Trophoblast lineage-specific differentiation and associated alterations in preeclampsia and fetal growth restriction

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Abstract

The human placenta is a poorly-understood organ, but one that is critical for proper development and growth of the fetus in-utero. The epithelial cell type that contributes to primary placental functions is called "trophoblast," including two main subtypes, villous and extravillous trophoblast. Cytotrophoblast and syncytiotrophoblast comprise the villous compartment and contribute to gas and nutrient exchange, while extravillous trophoblast invade and remodel the uterine wall and vessels, in order to supply maternal blood to the growing fetus. Abnormal differentiation of trophoblast contributes to placental dysfunction and is associated with complications of pregnancy, including preeclampsia (PE) and fetal growth restriction (FGR). This review describes what is known about the cellular organization of the placenta during both normal development and in the setting of PE/FGR. It also explains known trophoblast lineage-specific markers and pathways regulating their differentiation, and how these are altered in the setting of PE/FGR, focusing on studies which have used human placental tissues. Finally, it also highlights remaining questions and needed resources to advance this field.

Keywords

Placenta; Cytotrophoblast; Extravillous trophoblast; Syncytiotrophoblast; trophoblast stem cells

Introduction

Trophoblast (derived from the Greek word "tropho," meaning to feed) is the epithelial cell in the placenta, a transient organ which plays a pivotal role in fetal growth and development during pregnancy [1]. Trophoblast are derived from trophectoderm (TE), the cells on the outer part of the blastocyst-stage embryo. In human, the TE expands during the early post-implantation period to form a shell around the embryo, composed mostly of proliferative cytotrophoblast (CTB), the putative trophoblast "stem" cell in the placenta [1,2]. CTB subsequently differentiate down two main lineages: in floating villi, they fuse to form syncytiotrophoblast (STB), which form the main barrier against pathogens while also serving as the gas and nutrient exchange interface, while in anchoring villi, they undergo a modified form of epithelial-to-mesenchymal transition to differentiate into invasive extravillous trophoblast (EVT) [1–3]. The latter are composed of two subtypes of cells, one of which invade deeply into the uterine wall as groups of cells, termed interstitial EVT, and another which remodel and line decidual arterioles as endovascular EVT, allowing for maternal blood flow to be established into the intervillous space [1]. Differentiation into both these lineages is important for development of a fully-functioning placenta, which can support growth of the fetus in utero. Abnormal differentiation and function of these two lineages has been associated with pregnancy disorders, including miscarriage, preterm birth, preeclampsia (PE), and fetal growth restriction (FGR) [4,5]. This review will focus on describing trophoblast subtypes as organized within the normal placenta, and discuss how the organization of these lineages is disturbed in pregnancy complications, with a focus on placental insufficiency associated with PE and FGR. Finally, it will also highlight gaps in knowledge, where more research is needed in understanding the relationship between abnormal trophoblast differentiation and placental function.

Trophoblast lineages and their organization within the placenta

Two commonly-used markers for distinguishing villous (CTB and STB) and extravillous trophoblast (EVT) are EGFR and HLAG, respectively [6]. Early in gestation, the trophoblastic shell surrounding the embryo is composed of primary villi [1–3], whose trophoblast is highlighted by EGFR, as well as multiple layers of HLAG+ EVT, among which are abundant multinucleated giant cells (Figure 1a–b). The origin of these giant cells has been debated: on one hand, their location within the EVT layers and increasing numbers within the uterine myometrium with increasing gestation [7] suggest they are part of this lineage. On the other hand, the predominant expression of EGFR in these cells suggests that, at least in early gestation, they are in fact of villous origin, potentially representing STB fragments, entrapped within the EVT shell (Figure 1c–d).

Later in gestation, EGFR and HLAG staining continue to represent villous and extravillous lineages, respectively. EGFR marks the trophoblast surrounding the floating villi, but also marks the layer of trophoblast at the chorionic plate (fetal surface), while HLAG primarily highlights EVT at the basal plate (maternal surface), but also marks EVT present just below the chorionic plate and surrounding some villi within intraplacental trophoblast islands (Figure 2a–c). Interestingly, while the majority of EVT at the basal plate express HLAG, occasional multinucleated giant cells can be seen that are double-positive for EGFR and

HLAG (Figure 2d), indicating a potentially different cell-of-origin of these cells in later gestation placenta.

Within the EGFR$^+$ villous trophoblast, CTB stem cells, visualized by TP63 immunoreactivity, are abundant in early gestation, comprising a layer that is adjacent to the basement membrane (Figure 3a) [8]. Little is known about the heterogeneity of this cell layer, although single cell RNAseq analysis is beginning to shed some light on this subject [9–11]. Our own work has pointed to one marker, CDX2, which appears to highlight a subset of these villous CTB (Figure 3b). In normal gestation, CDX2$^+$ CTB are present mostly near the chorionic plate and disappear after first trimester [12]. The CTB layer itself starts to become discontinuous in the second trimester; by term, only 1–2 CTB can be found per terminal villus [8]. This is reflected in a decrease in PCNA staining over gestation, as CTB are the proliferative cells within the trophoblastic compartment [13].

While there are likely many more human trophoblast subtypes than described above, few have been described or characterized, beyond mere identification with a single marker, and fewer still have been functionally defined. Nevertheless, in the remainder of this review, we will describe changes in trophoblast differentiation and related regulatory pathways, as revealed by a combination of morphology, lineage-specific marker expression, and functional studies (where available), associated with disorders of placental insufficiency, with a focus on PE and FGR.

Preeclampsia and Fetal Growth Restriction: Clinical Synopsis

Pre-eclampsia (PE) is a common pregnancy disorder that is characterized by new-onset hypertension and proteinuria in the latter half of pregnancy, and affects 5–8% of all pregnancies worldwide [14–16]. The incidence of PE in the United states has been on the rise since the 1990s, and it is currently one of the leading causes of maternal and neonatal morbidity and mortality [14]. PE is a heterogeneous disease, and can manifest itself at different gestational ages, with varying degrees of severity. Recently, a "two-stage" model has been developed to describe the underlying pathophysiology, with the first stage being deficient placentation and second stage characterized by systemic vascular inflammation [17]. PE, particularly severe early-onset PE, presenting before 34 weeks gestational age, can be accompanied by fetal growth restriction (FGR), defined as birthweight less than 10th percentile adjusted for sex and gestational age [18]. However, FGR can also be present in isolation, without a simultaneous maternal syndrome [5, 18,19]. While FGR can also be multifactorial, PE and isolated FGR have in common, both some clinical features, including abnormal umbilical doppler studies, as well as underlying etiologies, including defective placentation and systemic maternal vascular dysfunction [5,19]. Both PE and FGR are often associated with a small placenta at delivery, and show various combinations of villous hypermaturity, infarction, and decidual vasculopathy, on histopathologic examination [1,2]. Recent studies have used a combination of gene expression profiling and histopathology to further separate PE and FGR into unique subtypes, with the potential to identify biomarkers with improved diagnostic accuracy [20–23]. However, trophoblast lineage-specific differentiation has yet to be described in these different disease subtypes. Below, we will describe abnormalities in both villous and extravillous trophoblast associated with PE/FGR.

Abnormalities of villous trophoblast associated with PE/FGR

Normal villous trophoblast development involves regular branching of the villous tree, with fusion of underlying CTB into the overlying syncytium within these structures [2]. The process of fusion is regulated through Glial Cells Missing-1 (GCM1), a transcription factor, which is expressed in a subgroup of CTB, induces cell cycle arrest, and mediates cell-cell fusion through induction of SYNCYTIN genes [24, 25]. Downregulation of GCM1 in denuded floating villi has been shown to promote CTB proliferation and inhibit syncytial regeneration [26]. Syncytialization involves induction of two fusogenic genes, SYNCYTIN 1 and 2, in human chorionic villi, both of which were acquired from human endogenous defective retroviruses [27,28]. The syncytium undergoes significant turnover through shedding of a combination of extracellular vesicles and microparticles (sometimes referred to as trophoblast "deportation"), which, over time, with decreased fusion of underlying CTB, results in formation of nuclear aggregates, called syncytial knots [29]. The number of syncytial knots increases with increasing gestational age, and is thus a reflection of villous maturity [30]. Syncytial knots are composed primarily of condensed nuclei with transcriptionally inactive heterochromatin [31]. The presence of syncytial knots in normal term placentas is also associated with a small spike in TUNEL staining, a further reflection of placental aging [13].

PE/FGR placentas often show poor villus branching, described as "distal villous hypoplasia," accompanied by increased numbers of syncytial knots adjusted for gestational age, consistent with "accelerated villous maturation" [32]. The latter changes were originally described by Tenney and Parker in 1940, as degenerative changes associated with "toxemia of pregnancy," and hence are often referred to in the literature as "Tenney-Parker changes" [33]. These changes are thought to arise due to more turbulent flow of maternal blood into the intervillous space of PE/FGR placentae, resulting in increased oxidative stress [34]; in fact, syncytial knots are positive for markers of oxidative damage, such as 8-oxo-deoxyguanosine [31]. The syncytial knots are also at least one source of circulating soluble VEGF receptor-1 (sVEGFR-1, also known as sFlt-1), an anti-angiogenic protein, whose increased expression has been associated with, and indeed may be diagnostic for, PE [35–37]. The syncytial knots in PE/FGR also show a decrease in the anti-apoptotic BCL-2 protein and an increase in cell death, as measured by TUNEL staining [38]. The increase in cell death has been attributed to caspase-independent apoptosis [39], increased levels of endoplasmic reticulum stress [40], as well as excessive autophagy [41,42]. The increase in syncytial knots and cell death leads to discontinuity of the syncytium, resulting in leakage of fetal proteins into maternal circulation [43]. It also likely leads to increased thickness (~two-fold) of the vasculosyncytial membrane, the exchange interface of chorionic villi, thus negatively affecting fetal growth [44]. Finally, at least in early-onset PE, this increase in syncytial knots and apoptosis is also associated with an increase in STB turnover/shedding, with over two-fold increase in circulating STB-derived microparticles, compared to gestational age-matched control cases [45].

At the molecular level, these alterations in STB morphology and function have been attributed to alterations in the GCM1-SYNCYTIN pathway, regulating syncytialization and STB organization. Specifically, both GCM1 and SYNCYTIN-1 proteins have been found to

be reduced in PE placentae [46,47], with SYNCYTIN-1 also being mis-localized to the apical, instead of the basal, membrane in STB [47]. Hypoxia has been proposed as the potential mechanism for GCM1 downregulation, acting through suppression of the PI3K-AKT signaling pathway to activation of GSK3β, with subsequent phosphorylation of GCM1, targeting it for ubiquitination and degradation [48]. A reduction in GCM1 leads to a decrease in SYNCYTIN expression, resulting, in turn, in reduced fusion and an altered syncytium.

Other functions that have been attributed to SYNCYTIN include both promotion of cell proliferation [49] and inhibition of apoptosis [39]. However, while the latter is consistent with the STB phenotype noted in PE, PE-associated alterations in villous CTB proliferation have been somewhat controversial. Intuitively, it is easy to assume that accelerated maturation must be accompanied by a loss in CTB proliferation. However, results of studies evaluating CTB proliferation in PE/FGR range from no differences in CTB proliferation [50,51], region-specific alterations in CTB proliferation [52], to an increase in CTB proliferation attributed to a damaged syncytium in need of repair and regeneration [53,54]. Given the heterogeneity of PE and FGR, as recently elaborated on by genome-wide expression profiling of placental tissues associated with these complications [20–23], it is possible that distinct subclasses of these diseases have different alterations at the cellular level, including differences in CTB proliferation, depending on the underlying pathophysiology.

Abnormalities of extravillous trophoblast in PE/FGR

Extravillous trophoblast (EVT) differentiate from CTB precursor cells within anchoring chorionic villi at the basal plate. This process occurs within trophoblast cell columns, where CTB gradually lose expression of EGFR and gain HLAG expression [6]. This process occurs early in gestation, in a relatively hypoxic microenvironment [55] and, in fact, is regulated by the hypoxia-inducible complex (HIF), at least in its initial transition from CTB to EVT [6]. HIF, in turn, induces expression of transcription factors ASCL2 and TEAD2, which are specifically expressed in EVT in the human placenta [6]. Hypoxia also induces NOTCH1, which inhibits expression of transcription factors involved in CTB self-renewal, including TEAD4 and TP63, represses syncytialization, and promotes proliferation and survival of EVT precursor cells [56]. GCM1 is another transcription factor induced during EVT differentiation, although it does not appear to be regulated by HIF in this context [6]. Because GCM1 is also involved in STB differentiation of CTB progenitor cells [24–26], cellular context/microenvironment likely plays a key role in distinguishing which lineage is induced downstream of GCM1 expression.

Aside from hypoxia/HIF, two other pathways known to be involved in EVT differentiation include epithelial-mesenchymal transition (EMT) and WNT signaling. As the cells move distally within the column, they lose their proliferative potential and undergo what has been described as a "partial EMT," losing some (but not all) of their epithelial markers, and gaining some mesenchymal markers [57,58]. This phenotype is accompanied by an induction of ZEB2 [57] and TEAD2 [6], as mentioned above, both of which are transcription factors known to be involved in EMT [59,60]. This process also involves a switch in cell

surface integrins, from ITGA6 in CTB, to ITGA5 in cell column/immature EVT, and ITGA1 in mature EVT [61], acquiring a vascular phenotype, including induction of VE-Cadherin, VCAM-1, and PECAM [62], as well as cytoskeletal remodeling [6,57]. WNT signaling is also important for EVT differentiation, requiring to be turned off during the initial transition from CTB to EVT, but subsequently needed to be reactivated, acting through the transcription factor TCF4 to promote EVT maturation [63,64].

EVTs are more difficult to evaluate, as they are left behind, for the most part, following delivery of the placenta (those in the decidua basalis). Nevertheless, evaluation of decidual vessels present within the fetal membranes (those in the decidua parietalis) are thought to reflect the general state of maternal uterine vessels, and by inference, the functional status of endovascular EVT, for that particular pregnancy [1,2]. At the same time, evaluation of the basal plate EVT (those in the decidua basalis--at the maternal surface of the placental disc) provides at least some insight into the differentiation and function of interstitial EVT [1,2]. Combined, these evaluations have identified lesions which suggest that both PE and FGR, particularly in pre-term and severe cases, are associated with abnormalities of EVT differentiation, vascular remodeling, and invasion [1,2]. These lesions include decidual vasculopathy, characterized by hypertrophy/hyperplasia of the muscular layer of the decidual arterioles, perivascular chronic inflammation, fibrinoid necrosis, and/or infiltration by foamy macrophages [2]. The presence of decidual vasculopathy, alongside multifocal infarction, within PE/FGR placentae is highly suggestive of a defect in endovascular EVT function, which leads to lack of proper maternal blood flow to the placenta [2]. In addition, at the basal plate, an increase in immature interstitial EVT has been confirmed by increased markers of proliferation and reduced immunostaining for human placental lactogen (HPL) [65].

Additional insight into EVT differentiation and function has come from placental bed biopsies, performed post-delivery for research purposes. These studies have pointed to limited vascular remodeling in both PE and FGR placentae, with the resulting physiologic conversion of the arteries being confined to the decidual portions of these vessels, and not extending into the myometrium [66–68]. The absence of remodeling in these biopsies has also been associated with a decrease in number of mature, HPL^{+} interstitial EVT [69], but with an increase in placental bed giant cells, though a specific origin for these cells has yet to be confirmed [70]. Evaluation of these biopsies by immunohistochemistry has also revealed a lack of integrin switching, as well as failure to acquire a vascular adhesion phenotype, with significantly reduced expression of VE-Cadherin and VCAM-1 in endovascular and interstitial EVT, respectively [71].

More recently, more specific markers for differentiated EVT subtypes have been identified and characterized in context of PE/FGR. One example is PLAC8, which has been identified as specific to interstitial EVT [72]. PLAC8 is an actin-associated protein which promotes migration and invasion of these cells, through activation of RAC1 and CDC42 [72]. However, its expression was found to be elevated in interstitial EVT of PE placentae, speculated to be a compensatory response to the shallow invasion of these cells in PE [72]. A second example is LAIR2 (Leukocyte-Associated Immunogloubin-like Receptor-2), which appears to be specific to the endovascular and vascular plug EVT [73]. LAIR2 has

been noted to be decreased in chorionic villus samples of placentae associated with PE development later in pregnancy [74]; however, it is not clear whether this finding is significant, particularly if this protein is in fact specifically expressed in endovascular EVT, as CVS would be unlikely to capture this cell population. Nevertheless, a decrease in LAIR2 in the placental bed of PE patients, if measured, may be explained by an increase in apoptosis in endovascular EVT, which has reported in context of early-onset PE accompanied by FGR [75]. Unfortunately, similar to the LAIR2 study above, many studies have used villous tissue from PE/FGR placentae to evaluate alterations in pathways involved in EVT differentiation, including EMT [76,77] and WNT signaling [78,79]. While this is understandable, given the paucity of material, particularly of placental bed biopsies, ideal for these studies, these results preclude definitive conclusions on the role of alterations of these pathways in the pathogenesis of PE/FGR.

Conclusions and Gaps in Knowledge

While much has been learned about pathways regulating human trophoblast differentiation over the past ~30 years, many questions remain. In particular, in comparison to mouse, where studies have led to the identification of numerous trophoblast subtypes in both the labyrinth and junctional zones (equivalent to villous and extravillous compartments in the human placenta) [80,81], we know very little about the heterogeneity of both CTB and EVT subtypes. Recent studies using single cell RNAseq are beginning to shed light in this area [9–11]; however, significant work remains, on identification and localization of different cell types, both within normal development of the placenta and in the setting of diseases such as PE and FGR. Perhaps the most important point for future studies would be to first assess the expression pattern of an individual gene product within the placenta across gestation, prior to evaluation of its expression (by qPCR, western blot, or other means) using banked tissues from diseased placentae, most of which consist of only villous portions of this organ. Alternatively, immunohistochemistry/in-situ hybridization can provide a means to evaluate cell- or region-specific expression, albeit in a less quantitative manner. In addition, detailed molecular studies of pathways involved in EVT differentiation, specifically evaluating both interstitial and endovascular EVT in the setting of PE/FGR, are currently scant and require widely available perinatal tissue banks which include placental bed biopsies. Finally, functional analysis of these pathways requires improved *in vitro* systems for studying human trophoblast differentiation, including recently-established trophoblast stem cells and organoids, as well as iPSC-based models of human placental disease [3,82,83]. With the availability of the above resources, this field is poised to significantly advance over the next few decades, accompanying development of useful biomarkers and therapies for diagnosis and treatment of placenta-based disorders of pregnancy.

Acknowledgement

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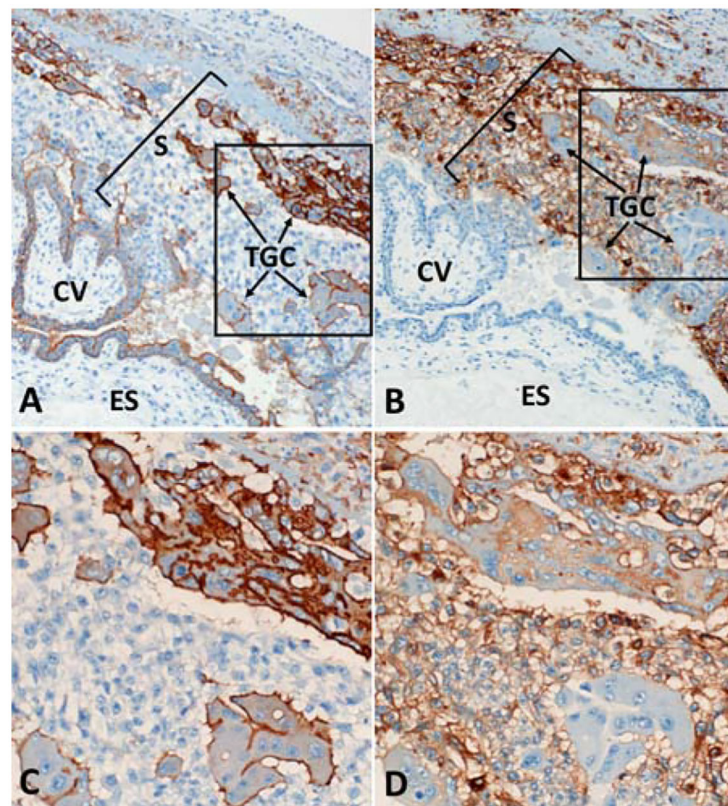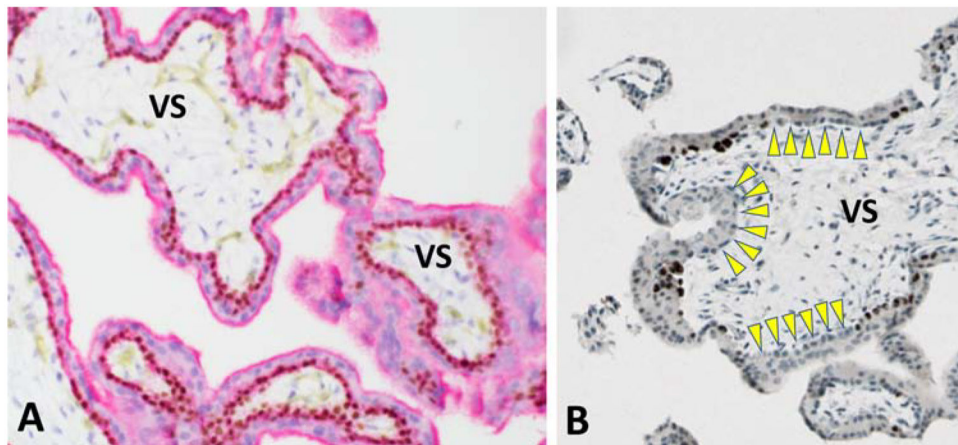
Figure 1.
Sequential sections of an archived day-16 human embryo stained with antibodies to EGFR (**A, C**) or HLAG (**B, D**). Formalin-fixed paraffin embedded (FFPE) tissue was sectioned and stained with a rabbit monoclonal antibody against EGFR (clone 5B7, Ventana/Roche) or a mouse monoclonal antibody against HLAG (4H84 clone, Abcam), using a Ventana Discovery Ultra automated immunostainer with standard antigen retrieval and reagents as per the manufacturer's protocol. Note that EGFR highlights both villous trophoblast surrounding the embryonic sac (ES) and primary chorionic villi (CV), as well as trophoblast giant cells (TGC) embedded within the trophoblastic shell (S), while HLAG predominantly highlights the mononuclear trophoblast within the trophoblastic shell. Immunostaining is shown in brown with blue (hematoxylin) counterstain. C and D represent magnification of the trophoblastic shell area with TGC, bracketed in a rectangular area in A and B, respectively. Magnification of 40x (for A and B) and 80x (for C and D).
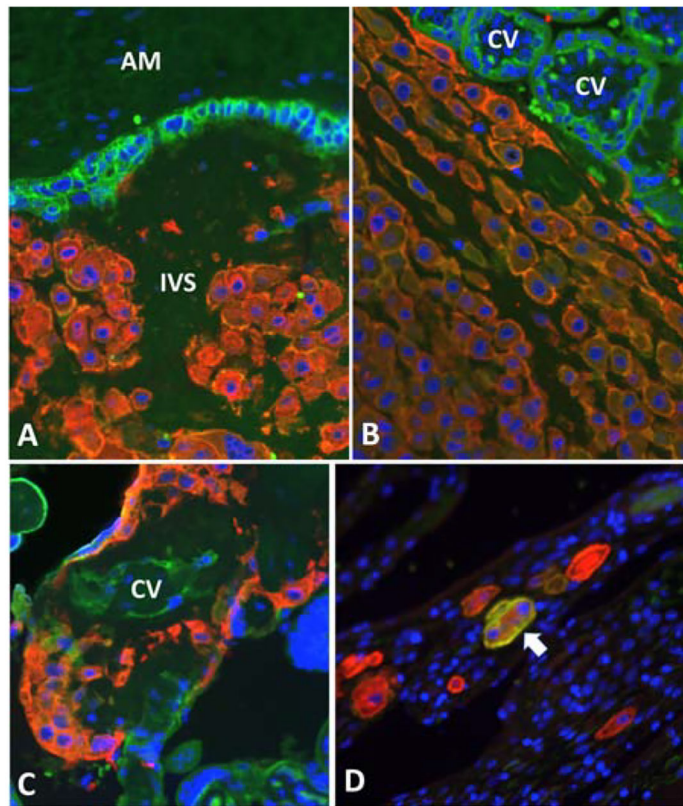
Figure 2.
Human placenta at term double-stained with antibodies to EGFR (green) and HLAG (red), counterstained with DAPI (blue). FFPE sections from 12 different term placentas were de-paraffinized and rehydrated, and antigen retrieval was performed using heat and antigen retrieval buffer (Dako) for 20 minutes. Tissues were incubated with the same primary antibodies as used for immunohistochemistry shown in Figure 1, then visualized by Alexa 488- or Alexa 594-conjugated secondary antibodies (Invitrogen) and counter-stained with the nuclear stain, DAPI. **A)** Chorionic plate (near the fetal surface), showing a layer of EGFR$^+$ cytotrophoblast (CTB) immediately below the amniotic mesenchyme (AM), with HLAG$^+$ cells emanating from the CTB layer, protruding into the intervillous space (IVS). **B)** Basal plate (near the maternal surface), showing EGFR$^+$ syncytiotrophoblast covering chorionic villi (CV, top right), below which are multiple layers of mature, HLAG$^+$ cells. **C)** An intraplacental trophoblast island within the placental disc, where a chorionic villus remnant (CV) gets surrounded by perivillous fibrin resulting in differentiation of CTB into HLAG$^+$ EVT. **D)** An EGFR/HLAG double-positive multinucleated cell (arrow) at the basal plate, surrounded by HLAG$^+$ EVT and unstained decidual cells. Magnification of 80x for A-D.

Figure 3.
Cytotrophoblast heterogeneity in early gestation (6-week gestational age) human placenta. FFPE sections from 5 different placentas were stained, either with antibodies against TP63 (mouse monoclonal antibody specific to p40, BC28 clone, Biocare Medical), EGFR (same antibody as in Figure 1), and CD31 (rabbit monoclonal, ab76533, Abcam) (shown in A), or against CDX2 (rabbit monoclonal, ab76541, Abcam) (shown in B), using a Ventana Discovery Ultra automated immunostainer with standard antigen retrieval and reagents as per the manufacturer's protocol. **A)** Section showing triple staining for TP63 (brown), EGFR (magenta), and CD31 (yellow-green), counter-stained with hematoxylin (blue). Note that TP63 is uniformly expressed in the CTB layer (trophoblast layer adjacent to the villous stroma/VS). **B)** Section showing staining for CDX2 (brown), counter-stained with hematoxylin (blue). Note stretches of CTB that lack CDX2 staining (yellow arrowheads). Magnification of 80x for A-B.