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Rapid Communication

Is Oncofetal Fibronectin a Trophoblast Glue for Human Implantation?

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Using an antibody probe specific for the class of fibronectins that contain the oncofetal domain, it was shown that oncofetal fibronectin (onfFN) is present wherever trophoblasts make contact with extraplacental extracellular matrix (ECM). In normal human implantation sites, onfFN was localized to a highly specific region—the ECM connecting extravillous trophoblasts and trophoblastic cell columns to the uterine decidua. This same zone of onfFN was present in an analogous location in extrauterine gestations. Like these in vivo ***extravillous trophoblasts, isolated cytotrophoblasts in primary culture synthesized and secreted onfFN as they underwent differentiation. Furthermore, when cocultured with an ECM gel, cytotrophoblast aggregates deposited onfFN at cell–ECM contact sites, resembling early implanting trophoblasts*** in vivo. ***In the presence of cyclic AMP agonists, onfFN synthesis was inhibited markedly. It is concluded from these results that onfFN is a trophoblast protein that, under cAMP regulation, could mediate implantation and placental–uterine attachment throughout gestation. (Am J Pathol 1991, 138:537–543)***

The precise biochemical mediators of human implantation are unknown. On a cellular level, the trophoblast shell of the early embryo establishes initial contact with the endometrial lining.[1] Subsequently an expanding zone of trophoblast adherence to the extracellular matrix (ECM) establishes the uteroplacental junction. Previous studies examining trophoblast behavior *in vitro*[2,3] suggested a significant role for fibronectin in modulating trophoblast contact, spreading, and syncytial formation. However more specific information about the significance of fibronectin in trophoblast–uterine interactions *in vivo* is not known.

Fibronectin molecules bearing a unique glycopeptide domain within the type III connecting segment (IIICS), defined as the oncofetal fibronectin (onfFN) class, recently were described in human tumors and pregnancy tissues.[4–7] To understand the biologic significance of these fibronectins in human gestation, we used the monoclonal antibody FDC-6[4–6] as a specific probe for onfFN immunohistochemical localization and immunoblot analyses. The unusual features of FDC-6 are the antibody's high specificity to an O-linked glycosylated hexapeptide within IIICS, negative reactivity with normal adult plasma fibronectins, and requirement for both the sugar and peptide moieties within the epitope binding site.

Unlike normal adult plasma and cellular fibronectins, we found that FDC-6–reactive fibronectins are specifically present wherever trophoblasts make contact with the ECM. Furthermore, by comparing the immunohistochemical staining of human implantation sites *in vivo* with trophoblast behavior *in vitro*, we found remarkable similarities in the way trophoblasts interact with the ECM and synthesize onfFN.

Materials and Methods

Immunohistochemical Studies

Five-micrometer–thick sections from Bouin's-fixed and paraffin-embedded tissue were placed on glass slides

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previously coated with a film of 1% poly-d-lysine, 30,000 to 70,000 molecular weight (Sigma Chemical Co., St. Louis, MO), dried at temperatures no greater than 60°C, and stored at room temperature until used. Immunoperoxidase staining was carried out as described previously.[8] Monoclonal antibody FDC-6 (gift from Adeza Biomedical, Sunnyvale, CA), was used at a concentration of 4 μg/ml. Control slides were incubated with undiluted ATCC P3X63Ag8 mouse myeloma cell line supernatant.

Immunoblotting

Human cytotrophoblasts were purified from the placentae of uncomplicated term pregnancies immediately after delivery or elective pregnancy termination by serial trypsin-DNase digestions followed by Percoll gradient centrifugation, as previously described by Kliman et al.[8] For first-trimester placentae, the digest times were reduced to 10 minutes. The cytotrophoblasts were cultured in Dulbecco's Modified Eagles' Medium (DMEM) containing 25 mmol/l (millimolar) glucose and 25 mmol/l HEPES (DMEM-HG) supplemented with gentamicin (50 μg/ml), glutamine (4 mmol/l), and 20% (v/v) heat-inactivated fetal calf serum. For preparation of cell extracts, cells were washed with phosphate-buffered saline (PBS), pH 7.4, scraped from the culture dish, and total cellular protein was extracted with a cell lysis buffer containing PBS, 1.0% sodium cholate, 0.1% sodium dodecyl sulfate (SDS), and 0.02% sodium azide. One hundred micrograms of total trophoblast cellular protein and 100 μl of unconcentrated conditioned media were electrophoresed on a 6% SDS-polyacrylamide gel under reducing conditions. The gels were electrotransferred to nitrocellulose (Schleicher and Schull, Keene, NH) overnight, incubated with FDC-6 as primary antibody (8 μg/ml), with immunodetection using a biotinylated anti-mouse secondary antibody (ABC Vectastain, Vector Labs, Burlingame, CA). This immunoblot method detects as little as 50 ng of intact onfFN subunit, with a molecular weight of approximately 250 kd.

Trophoblast–Matrigel Suspension Coculture

Ice-cold Matrigel™ (Collaborative Research, Bedford, MA) was applied to a sterile petri dish, the dish was tilted to spread the solution out, and then placed into a humid 37°C incubator for 1 hour to promote gelling. The ECM gel was minced into 3- to 5-mm cubes with a sterile scalpel and individual pieces were placed into 1 ml of a 1×10^6 cells/ml suspension of cytotrophoblast and cocultured as previously described.[9] After 48 hours of suspension culture, the Matrigel fragments were fixed in Bouin's solution and processed for immunohistochemistry as described above.

Results

Oncofetal Fibronectin Immunohistochemistry

When pregnancy tissues from previously fixed specimens were analyzed immunohistochemically for FDC-6 reactivity, a consistent staining pattern was found. As shown in a histologic section from an intact 16-week implantation site, specific and intense staining for onfFN was noted within the attachment zone of the placental–uterine junction (Figure 1a). At higher magnification (Figure 1b), it can be seen that this dark staining is localized to the ECM connecting extravillous anchoring trophoblasts and trophoblast cell columns to the uterus. Both placental villi and uterine tissue remote from the implantation site were negative for onfFN. We observed this specificity of staining in different placental attachment sites across multiple gestational ages, ranging from 20 days after conception to term. An analogous zone of onfFN was present within the ECM of anchoring trophoblasts from pregnancies that implanted outside the uterus, including gestations within the fallopian tube (Figure 1c), ovary, and cervix. These results clearly link onfFN deposition in the ECM with implanting trophoblasts, whether intra- or extrauterine in location. In one unusual specimen from a uterine curettage collected during a cycle of conception (postfertilization day 20) (Figure 1d), cytotrophoblasts at the edge of the trophoblastic shell contained intracytoplasmic- and membrane-associated onfFN, indicating very early trophoblast production of this implantation-site protein. OnfFN also was prominent within the ECM of the chorionic membrane at the chorionic-decidual junction (not shown), an expected finding because the chorion is actually the remnant of the trophoblastic shell that did not form a placenta and only contains extravillous trophoblasts attached to maternal decidual stroma.

Oncofetal Fibronectin Secretion by Cultured Trophoblasts

Although onfFN appears to be synthesized by anchoring trophoblasts *in vivo*, we wished to analyze directly human trophoblast fibronectin and confirm the presence of the oncofetal domain. Therefore primary cultures of human trophoblasts were used as an *in vitro* model to determine if these cells both synthesize and deposit onfFN at sites of trophoblast–ECM contact. Trophoblasts isolated from

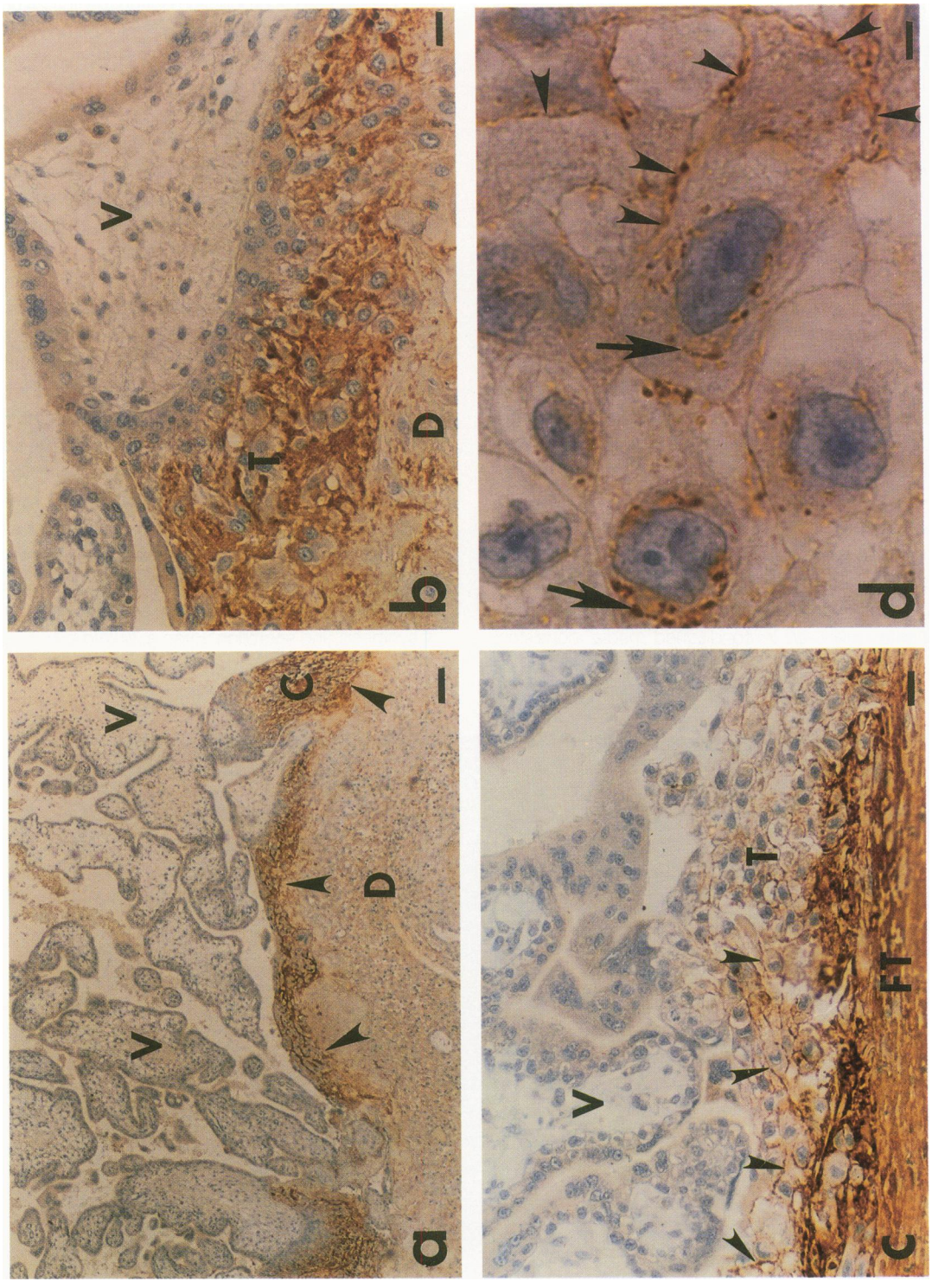

Figure 1. *Oncofetal fibronectin (onfFN) immunohistochemistry.* **a**: *Uteroplacental junction from a 16-week gestation exhibits distinct band of onfFN staining (arrow heads) at zone of contact between extravillous trophoblasts and decidualized endometrium (D). Note positively staining cell column (C) emanating from the negatively stained chorionic villi (V).* **b**: *Higher power of same uteroplacental junction reveals that the onfFN staining is largely extracellular around the extravillous trophoblasts (T). Decidua (D), villous (V).* **c**: *A distinct band of onfFN staining can be seen at the junction of the extravillous trophoblasts (T) and fallopian tube (FT) in a tubal pregnancy. The extravillous trophoblasts nearest the junction have a heavy ECM deposit of onfFN, while the trophoblasts farther away from the trophotubal junction appear to have a delicate membrane-stained pattern (arrow heads). Note the negatively stained cytotrophoblasts and syncytiotrophoblasts of the chorionic villi (V).* **d**: *High-power view from the edge of the trophoblastic shell of a 20-day postconception gestation. Note the intracytoplasmic, perinuclear onfFN staining (arrows) and the delicate intercellular-membrane staining (arrow heads). Bars represent 100 μm* (**a**), *20 μm* (**b,c**), *and 5 μm* (**d**).

term placentae, while initially appearing as undifferentiated mononuclear cytotrophoblasts, undergo profound morphologic and biochemical changes, including formation of true syncytia and *de novo* synthesis of human chorionic gonadotropin (hCG), human placental lactogen, and progesterone.[8,10] In addition, we previously demonstrated that these trophoblasts attach to, penetrate, and degrade the ECM in an *in vitro* assay of cellular–ECM interactions.[2]

Freshly purified placental villous cytotrophoblasts contained barely detectable onfFN (less than 50 ng/mg cell protein), in accord with the negative villous staining for onfFN in the placenta. A small quantity of onfFN was present intracellularly after 24 hours (125 ng/mg cell protein), suggesting that onfFN synthesis had been initiated by the cultured cells. After 96 hours, trophoblasts contained 18 times more onfFN (2200 ng/mg cell protein) than the 24-hour cells, representing 0.2% of total trophoblast intracellular protein (Figure 2a). Thus villous cytotrophoblasts, while not containing significant onfFN *in vivo*, were induced in culture to produce significant onfFN. We also determined that these cultured trophoblasts secreted onfFN (Figure 2b). In the first 24 hours, very little onfFN was measured in conditioned media, consistent with the low level of production during the first 24 hours of culture. However, during the time interval from 24 to 48 hours the media concentration of onfFN averaged 4.5 μg/ml, demonstrating that trophoblasts secreted abundant amounts of newly synthesized onfFN. onfFN continued to be present until 120 hours when the cultures were terminated. We also assayed by immunoblot the ability of first-trimester cytotrophoblasts to synthesize and secrete onfFN *de novo*. In an identical pattern to term cytotrophoblasts, we found that onfFN production was predominantly induced between 24 and 48 hours of culture. Based on sensitive enzyme-linked immunoassays using FDC-6 and a polyclonal anti-plasma fibronectin antibody in parallel, close to 100% of trophoblast secreted fibronectin was found to contain the oncofetal domain.

Cyclic AMP agonists are major stimulators of trophoblast hCG synthesis and secretion *in vitro*.[10] The addition of 1.5 mmol/l 8-bromo-cAMP to trophoblast cultures, a concentration that maximally stimulates hCG synthesis, resulted in almost complete inhibition of cellular and secreted onfFN at all time points examined. Treatment of freshly isolated cytotrophoblasts for only 6 hours with 1.5 mmol/l 8-bromo-cAMP resulted in an equivalent inhibition of synthesis and secretion. Even a 30-fold lower concen-

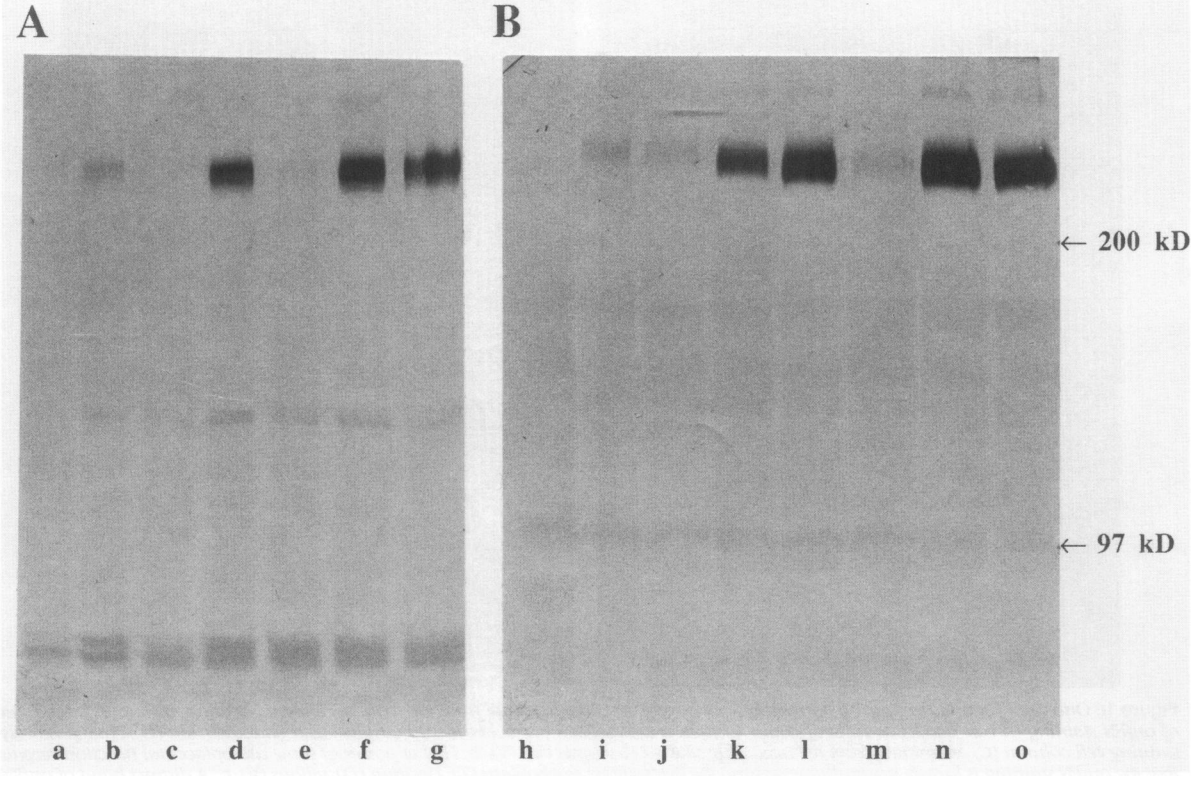

Figure 2. *Immunoblots of trophoblast cell extract and conditioned media for oncofetal fibronectin.* **A**: *Trophoblast cell extract contains barely detectable onfFN at time zero (lane a), with some synthesis initiated after 24 hours in culture (lane b). Significantly more onfFN was present in the cell extracts after 48, 72, and 96 hours (lanes d, f, g), whereas 1.5 mmol/l cAMP significantly inhibited trophoblast onfFN synthesis (lanes c, e).* **B**: *Trophoblast-conditioned media mirrored the cell extract for onfFN content. No onfFN was detected in control media containing 20% fetal calf serum (lane h), but a faint onfFN signal was present in media from 24-hour cells (lane i). Significant onfFN secretion was noted at 36, 48, 72, and 96 hours (lanes k, l, n, o) but not in the presence of 1.5 mmol/l 8-bromo-cAMP (lane m).*

tration of 8-bromo-cAMP (0.05 mmol/l) resulted in 20-fold inhibition of onfFN after 48 hours and 30-fold reduction after 72 hours. Forskolin (100 μmol/l [micromolar]) also inhibited onfFN production. No regulatory effect on onfFN synthesis was noted with 1.5 mmol/l 8-bromo-cyclic GMP or 155 nmol/l (nanomolar) of the phorbol ester 12-O-tetradecanoyl-phorbol 13-acetate, suggesting specificity of the cAMP response. Immunocytochemical staining of fixed cultured trophoblasts after 48 hours demonstrated onfFN within the cytoplasm and on the cell surface, whereas those cells treated with 1.5 mmol/l 8-bromo-cAMP contained no detectable onfFN. Inhibition of onfFN synthesis by cAMP agonists may explain previous observations that 8-bromo-cAMP abolishes trophoblast spreading on and degradation of Matrigel™.[2] Transcriptional down regulation by cAMP agonists is likely to be one important mechanism for onfFN modulation.[11]

In Vitro *Model for Implantation*

Trophoblasts cultured in suspension form aggregates.[9] When trophoblasts were cocultured with fragments of Matrigel,™ multiple aggregates attached and burrowed into the matrix (Figure 3a). Immunohistochemical analysis of these sections with FDC-6 revealed a specific band of onfFN at the trophoblast–ECM interface, resembling the staining pattern seen at the edge of the early trophoblastic shell specimen (Figure 1d). Prominent onfFN was

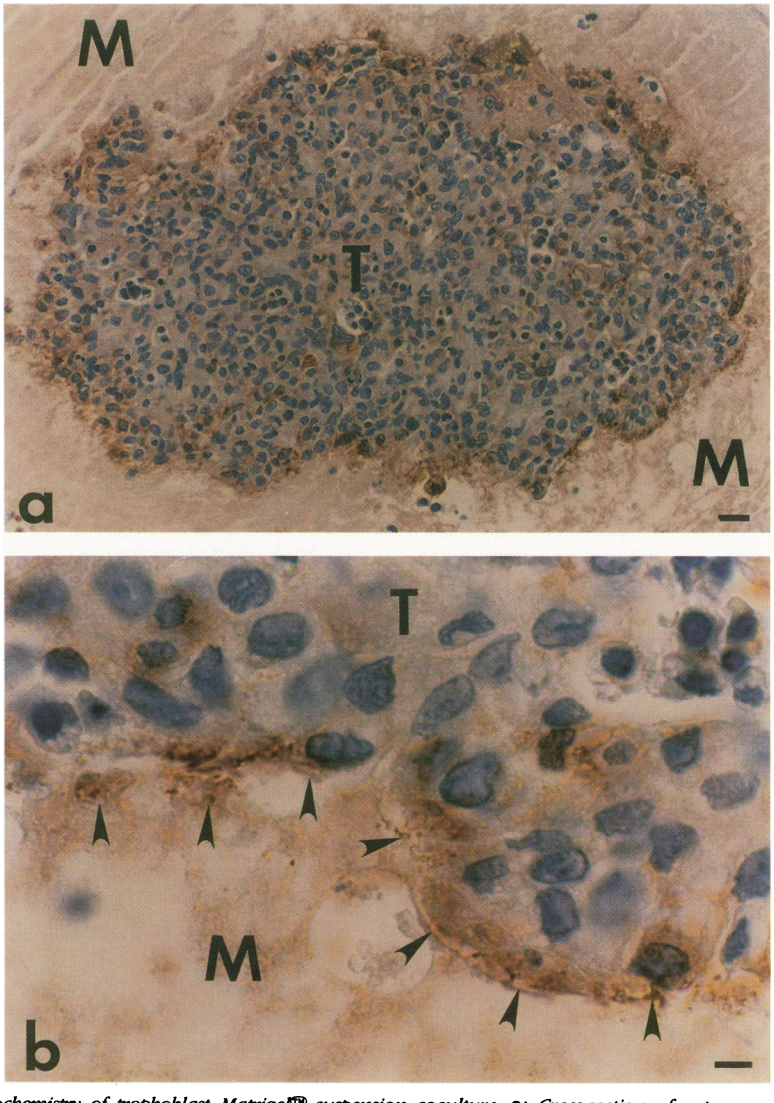

Figure 3. *Immunohistochemistry of trophoblast–Matrigel™ suspension coculture.* **a**: *Cross-section of a tongue of trophoblasts (T) that penetrated into the Matrigel (M) fragment. The majority of the onfFN immunoreactivity is restricted to the periphery of the trophoblastic aggregate, at the junction with the surrounding Matrigel.* **b**: *High-power view of the edge of the aggregate at the trophoblast–Matrigel (M) junction. Note that the trophoblasts (T) have synthesized and secreted onfFN into the ECM adjacent to the Matrigel (arrow heads). Bars represent 20 μm* **(a)** *and 5 μm* **(b)**.

seen primarily within the extracellular space at the trophoblast–Matrigel™ junction (Figure 3b).

Discussion

Fibronectins are ubiquitous glycoproteins that are thought to be responsible for cellular–ECM interactions,[12] including trophoblast attachment and spreading *in vitro*.[3] Our results provide further evidence that a specific class of fibronectins—those containing the oncofetal domain—function as a *trophouteronectin,* or trophoblast glue, for implantation of the early conceptus and anchorage of the developing placenta to the uterine wall.

Previous studies identified fibronectin staining in a variety of locations in pregnancy tissue, including ECM of the placental–uterine junction, uterine stroma, connective tissue core of placental villi, fetal membranes, and in walls of fetal blood vessels.[13–15] These immunolocalization studies, while specific for fibronectin, were performed with antibodies that may have reacted with more ubiquitous, less well-characterized fibronectin epitopes. The specificity of staining that we identified with FDC-6, unlike other anti-fibronectin antibodies, implicates the oncofetal domain or closely adjacent portions of IIICS as critical moieties associated with implantation and trophoblast attachment.

The functional significance of IIICS, while not yet defined in trophoblasts, has been examined closely in other human cells. Humphries et al[16,17] studied human melanoma cell attachment and found two distinct regions within IIICS that promoted melanoma adhesion. The C terminus of one of these peptide attachment sites, defined as CS1, is separated by only seven amino acids from the FDC-6–reactive hexapeptide. More recently, Kocher et al[18] demonstrated that fibronectins derived from alternative splicing of IIICS can alter significantly the spreading and migration characteristics of cultured endothelial cells. As in these systems, variable splicing within trophoblast IIICS RNA could yield fibronectins that modulate the attachment and migratory capabilities of implanting trophoblasts.

What factors regulate trophoblast differentiation *in vivo* toward a phenotype of onfFN expression? Previously trophoblast differentiation *in vitro* was characterized morphologically by syncytial formation and biochemically by the production of placental hormones.[3,8,10] We have shown now that onfFN is another important marker both *in vivo* and *in vitro* that defines a differentiated form of trophoblast capable of penetrating and anchoring to the ECM. Cyclic AMP agonists act as intracellular second messengers to increase hCG[10] and inhibit onfFN synthesis in cultured trophoblasts. This coordinated response to cAMP *in vitro* suggests that trophoblast differentiation toward a syncytial villous phenotype *versus* an extravillous anchoring phenotype could be regulated by cAMP *in vivo*. Support for this model is based on the fact that in the placenta only villous syncytiotrophoblasts produce hCG.[8] In the uteroplacental junction, anchoring trophoblasts and cell columns, differentiated extravillous cells attached to the uterus, synthesize onfFN but not hCG.

It is not known yet what agents may, in an opposite manner to cAMP, stimulate trophoblast onfFN synthesis. The goal of future studies will be to identify the endogenous factors in the milieu of the implanting trophoblasts that modulate trophoblast onfFN synthesis and trophoblast–endometrial interactions. Because *in vitro* onfFN production parallels *in vivo* onfFN immunolocalization, the human trophoblast system should provide a unique *in vitro* model for identifying those factors that regulate normal human implantation.

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