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Pinopodes: Recent Advancements, Current Perspectives, and Future Directions

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Abstract

Successful embryo implantation is a complex and highly regulated process involving precise synchronization between the fetal-derived trophoblast cells and maternal uterine luminal epithelium. Multiple endocrine-driven factors are important for controlling the timely receptivity of the uterus, and this complexity underscores implantation failure as a major cause of recurrent infertility associated with assisted reproductive technologies. One particular cellular structure often hypothesized to promote receptivity is the pinopode or uterodome - a hormonally regulated, large cellular protrusion on the uterine epithelial surface. Recent clinical studies associate pinopodes with favorable fertility outcomes in women, and because they are directly linked to an increase in progesterone levels, the potential utility of these hormone-regulated cell biological structures in predicting or improving implantation in a clinical setting holds promise. In this review, we aim to generate interest in pinopodes from the broader cell biology and endocrinology communities, re-examine methodologies in pinopode research, and identify priorities for future investigation of pinopode structure and function in women's reproductive health.

Graphical Abstract

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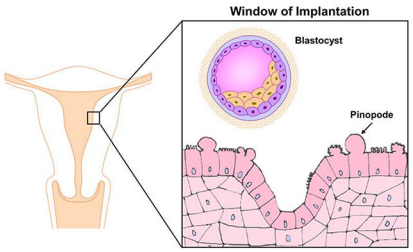
The authors have declared that no conflict of interest exists.



Keywords

progesterone; endometrial receptivity; implantation; pinopodes; uterodomes

Introduction

Recurrent implantation failure is a major clinical problem in reproductive medicine, estimated to persist in about 48% of couples undergoing *in vitro* fertilization (IVF), resulting in extensive physical and emotional trauma (Coughlan et al., 2014; Coughlan et al., 2013). Two main causes of implantation failure include aberrant embryonic development and poor endometrial receptivity (Brosens et al., 2014). Endometrial receptivity is defined as the period during which the uterus undergoes changes to provide an adequate and receptive environment for the incoming fertilized embryo to implant (Teh et al., 2016).

In humans, the endometrial receptivity period occurs during the mid-luteal phase of the menstrual cycle (days 19-23) (Paria et al., 2001). A typical menstrual cycle in women consists of 28 days but can range from 21-35 days (Hawkins and Matzuk, 2008). The menstrual cycle is comprised of two parallel cycles: the ovarian cycle, which includes the follicular and luteal phases, and the uterine cycle consisting of menses and proliferative and secretory phases (Messinis et al., 2014). In the mid-luteal phase, the endometrium undergoes changes in response to a rise in progesterone levels to ensure receptivity of the fertilized embryo (Hawkins and Matzuk, 2008). There is a finite time during which the endometrium becomes receptive for blastocyst attachment, known as the “window of implantation” (WOI) (Harper, 1992).

The events leading up to the WOI are critically important for the establishment of a healthy pregnancy and are of clinical interest to the field of reproductive endocrinology and infertility (Murray et al., 2004). In particular, there has been a strong movement in the reproductive field to better define factors related to endometrial receptivity. The advent of the endometrial receptivity array (ERA) several years ago provided an initial tool to diagnose the molecular status of the receptive endometrium using an expression array panel of approximately 250 genes associated with endometrial receptivity (Diaz-Gimeno et al., 2011). This diagnostic tool also helped elucidate that each woman has a personalized WOI that could be utilized for optimizing timing of embryo transfer (Diaz-Gimeno et al., 2011; Diaz-Gimeno et al., 2013). However, there are limitations to utilizing this tool, including expense, inaccurate results, and the invasive nature of the test (Bassil et al., 2018; Mahajan, 2015). Therefore, identification of additional receptivity markers, including cellular

modifications that may enable endometrial receptivity at the WOI and therefore improve clinical outcomes, is highly desirable.

Over the years, a handful of groups have commented on cellular modifications of the uterine luminal epithelium that may be associated with embryo implantation and fertility. Some of the first transmission electron microscope (TEM) studies illustrated surface modifications of the uterine luminal epithelium of rodents following administration of steroid hormones (Bergstrom and Nilsson, 1972; Nilsson, 1966; Warren and Enders, 1964). The observed changes in microvilli and formation of uterine protrusions in the endometrium were believed to be associated with a type of secretory mechanism to nurture the blastocyst at the time of implantation (Nilsson, 1958, 1966; Warren and Enders, 1964). These structures were first described as “mushroom-like” or “sea-anemonae-like” protrusions during the peri-implantation period (Nilsson, 1972; Psychoyos and Mandon, 1971). Originally termed “pinopods” (“drinking foot” in Greek) following the observation that these protrusions took up lead citrate-labeled tracers in vacuoles, these endometrial structures have proven mysterious and controversial for several decades (Enders and Nelson, 1973; Quinn and Casper, 2009). The term “uterodome” has also been used to divorce the name of these protrusions from an implied function. However, in this review, we will refer to these structures as “pinopodes” for the sake of consistency with the majority of the available literature.

Pinopodes are generally considered to be 5-10 μm cellular protrusions of the apical plasma membrane of uterine epithelial cells (Figure 1). They bear some resemblance to cellular blebs, which are ~2 μm transient plasma membrane extravasations that stochastically appear and disappear within seconds to minutes on the surface of migrating cells. Therefore, in comparison to other epithelial plasma membrane protrusions, including blebs and others like cilia and microvilli, which are approximately 0.2 μm to 90 nm in size, respectively, pinopodes are uniquely large and present during the WOI (Nikas, 2000)— a period of ~1-2 days in rodents and ~4 days in humans.

Pinopodes occur in multiple species, including mice, rats, and humans, suggesting that they serve an evolutionary conserved purpose. Initially hypothesized to form at the sites of blastocyst attachment (Johannisson and Nilsson, 1972; Nilsson, 1958; Psychoyos and Mandon, 1971), pinopodes have typically been correlated with successful implantation (Nilsson, 1958; Usadi et al., 2003) and are strongly regulated by the presence of the ovarian steroid hormones.

Pinopode Density and Structure: Hormonal Determinants

A strong area of recent research interest has focused on identifying correlations between hormonal and other factors and pinopode development (Table 1) (Nikas et al., 1995; Nikas and Psychoyos, 1997). Indeed, several studies have emphasized the dependence of pinopode formation on hormonal control by the two most studied hormone contributors, estrogen and progesterone (Bentin-Ley, 2000; Lopata et al., 2002; Nardo et al., 2002; Rarani et al., 2018). The majority of literature reports that progesterone is responsible for stimulation of pinopode development, whereas an increase in estrogen parallels pinopode regression (Martel et al., 1991; Psychoyos and Mandon, 1971; Sarantis et al., 1988; Singh et al., 1996).

To evaluate pinopodes as a marker of endometrial receptivity, many have tracked pinopode formation during the luteal/secretory phase of the menstrual cycle in women (Figure 2A) (Develioglu et al., 1999; Nikas et al., 1999; Nikas et al., 1995; Nikas and Psychoyos, 1997). Although the timeframe of pinopode formation during the luteal phase varies in the literature, on average, prominent formation occurs on days 20-22 of the natural menstrual cycle and 1-2 days earlier in stimulated cycles, coinciding with the WOI.

Based on scanning electron microscopy (SEM) analysis of pinopodes throughout the menstrual cycle, pinopodes are typically classified as developing/immature, fully developed/mature, and regressing according to their ultrastructural morphology (Table 2). However, it is important to reiterate that a specific cycle day does not guarantee that all pinopodes will encompass similar morphology (Aunapuu et al., 2018; Nikas et al., 1995; Stavreus-Evers et al., 2001). Generally, during days 17-19 of the menstrual cycle, cellular bulging increases with the formation of small (1-2 μm) pinopodes. By day 20, fully developed pinopodes, described as spherical, smooth structures with no microvilli, cover the endometrium (Lopata et al., 2002; Quinn and Casper, 2009; Rarani et al., 2018). By the end of the secretory phase in women (days 23-25), pinopodes regress with a wrinkled appearance resembling a raisin or deflated balloon (Nikas, 2000; Nikas et al., 1995). There are discrepancies in the literature regarding pinopode morphology on specific days of the cycle, and of course, these changes may vary between individuals (Table 2) (Nikas, 2000).

Variations in pinopode morphology are also observed in the clinic. Women experiencing poor IVF outcomes commonly have alterations in pinopode shape and poor pinopode development (Aunapuu et al., 2018). Previous research has taken into consideration pinopode variance in women by modifying the standard clinical IVF protocol (5 day-old blastocyst transferred on day 7 of progesterone treatment) to include progesterone treatment based on pinopode morphology. When pinopode morphology was evaluated in women undergoing IVF treatment and fully developed pinopodes were synchronized with progesterone treatments, the pregnancy success rate increased, further validating an important stimulatory relationship (Sudoma et al., 2011). Although pinopode development appears to correlate with increasing progesterone, it has yet to be established whether pinopodes themselves secrete hormones. Additional hormones such as adrenomedullin and gonadotropin releasing hormone agonist correlate with increased pinopode growth, whereas testosterone results in a decrease in pinopodes (Matson et al., 2017; Mokhtar et al., 2014; Zhou et al., 2017). Based on these studies, hormonal regulation is an important aspect of pinopode growth and morphology, and further investigation into these correlations could help define the proper period for embryo transfer (Pantos et al., 2004; Sudoma et al., 2011).

Several clinical studies, including two recent publications, demonstrate promising observations of the potential for pinopodes to serve as indicators of endometrial receptivity at the WOI (Jin et al., 2017; Pantos et al., 2004; Qiong et al., 2017; Sudoma et al., 2011). The most recent clinical trials reported that women undergoing IVF with a greater pinopode score exhibited a higher embryo implantation and pregnancy rate compared to those with a lower score (Jin et al 2017; Qiong et al., 2017). Although these studies did not evaluate natural cycles, their extensive patient recruitment and outlining of new strategies for pinopode measurement and quantitation further ratifies pinopodes as a reliable endometrial

receptivity marker (Jin et al., 2017; Qiong et al., 2017). Others have reported that women with ample pinopode coverage (greater than 10%) have an increased likelihood of pregnancy success compared to those with sparse or no pinopode coverage (Nikas and Aghajanova, 2002).

However, some argue that although pinopodes are present during the WOI, they do not directly correlate to embryo implantation and pregnancy success in women (Quinn et al., 2007a; Quinn and Casper, 2009). This notion is due to the expanded period of pinopode presence throughout the luteal phase, rather than a specific growth period at the WOI, which would rule out their possible benefit in embryo receptivity (Quinn et al., 2007a). In addition, women with recurrent implantation failure and women experiencing infertility do not exhibit a significant difference in pinopode coverage or morphology (Da Broi et al., 2017; Xu et al., 2012). However, the possibilities remain that there are differences in these patients in pinopode function or in other implantation-related events that are unrelated to pinopodes, underscoring the complexity of the implantation process.

Consistent with human studies, we have also observed changes in pinopode formation and morphology in mice based on the day of pseudopregnancy (Figure 2B). In pseudopregnant mice, the WOI ranges from days 2.0-3.5 and is characterized by an increase in progesterone levels (Ueda et al., 2003). By day 3.5 of pseudopregnancy, an increase in spherical, smooth, “healthy” pinopodes were observed rising above short microvilli. By day 4.5 (indicative of the timeframe of embryo implantation), the pinopodes exhibited an increase in size, yet with a deflated appearance. These findings are consistent with observations in rats and humans, confirming murine pseudopregnancy as a valuable model for studying pinopode formation and functionality in association with hormonal regulation. We have previously reported that mice experiencing impaired embryo implantation and sub-fertility due to haploinsufficiency of the peptide hormone adrenomedullin have fewer pinopodes compared to their wildtype counterparts (Li et al., 2008). In contrast, one group has concluded that infertile mice do not exhibit a difference in pinopode formation, yet this particular cause for infertility may be independent of pinopode function. (Quinn et al., 2007a; Quinn et al., 2007b). Therefore, a direct causal association between pinopodes and embryo receptivity across species is not currently available.

The duration of the pinopode lifespan may be varied, as some have specified a limited lifespan of up to 48 hours, whereas others observe pinopodes persisting beyond 48 hours (Acosta et al., 2000; Usadi et al., 2003). The discrepancies in lifespan could be due to variations in number or location of biopsies collected, in cycle duration, and in individual patient-level physiology. Research has identified a handful of promising molecular markers of endometrial receptivity, but our knowledge of the chronological events establishing endometrial receptivity and how they affect fertility is still naïve. To this extent, it is of utmost importance to further our understanding of factors that define the receptivity period, including establishing better methodologies to study pinopodes. If additional studies are completed to describe how pinopodes correlate with hormonal surges and timely reception, the development of additional tools could advance the clinical reproduction field, especially in IVF patients.

Unfortunately, very little is known about the molecular architecture and organization of pinopodes. One group has identified ezrin, a plasma membrane protein-actin cytoskeleton cross-linker present in ruffled membranes, in pinopode-laden uterine luminal epithelial cells by immunohistochemistry (Tan et al., 2012). Otherwise, whether cell adhesion proteins like integrins are present on the surface of pinopodes, potentially in interaction with the implanting blastocyst, remains to be determined. As described in more detail below, our ability to develop models to study pinopode structure, function, and dynamics comparable to those used to study microvilli, cilia, and blebs has unfortunately been limited. Thus, further investigation into the variation in pinopode morphology is merited.

Pinopode Function

Regulation of Uterine Luminal Contents

As previously discussed, the term “pinopode” was derived from a hypothesized function for these structures in pinocytosis of uterine luminal fluid. Regulation of luminal fluid volume is critically important before and during embryo implantation. Prior to implantation, uterine fluid volume increases to promote embryo transportation and appropriate intrauterine position (Zhang et al., 2017). Following embryo transportation, uterine fluid levels decrease, allowing luminal closure around the embryo to facilitate attachment to the uterus (Yoshinaga, 2013). In rats, endocytic activity peaks between days 4-6 of pregnancy, coinciding with embryo implantation (Parr, 1980). Towards the end of this timeframe, pinopodes begin to regress, a process thought to be functionally important for the movement of material into the apical cytoplasm of epithelial cells (Enders and Nelson, 1973).

Early studies described absorption of trypan blue by epithelial cells of the rat endometrium and subsequent storage in the cytoplasm (athrocytosis), a process that was hypothesized to play a role in establishing an ideal maternal environment for implantation (Sartor, 1972). At the time, pinopodes were not fully classified or named, but this study set the stage for a connection between progesterone, a predominant hormonal driver of pinopodes, and fluid absorption in the endometrium (Bentin-Ley et al., 1999; Sartor, 1972). The process of material migration and removal from the endometrium was then later described as endocytosis and pinocytosis, involving material ingestion into epithelial cells and formation of vacuoles and small vesicles (Enders and Nelson, 1973; Parr and Parr, 1974). This material was often observed to be translocated into lysosomes or dispersed into the uterine stroma (Parr, 1980; Parr and Parr, 1977, 1986; Tung et al., 1988). Additionally, pinopodes were observed to uptake ferritin and horseradish peroxidase into vacuoles in rats and mice. There was therefore speculation that these epithelial projections were important for altering uterine luminal fluid contents to provide an environment conducive to implantation for the blastocyst (Nilsson, 1972).

Another hypothesis suggested that an endocytic function for pinopodes promoted uterine closure (Enders and Nelson, 1973) and the establishment of a proper “runway” for the incoming embryo to adhere to the uterine epithelium (Lessey et al., 2000; Nikas, 1999). A potential endocytic function was supported by a study demonstrating that expression of water channel proteins, aquaporins (AQP), positively correlated with pinopodes in the rat (Lindsay and Murphy, 2007). Some have speculated that pinopodes express AQPs on their

surface. Indeed, one group observed localization of AQP2 to pinopodes by confocal microscopy during the mid- to late secretory phase in women (Hildenbrand et al., 2006), and quercetin (a plant-derived polyphenolic compound) causes a decrease in AQPs, which correlates with a decrease in pinopodes (Table 1) (Shahzad et al., 2017). Consistent with a hypothesized role for pinopodes in water transport in the context of embryo attachment and implantation, knockdown of AQP2 decreased embryonic spheroid attachment to endometrial cells *in vitro* (He et al., 2019). However, the same study also demonstrated that estradiol treatment of human endometrial biopsies increases AQP2 levels, suggesting a pinopode-independent mechanism of AQP2 upregulation (He et al., 2019).

We have also observed a correlation between pinopode formation and uterine fluid transport following treatment with the peptide hormone adrenomedullin (Matson et al., 2017). Intrauterine injection of adrenomedullin during the peri-implantation period in mice stimulates water accumulation correlating with an increase in pinopode size and number and embryo implantation rate. In addition to adrenomedullin, known hormonal drivers of uterine fluid homeostasis include estradiol and progesterone (Zhang et al., 2017). Even a small physiological increase in estradiol in mice can disrupt intrauterine fluid accumulation and disturb efficient embryo implantation (Zhang et al., 2015). Dynamic changes in estradiol and progesterone signaling may result in decreased pinopode formation and thus abnormal fluid accumulation in the uterus prior to implantation, which may impair the establishment of a healthy maternal-fetal environment for embryo attachment (Parr, 1983).

The significance of fluid uptake and secretion and pinopode participation in these processes during pregnancy are continuously debated among researchers, particularly when examining differences between rodents and humans (Adams et al., 2002; Murphy, 2000; Quinn and Casper, 2009). Currently, vacuoles have not been identified in human pinopodes, therefore it is thought that they do not participate in pinocytotic activity and could elicit a different function in the uterus (Adams et al., 2002). However, failure to observe pinopode pinocytotic activity in humans could stem from ethical limitations, causing the distinct activities and the functionality of pinopodes to remain questionable (Adams et al., 2002).

In addition to participation in endocytosis of uterine luminal fluid, pinopodes may participate in exocytosis by moving materials from the stroma to the uterine lumen (Parr, 1980). Parr discovered that administration of horseradish peroxidase as a tracer during day 5 of pregnancy in rats caused localization of the tracer to vesicles close to stromal cells at the basement membrane (Parr, 1980). Later, this tracer was tracked to vesicles adjacent to the apical membrane in uterine epithelial cells. They hypothesized that these vesicles translocate to the luminal surface and fuse with the apical membrane to release material directly into the uterine lumen (Parr, 1980). Unfortunately, they did not specifically determine whether the uterine epithelial cells containing the tracer included pinopodes. However, using SEM, we have observed potential release of vesicular material from pinopodes on day 4 of pregnancy in mice, which may be similar to Parr's previous findings (Figure 3).

It is possible that this secreted material contains exosomal structures. Much smaller than pinopodes, exosomes are extracellular vesicles ranging in size from 30 nm to 150 nm. They play a role in cell-cell communication through packaging of lipids, proteins, mRNAs, and

miRNAs, and their cargo is directly influenced by estrogen and progesterone (Greening et al., 2016; Nguyen et al., 2016). The field of exosomes is quickly evolving, with preliminary evidence demonstrating that exosome release from epithelial cells of the endometrium mediates dialogue between maternal and fetal cell communication during implantation (Machtinger et al., 2016). The process of exocytosis by pinopodes and the hypothesis that pinopodes may release exosomes could be important for communication with the blastocyst or involvement in degradation of the uterine luminal epithelium and remodeling of the endometrial stroma (Enders and Nelson, 1973; Moulton et al., 1978; Parr, 1983). Therefore, pinopode exocytosis at the uterine luminal epithelial surface is an intriguing area of endometrial research, requiring further exploration.

Regulation of Proteins Involved in Implantation

Because pinopodes appear during the WOI, it is not surprising that the majority of hypothesized pinopode markers are related to implantation genes (Bagot et al., 2001; Shimizu et al., 2008; Stavreus-Evers et al., 2002a; Stavreus-Evers et al., 2002b). Unfortunately, few have demonstrated localization of these proteins either on the surface of or within pinopodes. One hypothesis is that pinopodes may express proteins like integrins on their surface that promote blastocyst attachment. For example, in women with high rates of embryonic loss, a decrease in pinopode density was associated with decreased integrin β3 expression (Aghajanova et al., 2003; Liu et al., 2017; Nardo et al., 2003; Xu et al., 2012). Others have hypothesized that pinopodes secrete leukemia inhibitory factor (LIF), which is involved in the epithelial transition to a receptive state (Kabir-Salmani et al., 2005). However, others deny that localization of integrins and LIF parallel that of pinopodes (Creus et al., 2003; Creus et al., 2002; Mikolajczyk et al., 2011).

Another dispute is whether anti-adhesive glycoproteins known as mucins (MUC1 and MUC16), which are thought to disappear and therefore facilitate the unmasking of integrins on epithelial cells (Aplin, 1997), are expressed on or in pinopodes. While one group found that extracellular MUC1 localization is not associated with pinopodes at the time of embryo attachment (Horne et al., 2005; Horne et al., 2002), others have identified MUC1 expression on pinopodes (Gipson et al., 2008). Most recently, MUC1 was identified by SEM in ciliated luminal epithelial cells but not in secretory cells or pinopodes (Wu et al., 2019). Expression of MUC16 also vanishes from pinopodes during endometrial receptivity, which could indicate that pinopode formation is beneficial in embryo adhesion (Gipson et al., 2008).

Additionally, environmental exposures and endocrine disruptors have long been an important topic in female infertility. More recently, these effectors have been an area of investigation with regard to pinopode development and embryo implantation. Studies in mice have demonstrated that environmental exposures or endocrine disruptors can decrease pinopode development and cause poorly developed pinopodes (Table 1) (Duran et al., 2014; Qu et al., 2018; Shahzad et al., 2017; Zhou et al., 2018). Further research pertaining to the association of these effectors with poorly developed pinopodes could provide more insights describing how pinopodes form, resulting in further characterization of pinopodes.

Current Perspectives on Analysis of Pinopodes

Quantitative Evaluation of Pinopodes

Here, we provide insight into the quantitative and qualitative assessment of pinopodes with an emphasis on establishing guidelines for improvement, standardization, and future direction. To date, the vast majority of studies on pinopodes across all species have used SEM to evaluate pinopode coverage in the endometrium (Chen et al., 2016; Jin et al., 2017; Li et al., 2010; Nikas et al., 1995; Qiong et al., 2017). However, there is certainly variability in what each individual observer determines to be the size threshold above which a pinopode is counted. Therefore, investigators should consider developing an overall standard when counting pinopodes regarding both pinopode size and number of fields to be counted. Commonly, the size range of pinopodes varies from 1.0-10.0 μm, with previous average reports around 4.0 μm in the rat and 6.0 μm in the mouse and human (Ljungkvist and Nilsson, 1971; Quinn et al., 2007a; Quinn et al., 2007b). To the second point, Jin et al. recently recommended using a running average of 60 fields in each specimen to reduce sample error and achieve a reproducible result for calculating pinopode coverage (Jin et al., 2017). Therefore, the quantitation methods outlined by Jin et al. stress that increased scientific rigor is required for accurate assessment of pinopode density.

An additional consideration regarding the quantitative evaluation of pinopode size is the qualitative evaluation of pinopode morphology. As previously discussed, pinopodes can be classified into developing/immature, fully developed/mature, and regressing pinopodes based on their morphology, which should be considered when assessing pinopode coverage. For example, Jin et al. calculated a percentage: fully developed pinopodes divided by the total number of pinopodes (including pinopodes in numerous stages) (Jin et al., 2017). A more recent study analyzed both pinopode subpopulations as well as other ultrastructural characteristics of surrounding cells like cilia and microvilli (Wu et al., 2019). An additional caveat to the quantitative evaluation of pinopode size is that differently sized pinopodes may demonstrate different functions. It is certainly possible that women experiencing infertility exhibit pinopodes of various sizes in different ratios and therefore different functions than women who do not experience infertility.

Historically, pinopode density has typically been quantitated by hand counting by a blinded observer (Nikas, 2000; Stavreus-Evers et al., 2001). One should take into consideration that this type of assessment is variable and somewhat imprecise, as the magnification or the subjective criteria of a pinopode could affect how many pinopodes are actually evaluated. Our laboratory recently provided the first to our knowledge automated pinopode quantitation method (Matson et al., 2017). This method eliminates human error and bias from traditional blind-observer counting, thus providing a more accurate pinopode count in a given sample. Briefly, by using the binary plugin in Fiji (Schindelin et al., 2012; Schneider et al., 2012), SEM images can be analyzed based on particle size and circularity to specifically quantitate pinopodes. The use of an automated quantitation method will enable the characterization of specific pinopode populations based on size, ultimately resulting in accurate results and reproducibility between studies.

Imaging Techniques

Because pinopodes are small, transient protrusions dependent on hormonal and potentially osmotic conditions of the extracellular environment, they are often difficult to image in space and time. Imaging pinopodes using confocal microscopy is therefore limited, and interpretations of specific cell staining should be taken with reservation. Uncertainty surrounding several potential markers as well as the overall paucity of markers underscore the importance of using imaging modalities, including SEM and additional tools, to characterize pinopodes. Although SEM and TEM provide superb resolution of pinopode structures, antibodies are often difficult to use in electron microscope imaging strategies (Horne et al., 2005; Stavreus-Evers et al., 2006; Tan et al., 2012). Thus, development and application of super-resolution imaging technologies combined with immunohistochemistry are needed to identify new markers of pinopode structures. In these ways, extracellular and intracellular expression patterns in pinopodes could be elucidated, strongly enhancing our knowledge of pinopode structure and function.

Concluding Remarks and Future Directions

Recently published clinical trials correlating pinopodes with implantation and pregnancy serve to generate renewed interest in pinopode biology (Jin et al., 2017; Qiong et al., 2017). The majority of available literature to date has described and analyzed density of pinopodes without subclassifying pinopodes into their developmental stages. Previous research has indicated that pinopode morphology, rather than absolute numbers, may be more relevant to endometrial receptivity (Jin et al., 2017; Sudoma et al., 2011). Encouragingly, though, studies are beginning to incorporate developmental stage into their analysis of pinopode density (Wu et al., 2019). Optimization of automated techniques to analyze pinopode density and morphology will allow for reproducibility in the field and understanding of the importance of pinopodes during the WOI.

It is still unknown whether pinopode function (e.g. endocytosis, exocytosis, expression of adhesion molecules) may differ between healthy patients and those with recurrent implantation failure. For example, an infertile patient may exhibit the same number of pinopodes as a fertile patient, yet the distribution of pinopodes and the material therein may actually determine embryo spacing and implantation success. We must also not neglect potential interaction of pinopodes with surrounding ultrastructural features of uterine epithelial cells, as some studies report that pinopodes allow for the entrapment of cilia, thereby preventing embryo movement and enabling close contact and adherence of the embryo during implantation (Bentin-Ley, 2000; Bentin-Ley et al., 1995; Bentin-Ley et al., 1999; Bergstrom and Nilsson, 1972).

One of the most pressing quandaries in the pinopode field is the accurate identification of a reliable marker for pinopodes. Identifying this marker could finally confirm the significance of pinopode expression at the WOI and clinically provide a new endometrial receptivity indicator. The WOI requires synchrony between maternal tissues and the embryo to establish appropriate attachment (Valles and Dominguez, 2006). Because there is debate in the literature concerning the formation and development of pinopodes during the WOI, perhaps, a “pinopode window” exists in which certain coverage, morphology, and protein expression

is critically important for embryo communication or contact. To examine this hypothesis, pinopode analysis methods must be rigorous and investigators should consider quantifying coverage and morphology in a given sample. Additionally, at least 10 fields (or emphasized by Jin et al., 2017 at least 60 fields) should be examined in a sample to achieve reproducibility. Implementation of a standardized, automated quantitation method would also eliminate variability among publications and could help to establish consistency across the literature.

To further study the function of pinopodes, especially their role in endometrial receptivity, advanced model systems must be implemented. Development of an *in vitro* culture system would be advantageous in improving our understanding of mechanisms controlling pinopode formation. Prior attempts in our lab and others of *in vitro* culture systems have identified pinopode-like structures; however, these structures may be easily confused with cellular bleb formation (Bentin-Ley et al., 1999; Fleming et al., 1998; Park et al., 2003). Advances in imaging modalities and the development of a reliable *in vitro* system will promote the inquiry of appropriate questions regarding pinopode structure and function and help to distinguish pinopodes from cell bleb structures. Future pinopode research will be instrumental to further our broad understanding of factors that mediate implantation failure and may eventually lead to approaches to combat poor pregnancy outcomes in women undergoing natural conception or IVF treatments.

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- Review of literature regarding the significance of pinopodes in endometrial receptivity and implantation.
- Quantitative and qualitative approaches for pinopodes are discussed.
- Clinical relevance of pinopodes is revisited.

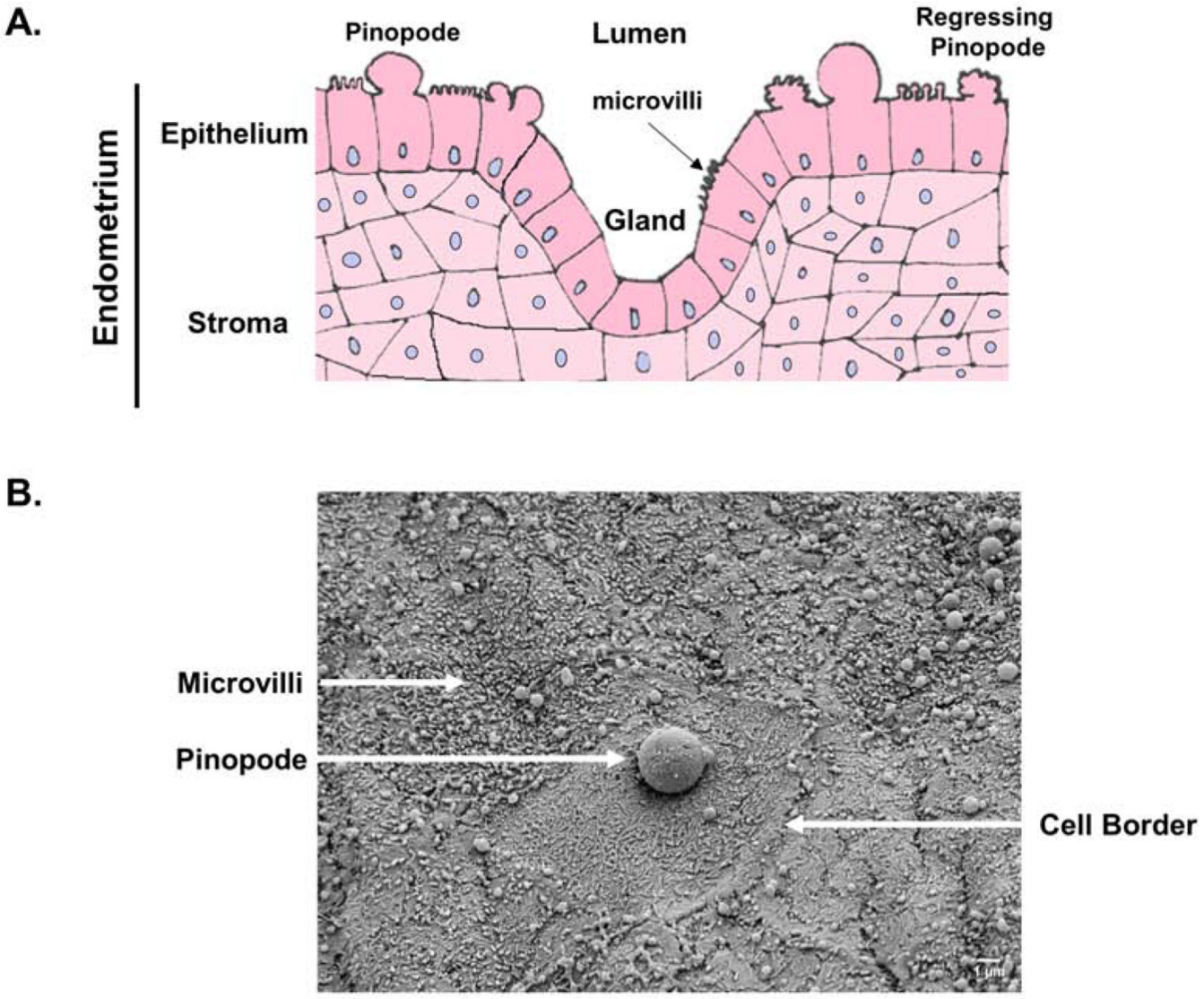

Fig. 1.

A) Pinopodes are large plasma membrane protrusions of uterine epithelial cells that transiently project toward the uterine lumen during the "window of implantation." **B)** Scanning electron micrograph of the murine uterine epithelial surface at pseudopregnancy day 3.5, the peak timing of the "window of implantation."

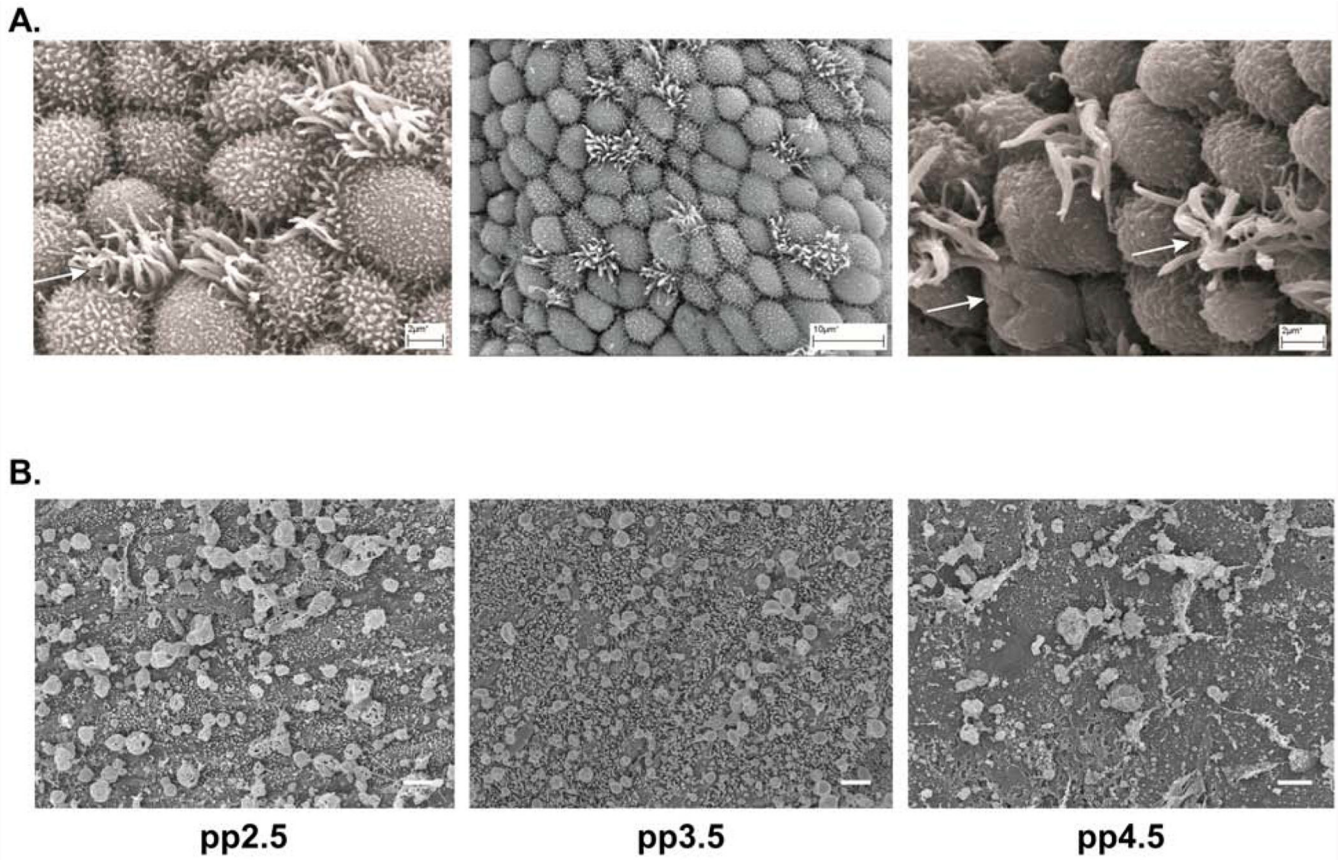

Fig. 2.

A) Representative scanning electron microscopy pinopode images from women experiencing infertility issues. Each image represents a different patient and demonstrates the variety of pinopodes in a given sample. Arrows denote regressing pinopode and cilia. Endometrial biopsies were collected between LH +6 to +10 in a natural menstrual cycle. Figure used from Aunapuu et al., 2018 (CC BY 4.0) under the creative commons attribution license. **B)** Representative stages of pinopode and microvilli development during pseudopregnancy (pp) in the mouse endometrium. On day 2.5 of pseudopregnancy, pinopodes appear to expand and have punctate holes. By day 3.5 of pseudopregnancy, pinopodes rise above microvilli and become smooth and spherical in shape. Few microvilli are present on day 4.5 of pseudopregnancy with pinopodes demonstrating more of a deflated and elongated appearance. Scale bars, 2 μm.

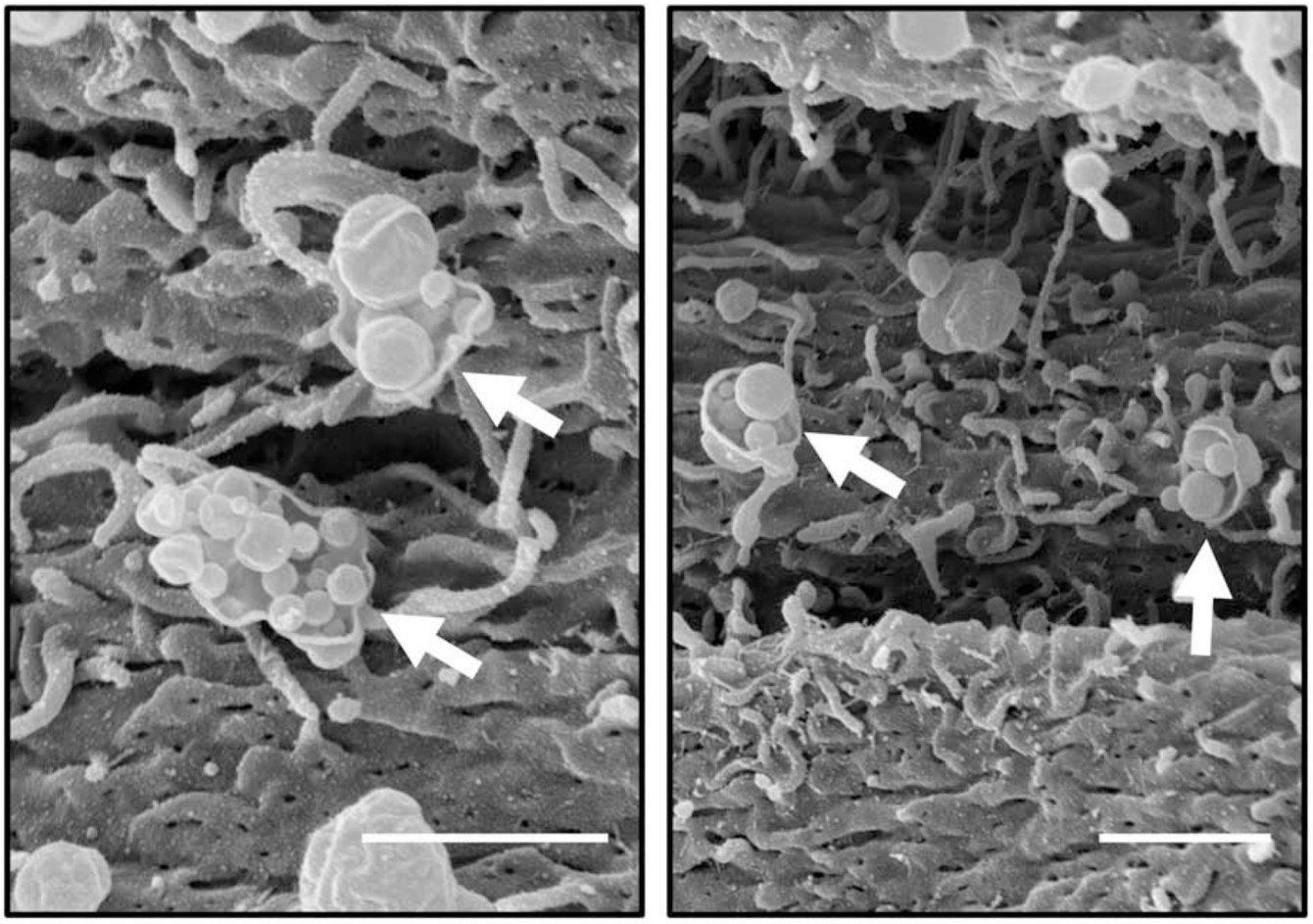

Fig. 3.

Scanning electron microscopy of the mouse endometrium on day 4.5 of pseudopregnancy. Some pinopodes (arrows) appear to rupture and release vesicular contents. Scale bars, 1 μm.

Table 1.

Regulators of Pinopodes

Protein/Treatment			
Hormones	**Species**	**Summary**	**Reference**
Progesterone (P4)	Human	Peak serum P4 levels correlates with increased pinopode numbers. High serum P4 levels results in early pinopode development.	Develioglu et al., 1999 Stavreus-Evers et al., 2001
Estrogen (E2)	Human Rat	High doses of E2 inhibit pinopode formation. Centchroman (E2 inhibitor) treatment decreases pinopodes.	Martel et al., 1991 Singh et al., 1996
Adrenomedullin (AM)	Mouse	Inhibition of AM and haploinsufficiency of Adm reduces pinopodes. Intrauterine delivery of AM increases pinopodes.	Li et al., 2008 Matson et al., 2017
Gonadotropin releasing hormone agonist (GnRH-a)	Human Mouse	Treatment of GnRH-a increased pinopode growth in women experiencing infertility. Pinopodes increase and are well-developed at day 4 of pregnancy following treatment of GnRH-a	Zhou et al., 2017 Guo et al., 2018
Testosterone	Rat	Administration decreases pinopodes.	Mokhtar et al., 2014
Secretion and Transcription Factors			
LIF and LIFR	Human	LIF and LIFR peak between LH days 6 to 9, coinciding with fully developed pinopodes.	Aghajanova et al., 2003
Glycodelin	Human	Increased staining in tissue with pinopodes.	Stavreus-Evers et al., 2006
Hoxa10	Mouse	Overexpression leads to an increase in pinopodes and blocking Hoxa10 in the uterus reduces pinopode formation.	Bagot et al., 2001
Endocrine Disrupters and Environmental Exposures			
Polychlorinated biphenyls	Mouse	Exposure leads to poorly developed pinopodes.	Qu et al., 2018
Quercetin (polyphenolic compound found in fruits and vegetables)	Rat	Administration causes poorly developed pinopodes.	Shahzad et al., 2017
Cigarette Smoke	Mouse	Decreased pinopode development.	Duran et al., 2014
Cypermethrin (CYP; type II pyrethroids pesticides)	Mouse	In mice with medium and high dosage of CYP, pinopodes are sparse.	Zhou et al., 2018

Table 2.

Human Pinopode Morphology

Day of Cyclicity	Microvilli Characteristics	Pinopode Morphology	Reference
15-16	Length and density increases	No appearance, cells begin to bulge	Nikas et al., 2000
17-19	Tall, long and thick	Small pinopodes appear (1-2 μm), endometrial surface bulging increases	Nikas et al., 2000
18-19	Size diminishes with the appearance of swollen tips	Distinct cell bulging with slender pinopodes rising from cell apex	Nikas et al., 2000
19-21	Short or no microvilli present	Smooth surface pinopodes develop and fold	Nikas et al., 2000; Stavreus-Evers et al., 2001; Quinn and Casper, 2009
20-23	Absent	Full protrusion of pinopodes, some appear with a wrinkled surface	Stavreus-Evers et al., 2001; Aunapuu et al., 2018
20-23	Increase in Microvilli	Pinopodes regress with a deflated balloon appearance	Nikas et al., 2000
23-24	Microvilli reappear on cell membranes	Pinopodes regress and/or disappear	Nikas et al., 2000; Stavreus-Evers et al., 2001