained for cytokeratins (A) and chromatin (B). Reaction for cytokeratins is seen in the cortical layer and the region of the germinal vesicle, including the karyosphere. The inside of the nucleolus remains unstained. Bar = 20  $\mu$ m



**Fig. 2.** Fluorescent image of a metaphase I oocyte stained for cytokeratins (A) and chromatin (B). Bright fluorescent reaction is seen in metaphase plate and the cap-shaped cortical region overlying it, while staining in the rest of the cortex is diminished. Bar = 20  $\mu$ m

ing complexity reported for hamster (KABASHIMA *et al.* 2010). The present findings, compared to the above cited studies on *Xenopus*, suggest that animals occupying most distant positions in the evolutionary tree of extant tetrapods (amphibians and mammals) share a conserved pattern of cytokeratin distribution in maturing oocytes from GV to metaphase II stages. It does not seem very likely that the mouse oocyte, while showing a pattern essentially similar to that of *Xenopus*, would differ dramatically from that of the hamster, a member of the same mammalian order (Rodentia). An alternative possibility is that the findings reported by KABASHIMA *et al.* (2010) could actually reflect a transition to a more dynamic structure. Our previous chemical dissection experiments have

shown that intermediate filaments of mature mouse eggs have lost the extraction resistance possessed by immature GV-stage oocytes (NIKOLOVA *et al.* 2011). A study on the mechanical properties of *Xenopus* egg cytoplasmic extracts has found that cytokeratins do not contribute to viscoelasticity (VALENTINE *et al.* 2005).

The observed keratin localisation in this study, combined with data obtained by other authors on nonmammalian vertebrates, implies structural uniformity across tetrapods, suggesting a possible similarity of functions. The maternal cytokeratins stored in the oocytes could be used by the early embryo. This hypothesis has been confirmed for *Xenopus*, where experimental depletion of oocyte cytokeratins

results in loss of the compacted epithelial surface of the blastula and defective gastrulation (TORPEY *et al.* 1992). It is more difficult to ascribe a function to cytokeratins during oogenesis. The well-developed keratin cortex of GV-stage oocytes in members of three tetrapod classes (amphibians, reptiles and mammals) suggests a mechanical function in supporting the periphery of the prophase oocyte and its contacts with the surrounding follicle cells. This keratin cortex, however, is relaxed after resumption of meiosis both in *Xenopus* and in the mouse oocytes studied by us. This probably reflects the disconnection of the oocyte from surrounding follicle cells and the increase of cytoskeletal dynamics needed to prepare the oocyte for polar body extrusion and zygote cleavage.

The other domain of prophase oocytes where keratins were found in our study, the nuclear periphery, corresponds to data reported for both *Xenopus* (GARD *et al.* 1997) and hamster oocytes (KABASHIMA *et al.* 2010). This suggests a possible contribution of cytokeratins to the meiotic reorganisation of nucleus and chromatin. It has been reported that keratin intermediate filaments in *Xenopus* eggs serve as docking sites for vesicles obtained from the nuclear envelope and facilitate its reassembly (ZHANG *et al.*

1998). Hence, the association of cytokeratins with the meiotic spindle and chromosomes at metaphase I and II observed in the present study could have the function of keeping nuclear envelope components in the vicinity of chromosomes. The bright cap-shaped staining observed in front of the spindle suggests that keratin filaments may also contribute to its anchoring in subplasmalemmal position. In light of the strikingly conservative keratin distribution in oocytes of some tetrapods, it could be expected that more detailed investigations will detect a similar association of keratin with spindle and chromosomes in nonmammalian metaphase oocytes.

## Conclusion

Our immunocytochemical study characterised the intracellular localisation of a set of cytokeratins in mouse oocytes and suggested uniform conservative keratin distribution pattern in oogenesis of most tetrapods, undergoing similar processes of reorganization from germinal vesicle stage to metaphase II.

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