rate calculations. To mitigate these sources of bias, we confined comparisons to a single group of birds, the oscine passerines. We compare taxa only when the overall mtDNA genome was sampled with restriction fragments [restriction fragment length polymorphisms (RFLPs)] or sequence data were available for coding genes. Differences between closely related species are primarily third base position transitions, and such changes probably evolve in a clocklike manner.

11. Independent clock calibrations for a diverse array of avian taxonomic orders support a mtDNA substitution rate of 2%/My. These include: geese {Anseriformes [G. F. Shields and A. C. Wilson, *J. Mol. Evol.* **24**, 212 (1987)], 2.0%/My}; Old-world partridges and fowl {Galliformes [E. Randi, *Mol. Phylogenet. Evol.* **6**, 214 (1996)], 2.0%/My}; cranes {Gruiformes [C. Krajewski and D. G. King, *Mol. Biol. Evol.* **13**, 21 (1996)], 0.7 to 1.7%/My}; albatrosses {Procellariiformes [G. B. Nunn, J. Cooper, P. Jouventin, C. J. R. Robertson, G. G. Robertson, *Auk* **113**, 784 (1996)], 0.65%/My}; Hawaiian honeycreepers {Passeriformes [C. L. Tarr and R. C. Fleischer, *ibid.* **110**, 825 (1993)], 2.0%/My}, and New-world quail [Galliformes (R. M. Zink, unpublished data), 2.0%/My].
12. This phenomenon is termed lineage sorting. Alternatively, haplotype trees from species isolated for a sufficient period do reflect species (taxonomic) limits (termed reciprocal monophyly).
13. Species studied traditionally have been considered sisters, although recent work has shown that some are not sisters but rather are members of species complexes. All pairs and complexes are thought to have Late Pleistocene origins (*5*). Three subspecies pairs are included because they qualify as phylogenetic species; their inclusion makes the tests more conservative. Nomenclature follows the most recent AOU Check-list [American Ornithologists' Union, *Check-list of North American Birds* (Allen Press, Lawrence, KS, ed. 6, 1983)] and subsequent supplements.
14. We compared both mtDNA coding gene sequences and RFLPs, which provide comparable estimates of songbird sequence divergence (*15*). A subset (n = 12; those shown in Fig. 1 with standard errors) of sequence data having an uncorrected average percent divergence of 6.3 (±0.76) has a Kimura two-parameter [S. Kumar, K. Tamura, M. Nei, *MEGA: Molecular Evolutionary Genetic Analysis* (Pennsylvania State Univ., University Park, PA, 1993), version 1.01] corrected distance of 6.7 (±0.86). The similarity of these values indicates little saturation.
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20. Species are younger than the ages suggested by the coalescence times of their respective haplotypes, but this bias is minimal [W. S. Moore, *Evolution* **49**, 718 (1995)]. Edwards [in *Avian Molecular Systematics and Evolution*, D. P. Mindell, Ed. (Academic Press, New York, 1997), pp. 251–278)] estimated an interspecific distance correction of 350,000 years, which would have the maximum effect of shifting values in Fig. 1 one column to the left.
21. Those 13 pairs are as follows: Mexican and Chestnut-backed chickadees (*Parus sclateri* versus *P. rufescens*, 4.75%), Black-capped and Mountain chickadees (*Parus atricapillus* versus *P. gambeli*, 4.1%) (*16*); Rusty and Brewer's blackbirds (*Euphagus carolinus* versus *E. cyanocephalus*, 5.4%), Hooded and Orchard orioles (*Icterus cucullatus* versus *I. spurius*, 4.6%), Bronzed and Shiny cowbirds (*Molothrus aeneus* versus *M. bonariensis*, 2.0%) (*19*), Baird's and Henslow's sparrows (*Ammodramus bairdii* versus *A. henslowii*, 4.9%), Seaside and Sharp-tailed sparrows (*Ammodramus maritimus* versus *A. caudacutus*, 2.15%) [R. M. Zink and J. C. Avise, *Syst. Zool.* **39**, 148 (1990)], Bell's and White-eyed vireos (*Vireo bellii* versus *V. griseus*, 5.7%), Philadelphia and Warbling vireos (*Vireo philadelphicus* versus *V. gilvus*, 7.85%), Black-capped and Solitary vireos (*Vireo atricapillus* versus *V. solitarius*, 10.8%), Red-eyed and Black-whiskered vireos (*Vireo olivaceus* versus *V. altiloquus*, 4.5%) [B. W. Murray, W. B. McGillivray, J. C. Barlow, R. N. Beech, C. Strobeck, *Condor* **96**, 1037 (1994)], Swamp and Lincoln's sparrows (*Melospiza georgiana* versus *M. lincolnii*, 3.6%), and Black-chinned and Field sparrows (*Spizella atrogularis* versus *S. pusilla*, 6.5%) (R. M. Zink, unpublished data).
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23. The AOU [see (*13*)] Committee on Classification and Nomenclature considers the Timberline Sparrow to be a subspecies of Brewer's Sparrow, although we consider it a species (J. Klicka *et al.*, unpublished data). This taxon pair is characterized by differences in morphology, ecology, and vocalizations [T. J. Doyle, *Western Birds* **28**, 1 (1997)].
24. DNA methods followed standard protocols (*15*). Primers for polymerase chain reaction included L14841 [T. D. Kocher *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6196 (1989)] and H4A [J. Harshman, thesis, University of Chicago (1996)] for cytochrome b (1050 bp), and LGL2 and H417 [C. L. Tarr, *Mol. Ecol.* **4**, 527 (1995)] for control region I (400 bp).
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Regulation of Human Placental Development by Oxygen Tension

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Cytotrophoblasts, specialized placental cells, proliferate early in pregnancy and then differentiate into tumor-like cells that establish blood flow to the placenta by invading the uterus and its vasculature. In this study, cytotrophoblasts cultured under hypoxic conditions (2 percent oxygen), mimicking the environment near the uterine surface before 10 weeks of gestation, continued proliferating and differentiated poorly. When cultured in 20 percent oxygen, mimicking the environment near uterine arterioles, the cells stopped proliferating and differentiated normally. Thus, oxygen tension determines whether cytotrophoblasts proliferate or invade, thereby regulating placental growth and cellular architecture.

The human placenta's unique anatomy (Fig. 1) is due in large part to differentiation of its epithelial stem cells, termed cytotrophoblasts (*1*). How these cells differentiate determines whether chorionic villi, the placenta's functional units, float in maternal blood or anchor the conceptus to the uterine wall. In floating villi, cytotrophoblasts differentiate by fusing to form multinucleate syncytiotrophoblasts whose primary function—transport—is ideally suited to their location at the villus surface. In anchoring villi, cytotrophoblasts also fuse, but many remain as single cells that detach from their basement membrane and aggregate to form cell columns (Fig. 1A). Cytotrophoblasts at the distal ends of these columns attach to and then deeply invade the uterus [interstitial invasion (Fig. 1, A and C)] and its arterioles (endovascular invasion). As a result of endovascular invasion, the cells replace the endothelial and muscular linings of uterine arterioles, a process that initiates maternal blood flow to the placenta and greatly enlarges the vessel diameter. Paradoxically, the cells invade only the superficial portions of uterine venules. How this unusual behavior is regulated is unknown.

Our laboratory is studying the differen-

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tiation pathway that gives rise to anchoring villi and the population of cytotrophoblasts that, much like tumor cells, invade the uterus. This process begins when cells form columns and ends when they have deeply invaded the uterus. In between, the cytotrophoblasts differentiate in stages. Those in columns react with an antibody against the Ki67 antigen (anti-Ki67) (Fig. 1B), which is indicative of DNA synthesis (*2*). Distal to this region, anti-Ki67 staining abruptly stops (Fig. 1D), and the cytotrophoblasts intricately modulate their expression of stage-specific antigens, including integrin cell adhesion molecules (*3*), matrix metalloproteinase-9 (*4*), HLA-G [a cytotrophoblast class Ib major histocompatibility complex molecule (*5*)], and human placental lactogen (*6*).

In considering mechanisms that could regulate this process, we took into account that blood flow to the placenta changes dramatically in early pregnancy. During much of the first trimester, there is little endovascular invasion, so maternal blood flow to the placenta is at a minimum. The mean oxygen pressures of the intervillous space (that is, at the uterine surface) and within the endometrium are estimated to be (mean ± SD) 17.9 ± 6.9 and 39.6 ± 12.3 mm Hg, respectively, at 8 to 10 weeks of gestation (*7*). Afterwards, endovascular invasion proceeds rapidly; cytotrophoblasts are in direct contact with blood from maternal spiral arterioles, which could have a mean oxygen pressure as high as 90 to 100 mm Hg. Thus, as cytotrophoblasts invade the uterus during the first half of pregnancy, they encounter a steep, positive gradient of oxygen tension. These observations, together with the results of initial experiments we conducted on isolated cytotrophoblasts (*8*), suggested that oxygen tension might regulate cytotrophoblast proliferation and differentiation along the invasive pathway.

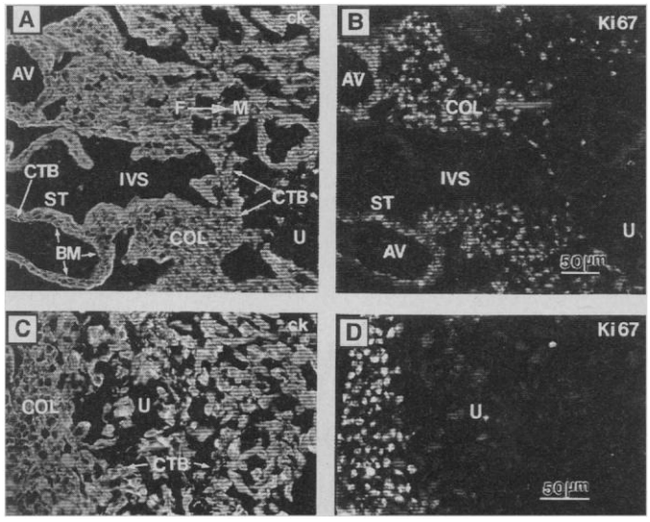

Fig. 1. Tissue sections of the maternal-fetal interface at 10 weeks of gestation (*20*). (**A**) Cytokeratin (ck) staining shows the placental trophoblast populations. In anchoring villi (AV), cytotrophoblast (CTB) stem cells are attached to the trophoblast basement membrane (BM). These cells fuse to form multinucleate syncytiotrophoblasts (ST), which cover most of the villus surface of the placenta, where they are in direct contact with maternal blood in the intervillous space (IVS). In certain areas, a subpopulation of cytotrophoblast stem cells aggregates to form cell columns (COL). These fetal cells invade maternal tissue (F→M), thereby attaching the anchoring villi to the uterus (U). (**B**) Cytotrophoblast stem cells and cytotrophoblasts in cell columns react with an antibody against Ki67, an antigen indicative of DNA synthesis. Cells in the column also begin to switch their expression of stage-specific antigens to those indicative of invasion. (**C**) Cytokeratin staining shows that once the cell columns contact the uterus, the cytotrophoblasts disaggregate and invade the uterus, where they intermingle with maternal cells. (**D**) Once they disaggregate, Ki67 staining abruptly stops.

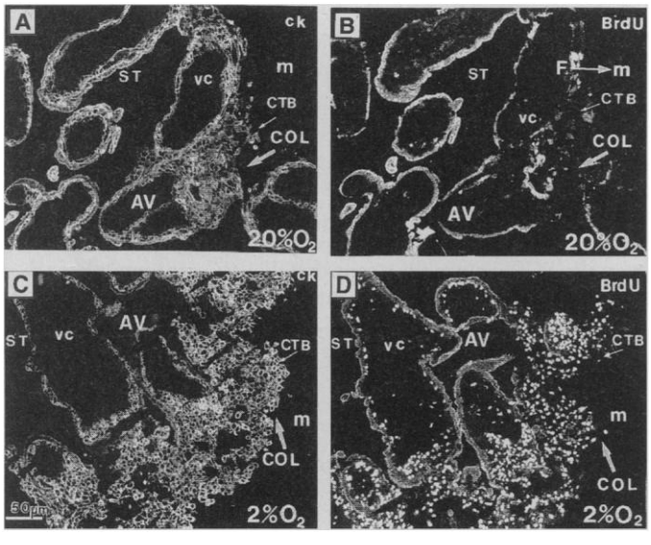

Fig. 2. Low oxygen (2% O_2) stimulates cytotrophoblast BrdU incorporation in vitro. Anchoring villi (AV) from 6- to 8-week-old placentas were cultured on Matrigel (m) for 72 hours in either 20% O_2 (**A** and **B**) or 2% O_2 (**C** and **D**) (*21*). By the end of the culture period, fetal cytotrophoblasts migrated into the Matrigel (F→m). To assess cell proliferation, BrdU was added to the medium (*22*). Tissue sections of the villi were stained with anti-cytokeratin (ck) (A and C), which recognizes syncytiotrophoblasts (ST) and cytotrophoblasts (CTB) but not cells in the villus core (vc), and with anti-BrdU (B and D), which detects cells in S phase. Villus explants maintained in 2% O_2 (C) formed much more prominent columns (COL) with a larger proportion of CTB nuclei that incorporated BrdU (D) than explants cultured in 20% O_2 (A and B).

As a model system for testing this hypothesis, we used organ cultures of anchoring villi explanted from early gestation (6 to 8 weeks) placentas onto an extracellular matrix substrate. Some of the anchoring villi were cultured for 72 hours in a standard tissue culture incubator (20% O_2 or 98 mm Hg). Figure 2A shows a section of one such villus that was stained with an antibody that recognizes cytokeratin to demonstrate syncytiotrophoblasts and cytotrophoblasts. The attached cell columns are clearly visible. To assess the cells' ability to synthesize DNA, the villi were incubated with 5-bromo-2′-deoxyuridine (BrdU), a thymidine analog. Incorporation was detected in the cytoplasm, but not the nuclei, of syncytiotrophoblasts. Few or none of the cells in columns incorporated BrdU (Fig. 2B). Other anchoring villi were maintained in a hypoxic atmosphere (2% O_2 or 14 mm Hg). After 72 hours, cytokeratin staining showed prominent cell columns (Fig. 2C), and the nuclei of many of the cytotrophoblasts in these columns incorporated BrdU (Fig. 2D). Because cytotrophoblasts were the only cells that entered S phase, we also compared the ability of anchoring villus explants cultured under standard and hypoxic conditions to incorporate [^{3}H]thymidine (*9*). Villus explants cultured under hypoxic conditions (2% O_2) incorporated 3.3 ± 1.2 times more [^{3}H]thymidine than villi cultured under standard conditions (20% O_2) (n = 21 per group; dissected from seven placentas). In contrast, [^{3}H]thymidine incorporation by explants cultured in a 6% O_2 atmosphere (40 mm Hg) was no different than that in control villi (n = 8 per group; dissected from four placentas). Taken together, these results suggest that a hypoxic atmosphere compa-

rable to that encountered by early gestation cytotrophoblasts in the intervillous space stimulates the cells to enter S phase.

Cytokeratin staining also showed that the cell columns associated with anchoring villi cultured under hypoxic conditions were larger than cell columns of control villi cultured under standard conditions (compare Fig. 2, A and C). We made serial sections of columns attached to two villus explants maintained for 72 hours in 20% O_2 and two that were maintained in 2% O_2, and then counted the number of cells in each column (n = 8 per group; dissected from two placentas). Under standard tissue culture conditions, the columns contained a mean of 516 ± 72 cells; under hypoxic conditions, they contained a mean of 1476 ± 156 cells. These results indicate that hypoxia stimulates the proliferation of cytotrophoblasts in cell columns.

We reasoned that the hypoxia-induced changes in the cells' proliferative capacity would be reflected by changes in their expression of proteins that regulate passage through the cell cycle. With regard to the G2-M cell cycle transition, we were particularly interested in their cyclin B expression because threshold levels of this protein are required for cells to enter mitosis (*10*). Immunoblotting of cell extracts (*11*) showed that after 3 days in culture, anchoring villi maintained in 2% O_2 contained 3.1 times more cyclin B than did villi maintained in 20% O_2 (Fig. 3A). Immunolocalization experiments confirmed that cyclin B was primarily expressed by cytotrophoblasts. Because $p21^{WF1/CIP1}$ abundance has been correlated with cell cycle arrest (*12*), we also examined the effects of oxygen tension on cytotrophoblast expression of this protein. Very little $p21^{WF1/CIP1}$ expression was detected in cell extracts of anchoring villi maintained for 72 hours in 2% O_2 (Fig. 3B), but expression increased 3.8-fold in anchoring villi maintained for the same time period in 20% O_2. Immunolocalization experiments confirmed that $p21^{WF1/CIP1}$ was primarily expressed by cytotrophoblasts. These results, replicated in five separate experiments, confirm that culturing anchoring villi in 20% O_2 induces cytotrophoblasts in the attached cell columns to undergo cell cycle arrest, whereas culturing them in 2% O_2 induces them to enter mitosis.

Changes in proliferative capacity are often accompanied by concomitant changes in differentiation. Accordingly, we investigated the effects of hypoxia on the ability of cytotrophoblasts to differentiate along the invasive pathway (Fig. 4). Under standard tissue culture conditions, cytotrophoblasts migrated from the cell columns and modulated their expression of stage-specific antigens, as they do during uterine invasion in vivo (*3*). For example, they began to express integrin α1, a laminin-collagen receptor that is required for invasiveness in vitro (*13*). Both differentiated cytotrophoblasts and villus stromal cells expressed this antigen (Fig. 4B). When cultured under hypoxic conditions, cytotrophoblasts failed to stain for integrin α1, but stromal cells continued to express this molecule, suggesting that the observed effects were cell type–specific (Fig. 4E). Hypoxia also reduced cytotrophoblast staining for human placental lactogen, another antigen that is expressed once the cells differentiate. However, lowering the O_2 tension did not change cytotrophoblast expression of other stage-specific antigens, such as HLA-G (Fig. 4, C and F) and integrins α5/β1 and αv/β3. These results suggest that hypoxia produces selective deficits in the ability of cytotrophoblasts to differentiate along the invasive pathway.

The effects of oxygen tension on the proliferative capacity of cytotrophoblasts could help explain some of the interesting features of normal placental development. Before cytotrophoblast invasion of maternal vessels establishes the uteroplacental circulation (≤10 weeks), the conceptus is in a relatively hypoxic atmosphere. During this period, the mass of the placenta increases much more rapidly than that of the embryo proper. Histological sections of early stage pregnant human uteri show bilaminar em-

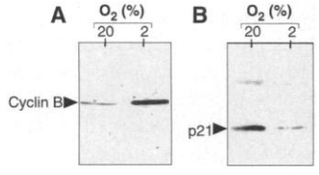

Fig. 3. Hypoxia induces changes in cytotrophoblast expression of proteins that regulate progression through the cell cycle. (**A**) Villus explants cultured for 72 hours in 2% O_2 contained 3.1 times more cyclin B than did villi maintained under standard culture conditions (20% O_2) for the same length of time. (**B**) Expression of p21 increased 3.8-fold in anchoring villi cultured for 72 hours in 20% O_2 as compared to those in 2% O_2.

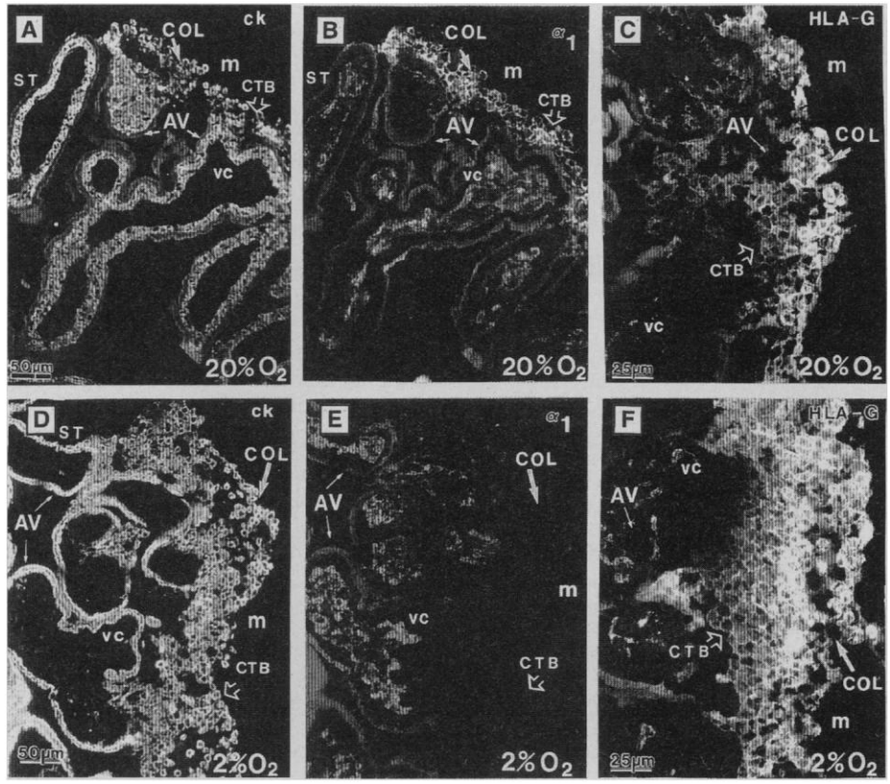

Fig. 4. Some aspects of cytotrophoblast differentiation and invasion are arrested in hypoxia. Anchoring villi (AV) from 6- to 8-week-old placentas were cultured on Matrigel (m) for 72 hours in either 20% O_2 (**A** through **C**) or 2% O_2 (**D** through **F**). Tissue sections of the villi (*20*) were stained with anti-cytokeratin (ck) (A and D), anti–integrin α1 (B and E), or anti–HLA-G (C and F). Cytotrophoblasts (CTB) that composed the cell columns (COL) of villus explants that were cultured in 20% O_2 up-regulated both integrin α1 (B) and HLA-G expression (C). In contrast, cytotrophoblasts in anchoring villus columns maintained in 2% O_2 failed to express integrin α1, although constituents of the villus core (vc) continued to express this adhesion molecule (E). However, not all aspects of differentiation were impaired: The cells up-regulated HLA-G expression normally (F). ST, syncytiotrophoblast.

bryos surrounded by thousands of trophoblast cells (*14*). The fact that hypoxia stimulates cytotrophoblasts but not most other cells (*15*) to undergo mitosis could help account for the discrepancy in size between the embryo and the placenta, which continues well into the second trimester of pregnancy (*16*). Although we do not as yet understand this phenomenon at a mechanistic level, we know that cytotrophoblasts within the uterine wall mimic a vascular adhesion molecule phenotype (*17*). In other tissues, hypoxia induces the production of vascular endothelial growth factor, which stimulates endothelial cell proliferation (*18*), raising the possibility that similar regulatory pathways operate during placental development.

We suspect that the effects of oxygen tension on cytotrophoblast differentiation and invasion could have important implications. Relatively high oxygen tension promotes cytotrophoblast differentiation and could help explain why these cells extensively invade the arterial rather than the venous side of the uterine circulation. Conversely, if cytotrophoblasts do not gain access to an adequate supply of maternal arterial blood, their ability to differentiate into fully invasive cells may be impaired. We suggest that the latter scenario could be a contributing factor to pregnancy-associated diseases, such as preeclampsia, that are associated with abnormally shallow cytotrophoblast invasion and faulty differentiation, as evidenced by their inability to up-regulate integrin α1 expression (*19*).

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20. Placental-bed biopsy specimens were obtained as previously described (*5, 17*). Sections of these specimens were incubated with anti-Ki67 (Dako, Carpinteria, CA) and anti–human cytokeratin (7D3) [S. J. Fisher *et al.*, *J. Cell Biol.* **109**, 891 (1989)]. Antibody binding was detected with fluorescein-conjugated rabbit anti–mouse immunoglobulin G (IgG) and rhodamine-conjugated goat anti-rat IgG (Jackson Immunoresearch). Tissue blocks of cultured anchoring villi were prepared like the biopsy specimens. Sections cut from these blocks were double stained with 7D3 and an anti–integrin α1 mouse IgG (T Cell Diagnostics, Woburn, MA). Different sections were stained with an anti–HLA-G mouse monoclonal antibody produced in our laboratory (*5*), anti–integrin α5 (*3*), anti-αv/β3 (LM609, D. Cherish, Scripps Research Foundation), anti–human placental lactogen (Harlan Bioproducts, Indianapolis, IN), anti–cyclin B, and anti-p21 (both from Santa Cruz Biotechnology, Santa Cruz, CA). Antibody binding was detected as described above.
21. Anchoring villi were dissected from 6- to 8-week-old human placentas and plated on Matrigel substrates (Collaborative Biomedical Research) [O. Genbacev, S. Schubach, R. K. Miller, *Placenta* **13**, 439 (1993)]. Control and hypoxic cultures were maintained for 3 days as previously described (*8*). Dissolved O_2 at the cell-medium interface, measured using a micro-oxygen electrode (MI-730; Microelectrodes, Inc., Londonderry, NH), was 20% (98 mm Hg) under standard tissue culture conditions, and either 6% (40 mm Hg) or 2% (14 mm Hg) in hypoxic conditions.
22. After 48 hours, the culture medium was aspirated, and medium containing 1 μM BrdU (Sigma) was added. After 24 hours, villi on filter substrates were washed with PBS for 20 min, fixed for 1 hour (4°C) in 4% paraformaldehyde, embedded in optimal-cutting-temperature medium, and frozen in liquid nitrogen (*5, 17*). Sections cut from these blocks were incubated with a fluorescence-labeled antibody to BrdU (Boehringer Mannheim, Indianapolis, IN). Adjacent sections were stained with 7D3.

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Proteolysis and DNA Replication: The *CDC34* Requirement in the *Xenopus* Egg Cell Cycle

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The cell division cycle gene, *CDC34*, is required for ubiquitin-mediated degradation of G_1 regulators and cell cycle progression through the transition from G_1 to S phase in budding yeast. A *CDC34* requirement for S phase onset in higher eukaryotes has not been established. Studies of the simple embryonic cell cycle of *Xenopus laevis* eggs demonstrated that Cdc34p in a large molecular size complex was required in the initiation of DNA replication. Cdc34p appears to regulate the initiation function of Cdk2–cyclin E, perhaps through the degradation of the *Xenopus* cdk inhibitor, Xic1.

Protein ubiquitination and degradation were linked to cell cycle control by the discovery that Cdc34p, an essential G_1- to S-phase regulator in budding yeast, is a ubiquitin conjugating enzyme (UBC3) (*1*). The Cdc34p-dependent degradation of a single substrate, p40sic1p [an inhibitor of cyclin-dependent kinase (cdk) Cdc28p complexed with cyclins Clb5p or Clb6p], appears to be sufficient to trigger the transition from G_1 to S phase (*2*). This degradation requirement in yeast is a key element coupling extrinsic control of cell proliferation to the cell cycle. The human homolog of *CDC34* can complement the yeast *cdc34-2* temperature-sensitive strain (*3*). Cdc34p in mammalian cells may also function to couple extrinsic control by growth factors to cell cycle progression. However, Cdc34p may play a more basic role in the intrinsic control of each cell cycle. A requirement for Cdc34p in the early embryonic cell cycle of the frog *Xenopus laevis*, where cell division is independent of extrinsic controls, may reveal a more fundamental role for protein degradation in regulating the initiation of DNA replication.

We tested whether Cdc34p is required for the onset of DNA replication in higher eukaryotes and examined the nature of such a requirement using an in vitro DNA rep-

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