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33 ***Abstract***
34

35 The human placenta is a poorly-understood organ, but one that is critical for proper development
36 and growth of the fetus in-utero. The epithelial cell type that contributes to primary placental
37 functions is called “trophoblast,” including two main subtypes, villous and extravillous
38 trophoblast. Cytotrophoblast and syncytiotrophoblast comprise the villous compartment and
39 contribute to gas and nutrient exchange, while extravillous trophoblast invade and remodel the
40 uterine wall and vessels, in order to supply maternal blood to the growing fetus. Abnormal
41 differentiation of trophoblast contributes to placental dysfunction and is associated with
42 complications of pregnancy, including preeclampsia (PE) and fetal growth restriction (FGR).
43 This review describes what is known about the cellular organization of the placenta during both
44 normal development and in the setting of PE/FGR. It also explains known trophoblast lineage-
45 specific markers and pathways regulating their differentiation, and how these are altered in the
46 setting of PE/FGR, focusing on studies which have used human placental tissues. Finally, it also
47 highlights remaining questions and needed resources to advance this field.
48

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

95 basement membrane (**Figure 3a**) [8]. Little is known about the heterogeneity of this cell layer,
96 although single cell RNAseq analysis is beginning to shed some light on this subject [9-11]. Our
97 own work has pointed to one marker, CDX2, which appears to highlight a subset of these villous
98 CTB (**Figure 3b**). In normal gestation, $CDX2^{+}$ CTB are present mostly near the chorionic plate
99 and disappear after first trimester [12]. The CTB layer itself starts to become discontinuous in
100 the second trimester; by term, only 1-2 CTB can be found per terminal villus [8]. This is
101 reflected in a decrease in PCNA staining over gestation, as CTB are the proliferative cells within
102 the trophoblastic compartment [13].
103 While there are likely many more human trophoblast subtypes than described above, few
104 have been described or characterized, beyond mere identification with a single marker, and fewer
105 still have been functionally defined. Nevertheless, in the remainder of this review, we will
106 describe changes in trophoblast differentiation and related regulatory pathways, as revealed by a
107 combination of morphology, lineage-specific marker expression, and functional studies (where
108 available), associated with disorders of placental insufficiency, with a focus on PE and FGR.
109
110 ***Preeclampsia and Fetal Growth Restriction: Clinical Synopsis***
111 Pre-eclampsia (PE) is a common pregnancy disorder that is characterized by new-onset
112 hypertension and proteinuria in the latter half of pregnancy, and affects 5-8% of all pregnancies
113 worldwide [14-16]. The incidence of PE in the United states has been on the rise since the 1990s,
114 and it is currently one of the leading causes of maternal and neonatal morbidity and mortality
115 [14]. PE is a heterogeneous disease, and can manifest itself at different gestational ages, with
116 varying degrees of severity. Recently, a "two-stage" model has been developed to describe the
117 underlying pathophysiology, with the first stage being deficient placentation and second stage
118 characterized by systemic vascular inflammation [17]. PE, particularly severe early-onset PE,
119 presenting before 34 weeks gestational age, can be accompanied by fetal growth restriction
120 (FGR), defined as birthweight less than 10th percentile adjusted for sex and gestational age [18].
121 However, FGR can also be present in isolation, without a simultaneous maternal syndrome [5,
122 18,19]. While FGR can also be multifactorial, PE and isolated FGR have in common, both some
123 clinical features, including abnormal umbilical doppler studies, as well as underlying etiologies,
124 including defective placentation and systemic maternal vascular dysfunction [5,19]. Both PE
125 and FGR are often associated with a small placenta at delivery, and show various combinations
126 of villous hypermaturity, infarction, and decidual vasculopathy, on histopathologic examination
127 [1,2]. Recent studies have used a combination of gene expression profiling and histopathology
128 to further separate PE and FGR into unique subtypes, with the potential to identify biomarkers
129 with improved diagnostic accuracy [20-23]. However, trophoblast lineage-specific
130 differentiation has yet to be described in these different disease subtypes. Below, we will
131 describe abnormalities in both villous and extravillous trophoblast associated with PE/FGR.
132
133 ***Abnormalities of villous trophoblast associated with PE/FGR***
134 Normal villous trophoblast development involves regular branching of the villous tree,
135 with fusion of underlying CTB into the overlying syncytium within these structures [2]. The
136 process of fusion is regulated through Glial Cells Missing-1 (GCM1), a transcription factor,
137 which is expressed in a subgroup of CTB, induces cell cycle arrest, and mediates cell-cell fusion
138 through induction of SYNCYTIN genes [24, 25]. Downregulation of GCM1 in denuded floating
139 villi has been shown to promote CTB proliferation and inhibit syncytial regeneration [26].
140 Syncytialization involves induction of two fusogenic genes, SYNCYTIN 1 and 2, in human

141 chorionic villi, both of which were acquired from human endogenous defective retroviruses
142 [27,28]. The syncytium undergoes significant turnover through shedding of a combination of
143 extracellular vesicles and microparticles (sometimes referred to as trophoblast "deportation"),
144 which, over time, with decreased fusion of underlying CTB, results in formation of nuclear
145 aggregates, called syncytial knots [29]. The number of syncytial knots increases with increasing
146 gestational age, and is thus a reflection of villous maturity [30]. Syncytial knots are composed
147 primarily of condensed nuclei with transcriptionally inactive heterochromatin [31]. The presence
148 of syncytial knots in normal term placentas is also associated with a small spike in TUNEL
149 staining, a further reflection of placental aging [13].
150 PE/FGR placentas often show poor villus branching, described as "distal villous
151 hypoplasia," accompanied by increased numbers of syncytial knots adjusted for gestational age,
152 consistent with "accelerated villous maturation" [32]. The latter changes were originally
153 described by Tenney and Parker in 1940, as degenerative changes associated with "toxemia of
154 pregnancy," and hence are often referred to in the literature as "Tenney-Parker changes" [33].
155 These changes are thought to arise due to more turbulent flow of maternal blood into the
156 intervillous space of PE/FGR placentae, resulting in increased oxidative stress [34]; in fact,
157 syncytial knots are positive for markers of oxidative damage, such as 8-oxo-deoxyguanosine
158 [31]. The syncytial knots are also at least one source of circulating soluble VEGF receptor-1
159 (sVEGFR-1, also known as sFlt-1), an anti-angiogenic protein, whose increased expression has
160 been associated with, and indeed may be diagnostic for, PE [35-37]. The syncytial knots in
161 PE/FGR also show a decrease in the anti-apoptotic BCL-2 protein and an increase in cell death,
162 as measured by TUNEL staining [38]. The increase in cell death has been attributed to caspase-
163 independent apoptosis [39], increased levels of endoplasmic reticulum stress [40], as well as
164 excessive autophagy [41,42]. The increase in syncytial knots and cell death leads to
165 discontinuity of the syncytium, resulting in leakage of fetal proteins into maternal circulation
166 [43]. It also likely leads to increased thickness (~two-fold) of the vasculosyncytial membrane,
167 the exchange interface of chorionic villi, thus negatively affecting fetal growth [44]. Finally, at
168 least in early-onset PE, this increase in syncytial knots and apoptosis is also associated with an
169 increase in STB turnover/shedding, with over two-fold increase in circulating STB-derived
170 microparticles, compared to gestational age-matched control cases [45].
171 At the molecular level, these alterations in STB morphology and function have been
172 attributed to alterations in the GCM1-SYNCYTIN pathway, regulating syncytialization and STB
173 organization. Specifically, both GCM1 and SYNCYTIN-1 proteins have been found to be
174 reduced in PE placentae [46,47], with SYNCYTIN-1 also being mis-localized to the apical,
175 instead of the basal, membrane in STB [47]. Hypoxia has been proposed as the potential
176 mechanism for GCM1 downregulation, acting through suppression of the PI3K-AKT signaling
177 pathway to activation of GSK3β, with subsequent phosphorylation of GCM1, targeting it for
178 ubiquitination and degradation [48]. A reduction in GCM1 leads to a decrease in SYNCYTIN
179 expression, resulting, in turn, in reduced fusion and an altered syncytium.
180 Other functions that have been attributed to SYNCYTIN include both promotion of cell
181 proliferation [49] and inhibition of apoptosis [39]. However, while the latter is consistent with
182 the STB phenotype noted in PE, PE-associated alterations in villous CTB proliferation have been
183 somewhat controversial. Intuitively, it is easy to assume that accelerated maturation must be
184 accompanied by a loss in CTB proliferation. However, results of studies evaluating CTB
185 proliferation in PE/FGR range from no differences in CTB proliferation [50,51], region-specific
186 alterations in CTB proliferation [52], to an increase in CTB proliferation attributed to a damaged

187 syncytium in need of repair and regeneration [53,54]. Given the heterogeneity of PE and FGR, as
188 recently elaborated on by genome-wide expression profiling of placental tissues associated with
189 these complications [20-23], it is possible that distinct subclasses of these diseases have different
190 alterations at the cellular level, including differences in CTB proliferation, depending on the
191 underlying pathophysiology.
192
193 ***Abnormalities of extravillous trophoblast in PE/FGR***
194 Extravillous trophoblast (EVT) differentiate from CTB precursor cells within anchoring
195 chorionic villi at the basal plate. This process occurs within trophoblast cell columns, where
196 CTB gradually lose expression of EGFR and gain HLAG expression [6]. This process occurs
197 early in gestation, in a relatively hypoxic microenvironment [55] and, in fact, is regulated by the
198 hypoxia-inducible complex (HIF), at least in its initial transition from CTB to EVT [6]. HIF, in
199 turn, induces expression of transcription factors ASCL2 and TEAD2, which are specifically
200 expressed in EVT in the human placenta [6]. Hypoxia also induces NOTCH1, which inhibits
201 expression of transcription factors involved in CTB self-renewal, including TEAD4 and TP63,
202 represses syncytialization, and promotes proliferation and survival of EVT precursor cells [56].
203 GCM1 is another transcription factor induced during EVT differentiation, although it does not
204 appear to be regulated by HIF in this context [6]. Because GCM1 is also involved in STB
205 differentiation of CTB progenitor cells [24-26], cellular context/microenvironment likely plays a
206 key role in distinguishing which lineage is induced downstream of GCM1 expression.
207 Aside from hypoxia/HIF, two other pathways known to be involved in EVT
208 differentiation include epithelial-mesenchymal transition (EMT) and WNT signaling. As the
209 cells move distally within the column, they lose their proliferative potential and undergo what
210 has been described as a “partial EMT,” losing some (but not all) of their epithelial markers, and
211 gaining some mesenchymal markers [57,58]. This phenotype is accompanied by an induction of
212 ZEB2 [57] and TEAD2 [6], as mentioned above, both of which are transcription factors known
213 to be involved in EMT [59,60]. This process also involves a switch in cell surface integrins,
214 from ITGA6 in CTB, to ITGA5 in cell column/immature EVT, and ITGA1 in mature EVT [61],
215 acquiring a vascular phenotype, including induction of VE-Cadherin, VCAM-1, and PECAM
216 [62], as well as cytoskeletal remodeling [6,57]. WNT signaling is also important for EVT
217 differentiation, requiring to be turned off during the initial transition from CTB to EVT, but
218 subsequently needed to be reactivated, acting through the transcription factor TCF4 to promote
219 EVT maturation [63,64].
220 EVTs are more difficult to evaluate, as they are left behind, for the most part, following
221 delivery of the placenta (those in the decidua basalis). Nevertheless, evaluation of decidual
222 vessels present within the fetal membranes (those in the decidua parietalis) are thought to reflect
223 the general state of maternal uterine vessels, and by inference, the functional status of
224 endovascular EVT, for that particular pregnancy [1,2]. At the same time, evaluation of the basal
225 plate EVT (those in the decidua basalis--at the maternal surface of the placental disc) provides at
226 least some insight into the differentiation and function of interstitial EVT [1,2]. Combined, these
227 evaluations have identified lesions which suggest that both PE and FGR, particularly in pre-term
228 and severe cases, are associated with abnormalities of EVT differentiation, vascular remodeling,
229 and invasion [1,2]. These lesions include decidual vasculopathy, characterized by
230 hypertrophy/hyperplasia of the muscular layer of the decidual arterioles, perivascular chronic
231 inflammation, fibrinoid necrosis, and/or infiltration by foamy macrophages [2]. The presence of
232 decidual vasculopathy, alongside multifocal infarction, within PE/FGR placentae is highly

233 suggestive of a defect in endovascular EVT function, which leads to lack of proper maternal
234 blood flow to the placenta [2]. In addition, at the basal plate, an increase in immature interstitial
235 EVT has been confirmed by increased markers of proliferation and reduced immunostaining for
236 human placental lactogen (HPL) [65].
237 Additional insight into EVT differentiation and function has come from placental bed
238 biopsies, performed post-delivery for research purposes. These studies have pointed to limited
239 vascular remodeling in both PE and FGR placentae, with the resulting physiologic conversion of
240 the arteries being confined to the decidual portions of these vessels, and not extending into the
241 myometrium [66-68]. The absence of remodeling in these biopsies has also been associated with
242 a decrease in number of mature, HPL^+ interstitial EVT [69], but with an increase in placental bed
243 giant cells, though a specific origin for these cells has yet to be confirmed [70]. Evaluation of
244 these biopsies by immunohistochemistry has also revealed a lack of integrin switching, as well as
245 failure to acquire a vascular adhesion phenotype, with significantly reduced expression of VE-
246 Cadherin and VCAM-1 in endovascular and interstitial EVT, respectively [71].
247 More recently, more specific markers for differentiated EVT subtypes have been
248 identified and characterized in context of PE/FGR. One example is PLAC8, which has been
249 identified as specific to interstitial EVT [72]. PLAC8 is an actin-associated protein which
250 promotes migration and invasion of these cells, through activation of RAC1 and CDC42 [72].
251 However, its expression was found to be elevated in interstitial EVT of PE placentae, speculated
252 to be a compensatory response to the shallow invasion of these cells in PE [72]. A second
253 example is LAIR2 (Leukocyte-Associated Immunogloubin-like Receptor-2), which appears to be
254 specific to the endovascular and vascular plug EVT [73]. LAIR2 has been noted to be decreased
255 in chorionic villus samples of placentae associated with PE development later in pregnancy [74];
256 however, it is not clear whether this finding is significant, particularly if this protein is in fact
257 specifically expressed in endovascular EVT, as CVS would be unlikely to capture this cell
258 population. Nevertheless, a decrease in LAIR2 in the placental bed of PE patients, if measured,
259 may be explained by an increase in apoptosis in endovascular EVT, which has reported in
260 context of early-onset PE accompanied by FGR [75]. Unfortunately, similar to the LAIR2 study
261 above, many studies have used villous tissue from PE/FGR placentae to evaluate alterations in
262 pathways involved in EVT differentiation, including EMT [76,77] and WNT signaling [78,79].
263 While this is understandable, given the paucity of material, particularly of placental bed biopsies,
264 ideal for these studies, these results preclude definitive conclusions on the role of alterations of
265 these pathways in the pathogenesis of PE/FGR.
266
267 ***Conclusions and Gaps in Knowledge***
268 While much has been learned about pathways regulating human trophoblast
269 differentiation over the past ~30 years, many questions remain. In particular, in comparison to
270 mouse, where studies have led to the identification of numerous trophoblast subtypes in both the
271 labyrinth and junctional zones (equivalent to villous and extravillous compartments in the human
272 placenta) [80,81], we know very little about the heterogeneity of both CTB and EVT subtypes.
273 Recent studies using single cell RNAseq are beginning to shed light in this area [9-11]; however,
274 significant work remains, on identification and localization of different cell types, both within
275 normal development of the placenta and in the setting of diseases such as PE and FGR. Perhaps
276 the most important point for future studies would be to first assess the expression pattern of an
277 individual gene product within the placenta across gestation, prior to evaluation of its expression
278 (by qPCR, western blot, or other means) using banked tissues from diseased placentae, most of

279 which consist of only villous portions of this organ. Alternatively, immunohistochemistry/in-situ
280 hybridization can provide a means to evaluate cell- or region-specific expression, albeit in a less
281 quantitative manner. In addition, detailed molecular studies of pathways involved in EVT
282 differentiation, specifically evaluating both interstitial and endovascular EVT in the setting of
283 PE/FGR, are currently scant and require widely available perinatal tissue banks which include
284 placental bed biopsies. Finally, functional analysis of these pathways requires improved *in vitro*
285 systems for studying human trophoblast differentiation, including recently-established
286 trophoblast stem cells and organoids, as well as iPSC-based models of human placental disease
287 [3,82,83]. With the availability of the above resources, this field is poised to significantly
288 advance over the next few decades, accompanying development of useful biomarkers and
289 therapies for diagnosis and treatment of placenta-based disorders of pregnancy.
290

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294

295 ***Author contributions***
296 OF and CT did the preliminary literature search. CN performed the immunohistochemistry
297 which contributed to the generation of figures. OF and MMP wrote the manuscript text.
298

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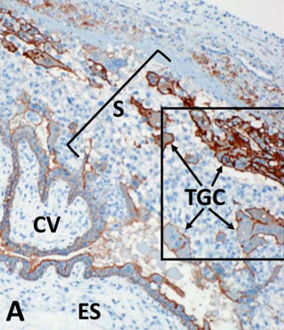
625 ***Figure Legends***
626
627 **Figure 1.** Sequential sections of an archived day-16 human embryo stained with antibodies to
628 EGFR (**A, C**) or HLAG (**B, D**). Formalin-fixed paraffin embedded (FFPE) tissue was sectioned
629 and stained with a rabbit monoclonal antibody against EGFR (clone 5B7, Ventana/Roche) or a
630 mouse monoclonal antibody against HLAG (4H84 clone, Abcam), using a Ventana Discovery
631 Ultra automated immunostainer with standard antigen retrieval and reagents as per the
632 manufacturer's protocol. Note that EGFR highlights both villous trophoblast surrounding the
633 embryonic sac (ES) and primary chorionic villi (CV), as well as trophoblast giant cells (TGC)
634 embedded within the trophoblastic shell (S), while HLAG predominantly highlights the
635 mononuclear trophoblast within the trophoblastic shell. Immunostaining is shown in brown with
636 blue (hematoxylin) counterstain. C and D represent magnification of the trophoblastic shell area
637 with TGC, bracketed in a rectangular area in A and B, respectively. Magnification of 40x (for A
638 and B) and 80x (for C and D).
639
640 **Figure 2.** Human placenta at term double-stained with antibodies to EGFR (green) and HLAG
641 (red), counterstained with DAPI (blue). FFPE sections from 12 different term placentas were de-
642 paraffinized and rehydrated, and antigen retrieval was performed using heat and antigen retrieval
643 buffer (Dako) for 20 minutes. Tissues were incubated with the same primary antibodies as used
644 for immunohistochemistry shown in Figure 1, then visualized by Alexa 488- or Alexa 594-
645 conjugated secondary antibodies (Invitrogen) and counter-stained with the nuclear stain, DAPI.
646 **A)** Chorionic plate (near the fetal surface), showing a layer of $EGFR^+$ cytotrophoblast (CTB)
647 immediately below the amniotic mesenchyme (AM), with $HLAG^+$ cells emanating from the CTB
648 layer, protruding into the intervillous space (IVS). **B)** Basal plate (near the maternal surface),
649 showing $EGFR^+$ syncytiotrophoblast covering chorionic villi (CV, top right), below which are
650 multiple layers of mature, $HLAG^+$ cells. **C)** An intraplacental trophoblast island within the
651 placental disc, where a chorionic villus remnant (CV) gets surrounded by perivillous fibrin
652 resulting in differentiation of CTB into $HLAG^+$ EVT. **D)** An EGFR/HLAG double-positive
653 multinucleated cell (arrow) at the basal plate, surrounded by $HLAG^+$ EVT and unstained
654 decidual cells. Magnification of 80x for A-D.
655
656 **Figure 3.** Cytotrophoblast heterogeneity in early gestation (6-week gestational age) human
657 placenta. FFPE sections from 5 different placentas were stained, either with antibodies against
658 TP63 (mouse monoclonal antibody specific to p40, BC28 clone, Biocare Medical), EGFR (same
659 antibody as in Figure 1), and CD31 (rabbit monoclonal, ab76533, Abcam) (shown in A) , or
660 against CDX2 (rabbit monoclonal, ab76541, Abcam) (shown in B), using a Ventana Discovery
661 Ultra automated immunostainer with standard antigen retrieval and reagents as per the
662 manufacturer's protocol. **A)** Section showing triple staining for TP63 (brown), EGFR
663 (magenta), and CD31 (yellow-green), counter-stained with hematoxylin (blue). Note that TP63
664 is uniformly expressed in the CTB layer (trophoblast layer adjacent to the villous stroma/VS).
665 **B)** Section showing staining for CDX2 (brown), counter-stained with hematoxylin (blue). Note
666 stretches of CTB that lack CDX2 staining (yellow arrowheads). Magnification of 80x for A-B.
667
668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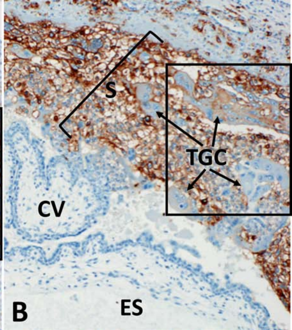
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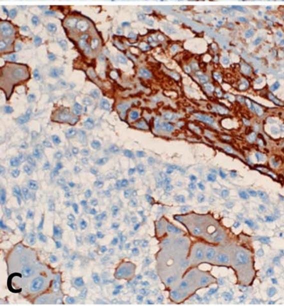
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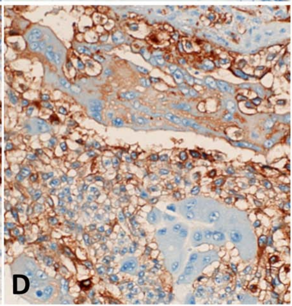
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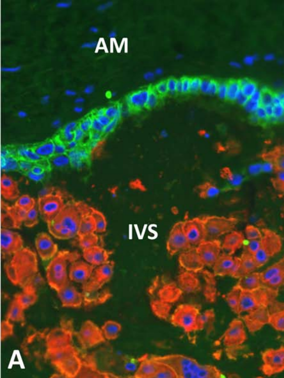
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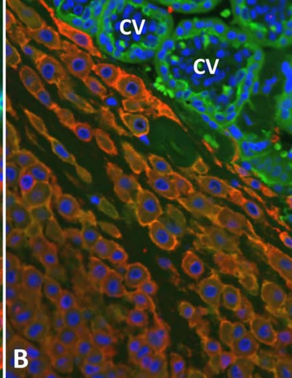
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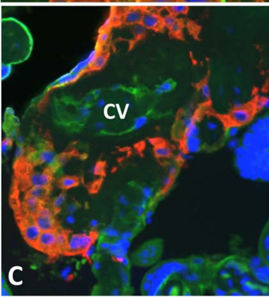
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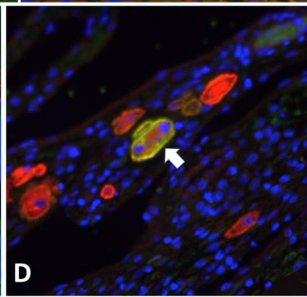
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