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REGULAR ARTICLE

CD marker expression profiles of human embryonic stem cells and their neural derivatives, determined using flow-cytometric analysis, reveal a novel CD marker for exclusion of pluripotent stem cells

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Abstract Human embryonic stem cells (hESCs) are pluripotent cells that can differentiate into neural cell lineages. These neural populations are usually heterogeneous and can contain undifferentiated pluripotent cells that are capable of producing teratomas in cell grafts. The characterization of surface protein profiles of hESCs and their neural derivatives is important to determine the specific markers that can be used to exclude undifferentiated cells from neural populations. In this study, we analyzed the cluster of differentiation (CD) marker expression profiles of seven undifferentiated hESC lines using flow-cytometric analysis and compared their profiles to those of neural derivatives. Stem cell and progenitor marker CD133 and epithelial adhesion molecule marker CD326 were more highly expressed in undifferentiated hESCs, whereas neural marker CD56 (NCAM) and neural precursor marker (chemokine receptor) CD184 were more highly expressed in hESC-derived neural cells. CD326 expression levels were consistently higher in all nondifferentiated hESC lines than in neural cell derivatives. In addition, CD326-positive hESCs produced teratomas in SCID mouse testes, whereas CD362-negative neural populations did not. Thus, CD326 may be useful as a novel marker of undifferentiated hESCs to exclude undifferentiated hESCs from differentiated neural cell populations prior to transplantation.

Introduction

Human embryonic stem cells (hESCs) are pluripotent cells derived from the inner cell mass of preimplantation embryos

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that can be expanded in large amounts in culture in an undifferentiated state (Reubinoff et al., 2000; Thomson et al., 1998). The expression of specific markers is linked to the maintenance of hESC pluripotency and self-renewal. Such markers include the transcription factors Oct-4 and Nanog (Hart et al., 2004; Reubinoff et al., 2000) and various cell surface markers, such as the stage-specific embryonic glycolipid antigens (SSEA) 3 and 4, the keratan sulfate-related antigens TRA-1-60 and TRA-1-81, and alkaline phosphatase (Inzunza et al., 2005; Reubinoff et al., 2000; Thomson et al., 1998). SSEA-3 and SSEA-4, however, are not critical for maintaining hESC pluripotency (Brimble et al., 2006). The results of a recent large study by the International Stem Cell Initiative (ISCI) indicated that a significant proportion of hESC lines do not express SSEA-3 (ISCI, 2007). Also, SSEA-4 is expressed by a subset of dorsal root ganglion cells (Holford et al., 1994), by dissociated fetal forebrain and spinal cord tissues (Piao et al., 2006), and by early neuroepithelial cells in the developing forebrain (Barraud et al., 2007).

Human ESCs can differentiate into all cell types of the human body. By following established differentiation protocols, hESCs may be induced to differentiate into cells of neuroectodermal lineage, producing cells of neural origin (Carpenter et al., 2001; Reubinoff et al., 2001; Zhang et al., 2001). Early neural differentiation is characterized by the expression of the neuroectodermal transcription factors Pax6 and Sox1, the intermediate filament protein nestin, and the neural cell adhesion molecule (NCAM, CD56) (Nat et al., 2007; Reubinoff et al., 2001; Zhang et al., 2001). Maturing neurons are distinguished by morphologic features and express the neural markers β-tubulin$_{III}$, microtubule-associated protein-2 (MAP-2), and neurofilament proteins, among others (Nat et al., 2007; Reubinoff et al., 2001; Zhang et al., 2001). Upon transplantation, hESC-derived neural progenitor cells positive for the markers Pax6, nestin, and NCAM integrate into the host brains of newborn mice, migrate, and differentiate into neurons, astrocytes, and oligodendrocytes (Reubinoff et al., 2001; Zhang et al., 2001). Thus, neural cells derived from hESCs are a possible donor source for cell transplants to be used in the treatment of various neurodegenerative injuries and diseases (Reubinoff et al., 2001; Zhang et al., 2001). Due to the risk of teratoma formation, however, the transplanted neural population should not contain any undifferentiated hESCs.

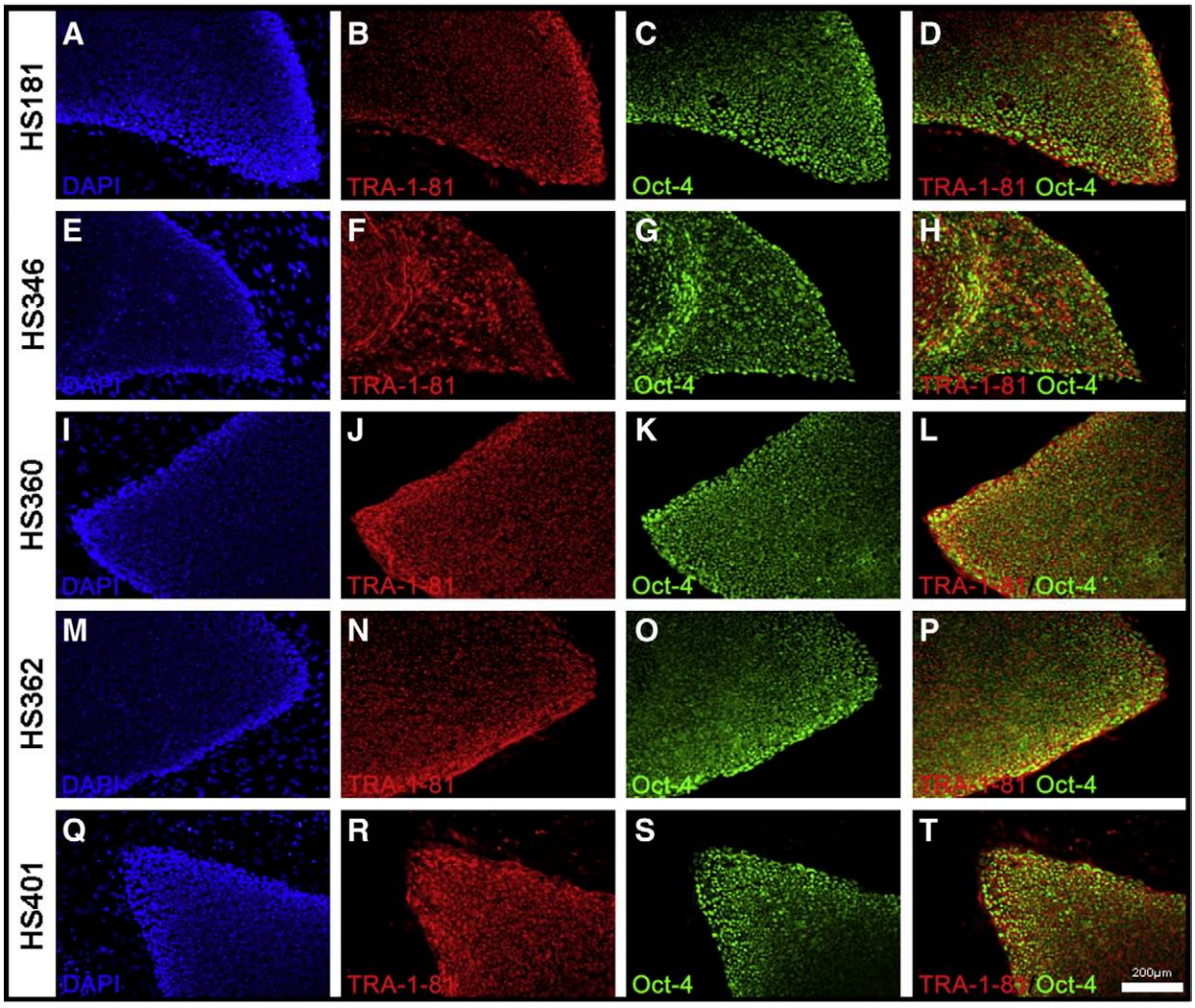

Figure 1 Immunofluorescent staining to characterize undifferentiated hESCs. hESCs expressing (B, F, J, N, R) TRA-1-81 and (C, G, K, O, S) Oct-4 and (D, H, L, P, T) merged TRA-1-81 and Oct-4 images. (A, E, I, M, Q) Nuclear staining is shown in blue (DAPI). (A–D) HS181, (E–H) HS346, (I–L) HS360, (M–P) HS362, (Q–T) HS401. Scale bar, 200 μm.

Cluster of differentiation (CD) markers are surface proteins that belong to several different classes, such as integrins, adhesion molecules, glycoproteins, and receptors. Antibodies recognizing CD markers are frequently used to identify and characterize various cell populations. The CD markers associated with pluripotent hESCs are CD9, CD24, and CD133 (Assou et al., 2007; Bhattacharya et al., 2004; Carpenter et al., 2004; Lian et al., 2006; Skottman et al., 2005; Zambidis et al., 2005). In addition, hESCs express markers such as CD29, CD90, and CD117 (Carpenter et al., 2004; Draper et al., 2002; Lian et al., 2006; Xu et al., 2001). These and other markers are also associated with neural stem cells (NSCs) and neural precursor cells (NPCs) and with mature neurons and/or glial cells in the adult human central nervous system (CNS) and peripheral nervous system (Supplemental Table 1).

Thus, CD markers are a useful tool for studying the differentiation of living cells (Pruszack et al., 2007). Furthermore, these markers can be used to isolate specific cell populations based on their surface marker expression profile using techniques such as immunopanning, magnetic cell sorting, and fluorescence-activated cell sorting (FACS). For example, $CD133^{+}$ human CNS stem cells isolated from fetal brain tissue using FACS differentiate into neural cells when engrafted into the brains of immunodeficient newborn mice (Tamaki et al., 2002; Uchida et al., 2000). The hESC-derived neural populations must be carefully characterized and purified prior to transplantation to avoid

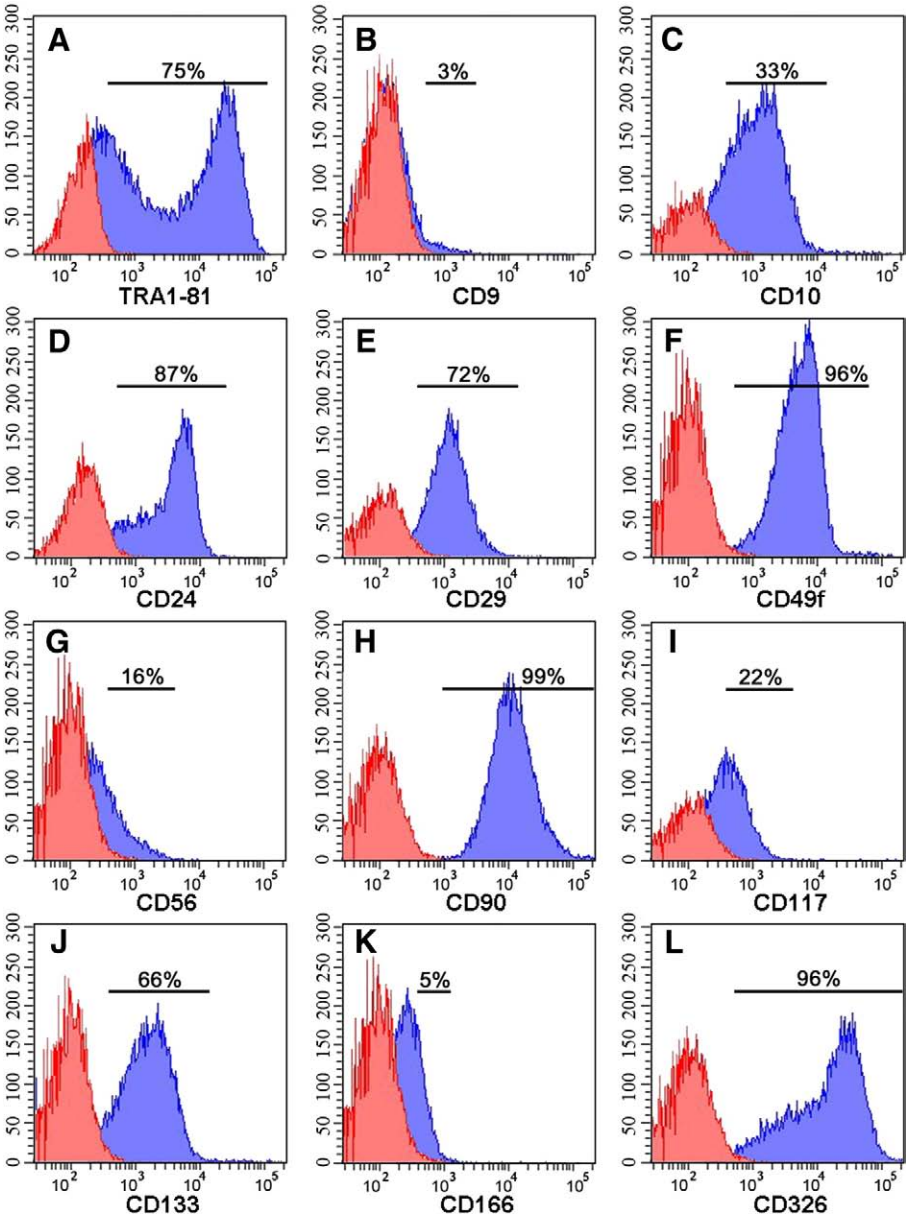

Figure 2 Flow-cytometric analysis of undifferentiated hESCs. Representative FACS plots of hESC line HS181 expressing (A) TRA-1-81, and CD marker expression by undifferentiated TRA-1-81-positive hESCs: (B) CD9, (C) CD10, (D) CD24, (E) CD29, (F) CD49f, (G) CD56, (H) CD90, (I) CD117, (J) CD133, (K) CD166, and (L) CD326.

tumor formation *in vivo* caused by undifferentiated hESCs. To characterize the cells, the expression patterns of specific markers in undifferentiated hESCs need to be validated.

In this study, the expression of a panel of CD markers, some of which were previously associated with hESCs, was compared in five hESC lines derived and cultured similarly (Hovatta et al., 2003, Inzunza et al., 2005). An expanded panel consisting of 30 surface markers was used to characterize undifferentiated TRA-1-81-positive hESCs and their neural derivatives. The expression patterns of the most interesting CD markers were further studied in two hESC lines derived and cultured in-house.

Results

hESC culture and neural differentiation

The undifferentiated state of hESCs was confirmed by positive staining for Nanog, Oct-4, and TRA-1-81 (Figs. 1 and 4), and SSEA-3 (data not shown), and by expression of *Nanog* and *Oct-4* (Supplemental Fig. 1). The neural cultures were monitored and characterized morphologically using the Cell-IQ online monitoring culture platform (Supplemental Movie 1) and immunocytochemically using MAP-2 (Supplemental Fig. 2), nestin, β-tubulin$_{III}$, BLBP, and GFAP (data not shown here, see Nat et al., 2007). The expression of *Pax6, nestin, Mash1,* and *MAP-2* was confirmed in neural cultures (Supplemental Fig. 1).

Surface protein expression profiles of hESCs

The hESCs (from lines HS181, HS346, HS360, HS362, and HS401) were labeled with TRA-1-81 to identify undifferentiated cells and colabeled with 11 CD markers (CD9, CD10, CD24, CD29, CD49f, CD56, CD90, CD117, CD133, CD166, and CD326). Representative FACS plots are presented in Fig. 2. The CD marker expression in undifferentiated hESCs from five hESC lines is shown in Fig. 3. The expression profile was categorized as follows: 0–2%, negative; 3–20%, weak; 21–50%, medium; 51–80%, medium high; >80%, high (Fig. 3). For analysis details see Supplemental Table 2.

Flow-cytometric analysis of undifferentiated hESCs (HS lines) showed high expression of CD24, CD90, and CD326 in all hESC lines (Fig. 3). As CD326 has not been previously associated with hESCs, its expression was confirmed immunocytochemically (Fig. 4). Staining showed that CD326 was expressed on the surface of Oct-4 and Nanog-positive hESCs (Fig. 4). Also, the hESC lines derived in REGEA expressed CD326 (Figs. 4 and 5). As Fig. 3 shows, expression of CD49f and CD133 was also high or medium high in all five hESC lines. Expression of CD10 was medium or medium high in all hESC lines, while that of CD9, CD56, CD117, and CD166 was weak or absent. Expression of marker CD29 was heterogeneous in the five hESC lines analyzed, varying from 13 to 72%.

Surface protein expression profiles of hESCs and their neural derivatives

The HS181 line was chosen for a broader surface marker study in which we compared the expression of 30 CD markers on TRA-1-81-positive hESCs and their neural derivatives. The surface protein expression profiles are shown in Fig. 5. Similar to HS181 hESCs, neural cells expressed high or medium high levels of CD24, CD29, CD49f, CD59, and CD90. Expression of CD10, CD133, and CD326 was substantially higher (>20%) in HS181 hESCs compared to the neural derivatives. The most prominent difference was in the expression of CD326, which was high in undifferentiated hESCs (>90%) and weak in neural cells (<20%). Compared to the hESCs, hESC-derived neural cells had higher expression of CD49b, CD56, CD117, CD166, CD184, and CD271. Of these markers, CD49b, CD146, and CD166 have not been previously associated with human NSCs or NPCs (for comparisons, see Fig. 5A and Supplemental Table 1). Supplemental Fig. 3 shows representative FACS plots of CD marker expression levels that were substantially increased in hESC-derived neural cells. Differentially expressed CD markers between hESCs and neural cells were further profiled in four other hESC lines: HS360, HS362, Regea040/06, and Regea023/08. This analysis showed that CD133 and CD326 were expressed at substantially higher levels in undifferentiated hESCs

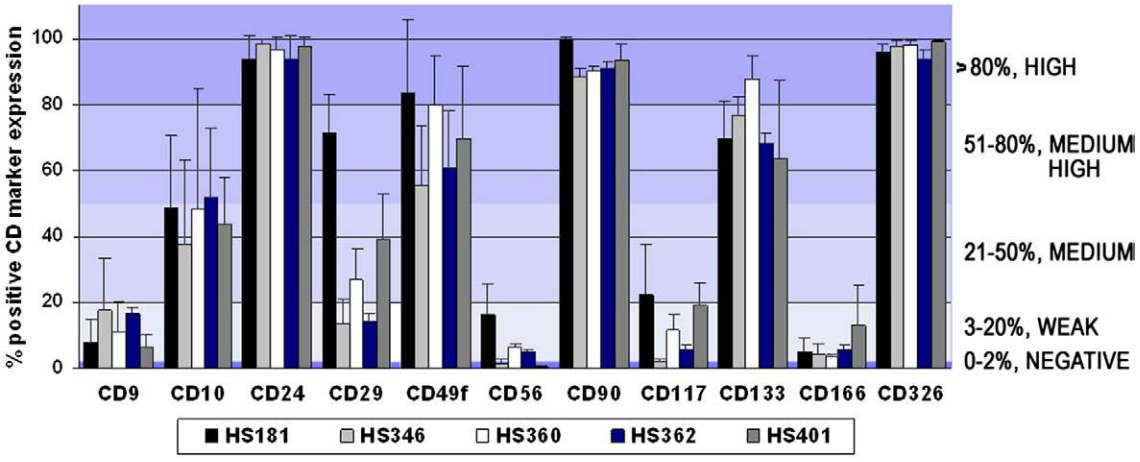

Figure 3 CD marker expression in five hESC lines. Expression of CD markers in TRA-1-81-positive hESCs in hESC lines HS181, HS346, HS360, HS362, and HS401, as determined by flow cytometry.

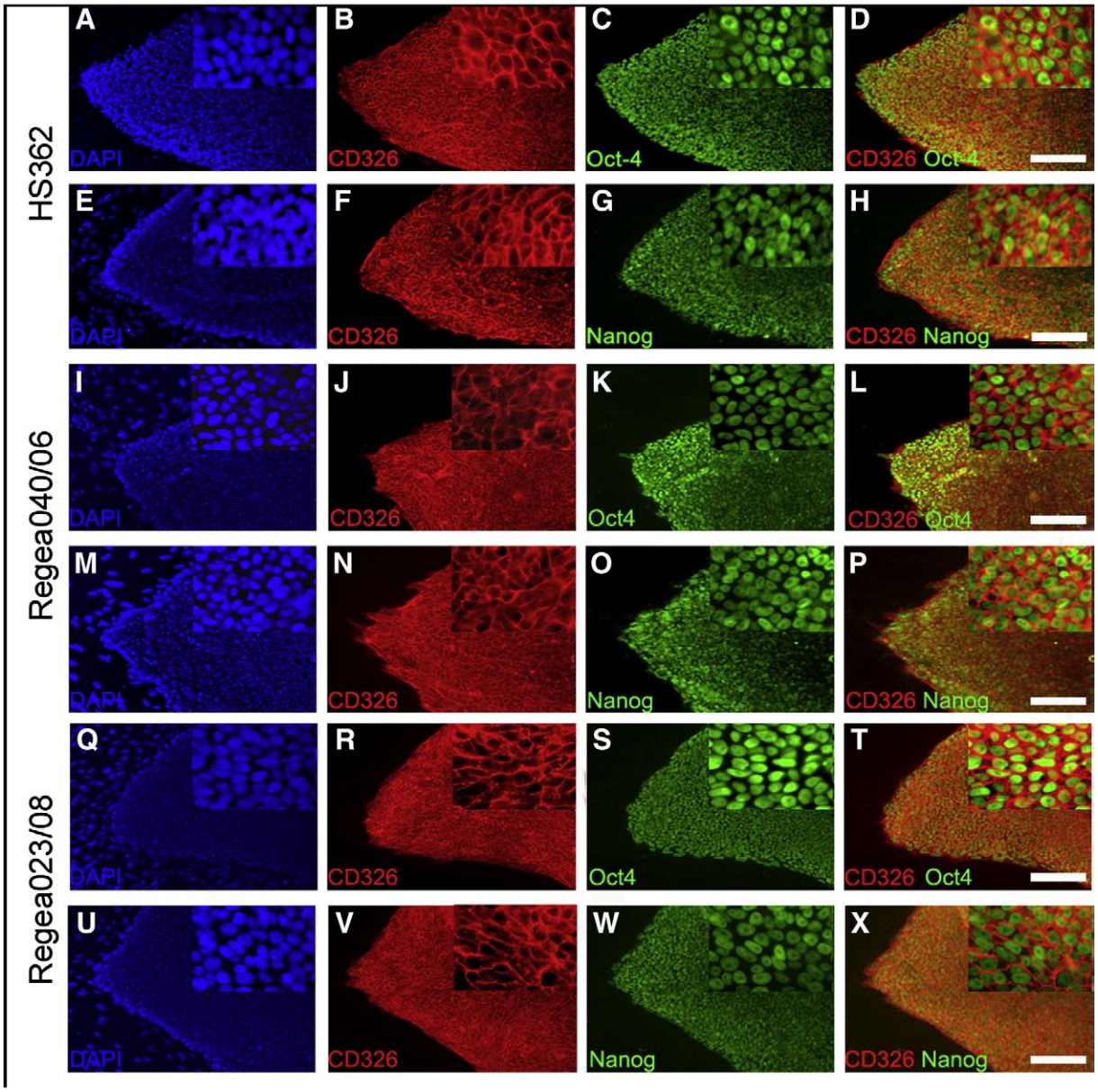

Figure 4 Immunofluorescent staining of hESC colonies expressing CD326, Oct-4, and Nanog. hESC lines: (A–H) HS362, (I–P) Regea040/06, and (Q–X) Regea023/08. hESCs expressed (B, F, J, N, R, V) CD326, (C, K, S) Oct-4, and (G, O, W) Nanog. (D, H, L, P, T, X) Merged CD326/Oct-4 and CD326/Nanog images. Each image also includes higher magnifications obtained with a 40× objective. (A, E, I, M, Q, U) Nuclear staining with DAPI. Scale bar, 100 μm.

compared to their neural derivatives, while CD56 and CD184 expression was higher in neural cells (Fig. 5).

Neural subpopulations and FACS sorting

Double labeling showed that CD56- (NCAM) and MAP-2-positive neural populations were not positive for CD326, a finding confirmed by combinatorial FACS analysis (Fig. 6). Subpopulations of CD56- and MAP-2-positive neural cells were positive for CD184 that especially localized to neurites in MAP-2-positive neurons (Fig. 6). Combinatorial FACS analysis showed that 48.8% of neural cells were $CD56^+/CD184^+$ (Fig. 6).

Neural cell sorting was performed using either positive selection for CD56, CD117, CD133, CD166, CD184, and CD271 or negative selection for CD326. The viability and neuronal morphology of sorted cells was monitored for 3 days after subcultivation (Supplemental Movie 2) and the results showed that cells in the original neural populations and in $CD326^-$, $CD56^+$, and $CD184^+$ populations resembled typical neurons (Fig. 6). FACS-sorted cells were 34 to 64% positive for MAP-2 (Supplemental Fig. 2). Especially, $CD56^+$ and $CD184^+$ cell populations were highly (>60%) positive for MAP-2. No GFAP-positive cells were identified.

Additional teratoma formation experiments with SCID mice revealed that undifferentiated hESCs formed teratomas, whereas neural cells and CD326-negative neural

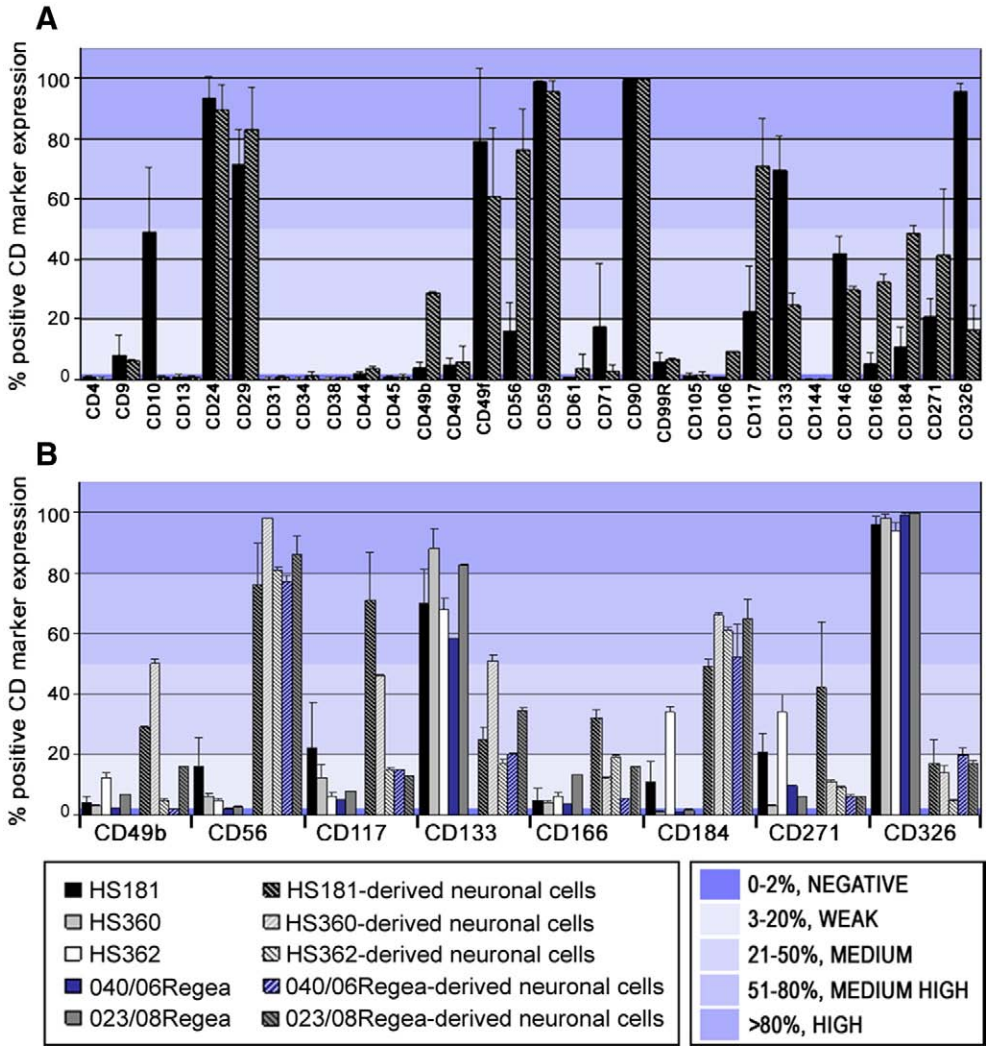

Figure 5 CD marker expression in hESCs and their neural derivates. (A) Expression of 30 different CD markers in TRA-1-81-positive hESCs (HS181) and neural derivatives determined by flow cytometry. (B) The expression of CD49b, CD56, CD117, CD133, CD166, CD184, CD271, and CD326 in TRA-1-81-positive hESCs and hESC-derived neural cells, determined by flow cytometry.

cells did not show any tumor formation after 2 months (Supplemental Fig. 4).

Downregulation of pluripotent markers and upregulation of neural markers during differentiation

Next, we studied the more detailed expression profiles of specific markers during the differentiation of hESCs toward neural cells. The expression of *Oct-4* and *Nanog* was gradually downregulated during the first 4 weeks of differentiation at which time the endo- and mesodermal markers also vanished. Expression of *Pax-6* and *nestin* was constant, while expression of *Mash1*, *MAP2*, and *CXCR4* appeared after 1 week of differentiation and remained quite constant thereafter (Supplemental Fig. 1).

The surface expression of the pluripotency marker TRA-1-81 was downregulated in parallel with CD326 during 3 weeks of neural differentiation. The expression of CD133 varied but was downregulated in 6 weeks' time. CD56 was highly upregulated already during the first week of differentiation and remained high during the 6-week time course. CD184 expression followed the expression curve of CD56 at a lower level (Fig. 7).

The combinatorial FACS analysis revealed that 88% of $CD133^+$ cells were positive for CD326 in hESCs (day 0). After 3 weeks, less than 8% of $CD133^+$ cells were CD326 positive. Interestingly, the $CD184^+$ subpopulation in $CD56^+$ cells increased from 28 up to 46% during 6 weeks of differentiation, further confirming that CD184 is expressed in differentiating neural cell populations (Fig. 7).

Discussion

In this study, the expression of 30 CD surface markers on TRA-1-81-positive hESCs and their neural derivatives was characterized. The CD expression profiles of seven hESC lines were analyzed and compared between undifferentiated hESCs and their neural derivatives. Here, we showed that similarly derived and cultured hESC lines had relatively

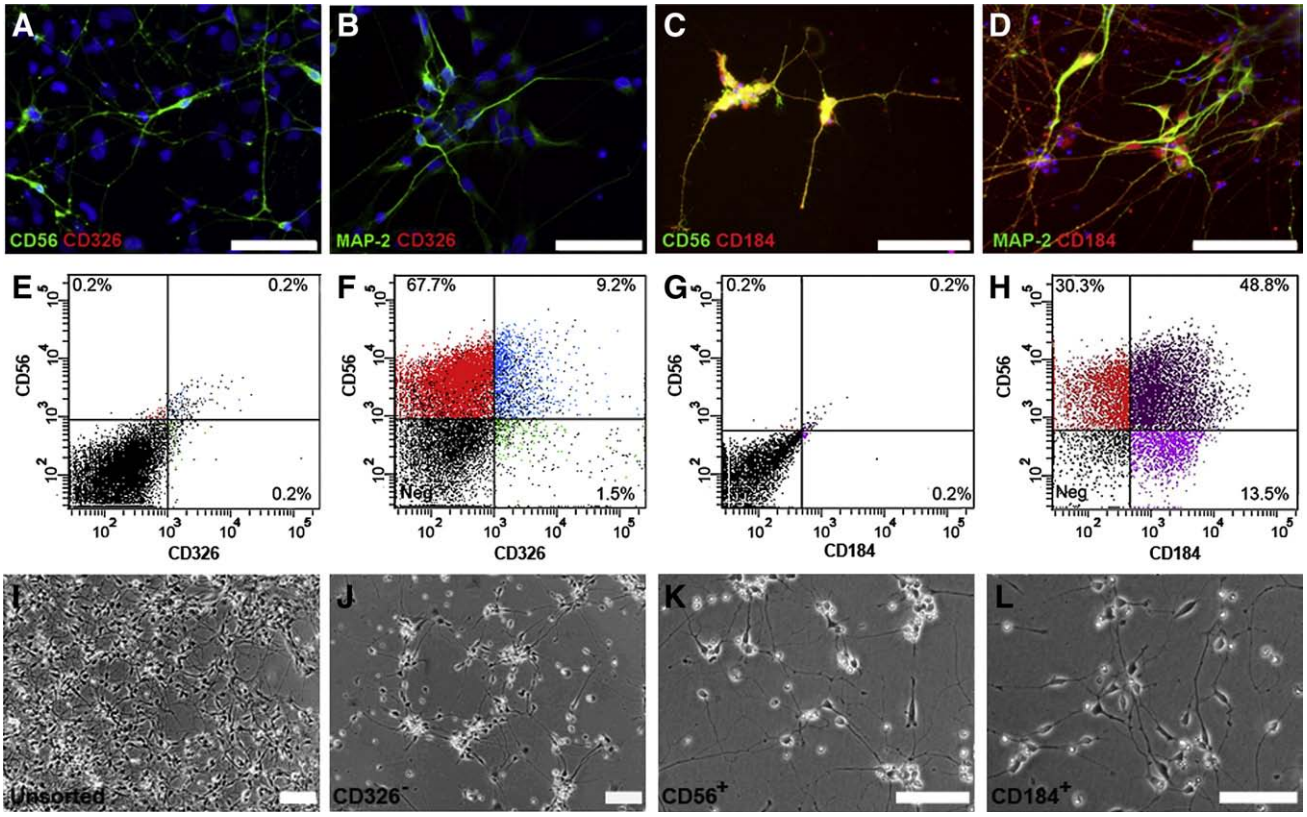

Figure 6 Immunofluorescent staining of neural populations showed that cells positive for (A) CD56 (NCAM, green) and (B) MAP-2 (green) were not positive for CD326 (red). (C, D) CD56- and MAP-2-positive cells coexpressed CD184 (red). Combinatorial FACS analysis showed that (F) the majority of neural cells were $CD56^+$ (~77%) and only a minor population was $CD56^+/CD326^+$ (~9%), (H) whereas ~49% of cells were $CD56^+/CD184^+$. (E, G) Unlabeled neural cells were used for population determination. The morphology of (I) unsorted neural cells and (J) $CD326^-$-, (K) $CD56^+$-, and (L) $CD184^+$-sorted populations is presented. Scale bar, 100 μm.

constant CD marker expression levels, whereas neural populations derived from different hESC lines had more variable CD marker expression levels. The expression of CD133 and CD326 was strong in undifferentiated hESCs and low in neural derivatives, and the expression of CD56 and CD184 was increased in hESC-derived neural cells. These CD marker expression results were similar in hESCs derived in two laboratories.

A detailed characterization of undifferentiated hESCs and their derivatives is important for many reasons. First, it is important to determine the similarity of hESCs derived and cultured by different laboratories, as recently investigated by the ISCI (ISCI, 2007). For example, their analysis of 59 hESC lines revealed large variability between different hESC lines in the expression of the surface markers SSEA-3 and SSEA-4 (ISCI, 2007). Here, we used TRA-1-81, which is more consistently expressed by undifferentiated hESCs (ISCI, 2007), as a comarker in CD marker screening for the selection of undifferentiated hESCs. Second, novel surface markers that are highly and consistently expressed in all undifferentiated hESC lines would enable separation of undifferentiated hESCs from their neural derivatives. In heterogeneous populations, this would enable the exclusion of pluripotent cells that can form teratomas *in vivo*. This negative selection may be needed to produce safe neural cell grafts for transplantation therapies. Further, negative-selected populations do not contain surface-bound antibodies, in contrast to positive-selected populations, a difference that may have a large impact on graft survival.

Here, we showed that all five of the undifferentiated hESC lines analyzed had a consistently high expression of CD24, CD90, and CD133, markers that were previously associated with hESCs. In this study, more than 80% of hESCs expressed CD24 and CD90, whose gene and protein products have been identified in hESCs (Draper et al., 2002; Skottman et al., 2005). CD24 can be used to distinguish hESCs from fibroblasts in culture (Assou et al., 2007) and to distinguish mouse multipotent fetal stem cells from neural progenitors and postmitotic neurons (Panchision et al., 2007). Pruszack and co-workers suggested that CD24 is a specific surface marker that is upregulated during neural differentiation (Pruszack et al., 2007). In contrast, our study shows that hESCs and hESC-derived neural cells expressed CD24 at equal levels (90% vs 94%, respectively). Also, high (>90%) expression of CD90 was detected in both undifferentiated hESCs and hESC-derived neural cells, which is consistent with the results of previous studies (Schwartz et al., 2003; Draper et al., 2002; Hamann et al., 1980). Hence, our data exclude the potential use of CD24 and CD90 as specific markers for undifferentiated hESCs or differentiating hESC-derived neural cells. In contrast, CD133 marker expression was over 65% in all undifferentiated hESC lines and was downregulated in hESC-derived neural cells. This finding is consistent with the literature and suggests that

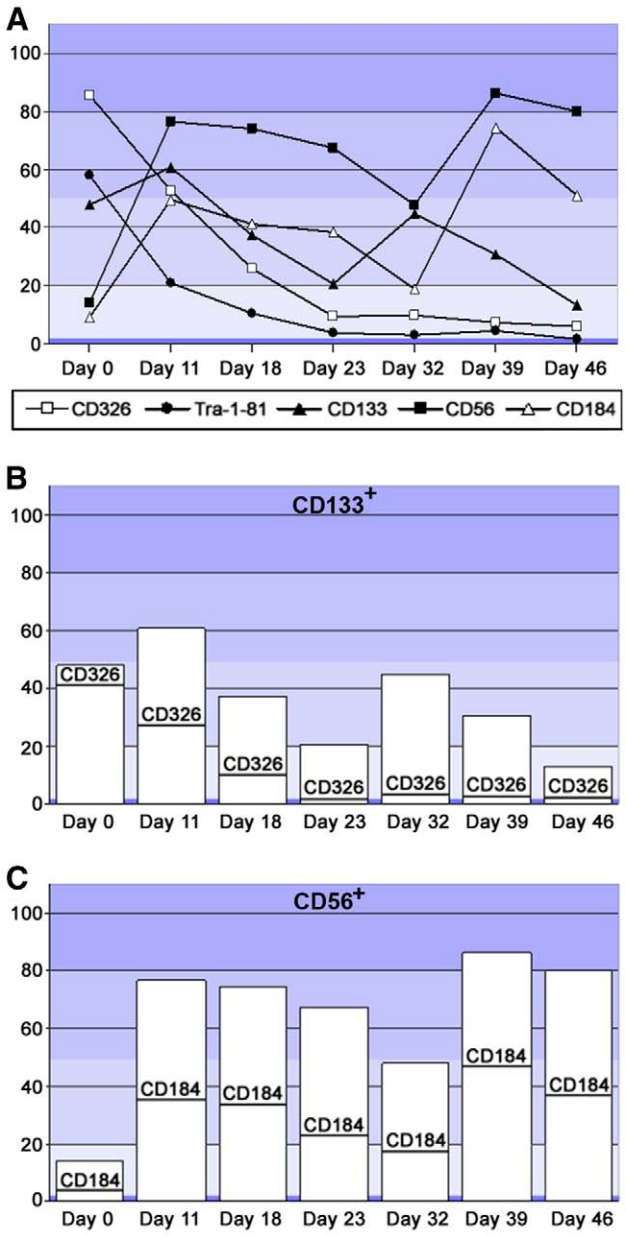

Figure 7 Time-point analysis of hESC (HS360) neural differentiation. (A) Expression curves of TRA-1-81$^+$, CD326$^+$, CD133$^+$, CD56$^+$, and CD184$^+$ populations from 0 to 46 days of neural differentiation. (B) CD326 expression in the CD133$^+$ subpopulation and (C) CD184 expression in the CD56$^+$ subpopulation.

CD133 is a reliable marker for stem and progenitor cells (Carpenter et al., 2004; Schwartz et al., 2003; Yin et al., 1997). CD133 can also be used as a selection marker for proliferating neural stem and precursor populations from postmitotic neurons (Uchida et al., 2000). Moreover, we suggest that the downregulation of CD133 expression in differentiating neural cells indicates their decreasing proliferation capacity compared to undifferentiated hESCs.

Most interestingly, we found a novel surface marker, CD326, consistently expressed in over 94% of the undifferentiated cells in all seven hESC lines studied. This result was constant between hESC lines derived in two independent laboratories. CD326, also known as epithelial cell adhesion molecule (EpCAM), is a glycoprotein involved in cell adhesion (Litvinov et al., 1997). The high expression of CD326 by hESCs might explain the tight cell-to-cell contacts of hESCs in colonies. CD326 is associated with stem cells in hepatic cell lineages (Schmelzer et al., 2006). CD326/EpCAM is also part of the same epithelial adhesion molecule family as E-cadherin, which is expressed in undifferentiated hESC colonies but not in differentiated embryoid bodies (Cai et al., 2005; Ullmann et al., 2007). In carcinoma cells, CD326/EpCAM affects upregulation of the proto-oncogene c-myc and cyclin A/E and thereby has direct impact on the cell cycle and proliferation (Munz et al., 2004). Thus, this molecule might also affect the ability of hESCs to proliferate efficiently. Importantly, both flow-cytometric and immunocytochemical analyses showed that hESC-derived neural populations did not contain CD362-positive cells. Moreover, expression of CD362 was downregulated in parallel with TRA-1-81 on hESC-derived populations during the first 4 weeks of neural differentiation. Further, combinatorial FACS analysis showed that the CD133$^+$/CD326$^+$ population was downregulated during differentiation. Thus, we propose that CD326 can be used as a novel marker for undifferentiated hESCs to facilitate the removal of undifferentiated hESCs from differentiated neural cell populations before cell transplantation.

Some markers previously associated with hESCs, such as CD9, CD29, and CD117 (Assou et al., 2007; Bhattacharya et al., 2004; Carpenter et al., 2004; Draper et al., 2002; Lian et al., 2006; Xu et al., 2001; Zambidis et al., 2005) were only weakly or heterogeneously expressed by our undifferentiated hESCs. CD marker expression during neural differentiation of hESCs appears to vary depending on both the differentiation protocol used and the initial expression profile in hESCs. Previously, Pruszack and co-workers performed flow-cytometric analysis and sorting on hESCs and neural cells differentiated in a coculture system (Pruszak et al., 2007). The expression levels of stem-cell-related markers CD24, CD29, and CD133 were less than 15% in the hESC lines HI and H7 (Pruszak et al., 2007), compared to much higher expression in our undifferentiated hESCs. These differences may reflect the different derivation and culturing methods of hESCs and further imply that universal, constantly highly expressed markers for hESCs are needed.

There are currently many differentiation protocols for neural differentiation of hESCs utilizing adherent, suspension, and cocultures with various supplements (Hoffman and Carpenter, 2005). Thus, the neural cell cultures are often heterogeneous, impure, and not necessarily comparable with one another. Here, we used simple methods for neural differentiation and compared the CD profiles in these cultures to the undifferentiated hESCs. In our study, the neural cells derived from five hESC lines had increased expression of CD56 and CD184 compared to undifferentiated hESCs, whereas the expression of CD49b, CD177, CD166, and CD271 was heterogeneously increased. CD56 is a marker of early neuroectodermal differentiation (Reubinoff et al., 2001) and has been used to identify neural differentiation of hESCs by flow-cytometric analysis and sorting (Pruszak et al., 2007). CD184, also known as the chemokine receptor CXCR4, is characterized as a receptor protein modulating cell growth and migration of neural cells in the CNS (Ni et al., 2004). Interestingly, in our study the hESC-derived neural cells had quite similar expression levels of CD184 compared

to NPCs derived from human fetal brain tissues (Ni et al., 2004), suggesting that these cell populations might have similar migration capacities.

The more detailed analysis of the differentiating neural populations revealed that both gene and surface protein markers for pluripotent cells disappeared during the first 4 weeks of differentiation. These time curves for TRA-1-81 and CD326 expression were very similar to those described for TRA-1-81, TRA-1-60, SSEA-3, and SSEA-4 in a coculture system (Pruszak et al., 2007). In addition, the expression of CD56 was rapidly upregulated in differentiating neural populations and remained high during 6 weeks of differentiation. These results are in line with a previous study (Pruszak et al., 2007), suggesting that CD56 can be used to monitor neural specification. Moreover, our neural cell population did not produce teratomas when transplanted into SCID mice. This implies that our hESC-derived neural cell cultures are valid populations for further production of more specialized neural cells or for transplantation.

As expression of CD184 was increased in parallel with CD56, we performed combinatorial FACS analysis with CD56 and CD184. These results showed that during 5 to 6 weeks of differentiation up to 50% of CD56-positive cells were CD184 positive. Further, CD184 localized in soma and neurites of $CD56^+$ cells and especially in MAP-2-positive neurons. FACS-sorted $CD184^+$ neural cells, followed with time-lapse imaging, matured into viable neurons. These results are in line with a previous study by Peng and co-workers (Peng et al., 2007), who showed that human fetal brain tissue-derived NPCs also express CD184 at high levels when differentiated into neuronal cells. Altogether, these results suggest that CD184 is an important factor in neurogenesis and in maturation of neuronal cells and should be studied in more detail in the future.

Fluorescence-activated cell sorting has been considered as a possible method for producing pure neural subpopulations to be used in transplantation therapies (Chung et al., 2006; Guzman et al., 2008). Here, we showed that neural subcultivation is possible after sorting with various CD markers, CD56, CD117, CD133, CD166, CD184, and CD271. Especially, by sorting with CD56 and CD184 viable neuronal populations can be produced. Moreover, pluripotent cells can be eliminated by negative sorting for CD326 if more purification of the neural population is needed prior to transplantation. Although there are milder and more sensitive ways to sort with FACS using a bigger nozzle (100 μm) and low sorting setups for fragile cells, this method needs to be better optimized before separating populations for cell grafting. For these types of studies, microfluidics-based cell sorting techniques may be a future prospect (Studer et al., 2004; Chen et al., 2008). In any case, FACS is a usable and reliable method for studies of neural subpopulations and their characteristics.

In conclusion, surface marker expression in previously established hESC lines varies to a great extent universally, whereas hESC lines derived and cultured similarly have quite constant CD marker profiles. Even though there are many commonly used markers for hESCs (SSEA-3, SSEA-4, TRA-1-60, TRA-1-81), their expression levels vary markedly in different hESC lines. In contrast, CD326 showed consistently high expression in all undifferentiated hESCs compared to their neural derivatives. CD326 has not, to our knowledge, been previously associated with hESCs, and therefore we consider this surface protein to be a novel marker for undifferentiated hESCs. As our transplantation experiments showed no teratoma formation with hESC-derived CD326-negative selected neural cells, we conclude that this marker might be useful for the production of differentiated, hESC-free neural populations.

Materials and methods

hESC culture

Five hESC lines were used in this study: HS181, HS346, HS360, HS362, and HS401. All hESC lines were derived from the inner cell mass of supernumerary blastocyst-stage embryos at the Karolinska Institute, Karolinska University Hospital Huddinge, Sweden, after approval by the ethics committee of the Karolinska Institute. The procedures for the derivation, characterization, and culture of these hESC lines were described previously (Hovatta et al., 2003). Studies utilizing the hESC lines derived at the Karolinska Institute and performed at REGEA, Institute for Regenerative Medicine, University of Tampere, Finland, were approved by the ethics committee of the Hospital District of Pirkanmaa (Hovatta, R05051). In REGEA, two novel hESC lines, Regea040/06 and Regea023/08, were derived and cultured (H. Skottman et al., unpublished data). REGEA has a supportive statement from the ethics committee of the Pirkanmaa Hospital district to derive and expand new human ESC lines from surplus embryos, which cannot be used in the infertility treatment of the donating couples (Skottman, R05116). All the hESCs were cultured on commercially available, mitotically inactivated human foreskin fibroblasts (CRL-2429, ATCC, Manassas, VA, USA) in hESC medium consisting of knockout Dulbecco's modified Eagle's medium (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 20% knockout SR (Gibco Invitrogen), 2 mM GlutaMax (Gibco Invitrogen), 0.1 mM minimal essential medium nonessential amino acids (Cambrex Bio Science, Karlskoga, Sweden), 0.1 mM β-mercaptoethanol (Gibco Invitrogen), 50 U/ml penicillin/streptomycin (Cambrex), and 8 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN, USA) at 37 °C in 5% CO_2 in a humidified atmosphere. The hESC colonies were passaged at 5- to 7-day intervals by mechanical splitting and replated on fresh feeder cells.

Neural differentiation of hESCs

HS181, HS360, HS362, Regea040/06, and Regea023/08 colonies were mechanically dissected and differentiated into neural cells in adherent or suspension culture using a modified protocol by Nat and co-workers (Nat et al., 2007). The neural proliferation medium contained Dulbecco's modified Eagle's medium/F-12 and neurobasal medium (1:1) supplemented with 1× B27, 1× N2, 2 mM GlutaMax (all from Gibco Invitrogen), 25 U/ml penicillin/streptomycin, and 20 ng/ml bFGF. Briefly, differentiating neural cells formed rosette-like structures in adherent culture (CellBIND Surface, Corning, Inc., Corning, NY, USA) or in suspension culture in proliferation medium after 7 to 10 days. These rosette structures were dissected and plated on laminin-coated (10 μg/ml; Sigma Chemical Co., St. Louis, MO, USA)

culture well plates (Nunc, Roskilde, Denmark) in the absence of bFGF (neural differentiation medium) for further adherent culturing for 6 to 8 weeks. In suspension culture, the spheres were mechanically split once a week and cultured for 6 to 8 weeks. Thereafter, spheres were dissected and replated on laminin-coated well plates for 1 week. bFGF (4 ng/ml) and brain-derived neurotrophic factor (5 ng/ml; Gibco Invitrogen) were added to cultures to support the growth and survival of maturing neural cells. All cultures were maintained in a humidified atmosphere at 37 °C and 5% CO_2.

Automated monitoring of hESC-derived neural cells

The growth and morphologic characteristics of hESC-derived neural cells were monitored prior to immunocytochemical and flow-cytometric analysis using an online cell culture platform (Cell-IQ, Chip-Man Technologies, Tampere, Finland) equipped with phase-contrast microscope optics (10×) and a CCD camera, as described previously for hESCs (Narkilahti et al., 2007). This software allows time-lapse imaging of 500 × 670-μm areas in the culture wells. Captured images (JPEG) were saved in separate folders and converted into movie format using Cell-IQ analysis software.

Immunocytochemical analysis of hESCs and hESC-derived neural cells

Subsets of hESCs and hESC-derived neural cells were fixed using 4% paraformaldehyde in phosphate-buffered saline (PBS; 0.01 M PBS, pH 7.4) for 20 min at room temperature and washed twice with PBS. The cells were permeabilized and blocked with 10% normal donkey serum (NDS; Sigma) and 1% bovine serum albumin (BSA; Sigma) in PBS containing 0.1% Triton X-100 for 45 min at room temperature. Primary antibodies were applied overnight at 4 °C in 1% BSA, 1% NDS in PBS containing 0.1% Triton X-100. Cells were washed three times and incubated with fluorescent secondary antibodies in 1% BSA in PBS for 60 min at room temperature in the dark. Cells were then washed two times with PBS and two times with phosphate buffer and mounted using Vectashield containing 4′,6-diamidino-2-phenylindole (DAPI) for nuclear staining (Vector Laboratories, Inc., Burlingame, CA, USA). The primary antibodies used for hESCs were mouse anti-epithelial-specific antigen IgG (EpCAM/CD326, 1:200; Chemicon, Temecula, CA, USA), goat anti-Nanog IgG (1:200; R&D Systems), goat anti-Oct-4 IgG (1:100; R&D Systems), and mouse anti-TRA-1-81 IgM (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Primary antibodies used for neural cells were mouse anti-CXCR4 IgG (1:2000; R&D Systems), sheep anti-GFAP IgG (1:600; R&D Systems), rabbit anti-MAP-2 IgG (1:600–1:800; Chemicon), and rabbit anti-NCAM IgG (1:800; Chemicon). Secondary antibodies used were Alexa Fluor 488 donkey anti-goat IgG (1:800), Alexa Fluor 488 donkey anti-rabbit IgG (1:400), Alexa Fluor 488 donkey anti-sheep IgG, and Alexa Fluor 568 goat anti-rabbit IgG (1:400) (all from Molecular Probes Invitrogen) and rhodamine red-conjugated donkey anti-mouse IgM and IgG (1:400; Jackson ImmunoResearch Europe Ltd., Cambridgeshire, UK). For negative controls, the primary antibodies were omitted from the immunostaining, which resulted in the disappearance of all staining. Stained cells were viewed and photographed using an Olympus IX51 phase-contrast microscope equipped with fluorescence optics and an Olympus DP71 camera.

Sample preparation for flow cytometry

The hESC cultures were washed once with ice-cold sterile PBS and trypsinized for 10 min at 37 °C (trypsin–EDTA; BioWhittaker, Fisher Scientific, Leicestershire, UK) and inactivated with 2% fetal bovine serum (FBS; Gibco Invitrogen) in PBS. Cell colonies were dissociated into single-cell suspensions by trituration, centrifuged, resuspended in 2% FBS in PBS, and counted using trypan blue exclusion to identify viable cells. Aliquots of 100,000 viable cells per sample were used for flow-cytometric analysis. hESC-derived neural cells were washed twice, trypsinized for 5 min at 37 °C, and inactivated with 5% human serum (HS) in PBS. Cells were dissociated into a single-cell suspension by trituration, centrifuged, resuspended in 5% HS in PBS, and filtered using 50-μm cell strainers (CupFilcons; BD Biosciences, Franklin Lakes, NJ, USA) prior to trypan blue counting. Aliquots of 100,000 viable cells per sample were used for flow-cytometric analysis.

Surface antigen expression analysis

To identify undifferentiated cells, hESCs were labeled for TRA-1-81 and colabeled with fluorochrome-conjugated anti-human CD markers. Briefly, hESCs were incubated for 15 min at 4 °C with TRA-1-81 antibody, followed by incubation with fluorescent secondary antibody and antibodies recognizing CD markers for 15 min at 4 °C in the dark. Cells were washed twice with 2% FBS in PBS between each step. Thereafter, labeled cells were suspended in 2% FBS in PBS. At least three parallel samples for each TRA-1-81 and CD marker combination were analyzed. hESC-derived neural cells were directly labeled with anti-human CD markers and suspended in 5% HS in PBS. Two to four parallel samples for each CD marker were analyzed.

The primary antibody used was mouse anti-TRA-1-81 IgM (1:200; Santa Cruz Biotechnology) with secondary antibody anti-mouse Alexa Fluor 488 IgM (1:6500; Molecular Probes Invitrogen) or anti-mouse phycoerythrin (PE) IgM (1:500; Caltag Invitrogen). Antibodies recognizing CD markers were CD4–fluorescein isothiocyanate (FITC), CD9–FITC, CD13–PE, CD24–FITC, CD31–FITC, CD34–allophycocyanin (APC), CD38–PE, CD44–FITC, CD49b–FITC, CD56–PE, CD59–FITC, CD61–FITC, CD71–PE, CD99R–FITC (ImmunoTools, Friesoythe, Germany); CD45–FITC, CD117–APC, CD133–PE, CD271–FITC, CD326–APC (Miltenyi Biotech, Bergisch Gladbach, Germany); CD9–PE, CD10–PE–Cy7, CD29–APC, CD49d–PE, CD49f–APC, CD90–APC, CD106–PE–Cy5, CD146–PE, CD166–PE, CD184–PE–Cy5 (BD Biosciences); and CD105–PE and CD144–PE (R&D Systems). Antibody concentrations were chosen according to the manufacturer's protocol or adjusted to the optimal concentration when necessary. Background fluorescence was excluded using unlabeled cells or isotype controls or by incubation with secondary antibodies only. Analyses were performed using FACSAria equipment with 488- and 633-nm lasers, a standard filter set, and FACSDiva software (BD Biosciences). The cell population of interest was determined and dead cells were excluded using forward-

and side-scatter parameters. For each sample run, 10,000 to 20,000 events were recorded and analyzed.

Fluorescence-activated cell sorting and subcultivation of hESC-derived neural cells

HS181-derived neural cell samples (400,000–1,000,000 cells/sample) were prepared as described above and labeled for CD56, CD117, CD133, CD166, CD184, or CD271. Cell sorting was performed using a FACSAria with a 100-μm nozzle, 20.00 psi sheath pressure, sort precision mode set for purity, flow rate 2, and plates voltage 5.0 (sort setup: low). Live cells were gated using forward- and side-scatter parameters, and cells positive for each CD marker were collected into 5-ml polystyrene tubes (BD Biosciences) in neural differentiation medium. Cells were centrifuged and resuspended in medium containing 4 ng/ml bFGF and 5 ng/ml brain-derived neurotrophic factor and plated onto laminin-coated culture plates. After 3 days, the FACS-sorted cells were monitored with CellIQ for 24 h, fixed, and stained using antibodies for MAP-2 and GFAP, as described above.

Teratoma formation

To study *teratoma formation, hESCs and hESC-derived neural cell populations* were transplanted into SCID mouse testes as previously described (Hovatta et al., 2003; Inzunza et al., 2005). After the cell injections of hESCs, 200,000 cells/testis ($n=3$); neural cells, 1,000,000 cells/testis ($n=7$); and CD326-negative neural cells, 1,000,000 cells/testis ($n=3$), the animals were followed for 2 months for teratoma formation.

Time-point analysis of specific markers during neural differentiation

The more detailed time-point analyses of specific markers were performed with HS360 during the neural differentiation. The cells were first grown in suspension for 0, 7, 14, 19, 28, 35, or 42 days. Thereafter, they were *in vitro* differentiated on laminin-coated wells for 4 days as described above. Thus, the samples for gene expression (RT-PCR) and FACS analysis were collected at days 0, 11, 18, 23, 32, 39, and 46.

For RT-PCR, the expression of the pluripotent markers *Nanog* and *Oct4;* the endodermal marker *α-fetoprotein;* the mesodermal marker *brachyury;* the neural markers *MAP-2, Mash1, nestin,* and *Pax6; C-X-C chemokine receptor type 4;* and the housekeeping gene *GAPDH* was analyzed. For RT-PCR, RNA was extracted using the RNeasy Micro Kit (Qiagen, Hilden, Germany). A total of 50 ng of RNA was used for cDNA synthesis (Sensiscript RT Kit; Qiagen) according to the manufacturer's instructions. Each PCR contained 700 ng of cDNA, 0.25 μM forward and reverse primers, 1× Taq buffer (−MgCl, +KCl) (Fermentas, Leon-Rot, Germany), 2.5 mM dNTP (Fermentas), 25 mM MgCl (Fermentas), dH_2O, and 0.6 U Taq DNA polymerase enzyme (Fermentas). PCR program parameters were denaturation at 95 °C for 3 min followed with 35 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min and a final extension for 5 min in 72 °C.

For FACS analysis, the expression of TRA-1-81, CD326, CD133, CD56, and CD184 was analyzed. In addition, combinatorial analyses for CD133/CD326, CD56/CD326, and CD56/184 were performed. All FACS analyses were performed as described above.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.scr.2008.08.001.

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