The nuclear mitotic apparatus (NuMA) protein: localization and dynamics in human oocytes, fertilization and early embryos

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Submitted on November 17, 2010; resubmitted on January 21, 2011; accepted on February 2, 2011

ABSTRACT: The oocyte’s meiotic spindle is a dynamic structure that relies on microtubule organization and regulation by centrosomes. Disorganization of centrosomal proteins, including the nuclear mitotic apparatus (NuMA) protein and the molecular motor complex dynein/dynactin, can lead to chromosomal instability and developmental abnormalities. The present study reports the distribution and function of these proteins in human oocytes, zygotes and early embryos. A total of 239 oocytes, 90 zygotes and discarded embryos were fixed and analyzed with confocal microscopy for NuMA and dynactin distribution together with microtubules and chromatin. Microtubule-associated dynein-dependent transport functions were explored by inhibiting phosphatase and ATPase activity with sodium-orthovanadate (SOV). At germinal vesicle (GV) stages, NuMA was dispersed across the nucleoplasm. After GV breaks down, NuMA became cytoplasmic before localizing at the spindle poles in metaphase I and II oocytes. Aberrant NuMA localization patterns were found during oocyte in vitro maturation. After fertilization, normal and abnormal pronuclear stage zygotes and embryos displayed translocation of NuMA to interphase nuclei. SOV treatment for up to 2 h induced lower maturation rates with chromosomal scattering and ectopic localization of NuMA. Accurate distribution of NuMA is important for oocyte maturation, zygote and embryo development in humans. Proper assembly of NuMA is likely necessary for bipolar spindle organization and human oocyte developmental competence.

Key words: oocyte quality / MII spindle / microtubules / NuMA / centrosomes

Introduction

Oocyte maturation is a complex process that is controlled by cellular and molecular mechanisms leading to cytoplasmic and nuclear maturation, a necessary step for successful meiosis, fertilization, embryo development and implantation (Schroeder and Eppig, 1984; Abbott and Ducibella, 2001; reviewed in Sun and Nagai, 2003; Combelles and Racowsky, 2005).

At birth, the mammalian oocyte is arrested in diakinesis of prophase I (germinal vesicle, GV). Upon meiotic resumption after puberty, the GV breaks down (GVBD) with subsequent polymerization of cytoplasmic tubulin into microtubules as chromosomes begin to condense. Interactions between microtubules and chromosomes are already established when oocytes enter metaphase I (MI). After a short anaphase and telophase I, the first polar body is extruded, the second meiotic spindle organizes and the oocyte becomes arrested at metaphase-II (MII) until fertilization takes place.

The majority of oocytes recovered after ovarian stimulation are arrested at MII; most of them (70–80%) fertilize and 50% of generated zygotes are able to complete development to the blastocyst stage (Jones, 2000). In contrast, when immature oocytes are recovered and matured in vitro, only a small fraction progresses to the blastocyst stage and eventually develops to term (Veeck et al., 1983; Nagy et al., 1996; Chen et al., 2000). Inefficient cytoplasmic maturation may be responsible for this failure. The events responsible for optimal coordination of nuclear and cytoplasmic maturation have gained increasing attention during the past few years.

The organization of a bipolar meiotic spindle provides the framework for translocation of chromosomes and regulatory components essential for final oocyte maturation (reviewed in Brunet and Maro, 2005).
2005; Wang and Sun, 2006; Sun and Schatten, 2006). Centrosomes are key organizers of the meiotic spindle. In somatic cells, they are typically composed of a pair of centrioles surrounded by a proteinaceous scaffold containing numerous centrosome and centrosome-associated proteins, including the centrosome core protein γ-tubulin and the nuclear mitotic apparatus (NuMA) protein. Meiotic spindle centrosomes in oocytes lack centrioles and are most commonly referred to as acentriolar centrosomes (Calarco-Gillam et al., 1983; Calarco, 2000; Lee et al., 2000; reviewed in Schatten, 2008, Schatten and Sun, 2009a,b, 2010; Schatten et al., 2011). Dynein/dynactin function is necessary for NuMA’s association with the centrosome for precise microtubule organization and subsequent accurate chromosome segregation (Merdes et al., 1996, 2000; Fan and Sun, 2004; reviewed in Sun and Schatten, 2006). Much of our knowledge on the organization of the female meiotic spindle has been obtained from laboratory and farm animal species (Merdes et al., 1996; Lee et al., 2000). Due to the scarcity of human oocytes, little is known about the cellular and molecular events that regulate spindle functions during in vivo and in vitro maturation (IVM) and its impact on developmental competence of human oocytes.

In the present study, we assessed the distribution of NuMA and dynactin by immunocytochemistry (ICC) and laser confocal microscopy in in vivo and in vitro matured human oocytes, zygotes, early embryos and fertilization failures. Sodium-orthovanadate (SOV, a known phosphatase and ATPase activity inhibitor) was used to explore the role of ATPase-dependent dynein transport and its effect on NuMA dynamics during human oocyte maturation.

Materials and Methods

Source of oocytes

All human oocytes, zygotes and embryos were donated for research with informed written consent by couples undergoing assisted reproduction procedures at CEGyR (Buenos Aires, Argentina). All human procedures were approved by the Internal Review Board and Ethics Committee of CEGyR. None of the work involving human gametes or zygotes was performed at the University of Missouri-Columbia, USA. All donors were <35 years old, with medical indication of ICSI and mostly male factor-related. None of the presumed normal ova donated for this project were allowed to cleave in vivo or transferred to recipients after ICSI. These ova were going to be discarded if not donated for research. Studied embryos were those not suitable for embryo transfer based on morphological criteria. These poor quality supernumerary embryos are sometimes not frozen and therefore donated for research. All oocytes were used immediately upon oocyte retrieval by ultrasound-guided transvaginal aspiration 36 h post-hCG administration. Supernumerary presumed normal and abnormally fertilized oocytes were obtained 18 h post-ICSI. Failed-to-fertilize oocytes (no pronuclei visualized 18–20 h after sperm injection) were obtained 48 h post-oocyte retrieval following ICSI. Oocytes recovered with inferior morphology were excluded from the study. The present report is based on the study of 239 human oocytes and 90 zygotes and embryos analyzed during 2 years. Table I gives a detailed account of the different sources of material.

Chemicals and antibodies

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated. NuMA and dynactin were recognized using anti-full-length recombinant human NuMA (rabbit polyclonal, dil: 1:100, Novus Biologicals Inc., Littleton, CO, USA) and anti-p150-glued antibody (mouse monoclonal, dil: 1:50, BD Biosciences, San José, CA, USA), respectively. Affinity purified sheep anti-tubulin antibody (dil: 1:200) was obtained from Cytoskeleton, Inc. (Denver, CO, USA). All antibodies were used at optimal concentrations.

**Table I Total numbers of oocytes, zygotes and early embryos analyzed under different conditions.**

<table>
<thead>
<tr>
<th>Source</th>
<th>Description</th>
<th>Totals</th>
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<tr>
<td>Oocytes</td>
<td></td>
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<tr>
<td>(a) Spontaneous maturation</td>
<td>GV: 25, GVBD: 23, MII: 21</td>
<td>69</td>
</tr>
<tr>
<td>(b) IVM</td>
<td>GV: 99</td>
<td>99</td>
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<tr>
<td>(c) SOV experiment</td>
<td>Control: 34, SOV-treated: 37</td>
<td>71</td>
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<tr>
<td>Total number of oocytes</td>
<td>239</td>
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<tr>
<td>Zygotes and embryos</td>
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<tr>
<td>(a) Normal fertilization</td>
<td>2PNs</td>
<td>15</td>
</tr>
<tr>
<td>(b) Abnormal fertilization</td>
<td>3–5PNs</td>
<td>19</td>
</tr>
<tr>
<td>(c) Fertilization failures</td>
<td>0PNs</td>
<td>33</td>
</tr>
<tr>
<td>(d) Fertilization of aged oocytes</td>
<td>4–8 cells</td>
<td>8</td>
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<tr>
<td>(e) Early embryos</td>
<td></td>
<td>15</td>
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<tr>
<td>Total number of zygotes and embryos</td>
<td>90</td>
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In vitro culture of human oocytes

After ~3 h post-retrieval, oocytes were stripped of cumulus cells with hyaluronidase and mechanical pipetting except for oocyte—oocyte complexes from small follicles (presumed to contain GV’s) that were set apart and lightly treated with hyaluronidase until an intact GV nucleus could be observed. GV oocytes used for IVM contained a significant amount of surrounding cumulus cells.

Within 2 h of complete removal of cumulus cells, GV’s, GVBDs and MI oocytes were fixed and studied for ICC (see below). Oocytes were classified by morphological criteria: presence of an intact GV, GVBD and lack of a polar body, and presence of a polar body (MII).

Medium for IVM was prepared on the day prior to oocyte retrieval for overnight equilibration. IVM culture medium consisted of M199 medium supplemented with 10% synthetic serum substitute (SSS), 0.075 IU/ml rFSH, 0.075 IU/ml hCG, 1.0 µg/ml estradiol, 0.25 mmol/l sodium pyruvate and 1 mmol/l glutamine (Chian et al., 1999). Cumulus-enclosed GV oocytes were assessed after 27 h in culture (in 5% CO2 and 100% humidity at 37°C); at that time oocyte maturation status was recorded and IVM terminated by fixation.

Pharmacological inhibition of ATPase and phosphatase activities during IVM was achieved by using 400 µM SOV added to GV-stage oocytes during 2 h (Racedo et al., 2009; Wang et al., 2004), then removed and maintained in IVM culture media without SOV until 27 h; at that time they were fixed and analyzed by immunofluorescence. Control IVM was performed without SOV, by using vehicle solution (ethanol) at identical concentrations.
ICC of human oocytes, zygotes and embryos

Cumulus cells and zona pellucida were removed from oocytes, zygotes and embryos with 1 mg/ml hyaluronidase and acid Tyrode’s solution at room temperature, respectively. Zona-free human oocytes, zygotes and embryos were then fixed for 40 min in 2% formaldehyde and permeabilized in 10 mM PBS + 0.1% Triton X-100 for an additional 40 min (method modified and based on Messinger and Albertini, 1991). After fixation and permeabilization, samples were blocked for 1 h in 10 mM PBS + 0.3% bovine serum albumin (BSA) + 1% fetal calf serum prior to incubation in humidified chambers with primary and secondary antibodies, overnight at 4°C and for 1 h at room temperature, respectively. Samples were counterstained for DNA using TOTO-3 at 10 μg/ml and RNase to eliminate RNA staining. Images were obtained using an Olympus spectral confocal microscope, with laser lines at 488-, 568- and 633-nm wavelengths (University of Buenos Aires, Faculty of Exact and Natural Sciences) and then processed using Adobe Photoshop 7.0. Negative controls were run in the absence of primary antibodies.

ICSI in spontaneously matured human oocytes

A total of eight denuded GVs donated for this research by consenting patients were incubated in Human Tubal Fluid supplemented with 15% of SSS and injected 27 h later when they reached the MII stage. All injected human oocytes were fixed at 16–18 h after ICSI, processed by ICC and studied by confocal microscopy as described above.

Statistical analysis

For each figure, representative images are shown for oocytes, zygotes and embryos. Pilot experiments designed to standardize the concentrations of antibodies are not included in calculations. The significance of observed differences in individual experiments was assessed using χ² test (software: Statistix 9.0, 2008 version). To estimate the magnitude of the relationship between SOV and each oocyte maturation stage, Cramer’s V values were calculated. A P-value of <0.05 was considered significantly different.

Results

Distribution of NuMA during human oocyte meiosis, fertilization and early embryo development

All the material analyzed and the sample sizes are summarized in Table I. Oocytes reported in Table I were at GV, GVBD, MI and MII at the time of oocyte retrieval after ovarian stimulation. In GV and early diakinesis, just prior to GVBD, oocytes show strong NuMA signal inside the nucleus and weak localization in the cytoplasm (Fig. 1A). Right after GVBD, NuMA disperses into the ooplasm (not shown), and when microtubules form the meiotic spindle and chromosomes align at the metaphase plate (MI) NuMA translocates to the poles (Fig. 1B). After first polar body extrusion and formation of the meiosis II spindle (MII), NuMA displayed similar staining patterns as shown for MI oocytes (Fig. 1C). Control oocytes (without first antibody) only displayed faint background staining in the cytoplasm (not shown).

To evaluate NuMA during fertilization and early embryo development, we analyzed normally fertilized zygotes (Table I) and showed NuMA staining inside male and female pronuclei (Fig. 1D). This is also true in cases of abnormal fertilization with multiple pronuclei (not shown). After syngamy and pronuclear envelope breakdown (mitotic prophase), NuMA became associated with paternal and maternal chromosomes (inset Fig. 1D). When the first mitotic metaphase plate was formed, NuMA re-localized to the spindle poles (Fig. 1E). Early embryos showed uniform localization in blastomere nuclei (Table I, Fig. 1F).

NuMA associates early with the developing male and female pronuclei

As an approach to understand NuMA dynamics during fertilization, we studied oocytes that failed to fertilize (Table I). After penetration, when sperm chromatin is still highly condensed, no association with NuMA is detected (Fig. 2A arrowhead, failure of sperm head decondensation). As soon as some degree of sperm chromatin decondensation is observed, NuMA is detected in the developing male pronucleus (arrowhead, Fig. 2B). In cases of premature chromosome condensation (PCC, Fig. 2C), multiple NuMA foci associate with microtubules around the paternal chromosome cluster (arrowhead). In all cases, NuMA is normally distributed at the poles of the oocyte’s meiotic spindle. Figure 2D shows diffusely localized NuMA in early apposed male and female pronuclei.
Abnormal patterns of NuMA distribution in in vitro matured and aged human oocytes

Sixty-three out of ninety-nine (63.4%) GV stage oocytes placed in IVM medium reached the MII stage by 27 h. Only 33% of these in vitro matured-MII oocytes displayed the typical localization of NuMA at the meiotic spindle poles (as shown for in vivo matured-MII oocytes in Fig. 1C). In contrast, the remaining 67% depicted various abnormalities such as the absence of NuMA (14%), faint staining at the meiotic spindle poles (36%) or ectopic localization in the ooplasm (50%) (Fig. 3A–C). These abnormalities were seen in association with meiotic spindle disarray or irregular chromosomal distribution.

Inhibition studies using SOV during IVM

SOV incubation induced a significant arrest of oocytes at the GV stage and during meiosis I, with only 16.2% of the oocytes reaching MII (Table II). In contrast, 58.8% control oocytes reached MII. Cramer’s V values for GV and MII stages were 0.3072 and 0.4418, respectively (‘medium’ and ‘strong’ effect size), showing the important impact of SOV treatment on these two stages. Regardless of the maturation stage reached, drastic changes were observed after SOV treatment. Figure 3E shows a normal NuMA and dynactin association with the second meiotic spindle of a control oocyte (Fig. 3E). This association was disrupted by SOV treatment with ectopic distribution of NuMA and scattering of chromosomes (Fig. 3F). Of the 14 SOV treated oocytes that were analyzed for dynactin, five oocytes were also evaluated for microtubule; these revealed the presence of spindle-like...
structures, all of which were disorganized. The subset of SOV-treated oocytes studied to explore dyactin distribution show the molecular motor absent from the meiotic spindle. SOV treatment resulted in oocytes with a higher incidence of NuMA distribution abnormalities (81% of the studied oocytes) when compared with IVM alone (Fig. 3A–C).

**Discussion**

In mammalian oocytes, accurate microtubule organization is critically important for progression to MI stages, fertilization and early embryo development. Minus end-directed dynein motor activities are necessary to transport the centrosomal protein NuMA along microtubules to the spindle poles for spindle-specific functions (reviewed in Sun and Schatten, 2006, 2007). NuMA is one of the essential centrosome proteins of meiotic and mitotic spindles that bundles and stabilizes microtubules at the centrosome-associated minus ends and ensures accurate organization of MI and MII spindles. In interphase, NuMA serves as a nuclear matrix protein and is not associated with interphase centrosomes.

Mechanisms of NuMA and cyclin B association/dissociation with mitotic centrosomes in somatic cells have been described in excellent detail (Gehmlich et al., 2004). However, the regulation of meiotic acentriolar centrosomes (oocytes) is different from mitotic centrosomes (reviewed in Schatten and Sun 2009a,b, 2010) and needs further investigation. Our study reports for the first time the presence and distribution of NuMA (together with a preliminary evaluation of dyactin) in human oocytes, zygotes and embryos. Results demonstrate strong NuMA localization at the GV, its dispersion into the ooplasm at GVBD and its subsequent reorganization at the MI and MII spindle poles. We have further determined that NuMA associates rapidly and strongly with developing pronuclei, is transported to first mitotic spindle poles and re-locates into blastomere nuclei.

During IVM, most oocytes displayed NuMA ectopically located, absence or faint localization with spindles; importantly, all of these abnormal NuMA patterns were associated with abnormal spindle architecture and dispersed chromosomes (Fig. 3). Similar abnormalities in NuMA and dynein organization at the meiotic poles have been reported for dysfunctional spindles in non-human primates (Simerly et al., 2003, 2004), which perhaps suggests a similar mechanism responsible for oocyte IVM abnormalities/failures in human and non-human primates.

To further explore these mechanisms, we resorted to pharmacological inhibition experiments using SOV, an inhibitor of phosphatase and ATPase activity with high selectivity for dynein inhibition (Niclas et al., 1996). Cytoplasmic dyneins control mitotic and meiotic progression via an ATPase-dependent protein transport from kinetochores to the spindle poles that involves phosphorylation/dephosphorylation mechanisms (Gehmlich et al., 2004; Zhang et al., 2007; Racedo et al., 2009). The incubation of human oocytes with SOV reported here yielded results similar to those obtained on mouse spindle structure and oocyte maturation rates under the same inhibition treatment (Carabatsos et al., 2000; Wang et al., 2004). As expected, by disrupting ATPase and phosphatase activities using SOV, the progression to MII is significantly affected. This arrest in meiosis is accompanied by severe NuMA, dynactin, spindle and chromosome abnormalities. On the basis of the need of intact microtubules for NuMA localization (reviewed by Cleveland, 1995), we could hypothesize that NuMA was unable to localize to the meiotic spindle poles during SOV treatment. Alternatively, spindles may be disorganized as a result of a failure in the recruitment of NuMA and/or dynactin to it. Contrary to bovine oocytes (Racedo et al.,

<table>
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<th>Table II Percentages of human oocytes reaching different maturation stages after SOV treatment.</th>
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<td><img src="image" alt="Graph showing percentages of human oocytes reaching different maturation stages after SOV treatment." /></td>
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</tbody>
</table>

GV, germinal vesicle; GVBD, germinal vesicle break-down; MI, metaphase I; AI, anaphase I; MII, metaphase II. Cramer’s V was calculated to measure the association between variables for the χ² test. Cramer’s V for GV = 0.3072, MII = 0.4418. *Statistically different compared with controls. Arrests at the GV and MII stages have a P-value of < 0.01.
we observed a significant arrest at the GV stage after SOV treatment during human IVM. Although SOV is an inhibitor of phosphatase and ATPase activity with high selectivity for dyneins (Niclas et al., 1996), the inhibition of other enzymes and cellular pathways cannot be ruled out. This is particularly relevant in our use of SOV at a 400 μM concentration. Gordon (1991) reported on the inhibitory activity of 100 μM SOV on protein-phosphotyrosine (as well as other) phosphatases. It is thus important to consider that the disturbances in spindle and chromosomes reported herein may not reflect a direct effect on dynein motors but rather a general disruption in kinase/phosphatase signaling. Future studies could extend into a selective inhibition of dynein function with decreased SOV concentration and/or the use of a specific inhibitor.

It is well known that two major regulatory components, the maturation promoting factor (MPF; a CDK1/cyclin B1 complex) and the mitogen-activated protein kinase (MAPK), are important for MII spindle dynamics. Reduced activity of MPF in in vitro cultured oocytes is associated with premature chromosomal centromere separation, which increases the probability of aneuploidy in zygotes (Jeffreys et al., 2003; Pellestor et al., 2003). Future studies are needed to determine whether decreases in MAPK and/or MPF play a role in human oocyte spindle and NuMA abnormalities, as balanced phosphorylation and dephosphorylation is required for accurate NuMA functions (reviewed in Sun and Schatten, 2006).

Until the present study, the association of NuMA with the meiotic centrosome had not been reported in humans. Our results suggest that defects in NuMA dynamics in maturing oocytes and after fertilization may contribute to fertilization failures during IVF treatments. Any failure in MII spindle functions can result in cell and developmental abnormalities resulting in abortion, disease or developmental defects (reviewed in Miao et al., 2009). The MII spindle is therefore an important key structure that requires precise regulation, receiving signals from the surrounding cells, the ooplasm and the sperm during fertilization. Just by studying the overall spindle structure (as often performed in IVF and oocyte cryopreservation studies), subtle molecular abnormalities may be overlooked. Evaluation of NuMA within the meiotic spindle could be an important criterion to assess the oocyte quality.

Some studies, including those by Lee et al. (2000), showed differing NuMA distribution patterns in the mouse when compared with other mammalian species such as the porcine or bovine models. Porcine and bovine oocytes often display the closest molecular similarities to human oocytes (Prather, 1993, 2007; Schatten and Sun, 2009a,b, 2010) and both systems have been used for heterologous fertilization to test oocyte and sperm quality. Although mechanisms underlying cytoskeletal and centrosomal regulation in the porcine and human model have similarities, important molecular differences can be expected to exist (reviewed in Prather, 2007; Schatten et al., 2011). These observations highlight the importance of species-specific differences and call for direct research on human oocytes to advance assisted reproduction techniques (Schatten et al., 2011).

To study how early NuMA becomes localized to male and female pronuclei, different time point experiments at short intervals are needed during fertilization. Because of the ethical restrictions on the use of human material, we decided to study several samples of human fertilization failures as an approach to understand NuMA dynamics (n = 33, Table I and Fig. 2). We were able to determine that NuMA rapidly associates with developing pronuclei. Fully apposed (Fig. 1) and developing male and female pronuclei (Fig. 2) show NuMA forming part of the nuclear matrix as it does in somatic cells. Information for NuMA has also been obtained in porcine zygotes and embryos (Liu et al., 2006). Interestingly, NuMA localized to both the oocyte and early embryo nuclei, but upon chromosome condensation, it was associated with mitotic but not meiotic chromosomes. The role of NuMA’s association with condensed chromosomes may thus differ between these distinct cell types and cellular divisions, a subject of relevant future investigation.

Even in natural cycles, failures in oocyte maturation or fertilization prevail although the molecular mechanisms leading to these defects are still largely unexplored. The numerous instances of failure in assisted reproductive procedures have allowed novel insights to be gained into the subcellular nature of oocyte abnormalities and mechanisms that lead to aberrant maturation. The understanding and characterization of oocytes’ deficiencies will help us to design strategies to improve human fertilization.

Authors’ roles

C.A.S. and V.Y.R. were involved in acquisition of all data, analysis and interpretation of data, conception and design of the study, drafting the article and final approval. C.C. and H.S. played a role in conception and design of the study, revising the article and final approval.

Acknowledgements

We would like to express our special thanks to Florencia Nodar and Mariano Lavolpe for their dedication in collecting the donated human gametes and to Dr Chenes for critical reading of the manuscript. We are most grateful to anonymous, consenting donors who kindly donated their supernumerary gametes and zygotes for this research. The technical assistance of Roberto Fernández (Confocal Microscopy at the University of Buenos Aires) is gratefully acknowledged.

Funding

This work was supported in part by CEGyR Foundation and Grants from CONICET (PIP 2565), ANPCyT (PICT 2005 38229).

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