

Localization of plasminogen activator inhibitor type 1 and 2 in preimplantation mouse development *in vitro*

Duygu MUTLUAY^{1,2,a,✉}, Yukiko YAMAZAKI^{2,b}, Kanani HOKUTAN^{3,4,5,c}, Charles J ROSSER^{3,4,5,6,d},
Hideki FURUYA^{3,4,5,6,e}

¹Mehmet Akif Ersoy University, Faculty of Veterinary Medicine, Department of Histology and Embryology, Burdur, Turkey;

²University of Hawaii, John A. Burns School of Medicine, Institute of Biogenesis Research; ³University of Hawaii, Cancer Center, Clinical and Translational Research Program; ⁴University of Hawaii Cancer Center, Cancer Biology Program; ⁵University of Hawaii, Department of Molecular Biosciences and Bioengineering, Honolulu, Hawaii; ⁶ Department of Surgery, Division of Urology, Cedars-Sinai Medical Center, Los Angeles, California, USA

^aORCID: 0000-0003-3286-130X; ^bORCID: 0000-0001-5089-1487; ^cORCID: 0000-0003-1494-2392;

^dORCID: 0000-0002-6052-4223; ^eORCID: 0000-0002-9536-8662.

✉Corresponding author: duygumutluay@gmail.com

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Abstract: Plasminogen activator inhibitor type 1 (PAI-1) and type 2 (PAI-2) are the major endogenous inhibitors of fibrinolysis, or thrombolysis, as it is effective in blocking the conversion of plasminogen to plasmin. In mammalian embryos, both PAI-1 and PAI-2 proteins are expressed in the trophoblasts during and after implantation, suggesting their critical roles in implantation and placentation during pregnancy. However, it remains unclear how both proteins localize in the early stage embryos before implantation. In this study, 2 cell stage embryos were flushed from the oviducts and cultured to specified stages in medium at 37 °C in a 5% CO₂ incubator. Embryos were fixed and double immunostained with anti-PAI-1 and anti- PAI-2 antibody. We determined the critical expression and localization patterns of PAI-1 and PAI-2 proteins in murine preimplantation embryos at 2 cell, 8 cell, morula and blastocyst stages by using confocal laser scanning microscope. We found that PAI-1 and PAI-2 constantly express in the embryos during preimplantation development, and these proteins localize in both the cytoplasm and the nucleus of each blastomere regardless of their developmental stage. Our results suggest that PAI-1 and PAI-2 proteins may play roles in early embryonic development before implantation.

Keywords: Embryo development, PAI-1, PAI-2, preimplantation.

Farelerde preimplantasyon embriyo gelişiminde plazminojen aktivatör inhibitör tip 1 ve 2'nin *in vitro* lokalizasyonu

Özet: Plazminojen aktivatör inhibitör tip 1 (PAI-1) ve tip 2 (PAI-2), plazminojenin plazmaya dönüşümünü bloke etmede etkili olduğundan fibrinolizis veya trombolizisin başlıca endojen inhibitörleridir. Memeli embriyosunda, hem PAI-1 hem de PAI-2 proteinlerinin implantasyon sırasında ve sonrasında trofoblastlarda eksprese ediliyor olması gebelikte bu proteinlerin implantasyon ve plasantasyonda kritik rolleri olduğunu düşündürmektedir. Bununla birlikte, her iki proteinin implantasyondan önce erken embriyo döneminde nasıl lokalize olduğu konusu hala açık değildir. Bu çalışmada, 2 hücre evrede bulunan embriyolar oviduktlar yıkanılarak çıkarıldı ve %5'lik CO₂ inkübatöründe 37 °C 'de belirtilen aşamalara göre kültüre edildi. Embriyolar tespit edildi ve anti-PAI-1 ve anti-PAI-2 antikorları kullanılarak ikili immunboyama yapıldı. PAI-1 ve PAI-2 proteinlerinin murin preimplantasyon dönemi embriyolarındaki kritik ekspresyon ve lokalizasyon paternleri 2 hücre, 8 hücre, morula ve blastosist aşamalarında konfokal lazer tarama mikroskobu kullanılarak belirlenmiştir. PAI-1 ve PAI-2'nin preimplantasyon gelişimi sırasında embriyolarda sürekli eksprese ettiğini ve bu proteinlerin gelişim aşamalarına bakılmaksızın her bir blastomerin hem çekirdeğinde hem de sitoplazmasında lokalize olduğu gösterilmiştir. Bu sonuçlar, PAI-1 ve PAI-2 proteinlerinin implantasyon öncesinde erken embriyo gelişimde rol oynuyor olabileceğini göstermektedir.

Anahtar sözcükler: Embriyo gelişimi, PAI-1, PAI-2, preimplantasyon.

Introduction

Plasminogen activators (PAs) are serine proteases that catalyze the activation of plasmin, which is a factor to breakdown fibrin polymers during blood clotting (22).

Two mammalian PA isoforms, tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA) are the central components of the plasmin/plasminogen activator system, which plays a

major role in benign disorders such as deep vein thrombosis, myocardial infarction, atherosclerosis, and stroke (9). Plasminogen activator inhibitor-1 and -2 (PAI-1 and PAI-2) are known to be the major inhibitors of this system (7, 29). PAI-1 expression is regulated by a number of intrinsic factors (*e.g.*, cytokines and growth factors) and extrinsic factors (*e.g.*, cellular stress) (29). In the early stage embryos, PAI-1 is expressed in trophoblasts, cytotrophoblasts, trophoblastic cells (5, 24) endothelial cells and placental cells (30). PAI-2 is also present in placental trophoblasts and macrophages and keratinocyte (2, 8). During the second trimester of pregnancy in humans, the concentration of PAI-1 in mother's plasma is gradually increased and it reaches a peak at 32-40 weeks of pregnancy (14). The PAI-2 concentration is also increased during pregnancy and birth (2).

Accumulating evidence suggests that PAs are implicated in oocyte meiotic maturation (16), ovulation (21), fertilization (17, 34) and embryo implantation (31). In rat embryos, uPA and tPA are expressed throughout their preimplantation development. While uPA is localized in the cell cytoplasm, tPA is detected only on cell surface and in the perivitelline space (1). In light of these studies, we hypothesize that the expression of PAI-1 and PAI-2 may be also important to maintain the normal embryogenesis. However, the critical expression patterns of PAI-1 and 2 in mammalian preimplantation embryos remain, unclear. In this study for the first time we obtained critical information about the localization of PAI-1 and 2 proteins throughout the embryo development *in vitro* by using immunofluorescence confocal microscopy.

Material and Methods

Animals and collection of embryos: C57BL/6J (Jackson Laboratory, Bar Harbor, ME) female and male mice were used in this study. Females at 6-8 weeks old were mated with males at 12-16 weeks old. After the mating, females with the vaginal plug were indicated as Day 1 of pregnancy. On Day 2 of pregnancy, females were sacrificed to dissociate their oviducts. Two-cell stage embryos were flushed out from the oviducts in M2 medium (MR-015P, Sigma-Aldrich). These 2-cell embryos were cultured in 20 μ l drops of KSOM-AA medium (MR-121, Millipore) overlaid with mineral oil at 37°C in 5% CO₂ in air for up to Day 5 (26). The embryos at 2-cell (Day 2), 8-cell (Day 3), morula (Day 4) and blastocyst (Day 5) stages were subjected to immunofluorescent staining. At least three pregnant females were used to collect each stage embryos. All relevant experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Hawaii with a Protocol No. 11-1160-8.

Double immunofluorescent staining: The embryos at different stages were fixed for 15 min at room temperature. After fixation, the embryos were permeabilized for 15 min and blocked with 5% bovine serum albumin. Then the embryos were incubated in the primary antibodies overnight at 4°C. The primary antibodies provided were rabbit polyclonal anti-PAI-1 (1:400 dilution; sc-8979, Santa Cruz) and goat polyclonal anti-PAI-2 (1:400 dilution; sc-6649, Santa Cruz). After the first antibodies treatment, the embryos were incubated with secondary antibodies for 2-3 h at room temperature. The secondary antibodies were conjugated with DyLight 488 (1:500; Thermo Fisher Scientific, Life Technologies), namely donkey anti-goat and Alexa Fluor 568 (1:500; Life Technologies), namely, goat anti-rabbit for 3 h at 25°C. Stained samples were mounted in ProLong Gold antifade reagent containing 4',6'-diamidino-2-phenylindole (DAPI) (Life Technologies) on a slide (25). Negative and positive control staining on 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 PAI-1 and 2 secondary antibody was determined that no PAI-1 and 2 staining was observed in negative control groups.

Microscopy and image analysis: Embryos were imaged using a Leica TCS SP5 confocal laser scanning microscope. For confocal microscopy, serial optical sections were imaged at 1-2 μ m intervals under a 60x objective lens with oil.

Results

Localization of PAI-1 and 2 proteins during the mouse preimplantation development: To determine the expression patterns of PAI-1 and 2, proteins during preimplantation development, mouse embryos from 2-cell to blastocyst stages were double immunostained with anti-PAI-1 and 2 antibodies. In the 2-cell stage embryos (n=25), both PAI-1 (red) and PAI-2 (green) proteins localized in the cytoplasm and the nucleus (Figure 1). In addition, polar bodies in the 2-cell embryos showed PAI-1 and 2, staining positive (Figure 1), suggesting that PAI-1 and 2 proteins are produced not only in the embryo after fertilization, but also in the female-specific oocytes. In the advanced embryos at the 8-cell (n=10) and the morula (n=25) stages, PAI-1 and 2, expressions were constantly observed (Figure 2). These proteins were entirely observed in the cytoplasm and the nucleus except a few nucleoli (Figure 2). In morula stage embryos (16-32 cells) strongly stained dotted structures were observed in each blastomeres regardless of their internal or external position (Figure 2).

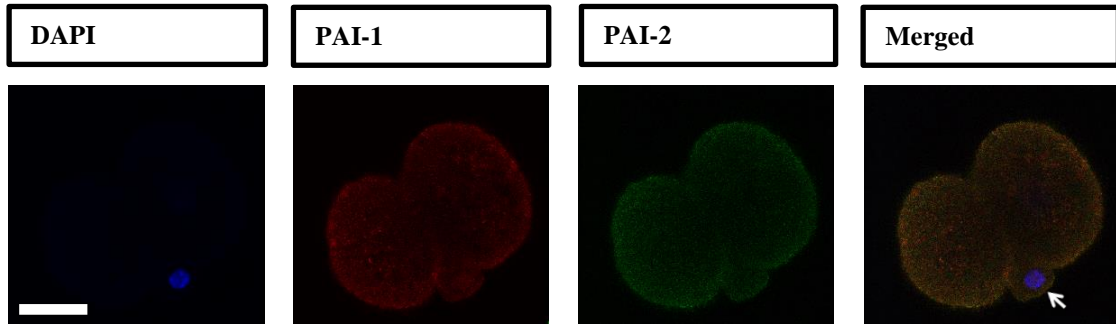


Figure 1. Localization of PAI-1 and PAI-2 protein at 2-cell stage embryo under a confocal microscopy. PAI-1(red) and PAI-2 (green) were localized in the polar body (arrow). Nuclei were stained with DAPI (blue). Scale bar represents 20 μ m. DAPI: 4',6' - Diamidino-2-phenylindole, PAI-1: Plasminogen Activator Inhibitor Type 1, PAI-2: Plasminogen Activator Inhibitor Type 2.

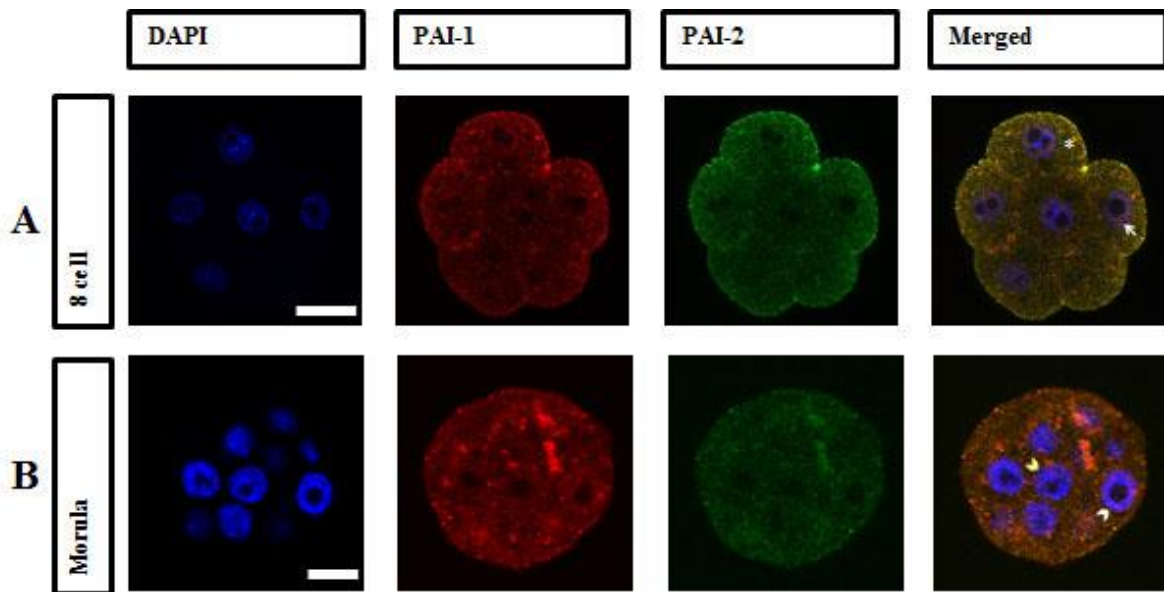


Figure 2. Localization of PAI-1 and PAI-2 in 8-cell and morula stage embryos. (A-B) PAI-1 and PAI-2 were present at the cytoplasm (asterisk) and nucleus (arrow). External (white arrowhead) and internal cells (yellow arrowhead) cytoplasm and nuclei were positively stained with PAI-1 and PAI-2. Confocal optical sections of embryos immunofluorescently stained with PAI-1 (red), PAI-2 (green) and nuclei stained with DAPI (blue). Scale bar represents 20 μ m.

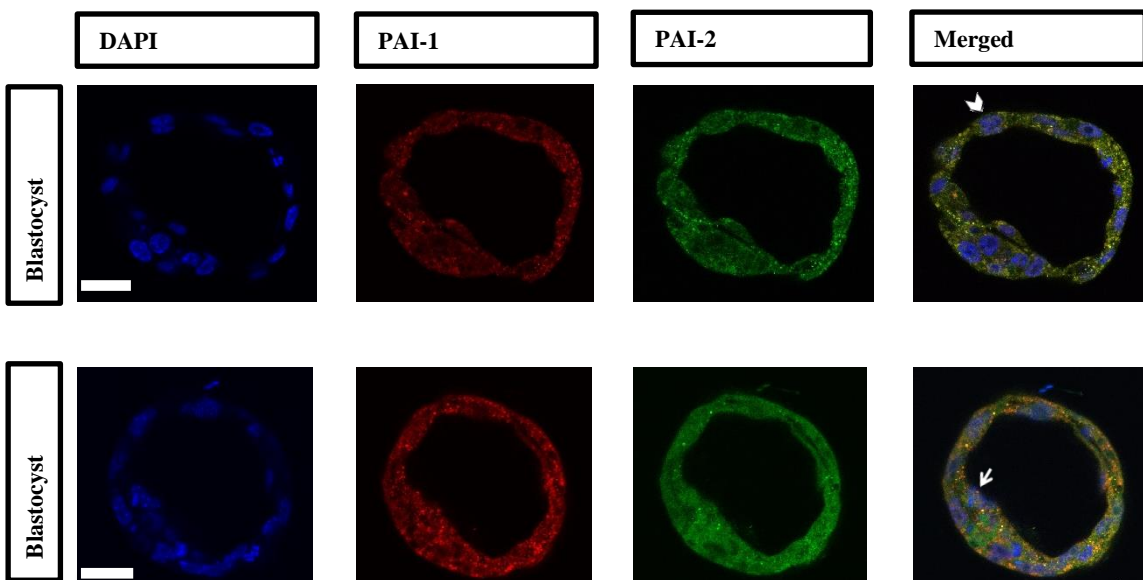


Figure 3. Localization of PAI-1 and PAI-2 proteins in blastocysts. Blastocysts were immunostained with PAI-1 (red) and PAI-2 (green). The nuclear and cytoplasmic staining on TE cells (arrowhead) and on ICM (arrow). Nuclei were stained with DAPI (blue). Scale bar represents 20 μ m.

At the blastocyst stage, the embryos differentiate into two different cell lineages, trophoblast (TE) and inner cell mass (ICM). After the double immunostaining of the blastocyst embryos, the blastomeres in TE and ICM were strongly stained with both PAI-1 and PAI-2 antibodies (Figure 3). Both proteins were localized in the cytoplasm and the nucleus (except a few nucleoli) in each blastomere at the blastocyst stage. In conclusion, PAI-1 and PAI-2 were detected in the early mouse embryos in all preimplantation stages, suggesting that PAI-1 and 2 proteins are constantly expressed in the early stage embryos before implantation in the mother's uterus.

Discussion and Conclusion

In the present study, we demonstrated that PAI-1 and 2 are constantly expressed and localized in both the cytoplasm and the nucleus in the mouse embryos throughout the preimplantation period. Our results indicate that PAI-1 and PAI-2 may contribute to normal preimplantation development essential for successful implantation and maintaining the pregnancy.

Previous studies reported that PAI-1 and 2 are expressed in human trophoblasts during and after implantation (10). Also, it was observed that both proteins are distributed in the cytoplasm of cytotrophoblasts and cytoplasm and plasma membrane of the syncytiotrophoblasts (15). In contrast, uPA and tPA expression in rat embryos has occurred during their early embryonic development, while uPA was distributed in the cell cytoplasm, tPA was appeared only on cell surface and in the perivitelline space (1). Because PAs are implicated in early stages of development and embryo implantation, we expected that the expression of PAI-1 and 2 (inhibitors of PAs) could be suppressed, or very limited during embryogenesis. However, our data indicate that both proteins are strongly and entirely expressed in the embryos at the 2-cell ~ the blastocyst stages, suggesting that PAI-1 and 2 proteins may not inhibit roles of PAs in oocyte meiotic maturation, ovulation, fertilization and embryo implantation. In fact, several studies showed that PAI-1 is a multi-functional protein that plays a role in physiological and pathological processes such as tissue remodeling, embryogenesis, regulating cell proliferation and migration, adhesion and tumor invasion, angiogenesis and metastasis (3, 19, 20, 28). Mashiko et al. (23) and Giacoia et al. (12, 13) suggested that PAI-1 inhibition promotes cell cycle arrest and apoptosis in ovarian and bladder cancer, and PAI-1 knockdown resulted in significant suppression of cell growth in cancer cells. The evidence suggests that PAI-1 may have some unique role associated with embryogenesis rather than inhibiting PAs.

It has been known that PAI-1 levels in the plasma gradually increased during normal pregnancy and reached maximum at 32-40 weeks of pregnancy, while PAI levels

fall again 5-8 weeks after the birth in healthy pregnant women (14). PAI-1 may also play a role in remodeling maternal uterine spiral arteries (11). Hypoinvasion and unsuccessful placental vascular remodeling are related with intrauterine growth restriction, maternal and fetal death (18, 32). Depending on this information, previous studies demonstrated that any pathological disturbance in PAI-1 concentrations can cause pregnancy complications (27) and PAI-1 expression is increased in recurrent pregnancy losses, pre-eclampsia, intrauterine growth restriction and gestational diabetes mellitus (33). Intriguingly, Carmeliet et al. (6) indicated that PAI-1 deficient mice were viable and could generate morphologically normal offspring with normal litter sizes. PAI-2 exists in human plasma and its expression levels are increased during normal pregnancy, suggesting a possible requirement for PAI-2 during early development and/or placentation (4). However, Dougherty et al. (8) reported that PAI-2 is not required for normal murine development, survival and fertility. Our findings showed the localization of PAI-1 and 2, in cytoplasm and nucleus in all developmental stages, suggesting possible roles of these genes in preimplantation embryo development.

In this study our results indicated that, PAI-1 and PAI-2 are expressed in both cytoplasm and nucleus during all stages of preimplantation development in mice, suggesting that they may play roles in early stages of embryonic development. Especially expression of PAI-1 and PAI-2 in the trophoblast supports the idea that these genes can be important for proper implantation and placentation. Further studies are required to fully understand the functions of PAI-1 and PAI-2 in embryogenesis.

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Conflict of Interest

The authors declared that there is no conflict of interest.

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