Expression of IL-1 β and implantation serine proteases is required for mouse blastocyst hatching

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Abstract

Mammalian blastocyst hatching is a critically indispensable process for successful implantation. One of the major challenges in IVF clinics is to achieve superior embryonic development with intrinsically potent hatching-competent blastocyst. However, the molecular regulation of hatching phenomenon is poorly understood. In this study, we examined the expression and function of one of the cytokines, IL-1 β during blastocyst hatching in the mouse. In particular, the expression of IL-1 β (Interleukin-1 β), IL-1ra (Interleukin-1 receptor antagonist) and their functional receptor IL-1rt1 (Interleukin 1 receptor type-1) in morulae, zona intact- and hatched-blastocysts was studied. Supplementation of IL-1 β to cultured embryos accelerated blastocyst development with improved hatching (treated: $89.6 \pm 3.6\%$ vs untreated: $65.4 \pm 4.1\%$). When embryos were treated with IL-1ra, blastocyst hatching was decreased (treated: $28.8 \pm 3.1\%$ vs untreated: $67.5 \pm 3.8\%$). Moreover, IL-1 β and IL-1ra influenced the expression of hatching enzymes viz., implantation serine proteases (ISP1 and ISP2). While IL-1 β increased the embryonic mRNA expression of ISPs (*Isp1*: 2–4; *Isp2*: 9- to 11-fold), IL-1ra decreased expression. The protein localization studies revealed increased nuclear presence predominantly of ISP 2 in IL-1 β -treated blastocysts. This is the first report to show the functional significance of embryonic IL-1 β in regulating hatching-associated proteases, particularly ISP2. These findings have implications in our understanding of molecular regulation of blastocyst hatching and implantation failure in other species including humans.

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Introduction

Mammalian blastocyst hatching is an exemplary early-development phenomenon preceding the implantation event, both being pre-requisites for subsequent embryo viability and successful pregnancy (Seshagiri *et al.* 2009, 2016, Shafei *et al.* 2017). It is well documented that the biological viability of *in vitro* cultured embryos is inferior to those developing *in vivo* (Harlow & Quinn 1982, Roth *et al.* 1994, Stokes *et al.* 2005, Chimote *et al.* 2013). Human infertility clinics perform assisted hatching techniques in a few indications, in order to achieve improved implantation rates (Ghannadi *et al.* 2011, Li *et al.* 2016). Such approaches pose unintended adverse effects on embryonic viability, hatching failure, monozygotic twinning and possibly mutations in the embryonic genome (Hammadeh *et al.* 2011, Huang *et al.* 2019, Lu *et al.* 2019). Therefore, it is essential to understand the biology of development of hatching-competent blastocysts and to determine the causes of hatching failure. Unlike extensive research on implantation biology (Singh *et al.* 2011), studies on the molecular regulation of blastocyst hatching biology

per se are scarce in most species studied (Seshagiri *et al.* 2009, 2016). Among animal models studied so far, the mouse has been a main-stay to study early peri-implantation development (Taft 2008).

Blastocysts of the mouse, similar to human, exhibit hatching behavior which includes expansion of blastocoel, its increased hydrostatic pressure, zona thinning and leading to rupture of the zona (Lin *et al.* 2001, Sathananthan *et al.* 2003, Leonavicius *et al.* 2018). These mechanical events, occurring during hatching, are believed to be accompanied by secretory zonalytic protease-factors (Perona & Wassarman 1986, Seshagiri *et al.* 2009, 2016, Shafei *et al.* 2017). In the mouse, blastocyst-hatching enzymes such as (1) trypsin-like serine protease (Sawada *et al.* 1990) and (2) a set of serine proteases ISP1 and ISP2 are implicated in blastocyst hatching (O'Sullivan *et al.* 2001, 2002, Sharma *et al.* 2006). Interestingly, the ISP1, exclusively expressed in the mouse, is hypothesized to have neo-functionality as a hatching enabling enzyme (Sharma *et al.* 2006). Besides, in non-embryonic cells, the expression of serine-like proteases is reported to be regulated by IL-1 β (Indalao *et al.* 2017).

A plethora of implantation-associated molecules, such as steroid hormones, growth factors, cytokines, enzymes and transcription factors (Singh *et al.* 2011, Bulun 2017, Raheem 2018), have been extensively studied. Ironically, they have not been examined, with any details, in the context of blastocyst hatching in species studied (Pan *et al.* 2015, Shafei *et al.* 2017, Zhan *et al.* 2018). In mouse, embryonic expression of various cytokines was examined and their potential involvement in the regulation of development of pre-implantation embryos has been reported (Gerwin *et al.* 1995, Krussel *et al.* 1998, Nakasato *et al.* 2004, Seshagiri *et al.* 2016, Robertson *et al.* 2018). Much of the functional data on cytokines and their signaling systems come from gene knockout studies (Stewart *et al.* 1992, Murphy *et al.* 2005, Uri-Belapolsky *et al.* 2014). However, observations made did not indicate any profound implantation-failure phenotypes and importantly, the hatching phenotype (Seymour *et al.* 1997), including an assessment of embryos during pre-implantation development and whether or not mutant blastocysts were capable of hatching. Of relevance, in this context, are reports on the expression and involvement of interleukin-1 β (IL-1 β), interleukin-1 receptor antagonist (IL-1ra) and interleukin-1 receptor type I (IL-1RtI) during the peri-hatching development (Kita *et al.* 1994, Krussel *et al.* 1997, 1998, Correia-Álvarez *et al.* 2015). In humans, both the embryo and the endometrium express IL-1 β and its increasing concentration is associated with successful implantation. In this regard, IL-1 β is thought to be a potent embryo biomarker for successful implantation (Sequeira *et al.* 2015, Salama *et al.* 2020). Moreover, the embryonic expression of IL-1ra cytokine, the natural antagonist of IL-1 β , has been shown to be associated with developmentally arrested embryos (Krussel *et al.* 1997, Simón *et al.* 1998).

However, the role of IL-1 β in blastocyst development and hatching remains poorly studied. In the mouse, there are contradictory reports on the functional significance of IL-1 β in the implantation process (Abbondanzo *et al.* 1996, Simón *et al.* 1998). In the context of pre-implantation development, reports on the involvement of IL-1 β are very scarce and inconclusive (Schneider *et al.* 1989, Tartakovsky & Ben-Yair 1991). For example, the administration of IL-1 β to mouse promoted the advanced development of morulae (Tartakovsky & Ben-Yair 1991). In contrast, interleukins (IL-1 β) did not promote the development of cultured mouse embryos to blastocysts (Schneider *et al.* 1989). These findings provide a compelling reason to investigate the potential involvement of IL-1 β in blastocyst development and hatching. Here, we present data on the embryonic expression and function of both IL-1 β and IL-1ra in the context of blastocyst development and hatching in the mouse.

Materials and methods

Animals

Albino mice (CD1 or FVB/N strain) were used for the study. Animals were provided with proper light-dark cycle, temperature, humidity, food and water. Sexually mature females (6–8 weeks old) were observed for regular estrus cyclicity, assessed by vaginal gross-morphology. Healthy, regularly cycling females were selected as embryo donors. The Institutional Animal Ethics Committee approved procedures for animal handling and experimentation, in accordance with guidelines on the use of laboratory animals for research (INSA, New Delhi).

Embryo recovery and culture

Embryo donor female mice ($n = 50$) were super-ovulated with an i.p. injection of 5 IU Pregnant Mare Serum Gonadotropin (PMSG, Sigma or Folligon, MSD) on the day of estrus. This was followed, post-48 h, by HCG injection. Females were then kept with sexually mature healthy males for mating. In the evening of post-day 3 of mating, *in vivo* developed, compacting eight cells or morulae were collected by flushing the excised uterine horns with pre-equilibrated M-2 medium contained in a blunted hypodermic flushing needle; recovered embryos were cultured *in vitro* in 50 μ L micro-drops of M-16 medium (prepared freshly using embryo culture grade components from Sigma) in 35 mm dishes (Greiner) at 37°C in a humidified gas atmosphere of 5% CO₂ in air incubator (Hogan *et al.* 1986). Embryo culture drops were overlaid with 2.5 mL of pre-equilibrated embryo-tested silicone oil (Sigma). For each experiment, a group of 4–10 embryos were cultured in each treatment drop. Embryo development was monitored microscopically every 12 h for a total of 60–72 h for development and hatching. Embryos, sampled for expression analysis, were either frozen for RNA isolation or fixed in 4% paraformaldehyde (Sigma) for immunocytochemistry (ICC) analysis. For IL-1 β or IL-1ra-treated embryos, samples collected were a pool of embryos from a single medium drop containing 4–10 embryos. Embryos were washed with PBS and were frozen (–80°C) in 10 μ L of PBS.

Supplementation of IL-1 β and IL-1ra in embryo culture

The influence of IL-1 β (PMC0814; GIBCO, ThermoFisher) and IL-1ra (cyt-658; Prospec) were tested on blastocyst hatching. Working solutions with test compounds were prepared in M-16 medium. About 4–10 embryos per treatment were cultured in M-16, without or with the test compounds, for 60 h and a minimum of four replicate experiments were performed. For IL-1 β and IL-1ra supplementation studies, a total of 15 and 10 females were used, respectively. A randomized distribution of an equal number of synchronously developed embryos from individual donors was adopted. All embryos were monitored for viability, gross-morphology, hatching rates and post-hatching trophoblast outgrowths. Total

cell numbers of (hatched) blastocysts were monitored. Photomicrographic images were captured, using a CCD camera and Image Cell Sens software program (Mishra & Seshagiri 1998, Sireesha *et al.* 2008). For dose-dependent studies, doses were selected based on published reports (Paula-lobes *et al.* 1998) and as recommended by the safety data sheet/product information from the company. Besides, experiments were also performed for dose-determination, which was selected for subsequent experiments.

Embryo mRNA isolation and cDNA synthesis

Using saved frozen embryos (10–20, pooled), direct polyA⁺ mRNA isolation was performed using Dynabead mRNA DIRECT Kit (61011, Invitrogen/Life Technologies; as per provided protocol). This relied on base-pairing between poly A residues at 3' end of mRNA and the oligo dT25 residues that were covalently coupled to the surface of Dynabeads. The RNA was then subjected to cDNA synthesis in 20 μ L reaction mixture, using Verso cDNA synthesis kit (AB-1453/A, ThermoFisher; as per the provided protocol). The synthesized cDNA was then subjected to gene amplification.

Quantification of embryonic gene expression using qPCR

The embryonic cDNA used per reaction was a half embryo equivalent (i.e. if cDNA from a pool of 10 embryos was reconstituted in 20 μ L solution, then, 1 μ L is considered as half embryo equivalent). The reaction was set in duplicates for each sample. Three biological replicates were performed with each one constituting a pool of 10–20 embryos of each stage (one sample) for stage-dependent expression and, for IL-1 β or IL-1ra-treated embryos, 5–10 embryos per replicate were used for analysis. The qPCR conditions used for all primers were 95°C for 2 min for initial denaturation, followed by 40 repetitive cycles consisted of steps: (1) 95°C for 15 s for subsequent denaturation, (2) annealing step of 58°C for 60 s and final step and (3) 72°C for 30 s for extension. This was followed by melting curve analysis at 95°C for 15 s, followed by 60°C for 15 s and 95°C for 15 s. The analysis was performed using Step One Plus Real Time PCR (Applied Biosystem) system using Syber Green PCR Master Mix (Cat. No. AB-1053/A, ThermoFisher). The result was analyzed using Stepone software 2.2.2 and Ct values were calculated from the amplification plot. The actin gene normalization was performed for each sample in each assay to minimize the sample to sample and assay to assay variations. The expression values were assessed by calculating normalized- Δ Ct Value (Ct (test gene) – Ct (*Actb* gene)) and plotting $2^{-\Delta\text{Ct}}$ values to show relative expression during different embryonic stages. In order to calculate fold change of IL-1 β /IL-1ra-treated embryos with respect to the control untreated embryo samples, the calibrated $\Delta\Delta$ Ct value was calculated by subtracting normalized Ct values of the two genes; Δ Ct (test, e.g. gene expression in treated embryos) – Δ Ct (calibrator, e.g. gene expression in untreated embryo samples). The $2^{-\Delta\Delta\text{Ct}}$ was plotted as fold change. The 'No Template Controls' were included in all experiments.

The nucleotide sequences of primers used were:

*Actb*_{for}: 5'-AGGTGACAGCATTGCTTCTG-3'
*Actb*_{rev}: 5'-GCTGCCTCAACACCTCACC-3' (188 bp)
*ISP1*_{for}: 5'-CAGTCCAGTCTCTCTGCCAAAA-3'
*ISP1*_{rev}: 5'-ATAAGCCCGCTTACAGCTCTTG-3' (166 bp)
*ISP2*_{for}: 5'-GCTGGCAGTGTCTGTACAATCC-3'
*ISP2*_{rev}: 5'-CTGGTGGCATTGTGTTACATCT-3' (213 bp)
IL-1 β _{for}: 5'-TACATCAGCACCTCACAAGCA-3'
IL-1 β _{rev}: 5'-CCCATACTTTAGGAAGACACGG-3' (107 bp)
*IL-1ra*_{for}: 5'-AGACAAGCGCTTTACCTTCATC-3'
*IL-1ra*_{rev}: 5'-TACAGGCCTCGGCAGTACTATT-3' (195 bp)
*IL-1rt1*_{for}: 5'-CGTGAGCTTCTTCGGAGTAAAA-3'
*IL-1rt1*_{rev}: 5'-GAGGGCTCAGGATAACAGGTCT-3' (182 bp)

Whole-mount immunocytochemistry (ICC) and quantification

Embryos, fixed in PFA (4% in PBS, Sigma), were rinsed in PBS-PVA and then permeabilized with 0.2% Triton X-100 for 15 min and blocked in 0.3% BSA for 2 h at room temperature. Embryos were rinsed again in PBS-PVA (Sigma) and then incubated with the corresponding anti-mouse primary antibody; polyclonal goat IgG IL-1 β (AB-401-NA, R&D systems), polyclonal goat IgG IL-1rt1 (ITAB11621, Immunotag), rabbit polyclonal IgG IL-1ra (32594, Signalling Antibody), Polyclonal Rat IgG ISP 2 (MBS1495206, Mybiosource), for overnight at 4°C. The dilution of the primary antibody used was 1:200 in blocking solution except for IL-1ra, which was used at 1:50 dilution. Control embryos were incubated in blocking solution without primary antibody. After washing in PBS-PVA, embryos were subjected to incubation with secondary antibody, that is, polyclonal donkey IgG alexa fluor 633/alexa 594 conjugated anti-goat IgG (Invitrogen) or anti-rabbit IgG-FITC conjugated or anti-rat alexa fluor 568 (Invitrogen/Life Technologies), at dilution of 1:500 for 45 min at room temperature. For nuclear staining, embryos were stained with DAPI (10 μ g/mL, Sigma) for 15 min at room temperature. Embryos were then washed a couple of times in PBS-PVA and then mounted using prolonged gold anti-fade mountant (Invitrogen) on the 0.2 mm coverslips, later inverted on concavity glass slides. The slides were imaged using Zeiss LSM 510/LSM 880 confocal laser scanning microscope (Carl Zeiss) using Zen 2.1 software. For immuno quantification, a total of 3–4 embryos of each stage was analyzed for embryonic stage-dependent protein expression level analysis. For IL-1 β /IL-1ra supplementation experiment, a total of 5–8 embryos was analyzed from each treatment group.

Zona thickness measurement studies, viability assay, differential cells (trophectoderm (TE)-inner cell mass (ICM) determination and cell attachment assay

Images of cultured zona-intact embryos, at different stages, were captured and subjected to zona thickness measurement using the software; the digital scale provided in the software was calibrated using a manual micrometer (10 μ m) scale. The thickness of the zona was measured using arbitrary line

drawn, spanning their width of the translucent zona layer. Measurements were made at four different quadrants of the zona, at two or more magnifications; their mean values were taken. For partially hatched embryos, measurements were carried out at three different points and the mean value was calculated for each embryo. The viability of embryos was assessed using 0.5% trypan blue dye exclusion test. Allocation of cells to TE and ICM of cultured blastocysts was determined by ICM-cell-specific staining using OCT-4 (Santa Cruz Biotechnology) antibody, followed by nuclear staining using DAPI (10 µg/mL, Sigma). Blastocyst attachment and TB outgrowth cultures and their morphometric analysis were performed, as described elsewhere (Mishra & Seshagiri 1998). The TB outgrowth was assessed under an inverted microscope (IMT-2; Olympus Co Ltd), using phase-contrast objectives. Morphometric measurements of TB outgrowth were made using an image analysis software provided by Cell Sens.

Statistical analysis

Mean values in all experiments were calculated from the total number of embryos cultured during a particular treatment. To control between-animal variations, a block design was used. For this, freshly recovered embryos from each donor female were distributed randomly and in similar numbers among all treatments and this constituted one replicate experiment. Moreover, all replicate experiments were performed under identical experimental conditions. This design considerably strengthened the statistical analysis of data. Hence, the statistics were based on the number of biological replicates, that is, a different set of experiments that were performed (independent of the number of donors used). A minimum of 4–10 replicate experiments (from minimum of three different animals) were performed. To assess the statistical significance of treatment effects, one-way ANOVA/Student's *t*-test/two-way ANOVA, followed by Bonferroni's *post hoc*, tests were performed to obtain *P*-value (Graph Pad Prism Software Version-4).

Results

Embryonic expression of IL-1β, IL-1ra and IL-1rt1 during peri-hatching development

We first analyzed the embryonic development stage-dependent expression patterns of IL-1β, its natural antagonist IL-1ra, and its functional receptor IL-1rt1 (Fig. 1). This was performed with freshly recovered morulae, expanded zona-intact blastocysts and hatched blastocysts. The analysis of mRNA levels of the above revealed a stage-dependent pattern with an increase in the expression of IL-1β (Fig. 1A-i); however, the differences in expression were not statistically significant. In contrast, a statistically significant decrease in the expression of IL-1ra was observed as the development of embryos progressed (Fig. 1A-ii, *P* < 0.05). Similar levels of expression of IL-rt1 were observed in three stages of embryo development (Fig. 1A-iii). When their protein expression and localization studies were performed,

it was observed that the IL-1β and IL-1ra were uniformly localized to the cytoplasm of all TE and ICM cells, whereas IL-1rt1 showed membrane-associated localization (Fig. 1B). The protein quantification (mean fluorescent intensity) of ICC data corroborated with the observed changes in mRNA levels (Fig. 1C).

Influence of IL-1β or IL-1ra on blastocyst hatching

To investigate the functional role of IL-1β during blastocyst hatching, supplementation of IL-1β or its antagonist (IL-1ra) in the M-16 medium was made during independent embryo culture experiments. The cultured embryos developed to expanded blastocysts by 24 h (Fig. 2A-i, A-iii, A-v) and eventually, they hatched by 48 h (Fig. 2A-ii, A-iv, A-vi). The supplementation of IL-1β led to enhanced blastocyst hatching rate in a dose-dependent manner. The observed increases in hatching percentages in the presence of IL-1β (1 ng/mL or 2 ng/mL) were $76.3 \pm 1.3\%$ (not significant) or $88.8 \pm 6.6\%$ (*P* < 0.01), when compared to untreated control ($58.7 \pm 5.9\%$; Fig. 2B-i). Additional experiments were also performed with only effective dose of 2 ng/mL of IL-1β. It was observed that the supplementation of IL-1β yielded significantly improved hatching percentages, that is, $89.6 \pm 3.6\%$, when compared to untreated control blastocysts ($65.4 \pm 4.1\%$; Fig. 2B-ii, *P* < 0.001).

Two independent set of experiments were performed to examine the influence of varying concentrations of IL-1ra on blastocyst hatching. In the first set, embryos were cultured in the presence of 0.5 or 1 ng/mL concentration of IL-1ra (Fig. 2C-i) and percentages of hatched blastocysts observed, respectively, were 33.3 ± 8.3 or 8.3 ± 8.3 (*P* < 0.05); the percentage of hatched blastocysts in the untreated control was 66.6 ± 6.6 (Fig. 2A-vi, C-i). To mitigate the possible observed embryo-toxicity of IL-1ra, subsequent experiments were performed using a lower concentration of IL-1ra, that is, 0.2 ng/mL. In this set of experiment, while $67.5 \pm 3.8\%$ of embryos hatched (60 h) in the untreated control, IL-1ra-treated (0.2 ng/mL) embryos showed reduced hatching percentage (28.8 ± 3.7 , *P* < 0.01; Fig. 2C-ii). When we analyzed the effect of IL-1β on cellular viability and differential cell counts of TE and ICM of cultured blastocysts and, the trophoblasts outgrowth capability of hatched blastocysts, we observed no significant effect on these parameters whether or not embryos were treated with the IL-1β (data not shown).

Influence of IL-1β on time-kinetic of hatching and zona thinning

To elucidate the hastening effect of IL-1β on blastocyst hatching, we performed the time kinetics of blastocyst hatching (Fig. 3A and B). The hatching rate trajectory was dose-dependent (Fig. 3A) and it was more pronounced with embryos treated with 2 ng/mL of IL-1β (Fig. 3B). The percentage of blastocysts hatched in the IL-1β-treated

Figure 1 Embryonic expression of *IL-1 β* , *IL-1ra* and *IL-1rt1* during peri-hatching stages of embryo development. Panel A depicts the quantification of embryonic transcript levels of *IL-1 β* (A-i), *IL-1ra* (A-ii) and *IL-1rt1* (A-iii), in peri-hatching embryonic stages. Panel B depicts immunostained images of morula, blastocyst and hatched blastocyst. Immunolocalization of three proteins are shown, namely embryonic *IL-1 β* (B-i), *IL-1ra* (B-ii) and *IL-1rt1* (B-iii). Right panels in each set show merged images of immunostainings with DAPI, with Scale bar: 20 μ m. Panel C depicts the quantification of fluorescent intensity levels of *IL-1 β* (C-i), *IL-1ra* (C-ii) and *IL-1rt1* (C-iii), in peri-hatching embryonic stages. Values represent mean $\pm$ S.E.M. for minimum of three biological replicates. Statistical difference was determined using one-way ANOVA, subjected to post-Bonferroni's test. * P < 0.05, ** P < 0.01. MO, morulae; eBC, expanded blastocysts; hBC, hatched blastocysts.

Figure 2 Effect of IL-1 β /IL-1ra treatment on blastocyst hatching. Panel A depicts representative morphology of development of freshly recovered morulae to blastocysts in the untreated group (A-i, A-ii) vs the IL-1 β -treated group (2 ng/mL) (A-iii, A-iv) and IL-1ra-treated group (0.2 ng/mL) (A-v, A-vi). Panel B depicts the graph showing the total percentage of hatched blastocysts, observed under untreated vs IL-1 β -treated group. Panel C shows the bar graph depicting the total percentage of hatched blastocysts, observed for the untreated vs IL-1ra-treated embryos at a different dosage. Photographs were captured with Nomarski optics. Magnification: 10 $\times$ and Scale bar: 20 μ m. Values in the graph represent mean $\pm$ S.E.M. for a minimum of four biological replicate experiments. Statistical difference was determined using one-way ANOVA, subjected to post-Bonferroni's test. ** $P < 0.01$, *** $P < 0.001$.

group, at the 24 h time point, was $24.3 \pm 4.7\%$ when none was observed in the untreated control group (Fig. 3B, $P < 0.01$). Blastocysts supplemented with IL-1 β were invariably associated with hastened hatching behavior when compared to untreated control embryos.

As the embryo culture progressed, a relatively non-significant and similar zona thickness ($3.7 \pm 0.7 \mu$ m) was observed in the IL-1 β -treated embryos or the control embryos, post-48 h of culture (Fig. 3C). The zona thickness measurements for partly hatched embryos were made separately at 48 hr (Fig. 3D). The zona thickness for the IL-1 β -treated, partly hatched embryos was $2.2 \pm 0.1 \mu$ m, when compared to the untreated control embryos ($3.3 \pm 0.4 \mu$ m; Fig. 3D). The average thickness of ruptured zona, left behind in the untreated embryos, was similar to IL-1 β -treated embryos ($3.7 \pm 0.5 \mu$ m vs $3.4 \pm 0.4 \mu$ m) at 48 h (Fig. 3D). The retention of elasticity of the ruptured zona was responsible for the observed increasing patterns in the zona thickness post-hatching. Hence, the effect of IL-1 β on zona-thinning was non-significant.

ISPs mRNA level in IL-1 β /IL-1ra supplemented embryos

The effect of IL-1 β or IL-1ra on the expression of hatching associated proteases, that is, *Isp1* and *Isp2* was examined (Fig. 4). Embryos cultured without or with IL-1 β were used for analysis. The embryonic transcript

levels of both *Isp1* and *Isp2* significantly increased in the IL-1 β -treated zona-intact blastocysts, that is, 3.5 ± 0.5 -fold for *Isp1* ($P < 0.05$) and 11.7 ± 2.6 -fold for *Isp2* (Fig. 4A-i, $P < 0.01$). With the IL-1 β -treated embryos, at the hatched stage, a statistically significant increase was observed in the transcript level of *Isp2* (9.1 ± 1.4 -fold, $P < 0.05$) than that (2.8 ± 1.1 -fold) of *Isp1* (Fig. 4A-ii).

Similarly, the expressions of *Isp1* and *Isp2* were quantitatively analyzed for embryos cultured in the presence and absence of IL-1ra (0.2 ng/mL). The transcript levels of both, *Isp1* and *Isp2* decreased in the IL-1ra-treated blastocysts. The reduction in the expression level observed for *Isp1* in IL-1ra-treated blastocyst was by $73.2 \pm 3\%$ (0.26 ± 0.03 -fold, $P < 0.05$; Fig. 4B) as opposed to the untreated embryos. For *Isp2* also, there was a statistically significant decrease in the expression level by $82.3 \pm 11\%$ (0.17 ± 0.11 -fold, $P < 0.01$; Fig. 4B) in the IL-1ra-treated zona-intact blastocysts (Fig. 4B), when compared with the untreated control embryos. Because, the numbers of embryos hatched in the presence of IL-1ra were too low, the mRNA expression analysis was not performed for the hatched blastocyst stage.

ISP2 protein levels in IL-1 β or IL-1ra supplemented embryos

To analyze protein levels of proteases, we focused on transcriptionally more abundant ISP2. Cultured

Figure 3 Time kinetics of development of blastocysts in the presence and absence of IL-1 β . (A) Represents % hatched blastocysts observed at different time points for IL-1 β -treated (1 ng/mL, blue squares or 2 ng/mL, dark blue triangles) embryos, compared to untreated control (pink circles). (B) Represents % hatched blastocysts observed at different time points for optimized concentration of 2 ng/mL (dark blue circles) as compared to untreated control (pink circles). (C) Represents the influence of IL-1 β on zona thickness of untreated (pink circles) and 2 ng/mL IL-1 β -treated (blue circles) zona-intact blastocysts, till 48 h. (D) Depicts the zona thickness of untreated (pink circles) and IL-1 β -treated (dark blue circles) zona-intact blastocysts, till 24 h and partly hatched blastocyst at 48 h. Dotted lines (...) in both line-graphs represent ruptured zona. Values in the graph represent mean $\pm$ S.E.M. for a minimum of four biological replicate experiments. Statistical difference was determined using one-way ANOVA from four replicate experiments for (A) and eight replicate experiments for (B). * P < 0.05, ** P < 0.01, *** P < 0.001.

blastocysts were subjected to protein localization studies. Localization of ISP2 was observed in the TE and ICM (predominantly nuclear) cells in the IL-1 β -treated as well as untreated embryos (Fig. 5A and C). Quantitative analysis of immunostained zona-intact blastocysts for ISP2 showed a differential level of fluorescent intensity among IL-1 β -treated and untreated embryos (Fig. 5B and D). Mean immunofluorescence intensity quantification showed an increased level of ISP2, following the treatment of IL-1 β . The mean intensity for ISP2 immunofluorescence was 4672 ± 177.4 in the IL-1 β -treated blastocyst, whereas its level was 3832 ± 70.20 in the untreated control embryos (Fig. 5B, P < 0.05). Protein localization for ISP2 was also performed in fully hatched embryos (Fig. 5C). Quantitative analysis showed increased levels of ISP2 protease following treatment with IL-1 β (Fig. 5D). The mean intensity for ISP2 immunofluorescence in hatched blastocyst was 4511 ± 136.2 in the IL-1 β -treated embryos, whereas its level was 3700 ± 90.17 in the untreated control embryos (Fig. 5D, P < 0.01). The observed increase in the ISP2 protein corroborated with the increase in its mRNA expression level.

Figure 4 Influence of IL-1 β or IL-1ra on mRNA level of ISPs in cultured blastocysts. Panel A-i depicts mRNA levels of *Isp1* and *Isp2* under IL-1 β -treated (2 ng/mL) vs untreated conditions in zona-intact blastocysts. Panel A-ii depicts mRNA levels of *Isp1* and *Isp2* under IL-1 β -treated vs untreated conditions in hatched blastocysts. Panel B depicts quantification of transcript levels of *Isp1* and *Isp2* under IL-1ra-treated (0.2 ng/mL) and untreated conditions in zona-intact blastocysts. Values represent mean $\pm$ S.E.M. of three biological replicate experiments. Statistical analysis was carried using two-way ANOVA subjected to post-Bonferonni's test. ** P < 0.01 * P < 0.05.

The colocalization studies for both the IL-1 β and ISP2 were performed under IL-1ra supplemented conditions. There was a significant reduction in the level of both proteins, IL-1 β and ISP2 (Fig. 6A and B). Quantitative analysis of immunostained zona-intact blastocysts, for IL-1 β and ISP2 showed a differential level of fluorescent intensity in IL-1ra-treated embryos when compared to the untreated ones (Fig. 6B, P < 0.05). Mean intensity for IL-1 β fluorescence was 3304 ± 1528 in treated embryos, whereas its level was 3931 ± 75.2 in the untreated control embryos (Fig. 6B). Mean intensity for ISP2 immunofluorescence was 3917 ± 152.8 in the IL-1ra-treated embryos, whereas its level was 4124 ± 292.8 in the untreated embryos (Fig. 6B, P < 0.05).

Discussion

This study shows, for the first time, the involvement of IL-1 β in the development-hatching of blastocysts and in modulating the hatching-associated proteases, that is, ISP 1 and ISP 2 in the mouse. Earlier, the role of IL-1 β during embryo implantation and in maintaining viable pregnancy was reported in the mouse (Sequeira *et al.* 2015, Salama *et al.* 2020) as well as in the human

Figure 5 Influence of IL-1 β on the ISP 2 protein localization. Panel A depicts ISP 2 localization in untreated and IL-1 β -treated blastocysts. Scale bar: 20 μ m. Panel B denotes protein quantification (in arbitrary units) for zona-intact blastocyst for ISP 2 staining intensity in the presence or absence of IL-1 β . Panel C depicts ISP 2 localization in untreated and IL-1 β -treated hatched blastocysts. Scale bar: 20 μ m. Panel D denotes protein quantification (in arbitrary units) for hatched blastocyst for ISP 2 staining in the presence or absence of IL-1 β ; the dose of IL-1 β used was 2 ng/mL. Values in the graph represent mean $\pm$ S.E.M. of minimum of three replicate experiments. Statistical analysis was performed using unpaired Student's t-test. * P < 0.05, ** P < 0.01.

(Kruessel *et al.* 1997, von Wolff *et al.* 2000, Salama *et al.* 2020). But, there were also contradictory reports indicating that the IL-1 β is not an essential requirement during the implantation process owing, to other cytokines' functional redundancy, with overlapping signaling occurring *in vivo* (Abbondanzo *et al.* 1996, Stewart & Cullinan 1997). Although IL-1 β is one of the key inflammatory cytokines involved in hatching (our findings), it is recognized that others could also be involved. Nevertheless, our data demonstrate the functional significance of IL-1 β during the peri-hatching embryo development in the mouse. This finding and those of others (Paula-Lopes *et al.* 1998, Robertson *et al.* 2001, 2018, Caluwaerts *et al.* 2002) emphasize the potential involvement of cytokines during early embryo development.

We observed dynamic reciprocally different relative levels of mRNA for *IL-1 β* and *IL-1ra* during the peri-hatching blastocyst development. While the expression of *IL-1 β* increased, it was concomitantly associated with decreased expression of *IL-1ra* in an embryonic stage-dependent manner. Moreover, we observed a positive effect of IL-1 β on the mRNA expression of both *Isp1* and *Isp2* genes. Consistent with the above, we established their respective protein localization of IL-1 β , IL-1ra and IL-1rt1 in specific developmental stages of morulae,

Figure 6 Influence of IL-1ra on the ISP2 protein localization. Panel A depicts the immunolocalization of ISP 2 along with IL-1 β in the absence and presence of IL-1ra (0.2 ng/mL). Scale bar: 20 μ m. Graph B denotes protein quantification (in arbitrary units) of IL-1 β and ISP 2 in the presence or absence of IL-1ra (0.2 ng/mL). Values represent mean $\pm$ S.E.M. of four biological replicate experiments. Statistical analysis was carried using two-way ANOVA subjected to post-Bonferonni's test. * P < 0.05.

blastocysts and hatched blastocysts. Interestingly, the ISP 2 protein level was higher in the IL-1 β -treated blastocysts and lower in IL-1ra-treated blastocysts. Moreover, the IL-1 β -inducing effect of the protease could be countered by its natural antagonist *IL-1ra*. Thus, indicating a direct regulatory effect of IL-1 β on the expression of hatching-associated proteases. This particular observation on the involvement of the IL-1 β -IL-1ra 'cytokine-set' in blastocyst hatching phenomenon in the mouse is a new finding and it is being reported for the first time. Previously, only a qualitative expression of *IL-1ra* in individual blastomeres was reported (Kruessel *et al.* 1997). Our observation is the first to report the successful demonstration on the quantitative expression of *IL-1ra* in peri-hatching blastocyst stages and, importantly, we have observed a negative effect of IL-1ra on the development and hatching of blastocysts. Of relevance here, is an earlier finding which showed that human embryos expressing *IL-1ra* failed to develop into blastocysts (Kruessel *et al.* 1998). Besides, because IL-1ra inhibits IL-1 β signaling, it consequently leads to diminished gene expression of other IL-1 β -mediated expression of developmentally important genes (Simon *et al.* 1998). From the foregoing, we conclude that, by the virtue of IL-1ra inhibiting embryo-trophic activity of embryonically expressed IL-1 β , either directly or by diminishing the expression of IL-1 β as well as ISPs, the IL-1 β -IL-1ra 'cytokine-set' is essential for blastocyst hatching.

We observed that the supplementation of IL-1 β to cultured embryos increased blastocyst hatching

percentage, accompanied by accelerated hatching by 24 h. This hatching accelerating effect observed with the IL-1 β -supplemented embryos is very significant, since *in vitro* cultured mammalian embryos normally show a substantial delay in blastocyst development, compared to their *in vivo* counterparts (Harlow & Quinn 1982, Roth *et al.* 1994, Stokes *et al.* 2005, Lange-Consiglio *et al.* 2020). Of relevance, here, is the recent report which showed an increased IL-1 β in cultured human embryos and it is considered as an early predictor of a successful pregnancy (Salama *et al.* 2020). Taken together, it appears that IL-1 β is important for improved development and hatching of blastocysts in humans and in other mammalian species as well. It is known that the behavior of hatching of mouse blastocysts is similar to humans and we did not observe any difference in the hatching process or zona thickness in the untreated or IL-1 β -treated embryos. This is in contrast to striking differences observed in the hamster species which show global dissolution of the zona during hatching (Seshagiri *et al.* 2009, 2016).

It will be intriguing to examine to what could be the mechanistic reasons for the observed effects of and IL-1 β and IL-1 α 'cytokine-pair' in regulating blastocyst hatching. It requires a detailed examination and it is beyond the scope of this study. However, based on earlier published reports with regard to the mechanistic involvement of signaling components, it is tempting to speculate that IL-1 β could manifest its effect by activating JNK, MAPK and NF- κ B in the blastocyst during hatching by virtue of these molecules being involved in survival, proliferation and inflammation (Lindroos *et al.* 1998, Lin *et al.* 2009, Weber *et al.* 2010). In this context, we earlier showed that, in hamsters, NF- κ B and COX-1 act as potent positive regulators of hatching-associated zonal proteases (Sen Roy & Seshagiri 2013, 2016). Drawing parallels with this, we, therefore, hypothesize that IL-1 β could, in part, enhance the expression of hatching-associated proteases (ISPs), by activating the PGE2 pathway and the NF- κ B transcription signaling system (Schmitz *et al.* 2003, Weber *et al.* 2010), thereby enabling blastocyst hatching in the mouse.

In conclusion, our study successfully demonstrates that the IL-1 β and IL-1 α 'cytokine-pair' modulates the hatching of mouse blastocysts via the regulation of expression of hatching-associated proteases, ISP 1 and ISP 2. The blastocyst development-hatching enhancing and accelerating effect of the IL-1 β strongly indicates the critical need for potential interleukins in the biological viability of blastocysts in terms of ensuing successful implantation and pregnancy outcome. This study provides a new, hitherto unexplored, role of IL-1 β in peri-hatching blastocyst development in addition to its established role in blastocyst implantation. These conclusions have implications in our understanding of hatching biology and in the potential management of infertility in the human.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

Madhulika Pathak and Venkatappa Vani designed and performed embryo culture experiments. Madhulika Pathak collected samples, performed data acquisition, analyzed the data and wrote the draft of the manuscript. Polani B Seshagiri conceived and conceptualized the research program, designed experimental strategies and supervised all experiments. Surendra Sharma contributed to editing and drafting the manuscript for submission. All authors contributed to the interpretation of the results, preparation and finalization of the manuscript and they approved submission of the manuscript.

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