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The Abelson tyrosine kinase (c-Abl) localization in preimplantation mouse development

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Abstract

The Abelson family member of non-receptor tyrosine kinase, c-Abl, has an important role in regulation of cellular processes like cell polarity, invasion, proliferation, survival and cell motility. In the present study, we investigated the localization of c-Abl protein during preimplantation mouse embryo development *in vitro* using immunofluorescence confocal microscopy. We have shown that c-Abl protein is expressed throughout all stages of preimplantation development. We found that c-Abl is asymmetrically localized in egg cortex, which suggests possible roles in positioning of the metaphase II spindle. We also observed strong c-Abl staining in external cells, especially enriched at the apical poles in morula stage and at the E3.5 stage blastocysts, cell membrane enrichment of c-Abl in trophectoderm (TE), the tissue responsible for implantation and placentation. The results indicate that c-Abl may play roles in preimplantation embryo development, especially in TE formation and differentiation.

Keywords: c-Abl, cell differentiation, embryo development, preimplantation.

Introduction

The Abelson tyrosine kinase (c-Abl) is a 150-kDa proto-oncoprotein, member of the Src family of non-receptor protein tyrosine kinases [1]. c-Abl gene was originally identified as the cellular homologue of Abelson murine leukemia virus protein [2]. The Abl family kinases are comprised of the normal cellular c-Abl and Arg and the oncogenic forms v-Abl, Bcr-Abl, Tel-Arg [3]. c-Abl protein includes SRC homology 3 domain (SH3), SH2 domain and tyrosine kinase domain (SH1). The SH3 domain binds to the polyproline containing linker sequence that connects the SH2 and SH1, and the SH2 domain interacts with C-terminal lobe of the SH1 to form an SH3-SH2-SH1 clamp structure [4, 5]. The carboxyl terminus of c-Abl contains a globular (G)-actin binding domain and the filamentous (F)-actin binding domain. Related to its nuclear and cytoplasmic distribution, c-Abl has three nuclear localization signal motifs and one nuclear export signal in its C terminus [4]. c-Abl is located at various subcellular sites like plasma membrane, cytoplasm, nucleus, mitochondria and endoplasmic reticulum [5].

Abl kinases have various roles in the regulation of cellular processes like cell polarity, invasion, survival, migration and proliferation [4]. Moreover, it has been suggested that c-Abl may have roles in G1/S cell cycle arrest response to DNA damage [6], modulation of apoptosis [5], and the development of female and male mouse germ cells [7].

Abl family of protein kinases is also important for embryonic development because its knockout in mice causes neonatal lethality [8, 9]. Although c-Abl and Arg have some overlapping physiological roles, single knockout of c-Abl causes developmental abnormalities, indicating that c-Abl may have important functions that cannot be replaced by Arg during mouse development [4, 10]. Studies showed that c-Abl knockout mice have reduced fertility, poor viability and reduced numbers of T- and B-cells, splenic and thymic atrophy, cardiac abnormalities, osteoporosis and premature senescence [4, 8, 9, 11].

Mammalian development is initiated by fusion of the sperm and egg, namely fertilization. Before fertilization, unfertilized egg is arrested at metaphase II (MII) of meiosis and subsequent fertilization converts two differentiated cells into a zygote. During mouse preimplantation development, the fertilization is followed by several cell and cleavage divisions to generate blastomeres. After first cleavage, the mouse embryo reaches the two-cell stage and continues to cleave until it moves to uterus. The first three cleavages result in the formation of eight cell stage embryo, after which compaction occurs at the late 8-cell stage and is completed at around 32-cell stage. With the compaction, blastomeres begin to form junctions with one another and develop distinct apical and basal membrane and cytoplasmic domain, leading the formation of the morula. Morula is a compacted cluster consisting of approximately 16–32 blastomeres where individual blastomeres cannot be easily distinguished. During compaction, blastomeres polarize and present an outward-facing apical surface, which assembles proteins like partitioning defective (Par) molecules, Par3/Par6 and atypical protein kinase C (aPKC). Also at this time, the serine/threonine kinase EMK1 (Par1) and E-cadherin/β-catenin complex are localized basolaterally. In morula stage, external polarized blastomers that are positioned at the surface retain apical and basal polarity, while internal non-polarized blastomeres that are surrounded by the neighboring cells are devoid of polarity [12, 13]. During this period, external cells tend to become trophectoderm (TE) cells whereas internal cells tend to become inner cell

mass (ICM). Then, the morula undergoes differentiation and one or more fluid-filled cavities begin to form an embryo called blastocyst. At E3.5, blastocyst consists of two distinct cell lineages, TE and ICM. TE is an epithelial-like outer layer, which is responsible for blastocyst implantation to the uterine wall and contributes to the placental tissues. The ICM is segregated from the small cluster of pluripotent cells, which gives rise to the fetus [12, 14, 15]. In mouse blastocysts, several transcription factors have been identified as essential for TE and ICM development. These transcription factors are POU-domain transcription factor Pou5f1 (Oct4) and a caudal-type homeodomain transcription factor Cdx2. Pou5f1 (Oct4), a key regulator of pluripotency, is strongly expressed in ICM, and Cdx2 is essential for maintenance of the TE lineage and suppresses the ICM lineage formation, specifically expressed in TE cells [16–18].

Despite its demonstrated role in embryonic development, there is hardly any information regarding the localization of c-Abl protein in preimplantation embryos in the literature. Therefore, this present study for the first time explored the localization of c-Abl protein during embryo development *in vitro* by using immunofluorescence confocal microscopy to understand the roles of c-Abl. Our data showed that c-Abl (c-19) is expressed throughout all stages of preimplantation period and might have some roles in preimplantation embryo development.

Materials and Methods

Animals

B6D2F1 (C57BL/6 females × DBA/2 males) mice strain were purchased from National Cancer Institute, USA. B6D2F1 strain was used in this study, as it is ideal for developmental embryology studies [19]. This research project was done at University of Hawaii, USA. The protocol of animal handling and treatment was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Hawaii on July 19, 2012, with a Protocol No. 05-029-8.

Oocyte collection

A total of 25 B6D2F1 female mice were superovulated by intraperitoneally injections of 5 IU of equine chorionic gonadotropin (pregnant mares' serum gonadotropin – PMSG) followed by human chorionic gonadotropin (hCG) 46 hours later and were mated with B6D2F1 male mice. Then, superovulated mice were sacrificed and their oviducts were dissected 13–15 hours after hCG injection. After dissection of the oviducts, the oviductal ampulae were broken to release the cumulus-oocyte complexes into FHM HEPES-buffered medium (Millipore) and these complexes transferred into FHM HEPES-buffered medium containing 0.5% hyaluronidase (SAGE, *In vitro* Fertilization Inc., Trumbull, CT, USA) to remove cumulus cells. Finally, MII oocytes (n=66) that were freed of cumulus cells were cultured in KSOM-AA medium at 37^0C with 5% CO_2 humidified air to use for the experiments [19].

Embryo collection

B6D2F1 female mice were superovulated as described above. About 46 hours after the hCG injection, inseminated females were selected by the presence of vaginal plug and these females were sacrificed. After that 2-cell stage, embryos (n=300) were flushed from the dissected oviducts with FHM HEPES-buffered medium under the stereo-microscope. After that, embryos were cultured to 8-cell stage [8-cell stage embryos fixed at various times, at 9:00 am, 12:30 pm and 4:00 pm and named like early (uncompacted), middle (during compaction) and late (compacted) 8-cell stage embryos respectively to determine distribution variation of c-Abl proteins], morula and blastocyst stage in KSOM-AA medium (EMD Millipore) at 37^0C with 5% CO_2 humidified air for the experiments [20].

Immunofluorescent staining

The *zona pellucida* was removed from MII oocytes and embryos by treating 0.5% pronase (Roche) in FHM for 10 minutes. Then, MII oocytes and embryos were fixed in 4% paraformaldehyde (PFA) solution in phosphate-buffered saline (PBS) for 20–30 minutes. Embryos were permeabilized in 0.5% Triton X-100 in PBS for 15 minutes. After blocking with 5% bovine serum albumin in PBS containing 0.1% Tween 20, samples were incubated overnight in primary antibody, at 4^0C, then incubated in secondary antibody for 1–2 hours, at room temperature. Primary antibodies used were, rabbit anti-c-Abl (c-19) (Santa Cruz), mouse anti-Cdx2 (BioGenex), goat anti-Pou5f1 (Santa Cruz). In blastocysts, TE cells were exclusively stained with TE-specific transcription factor Cdx2 protein and ICM cells were stained for Oct4 protein to demonstrate the cell lineages. Secondary antibodies (used were donkey anti-rabbit and goat anti-mouse used were conjugated with Alexa Fluor 488 (Life Technologies) and goat anti-rabbit conjugated with Alexa Fluor 546 (Life Technologies). To visualize actin filaments, phalloidin conjugated with Alexa Fluor 546 was used at a final concentration of 33 nM. Stained samples were mounted in ProLong Gold antifade reagent with 4',6'-diamidino-2-phenylindole (DAPI; Thermo Fisher Scientific) [18]. Negative and positive control staining on oocytes and embryos were done during this study. The negative control groups underwent the same staining protocol as the positive control group with the absence of the primary antibody. Moreover, the specificity of the c-Abl secondary antibody was determined that no c-Abl staining was observed in negative control groups.

ICM isolation by immunosurgery

Procedures were performed as previously described [20]. Briefly, E3.5 blastocysts still inside the *zona pellucida* were incubated in rabbit anti-mouse serum diluted in KSOM-AA followed by guinea pig complement (Invitrogen) diluted in KSOM-AA. Embryos were transferred in 0.5% pronase in FHM to remove the lysed TE cells and to dissolve the *zona pellucida* completely around the blastocyst. Afterwards, isolated ICMs were fixed for the immunofluorescent staining.

Microscopy and image analysis

Oocytes and embryos were observed with an FV1000 laser scanning confocal microscope (Olympus). With confocal microscopy, serial optical sections were imaged at 2 μm intervals under a 40× objective lens with oil.

Results

Asymmetrical localization of c-Abl in the egg cortex

To determine the localization of c-Abl protein in MII oocytes, the localization of the c-Abl protein was examined by immunofluorescence staining with an anti-c-Abl (c-19) specific antibody. We detected that c-Abl (c-19) protein was localized at the spindle apparatus and cytoplasm. c-Abl protein was present at a low concentration uniformly throughout the cytoplasm when compared to the cortex of the egg. An important data that were observed was c-Abl (c-19) prominently concentrated at the egg cortex as a crescent shape while it was absent where the actin-rich cortical domain (Figure 1A). This data showed that c-Abl (c-19) has an asymmetrical localization in the unfertilized egg cortex.

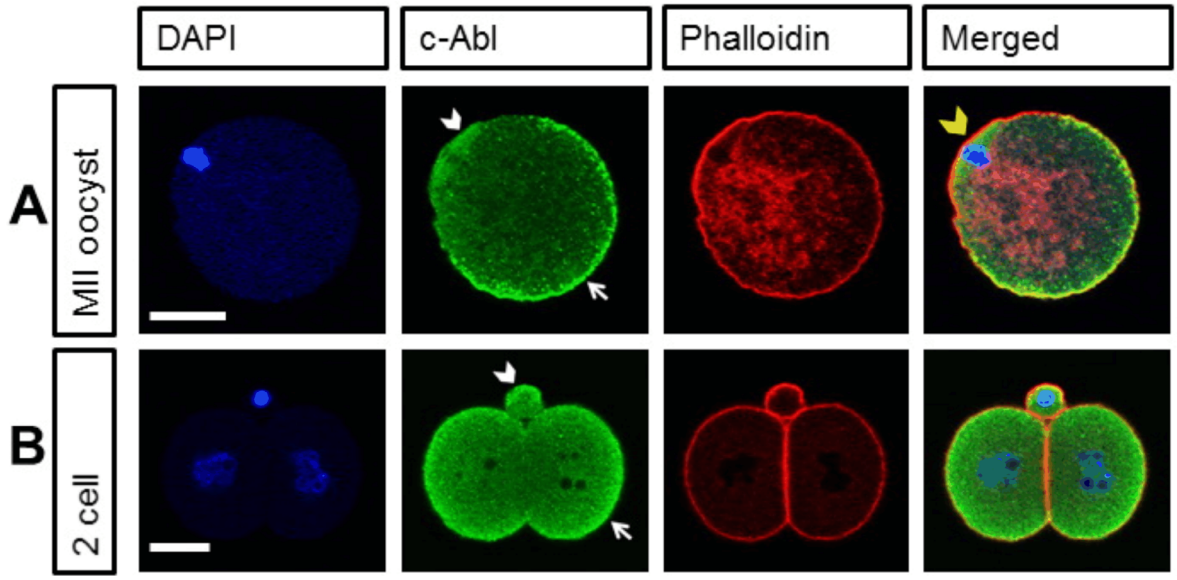

Figure 1 – ***Localization of c-Abl protein at MII oocyte and 2-cell stage embryo under a confocal microscopy: (A) MII arrested oocytes c-Abl protein was enriched in the egg cortex as a crescent shape (arrow) it was localized at the spindle apparatus (white arrowhead) and it was absent at the actin-rich cortical domain (yellow arrowhead); (B) c-Abl protein was concentrated in the periphery of the blastomeres (arrow). c-Abl localization in polar body (arrowhead). Confocal images of MII oocyte and 2-cell stage embryo was stained with c-Abl (green) phalloidin (showing actin filament staining) (red) and nuclei were stained with DAPI (blue). Scale bar represents 20 μm. c-Abl: Abelson tyrosine kinase; MII: Metaphase II; DAPI: 4',6'-Diamidino-2-phenylindole.***

Localization of c-Abl protein during preimplantation development

To investigate whether c-Abl is involved in preimplantation development, we examined the localization of c-Abl protein by immunofluorescence staining from 2-cell to the blastocyst stage. The c-Abl (c-19) was detected in all stages of embryo development (Figures 1–4). In addition, c-Abl (c-19) staining was observed both at the blastomeres during preimplantation development and within the polar bodies (Figure 1B), suggesting that c-Abl is not only produced in the embryo but also expressed by the polar body. In the 2-cell embryo, c-Abl (c-19) was expressed in the cytoplasm and nucleus with a granular staining and was more concentrated in the periphery of the blastomeres (Figure 1B). We performed a set of experiments to show the distribution variation of c-Abl proteins on early, mid and late 8-cell stage embryos. The cytoplasm and nuclei were positively stained during these stages. However, distribution results indicate there was no dramatic difference in c-Abl localization comparing early, mid and late 8-cell stage embryos (Figure 2).

In the morula stage, embryos exhibited membranous, nuclear and cytoplasmic staining. We observed c-Abl (c-19) protein had different subcellular distributions in the external and internal cells. By looking at the confocal optical sections of external blastomeres, c-Abl (c-19) was seen to be present at the baso-lateral membrane while it was enriched at the apical membrane (Figure 3A). In the internal cells, c-Abl (c-19) was distributed throughout the plasma membrane and appears in the cytoplasm and nucleus. c-Abl (c-19) was localized more intensely in the cytoplasm and nucleus in outer cells than in inner cells (Figure 3A).

At the blastocyst stage (E3.5), c-Abl became concentrated in the plasma membrane of the both mural and polar TE (Figure 3A). We also detected nuclear and cytoplasmic staining in TE cells. In ICM cells, c-Abl (c-19) staining was not observed at the membrane; however, it was present at cytoplasm and nucleus very weakly compared to TE cells (Figure 4, A and 4B). To identify and present this data, embryos were stained for c-Abl as well as transcription factors Cdx2 (specifically expressed in TE cells) and Oct4 (strongly expressed in ICM cells) respectively at E3.5 blastocyst. Especially in embryos stained by c-Abl and Cdx2, we clearly observed strong membranous staining at TE cells whereas no membranous staining at ICM cells (Figure 4).

Apical localization of c-Abl protein in the isolated ICMs

To test the c-Abl localization in ICMs, we isolated and cultured the ICMs from E3.5 blastocysts. After the isolation of the cells, we directly fixed and applied

immunohistochemistry staining on ICMs and intact E3.5 blastocysts (control) (Figure 5). In isolated ICMs, nucleus and cytoplasm were positively stained with c-Abl and it was strongly enriched in the apical membrane domain of external cells, whereas internal cells showed a nuclear and cytoplasmic distribution with weak membrane enrichment. In control blastocysts, we observed that c-Abl was enriched in the plasma membrane of the TE. Moreover, nucleus and cytoplasm of the TE and ICM cells were positively stained by c-Abl protein (Figure 5).

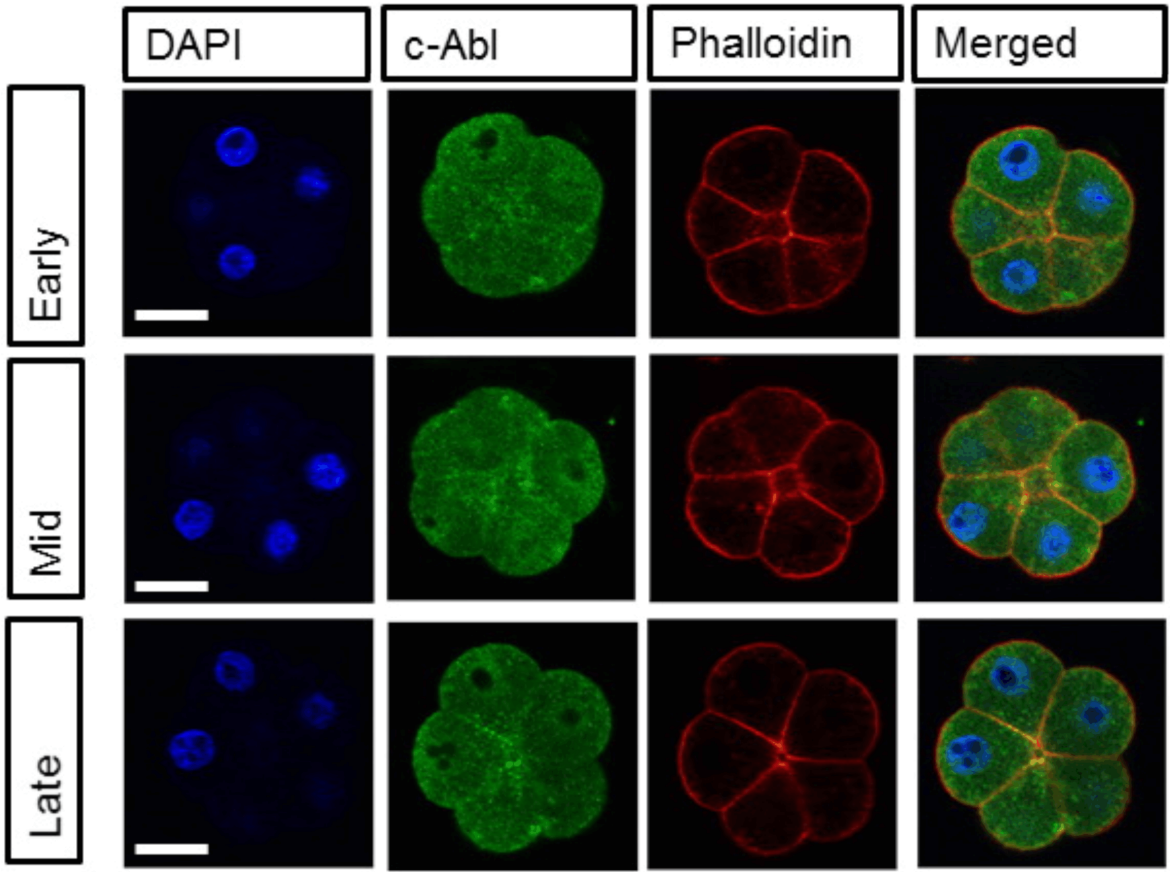

Figure 2 – ***Distribution of c-Abl protein during 8-cell stage embryo development. Eight-cell stage embryos showed no difference between early, mid and late stage. Confocal optical sections of embryos immunofluorescently stained with c-Abl (green), phalloidin (red), DAPI (blue). Scale bar represents 20 µm. c-Abl: Abelson tyrosine kinase; DAPI: 4',6'-Diamidino-2-phenylindole.***

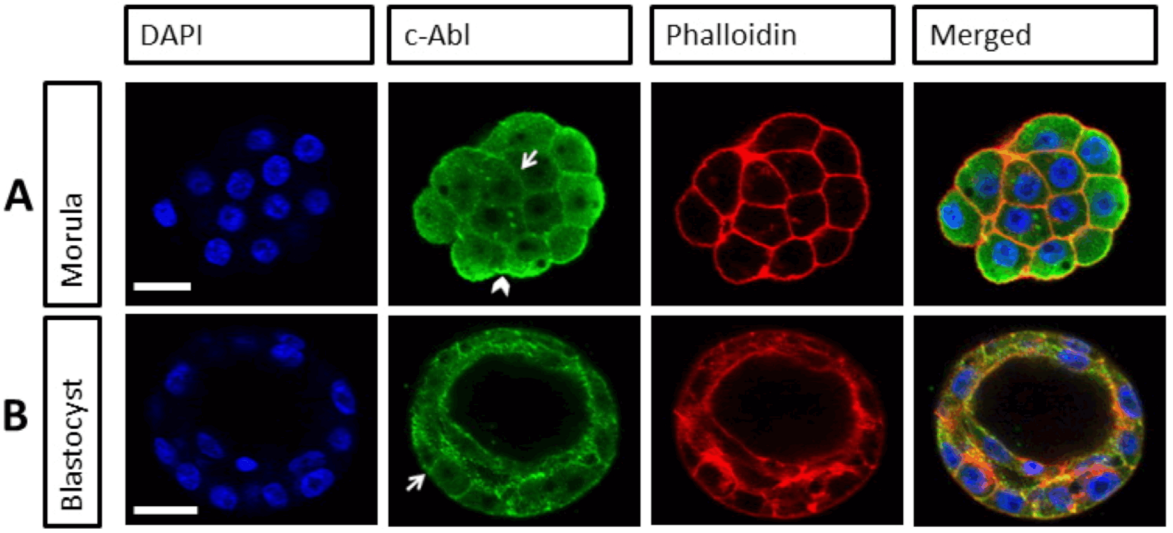

Figure 3 – ***Membranous staining of c-Abl in morula and E3.5 blastocyst stage embryos: (A) c-Abl was present at the baso-lateral membrane (arrow) and concentrated at the apical membrane of the external cells (arrowhead); (B) TE cells were examined strong membranous c-Abl distribution (arrow). Confocal optical sections show c-Abl (green), phalloidin (red), nuclei (blue). Scale bar represents 20 µm. c-Abl: Abelson tyrosine kinase; TE: Trophectoderm; DAPI: 4',6'-Diamidino-2-phenylindole.***

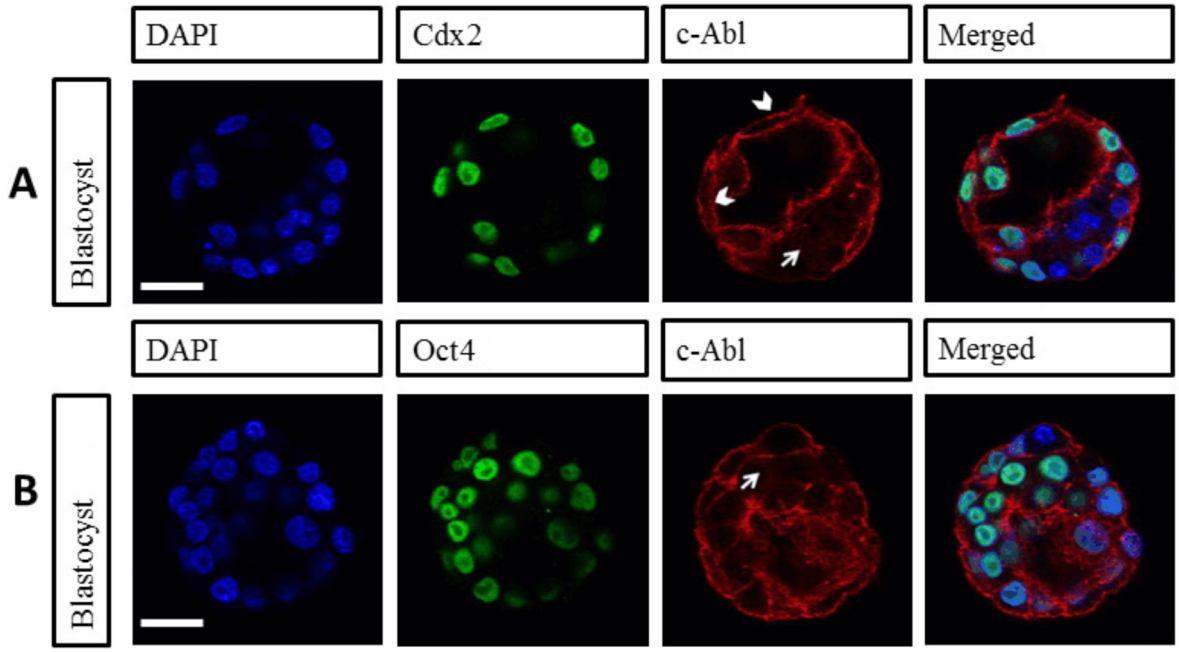

Figure 4 – ***Localization of c-Abl protein in E3.5 blastocysts: (A) Blastocysts were immunostained with c-Abl (red) and TE cells were stained specifically with Cdx2 (red) protein. Strong membranous staining at TE cells (arrowheads) and no membranous staining on ICM (arrow); (B) Blastocyst was immunostained for c-Abl (red) and pluripotency marker Oct4 (green) protein. ICMs cytoplasm and nuclei were stained very weakly (arrow). Nuclei were stained with DAPI (blue). Scale bar represents 20 µm. c-Abl: Abelson tyrosine kinase; TE: Trophectoderm; ICM: Inner cell mass; DAPI: 4',6'-Diamidino-2-phenylindole.***

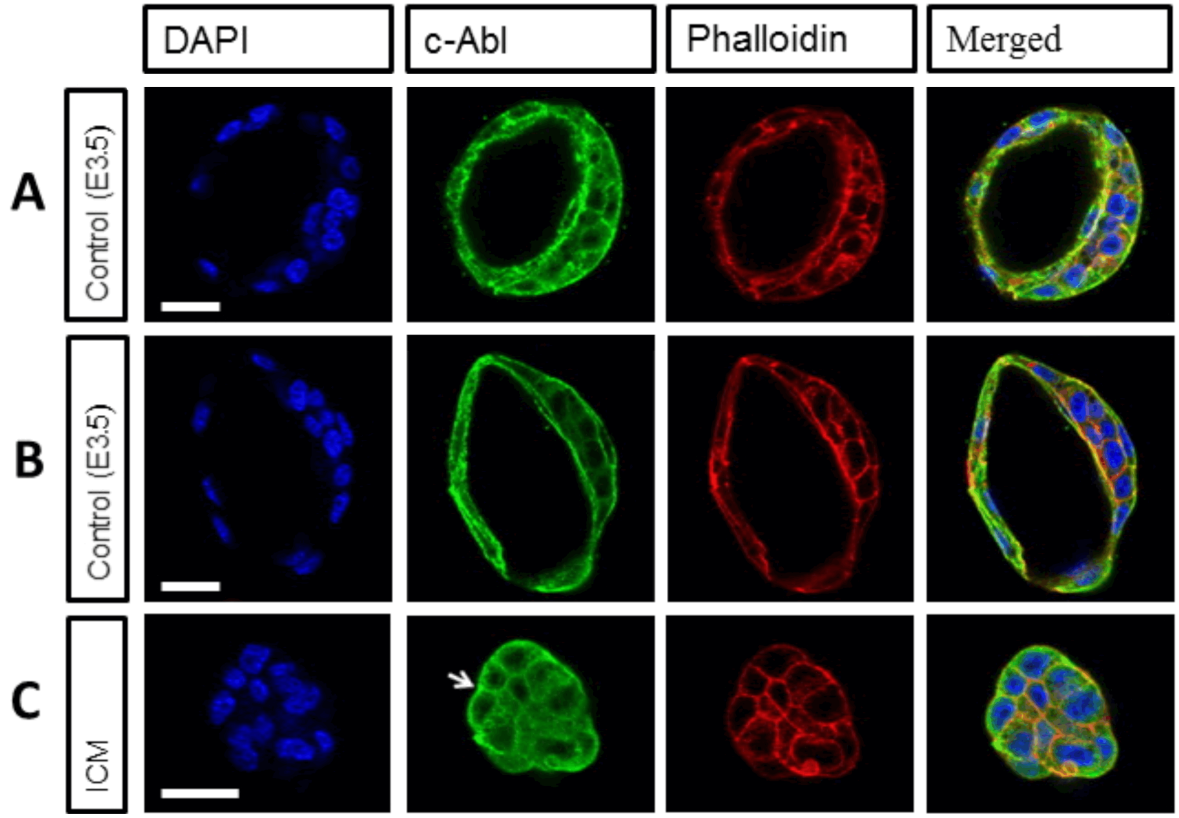

Figure 5 – ***Intact E3.5 blastocysts and isolated ICMs stained with c-Abl protein: (A and B) TE cells from intact E3.5 blastocyts showed membranous staining; (C) In isolated ICMs, c-Abl protein was concentrated in the apical membrane domain of the external blastomers (arrow). Confocal optical sections show c-Abl (green), phalloidin (red), nuclei (blue). Scale bar represents 20 µm. c-Abl: Abelson tyrosine kinase; ICM: Inner cell mass; TE: Trophectoderm; DAPI: 4',6'-Diamidino-2-phenylindole.***

Discussion

Here, we characterized the localization of c-Abl protein tyrosine kinase at MII oocytes and during the preimplantation mouse development. Our study demonstrated that c-Abl (c-19) is expressed at MII oocytes and throughout all stages of preimplantation development of mouse embryos. These findings provide evidence that c-Abl protein has some roles in early stages of embryonic development.

Previous studies assessed c-Abl may have roles in preimplantation embryonic development, uterine remodeling during decidualization, implantation and placentation throughout gestation [21, 22]. Mice with a disrupted c-Abl gene exhibit neonatal lethality, lymphopenia and low viability [8, 9]. In this study, we demonstrated the localization of c-Abl protein in MII oocytes and during preimplantation embryo development.

Our findings showed that c-Abl protein is asymmetrically localized in egg cortex and absent at region where the actin-rich domain and first polar body exist. We also found that c-Abl was localized on the mitotic spindle in the MII oocyte. These data indicate that c-Abl has possible roles in positioning of the MII spindle. Previous studies also reported that c-Abl protein enriched in the sea urchin egg cortex and suggesting it may play a role in the egg activation process following fertilization [23, 24]. Supporting our data, Matsumura *et al.* (2012), showed that c-Abl regulates spindle orientation in epithelial cells [25] and loss of c-Abl induces spindle misorientation [26].

Yaba *et al.* (2011) reported gradually increased expression of c-Abl protein in uterus gestation day 1 to day 5, when the implantation occurs in mouse, suggested that c-Abl may be involved in uterine remodeling [21]. They also observed immunostaining in the trophoblast cells and in the ICM of the blastocysts. However, there is no detailed information about localization of this gene during mouse preimplantation period. Our results showed localization of c-Abl in cell cytoplasm and nucleus in all developmental stages while membranous staining was detected at the morula and blastocyst stage, suggesting that c-Abl plays a role in preimplantation development. It has been known that c-Abl localizes at the plasma membrane, cytoplasm and nucleus, where c-Abl interacts with various cellular proteins, including signaling adaptors, kinases, cell cycle regulators, transcription factors and cytoskeletal proteins [5].

Previous studies indicated that external polar cells generate an epithelial monolayer, that will form TE whereas internal apolar cells now defined as ICM [12, 15, 27]. In this study, we observed strong c-Abl staining in external cells, especially enriched at the apical poles to compare with internal cells in morula stage, suggesting that c-Abl may have some roles on TE differentiation. Moreover, by the E3.5 stage blastocysts we found strong membranous staining in TE, the tissue responsible for implantation and placentation, suggesting a possible role in TE formation. After demonstrating the c-Abl staining pattern in TE cells, we isolated ICM cells by immunosurgery to investigate whether c-Abl is an epithelial marker for TE or not. We observed almost the same distribution pattern of c-Abl in morula stage, membranous staining in internal and external cells that c-Abl was concentrated at the apical cortex of the external blastomeres. Based on the literature, in experimentally manipulated embryos-like isolated ICMs, embryos will regain apical/basal polarity [12, 28, 29]. Firstly, apolar cells are forced to localize at the outside and lead to polarization of these cells, then cells adopt TE fate. Next, TE cells are regenerated from outer cells of the isolated ICMs, and polarity proteins relocalize at the apical poles [12, 28]. Stephenson *et al.* (2010) also indicated that repolarization can occur in isolated early ICMs from wild type embryos [29].

It has been reported that c-Abl protein was tightly associated with cytoskeletal components [23]. Abl family kinase's localization to cytoskeletal structures indicate that these proteins have related functions in cytoskeletal regulation [30]. The carboxyl terminus of c-Abl contains filamentous (F)-actin and globular (G)-actin binding domain binds to apical actin filaments in neuroepithelial cells [31, 32]. c-Abl regulates actin polymerization and has role in epithelial cell–cell adhesion, polarity and migration [10]. Based on these reports and our findings, enriched c-Abl staining in apical membrane of the external cells suggests that c-Abl may play a role on regulating apical cytoskeleton and cell structure during preimplantation development.

Conclusions

Our findings in mouse oocytes and preimplantation have highlighted localizations for c-Abl protein. We have shown that c-Abl (c-19) is detected in all stages of embryo development. Our data suggested that c-Abl may play roles in positioning of the MII spindle, TE differentiation and formation during early embryonic development.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this article.

Acknowledgments

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