Role of the Extracellular Matrix Protein Thrombospondin in the Early Development of the Mouse Embryo

K. S. O'Shea,* L.-H. J. Liu,* L. H. Kinnunen,‡ and V. M. Dixit§

Departments of *Anatomy and Cell Biology, ‡Human Genetics, and §Pathology, University of Michigan Medical School, Ann Arbor, Michigan 48109-0616

Abstract. The distribution of the extracellular matrix protein thrombospondin (TSP) in cleavage to egg cylinder staged mouse embryos and its role in trophoblast outgrowth from cultured blastocysts were examined. TSP was present within the cytoplasm of unfertilized eggs; in fertilized one- to four-cell embryos; by the eight-cell stage, TSP was also densely deposited at cell–cell borders. In the blastocyst, although TSP was present in all three cell types; trophectoderm, endoderm, and inner cell mass (ICM), it was enriched in the ICM and at the surface of trophectoderm cells. Hatched blastocysts grown on matrix-coated coverslips formed extensive trophoblast outgrowths on TSP, grew slightly less avidly on laminin, or on a 140-kD fragment of TSP containing its COOH terminus and putative cell binding domains. There was little outgrowth on the NH_2 terminus heparin-binding domain. Addition of anti-TSP antibodies (but not GRGDS) to blastocysts growing on TSP strikingly inhibited outgrowth. Consistent with its early appearance and presence in trophoblast cells during implantation, TSP may play an important role in the early events involved in mammalian embryogenesis.

G ENETIC and epigenetic events combine to direct the early development of the vertebrate embryo. Of the latter, selective adhesive interactions, both cell–cell as well as between cells and the extracellular matrix (ECM),[1] play a particularly important role in determining the final organization of the early embryo. Despite the obvious importance of both adhesion and deadhesion in morphogenetic processes such as ovulation, fertilization, cleavage, compaction, cell migration, and trophoblast invasion, few data are available on the pattern of synthesis and deposition of ECM and other cell surface constituents during these critical phases of embryonic development. To date laminin and nidogen (Dziadek and Timpl, 1985), heparan sulfate proteoglycan (Dziadek et al., 1985), fibronectin (Wartiovaara et al., 1979), collagens (Leivo et al., 1980; Wartiovaara et al., 1979), and uvomorulin (Damjanov et al., 1986), have been localized during cleavage stages of development.

Thrombospondin (TSP) is a trimeric adhesive glycoprotein present in the extracellular matrix and on the surface of many cell types. Like other ECM components, it binds a number of ligands including fibronectin (Lahav et al., 1982, 1984), collagens (Lahav et al., 1982; Mumby et al., 1984), glycosaminoglycans (Dixit et al., 1984; Lawler et al., 1985), as well as plasminogen and plasminogen activators (Silverstein et al., 1984). TSP may play an important role in mor-

phogenesis by both determining the organization of the matrix via its binding of other ECM components and by directing local protease activity via its binding of plasminogen activators (Silverstein and Nachman, 1987). It is involved in migration of a variety of cell types including: melanoma cells (Taraboletti et al., 1987), smooth muscle cells (Majack et al., 1986), keratinocytes (Varani et al., 1988), cerebellar granule cells (O'Shea et al., 1990), and neural crest cells (Boyne, L. J., K. S. O'Shea, and V. M. Dixit. 1989. *J. Cell Biol.* 109:112a). TSP is present during post-implantation development in a highly developmentally regulated pattern (O'Shea and Dixit, 1988).

In this investigation, we examined the pattern of TSP deposition in the developing follicle, and in cleavage to egg cylinder–stage embryos and its role in trophoblast outgrowth. TSP was present before fertilization and at earliest cleavage stages; with compaction, TSP was present on the cell surface of blastomeres and was particularly enriched in areas of contact between cell membranes. TSP substrates supported trophoblast outgrowth from isolated blastocysts to a greater extent than laminin, and than a 140-kD fragment of the TSP molecule containing the COOH terminus and putative cell binding domains. The amino terminus heparin-binding domain (HBD) was a less efficient substrate. Addition of anti-TSP antibodies to blastocysts growing on TSP inhibited outgrowth; addition of the synthetic peptide GRGDS had little effect on outgrowth. Aspects of this work have been presented previously (O'Shea, K. S., L-H. J. Liu, L. H. Kinnunen, and V. M. Dixit. 1988. *J. Cell Biol.* 107:596a).

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1. *Abbreviations used in this paper*: ECM, extracellular matrix; HBD, heparin-binding domain; TSP, thrombospondin.

Materials and Methods

Tissue

Embryos were obtained from matings of (C57Bl/6J × SJL) F1 mice (Jackson Laboratories, Bar Harbor, ME); day of finding a vaginal plug was considered the first day of gestation. Females were superovulated by intraperitoneal injection of pregnant mare's serum gonadotropin (PMSG, 5 units; Sigma Chemical Co., St. Louis, MO), followed 46 h later by injection of 5 units of human chorionic gonadotropin (hCG; Sigma Chemical Co.), then were mated with males of the same strain. One- and two-cell embryos were flushed from the oviducts and collected in M16 medium (below) supplemented with 0.5 mg/ml hyaluronidase to remove adherent cumulus cells. After 5 min incubation at 37°C, the embryos were transferred to M16 and processed for immunocytochemical localization of TSP. Additional embryos were cultured in M1 medium in an atmosphere of 5% O_2, 5% CO_2, 90% N_2 until the desired stage of development was reached (to 4 d) in microdrops under paraffin oil. Unfertilized eggs were obtained by mincing ovaries from stimulated (PMSG + hCG) and unstimulated mice or by mating similar females with vasectomized males and recovering eggs by flushing as described above.

Culture medium was Whitten's medium (Whitten and Biggers, 1968) supplemented with 0.1 mM EDTA (M1 medium) or modified by substitution of bicarbonate with 20 mM Hepes (M16) for embryo manipulation.

Sections of embryos implanting in the uterine endometrium (day 6 1/2) and sections through pre- and postovulatory follicles were also examined. Ovaries and implantation sites dissected from CD-1 strain mice (Charles River Laboratories, Portage, MI) were frozen in OCT (Miles Scientific, Inc., Kankakee, IL) in hexane cooled over an acetone-dry ice slurry. 6–8-μm sections were cut using a Zeiss Microm cryostat and collected on 0.1% polylysine-coated slides. Slides were stored at −20°C before immunocytochemical localization of TSP as described below.

Immunocytochemistry

Light Microscopy. For immunocytochemical localization of TSP in unfertilized eggs and during cleavage stages, embryos (with and without zona pellucidae) were fixed briefly (1–2 min) in 2% paraformaldehyde in 0.1 M phosphate buffer, washed in PBS also containing 1% BSA (PBS/BSA), exposed to normal goat serum (1:20) for 30 min at room temperature. They were then rinsed in PBS/BSA and then permeabilized in PBS/BSA containing 0.5% Triton X-100. Embryos were exposed to anti-TSP IgG (1:20) for 2 h at room temperature; washed in PBS/BSA, then exposed to goat anti–rabbit IgG-FITC (Miles Scientific, Inc.) at a dilution of 1:40 for 30 min at room temperature. Sections and embryos were rinsed, then coverslipped with glycerol containing phenylenediamine (0.1%). To improve antibody penetration, zonas were removed using a brief (60 s) exposure to acidic tyrodes (pH = 2.5) or brief incubation in pronase (0.5% in M16). Controls were exposed to PBS in place of the primary antibody, to anti-TSP antibodies to which a 10-fold excess of TSP had previously been added (preabsorption control) or to normal rabbit serum (1:25), and additional embryos were processed without fixation or permeabilization. The anti-TSP antibodies used in the current investigation have been characterized previously (Varani et al., 1986; O'Shea, Dixit, 1988). In immunoblots of protein extracts from day 13 mouse embryos, these antibodies detected a single protein that comigrated with purified human platelet thrombospondin (O'Shea, K. S., L-H. J. Liu, L. H. Linnunen, and V. M. Dixit. 1988. *J. Cell Biol.* 107:596a).

Sections and embryos were examined and photographed using a Leitz Aristoplan photomicroscope using epifluorescence and phase contrast microscopy. Embryos were also examined using confocal microscopy, with the Zeiss LSM system equipped with an argon laser. Micrographs of individual optical sections were obtained, or sections processed to obtain stereopairs to better visualize the distribution of TSP.

Blastocyst Outgrowth Assay

One- and two-cell embryos were flushed from the oviducts and cultured to the late blastocyst stage as described above. There was some variation in developmental stage at this time, the majority of the blastocysts were hatching from the zona pellucida by this stage, and attached to the substrate, although active outgrowth required an additional 18–24 h. A real attempt was made to use developmentally synchronous embryos in these assays. 5–10 blastocysts were transferred to each matrix-coated well of 24-well plates (Costar Corp., Cambridge, MA). Blastocysts were cultured in serum-free

DMEM containing 1% hybridoma HU (an additive containing a patented mixture of insulin, selenium, transferrin, and other factors [no TSP]) (Boehringer Mannheim Biochemicals, Indianapolis, IN) and 4 mg/ml BSA (Armant et al., 1986b; Sutherland et al., 1988) in 5% CO_2 in air for 72 h. Outgrowth was monitored and recorded photographically after 24, 48, and 72 h and medium was changed at those times.

Substrates. 24-well plates were washed three times in sterile water, coated with polylysine (0.1%) for 15 min, washed three times in medium, then incubated overnight in DMEM containing the protein of interest, followed by three washes in DMEM with 5 mg/ml BSA. Test proteins were: laminin (25 μg/ml: Collaborative Research, Lexington, MA), TSP (10 μg/ml), an 140-kD fragment of the TSP molecule containing the COOH terminus and putative cell binding domains (25 μg/ml; Varani et al., 1988), the NH$_2$ terminus HBD (25 μg/ml) or BSA (25 μg/ml). Additional blastocysts were grown on substrates coated with TSP (10 μg/ml) to which anti-TSP antibodies were added (50 μg/ml) or to which a synthetic peptide containing a three–amino acid sequence (RGD) involved in the cell binding of many ECM proteins (Ruoslahti and Pierschbacher, 1987; GRGDS, 500 μg/ml; Penninsula Laboratories, Belmont, CA) was added. Blastocysts were allowed to attach for 2 h, then antibodies or peptides were added to the culture medium to test their effects on outgrowth. Previous studies in our laboratories using ^{125}I-labeled TSP and its fragments indicated that the intact molecule and the 140-kD fragment bound in equimolar amounts to tissue culture plastic. The isolated 25-kD HBD bound in 20-fold molar excess likely reflecting the hydrophobic nature of this domain (Dixit et al., (1984).

Data Acquisition and Analysis. Photomicrographs were taken of blastocysts after 24, 48, and 72 h in vitro from at least 25 embryos per substrate. Outgrowth area was measured from resulting photographic prints using a digitizing tablet interfaced with a microcomputer and data analyzed using the z test (Guilford and Fruchter, 1973). For ease in interpretation and to control for slight asynchronies in development, data have been expressed as mean percent increase in outgrowth after 48 and 72 h in vitro (Fig. 6). Embryos were excluded from analysis if their outgrowth touched that of another embryo or the edge of the culture well, or if the embryo detached from the substratum.

Results

Immunocytochemistry

TSP was diffusely present in the cytoplasm of unovulated oocytes (Fig. 1, *A* and *B*), and in ovulated, unfertilized eggs. With fertilization, TSP remained present diffusely within the cytoplasm of the zygote, most densely in cell bodies and in the perinuclear cytoplasm (Fig. 1, *C* and *D*). During early cleavage stages, this pattern did not alter; some TSP was present on the surface of two-, four-, and eight-cell embryos and was diffusely present within the cytoplasm during these early stages (Fig. 1, *E–H*, Fig. 2, *A* and *B*). Fortuitous focal planes also indicated some intracellular packaging of TSP into vesicular structures (Fig. 1, *F* and *H*), and enrichment in regions of blastomere contact.

With compaction, the pattern changed somewhat. Although TSP remained present within the cytoplasm, it was most densely deposited on cell surfaces, particularly in regions of contact between individual blastomeres (Fig. 2). In the early blastocyst, TSP was present on cell surfaces of both trophectoderm as well as cells of the inner cell mass (ICM); however, the ICM contained more cytoplasmic TSP than trophectoderm cells (Fig. 2, *C–F*). With hatching of the blastocyst through the zona pellucida, there was an increase in TSP near the region emerging from the zona pellucida (Fig. 2, *G*, and *H*). The zona pellucida and blastocele were uniformly negative for TSP. In unfixed embryos, the pattern was somewhat similar to that seen in fixed embryos. TSP was present along the surface in patchy deposits (Fig. 3, *A* and *B*). Even in nonpermeabilized embryos, through focus observation indicated that there was slight cytoplasmic staining
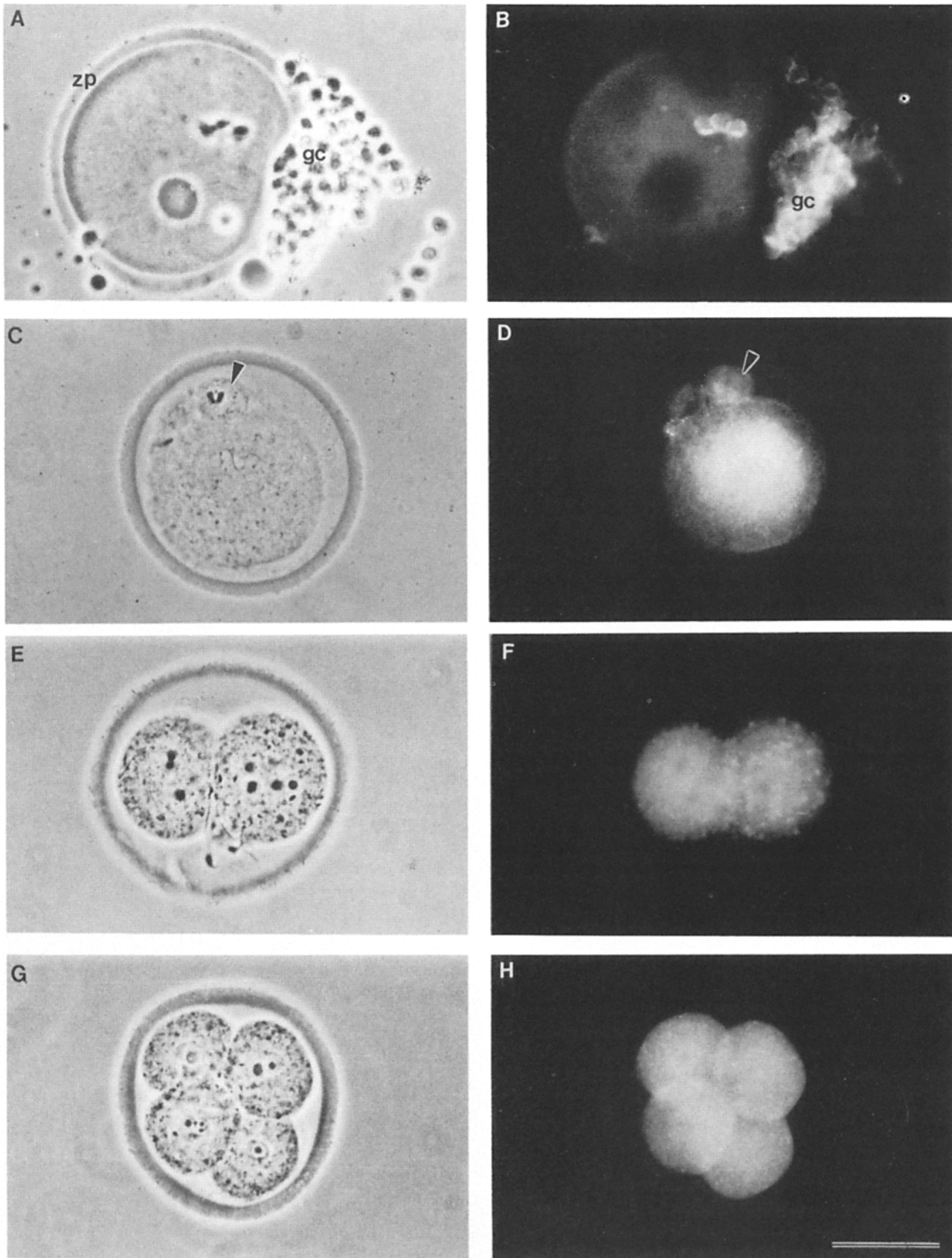
Figure 1. Immunocytochemical localization of TSP in unfertilized (*B*), and fertilized eggs (*D*), and early cleavage staged embryos (*F* and *H*). (*A, C, E,* and *G*) Corresponding phase-contrast micrographs. Bars, 100 μm. (*A* and *B*) There was consistent TSP immunoreactivity in unfertilized eggs, particularly in the perinuclear cytoplasm. Adherent granulosa cells (*gc*) stained intensely for TSP. *zp*, zona pellucida. (*C* and *D*) In the zygote, TSP was diffusely distributed in the cytoplasm, with immunoreactivity also present in the second polar body (*arrowhead*). (*E* and *F*) The two-cell embryo similarly exhibited diffuse cytoplasmic staining for TSP, although there was some vesicular TSP within the cytoplasm. (*G* and *H*) Four-cell embryo illustrating the diffuse intracellular deposition of TSP.
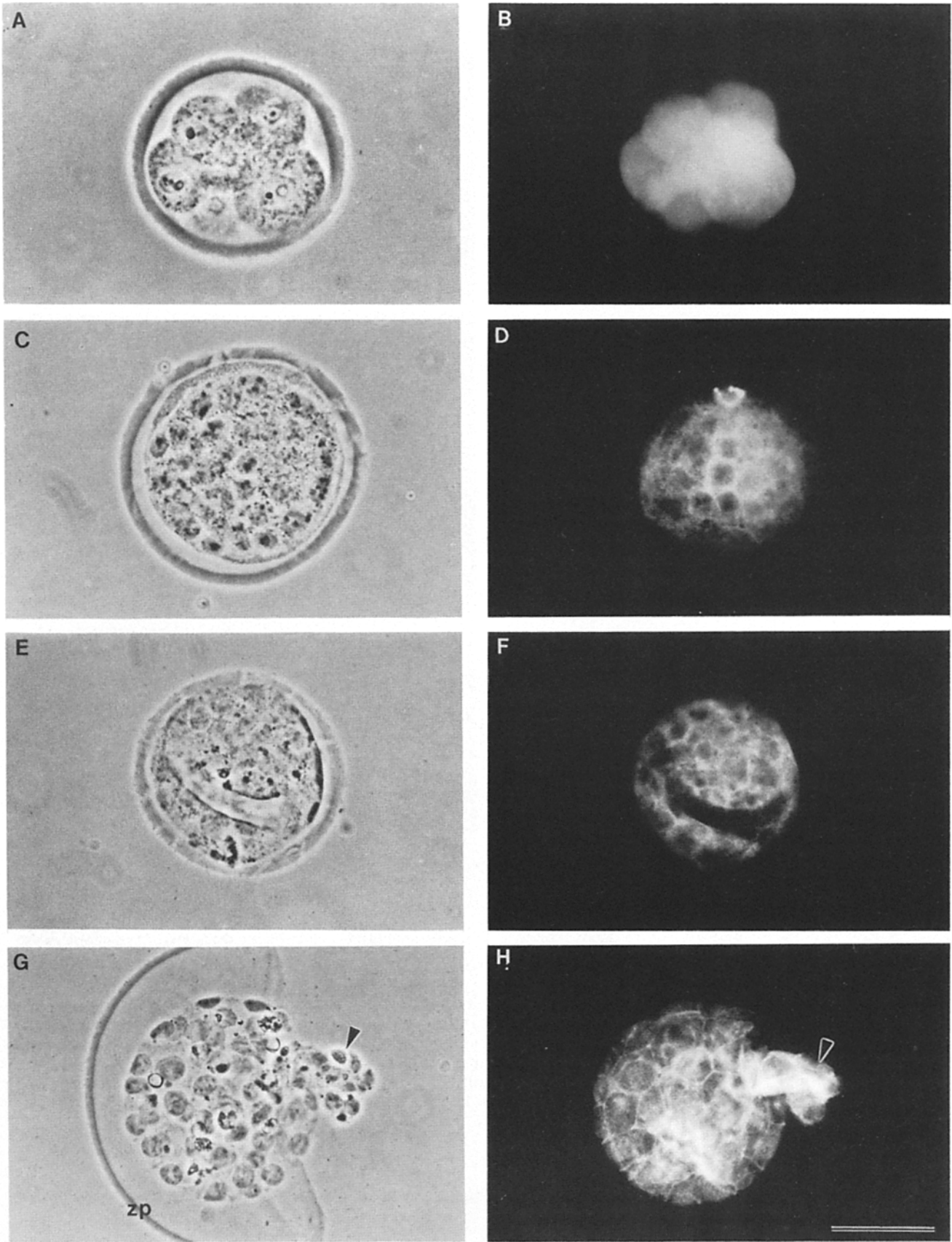
Figure 2. Localization of TSP in later cleavage staged embryos from eight-cell (*B*) to compacted morula (*D*), to early blastocyst (*F*) to hatching blastocyst (*H*) stages of development. *A, C, E,* and *G* are companion phase-contrast micrographs for comparison of structures seen using immunocytochemistry (*B, D, F,* and *H*). Bars 100 μm. (*A* and *B*) TSP was present throughout the cytoplasm of eight-cell embryos. (*C* and *D*) Compacted morula illustrating the localization of TSP within the cytoplasm and particularly on the cell surface and
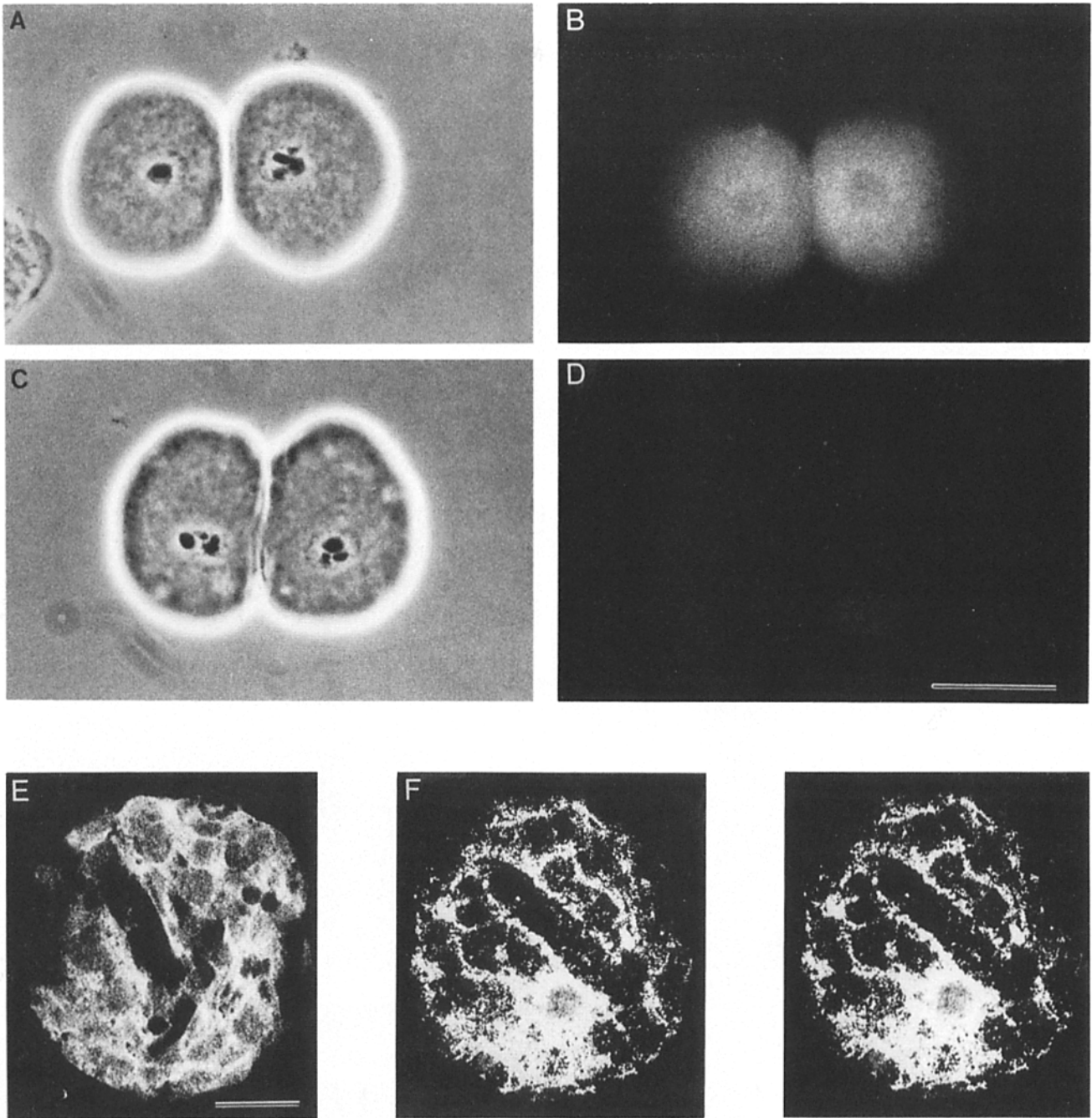
Figure 3. Localization of TSP in unfixed, unpermeabilized embryos (*A* and *B*), a control embryo exposed to normal rabbit serum in place of the primary antibody (*C* and *D*), and images produced using confocal microscopy to further illustrate the deposition of TSP in the blastocyst. (*E* and *F*). Bars, 50 μm. (*A* and *B*) TSP was present in a patchy distribution on the surface of unfixed embryos not exposed to Triton X-100. *A* illustrates the phase-contrast image of the two-cell embryo. (*C* and *D*) A two-cell embryo exposed to normal rabbit serum in place of the primary antibody, exhibited no fluorescence. (*E*) Optical section through a fixed, permeabilized early blastocyst taken using confocal microscopy. TSP is present within the blastomeres and is particularly enriched on their surfaces. (*F*) Stereopair of the blastocyst illustrated in *E*, indicating the very dense deposition of TSP on the cell surface.

between individual blastomeres. The inner cell mass, below the focal plane in this and the following illustrations, contained considerable TSP. (*E* and *F*) Early blastocyst in which the forming blastocoele has partially collapsed due to processing. TSP is present on the surface of trophectoderm, and is particularly densely deposited in the inner cell mass. (*G* and *H*) As the blastocyst begins to hatch, cells emerging from the zona pellucida (*zp*) are particularly enriched in TSP, although the cytoplasmic distribution and deposition between cells persisted. Although it is out of the focal plane, the inner cell mass stained intensely for TSP.
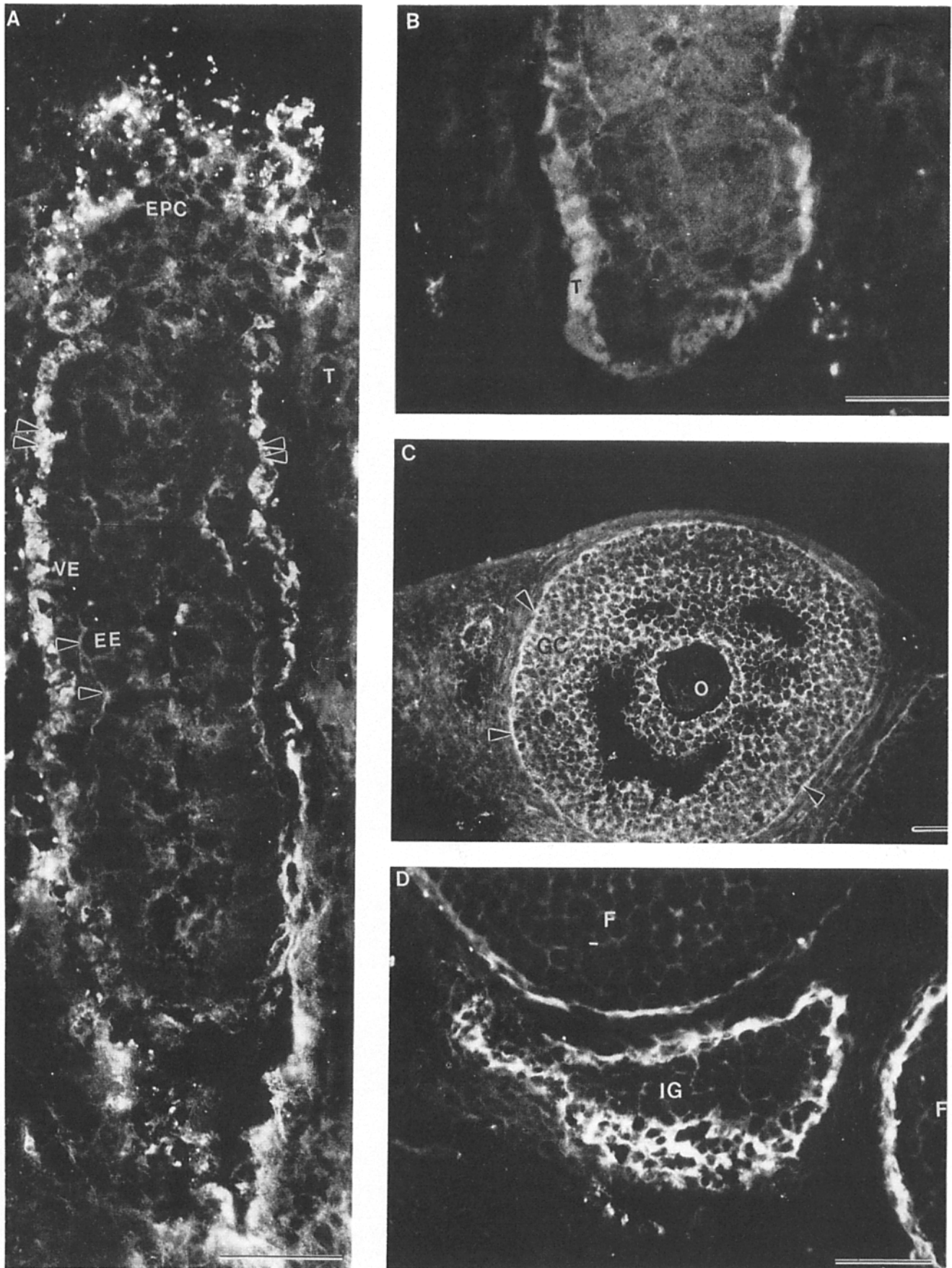
Figure 4. Immunocytochemical localization of TSP in day 6.5 egg cylinder (*A* and *B*), in a developing follicle (*C*), and an interstitial gland (*D*). Bars, 50 μm. (*A*) Transverse section through a late egg cylinder staged embryo illustrating the deposition of TSP in the various cell layers. TSP was present in the embryonic ectoderm (*EE*), in the visceral endoderm (*VE*) and particularly in the basement membrane separating these layers (*arrowheads*). It was present in the ectoplacental cone region (*EPC*) and in the maternal blood bathing this region. TSP was densely deposited in the Reichert's membrane (*double arrowheads*), and surrounding the trophoblast (*T*) cells. TSP was also present in the decidualizing endometrium. (*B*) Slightly higher magnification view of the base of a slightly older embryo illustrating the dense deposition of TSP in the trophoblast (*T*). (*C*) Section through a developing (secondary) follicle illustrating the depositional pattern
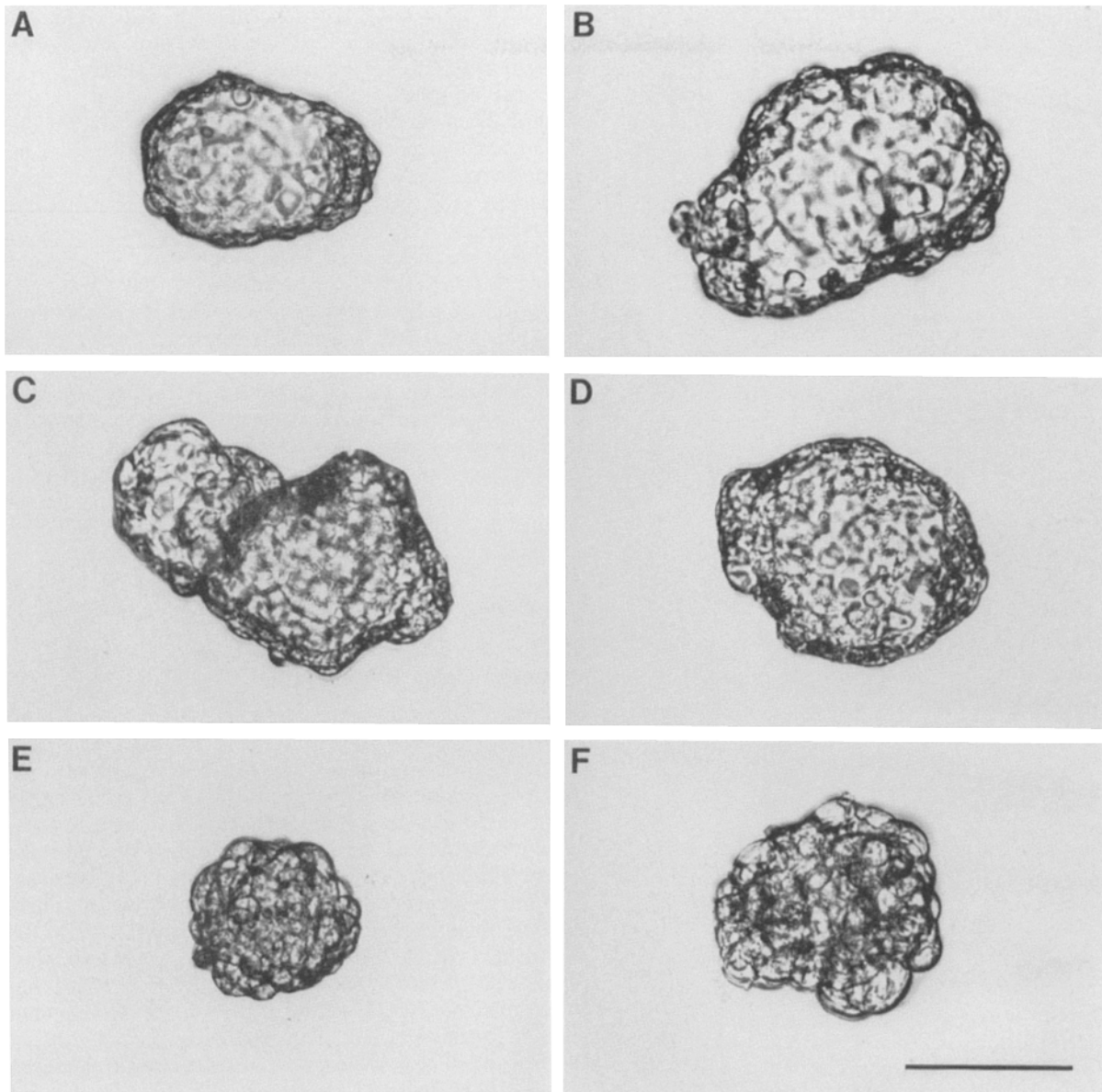
Figure 5. Phase-contrast micrographs of blastocysts grown on various ECM substrates after 24 h (*A*), or 48 h in vitro (*B–F*). Bar, 100 μm. (*A*) Blastocyst cultured on TSP (10 μg/ml) for 24 h, just beginning trophoblast outgrowth. (*B*) Appearance of a blastocyst grown on 25 μg/ml laminin after 48 h in vitro. (*C*) Outgrowth on TSP after 48 h in vitro was more extensive. (*D*) There was less outgrowth on the 140-kD fragment (25 μg/ml) than on the intact molecule after 48 h. (*E*) Addition of anti-TSP antibodies (50 μg/ml) after 2 h to embryos growing on TSP (10 μg/ml) significantly inhibited outgrowth. Embryos were often collapsed as well. (*F*) Addition of GRGDS (500 μg/ml) after 2 h to blastocysts grown on TSP (10 μg/ml) resulted in outgrowth, but also a collapse of the expanded blastocyst itself.

in these preparations as well. Control embryos and sections (fixed and unfixed), whether exposed to PBS, normal rabbit serum, or to anti-TSP antibody previously incubated with TSP, were uniformly negative for staining. Fig. 3, *C* and *D* illustrate this lack of staining in an embryo that was exposed to normal rabbit serum in place of the primary antibody.

To study the distribution of TSP in more detail, additional fixed, permeabilized embryos were also examined using confocal microscopy. Although providing greater resolution and detail, these observations confirmed those made using routine fluorescence microscopy and are presented here to illustrate that pattern in the absence of the somewhat confus-

of TSP in this region. There was little TSP present in the oocyte (*O*), none in the zona pellucida, but there was considerable TSP present on the surface of stratified granulosa cells (*GC*). Antral fluid was negative for TSP; the basement membrane separating the granulosa cells from the theca lutein layers (*arrowhead*) was heavily stained. There was some TSP also present within the ovarian stroma. (*D*) Section through the edge of two developing follicles (*F*), and an interstitial gland (*IG*) illustrating the dense deposition of TSP at the margin of the gland.

BLASTOCYST OUTGROWTH
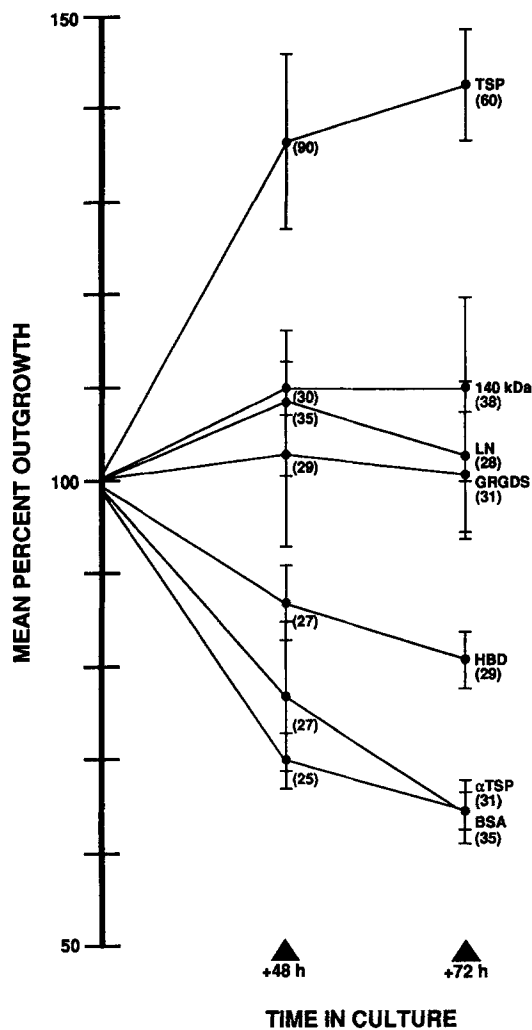
Figure 6. Outgrowth of blastocysts on various substrates. Area at 24 h was considered to be the initiation of outgrowth (100%). Data are expressed as mean percent increase in outgrowth ± SEM. The intact TSP molecule was most efficient in promoting trophoblast outgrowth; laminin (*LN*) and the 140-kD fragment were also good substrates for outgrowth; the HBD was significantly less effective. Addition of anti-TSP antibodies to blastocysts growing on TSP significantly inhibited trophoblast outgrowth; addition of GRGDS had little effect. Blastocysts that were able to attach initially to BSA did not undergo any additional outgrowth. $LN = 25$ μg/ml laminin; $TSP = 10$ μg/ml TSP; *140 kD* $= 25$ μg/ml 140-kD fragment COOH terminus of the TSP molecule; $HBD = 25$ μg/ml fragment containing the amino terminus heparin binding domain of the molecule; $GRGDS$ = blastocysts were allowed to attach to 10 μg/ml TSP coated substrates then GRGDS added after 2 h to a final concentration of 500 μg/ml; αTSP = blastocysts were allowed to attach to 10 μg/ml TSP for 2 h, then anti-TSP IgG was added to a final concentration of 50 μg/ml; $BSA = 25$ ug/ml BSA. Bracketed numbers indicate number of embryos examined. Significance, determined using z test at 48 h, was TSP > 140, LN, GRGDS > HBD, αTSP, BSA, $P \leqslant 0.001$. At 72 h, TSP > 140, LN, GRGDS > HBD, > αTSP, BSA, $P \leqslant 0.001$.

ing fluorescein spread. Fig. 3 *E* is a confocal image of one optical section taken through a region containing a portion of the blastocele, illustrating the deposition of TSP within cells and particularly on the surface of these embryos. A

stereopair illustrating the deposition of TSP within the blastocyst is also included to illustrate the three dimensional deposition of TSP as visualized in the absence of FITC spread (Fig. 3 *F*).

Thrombospondin was found in a number of tissues within the implanting embryo on day 6.5 of gestation (Fig. 4, *A* and *B*). It was present within the embryonic and extraembryonic ectoderm, there was staining in the basement membrane separating the visceral endoderm from the ectoderm, the Reichert's membrane, and in the trophoblast. The decidualizing endometrium also contained some TSP.

The follicle contained considerable TSP. In growing follicles (Fig. 4 *C*), TSP was densely deposited on the surface of granulosa cells, there was diffuse TSP in the oocyte, the zona pellucida and antrum contained no TSP immunoreactivity. TSP was particularly densely deposited in the basement membrane separating the granulosa cells from the theca interna, and was found in the ovarian stroma (Fig. 4 *C*). After ovulation, the basement membrane separating granulosa and theca interna contained considerable TSP, as did the endothelium of blood vessels. After involution of the follicle, the remnant interstial glands (Fig. 4 *D*) and basement membrane (glassy membrane) were particularly enriched in TSP.

Blastocyst Outgrowth Assay

Blastocysts grown in matrix-coated 24-well plates attached and an outgrowth of trophoblast cells formed over a 72-h period. Blastocysts attached initially, but outgrowth in serum-free medium typically began after a somewhat quiescent period of 18–24 h (Fig. 5 *A*). The 24-h timepoint was therefore considered the initiation of outgrowth and measurements taken at consecutive 24-h intervals. Growth in serum free medium is nonideal, and outgrowth was considerably less than when similar blastocysts were grown in the presence of serum (10%). The morphology of trophoblast cells is also affected by serum components; trophoblasts become much more highly spread than in the defined medium required for antibody inhibition experiments, although using Hoffman illumination, flattened, characteristic trophoblast cells can be visualized.

Blastocysts attached rapidly on laminin coated substrates and there was extensive outgrowth after 48 h (Fig. 5 *B*) increasing with an additional 24-h culture (Fig. 6). Outgrowth of trophoblast cells on TSP was rapid and even more extensive (Fig. 5 *C*, Fig. 6); the 140-kD fragment produced slightly less avid outgrowth compared with the intact molecule (Fig. 5 *D*, Fig. 6). Addition of anti-TSP antibodies (50 μg/ml) to blastocysts growing on 10 μg/ml TSP significantly inhibited outgrowth (Fig. 5 *E*, Fig. 6). In addition, with increasing time in culture, many of these embryos detached from the substrate. Addition of a vast molar excess of the synthetic peptide GRGDS had a variable effect on outgrowth (Fig. 5 *F*, Fig. 6). Blastocysts remained attached to the TSP substrate, but often appeared collapsed (Fig. 4 *F*). There was no effect of adding the "inactive" peptide GRGES (500 μg/ml) on outgrowth of similar embryos (not illustrated). Attachment and subsequent outgrowth of blastocysts on the HBD or on BSA was poor (Fig. 6).

Discussion

Although TSP has previously been reported to be present

during postimplantation stages of development (O'Shea and Dixit, 1988) and in a limited distribution in adult tissues (Wight et al., 1985), this is the first report of its presence during early embryogenesis. Relatively few ECM or cell surface components have been localized during these early cleavage stages. Laminin (Dziadek and Timpl, 1985) and heparan sulfate proteoglycan (Dziadek et al., 1985) appear as early as the 2-cell stage, with nidogen appearing in the 8- to 16-cell compacted morula. Both fibronectin (Wartiovaara et al., 1979) and type IV collagen (Leivo et al., 1980) were first observed between cells of the ICM in the late blastocyst. Unlike the broad deposition of TSP, these matrix components appeared initially in patchy distribution on the cell surface, but like TSP, laminin was enriched in areas of contact between blastomeres (Leivo et al., 1980; Wu et al., 1983).

Of the cell adhesion glycoproteins, uvomorulin has been studied extensively during early cleavage and implantation periods of development. It is present on the surface of unfertilized and fertilized eggs, but is not synthesized until the two-cell stage. Its uniform surface distribution changes with compaction to an enrichment at basolateral surfaces of individual blastomeres (Vestweber et al., 1987), then is gradually lost from trophectoderm as it is transformed into trophoblast (Damjanov et al., 1986; Vestweber et al., 1987). Uvomorulin plays a critical role in compaction (Damsky et al., 1983; Hyafil et al., 1983) as well as in segregation of endoderm from ICM (Richa et al., 1985); then it is progressively restricted to cells of epithelial organization (Damjanov et al., 1986).

In the egg cylinder, TSP was present in the embryonic ectoderm and endoderm, and was particularly enriched in the basement membrane separating them. Laminin (Leivo et al., 1980) is similarly present in both the endoderm and ectoderm, while type IV collagen (Leivo et al., 1980) and fibronectin (Wartiovaara et al., 1979) were present in endoderm but not ectoderm. Like the above components, heparan sulfate proteoglycan (Dziadek et al., 1985) and entactin were densely deposited in the basement membrane between ectoderm and endoderm and particularly in the Reichert's membrane (Wu et al., 1983). The precise roles of TSP in both cleavage stages as well as in the egg cylinder remain to be determined.

Hatched blastocysts, after a period of quiescence, are capable of attaching to and forming extensive trophoblast outgrowth on a number of ECM substrates; this paradigm has been used as a model of implantation (see Enders et al., 1981). Substrate adsorbed fibronectin, laminin, types I and IV collagen (Sutherland et al., 1988; Armant et al., 1986a; Wilson and Jenkinson, 1974) have been previously shown to support blastocyst attachment and trophoblast outgrowth. Outgrowth, but not attachment, on fibronectin and laminin substrates was inhibited by addition of RGD-containing peptides (Sutherland et al., 1988). Addition of heparin to blastocysts growing on laminin or fibronectin or on uterine epithelial cells affected both outgrowth and attachment to a much greater extent than RGD peptides, suggesting an important role for heparin/heparan sulfate containing molecules in this process (Farach et al., 1987).

In the current investigation, the HBD of the TSP molecule supported very limited blastocyst attachment and outgrowth. TSP is rapidly incorporated into the matrix via the HBD (McKeown-Longo et al., 1984), then it is rapidly cleaved from molecule, producing the 140-kD fragment (Roberts et al., 1987; Prochownik et al., 1989). It is of considerable interest therefore that the intact molecule produced significantly greater outgrowth than the 140-kD fragment. This particular aspect of TSP interaction with trophoblast cells varies from observations of neurite outgrowth on these fragments, in which the HBD was neither adhesive nor neurite promoting, while the 140-kD fragment supported both to a greater extent than the intact molecule (O'Shea, K. S., L. H. J. Liu, and V. M. Dixit. 1989. Soc. Neurosci. Abstr. 15:876a). These observations suggest a considerable degree of specificity in cell–TSP interactions.

The inability of the GRGDS peptide to inhibit trophoblast outgrowth on TSP substrates is consistent with results in certain cell systems (Tuszynski et al., 1987; Varani et al., 1988; O'Shea, K. S., L.-H. J. Liu, and V. M. Dixit. 1989. Soc. Neurosci. Abstr. 15:876a) but not others (Lawler et al., 1988). The mechanisms by which TSP promotes trophoblast outgrowth may reflect both its known adhesive role (Varani et al., 1986; Roberts et al., 1987; Tuszynski et al., 1987) as well as its role in cell attachment/deattachment (Vischer et al., 1988; Murphy-Ullrich and Hook, 1989) and migration of cells (Majack et al., 1986; Taraboletti et al., 1987; O'Shea et al., 1990). The latter is likely mediated by the increased plasmin production after TSP binding to plasminogen and both urokinase-type plasminogen activator (u-PA) and to tissue plasminogen activator (t-PA) (Silverstein et al., 1984, 1985; Silverstein and Nachman, 1987), thereby creating a local nidus of protease activity for directed matrix proteolysis and protection from inactivation by matrix protease inhibitors.

Proteases are similarly present during pre-ovulation oocyte development; t-PA in oocytes (Huarte et al., 1985, 1987; Sappino et al., 1989), t-PA and u-PA in granulosa and theca cells (Liu et al., 1987; Sappino et al., 1989) in a pattern strikingly similar to that of TSP. Interestingly, interstitial glands also contained considerable t-PA (Liu et al., 1987), in the current investigation, both interstitial glands and glassy membranes had dense deposits of TSP. A likely role for proteases in follicular development may relate to breakdown of antral proteoglycan before ovulation, to prevent clot formation after ovulation (Saksela and Rifkin, 1988), disruption of the follicle wall (Beers, 1975), and involution of the follicle. The role of proteases during cleavage stage development is unclear. Liedholm and Astedt (1975) have suggested a possible function in inhibiting ectopic (oviduct) implantation; it may also be that cell adhesion must be modulated during cleavage.

The role of matrix and protease in producing the sequential adhesion and deadhesions for trophoblast invasion is more obvious. TSP was densely deposited in trophectoderm, and trophectoderm derivatives, ectoplacental cone and invading trophoblast giant cells. Of the ECM components, only fibronectin (Wartiovaara et al., 1979) was present on trophoblast giant cells with implantation, although receptors for matrix proteins are present on implanting, but not cells of newly hatched trophoblast (Sutherland et al., 1988). Trophoblast cells also produce a number of proteases (Strickland, 1976; Queenan et al., 1987), some in a strikingly developmentally regulated manner (Fisher et al., 1989; Sappino et al., 1989). Inhibition studies have demonstrated that attachment of mouse embryos requires a trypsin-like activity, whereas trophoblast outgrowth requires both

trypsin-like and plasminogen activator activity (Kubo et al., 1981). Although a more detailed study of the deposition of TSP in trophoblast remains to be carried out, its presence in both trophectoderm and trophoblast, as well as the ability of TSP to support trophoblast outgrowth observed in the current investigation suggest that TSP may also play an important role in implantation.

The unique pattern of deposition of TSP in preovulatory follicles through egg cylinder stages of development suggests a multitude of possible roles for this important matrix component, the majority of which remain to be determined. Its presence emphasizes the highly conservative nature of early embryonic development, particularly the multiplicity of overlapping adhesion and deadhesion systems involved in the establishment of the adult, differentiated form.

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