

Human cytotrophoblasts acquire aneuploidies as they differentiate to an invasive phenotype

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Received for publication 26 October 2004, revised 7 December 2004, accepted 8 December 2004

Available online 29 January 2005

Abstract

Through an unusual differentiation process, human trophoblast progenitors (cytotrophoblasts) give rise to tumor-like cells that invade the uterus. By an unknown mechanism, invasive cytotrophoblasts exhibit permanent cell cycle withdrawal. Here, we report molecular cytogenetic data showing that ~20 to 60% of these interphase cells had acquired aneusomies involving chromosomes X, Y, or 16. The incidence positively correlated with gestational age and differentiation to an invasive phenotype. Scoring 12 chromosomes in flow-sorted cytotrophoblasts showed that more than 95% of the cells were hyperdiploid. Thus, aneuploidy appears to be an important component of normal placentation, perhaps limiting the proliferative and invasive potential of cytotrophoblasts within the uterus.

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Keywords: Placenta; Cytotrophoblast; Aneuploidy; Proliferation; Differentiation; Invasion; Fluorescence in situ hybridization

Introduction

Development of eutherian mammals depends on the placenta. Formation of this transient but vital organ presents an interesting opportunity to study seemingly unique processes. For example, uterine attachment and acquisition of a maternal blood supply require the organ's specialized epithelial cells (i.e., cytotrophoblasts [CTBs]) to aggressively invade maternal tissues (Fisher et al., 1989; Librach et al., 1991). Except for the fact that CTB invasion is limited to the decidualized endometrium and the inner third of the myometrium, this process is more akin to tumorigenesis than to organogenesis.

What is the origin of the CTB subpopulation with tumor-like properties? These cells arise by differentiation of a progenitor population that is anchored to the trophoblast basement membrane surrounding the mesenchymal cores of chorionic villi. In one pathway, CTBs leave this basement membrane and fuse to form a continuous layer of syncytiotrophoblasts (STBs) that cover the chorionic villi (Fig. 1). These floating villi, so named because they float in maternal blood, are the site of hormone production as well as nutrient, gas, and waste exchange between the mother and the fetus. In the second pathway, the focus of this study, CTBs at the tips of anchoring chorionic villi leave the trophoblast basement membrane and form columns of nonpolarized cells that attach to and then penetrate the uterine wall. The ends of the columns terminate within the superficial decidua, where they give rise to invasive CTBs (Fig. 1). During interstitial invasion, a subset of these cells, either individually or in small clusters, commingles with resident decidual, myometrial, and immune cells. During

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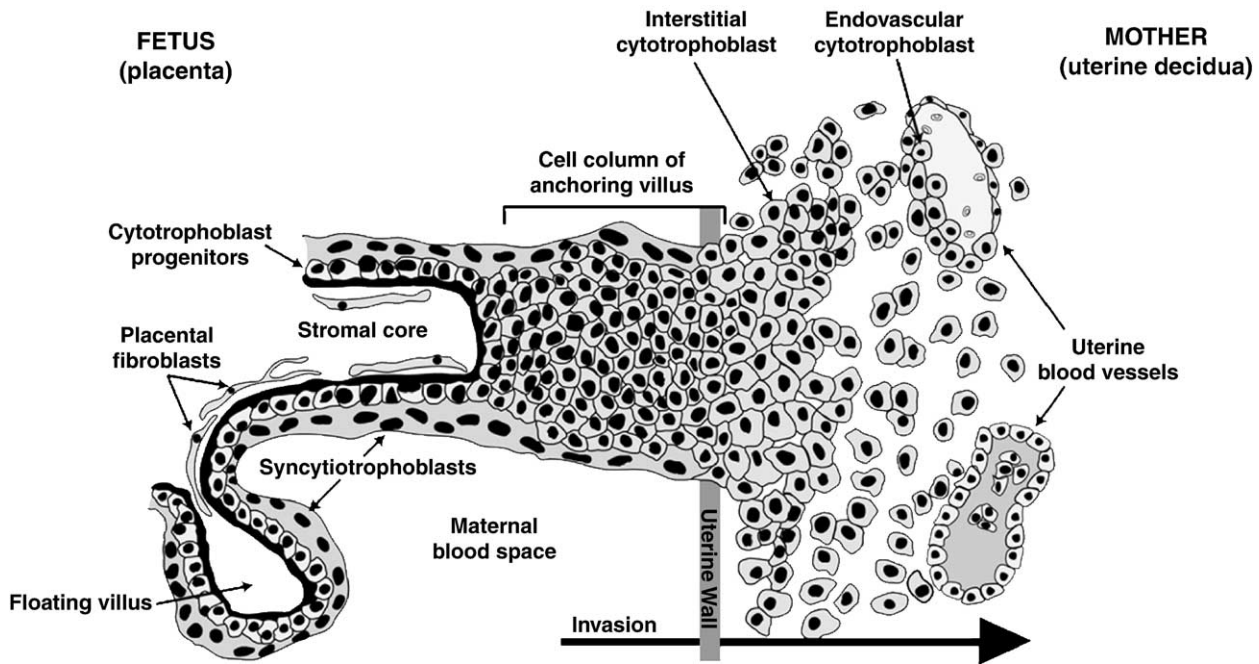


Fig. 1. Diagram of the maternal–fetal interface at about 10 weeks of gestation. The floating villi, which are suspended in the intervillous space, are bathed by maternal blood. The anchoring villi, which function as a bridge between the fetal and maternal compartments, form cell columns that give rise to the subpopulation of cytotrophoblasts (CTBs) that invade the uterine interstitium (decidua and first third of the myometrium, interstitial CTBs) and maternal vasculature (endovascular CTBs), thereby anchoring the fetus to the mother and accessing the maternal circulation.

endovascular invasion, masses of CTBs migrate into the vessels before the lumina eventually recanalize (Brosens et al., 2002; Zhou et al., 1997). Together, these two components of CTB invasion anchor the placenta to the uterus and divert uterine blood flow to the intervillous space.

There are several critical transitions in CTB differentiation along the invasive pathway that are marked by the cells' expression of stage-specific antigens (Damsky and Fisher, 1998). Before differentiation, the CTB progenitor subpopulation, which is attached to the trophoblast basement membrane, is actively proliferating; many of these cells stain positively for Ki67, an antigen expressed throughout the active phases of the mitotic cell cycle (Schwartz, 1993). CTB expression of this antigen is abruptly downregulated as the cells invade the uterus, thus uncoupling invasion from proliferation (Genbacev et al., 1997). Additionally, the cells upregulate expression of molecules that mediate invasion (e.g., matrix metalloproteinase-9) as well as immune regulators (e.g., HLA-G, a class Ib major histocompatibility complex molecule). Although the mechanisms are not yet fully understood, HLA-G is thought to play an important role in maternal tolerance of the hemiallogeneic CTB subpopulation that invades the uterine wall, i.e., the cells to which expression of this antigen is restricted (McMaster et al., 1995).

With regard to the genetic aspects of placental development, chromosomal abnormalities—the major cause of spontaneous abortions—are surprisingly common (Hassold and Jacobs, 1984). Cytogenetic studies of human preimplantation embryos have shown that mitotic errors such as non-

disjunction or anaphase lag occur frequently. In some patients, at least 50% of in vitro fertilized embryos show evidence of aneuploidy and chromosomal mosaicism (Munné, 2002). Generally, about one third of all embryos are lost in the first trimester, with the majority of cases occurring between implantation and recognition of pregnancy (Miller et al., 1980; Wilcox et al., 1988). Thus, it appears that abnormal embryos are selected against in the first few weeks of life by mechanisms that involve failures in critical elements of implantation and placental development.

In most pregnancies, the fetus and the placenta have the same karyotype because both lineages are descendants of the same zygote. However, in ~1 to 2% of pregnancies, chorionic villus sampling (CVS) reveals confined placental mosaicism (CPM) in which the placenta and the fetus have different karyotypes (Kalousek and Vekemans, 1996; Ledbetter et al., 1992). CPM can occur as a result of postzygotic errors in mitosis, in which case the conceptus may have a normal karyotype. Alternatively, a trisomic blastocyst may be rescued by chromosome loss within the embryo, leaving the extraembryonic lineages trisomic.

To date, many genetic studies have examined floating villi and the cells they contain, including the trophoblast populations. Very little is known about the karyotype of human CTBs that arise from anchoring villi and subsequently invade the uterine wall. Interestingly, in mice, the analogous population of invasive trophoblasts undergoes endoreduplication (MacAuley et al., 1998; Nakayama et al., 1998). A few reports suggest the possibility that invasive human CTBs have an elevated ploidy level (hypertetraploid

and hyperoctaploid) (Wakuda and Yoshida, 1992; Zybina et al., 2002). In this context, we performed a systematic correlation of CTB genotype (by fluorescence in situ hybridization, FISH) and phenotype (by evaluating cell proliferation and HLA-G expression) during the first and second trimesters of pregnancy, the critical period during which the placenta forms. We also studied CTBs at term. In all cases, the results showed an unexpectedly high rate of aneuploidy that is unlikely to occur by endoreduplication.

Materials and methods

Placental tissues

Portions of the placenta and basal plate (maternal–fetal interface) were collected immediately after elective pregnancy terminations for non-medical reasons during the first or second trimester. The gestational age ranged from 5 to 12 weeks and 15 to 23 weeks for first- and second-trimester placentas, respectively. Term placentas were obtained immediately after normal deliveries (37–40 weeks of gestation). Areas with floating villi and portions of anchoring villi together with their uterine attachment sites were biopsied. Several specimens of $1 \times 1 \times 0.5 \text{ cm}^3$ were excised and divided into five equal-sized pieces. All samples were washed in ice-cold phosphate-buffered saline (PBS), then fixed with freshly prepared 3% paraformaldehyde (PFA) in PBS for 30 min at 4°C. After two wash steps in PBS at 20°C, tissues were immersed in a sucrose series (5%, 10%, and 15% in PBS; 15 min/step, at 4°C). Finally, the samples were incubated for 20 min at 4°C in a 1:1 (vol/vol) mixture of 15% sucrose in PBS and “optimum cutting temperature” formulation of water-soluble sucrose and resins (Sakura Tissue-Tek OCT Compound), embedded in OCT, frozen in liquid nitrogen, and stored at -80°C (Damsky et al., 1992). Placental tissue sections (5–8 μm thick) were cut using a cryostat (Slee International Inc., Tiverton, RI), collected on precleaned ProbeOn Plus microscope slides (Fisher Scientific, Santa Clara, CA), and stored at -20°C .

CTBs and placental fibroblasts (described below) were isolated according to our published methods (Librach et al., 1991). For each isolation, 6–8 first trimester placentas were pooled; second trimester and term placentas were processed individually. The major steps included removal of the syncytium and release of CTBs by sequential enzymatic digestions (collagenase followed by trypsin digestion). The resulting cells were enriched by using Percoll gradient centrifugation. Isolated cells were counted and adjusted to 10^6 cells/ml in serum-free medium (SFM). Ten-microliter aliquots of CTBs, 10 μl of fetal bovine serum, and 200 μl of SFM were combined, spun onto Cytospin[®] microscope slides (Thermo Shandon, Pittsburg, PA), and stored at -20°C . The purity of CTB preparations, which ranged from 89 to 99%, was determined by staining an aliquot of the

cells with the anti-cytokeratin antibody 7D3 (Damsky et al., 1992).

Placental fibroblasts were isolated by trypsin digestion of the (first-trimester) chorionic villous stromal cores after they were denuded of trophoblasts (as described above). Then, the cells were pelleted by centrifugation (800 rpm, 5 min) before they were resuspended in 10 ml of a hypotonic solution of 0.3% KCl for 30 min at 37°C. A 200- μl aliquot of ice-cold Carnoy's fixative (methanol-acetic acid, 3:1, vol/vol) was added and gently mixed before the cells were centrifuged. The pellet was resuspended in ice-cold Carnoy's fixative, and the cells were once again isolated by centrifugation. The latter procedure was repeated three times. Then, the cells were dropped on fixative-cleaned slides that were placed above a boiling water bath. The slides were air-dried and stored at -20°C .

FACS

For flow-sorting of subpopulations according to DNA content, 10^3 – 10^7 CTBs in single-cell suspensions were fixed overnight in 70% ethanol at 4°C, washed with PBS at 20°C, and resuspended in propidium iodide (PI) staining medium (50 $\mu\text{g}/\text{ml}$ PI, 100 U/ml RNase A, 1 mg/ml glucose in PBS without Ca^{2+} or Mg^{2+}). Cell samples were analyzed on a FACS DIVA or FACS ARIA cell sorter (Becton Dickinson Immunocytometry Systems, San Jose, CA) using a standard filter configuration with the 488 nm line of an argon ion laser set to 100 mW output and PI fluorescence detected at 620 nm. Analyses of single cells were performed by eliminating cell debris and aggregates using doublet discrimination based on PI emission pulse width versus area. All flow-sorting data were analyzed using the peak deconvolution program ModFit (Verity House, Topsam, MA). Cells sorted from different PI fluorescence intervals (Fig. 3A) were spun onto Cytospin microscope slides for FISH analyses.

FISH

DNA probes chosen to bind specifically to chromosome X (CEP X SpectrumGreen[™]), chromosome Y (CEP Y SpectrumAqua), or chromosome 16 (CEP 16 SpectrumOrange) were purchased from Vysis Inc. (Downers Grove, IL). We also used two probe sets specific for the subtelomeric regions of chromosomes X and Y (TelVysion Xp/Yp SpectrumGreen, TelVysion Xq/Yq SpectrumOrange). The hybridization mixture was prepared following the probe manufacturer's protocol using 1 μl of each probe per 10 μl of hybridization mixture (Vysis).

Isolated CTBs and normal lymphocytes on slides (ProbeChek 0% trisomy8/12, Vysis) were incubated in Carnoy's fixative for 10 min followed by immersion in $2\times$ SSC (0.3 M NaCl, 0.03 M Na_3 citrate $\cdot 2\text{H}_2\text{O}$, pH 7.0) for 1 h at 37°C. Then, the cells were pretreated with 50 $\mu\text{g}/\text{ml}$ pepsin (Amresco, Solon, OH) in 0.01 N HCl for 5–13

min at 37°C before immersion in PBS for 5 min. Afterwards, the slides were incubated in 1% PFA/PBS for 5 min, PBS for 5 min, and sequential changes of ethanol (70%, 80%, 100%; 2 min per step), then air-dried.

Isolated fibroblasts on slides were incubated in 2× SSC for 20 min at 37°C and pretreated with pepsin (50 µg/ml in 0.01 N HCl) for 13 min at 37°C. At a constant temperature of 20°C, they were washed in PBS for 5 min, postfixed for 5 min with 1% PFA dissolved in 1× PBS/0.05 M MgCl₂, washed in PBS for 5 min followed by 0.5% Tween 20/PBS for 5 min. Finally, the slides were dehydrated in 70%, 80%, and 100% ethanol for 2 min each before they were used for FISH.

Isolated cells fixed on slides were denatured for 5 min at 76°C in 70% formamide (FA)/2× SSC, pH 7.0, and then dehydrated in 70%, 80%, and 100% ethanol (2 min per step) before air-drying. Meanwhile, the hybridization mixture was denatured for 5 min at 76°C. Two microliters of denatured hybridization mixture was then applied to each slide, and cover slips were added and sealed with rubber cement. The hybridization proceeded at 37°C for 40 h.

Slides with tissue sections were incubated in Carnoy's fixative for 5 min at 20°C and then placed on a hot plate at 45°C for 5 min. Unless otherwise noted, all reactions were carried out at 20°C. Next, the slides were immersed in two changes of 2× SSC for 5 min each, treated with 50–100 µg/ml pepsin in 0.01 N HCl for 10–20 min at 37°C, washed twice with 2× SSC for 5 min, and transferred back to the hot plate at 45°C for 5 min. After incubation in 4% PFA in PBS for 10 min, the slides were washed twice with 2× SSC (5 min each) and dried on a hot plate for 5 min at 45°C. Then, the tissue sections were dehydrated in sequential changes of 70%, 80%, and 100% ethanol (2 min per step), allowed to air-dry, overlaid with 3–5 µl of hybridization mixture, covered with a 12-mm-diameter round cover slip, and sealed with rubber cement. Finally, the DNA was denatured by incubation for 10 min on a hot plate at 85°C before the hybridization was allowed to proceed for 40 h at 37°C.

After hybridization, all slides were washed three times (10 min each time, 43°C) in 50% FA/2× SSC (pH 7.0), then twice in 2× SSC (10 min each time, 43°C). The slides were mounted with 8 µl of 4',6-diamidino-2-phenylindole (DAPI, 0.5 µg/ml, Calbiochem, La Jolla, CA) dissolved in anti-fade medium (Fung et al., 2001). Signals were visualized by using fluorescence microscopes equipped with filters for DAPI, FITC, rhodamine, Cy5, and Spectrum Aqua excitation and detection.

We also performed repeated hybridizations using five different sets of chromosome-specific probes to score 12 chromosomes (3, 6, 8, 9, 10, 11, 12, 16, 17, 18, X, and Y). After hybridizing the first probe set (CEP X SpectrumGreenTM, CEP Y SpectrumAqua, CEP 16 SpectrumRed) and acquiring images from six different areas of the slide, we removed the probes by twice incubating the slide in 0.01× SSC (5 min each time; 43°C), followed by denaturation for 2 min at 76°C and

dehydration in a graded ethanol series (70%, 80%, 100%). Next, we denatured a mixture of CEP 8 SpectrumOrange and CEP 12 SpectrumGreen probes (set 2) and allowed hybridization overnight. The process of probe stripping, denaturation, and re-hybridization was performed three more times. Subsequent probe sets used were CEP 18 SpectrumOrange and CEP 17 SpectrumGreen (set 3), CEP 10 SpectrumOrange, and WCP 9 SpectrumGreen (set 4), and a combination of a Cy5-labeled probe for chromosome 3 (Oncor, Gaithersburg, MD) with CEP 6 SpectrumOrange and CEP 11 SpectrumGreen (set 5).

For scoring, hybridization signals were counted according to the criteria published by Hopman et al. (1986): pairs that were spaced less than the diameter of a signal domain were counted as one chromosome, and pairs that were farther apart than the diameter of a signal domain were counted as two chromosomes. For each analysis of isolated CTBs, we analyzed at least 100 cells. For each tissue section, 40 individual cells were analyzed from three different areas in which single cells could be resolved (mesenchyme, STBs, and CTBs in the uterine wall). Chromosome-specific aneuploidy included hypodiploidy (any chromosome with fewer copies than diploidy) and hyperdiploidy (any chromosome with more copies than diploidy). All results are expressed as mean ± SD. Statistical analyses were performed using Student's *t* test; a *P* value of <0.05 was considered significant.

HLA-G staining and FISH

Isolated CTBs from Cytospin preparations were fixed in methanol for 10 min, air-dried, and stored at –20°C. Prior to hybridization, the cells were maintained at 4°C while they were fixed in methanol for 30 min, rinsed in PBS for 30 min, and then dehydrated in a graded ethanol series (70%, 80%, and 100%; 2 min per step). After air-drying, the slides were overlaid with 3–5 µl of the hybridization mixture, then covered with a 12-mm-diameter round cover slip and sealed with rubber cement. The DNA was denatured for 5 min at 73°C, and the hybridization was performed for 40 h at 37°C. Afterwards, the slides were washed in 0.4× SSC/0.3% NP40 for 2 min at 73°C, 2× SSC/0.1% NP40 for 2 min at 20°C, and three times with PBS for 5 min at 20°C. Then, they were incubated in the blocking medium (0.2% BSA/PBS) for 30 min, followed by addition of the primary mAb (anti-HLA-G diluted [1:25, vol/vol] in blocking medium; clone 4H84 (McMaster et al., 1995)). After incubation for 1 h, the slides were washed, and binding of the primary antibody was detected with a secondary antibody conjugated to fluorescein (FITC donkey anti-mouse IgG, diluted [1:100, vol/vol] in blocking medium; Jackson ImmunoResearch Labs, West Grove, PA). The slides were washed three times in PBS (5 min each time) and mounted with DAPI in anti-fade solution as described above.

Cell proliferation and FISH

Isolated CTBs were cultured on Matrigel-coated substrates (Collaborative Biomedical Products, Bedford, MA) in serum-free high-glucose DMEM with 2% Nutridoma (Roche Applied Science, Indianapolis, IN), 1% glutamine (Atlanta Biologicals, Atlanta, GA), 1% HEPES, 1% sodium pyruvate, 1% penicillin/streptomycin, and 50 µg/ml gentamycin in the presence of BrdU (10 µM, Sigma). After 12 h, the cells were trypsinized and resuspended in PBS. Then, 10-µl aliquots of cells, 10 µl fetal bovine serum, and 200 µl SFM were combined and spun onto Cytospin microscope slides. The cells were fixed in 70% ethanol for 30 min before they were air-dried. Then, the slides were immersed in 0.07 N NaOH (2 min at 20°C), washed in PBS (5 min at 20°C), and incubated in 2× SSC for 20 min at 37°C. Afterwards, the cells were pretreated with 50 µg/ml pepsin in 0.01 N HCl for 7 min at 37°C and rinsed in PBS at 37°C for 5 min. Then, the slides were transferred to the fixative (1% PFA in PBS) for 5 min and rinsed in PBS for 5 min followed by dehydration in 70%, 80%, and 100% ethanol (2 min per step). After air-drying, the denaturation, hybridization, and washing steps were performed as described above for FISH. Then, the slides were incubated with FITC-conjugated anti-BrdU (BD Biosciences, San Diego, CA; 20 µl of the mAb and 50 µl of 0.5% Tween 20 in PBS) for 30 min. Finally, they were washed three times in PBS (5 min each) and mounted with DAPI in anti-fade solution as described above.

Results

A subset of freshly isolated CTBs have numerical chromosomal abnormalities

The CTB population that is isolated from second-trimester placentas includes progenitors and cells that have begun to differentiate along the invasive pathway. When hybridized with three chromosome enumerator DNA probes (CEP X, CEP Y, and CEP 16), a majority of cells appeared to have a normal male or female chromosome complement (X, Y, 16, 16 or X, X, 16, 16). However, we frequently observed cells with abnormal sets of FISH signals. Fig. 2A shows a FISH analysis of five CTBs: three appeared diploid (X, Y, 16, 16), one carried a trisomy 16 (X, Y, 16, 16, 16), and one was hyperdiploid (X, X, Y, Y, Y, 16, 16, 16, 16). We also noted a large fraction of cells that exhibited paired arrangements of chromosome-specific FISH signals (Fig. 2B). Following established scoring criteria based on the distance of hybridization domains in paired arrangements (Hopman et al., 1986; Jenkins et al., 1992; Munné and Weier, 1996), the closely spaced paired signals (Fig. 2B, yellow arrows) were counted as one chromosome and attributed to replicated centromeres. Paired signals that were

separated by more than one signal diameter were scored as two chromosomes (Fig. 2B, white arrows). Thus, in this field, four cells were diploid, one cell was tetraploid (X, X, X, X, 16, 16, 16, 16) and another cell had an aneuploidy involving X (X, X, X, X, 16, 16) (Fig. 2B). We also observed that many CTBs displayed a continuum of CEP X signals that ranged from closely spaced pairs to widely separated CEP X signals (Figs. 2B–G).

To further investigate the phenomenon of paired signals, we utilized two approaches. The first was FISH analyses with a combination of CEP probes, which target tandemly repeated DNA sequences, and whole chromosome painting (WCP) probes. The results suggested that the signals were derived from two apparently complete copies of the X chromosome (Fig. 2C). Stripping the WCP X/CEP X probes and rehybridizing with CEP X, CEP Y, and CEP 16 probes revealed a Y chromosome-specific signal (Figs. 2D and E). In one cell (Fig. 2D, upper left corner), two signals for the CEP X probe and five signals for the CEP 16 probe were observed. The other cell (Fig. 2D, lower right corner) was karyotyped as X, X, Y, 16, 16.

The second approach was FISH analyses using CEP X/CEP Y probes in combination with a subtelomeric probe set for chromosomes X and Y (Figs. 2F and G). The left panel of Fig. 2F shows an ideogram of chromosomes X and Y with the color scheme of the FISH probes indicated. The right panel of Fig. 2F shows two male cells with one chromosome Y (one CEP Y [blue] and one Yqtel [red] signal) and two X chromosomes (two CEP X [green] and two Xqtel [red]) signals. Concordant results were obtained when the cells were hybridized with CEP X and CEP Y probes together with subtelomeric probes that map to Xptel and Yptel (Fig. 2G). These results provided further evidence that the paired CEP X signals represented two copies of the entire X chromosome.

In a complementary approach, we used fluorescence-activated cell sorting (FACS) to study the DNA content of CTBs isolated from second-trimester placentas. The propidium iodide (PI) DNA histogram (Fig. 3A) suggested cohorts of cells: nondividing CTBs likely to be in the G₀/G₁ phase of the cell cycle (59.2%, interval A); a population that corresponds, in part, to the G₂/M phase (34.0%, interval C); and cells with an intermediate DNA content that appeared to be in S phase (4.5%, interval B). We also found a substantial number of cells that were hypertetraploid (2.3%, interval D). The DNA histograms of CTBs isolated from first-trimester placentas showed a similar distribution pattern (data not shown). FISH analyses of second-trimester CTBs ($n = 4$ [2 female and 2 male]) from intervals A to D revealed an interesting spectrum of aneuploid cells in all fractions (Fig. 3B). Interval A had the highest number of cells with chromosome-specific signals expected from diploid cells (87.8%). In the other groups, the majority of aneuploid cells were hyperdiploid, with up to 10 copies of chromosome X and 7 copies of chromosome 16 per cell. In general, the copy

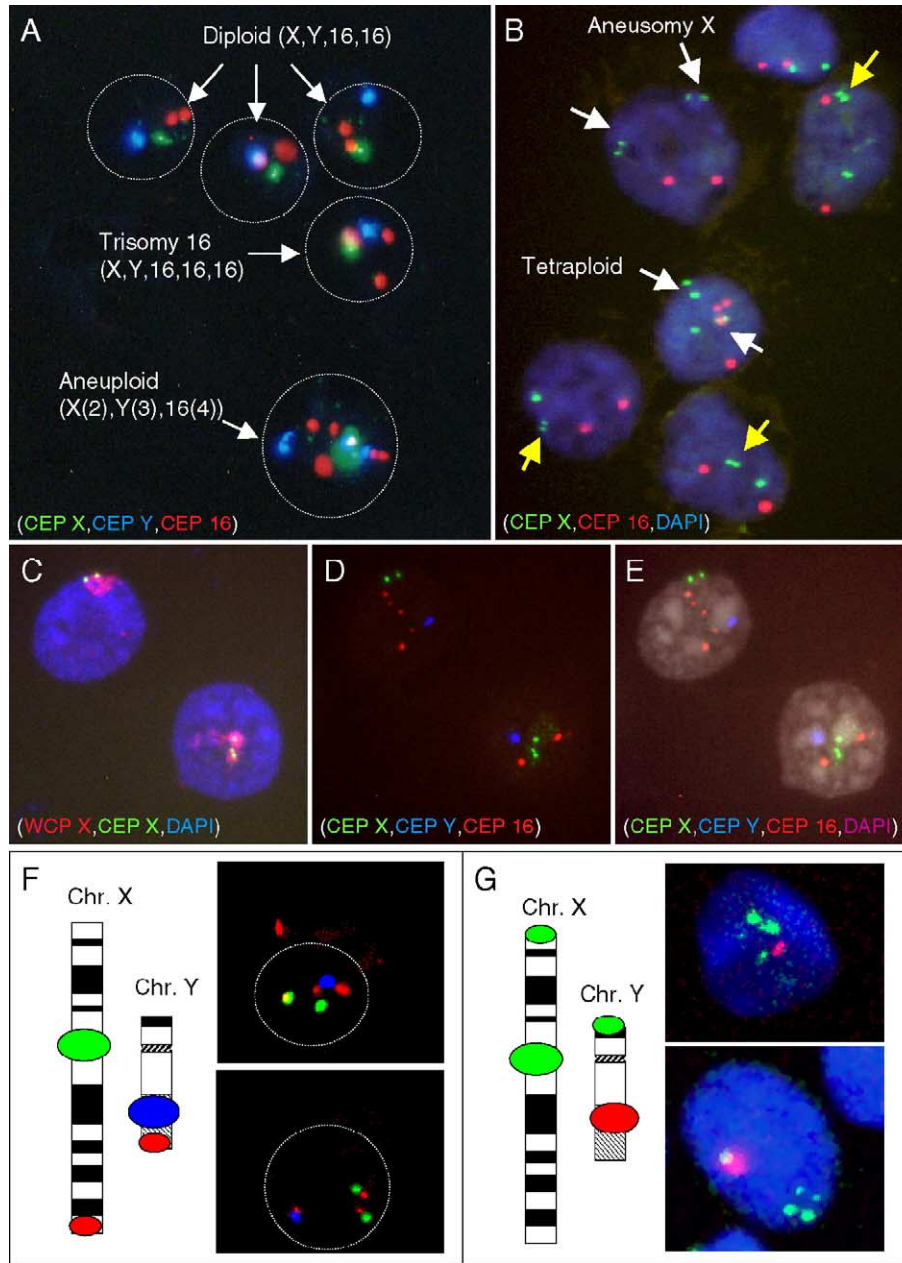


Fig. 2. Fluorescence in situ hybridization analysis of isolated cytotrophoblasts (CTBs; second trimester) reveals cytogenetic abnormalities such as supernumerary chromosome complements. (A) The red–green–blue (RGB) pseudo-color image of five male CTBs hybridized with three CEP probes (green signals, chromosome X; blue signals, chromosome Y; red signals, chromosome 16). White circles show the area of the cell that was occupied by the nucleus as determined by DAPI staining (staining not shown). (B) The same set of probes was used to analyze six female CTBs. The yellow arrows indicate the closely paired X–X signals that were counted as one chromosome. The white arrows indicate the paired X–X signals that were counted as two chromosomes. The DAPI counterstain is shown in blue. (C) Male CTBs were hybridized with a CEP X probe (green; signals appear yellow in the figure because of the overlap with red fluorescence) and an X-specific whole chromosome painting probe (red). (D) The probes shown in panel C were removed, and the cells were hybridized with three different CEP probes. Both CTBs were aneuploid. (E) The hybridization image shown in panel D was merged with DAPI staining. (F, G) The ideograms of chromosomes X and Y and the hybridization scheme are shown on the left of each panel, together with the FISH images on the right. The data generated by using a combination of CEP and subtelomeric probes are consistent with the presence of closely paired whole chromosome X signals and the absence of telomere fusion.

number of chromosome X was greater than the copy number of chromosome 16, especially in cells sorted from interval D. In conclusion, a significant fraction of CTBs isolated from normal human placentas was hyperdiploid, with gains of chromosome X and, to a lesser extent, chromosome 16.

Among isolated CTBs, the rate of aneuploidy increases as gestational age advances

Since the subpopulation of replicating (presumably diploid) CTB progenitors is rapidly depleted after the first trimester of pregnancy, we analyzed aneuploidy as a

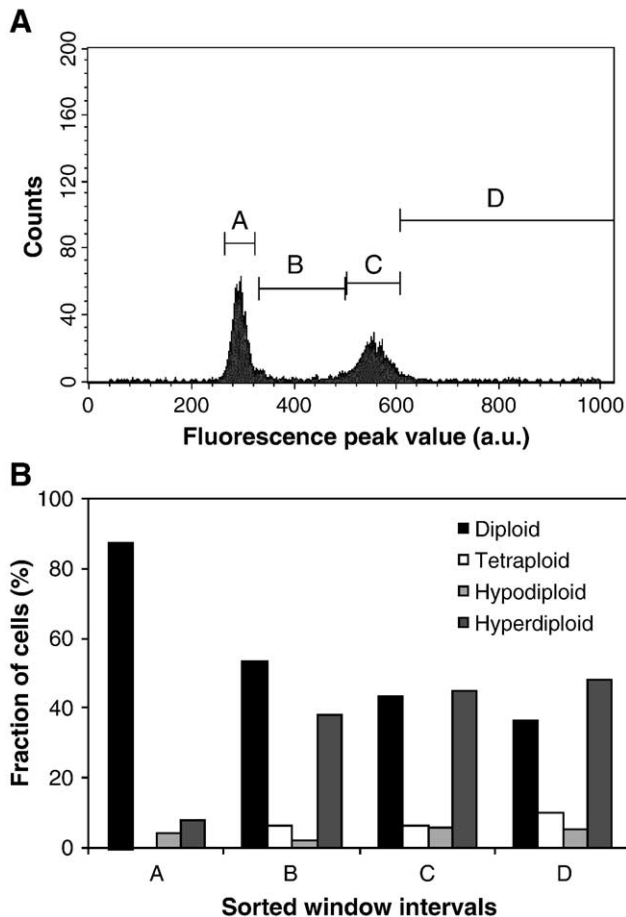


Fig. 3. Fluorescence in situ hybridization analysis of flow-sorted cytotrophoblasts (CTBs) revealed aneuploidies in every window. (A) A typical analysis in which flow-sorting was used to separate CTBs isolated from a second-trimester placenta according to their DNA content prior to FISH analyses. Horizontal bars indicate the windows defined for flow-sorting. Using PI counterstaining, we identified intervals for what initially appeared to be nondividing cells (interval A), cells in S phase (interval B), and cells with approximately twice the DNA content of those in interval A (interval C). However, we also found a substantial number of potentially hypertetraploid cells (interval D). (B) The karyotype of CTBs from sort windows A to D as determined by hybridization with three CEP probes (X, Y, and 16).

function of gestational age. In these experiments, 100 to 200 isolated cells were analyzed with three FISH probes (CEP X, CEP Y and CEP 16). Analyses of normal male lymphocyte preparations ($n = 2$) and fibroblasts isolated from first-trimester placentas ($n = 5$) served as controls

(Table 1). Ninety-five percent of the lymphocytes carried one CEP X-specific signal; 95.5 and 86% of these cells showed the expected number of signal domains for the CEP Y and the CEP 16 probes, respectively. Overall, 82.2% of lymphocytes were scored as diploid cells, a result that is in line with published findings (Jalal and Law, 1997). Similarly, 85.2% of placental fibroblasts were diploid.

Next, using the same approach, we analyzed 25 preparations of CTBs isolated from placentas of different gestational ages: 10 first trimester, 10 second trimester, and 5 term. Approximately 20 to 60% of CTBs in every isolate had numerical chromosomal aberrations. Most had a hyperdiploid rather than a tetraploid karyotype. Table 1 presents the results stratified by gestational age. CTBs isolated from first-trimester and term placentas had the lowest and highest mean rates of aneuploidy, respectively ($22.2 \pm 8.5\%$ vs. $40.5 \pm 9.0\%$), whereas second-trimester cells had an intermediate value ($35.8 \pm 12.5\%$). The increase in the number of chromosomally abnormal and hyperdiploid CTBs between the first and second trimesters was significant ($P < 0.02$), but the increase between second-trimester and term cells was not. Altogether, the percentage of hyperdiploid cells at every gestational age was significantly higher in CTBs than in fibroblasts (Table 1, $P < 0.05$). The most common aneusomy was a gain of chromosome X (“Hyper X”) observed in ~40% of aneuploid CTBs (Fig. 4A). Approximately 30% of the cells exhibited an aneuploidy that involved more than one chromosome (“Multi,” Fig. 4A). Surprisingly, cells with tetra- or higher ploidy levels as shown by three-probe FISH were sparse in our samples, suggesting that CTBs are not completing a typical endoreduplication cycle.

Finally, the overall rate of aneuploidy was significantly different between male and female CTBs [male vs. female: $37.2 \pm 13.3\%$ (11 samples) vs. $26.6 \pm 10.2\%$ (14 samples), $P < 0.05$]. The total rate of hyperdiploid CTBs was also significantly different [male vs. female: $34.2 \pm 13.9\%$ (11 samples) vs. $20.0 \pm 9.2\%$ (14 samples), $P < 0.05$].

Among CTBs in situ, the rate of aneuploidy increases as gestational age advances

To determine whether a similar phenomenon occurs in vivo, we analyzed tissue sections from first-trimester ($n = 7$), second-trimester ($n = 7$), and term ($n = 5$) placentas with the

Table 1
FISH analyses of human placental fibroblasts and cytotrophoblasts^a

Cell type	Number of samples	Diploid (%)	Tetraploid (%)	Hypodiploid (%)	Hyperdiploid (%)
Normal male lymphocytes	2	82.2	1.5	8.7	7.6
Placental fibroblasts (first trimester)	5	85.2	0	10.1	4.7
CTBs (first trimester)	10	77.8	1.8	3.5	16.9
CTBs (second trimester)	10	64.2	1.4	1.9	32.5
CTBs (term)	5	59.5	2.9	5.1	32.5

^a The cells were hybridized with three CEP probes (X, Y, and 16). At least 100 cells were analyzed from each sample.

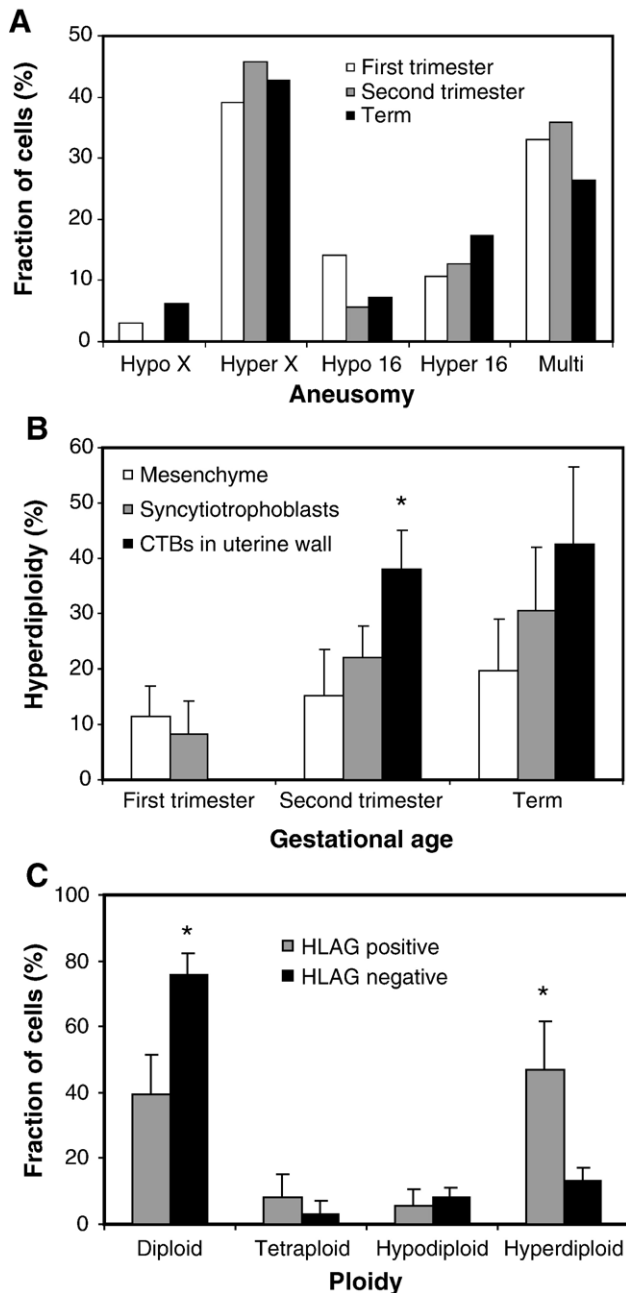


Fig. 4. Fluorescence in situ hybridization analysis of cytotrophoblast (CTB) aneuploidy. (A) The rates of the different aneusomies did not significantly change as a function of gestational age. (B) Analysis of tissue sections allowed calculation of the percentage of hyperdiploid cells in the various placental compartments during the first and second trimesters and at term. Samples of CTBs embedded in the uterine wall could not be obtained during the first trimester. During the second trimester, hyperdiploid cells were found more often within the uterine wall than in association with the floating villi (*) ($P < 0.002$). (C) The percentage of cells in the hyperdiploid categories positively correlated with acquisition of an invasive phenotype as demonstrated by HLA-G expression. HLA-G-negative CTB progenitors were more likely to be diploid, whereas the HLA-G-positive CTBs were more likely to be hyperdiploid (*) ($P < 0.002$).

same probe set (CEP X, CEP Y, and CEP 16). We scored three cell types: mesenchymal cells in the central cores of chorionic villi, multinucleated STBs that cover these villi, and CTBs

within the uterine wall (Fig. 1). The only exception was first-trimester placental samples in which the decidua detached from the placenta, precluding analysis of the invasive CTB subpopulation. In every tissue section, at least 40 cells of each type were analyzed. Progenitor CTBs and CTBs in the columns of anchoring villi, which were too tightly packed to resolve individual nuclei, were not scored.

The results are graphed in Fig. 4B. The average rate of hyperdiploidy among mesenchymal cells was $11.6 \pm 5.4\%$, $15.3 \pm 8.3\%$, and $19.3 \pm 9.3\%$ in tissue sections of first-trimester, second-trimester, and term placentas, respectively. In comparison, STBs showed a higher rate that increased with advancing gestational age ($8.2 \pm 6.1\%$, first trimester; $22.0 \pm 5.7\%$, second trimester; and $30.4 \pm 11.7\%$ at term). Of all the cells that were scored, CTBs in the uterine wall were more likely to be hyperdiploid: $38.1 \pm 7.0\%$ of cells in the second trimester and $42.6 \pm 13.8\%$ of cells at term had extra chromosomes. Finally, analysis of tissue sections showed that the spatial distribution of the aneuploid trophoblasts appeared to be random, suggesting that the cells acquire aneusomies sporadically as opposed to clonal expansion of an aneuploid CTB subset.

CTBs accumulate aneuploidies as they differentiate along the invasive pathway

To examine aneuploidy as a function of CTB differentiation, we combined FISH-based ploidy analysis with immunolocalization to assess the cells' expression of stage-specific antigens. Specifically, CTB progenitors are HLA-G-negative (McMaster et al., 1995). Once they are allocated to the invasive pathway, they rapidly upregulate HLA-G expression (Figs. 5A and B). The analysis of CTBs isolated from first-trimester ($n = 2$), second-trimester ($n = 2$), and term placentas ($n = 1$) showed that more than 50% of CTBs in every sample expressed HLA-G. The rate of diploidy was significantly higher in the HLA-G-negative population than in the HLA-G-positive population ($75.6 \pm 6.8\%$ vs. $39.4 \pm 12.2\%$; $P < 0.002$ [Fig. 4C]). Conversely, the rate of hyperdiploidy was significantly higher in the HLA-G-positive population than in the HLA-G-negative population ($47.2 \pm 14.6\%$ vs. $13.2 \pm 3.9\%$; $P < 0.002$ [Fig. 4C]). In contrast, HLA-G staining did not distinguish between CTBs in the tetraploid or hypodiploid groups (Fig. 4C). In summary, these data show that a large fraction of CTBs that are fated to invade the uterine wall are hyperdiploid.

As CTBs differentiate along the invasive pathway, they complete mitosis and exit the cell cycle. Therefore, we also investigated a possible negative correlation between CTB aneuploidy and mitosis. The DNA histogram of isolated CTBs suggested that 4.5% of cells had a DNA content between $2n$ and $4n$ (Fig. 3A). In the context of the aforementioned results, interpretation of the FACS data was equivocal. Therefore, we performed FISH analyses (CEP 16, CEP X, and CEP Y) on BrdU-labeled second-trimester CTBs. Duplicate experiments showed that fewer

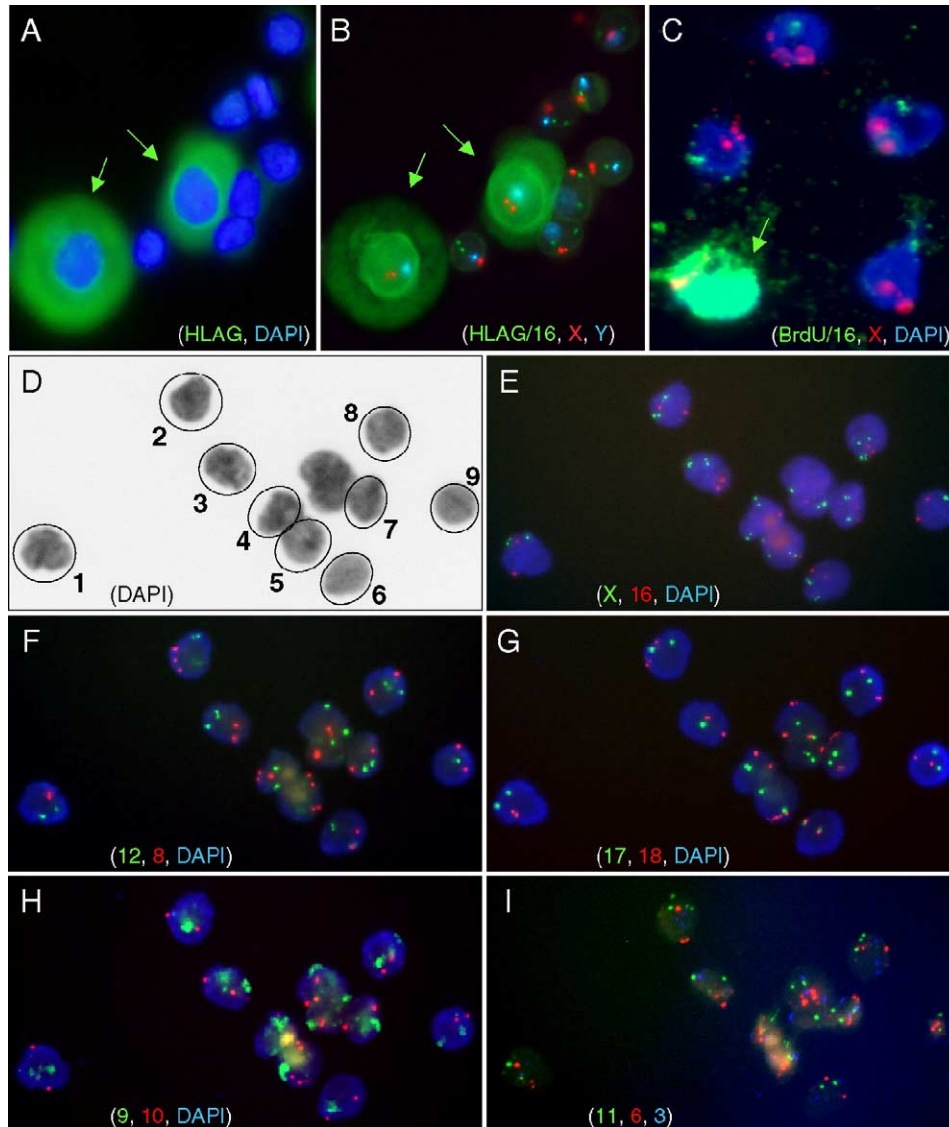


Fig. 5. (A–C) Cytotrophoblast (CTB) aneuploidy positively correlates with differentiation along the invasive pathway during the second trimester of human pregnancy. The cells (second trimester) were analyzed by FISH before they were stained with anti-HLA-G and DAPI. (A) Two HLA-G-positive cells (green arrows). (B) FISH signals acquired from the cells shown in panel A. (C) FISH signals combined with BrdU detection. The image shows one cell that incorporated BrdU (green arrow). The four BrdU-negative cells had two or more CEP X signals. (D–I) Repeated hybridizations to CTBs revealed chromosome-specific aneusomy patterns. CTBs isolated from a second-trimester placenta (female) were sorted from interval C (Fig. 3A) and hybridized with 12 different probes. (D) The nuclei were visualized by DAPI staining. (E–I) FISH results of five repeated hybridizations of the chromosomes indicated (see Table 2 for details).

than 1% of the cells incorporated BrdU (Fig. 5C), indicating that the majority of CTBs were not proliferating. The rate of hyperdiploid cells as compared with the rate of mitoses suggested that the aneuploidies we observed accumulated in CTBs that had exited S phase of the cell cycle.

Repeated hybridizations reveal additional chromosomal abnormalities

Next, we asked whether the aneuploidies we observed involved chromosomes in addition to X, Y, and 16. Second-trimester CTBs sorted from interval C (Fig. 3A) were first hybridized with a set of three CEP probes (X, Y, and 16)

before they were subjected to repeat hybridizations with four different probe sets that allowed the scoring of 12 different chromosomes (Figs. 5D–I, Table 2). The corresponding DAPI image (Fig. 5D) showed nine aneuploid cells with nuclei that were either well separated or minimally overlapping (Table 2).

Analysis of an additional 68 cells from the same CTB preparation revealed an aneuploidy rate of 97.3%, with only two CTBs showing the number of signals expected from diploid female cells. The number of copies of the autosomes per cell ranged from 1 to 5. Interestingly, none of the chromosomes was completely lost. Again, some of the extra signals appeared in paired arrangements, most notably for

Table 2
Number of hybridization domains in FACS-sorted second-trimester CTBs^a

Cell #	Green Chr.X ^b	Red Chr.16	Green Chr.12	Red Chr.8	Green Chr.17	Red Chr.18	Green Chr.9	Red Chr.10	Green Chr.11	Red Chr.6	Cy5 Chr.3
Cell 1	3	2	2	1	2	1	2	2	4	3	2
Cell 2	2	2	3	3	2	2	1	2	4	2	3
Cell 3	2	2	2	2	1	2	2	2	3	3	2
Cell 4	4	2	2	3	1	2	2	2	2	4	2
Cell 5	4	3	2	4	2	2	2	4	2	2	2
Cell 6	2	2	2	2	2	2	1	2	2	2	2
Cell 7	2	2	2	2	2	2	2	2	3	3	3
Cell 8	3	2	3	2	2	2	2	3	3	3	4
Cell 9	1	2	1	2	2	2	2	2	2	1	2

^a Results are for the nine cells shown in Figs. 5D–I. Cells were sorted from FACS interval C as defined in the legend of Fig. 3A.

^b All the cells were female; no Y signals (CEP Y SpectrumAqua) were detected.

the C-group chromosomes 6, 10, 11, 12, and X. All 10 autosomes and the X chromosome were involved in aneuploidies, albeit at different rates. The average copy number of each chromosome was as follows: chromosome 3, 2.51 ± 0.89 ; chromosome 6, 2.66 ± 0.87 ; chromosome 8, 2.24 ± 0.83 ; chromosome 9, 2.01 ± 0.70 ; chromosome 10, 2.56 ± 0.92 ; chromosome 11, 2.60 ± 0.92 ; chromosome 12, 2.43 ± 1.07 ; chromosome 16, 2.13 ± 0.77 ; chromosome 17, 1.91 ± 0.71 ; chromosome 18, 2.31 ± 0.77 ; and chromosome X, 2.71 ± 1.09 . The gain of extra copies of chromosomes 3, 6, 10, 11, 12, 18, and X was significant ($P < 0.05$) as compared with chromosome 9 (Fig. 6). Chromosomes 3, 6, 11, and X had the highest rates of gain, while chromosomes 9, 16, and 17 had the highest rates of loss (Fig. 6). Overall, X chromosome gains were the most common aneuploidy.

Discussion

The results of this study demonstrate an unexpectedly high rate of aneuploidy among the human CTB subpopu-

lation that exits the cell cycle and invades the uterus. This observation suggests that these aberrations in chromosome number are a normal part of CTB differentiation rather than an anomaly. When we screened 12 chromosomes by repeated hybridizations, we found that almost all the invasive CTBs were chromosomally abnormal, with aneusomies involving X as the most common aberration. This finding suggested that all of the autosomes will likely exhibit gains. Although the significance of this observation is not entirely clear, several interesting possibilities exist. Foremost, we speculate that the accumulation of chromosome gains and losses limits the cells' proliferative and invasive potential. Additionally, this mechanism could also limit the cells' lifespan. To our knowledge, this is the first plausible mechanism for holding these fetal cells with tumor-like properties in check. Additionally, an aneuploid state might provide other selective advantages, such as enhancing protein production. This phenomenon could play an important role in STB function. Amplification at the chromosome level might be functionally analogous to the recently reported existence of copy number polymorphisms in the human genome (Sebat et al., 2004).

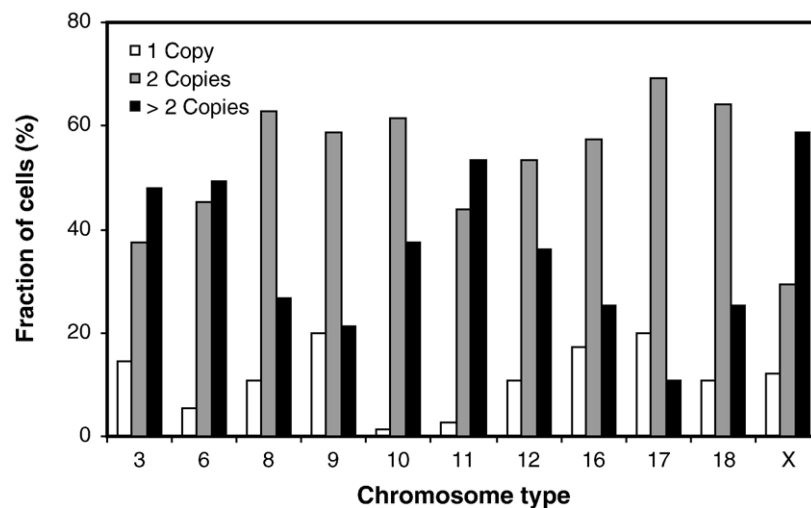


Fig. 6. Gains and losses of 11 chromosomes in 75 interphase cytotrophoblasts isolated from a second-trimester placenta. Repeat hybridizations were performed as described in the legend to Figs. 5E–I.

Interestingly, the copies of chromosome X or other numerically abnormal autosomes often appeared as paired signals separated by variable distances (Figs. 2 and 5). Several lines of evidence suggest that it is very unlikely that the paired arrangements of CEP signals are attributable to replicated centromeric DNA domains. First, during the mitotic cell cycle, the sister chromatids of each chromosome remain connected in the centromeric region until they separate in late metaphase during the transition to anaphase (Koshland, 1994). Thus, the signal pairs obtained by hybridization of CEP probes to chromosomes that have completed replication in late S phase are closely spaced (Boggs and Chinault, 1994; Hultdin et al., 2001; Selig et al., 1992). In comparison, the CEP signals in CTB nuclei are separated by relatively large distances. Second, only one rare condition, Roberts Syndrome, (Musio et al., 2004; Roberts, 1919) is associated with separation of centromeres in metaphase. In contrast, we found numerous paired CEP signals in virtually every CTB sample. Finally, the observation that only a fraction of the autosomal CEP probes generate paired signals in any nucleus provides further evidence in support of the notion that the cells have not completed a normal DNA replication program.

In general, the presence of aneuploid cells in the extraembryonic lineages is thought to be associated with poor pregnancy outcome, even when the chromosomal anomaly is exclusively confined to the placenta (CPM). Approximately 20% of pregnancies complicated by idiopathic intrauterine growth restriction are associated with CPM (Kalousek, 2000; Robinson et al., 1997; Wolstenholme et al., 1994). In these cases, genetic anomalies are detected in CTBs and/or villus stroma (Henderson et al., 1996; Kalousek and Vekemans, 1996), a fact that reflects the different embryologic origins of the two cell types. In many instances, the level of mosaicism detected by CVS does not reflect the level in the term placenta as a whole, which can vary considerably (Henderson et al., 1996). Additionally, abnormal cells can arise in any portion of the placenta, suggesting that CVS, especially in combination with G-banding analyses, underestimates the rate at which this phenomenon occurs. In our studies, we found that more than 50% of invasive (e.g., HLA-G-positive) CTBs in uncomplicated pregnancies were chromosomally abnormal. We note that this population is rarely studied by any means because of the difficulty of obtaining these samples. Thus, it is not surprising that this phenomenon was not described earlier. Here, we used an *in vitro* model of CTB differentiation along the invasive pathway together with tissue biopsies of the maternal–fetal interface to circumvent this problem.

Endoreduplication, in which chromosomal duplication takes place in the absence of mitotic events, has been reported in both animals and plants (Hartman and Southern, 1995; Klisch et al., 1999; MacAuley et al., 1998; Malinowski and Maszewski, 1994; Zybina et al., 2000). For example, murine trophoblast giant cell differentiation is

associated with exit from the normal mitotic cell cycle and onset of endoreduplication. In accord with this finding, the number of Barr bodies in rat trophoblasts increases in parallel with the ploidy level (Zybina et al., 1984). Using *in situ* hybridization, Varmuza et al. (1988) showed one hybridization signal per nucleus in both diploid and giant (murine) trophoblast cells, suggesting that sister chromatids, but not homologous chromosomes, remain closely associated during endoreduplication. Studies of human placental specimens suggest that as CTBs invade the uterine wall the cells acquire an optimal ploidy level, whereas highly polyploid cells may form a subpopulation at the border between the maternal and fetal parts of placenta (Wakuda and Yoshida, 1992; Zybina et al., 2002). The majority of these polyploid cells were deemed to be tetraploid by cytometry (Zybina et al., 2002). In contrast, our data, which were generated by using FISH methodologies, show that invasive CTBs are hyperdiploid rather than tetraploid, suggesting that a mechanism other than conventional endoreduplication is responsible for increasing the chromosome number of these cells. Furthermore, application of subtelomere-specific and WCP probes suggested that many invasive CTBs preferentially gained copies of the X chromosome, another observation that points to a potentially novel mechanism.

When do CTBs that invade the uterus become aneuploid? Our previous work shows that in this differentiation pathway, fate specification and cell cycle entry are tightly coordinated. Specifically, CTBs undergo a final mitotic cycle as they enter the cell columns, which are structures that bridge the gap between the chorionic villi and decidua (Fig. 1) (Genbacev et al., 2000). Subsequently, uterine invasion is coordinated with permanent withdrawal from the cell cycle. Here, we showed that the aneuploid cells, which fail to incorporate BrdU, are HLA-G-positive. Additionally, the rate of hyperdiploidy increased with gestational age, most likely reflecting the fact that the population of progenitor cells is largely depleted by mid second trimester as a result of their differentiation to STBs or invasive CTBs. Together, these findings suggest that the aberrations in chromosome number arise during the last mitotic cycle, a conclusion that is bolstered by our *in situ* analyses. The sequestration of the aneuploid cells within the uterine wall likely explains why hyperdiploid CTBs are not found by CVS. Furthermore, the cells' inability to proliferate explains why they are not detected using *in vitro* culture-based karyotyping methods.

The results of this study have several interesting clinical implications. For example, fetal trophoblasts, which can be found in the maternal circulation from the early stages of pregnancy onward, are a potential source of DNA for minimally invasive prenatal diagnosis (Oudejans et al., 2003; Vona et al., 2002). Our data show that, even in uncomplicated pregnancies, HLA-G-positive trophoblasts are chromosomally abnormal. Given their invasive properties, it is likely that a number of these CTBs could reach the

maternal circulation, but there is a high likelihood that they will not have the same chromosomal make-up as the fetus. Additionally, the process by which CTBs become hyperdiploid appears to be random, involving different chromosomes in different cells. Thus, it seems possible that certain gains will be beneficial, while others could be deleterious. In the latter regard, it will be interesting to learn whether particular combinations could trigger apoptosis in a subset of invasive CTBs thus affected, a phenomenon that is associated with certain pregnancy complications, including preeclampsia (DiFederico et al., 1999). Finally, once we understand the mechanisms involved, it may be possible to use this knowledge to trigger, at the whole chromosome level, aneuploidies in other cells, such as tumors, which could limit their replicative capacity.

Acknowledgments

This work was supported by grants from the National Institutes of Health (CA88258, HD30367, HD44313, HD41425, and HD45736), the UCSF Research Evaluation and Allocation Committee, a fellowship from the DOD Prostate Cancer Research Program (DAMD17-03-1-0157), and a Howard Hughes Medical Institute Student Research Fellowship (57001674).

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