Congenital human cytomegalovirus (HCMV) infection is associated with neurodevelopmental disabilities. To dissect the earliest events of infection in the developing human brain, we studied HCMV infection during controlled differentiation of human embryonic stem cells (hESC) into neural precursors. We traced a transition from viral restriction in hESC, mediated by a block in viral binding, toward HCMV susceptibility in early hESC-derived neural precursors. We further revealed the role of platelet-derived growth factor receptor alpha (PDGFRα) as a determinant of the developmentally acquired HCMV susceptibility.

Human cytomegalovirus (HCMV) is a leading cause of congenital infection (1), associated with neurodevelopmental disabilities (2, 3). The ability of the virus to infect the developing fetal brain is a key factor in its neuropathogenesis (3–8). While considerable experimental data were obtained from newborn and embryonic mouse models (6, 9–15), the strict species specificity precludes animal models of HCMV. Recent studies in human neuronal progenitor cells (NPC) derived from fetal and neonatal brains have revealed productive HCMV infection of NPC, with resultant functional alterations (16–20). Notably, NPC do not represent early neural embryonic development but rather a more advanced stage along the neuronal differentiation route. Hence, the earliest events defining the susceptibility of the developing human nervous system to HCMV have remained largely unknown.

Human embryonic stem cells (hESC) provide an opportunity to study early human neural development (21–23). We have developed highly reproducible protocols for controlled induction of differentiation of hESC toward a neural lineage, giving rise to enriched populations of proliferating, developmentally multipotent early NPC (24, 25).

Here, we have employed experimental HCMV infection in a dynamic model of controlled differentiation of hESC into neural precursors, to gain insight into the molecular events mediating HCMV infection during early human neurodevelopment.

We first sought to track the earliest stage of neural differentiation at which the cells become susceptible to HCMV. To this end, differentiation of hESC was induced in floating cell clusters toward the formation of neural spheres (Fig. 1A) as detailed previously (24, 25). The emerging neural spheres were infected at sequential time points along the neural differentiation process with the broadly tropic HCMV TB40/E strain expressing green fluorescent protein (GFP) (26, 27) (Fig. 1B). In accordance with previous studies (25), immunostaining analyses for the pluriplont stem cell marker TRA 1-81 and the neural differentiation marker PSA-NCAM showed that the majority of the cells underwent early neural differentiation within 11 days (Fig. 1C). Importantly, we have identified a transition toward HCMV susceptibility occurring between 4 and 11 days post-differentiation induction (Fig. 1B). A similar susceptibility pattern was observed for low-passage-number, cell-associated, clinical strains isolated from urine samples of congenitally infected neonates, with viral gene expression first detected following infection of 11-day-old hESC-derived NPC (Fig. 1D). Our findings trace the switch toward HCMV susceptibility to early neural precursors, representing the primitive neuroepithelial cells (24, 25, 28), which form the neural plate and neural tube as early as 4 weeks of gestation—later giving rise to the progenitors of neural stem cells (28–32). Viral targeting of these primary predecessors of the developing nervous system could underlie the progressive neurodevelopmental disabilities associated with congenital HCMV infection.

In line with the initial restriction to HCMV infection in hESC-derived NPC, we found that undifferentiated hESC (lines hES1 and HAD-C 102 [22, 33]) are resistant to infection by broadly tropic HCMV laboratory-derived and low-passage-number clinical strains. In contrast, hESC were susceptible to herpes simplex virus 1 (HSV-1) and adenovirus (data not shown). These findings, along with reports of their susceptibility to HSV-1, pseudorabies virus (PrV), coxsackie virus, and varicella-zoster virus (VZV), when grown in suspension (34, 35), suggest a virus-specific restriction mechanism(s).

We next studied the nature of the impediment to HCMV infection in hESC. Interestingly, we identified a block at the initial stage of viral binding, demonstrated by (i) failure of the major viral tegument protein pp65 to transport into the nucleus or cytoplasm (Fig. 2A and B), reflecting absence of viral internalization, and (ii) lack of HCMV DNA accumulation during viral infection with hESC (under conditions favoring HCMV and HSV-1...
binding onto susceptible human foreskin fibroblasts [HFF] and hESC, respectively) (Fig. 2C and D), compatible with HCMV-specific binding restriction in hESC. While we show a dominant viral entry block, we cannot rule out additional intracellular constraints, targeting downstream steps in the virus life cycle, including the recently demonstrated suppression of HCMV major immediate-early promoter (MIEP) in hESC lines Wisconsin H1 and H9, different from the ones used here (36, 37).

Having revealed the restriction at the stage of viral binding, we proceeded to identify the candidate cellular factor which mediates

**FIG 1** Transition toward HCMV susceptibility in early hESC-derived NPC. (A) Schematic presentation of induction of hESC differentiation into NPC. Undifferentiated hESC were maintained in culture medium supplemented with basic fibroblast growth factor 2 (bFGF). Upon initiation of neural differentiation (days 0 to 4), bFGF was withdrawn from the culture medium. The medium was supplemented during days 0 to 4 with two inhibitors of the SMAD signaling pathway (Noggin and SB-431542). From day 5, the SMAD signaling inhibitors were removed, and the medium was supplemented with bFGF. From day 12, the medium was further supplemented with epidermal growth factor (EGF) (25, 28). (B) Neurospheres formed at the indicated time points post-induction of differentiation were infected with HCMV strain TB40/E expressing the late gene pp150 fused to GFP (multiplicity of infection of 1 PFU/cell) and visualized at 7 days postinfection by inverted-phase (top row) and fluorescence (bottom row) microscopy. Similar findings were obtained using HCMV strain TB40/E expressing immediate-early GFP (not shown). (C) Expression of cell differentiation markers by neurospheres formed at the indicated times post-induction of differentiation, as analyzed by immunostaining and flow cytometry. (D) Neurospheres at day 11 post-induction of differentiation were infected with the low-passage-number clinical strain CI5006, recovered from the urine of a congenitally infected newborn. Shown are the viral IE-1 and late RNA R160461 mRNA levels, determined as described previously (27) at the indicated times postinfection and normalized by the housekeeping gene for glucose-6-phosphate dehydrogenase (G6PD) (similar results were obtained with three additional low-passage-number clinical isolates).
the transition toward viral susceptibility upon neural differentiation from hESC. Since platelet-derived growth factor receptor alpha (PDGFRα) has been shown to mediate HCMV entry in a cell-type-dependent manner (38, 39), we hypothesized that it plays a role in viral entry into NPC; we found a close correlation between PDGFRα expression along the neural differentiation route and the HCMV susceptibility pattern (Fig. 3A). Furthermore, we have shown a clear dose-dependent competitive inhibi-
PDGFRα mediates HCMV susceptibility in early hESC-derived NPC. (A) Expression of PDGFRα, as analyzed by immunostaining and flow cytometry, is shown in hESC, in neural spheres formed at the indicated times post-induction of differentiation, and in control HFF. (B) Pretreatment of cells with PDGF-AA inhibits HCMV infection in hESC-derived NPC. Neural spheres at 18 days postinduction were infected with HCMV strain TB40/E following pretreatment with increasing concentrations of PDGF-AA. Control cultures were mock infected or infected in the presence of heparin. (C) Pretreatment of HCMV with soluble PDGFRα inhibits HCMV infection in hESC-derived NPC. HCMV was preincubated with increasing amounts of soluble PDGFRα prior to infection of neural spheres. Control experiments revealed no inhibition of infection following preincubation of the virus with similar amounts of bovine serum albumin (not shown). (B and C) Viral IE-1 mRNA levels in the cell lysates at 24 h postinfection, normalized by the housekeeping gene for glucose-6-phosphate dehydrogenase (G6PD) or β-actin.
tion of HCMV infection following pretreatment of early hESC-derived NPC with PDGFRα ligand PDGF-AA (Fig. 3B). An independent experimental approach consistently revealed that pretreatment of HCMV with increasing amounts of soluble PDGFRα resulted in remarkable dose-dependent inhibition of HCMV infection in hESC-derived NPC (Fig. 3C). These combined findings directly support the role of PDGFRα as a determinant of the developmentally acquired HCMV susceptibility. The mechanism by which PDGFRα regulates HCMV infection in hESC-derived NPC remains to be determined. In view of the documented functions of PDGFRα during neurodevelopment (30, 40–47), it is tempting to speculate that its engagement by HCMV and its virus-induced downregulation (48) could be a key factor in the neuropathogenesis of congenital HCMV. Our findings do not exclude a role for other potential cofactors (including additional surface receptors) in modulating the increased viral susceptibility during differentiation, which could be further analyzed by global gene expression studies. In fact, we observed a gradual increase in viral susceptibility along subsequent differentiation time points (Fig. 1), suggesting a dynamic process with temporal release of additional restriction checkpoints. This finding is in agreement with a recent report demonstrating multiple viral restriction levels in hESC-derived neural differentiating cells, which were gradually alleviated during differentiation progression (49).

The novel mechanism that we have discovered here, which restricts viral infection in hESC and confers a developmental window of susceptibility, unveils a potential survival strategy by which the virus avoids perturbing embryo genesis and instead targets early lineage-unrestricted neuroepithelial precursors. Future studies that will examine the effect of HCMV infection on the fate and function of the primitive neuroepithelial cells could pave the way to new directions for prevention and therapy of congenital HCMV.

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