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ORIGINAL ARTICLE

Trisomy 21 is Associated with Caspase-2 Upregulation in Cytotrophoblasts at the Maternal-Fetal Interface

Daisy Leon-Martinez[1] · Joshua F. Robinson[2,3] · Tamara Zdravkovic[2,3] · Olga Genbacev[2,3] · Matthew Gormley[2,3] · Michael Mcmaster[2,3] · Susan J. Fisher[2,3] · Katherine Bianco[4]

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Abstract

Impaired placentation is implicated in poor perinatal outcomes associated with Trisomy 21. Earlier studies revealed abnormal cytotrophoblast differentiation along the invasive pathway as a contributing mechanism. To further elucidate the causes, we evaluated Caspase-2 expression at the protein level (immunolocalization and immunoblot) in samples from Trisomy 21 ($n = 9$) and euploid ($n = 4$) age-matched placentas. Apoptosis was investigated via the TUNEL assay. An immunolocalization approach was used to characterize Caspase-3, Fas (CD95), and Fas ligand in the same samples. Caspase-2 was significantly overexpressed in Trisomy 21 placentas, with the highest expression in villous cores and invasive cytotrophoblasts. Immunolocalization showed that Caspase-3 had a similar expression pattern as Caspase-2. Using the TUNEL approach, we observed high variability in the number of apoptotic cells in biopsies from different regions of the same placenta and among different placentas. However, Trisomy 21 placentas had more apoptotic cells, specifically in cell columns and basal plates. Furthermore, Caspase-2 co-immunolocalized with Fas (CD95) and FasL in TUNEL-positive extravillous cytotrophoblasts, but not in villous cores. These results help explain the higher levels of apoptosis among placental cells of Trisomy 21 pregnancies in molecular terms. Specifically, the co-expression of Caspase-2 and Caspase-3 with other regulators of the apoptotic process in TUNEL-positive cells suggests these molecules may cooperate in launching the observed apoptosis. Among trophoblasts, only the invasive subpopulation showed this pattern, which could help explain the higher rates of adverse outcomes in these pregnancies. In future experiments, this relationship will be further examined at a functional level in cultured human trophoblasts.

Keywords Caspase-2 · Trisomy 21 · Placenta · Trophoblasts · Apoptosis

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✉ Katherine Bianco
kbianco@stanford.edu

Daisy Leon-Martinez
Daisy.Leon-Martinez@yale.edu

Joshua F. Robinson
Joshua.Robinson@ucsf.edu

Tamara Zdravkovic
Tamara.Zdravkovic@ucsf.edu

Olga Genbacev
Olga.Genbacev-Krtolica@ucsf.edu

Matthew Gormley
Matthew.Gormley@ucsf.edu

Michael Mcmaster
Michael.Mcmaster@ucsf.edu

Susan J. Fisher
Susan.Fisher@ucsf.edu

[1] Department of Obstetrics and Gynecology, Yale University, New Haven, CT, USA

[2] Center for Reproductive Sciences, Department of Obstetrics, Gynecology and Reproductive Sciences, University of California, San Francisco, San Francisco, CA 94143, USA

[3] The Eli and Edythe Broad Center of Regeneration Medicine and Stem Cell Research, University of California, San Francisco, San Francisco, CA 94143, USA

[4] Division of Maternal-Fetal Medicine, Department of Obstetrics and Gynecology, Stanford University, 300 Pasteur Dr. HH333 MC 5317, Stanford, CA 94305, USA

Introduction

Trisomy 21 (T21), the genetic cause of Down syndrome (DS), occurs in 1 of every 691 births in the USA [1]. The triplication of human chromosome 21, DS is the most common chromosomal disorder of live-born infants. DS is characterized by over 80 clinically distinct phenotypes of varying penetrance and expressivity [2–5]. Moreover, the presence of aneuploid placental cells has been associated with poor pregnancy outcomes [6, 7]. For example, T21-affected pregnancies have higher rates of preeclampsia, intrauterine growth restriction, and stillbirth [8]. However, vast heterogeneity is observed. While many pregnancies continue to term, some end in pregnancy loss before the fetus is viable.

T21 has also been associated with abnormal placentation. Although some maternal serum markers used for the detection of T21 are placental in origin, little is known about the effects of T21 on placental development [9, 10]. Recent literature suggests that T21 placentas are characterized by abnormal trophoblast differentiation and impaired syncytiotrophoblast formation [11–13]. Other studies have demonstrated villous hypovascularity and delayed maturation [14]. Our previous work revealed the compromised ability of T21 cytotrophoblasts (CTBs) to differentiate along the invasive pathway, likely a contributor to poor placentation in this syndrome. Increased apoptosis of invasive CTBs at the maternal-fetal interface (MFI) was also noted [15]. Lastly, in an effort to further understand the molecular mechanisms at play, Bianco et al. investigated differences in global gene expression between T21 and euploid placentas. Caspase-2 (CASP2) was among the genes that were differentially expressed. Specifically, CASP2 expression was fourfold higher in T21 [16]. Due to CASP3's pathway interaction with CASP2 and previous publications by Bianco et al which showed the presence of CASP3 at the maternal-fetal interface [16], we thought to investigate the relationship between CASP2 and CASP3.

Normal placental development and trophoblast invasion is facilitated by apoptosis [17–19]. However, when dysregulated, modulators of the apoptotic pathway have been associated with aberrant trophoblast turnover and placental pathologies [17, 20–24]. Increased apoptosis has been found in pregnancies complicated by fetal growth restriction [25], preeclampsia [26], and premature rupture of membranes (PPROM) [27] and thereby thought to be a key mechanism in placental dysfunction. Placental apoptosis may be initiated by a variety of stimuli, including hypoxia and oxidative stress. For a table of the various factors that control placental apoptosis in health and disease, see Sharp et al, 2010 [28]. Members of the cysteine-aspartic acid protease family play a central role in apoptosis. One such molecule with important contributions is CASP2. In prior studies, CASP2 is associated with apoptosis-related cytoskeleton degradation, oxidative stress-induced cell death, and cell cycle regulation [29–32]. However, data are lacking regarding the role of CASP2 in placentation. Therefore, this study aimed to further characterize CASP2 expression in T21-affected pregnancies in the context of other molecules that function in the apoptotic pathway. Ultimately, better understanding of the pathophysiology of T21 can clarify the impact of placental abnormalities on fetal development.

Materials and Method

Study Subjects and Biological Specimen Collection

This study was approved by the Committee of Human Research at the University of California, San Francisco. Volunteers provided written informed consent in accordance with institutional guidelines for the protection of human subjects prior to sample collection. Healthy women without medical complications were recruited from University of California, San Francisco, at the time of voluntary pregnancy termination. Human placental specimens were collected (T21, $n = 9$, 12-23 weeks; Euploid, $n = 4$, 13–22 weeks). Ten basal plate biopsies (5 × 5 mm) were taken from each placenta, rinsed in PBS, snap frozen in liquid nitrogen, and stored at− 80 °C per previously published protocol [33].

Immunolocalization

Placental biopsies were taken from the center and the periphery. At least two specimens were obtained from each area. It is our protocol to do comparative histology of the placenta by H&E [15, 34]. Subsequently, for double-indirect immunolocalization, placental samples were processed as described previously [33]. Briefly, tissues were fixed in 3% paraformaldehyde for 30 min, infiltrated with 5–15% sucrose, and frozen in optimal cutting temperature (OCT) medium. A Leica cryostat (CM3050S, Leica Microsystems, Inc., Buffalo Grove, IL) was used to cut 5 μm frozen sections of OCT-embedded samples, which were stored at− 20 °C overnight. Tissue sections were washed three times in PBS for 5 min each then placed in 5% BSA/0.1% Tween-20 blocking solution for 45 min. Sections were incubated at 37 °C for 1 h with a mixture of two primary antibodies (rat antihuman cytokeratin to label CTBs and an antibody produced in a different species against the antigen of interest) serving as a positive and negative control, respectively (data not shown). Slides were washed, and binding of primary antibodies was detected using species-specific secondary antibodies

conjugated to fluorescein or rhodamine. The antibodies, catalogue numbers, sources and dilutions employed are listed in Table 1. Samples were washed in PBS, rinsed in dH_2O, dried, and mounted using ProLong Gold Antifade Mountant with DAPI (Life Technologies, Grand Island, NY). Immunoreactivity was detected by using a Leica DM 5000B fluorescence microscope equipped with a Leica DFC 350FX digital camera.

Scoring of Immunoreactivity

A semi-quantitative analysis of CASP2 expression and TUNEL positivity was evaluated with a scoring system previously described by our laboratory.[24] T21 and age-matched euploid placental specimens were analyzed in parallel. Briefly, we scored the relative strength of the fluorescence signal in each of the following placental structures: floating villous CTBs, floating villous mesenchyme, anchoring villous mesenchyme, CTB cell columns, and CTBs in the basal plate. A minimum of two tissue sections were analyzed per placenta. Twenty fields were randomly selected and scored for each tissue section (10× magnification). Scores were assigned as follows: 0 (fluorescence not detected); − (0–5% of cells were immunoreactive); + (5–15% of cells are immunoreactive); ++ (15–30% of cells are immunoreactive); +++ (30–50% of cells are immunoreactive) (see Figures 1G and 3I).

Immunoblotting

Immunoblotting of placental tissue biopsy specimens was performed as previously described [36]. Briefly, protein was extracted from placental specimens using tissue lysis buffer (NaCl 150 mM, Tris 50 mM, EDTA 2 mM, NP-40 1%). Tissue extracts underwent three freeze-thaw cycles. Then, the lysates were centrifuged at 800 rpm for 5 min to remove insoluble materials. Samples containing equal amounts of protein were mixed with SDS-PAGE sample buffer and electrophoretically separated. After the proteins were transferred to nitrocellulose, the membranes were incubated first with primary antibody,then with peroxidase-conjugated secondary antibodies. Immune complexes were visualized by using enhanced chemiluminescence and Hyperfilm (Amersham Life Sciences-USB, Arlington Heights, IL). The entire experiment was done in triplicate.

Results

T21 Was Associated with Overexpression of CASP2 at the Maternal-Fetal Interface

As previously published by our group, Bianco et al (2016) showed that global expression profiling of human placental biopsies that included the uterine attachment sites showed a four-fold increase in CASP2 expression in T21 samples compared to age-matched euploid control. qRT-PCR analyses validated this observation [16, 37]. To determine whether these differences held at the protein level, CASP2 expression was characterized by immunolocalization and immunoblot approaches. Immunoreactivity was present in the cytoplasm of villous trophoblasts (CTBs and syncytiotrophoblasts) and extravillous CTBs (Fig. 1C, and F). A semi-quantitative assessment was used to compare relative expression of CASP2 in five placental compartments— floating villous CTBs, floating villous mesenchyme, anchoring villous mesenchyme, CTB cell columns, and CTBs in the basal plate. In euploid samples, weak CASP2 immunoreactivity was detected (Fig. 1C). In relative terms, more intense signals were detected in association with the T21 placentas. CASP2 expression was highest in the villous cores (mesenchyme) of floating and anchoring villi (Fig.1G). Furthermore, a decreasing gradient of immunoreactivity was observed in the cell columns, whereby expression was highest in the proximal region and decreased distally near the uterine wall. Immunoblot analyses of the MFI from euploid and T21 pregnancies revealed a ~ 35 kDa band that corresponded to the estimated molecular weight of CASP2 [38]. With the amount of protein that was loaded on the gel (10 μg), bands were visible only in the T21 samples, and the levels varied relative to β–actin [Fig. 1H (left and middle panels)]. This result was in accord with our previous report of wide variations in pathological alterations at the MFI in T21 samples [24]. These results are summarized and quantified in Fig. 1H (right panel). A Mann-Whitney test indicated that the T21 specimens exhibited a 46-fold higher

Table 1 Primary antibodies

Target protein	Catalog #	Species/source	Dilution
B-actin	A4700, clone AC-40	Mouse/Sigma-Aldrich, St Louis, MO	1:100
Caspase-2	ab2251	Rabbit/Abcam, Cambridge, MA	1:100
Caspase-3	ab32042	Rabbit/Abcam, Cambridge, MA	1:50
Cytokeratin (7D3)	N/A	Rat/Fisher Lab*	1:100
Fas/CD95	ab126821	Mouse/Abcam, Cambridge, MA	1:100
Fas Ligand	ab16104	Mouse/Abcam, Cambridge, MA	1:100

*Produced by the Fisher Lab. See reference for further detail [35]

expression of CASP2 as compared to euploid samples, p = 0.05, confirming the expression pattern predicted by the transcriptomic analyses at the protein level.

Expression of the Effector CASP3 at the MFI Mirrored that of CASP2

CASP2 and CASP3 have been associated with apoptosis in other cell types, such as hematologic blood stream and neuroectoderm [39–43]. For this reason, an immunolocalization approach was used to investigate the effector caspase, CASP3, in the same placental regions. CASP2 (Fig. 2C and G) and CASP3 (Fig. 2D and H) had very similar patterns of immunoreactivity at the MFI in euploid and T21 specimens. As with CASP2, expression of CASP3 was consistently highest in the villous core. Immunoreactivity was also observed in association with invasive CTBs. However, only a portion of the columns stained. Immunoblot analyses confirmed enhanced expression of CASP3 (~ 29 kDa) in T21 samples relative to euploid specimens [Fig. 2J (left and middle panels)]. Again, intersample variation was noted in the setting of aneuploidy. Interestingly, samples that had high CASP2 levels (Fig. 1H) tended to have lower CASP3 levels (Fig. 2J) and vice versa. Overall, a 17-fold increase in CASP3 expression was observed in the T21 samples, p = 0.05 [Fig. 2J (right panel)].

T21 was Associated with Greater Levels of CTB Apoptosis in the Cell Columns and Within the Uterine Wall

Apoptosis was assessed in age-matched T21 and euploid placental specimens via the TUNEL assay (Fig. 3A through H). A semi-quantitative approach was used to compare the numbers of TUNEL-positive cells in five placental compartments— floating villous CTBs, floating villous mesenchyme, anchoring villous mesenchyme, CTB cell columns, and CTBs in the basal plate (Fig. 3I). Consistent with our previous findings, significant variability was observed in the number of apoptotic cells in various regions of the same placenta and among different placentas [24]. However, T21 tissue specimens had significantly greater numbers of apoptotic cells. The placental compartments that were most affected were the cell columns and basal plate (Fig. 3I). CASP2 expression, which was assessed in the same samples, colocalized with TUNEL reactivity only in the cell columns and basal plate (Fig. 3C and D, G and H). No double-positive cells were observed in the villous cores. This finding implied that CASP2 may activate CTB apoptosis as the cells emigrate from the placenta to the uterus.

Given that T21 is associated with an increased abundance of apoptosis (as demonstrated by TUNEL assay), we used an immunolocalization approach to investigate the expression of Fas (CD95) and Fas Ligand (FasL), which are also associated with apoptosis and known to be expressed in this region [44, 45]. Among the CASP2-positive cells (Supplemental Fig. 1C and G), Fas (CD95), expression was most often detected in association with a subset of CTBs within the uterine wall (Supplemental Fig. 1D and H). Investigation of Fas (CD95) expression in relation to FasL immunoreactivity revealed colocalization of the receptor-ligand pair among the extravillous CTBs (Supplemental Fig. 1K, O and L, P). These results suggested that the consequences could include initiation of apoptosis.

Comment

Down Syndrome, also known as T21, is characterized by abnormal placentation. In the early stages of pregnancies complicated with T21, aberrations may result in miscarriages and, later on, the great obstetrical syndromes such as IUGR/preterm birth and hypertensive disorders of pregnancy [6, 16]. Since these aneuploidies are associated with an increased risk of pregnancy complications, we were also interested in learning whether the results of our analysis might add to the understanding of the etiology of syndromes such as preeclampsia and preterm birth [34, 46]. In terms of clinical implications, given that the development of the placenta is linked to gestational age [47], chorionic villi sampling could provide the discrete amount of tissue needed for the assays we performed. In our study, the youngest gestational age was 15 weeks; however, previous publications have shown a variety of immunohistochemistry, and immunoblotting experiments can be achieved as early as 12 weeks' gestation [13, 48]. Future studies should stratify by gestational age; however, we anticipate the majority of the changes would be seen after 24 weeks. Recent work by our group showed that aberrant CTB differentiation and dysregulated apoptosis at the MFI may contribute to impaired uterine invasion [16, 24]. In the present study, with the use of immunostaining and immunoblotting, we demonstrated for the first time that the apoptotic marker CASP2 was overexpressed in T21 placentas. Furthermore, the majority of cells expressing CASP2 also expressed cytokeratin (CK), an established trophoblast marker [49]. Specifically, the invasive trophoblast lineage was most affected, with the highest level of CASP2 expression noted in the mesenchyme of floating and anchoring villi, followed by the cell columns of anchoring villi and finally the basal plate. This pattern of expression spatially recapitulated the path along which trophoblast progenitor cells residing in the villous core migrate out and differentiate into the invasive phenotype, ultimately entering the uterine wall [35, 50–55]. More importantly, it suggested that CASP2 misregulation occurs at the MFI of T21 placentas.

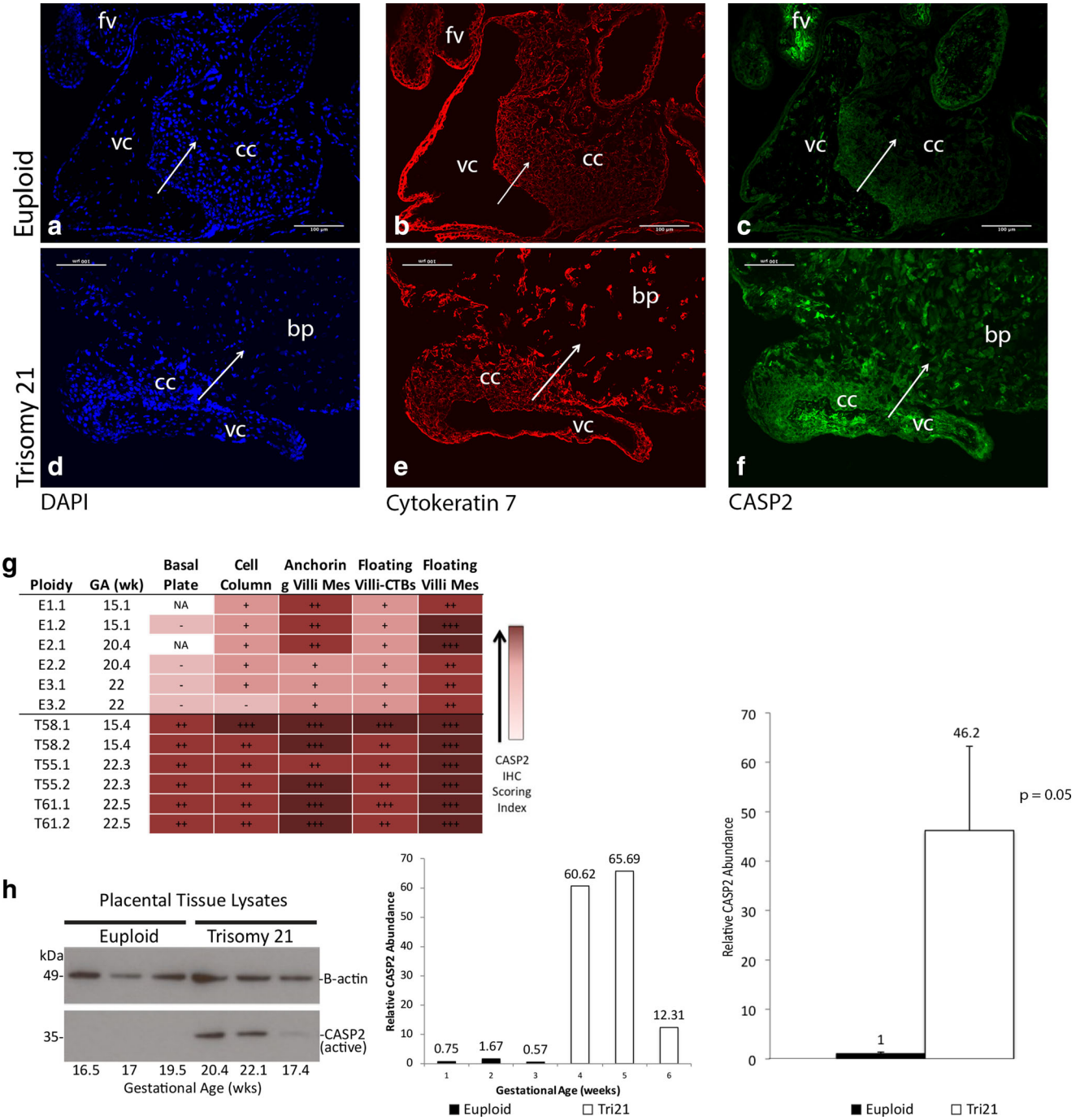
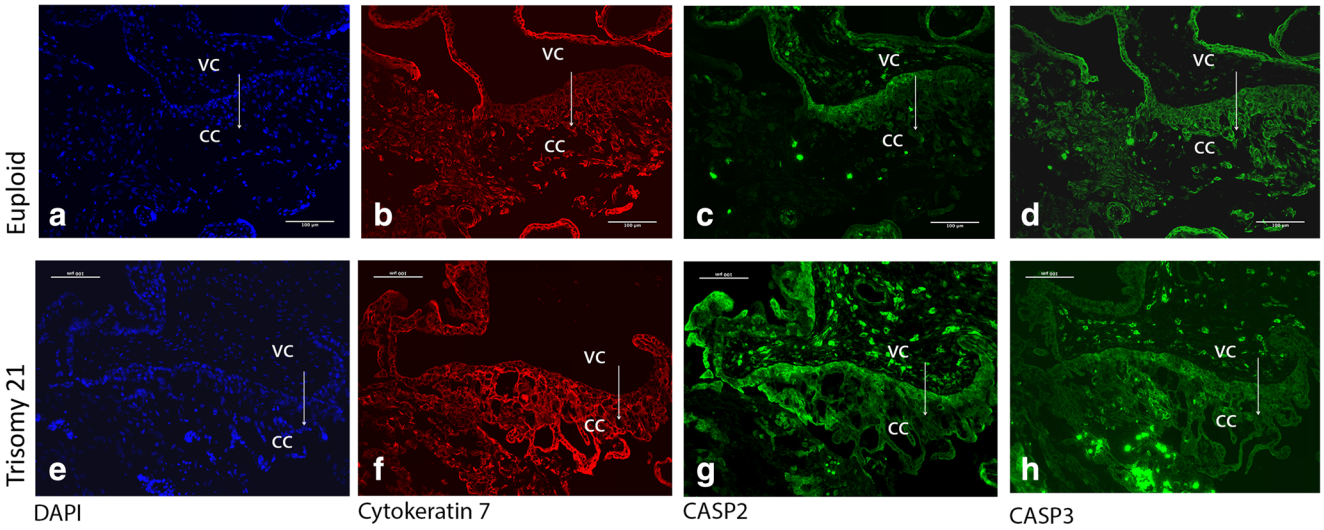


Figure 1 In T21, CASP2 expression was upregulated in the villous cores and among invasive CTBs. CASP2 expression was confirmed by qRT-PCR (not shown) and by immunolocalization (A–F) and immunoblot (H). Overall, overexpression of CASP2 was observed in T21 placentas compared to euploid placentas. Semi-quantitative assessment used to compare relative expression of CASP2 in five placental compartments: floating villi cytotrophoblasts (CTBs), floating villi mesenchyme, anchoring villi mesenchyme, cell columns, and basal plate (G). Degree of CASP2 presence is indicated by red intensity and crosses (+). CASP2 was present in the cytoplasm of villous trophoblasts (TBs) (CTBs and syncytiotrophoblasts) and extravillous TBs with greatest expression in the villous core. Quantification of CASP2 expression by immunoblot demonstrates greater abundance in T21 placentas as compared to euploid placentas (H). Arrows indicate direction of CTB invasion. Abbreviations: *vc* villous core, *cc* cell column, *bp* basal plate, *fv* floating villi. Scale bar = 100 μm

CASP2 mediates apoptosis in response to such insults as DNA damage, administration of TNF, and exposure to pathogens and viruses [39, 56–59]. Although its role in the apoptotic pathways is not yet fully defined, it is believed to have an amplifying rather than an effector function [59]. Our investigation suggests that CASP2 misregulation may mediate cell death in T21 placentas. As with CASP2, T21 placental specimens were noted to have an overabundance



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Ploidy	GA (wk)	Basal Plate	Cell Column	Anchoring Villi Mes	Floating Villi-CTBs	Floating Villi Mes
E10a	15.1	-	+	+	+	++
E10b	15.1	-	-	+	+	++
E17a	17	-	NA	NA	-	+
E17b	17	-	NA	NA	-	+
E12a	22	NA	NA	NA	-	+
E12b	22	NA	-	-	-	+
T22a	13.3	++	+	+++	++	+++
T22b	13.3	+	+	+++	++	+
T25a	17.6	++	+	++	++	+++
T25b	17.6	++	+	+++	++	++
T19a	20.4	NA	NA	NA	+	++
T19b	20.4	NA	NA	NA	+	++

Casp3 IHC Scoring Index

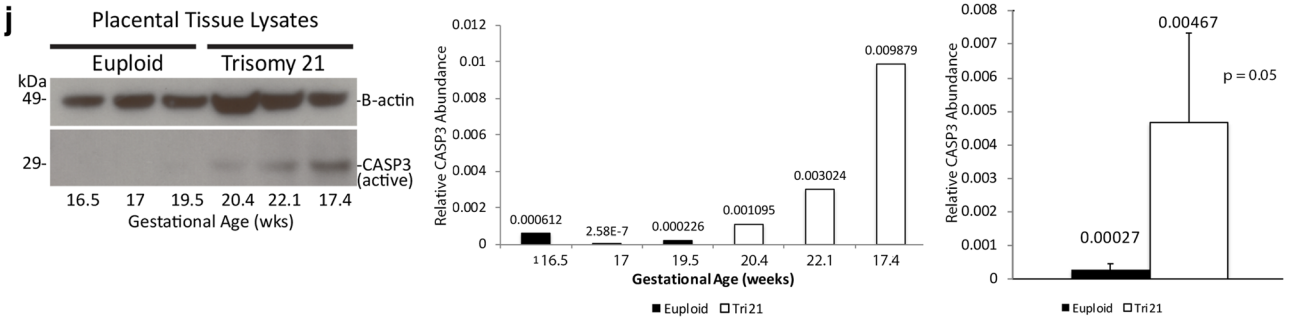
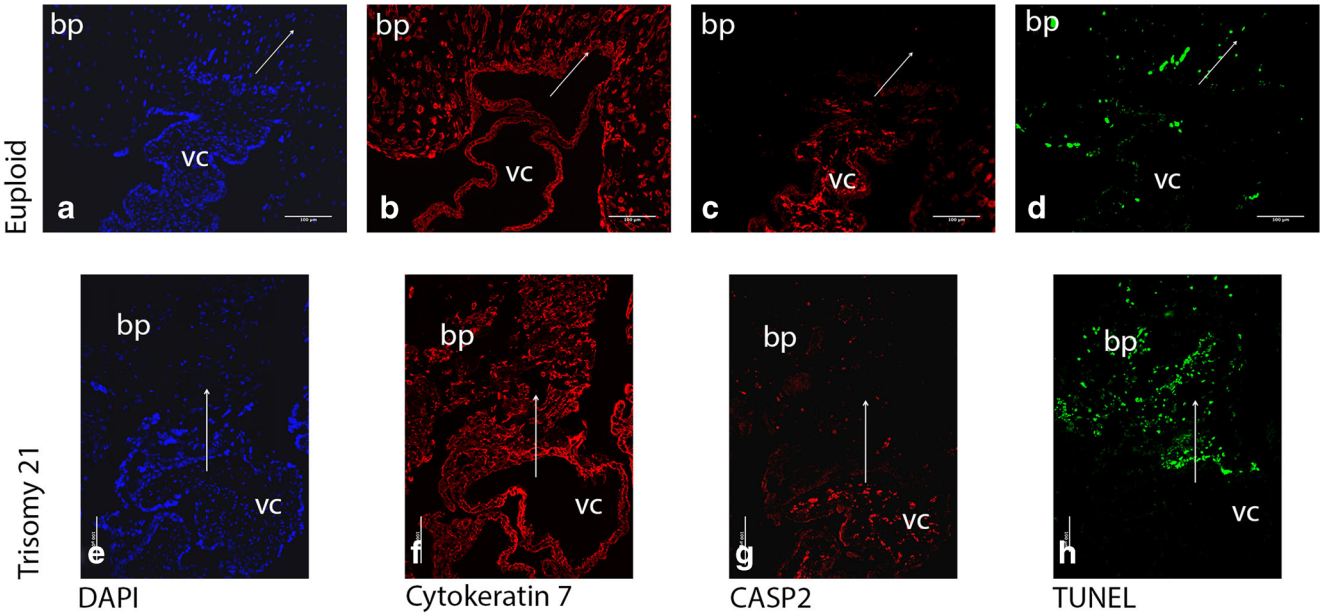


Figure 2 Expression of effector CASP3 mirrored that of CASP2 at the maternal-fetal interface. CASP2 and CASP3 expression was confirmed by immunolocalization (A–H) and immunoblot (J). Expression pattern of both proteins was very similar with the greatest level of immunoreactivity in the villous core. A gradient of expression was observed in the cell columns—highest in most proximal CTB and decreasing in CTBs most proximal to the uterus (basal plate). Overall, overexpression of CASP3 was observed in T21 placentas compared to euploid placentas. Semi-quantitative assessment used to compare relative expression of CASP3 in five placental compartments: floating villi cytotrophoblasts (CTBs), floating villi mesenchyme, anchoring villi mesenchyme, cell columns, and basal plate (I). Degree of CASP3 presence is indicated by red intensity and crosses (+). CASP3 was present in the cytoplasm of villous trophoblasts (TBs) (CTBs and syncytiotrophoblasts) and extravillous TBs with greatest expression in the villous core. As demonstrated by immunoblot, CASP2 and CASP3 protein abundance was greater in T21 than euploid placental specimens (Fig. 1H, Fig. 2J). Arrows indicate direction of CTB invasion. Abbreviations: *vc* villous core, *cc* cell column. Scale bar = 100 μm

of the effector caspase, CASP3, relative to euploid controls. Moreover, both had similar expression patterns at the MFI, with the greatest protein levels at the villous cores and decreasing expression as cells approached the uterine wall. This suggests that CASP2 is associated with progression of the apoptotic pathway in invasive CTBs. Further supporting



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Ploidy	GA (wk)	Basal Plate	Cell Column	Anchoring Villi Mes	Floating Villi-CTBs	Floating Villi Mes
E-1	15.1	+	+	-	+	-
E-3	17	+	-	-	+	-
E-4	20.4	-	-	-	+	-
E-5	22	+	-	-	+	-
T21-1	20.4	+++	NA	NA	+++	++
T21-2	17.2	+++	+++	++	++	++
T21-3	22.3	+++	+++	+	++	+
T21-4	15.4	++	++	+	++	+
T21-5	22.5	+++	+++	+	++	+

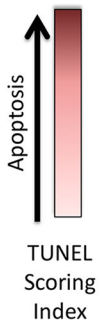

Figure 3 In T21, the uterine wall has a greater level of apoptotic CTBs. Age-matched euploid and T21 placental biopsies were assessed for the presence of apoptotic cells using the TUNEL assay. High variability in the number of apoptotic cells was observed among biopsies from different regions of the same placenta and between biopsies from different placentas. However, T21 placentas had more apoptotic cells—specifically in the cell columns and basal plates (D, H). CASP2 expression colocalizes with TUNEL in the cell column and basal plate. Arrows indicate direction of CTB invasion. Degree of TUNEL presence is indicated by red intensity and crosses (+). TUNEL was present in the basal plate and cell column (I). Abbreviations: *vc* villous core, *cc* cell column, *bp* basal plate. Scale bar = 100 μm

this idea, placental specimens with high CASP2 content had low CASP3 content and vice versa. This may suggest that CASP2 functions upstream of CASP3—early stages of apoptosis —have an overabundance of CASP2, while later stages of the apoptotic pathway will have a predominance of CASP3. Collectively, these findings support prior studies that report CASP2 activation of CASP3 leading to cell death [42, 43]. Interestingly, protein quantification by immunoblot revealed that although both CASP2 and CASP3 were overexpressed in T21 specimens, the degree of overabundance of CASP2 was greater than that of CASP3. This suggests that not all cells with CASP2 misregulation commit to completing the apoptotic pathway.

Fas (CD95) and Fas Ligand (FasL) expression patterns at the MFI further elucidated the potential contribution of CASP2 to placental pathology in T21. A membrane-associated receptor, Fas (CD95) is the best-characterized member of the TNF superfamily of receptors. When activated by FasL, Fas (CD95) triggers the extrinsic apoptotic pathway [45, 60, 61]. In the placental specimens studied, Fas (CD95) and FasL expression was confined to a subset of CASP2-positive CTBs within the uterine wall. Therefore,

consequences could include initiation of apoptosis within this defined CTB population. Further supporting this conclusion is the observation that TUNEL-positive cells (undergoing cell death) were also largely limited to CTBs within the uterine wall.

Increased expression of Fas (CD95) and FasL has been previously associated with trophoblast apoptosis in perinatal complications such as missed miscarriage and recurrent pregnancy loss [62–64]. Similarly, trophoblast in vitro studies showed that proinflammatory cytokines (e.g., IFN-g and TNFa) promote increased Fas (CD95) expression and sensitivity in trophoblasts, leading to apoptosis and ultimately pregnancy failure [65]. Furthermore, Olsson et al. have shown that CASP2 facilitates apoptosis in DNA-damaged cells via activation of Fas (CD95) [66]. Taken together, it is possible that in T21-affected placentas, one of the effects of CASP2 may be to increase Fas (CD95) expression and activity, ultimately leading to trophoblast apoptosis.

It is important to recognize that the strength of our findings may be limited by the small number of placental specimens available. As previous studies have shown, marked variation exists in the amount of apoptosis observed both within and between specimens. It is also important to note that this study was completed using placental tissue biopsies. Therefore, specimens are composed of mixed cell populations that are both maternal and fetal in origin. It is possible that some findings may be representative of cell populations other than the invasive cytotrophoblasts (CTBs).

Ultimately, this study provides evidence that CASP2 misregulation in T21 trophoblasts is associated with placental pathology. Although these results do not definitively describe the molecular pathways by which it happens, they give support to the idea that CASP2 plays an essential role in increased trophoblast apoptosis leading to poor invasion of decidua. Furthermore, this study raises the question of why the great majority of the observed cell death occurs in the uterine wall. The interplay between maternal and fetal cells, specifically the mechanism behind aberrant placentation, poses a challenge to future research and merits further investigation. This may be the key to understanding the various perinatal complications associated with this common aneuploidy. More importantly, it may lead to new ways to treat or prevent such complications, perhaps via small molecule inhibitor of CASP2 or other targets.

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Compliance with Ethical Standards

Conflict of Interests The authors declare that they have no conflict of interest. Susan J. Fisher was a consultant for Verinata Health, Inc an Illumina Company. Katherine Bianco was a consultant for SeraCare Life Science Inc.

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