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Trophectoderm projections: a potential means for locomotion, attachment and implantation of bovine, equine and human blastocysts

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The behaviour of bovine, equine and human blastocysts was studied *in vitro* by time-lapse videomicrography and computer imaging. This study revealed that cytoplasmic extensions of the trophectoderm ['trophectoderm projections' (TEP)] were expressed by embryos of all three species, prior to or during zona escape. Bovine and human blastocysts escaped their zonae with a combination of blastocoele expansion, collapse and re-expansion coupled with the penetration of the zona pellucida by TEP. In equine embryos, after several cycles of blastocoele expansion and collapse, trophectoderm ruptured the zona with the concomitant appearance of TEP. This study provides documentation that TEP are expressed by a diverse range of mammalian species, bringing the total number of species in which this phenomenon is found to six, since TEP are also known to be expressed by guinea-pig, hamster and rhesus monkey blastocysts, representing rodents, ungulates and primates. In all species studied, the dynamic nature (extension, retraction, and angular movement) of the TEP was similar, moving in an undulating manner with rapid cycles of extension and retraction. Because TEP appear to be a general feature of mammalian blastocysts, they are implicated in one or more key events in early development, namely zona escape, attachment and/or implantation.

Key words: embryo culture/embryo movement/preimplantation embryo/time-lapse videomicrography/zona pellucida escape

Introduction

Mammalian embryo development depends on an exchange of nutrients and chemical signals between the embryo and the maternal epithelium. During development, a mechanism for this exchange must be established. In ungulates, such as cattle or horses, a superficial implantation in the form of an epitheliochorial placentation is created via interdigitations of uterine and trophoblastic microvilli at the eventual site of cotyledon (discrete in bovine) or micro-cotyledon (diffuse in equine) formation (Silver *et al.*, 1973). In these types of implantation, uterine invasion by trophoblasts does not occur, although there is a transitory attachment as the trophoblasts develop finger-like papillae that project into the lumen of the uterine glands (Hafez, 1987), providing a temporary anchor (Guillomot and Quay, 1982). In rodents and primates, implantation is more invasive: a haemochorial placentation forms in which the trophectoderm actively invades the uterus (Perry, 1981) For successful implantation, the trophoblast cells must cross the basement membrane of the uterine epithelium (Strickland and Richards, 1992). In either case (superficial or invasive), implantation is necessary for the continued survival of the embryo.

Prior to implantation, the mammalian embryo must escape from its zona pellucida, become juxtaposed to the maternal epithelium, and make initial contact with the uterus. The mechanism of zona escape is not clear. Many studies have described a process of zona escape *in vitro* in which the blastocyst expands, causing the zona pellucida to become stretched very thin and then to rupture, allowing the blastocyst to extrude in a characteristic 'dumb-bell' shape, the so-called 'hatching' process. However, the normality of this mode of zona escape is questionable (Gonzales and Bavister, 1995), as is its usefulness as an index of embryo viability (Bavister, 1995). Cytoplasmic projections emanating from the trophectoderm, termed 'trophectoderm projections' (TEP), are involved in zona escape *in vitro* in the hamster (Gonzales and Bavister, 1995) but it is unknown if they function in this way *in vivo*. Following contact of the blastocyst with the uterine epithelium, implantation is potentially promoted by either uterine and/or trophoblast derived enzymes (Strickland and Richards, 1992). Herz *et al.* (1992) proposed a model in which a receptor externalized by trophoblast binds plasminogen activator, conferring some directionality to the invasive properties of the trophectoderm (Zini *et al.*, 1992; Carroll *et al.*, 1993). Increased protease inhibitor production by the embryo and the uterus restrict these invasive properties (Strickland and Richards, 1992), thereby controlling the depth of invasion in either superficial or invasive implantation. What is the mechanism for the directed delivery of the invasive properties by trophoblasts? The TEP may serve such a function during implantation (Gonzales *et al.*, 1996).

Research on TEP expression and function has been minimal and detailed studies are restricted to rodents Spee (1883) first described TEP penetrating the zona pellucida in guinea-pig blastocysts and many years later, Blandau (1949) verified their

existence in this species. In a subsequent study, Blandau used time-lapse micro-cinematography to record the dynamics of TEP movement (Blandau and Rumery, 1957) and described TEP as small mobile projections penetrating the zona pellucida prior to zona escape. The only other species in which TEP have been studied in detail is the golden hamster (Gonzales and Bavister, 1995; Gonzales *et al.*, 1996). Two studies have documented the existence of TEP during the zona escape/attachment developmental time window *in vivo* (Parr, 1973; Gonzales and Bavister, 1995), indicating that TEP may play a physiological role in early development. The present study shows that TEP occur in embryos of other species, specifically bovine, equine and human, suggesting that they may be of general importance for mammals. Additionally, this study compares the dynamics (extension, retraction and angular movement) of TEP in these species with TEP in the hamster (Gonzales *et al.*, 1996). Some possible physiological roles of TEP during development, including locomotion of preimplantation embryos, initial attachment to maternal epithelium and implantation, are also discussed.

Materials and methods

Embryo collection and/or production

Bovine embryo production and culture

Bovine embryos were generated by in-vitro maturation and in-vitro fertilization as previously described (Pinyopummintr and Bavister, 1994). Briefly, cumulus–oocyte complexes aspirated from 2 to 7 mm follicles were matured in 50 μl drops of tissue culture medium-199 (TCM-199, Gibco Laboratories, Grand Island, NY, USA) for 24 h at 39°C under 5% CO_2 in air and saturated humidity (Pinyopummintr and Bavister, 1991).

Following maturation, ova were inseminated with 10^6 spermatozoa/ml (from American Breeders Service, DeForest, WI, USA) in 50 μl drops of sperm-TALP (Parrish *et al.*, 1988) and co-incubated for 18 h at 39°C under 5% CO_2 in humidified air.

Resulting embryos were cultured in TCM-199 (Pinyopummintr and Bavister, 1991) until the blastocyst stage. To increase the chances of TEP expression, only expanded blastocysts were selected for further culture; all others were discarded. Only expanded blastocysts were transferred to 80 μl drops containing fresh medium for videotaping and computer imaging, as described below.

Equine embryo collection and culture

One embryo from each of eight mares was collected at 36 ± 12 h post-ovulation, as previously described (Peyrot *et al.*, 1987; Carnevale *et al.*, 1993) Briefly, mares were pre-medicated with xylazine (Rompun; Mobay Corp., Shawnee, KS, USA), and general anaesthesia was induced with ketamine hydrochloride (Ketaset; Aveco Co., Inc., Fort Dodge, IA, USA) and maintained with halothane. The oviduct was exposed through a flank laparotomy, dissected from the meso-salpinx, and severed at the utero–tubal junction. Embryos were recovered by retrograde-flushing the oviduct into 60×15 mm Petri dishes (Falcon Plastic no. 1007; Becton Dickinson and Co., Lincoln Park, NJ, USA) with 10 ml of TCM-199 containing 1% fetal calf serum (FCS; HyClone Laboratories, Inc., Logan, UT, USA) at 37°C. Embryos were located with a dissecting microscope and evaluated with a Diaphot (Nikon, Tokyo, Japan) inverted microscope, using phase-contrast optics. For transport from surgery to the laboratory, the embryos were placed in a test tube (Falcon Plastic no. 2054; Becton Dickinson and Co.) containing TCM-199 maintained at 37°C. Transport time was <30 min. Embryos were then placed in 80 μl culture drops of TCM-199 + serum overlaid with 10 ml silicone oil in 60×15 mm Petri dishes and cultured for ≥6 days. The culture drop and Petri dishes were prepared as previously described (Gonzales *et al.*, 1996).

Human embryo production and culture

Multiple follicular development was induced using a regimen of down-regulation with gonadotrophin-releasing hormone analogue (Lupron; Tap Pharmaceuticals, Abbott Laboratories, Chicago, IL, USA) followed by stimulation with human menopausal gonadotrophin (Pergonal; Serono Laboratories, Norwell, MA, USA). Final follicular maturation and ovulation was induced with human chorionic gonadotrophin (Profasi; Serono Laboratories) when the mean diameter of the lead follicle measured ≥18 mm. Oocyte recovery was carried out 34 h later by transvaginal aspiration. Cumulus enclosed oocytes were washed in Ham's F-10 medium (Gibco) containing 7.5% heat-inactivated fetal cord serum (FCS) and incubated in 2.0 ml of the same medium in organ culture dishes (Falcon 3037; Becton Dickinson and Co.) for 5–8 h prior to insemination. A fresh semen sample, prepared as described previously (Gerrity and Shapiro, 1985), was used to inseminate oocytes in each culture dish at a final sperm concentration of 5.0×10^4 motile spermatozoa/ml. At 16–18 h after insemination, the cumulus cells were mechanically removed and the oocytes examined for evidence of normal fertilization (two pronuclei and two polar bodies). From each stimulation cycle, up to eight fertilized oocytes from two women were cultured for 24 h in Ham's F-10 medium supplemented with 15% FCS. All additional fertilized oocytes were frozen at the pronuclear stage (Testart *et al.*, 1986) for use in subsequent cycles. Following 24 h of embryo culture, the best looking embryos (up to five) were selected for embryo transfer and the remaining embryos were cultured (n = 9) (IRB Protocol # 94-952-051) to assess embryo viability and, secondarily with patients' consent, to examine the morphology and timing of embryo development. For imaging, these embryos were transferred to 80 μl culture drops of Ham's F-10 medium supplemented with 15% fetal calf serum under silicone oil in 60×15 mm Petri dishes. The cultures were maintained in 5 5% CO_2 in air with saturated humidity at 37°C.

Dynamic and static data collection

Time-lapse and digital imaging

The methods for culturing embryos while simultaneously gathering time-lapse video images (dynamic data) and computer-generated digitized images (static data) have been described (Gonzales and Bavister, 1995). Briefly, embryos were cultured on the stage of a Diaphot inverted microscope (Nikon, Tokyo, Japan) as described by Bavister (1988). Embryo cultures were imaged using ×10 or ×20 Nomarski optics. Only one plane of focus (the equatorial plane) could be examined by time-lapse during embryo culture, due to equipment limitations (lack of an automated Z-drive). The microscope light source was covered with orange-red filters to protect the embryos from light toxicity (Umaoka *et al.*, 1993). Images were obtained with a video camera fitted with a Newvicon detector tube (Series 70, Dage-MTI Inc., Michigan City, IN, USA) attached to the camera port. A zoom lens attached to the detector tube was set at ×1 or ×1.5, resulting in final optical magnifications of ×10, ×15, ×20 or ×30. Time-lapse videomicrography was done using a Super-VHS recorder (S-VHS, AG-6720A; Panasonic, Secaucus, NJ, USA) Digitized images were gathered with a personal computer (486/33 Mhz; Gateway 2000, N. Sioux City, SD, USA) equipped with a PCVISION*plus* frame grabber (Part no. 47-H00010-E2; Imaging Technology Inc., Woburn, MA, USA) and temporarily stored on the computer hard drive. Digitized image collection and analysis were performed using Optimas image-processing software (BioScan Optimas Corp.,

Edmonds, WA, USA). Utilizing a macro created with the software, the computer was placed in time-lapse mode to collect digitized images at fixed intervals of 30 s to 10 min, depending on events examined. Relevant digital images were archived to an external optical disk drive (PMO-650, Pinnacle Micro Optical Storage Co., Irvine, CA, USA). Time-lapse video and computer digital images were examined on a Trinitron PVM-1343MD video monitor (Sony Corp , Tokyo, Japan).

Data acquisition

The method of acquiring spatial data from digital images has been described (Gonzales *et al* , 1996). Briefly, linear measurements, such as lengths and angles, were measured using macros provided with the imaging software. This system was calibrated when the images were created, using a microscope micrometer at the same magnification as the images. Measurements were made only on TEP that remained in the plane of focus. The velocities of extension and retraction, and of angular movement of TEP, were determined by comparing sequential computer images. All temporal measurements are given in real time. Comparisons of mean parameters measured were done by analyses of variance (Snedecor and Cochran, 1989) Hard copies for publication were made by photographing images on the video monitor with a Nikon N-2000 camera using a 55 mm macro lens and TMAX 400 film (Eastman Kodak Co., Rochester, NY, USA).

Results

Developmental timing of appearance of TEP

Bovine embryos

After selecting only expanded blastocysts for imaging (n = 25, in three cultures), eight of these blastocysts (32%) visibly expressed TEP while seven (28%) escaped from their zonae. Therefore, almost all embryos that produced TEP escaped from their zonae (7/8 = 88%) while no blastocysts escaped from their zonae without visible TEP expression. In these blastocysts, TEP first appeared, at multiple sites, as undulating cytoplasmic finger-like protrusions penetrating the zona pellucida (Figure 1). These TEP retracted and then reappeared at the site of initial penetration. Eventually, associated with the combined effect of blastocoele expansion, collapse and re-expansion, and zona penetration by TEP, a laceration appeared in the zona and the embryos extruded through the resulting hole. In addition, blastocysts underwent cycles of blastocoele collapse and re-expansion following zona escape.

The mean velocity of extension and retraction of the TEP was 0.3±0.07 and 0.2±0.03 μm/s (n = 11 observations each) respectively, and the mean maximal length of the TEP observed was 29.1±0.3 μm (n = 11). Bovine TEP spanned angles of 2–146° at velocities that ranged from 0.5 to 13.0°/s with a mean angular velocity of 6.4±1.0°/s (n = 11 observations). These observations (Table I) were made over 2.2 h However, not included in the calculations of these data was one TEP that penetrated the zona but never retracted It separated from the embryo when the blastocyst collapsed within the zona. It then broke away from the zona and 'crawled' around the culture dish like a worm for several minutes, and eventually degenerated.

Equine embryos

In eight cultures of one embryo each, four blastocysts escaped their zonae, while two of these expressed TEP After several cycles of blastocoele expansion and collapse, a portion of the trophectoderm herniated through the zona at the site of eventual zona rupture. In two embryos, this penetration was followed by the appearance of large appendage-like TEP with undulating movement, associated with the extruded portion of the embryo (Figure 2). There were at least four TEP in the first embryo and at least six in the second; the exact number could not be determined since not all of the TEP activity was in the plane of focus. The mean velocity of extension and retraction of the TEP observed was 0.3 ± 0.1 and 0.2 ± 0.05 μm/s (n = 6 observations each) respectively. The TEP reached a mean maximal length of 27.4 ± 0.3 μm (n = 10). During their undulating movement, the TEP spanned angles of 8–112° at velocities from 1.3 to 12.5°/s. The mean angular velocity was 7.2 ± 1.1°/s (n = 8 observations; Table I). One TEP paused with no extension, retraction, or angular velocity for 85 s and then resumed movement. These observations were made over periods of 5.7 and 8.9 min on the first and second embryos respectively. However, the exact duration of TEP expression could not be determined because the cultures were terminated prematurely to accommodate a different study.

Human embryos

Only 11% (one of nine) of the embryos escaped from the zona pellucida and 22% (two of nine) expressed TEP activity (Figure 3). One blastocyst that showed TEP was derived from a frozen–thawed embryo. In association with three cycles of blastocoele expansion, collapse and re-expansion, as well as zona penetration by TEP, a laceration appeared in the zona, through which the expanded blastocyst extruded. Following zona escape, the blastocyst underwent three more collapse and re-expansion cycles.

The dynamic data were obtained from five TEP that were expressed by two collapsed blastocysts, one zona-escaped and the other zona-enclosed. However, some TEP activity out of the plane of focus could not be measured in the zona-escaped blastocyst. Also, only three measurements of angular velocity could be made because all other angular movements were not in the plane of focus. Over 193 min, all of the TEP activity emanated from the inner cell mass (ICM) side of the zona-escaped embryo. It could not be determined from which side of the zona-enclosed embryo the TEP activity emanated. The mean maximal TEP length was 27.7 ± 0.5 μm (n = 5). The mean extension and retraction velocity was 0 3 ± 0 1 and 0.2 ± 0.04 μm/s (n = 5) respectively. TEP spanned angles of 0–145°, with a mean angular velocity of 7.3 ± 2 0°/s (n = 3; Table I).

Finally, in all three species observed, only the zona-escaped embryos moved about in the field of view while zona-intact blastocysts and empty zonae remained stationary.

Discussion

Embryos of all three species (equine, bovine and human) examined by time-lapse videomicrography underwent cycles of blastocoele expansion and collapse prior to escape from their zona pellucidae. This cyclic activity in human embryos was previously documented in a time-lapse video recording (Gonzales *et al*, 1995). The present study showed that in

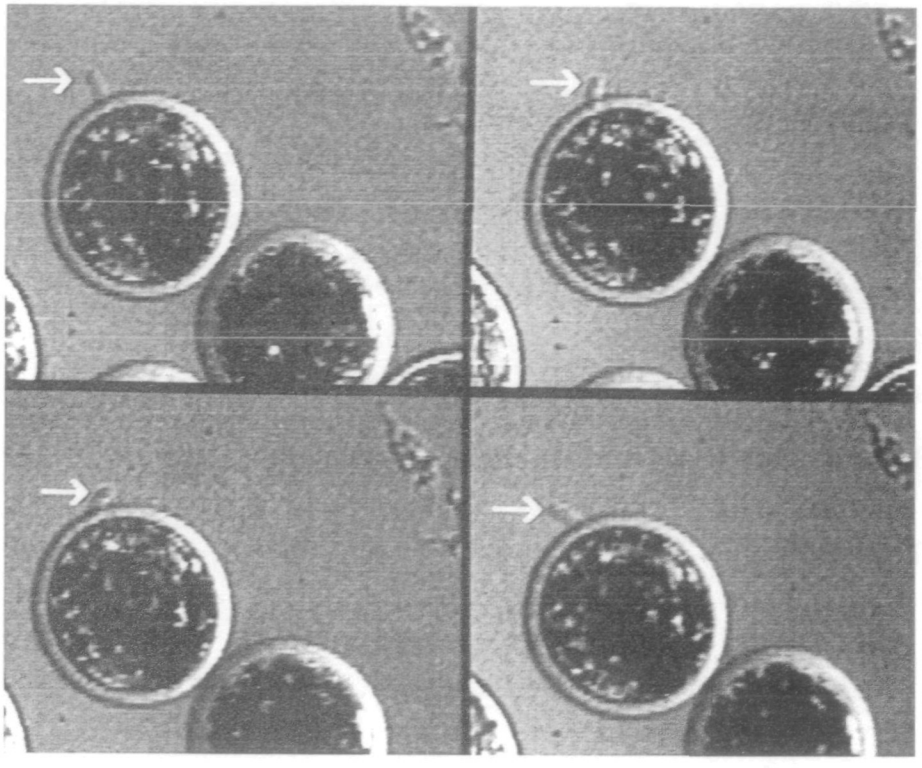

Figure 1. Time series of a bovine blastocyst expressing an appendage-like trophectoderm projection (TEP, arrow) undergoing angular movement while penetrating the zona pellucida. The sequence is top left to right then bottom left to right Frames are 6 min apart. Computer digital images converted from video images, ×15 original magnification

Table I. Mean maximal lengths (± SEM) and dynamics of trophectoderm projections (TEP) expressed by bovine, equine and human blastocysts[a]

	Maximal length (μm)	Extension velocity (μm/s)	Retraction velocity (μm/s)	Angular velocity (degrees/s)
Bovine	29 1 ± 0 3 (11)	0 3 ± 0 07 (11)	0 2 ± 0 03 (11)	6 4 ± 1 0 (11)
Equine	27 4 ± 0 3 (10)	0 3 ± 0 1 (6)	0 2 ± 0 05 (6)	7 2 ± 1 1 (8)
Human	27 7 ± 0 5 (5)	0 3 ± 0 1 (5)	0 2 ± 0 04 (5)	7 3 ± 2 0 (3)

[a]There was no significant difference of related mean parameters measured between species (all *P*-values ≥ 0 40)
Values in parentheses are numbers of observations

association with this cyclic activity, as well as zona penetration by TEP in bovine and human embryos, a tear appeared in the zona, at the site of eventual zona escape. Therefore, TEP appeared to be a component of zona escape in these cultured embryos. This could not be conclusively determined in equine embryos since zona penetration by TEP prior to zona escape was not detectable; however, TEP activity was clearly present at the time of zona escape. Penetration of the zona by TEP in the equine embryos could have been missed if the TEP were out of the plane of focus. Similarly, in the one bovine blastocyst in which TEP activity was not detected prior to zona escape, TEP may have been present out of the plane of focus. This is a limitation of two-dimensional (*x* and *y* axes) recording. Following cycles of expansion and collapse of the equine blastocysts, trophectodermal cells herniated through a break in the zona, followed by activity of TEP associated with these extruded cells (Figure 2). In all three species, TEP appeared in blastocysts only; there was no detectable TEP expression in cleavage-stage, retarded, or degenerate embryos. This is consistent with our previous reports on TEP activity in hamster blastocysts (Gonzales and Bavister, 1995).

These observations, at least for the cow and human, as well as for guinea-pig, hamster and rhesus monkey in which TEP penetration of the zona pellucida has been observed (Spee, 1883; Blandau, 1949; Boatman, 1987; Gonzales and Bavister, 1995; Gonzales *et al.*, 1996), appear strongly to implicate TEP activity in the process of zona escape. However, while this may well be true in cultured embryos, a detailed study on the timing of TEP expression in the hamster indicated that involvement of TEP in escape from the zona may be abnormal, due to a temporal displacement of zona loss caused by absence of the uterine environment (Gonzales and Bavister, 1995). Under normal conditions *in vivo*, the uterine secretions may play a crucial role in zona escape, so that the commonly observed mode of zona escape *in vitro*, so-called 'hatching', may well be an artefact of culture. Whether or not TEP play a role in zona escape *in vivo* in other species remains to be seen, but their true function may be in attachment/implantation, and possibly also in embryo locomotion (Gonzales and Bavister, 1995; Gonzales *et al.*, 1996).

The possible role of TEP in embryo locomotion was first indicated in a kinematic study with hamster blastocysts *in vitro*

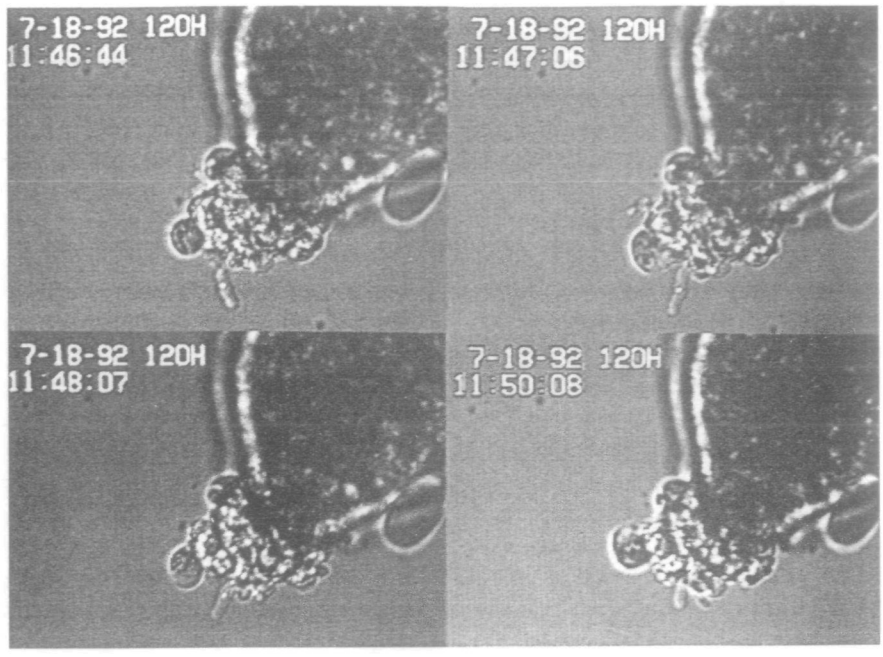

Figure 2. Montage of sequential images of a hatching equine blastocyst showing the dynamics of extension, retraction and angular movement of a single trophectoderm projection (TEP) associated with trophectoderm that has extruded from the zona pellucida Total elapsed time (top left to bottom right) 3 min and 22 s Frame averaged digitized images, ×30 original magnification

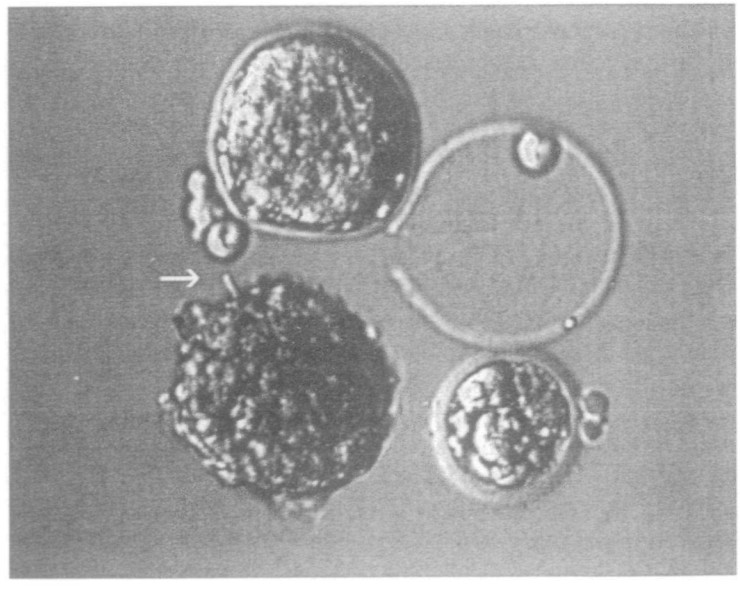

Figure 3. Digitized image of a collapsed, zona-escaped human blastocyst expressing one of several TEP (arrow) This TEP was monitored over several min and showed extension, angular movement and retraction (see text), ×10 original magnification

(Gonzales *et al.*, 1996). These cultured hamster blastocysts, after escape from their zonae, moved about by an endogenous means, most likely involving TEP activity. In the present study, time-lapse videomicrography revealed zona-escaped bovine, equine and human embryos moving about in the field of view (with reference to empty zonae and zona intact blastocysts that remained stationary) Although TEP were not observed during this event, this may be because only the equatorial plane of focus was examined during data collection However, because TEP are the only structures yet identified capable of conferring mobility on embryos, it is reasonable to postulate that TEP are involved in the locomotion of embryos, at least *in vitro*. If zona-free blastocysts *in vivo* are likewise capable of locomotion, which is consistent with the observation that TEP are expressed by blastocysts *in vivo* in the guinea-pig and golden hamster (Parr, 1973; Gonzales *et al.*, 1996), this could contribute to uniform spacing of embryos along the length of the uterus in polytocous animals such as rodents and pigs. In monotocous species like the three examined in this study, blastocyst mobility could still be an important factor contributing to transportation of the embryo within the uterus to the optimal site for implantation, and/or for distribution of chemical signals emanating from the embryo (see below). Moreover, in animals such as cattle in which initial attachment to the uterine epithelium is very superficial, TEP could play a role in absorption of nutrients, at least until extensive elongation

and corresponding increase in surface area of the trophectoderm would supersede such a function of TEP.

In higher primates, which are generally monotocous, blastocysts may not have much need for locomotion or for a system to assist with their nutrition prior to implantation, which begins soon after zona escape. What then is the function of TEP in human and rhesus monkey blastocysts? In both species, TEP are expressed, at least *in vitro*, immediately prior to zona escape (Boatman, 1987; present study) and thus most likely represent the cellular component of the embryo that makes first contact with the uterine epithelium, even while the embryo is still zona-enclosed. This makes TEP probable candidates for contributing to the 'embryo–maternal dialogue', i.e. a chemically-mediated recognition system that prepares the site of implantation (Hearn *et al.*, 1988; Hartshorne and Edwards, 1991) and possibly the embryo itself. If TEP are an important component of this signalling system, then failure to express TEP by otherwise normal-looking blastocysts might be predictive of implantation failure (Edwards, 1986). More data on human TEP activity related to zona escape are needed; in the present study, only one blastocyst escaped successfully, possibly because other embryos were damaged by freeze-thawing and/or because of suboptimal culture conditions.

Another possible function of TEP may be to control the invasiveness of the trophectoderm. The process of implantation requires initial contact with maternal epithelium, attachment, and to varying degrees depending on the species (Perry, 1981), implantation into the uterine epithelium (Hafez, 1987). In order for the embryo to implant, the trophectoderm must become invasive. This property of the trophectoderm must be directed and tightly controlled to restrain the location and depth of invasion, regardless of whether invasion is superficial and discontinuous or diffuse, as in ungulates (Silver *et al.*, 1973) or deep, as in rodents and primates (Perry, 1981). The control of invasiveness could reside in the trophectoderm, the uterine epithelium or both. The TEP may be the cellular components of the blastocyst that make initial contact, attach and implant into the uterine epithelium, and may be responsible for the directed delivery of invasive enzymes necessary for implantation (deep or superficial). Two enzyme systems are candidates for the biochemical aspects of trophectoderm invasiveness: the plasminogen–plasmin system (Strickland *et al.*, 1976; Dyk and Menino, 1991, Carroll *et al.*, 1993) and the metallo-proteinases such as stromelysin and/or type IV collagenase (Behrendtsen *et al.*, 1992). To control enzymatic activity, trophectoderm produces plasminogen activator inhibitor, a low density lipoprotein-related receptor that binds plasminogen activator–inhibitor complexes and internalizes them (Nykjaer *et al.*, 1992; Orth *et al.*, 1992). The trophectoderm also produces tissue inhibitors of metallo-proteinase (Brenner *et al.*, 1989) which stoichiometrically inhibit collagenase and stromelysin. In addition, transforming growth factor β-1, produced by trophectoderm and uterine epithelium, induces differentiation of invasive human cytotrophoblast into non-invasive syncytiotrophoblasts (Graham *et al.*, 1992), giving rise to another means for controlling the invasive property of trophectoderm.

The hypothesis that TEP are involved in or possibly responsible for the earliest stages of implantation (i.e., attachment) is strengthened by observations that TEP expression by zona-escaping blastocysts is not global but localized to a particular region of the embryo. In species in which attachment begins at the abembryonal trophectoderm, TEP expression was opposite the inner cell mass (guinea-pig: Blandau and Rumery, 1957; Spee, 1883; hamster: Gonzales *et al.*, 1996). In primates, however, attachment occurs at the embryonal region of the trophectoderm, so TEP expression would be expected in this region. Limited data on human (present study; Figure 3) and rhesus monkey embryos (Boatman, 1987) indicate that TEP were indeed localized at the embryonal pole. This must be confirmed by future studies.

It must be emphasized that our observations on human embryos were based on only two specimens that showed TEP activity, one of which escaped from the zona. We hope that these intriguing observations, together with strong data from the five other species examined here and in previous studies, will stimulate more intensive examination of the role of TEP in human blastocyst zona escape and possibly in attachment and implantation.

In conclusion, this study demonstrates that there were no detected differences in the dynamic nature of TEP (extension, retraction and angular movement) among the three species examined. The TEP were morphologically similar and were expressed at the same developmental stage (late blastocysts, during or immediately following zona escape) in equine, bovine and human embryos, exactly as reported in rhesus monkey, guinea-pig and hamster embryos (Blandau and Rumery, 1957; Spee, 1883; Boatman, 1987; Gonzales and Bavister, 1995; Gonzales *et al.*, 1996). Since the phenomenon of TEP expression was conserved across all six species, and is known to occur *in vivo* in at least two of them, TEP most likely play a key role in normal development of rodent, ungulate and primate embryos. More studies of TEP activities are warranted to elucidate their physiological role(s) as well as their possible usefulness for indicating blastocyst viability and/or competence to implant.

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