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REVIEW

Molecular and cellular regulators of embryo implantation and their application in improving the implantation potential of IVF-derived blastocysts

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Abstract

Background: In vitro fertilization (IVF) and embryo transfer (ET) are widely used in reproductive biology. Despite the transfer of high-quality blastocysts, the implantation rate of IVF-derived blastocysts remains low after ET.

Methods: This article provides a comprehensive review of current research on embryo implantation regulators and their application to improve the implantation potential of IVF-derived blastocysts.

Main Findings: The in vivo mouse model revealed selective proteolysis immediately after expression in activated blastocysts, that is, degradation of ERα expression in activated blastocysts regulated by the ubiquitin-proteasome pathway, followed by completion of blastocyst implantation. Treatment of blastocysts to induce appropriate protein expression during in vitro culture prior to ET is a useful approach for improving implantation rates. This approach showed that combined treatment with PRL, EGF, and 4-OH-E_2 (PEC) improved the blastocyst implantation rates. Furthermore, arginine and leucine drive reactive oxygen species (ROS)-mediated integrin α5β1 expression and promote blastocyst implantation.

Conclusion: Findings based on analysis of molecular and cellular regulators are useful for improving the implantation potential of IVF-derived blastocysts. These approaches may help to elucidate the mechanisms underlying the completion of the blastocyst implantation, although further investigation is required to improve the success of implantation and pregnancy.

KEYWORDS
blastocyst, embryo transfer, implantation, in vitro fertilization, mice

1 | | INTRODUCTION

During the implantation process in mammalian reproduction, cross-talk between the blastocyst and uterine luminal epithelium is essential.[1–5] For successful pregnancy, it is essential to synchronize embryonic development and uterine receptivity.[1–3,5–17] The receptivity of the uterus is time-limited and supports blastocyst growth, attachment, and subsequent events of implantation. Implantation is

a complex process that involves spatiotemporally regulated endocrine, paracrine, autocrine, and juxtacrine modulators that mediate cell–cell and cell–matrix interactions.[1–3,5–17]

Blastocysts are also active units in this process, with their own molecular program of cell growth and differentiation.[18–20] In implantation-competent blastocysts, the trophectoderm (TE) alters functional programming by altering the molecules on the cell surface.[20,21] The invasive trophoblast in blastocyst adhere, spread, and migrate on extracellular matrix (ECM) substrates[22–25] and penetrate three-dimensional ECM structures in mice.[26] During peri-implantation, proliferation and differentiation of uterine endometrial cells are also crucial steps. In this process, many factors such as transcription factors, lipid mediators, adhesion molecules, and ECM are involved.[3–5] For implantation to be successful, the blastocyst must achieve implantation competence in the receptive uterus.[3,4] However, our understanding of the underlying molecular pathways is hampered by their complexity.

In vitro fertilization (IVF) and embryo transfer (ET) are technologies commonly used in reproductive biology, including assisted reproductive technology (ART) in humans. Despite the transfer of high-quality blastocysts, the implantation rate of IVF-derived blastocysts after ET remains low,[27–30] with poor blastocyst implantation potential being one of the limiting factors for low pregnancy success in IVF. The inadequate expression of specific proteins in culture-induced IVF-derived blastocysts contributes to low implantation rates.[4,18,19] We have previously reported that proper treatment to induce proper regulation in in vitro culture prior to ET improves implantation rates.[19,20]

This review focuses on the molecular and cellular events during blastocyst implantation and their application in improving the implantation potential of IVF-derived blastocysts in a mouse model.

2 | | REGULATION OF THE "WINDOW" OF UTERINE RECEPTIVITY BY OVARIAN STEROID HORMONES

Successful pregnancies in mice require the uterus to be receptive to implantation for a limited period (Figure 1).[1,6,31,32] This receptive phase is also known as the "implantation window." At this stage, the uterine environment is able to support blastocyst growth, attachment, and subsequent implantation events. The ovarian steroids, progesterone (P_4) and 17β-estradiol (E_2) are crucial for implantation in mice (Figure 1).[33,34]

The coordinated actions of P_4 and E_2, which regulate the proliferation and/or differentiation of uterine cells in a spatiotemporal manner, establish the window for implantation, that is, on the first day of pregnancy (as indicated by a vaginal plug = day 1) in mice, preovulatory E_2 secretion induces the proliferation of uterine epithelial cells, and increasing levels of P_4 secreted by the freshly formed corpora lutea initiate stromal cell proliferation from day 3 onwards.[35] The prereceptive uterus on day 3 of pregnancy becomes receptive on day 4 due to rising P_4 levels and a small increase in ovarian E_2 secretion (Figure 1).[34] While E_2 causes epithelial cell proliferation, the coordinated effects of P_4 and E_2 stop uterine epithelial cell proliferation and initiate differentiation.[35] An active blastocyst in the uterus stimulates implantation in a normal pregnancy. The implantation process is divided into three stages: apposition, attachment (adhesion), and penetration.[1] In mice, around midnight on day 4 of pregnancy, the attachment reaction between the blastocyst TE and the luminal epithelium of the uterus occurs.[36,37] After the onset of attachment on day 4 at 2400 h, stromal cells surrounding the blastocyst undergo extensive proliferation and differentiation into decidual cells (decidualization).[1,3,4,6]

3 | | MOUSE PREFERRED TO STUDY IMPLANTATION

The mouse is widely used in animal research because of its small size, resistance to infection, relatively short generation time, and large litter size.[38] It is also uniquely suited for reproductive studies; that is, female mice begin to mature at approximately 6 weeks of age, and vaginal cytology shows 4–5 day estrous cycles.[38]

The morphology of the female reproductive tract varies greatly among mammalian species, most notably the mouse, which has a long duplex uterus with a double cervix.[39–42] The uterine horns are completely separated and have separate cervixes that

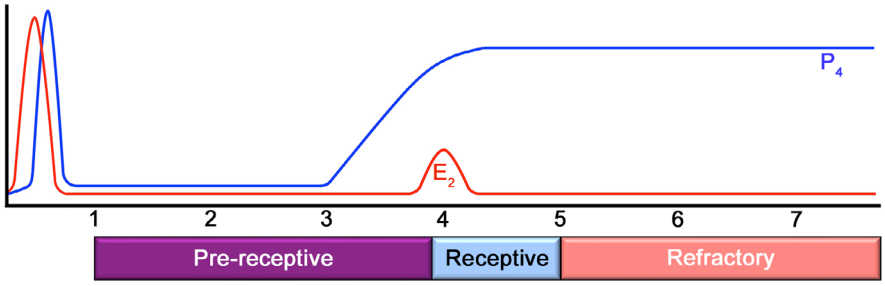

FIGURE 1 The window of uterine receptivity regulated by P_4 and E_2 in mice. Uterine implantation sensitivity is classified as prereceptive, receptive, and refractory (nonreceptive). The uterus is prereceptive on days 1–3 of pregnancy or pseudo-pregnancy (vaginal plug = day 1) and receptive on day 4. Due to rising P_4 levels and a slight increase in ovarian E_2 secretion, the prereceptive uterus on day 3 of pregnancy becomes receptive on day 4. It has become refractory to implantation by the afternoon of day 5.

open into the vagina (Figure 2). The mouse duplex uterus does not allow transuterine migration of embryos from one horn to the other.[39–41] This feature is advantageous for studying the potential of transferred blastocysts,[4,18–20,43] for example, ET of blastocysts untreated control or treated are transferred into one (left or right) or the other uterine horn (right or left), respectively, for the same pseudo-pregnant recipient mouse, followed by comparison of implantation rates between untreated control and treated (Figure 2).[4,18–20] These mouse characteristics and methods provide insight into both embryo implantation rates and recipient mouse pregnancy rates.

4 | | DETERMINING BLASTOCYST COMPETENCY USING THE DELAYED IMPLANTATION MOUSE MODEL

Blastocysts are the endpoint stage during pre-implantation development. Meanwhile, the blastocyst stage includes a variety of states and undergoes cell proliferation, expansion, hatching from the zona pellucida, and acquisition of implantation competence within a short period (Figure 3A). Prior to blastocyst implantation, hatching from the zona pellucida is necessary, whereas a delayed implantation mouse model indicates that hatched blastocysts are insufficient for complete implantation.[44]

Embryonic diapause is a temporary arrest of embryonic development and is characterized by delayed implantation in the uterus.[45–49] The factors that control diapause via uterine secretions can provide direct communication between the endometrium and the blastocyst, although they are complex and not fully defined. Embryonic diapause has subsequently been reported to occur in more than 130 different mammalian species across 10 orders of over 5000 species of mammals.[48,50,51] Delayed implantation does not normally occur in certain species, including sheep, hamsters, rabbits, guinea pigs, and pigs.[47] Meanwhile, an interspecies embryo transfer study found that sheep IVF-derived blastocysts remained dormant when transferred to delayed-implantation mouse uteri.[45] When these dormant blastocysts were transferred back into the donor sheep uterus, they underwent activation and implantation, with the birth of apparently normal lambs.[45] These results demonstrate the flexibility of blastocyst survival and implantation competence. Furthermore, it has been suggested that this is an ancestral trait common to all mammals, including humans.[46,52] The occurrence of embryonic diapause as a result of maternal stress has also been hypothesized in humans, although it is difficult to reconcile the suggestion that humans once had (or still have) evidence of embryonic diapause with current knowledge of human reproduction.[50]

In mice, an ovariectomy on the morning of day 4, prior to pre-implantation E_2 secretion (Figure 1), suspends implantation and induces blastocyst dormancy within the uterine lumen (Figure 3B).[53] Delayed implantation is maintained by continuous P_4 treatment and then terminated by E_2 injection to induce blastocyst activation and subsequent implantation in the uterus approximately 24 h later

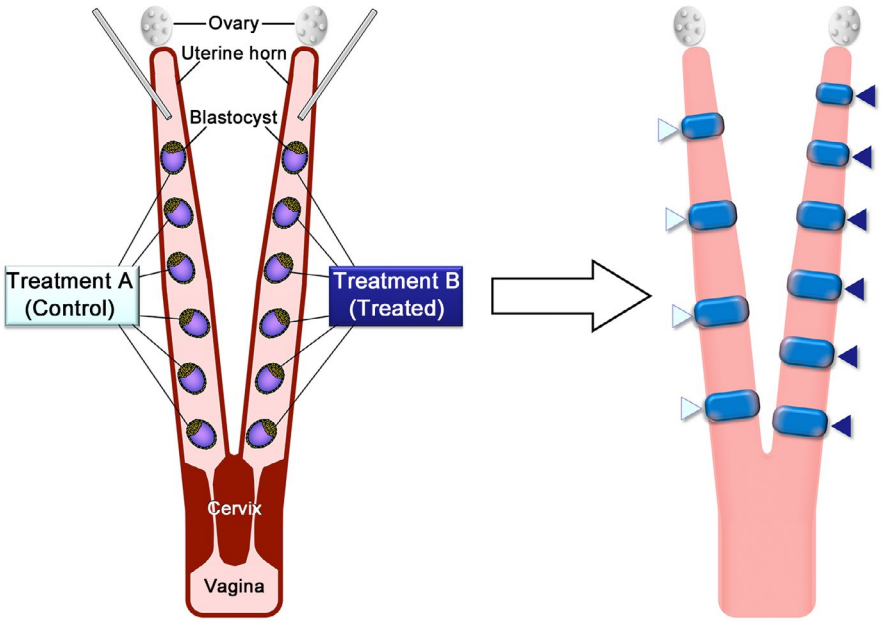

FIGURE 2 The traits of mouse uterus and paradigm of research for blastocyst implantation. Mice have a long duplex uterus, that is, the uterine horns are completely separated and have separate cervixes that open into the vagina. In a duplex uterus, embryos cannot migrate from one horn to the other. This trait allows the study of embryonic potential during the peri-implantation period for transferred embryos in the same recipient, that is, treatment A (untreated control) blastocysts (light blue) or treatment B (treated) blastocysts (dark blue) can be transferred separately to one (left side) or the other (right side) uterine horn. Blastocysts are transferred on the morning of day 4 of pseudo-pregnancy (vaginal plug = day 1). Implantation sites of transferred blastocysts are determined by the uterine blue reaction 2 days later, on day 6, for treatment A (untreated control) blastocysts (light blue arrowheads) or treatment B (treated) blastocysts (dark blue arrowhead).

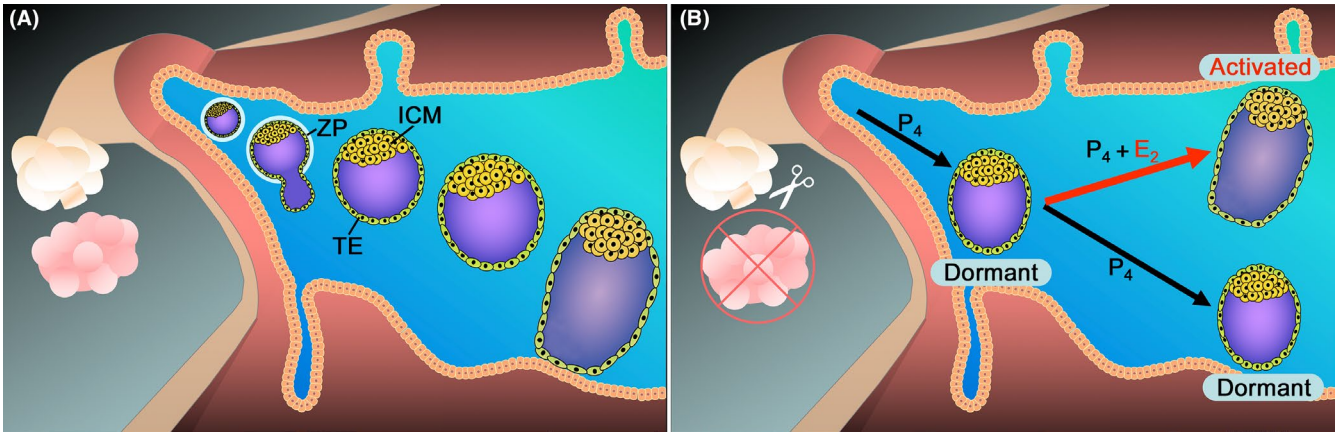

FIGURE 3 Development of the blastocyst and delayed implantation model in mice. (A) From blastocyst formation to implantation. Transformation to blastocysts occurs in the uterine lumen on day 4 of pregnancy (vaginal plug = day 1). Blastocysts contain a cavity (blastocoel) with two distinct cell populations, the inner cell mass (ICM) and trophectoderm (TE). The blastocyst stage includes a variety of states and undergoes cell proliferation, expansion, hatching from the zona pellucida (ZP), and acquisition of implantation competence within a short period until midnight on day 4. (B) From dormancy to activation and subsequent implantation. Ovariectomy on the morning of day 4, prior to pre-implantation E_2 secretion, prevents implantation and initiates blastocyst dormancy within the uterine lumen. Delayed implantation can be maintained by continuous P_4 treatment but is terminated upon E_2 injection, which leads to blastocyst activation and subsequent implantation in the uterus approximately 24 h later.

(Figure 3B). Blastocyst must achieve implantation competence in the receptive uterus, and blastocyst activity determines the window of implantation in the receptive uterus for successful implantation.[34] Therefore, the delayed implantation model is a powerful tool for determining the molecular signaling components that control blastocyst activation or dormancy.

Analysis of global gene expression in this model showed that these two different physiological states of the blastocyst can be distinguished at the molecular level and identified related genes involved in the cell cycle, cell signaling, and energy metabolism.[44] The study revealed an upregulation of *Hbegf* expression, encoding heparin-binding EGF-like growth factor (HBEGF) and HBEGF receptors, Egfr (ErbB1) and ErbB4, in blastocysts.[44,54,55] Another lipid signaling molecule is the endocannabinoid anandamide, and endocannabinoid signaling is critical for blastocyst implantation in mice.[56,57] CB1, the cannabinoid receptor, in blastocysts and uterine anandamide are coordinately downregulated during blastocyst activation and uterine receptivity, while both are elevated in dormant blastocysts and nonreceptive uteri.[57–59] Anandamide differentially modulates Ca^{2+} channel activity via CB1 and mitogen-activated protein kinase (MAPK) signaling, followed by blastocyst function.[59] In addition, the MAPK and phosphatidylinositol 3-kinase (PI3K)/Ca^{2+} signaling cascades are also critical for the development and activation of the blastocyst.[60–63]

The PI3K-Akt pathway is important for ensuring the survival of pre-implantation embryos.[64,65] Our recent study showed that Ca^{2+} independent nitric oxide synthase (NOS), iNOS, and phosphorylated eNOS (p-eNOS), are upregulated by E_2 in implantation-induced blastocysts in mice.[66] Since p-Akt is upstream of p-eNOS in implantation-induced blastocysts, it has been suggested that the embryonic survival signaling PI3K-Akt pathway is also associated with p-eNOS in blastocysts during peri-attachment to the uterine luminal epithelium.[66] However, neither iNOS nor p-eNOS are associated with cell proliferation during the transition from dormancy to the activated phase of the blastocyst.[66]

5 | | IMPLANTATION IS ASSOCIATED WITH DEGRADATION OF ESTROGEN RECEPTOR A IN ACTIVATED BLASTOCYSTS

Targeted disruption of estrogen receptor α (*ERα*, *Esr1*) does not affect embryonic development or implantation, whereas the ERα protein is expressed in blastocysts.[67,68] Therefore, for the peri-implantation period, ERα expression in blastocysts was not considered necessary. In contrast, overexpression of ERα resulted in a reduction in the number of implantation sites and litter size.[69] These results suggested the possibility of optimal ERα downregulation during blastocyst implantation in the peri-implantation period. Our study showed that the highly expressed ERα protein in implantation-induced (activated) blastocysts decreased within 6 h in culture, whereas the other protein expression, such as breast cancer 1 (BRCA1), was maintained in blastocysts in culture (Figures 4 and 5A).[70] This selective degradation of the ERα protein in activated blastocysts is regulated by the ubiquitin-proteasome pathway (Figures 4 and 5A). Furthermore, the completion of blastocyst implantation requires ERα downregulation through selective proteolysis (Figures 4 and 5A).[70] The reason is still unclear why the need for selective proteolysis immediately after expression in activated blastocysts and the elimination of transcription factors, including ERα, could be related to the avoidance of inadequate protein expression for the completion of blastocyst implantation.

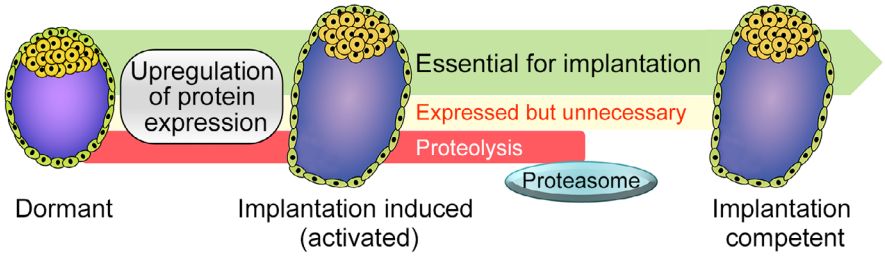

FIGURE 4 Progression from dormant to implantation-competent blastocysts. Expressed proteins in activated blastocysts are categorized into three groups to complete blastocyst implantation: essential (green), expressed but unnecessary (yellow), and proteolysis (red). In implantation-induced (activated) blastocysts, protein expression (green, yellow, and red) is upregulated. A critical step for successful implantation in activated blastocysts is the downregulation of specific proteins (red), including ERα degradation.

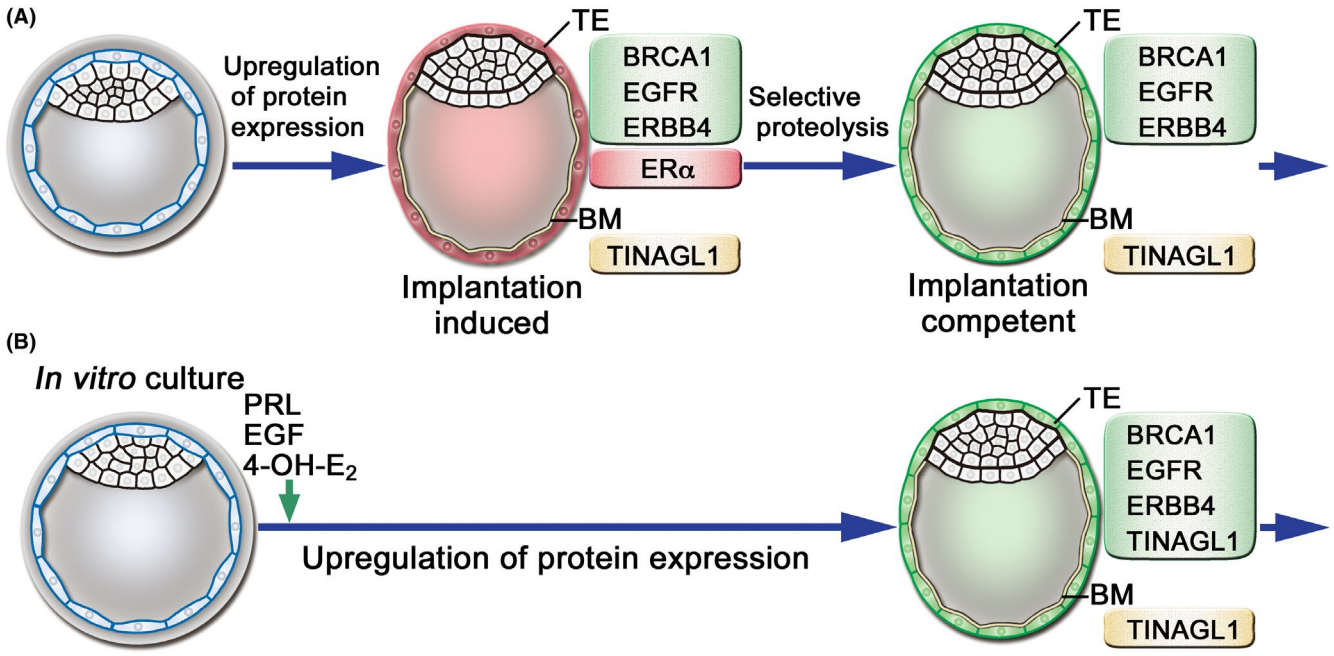

FIGURE 5 From implantation mechanisms to enhancing implantation potential in mouse blastocysts. (A) Analysis of blastocyst implantation mechanisms using an in vivo delayed implantation model. (B) Improvement of implantation potential in blastocysts during in vitro culture. Treatment with appropriate regulation improves the blastocyst implantation rate, that is, combined treatment with prolactin, epidermal growth factor, and 4-hydroxyestradiol (PEC) improves the implantation potential of IVF-derived blastocysts. TE, trophectoderm; BM, basement membrane.

6 | | IMPROVEMENT OF IMPLANTATION POTENTIAL OF IVF-DERIVED BLASTOCYSTS BY COMBINED TREATMENT WITH PROLACTIN, EPIDERMAL GROWTH FACTOR, AND 4-HYDROXYESTRADIOL (PEC)

During delayed implantation in vivo in mice caused by ovariectomy followed by continued progesterone administration, blastocyst dormancy is maintained and then rapidly activated by estrogen to an implantation-induced state (Figure 3B). Although the expression of many proteins is upregulated in implantation-induced blastocysts, selective proteolysis via the ubiquitin-proteasome pathway is required to achieve implantation-competent status (Figures 4 and 5A). Because the low quality of IVF-derived blastocysts causes implantation failure, the evaluation of expressed proteins and their regulation by humoral factors will provide useful information to improve the implantation potential of IVF-derived blastocysts (Figure 5).

Using immunohistochemical analysis, previous studies have shown higher levels of BRCA1 protein expression in the TE of implantation-induced blastocysts (Figure 5A).[18,44] *Brca1* is a tumor suppressor gene, and its mutations confer an increased risk for the development of various E_2-responsive tumors.[71,72] In the human breast cancer cell lines MCF-7 and T-47D, prolactin (PRL) stimulated the expression of BRCA1 protein.[73] Epidermal growth factor (EGF) also increased BRCA1 expression in MCF-7 cells.[74] Although E_2 is essential for blastocyst implantation, the catechol estrogen 4-hydroxyestradiol (4-OH-E_2), a catechol metabolite produced from primary E_2 in the uterus, is involved in the activation of dormant

blastocysts.[75] Therefore, 4-OH-E_2 is essential for implantation ability in blastocysts, while E_2 is necessary for uterine preparation. Furthermore, in activated blastocysts, EGF receptor (EGFR, also known as ERBB1) and ERBB4 (erb-b2 receptor tyrosine kinase 4) are increased in the TE, and tubulointerstitial nephritis antigen-like 1 (TINAGL1) is localized to the blastocyst basement membrane (Figure 5A).[18,55,76,77]

Our previous study revealed that PRL, EGF, or 4-OH-E_2 promoted the expression of BRCA1 protein in the TE in vitro.[19] PRL increased EGFR expression, whereas EGF induced both EGFR and ERBB4 expression in blastocyst TE.[19] 4-OH-E_2 increased TINAGL1 expression in the TE and localized this protein to the basement membrane.[19] Meanwhile, the combination of PRL, EGF, and 4-OH-E_2 promoted the expression of EGFR, ERBB4, TINAGL1, and BRCA1 in TE, whereas ERα was not increased in treated blastocysts (Figure 5B).[19] In this approach, combined treatment with PRL, EGF, and 4-OH-E_2 (PEC) improved the implantation potential of the mouse blastocysts, whereas each factor alone was ineffective (Figure 5B).[19]

7 | | ARGININE WITH LEUCINE STIMULATES ROS-MEDIATED INTEGRIN A5B1 EXPRESSION AND PROMOTES IMPLANTATION IN IVF-DERIVED BLASTOCYSTS

Trophoblast outgrowth is a reliable marker of differentiating and migrating trophoblasts.[22,78] This in vitro outgrowth assay demonstrated that L-arginine (Arg) and L-leucine (Leu) were necessary and sufficient to induce trophoblast motility.[79] Arg is required for conceptus growth and development during pregnancy, with cell signaling and metabolic functions as precursors for the synthesis of molecules (e.g., nitric oxide, polyamines, and creatine).[80] Nitric oxide synthase (NOS) metabolizes Arg to nitric oxide (NO) and has three isoforms: type I, neuronal NOS (nNOS); type II, inducible form (iNOS); and type III, endothelial form (eNOS).[81,82] Our previous results showed that both iNOS and phosphorylated eNOS were expressed in implantation-induced blastocysts in vivo.[66] Therefore, Arg and Leu may be involved in the induction of blastocyst implantation competence.

Our recent study revealed that treatment with Arg and Leu improved the implantation potential in blastocysts derived from IVF (Figure 6).[20] In blastocysts treated with Arg and Leu, integrin α5β1 expression was upregulated. Arg with Leu also upregulated reactive oxygen species (ROS) levels. ROS levels were positively correlated with integrin α5β1.[20] In addition, ascorbic acid, an antioxidant, reduced ROS, which was followed by a reduction in integrin α5β1 levels, with a positive correlation between ROS and integrin α5β1.[20] Blastocysts treated with Arg and Leu showed lower ROS scavenging activities with glutathione peroxidase (GPx) and glutathione (GSH) as reductants, that is, decreased GPx4 and GSH levels induced by Arg with Leu resulted in ROS accumulation (Figure 6).[20] Meanwhile, the mitochondrial membrane potential (ΔΨm) in blastocysts did not differ, suggesting that ROS were not due to increased production by oxidative phosphorylation but rather an accumulation by reduced ROS degradation due to decreased GPx4 and GSH levels (Figure 6).[20] This study demonstrated that accumulated ROS levels via decreased GPx4 and GSH induced by Arg with Leu stimulated integrin α5β1 expression, thereby enhancing blastocyst implantation potential (Figure 6).[20]

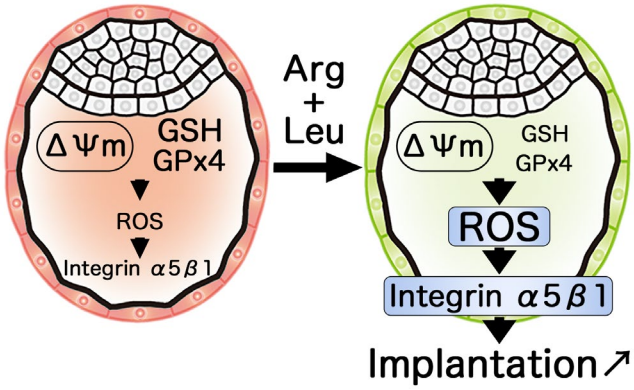

FIGURE 6 Arginine with leucine stimulates reactive oxygen species (ROS)-mediated integrin α5β1 expression and promotes implantation in IVF-derived blastocysts. Elevated ROS levels via decreased glutathione peroxidase 4 (GPx4) and glutathione (GSH) induced by Arg with Leu stimulated integrin α5β1 expression, thereby enhancing blastocyst implantation potential. The mitochondrial membrane potential (ΔΨm) in blastocysts did not differ, suggesting that ROS were not due to increased production by oxidative phosphorylation but rather an accumulation by reduced ROS degradation due to decreased GPx4 and GSH levels.

8 | | CONCLUSIONS

Although many important discoveries have been made in this field, knowledge of the complex events that occur during implantation is insufficient for preventing infertility caused by implantation failure. This review article describes the molecular and cellular events that occur during blastocyst implantation. To elucidate the mechanisms underlying the completion of blastocyst implantation, the delayed implantation mouse model is a powerful tool for defining the molecular signaling components that direct blastocyst activation or dormancy (Figure 3). This model revealed selective proteolysis immediately after expression in activated blastocysts, that is, degradation of ERα expression in activated blastocysts under the regulation of the ubiquitin-proteasome pathway, followed by completion of blastocyst implantation (Figure 4).[3,4,18] The implantation rate of IVF-derived blastocysts after ET remains low. The limiting factor for low pregnancy success in IVF is poor embryo quality. Therefore, insufficient protein expression induced by culture in IVF-derived blastocysts is one of the reasons for the low implantation rate. Blastocyst treatment to induce appropriate protein expression during in vitro culture prior to ET is a useful approach for improving implantation rates (Figure 5). Using this approach, we reported that combined

treatment with PRL, EGF, and 4-OH-E_2 (PEC) improves blastocyst implantation rates (Figure 5).[19] We also showed that implantation rates can be increased or decreased depending on simple amino acid combinations (Figure 6).[20] Arg with Leu drives ROS-mediated integrin α5β1 expression and promotes implantation in blastocysts (Figure 6).[20] These findings may help to elucidate the mechanisms underlying the completion of the blastocyst implantation, although further investigation is required to improve the success of implantation and pregnancy.

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CONFLICT OF INTEREST STATEMENT

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