Derivation of Multiple Cranial Tissues and Isolation of Lens Epithelium-Like Cells From Human Embryonic Stem Cells

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Key Words. Embryonic stem cells • Differentiation • Fluorescence-activated cell sorting • Lens • c-Met • p75

ABSTRACT

Human embryonic stem cells (hESCs) provide a powerful tool to investigate early events occurring during human embryonic development. In the present study, we induced differentiation of hESCs in conditions that allowed formation of neural and non-neural ectoderm and to a lesser extent mesoderm. These tissues are required for correct specification of the neural plate border, an early embryonic transient structure from which neural crest cells (NCs) and cranial placodes (CPs) originate. Although isolation of CP derivatives from hESCs has not been previously reported, isolation of hESC-derived NC-like cells has been already described. We performed a more detailed analysis of fluorescence-activated cell sorting (FACS)-purified cell populations using the surface antigens previously used to select hESC-derived NC-like cells, p75 and HNK-1, and uncovered their heterogeneous nature. In addition to the NC component, we identified a neural component within these populations using known surface markers, such as CD15 and FORSE1. We have further exploited this information to isolate the lens and CP derivatives by FACS of a CP derivative, the lens, from differentiating hESCs. Two surface markers expressed on lens cells, c-Met/HGFR and CD44, were used for positive selection of multiple populations with a simultaneous subtraction of the neural/NC component mediated by p75, HNK-1, and CD15. In particular, the c-Met/HGFR allowed early isolation of proliferative lens epithelium-like cells capable of forming lentoid bodies. Isolation of hESC-derived lens cells represents an important step toward the understanding of human lens development and regeneration and the devising of future therapeutic applications.

INTRODUCTION

Human embryonic stem cells (hESCs) provide an invaluable tool for investigating the developmental events that take place during the first 4 weeks of human embryonic development. One of these events is the specification of the neural plate border (NPB), a U-shaped area of cells that surrounds the thickened neural plate and, despite its transient existence, has a complex organization in terms of cell fate potential. The convergence of multiple molecular signals deriving from the neural plate, the non-neural epiblast, and the underlying mesoderm [1–3] allows cells located within different areas of the NPB to be specified as neural crest cell (NC) and cranial placode (CP) progenitors [4]. In the last two decades, the use of model organisms such as zebrafish, frog, chick, and mouse empowered comprehensive analyses of the inducing signals and the gene regulatory networks underlying NC [5] and, to a lesser extent, CP specification and differentiation [4, 6]. With regard to the formation of NC and CP in humans, several groups reported the derivation and isolation of NC-like cells from hESCs [7], which enabled investigations of their molecular characteristics. However, very little is currently known at cellular and molecular levels about the formation of CP derivatives in humans. Cranial placodes are spatially restricted thickenings of the non-neural ectoderm derived from a common preplacodal region (PPR) that forms only cranially and is roughly located at the more lateral side of the NPB [4, 6]. As early as gastrulation, complex signals to the PPR induce the formation of localized thickened areas that become visible at the end of neurulation and possess specific fate identity (lens, olfactory, hypothalamus, otic, epibranchial, trigeminal, lateral line). Placodal cells contribute to the sense organs and sensory ganglia of the head [3]. So far, none of the CP derivatives has ever been isolated from differentiating hESCs in vitro, although recent works have reported the conditions for enrichment of lens progenitor cells and lentoid bodies [8, 9] from hESC cultures.

Here, we report the establishment of hESC culture conditions that reproducibly define the
formation of neural and non-neural ectoderm, and to a much lesser extent mesoderm, with anterior identity up to anterior hindbrain level. We further defined the heterogeneous nature of populations isolated using the p75 (low-affinity nerve growth factor receptor, here referred to as p75) and HNK-1 antibody (directed against a carbohydrate moiety present on cell adhesion molecules), markers that have been often used to isolate hESC-derived NC-like cells [10, 11]. We found that, besides the NC component, the p75+/HNK-1+ population includes cells with anterior neural identity and, to a minor extent, paraxial mesoderm identity. More importantly, on the basis of this finding, we designed a sorting strategy using a combination of surface markers that enabled us to isolate different cell populations with lens cell fate. Specifically, the c-Met/HGFR [12, 13] and the CD44 [14, 15] surface molecules were used for positive selection of lens cell fate. Particularly, the c-Met/HGFR [12, 13] and the CD44 [14, 15] were used to subtract the neural component. To our knowledge, this is the first report of fluorescence-activated cell sorting (FACS)-mediated isolation of cells with lens fate derived from in vitro differentiating hESCs.

**Materials and Methods**

**Culture of Undifferentiated hESCs**

H9 (WA-09) cells, passages (p) 40–65, and HES3 cells, p37–p65, were initially maintained on mouse embryonic fibroblasts, as previously described [16], and then passaged on hESC-qualified matrix (Matrigel; BD Biosciences, San Diego, CA, http://www.bdbiosciences.com) in the presence of mTeSR1 medium (StemCell Technologies, Vancouver, BC, Canada, http://www.stemcell.com). Cells cultured for more than eight passages in Matrigel were used for the experiments.

**Differentiation of hESCs**

When colony size reached >800 μm in diameter and colony density on the plate was approximately 70%–80%, differentiation of hESCs was induced by switching the culture medium from mTeSR1 to a chemically defined, serum-free medium, insulin-transferrin-selenium (ITS), which contains human apo-transferrin, human insulin, and sodium-selenite (all from Sigma-Aldrich, St. Louis, MO, http://www.sigmaaldrich.com) [16]. Medium was replaced daily until the day of the analysis.

**Immunocytochemistry**

Differentiating hESCs were fixed with 4% paraformaldehyde for 10–15 minutes at room temperature and permeabilized with 0.3% Triton X-100 in phosphate-buffered saline (PBS) for 30 minutes. A complete list of primary and labeled secondary antibodies used in this study is provided in supplemental online Table 1. Primary antibody incubation and the subsequent secondary antibody incubation were performed in incubation buffer (0.1% bovine serum albumin, 2% fetal bovine serum, 0.1% Triton X-100 in PBS) for 30 minutes at 37°C. Image acquisition was performed on an inverted Nikon Eclipse Ti epifluorescence microscope (Nikon, Tokyo, Japan, http://www.nikon.com) with the appropriate filter sets using single channel acquisition on a Nikon digital sight DS-U2 camera. Images were analyzed with Nikon NIS-Elements 3.2 software.

**Reverse Transcription-Polymerase Chain Reaction and Quantitative Reverse Transcription-Polymerase Chain Reaction**

Total RNA was extracted using miRNeasy Mini kit (Qiagen, Hilden, Germany, http://www.qiagen.com) following the manufacturer’s protocol, and DNase I treatment (Qiagen) was performed to avoid genomic DNA contamination. The SuperScript VILO cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, http://www.invitrogen.com) was used to retrotranscribe 150 ng of total RNA per sample. Polymerase chain reaction (PCR) was performed in 33 cycles using a Mastercycler ProS (Eppendorf AG, Hamburg, Germany, http://www.eppendorf.com). Primer sequences and annealing temperatures are provided in supplemental online Table 2. Primers for Six1, Eya1, Dlx3, Dlx5, Six4, Dach1, Pax2, Pax8, FoxI1, Six3, Sox10, FoxG1, Pax7, Otx2, Gbx2, Krox20, HoxB4, Nkx6.1, and Prox1 were from Harvard Primer Bank [17, 18]; primers for Gapdh and Tbx6 [19], Pax6 [20], and CRAYA and Filensin [8] were from the cited references. For quantitative PCR, Gapdh was used as a reference gene, and reactions were run using LightCycler480 SYBR Green I Master (Roche Applied Science, Indianapolis, IN, https://www.roche-applied-science.com) on a LightCycler 480 system (Roche Applied Science). Relative quantification of gene expression was performed calculating primers’ efficiencies and applying the published formula [21] for relative gene expression.

**FACS**

Cells were dissociated with 0.25% trypsin (Invitrogen) to a single-cell suspension and incubated with fluorochrome-labeled antibodies (supplemental online Table 1) at a concentration of 10^6 cells per milliliter for 30 minutes at 4°C on a rocking platform. The primary antibody directed against FORSE1 was labeled with fluorescein isothiocyanate (FITC) using the Proton Fluorescein Labeling Kit (Vector Laboratories, Burlingame, CA, http://www.vectorlabs.com) following the manufacturer’s instructions. Labeled cells were sorted through the BD Influx (five lasers) flow sorter (BD Biosciences), according to the excitation requirements of the fluorochromes. Sorted populations were analyzed using FlowJo software (Tree Star, Ashland, OR, http://www.treestar.com).

**Postsorting Cell Culture**

Sorted cells were plated at a density of 8 × 10^4 cells per cm² on plates coated with 2 μg/ml fibronectin (Gibco/Invitrogen, Grand Island, NY, http://www.invitrogen.com), 2 μg/ml laminin (Invitrogen), and 5 μg/ml collagen IV (Millipore, Billerica, MA, http://www.millipore.com) in ITS supplemented with 10 μM Rock Inhibitor Y-27632 (Sigma-Aldrich), 10 ng/ml fibroblast growth factor 2 (FGF2) (Invitrogen), and 20 ng/ml epidermal growth factor (EGF) (Peprotech, Rocky Hill, NJ, http://www.peprotech.com) (here defined as ITS²). For lens, sorted cells were plated in ITS supplemented with 10 μM Rock Inhibitor Y-27632, 2 ng/ml FGF2, 10 ng/ml EGF, 20 ng/ml hepatocyte growth factor (Peprotech), and 10 ng/ml vascular endothelial growth factor (Peprotech). Myogenic differentiation occurred in sorted cells grown postsorting in ITS supplemented with 2% B27 (Invitrogen), 10 ng/ml FGF2, 10 ng/ml EGF, and 10 μM Rock Inhibitor Y-27632 (kept for 5 days) after 40–45 days of culture. For osteogenic differentiation, cells were kept for 4 days in ITS² and then treated as previously described [16].

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RESULTS

Neural Ectoderm, Non-Neural Ectoderm, and Mesoderm Spontaneously Form During Differentiation of hESCs in ITS Medium

Formation of the NPB and its derivatives (NCs and CPs) requires signaling from surrounding tissues, the neural ectoderm, non-neural ectoderm, and underlying mesoderm. Therefore, we induced hESC differentiation into these latter tissues at large colony size (diameter >800 mm) and high colony density in ITS medium, without adding neuralizing factors and/or Smad inhibitors. In these conditions, hESCs were capable of generating neural rosette structures, as well as non-neural ectoderm and mesoderm-like tissue. Neural rosettes positive for the neural markers Pax6 and Sox1 could be visualized as early as days 7–8, although more frequently from days 12–14 of in vitro differentiation (Fig. 1A). The presence of non-neural ectoderm was confirmed by the expression of the transcription factor p63 [22, 23] in a mutually exclusive distribution with Pax6 (Fig. 1B). Recent studies based on an immunohistochemical analysis of early-stage (CS12) human embryos revealed expression of the transcription factor AP2α in non-neural ectoderm and NPB [24]. In our in vitro differentiation system, at day 11, the AP2α transcription factor was detected in areas that only partially overlapped with Pax6-positive and Sox9-positive cells (Fig. 1C, 1D). During very early stages of vertebrate embryonic development, both Pax3 and Sox9 play a role in NPB and NC specification [5]. Therefore, their partial colocalization with AP2α demonstrated the presence of NPB-like areas and tissue with non-neural ectoderm identity. Furthermore, we could observe the formation of mesoderm, as underlined by the presence of cells expressing the paraxial and somitic mesoderm marker Paraxis. As shown in Figure 1E and 1F, Paraxis-positive cells did not coexpress the neural marker Pax6; in contrast, Pax3, which is also a somite marker, was coexpressed with Paraxis. Thus, our differentiation system promoted the formation of neural and non-neural ectoderm and to a lesser extent paraxial mesoderm.

The Genes Associated With the Acquisition of NC and PPR Identity Show an Early Onset of Expression During hESC Differentiation in ITS Medium

Fundamental studies on the formation of the NPB derivatives NCs and CPs in a variety of model organisms [4, 25, 26] have identified sets of genes whose expression defines a molecular signature for NCs and CPs. These genes include AP2α, Pax3, and Pax7 as NPB specifiers and Slug, Snai1, Sox9, and Sox10 as some of the most relevant NC specifiers in multiple organisms [5]. The PPR is known to express genes such as Six1, Six4, Eya1/2, and Dach1, as well as Dlx3 and Dlx5 [25]. Very little is known about the time and pattern of the expression of all these genes in differentiating hESC. We found that many NC-associated genes were expressed early during differentiation (Fig. 2A). In addition, we observed that expression of Pax3, Pax7, and SLUG had a defined time pattern. The early appearance of the anterior neural marker Pax6 and non-neural epithelium marker p63 underlined the initial formation of these tissues in differentiating hESC in vitro. Similarly, we detected a very early onset of PPR-associated genes (Fig. 2B). As previously reported, we observed the expression of Six4 [27] and ventral forebrain/placititary placode marker Hesx1 [28] before induction of differentiation. Two genes that are associated with early development of the otic placode, Pax2 and Pax8, exhibited a peak of expression between days 7 and 12 of in vitro differentiation, although this early onset may have reflected their hindbrain expression [29]. Furthermore, we started detecting a weak expression of Fox1, the earliest gene known to mark the otic anlage and otic precursor cells in zebrafish, before the placode thickening [30] at day 23 of in vitro hESC differentiation (Fig. 2B), which corresponds roughly to the 4th week of development.

Anterior-Posterior Patterning of Tissues Generated During Early hESC Differentiation in ITS Medium

To define the rostro-caudal identity of the tissues formed in our differentiation conditions, we analyzed the expression of genes associated with specific anterior-posterior regions of the developing central nervous system (CNS) by reverse transcription-polymerase chain reaction (RT-PCR) during the day 0 to day 23 interval of in vitro differentiation (Fig. 2C). We observed that forebrain markers, such as Pax6 (Fig. 2A) and FoxG1 (Fig. 2C), showed an early expression and were clearly detected around day 7. Furthermore, we observed an earlier and strong induction of Otx2, a known marker of both forebrain and midbrain [31], which is also expressed in the mouse inner cell mass [32], in unfertilized frog eggs [33], and in zebrafish embryos during gastrulation [34]. All three of these genes remained strongly expressed until the last day tested (day 23). Genes expressed at the hindbrain level, such as Gbx2 and Krox20, showed an early although weaker expression at day 7. Both hindbrain-associated genes, as well as another hindbrain-expressed gene, Pax2 (Fig. 2B), were expressed between day 7 and 16. However, their expression was not strongly sustained over the entire time window analyzed, in contrast to the expression pattern of most anterior markers. We were unable to detect expression of HOX genes that characterize more posterior portions of the hindbrain, such as Hoxb1 (rhombomere 4) and Hoxb4 (from rhombomere 7 to spinal cord), within the chosen time window. Moreover, we never detected Hoxb4 expression by immunostaining in differentiating hESCs for up to 40 days. Interestingly, the generation of cells of the anterior forebrain appeared to be more robust and stable in time than the production of anterior hindbrain cells. This was indicated by the strong, sustained expression of FoxG1, Otx2 (Fig. 2C), Pax6 (Fig. 2A), and Six3 (Fig. 2B) seen by RT-PCR and confirmed by the widespread expression of Pax6, FoxG1, and Otx2 detected by immunostaining (supplemental online Fig. 1).

Differentiating hESCs Display Different Levels of p75 Expression, Revealing the Existence of a Neural/Placodal p75-Positive Subpopulation

The p75 and HNK-1 surface markers have been used to isolate by FACS hESC-derived NC-like cells [10]. Because p75 is also expressed in a wide variety of cell types, including different subtypes of CNS neurons and cells of the ventral neural tube in early human embryos [24], we investigated further the nature of the p75+/HNK-1+ subpopulations.

We identified by FACS and immunocytochemistry two cell populations, p75hi and p75lo, that display different levels of p75-associated fluorescence intensity. These two populations first observed at around day 7 were still distinguishable until day 23 of in vitro differentiation (Fig. 3A–3D). Interestingly, these different levels of p75 expression were detected but not analyzed by other
investigators [11]. In order to characterize the two p75-positive cell subpopulations, we selected additional surface markers that are known to be expressed in neural cells. The CD15 (SSEA1, Lewis X) antigen is known to be expressed by neural stem cells but is absent from populations of NCs [35]. Moreover, the forebrain surface embryonic 1 (FORSE1) antigen has been found to be expressed in early neural rosettes that form during in vitro hESC differentiation [36]. Therefore, we simultaneously stained differentiating hESCs with antibodies directed against p75, HNK-1, CD15, and FORSE1 and isolated different subpopulations by FACS. Because the time-course, RT-PCR analysis of NC, PPR, and anterior-posterior genes (Fig. 2A–2C) showed expression of

**Figure 1.** Expression of neural ectoderm, non-neural ectoderm, and mesoderm genes in differentiating H9 cells. Shown is the detection by immunocytochemistry of genes expressed in neural progenitor cells (Pax6, Sox1) upon 16 days of differentiation in insulin-transferrin-selenium (A); non-neural epithelium (p63) (B); neural plate border (Sox9, AP2α) (C); non-neural (AP2α) and neural epithelium/neural plate border (Pax3) (D); and neural epithelium (Pax6), mesoderm, and paraxial mesoderm (Pax3, Paraxis) upon 11 days of differentiation (E, F). Scale bars = 50 μm (A) and 100 μm (B–F).
Almost all tested genes at or around day 16 of in vitro hESC differentiation in ITS medium, we decided to perform the FACS and to analyze the sorted populations at this time point. At day 16, 30%-50% of cells were p75+. From these p75+ cells, we isolated different subpopulations exhibiting the following surface antigen combinations and different levels of p75-associated fluorescence intensity: p75+hi/HNK-1− (population 1), p75+hi/ HNK-1+ (population 2), and p75+lo/HNK-1+. When p75+lo/ HNK-1+ cells were analyzed for the expression of CD15 and FORSE1, 80.1 ± 1.33% were CD15−/FORSE1− (population 3) and 13.6 ± 0.31% were FORSE1−/CD15+ (population 4) (Fig. 3E). In contrast, only a very small fraction of both p75+lo/ HNK-1− and p75+hi/HNK-1+ populations were also positive for CD15+ and FORSE1+ (1% and 2% of populations 1 and 2, respectively) (supplemental online Fig. 2). Since the HNK-1 epitope had been detected only in a very small portion of NC-derived cells analyzed in sections of human embryos [24] and we often observed stronger expression of NC-associated genes within the p75+lo population (compared with the p75+hi), we analyzed both HNK-1+ and HNK-1− fractions of the p75+lo population. To investigate the nature of the isolated subpopulations (1, 2, 3, and 4), we performed an RT-PCR analysis of specific molecular markers. In population 4, we observed a reduced expression of several genes associated with NC specification together with increased expression of genes associated with anterior neural ridge specification (Dlx5, Pax6, FoxG1, Sox1) [37–39] (Fig. 3F). This observation was in agreement with the reported expression of CD15 on neural stem cells [35], as well as in CP in the mouse [40]. Accordingly, the most anterior portion of NP, directly facing the neural tube, where the anterior CPs form, is known not to generate NCs [41, 42]. Populations 3 and 4 both displayed low levels of p75 (p75+lo/HNK-1+) and were distinguishable only by the presence of the CD15 surface marker. These observations showed that the p75+lo/HNK-1+ population was indeed heterogeneous and included a subpopulation that was likely composed of mixed cells with neural and anterior CP fate characteristic of the anterior neural ridge domain. In contrast, a robust expression of NC-associated genes was detected in the other three p75+ populations (1, 2, and 3), with stronger expression of FoxD3, Snai1, Slug, and Sox10 in the p75+hi/HNK-1+ populations (especially population 2, Fig. 3F). Of particular interest was the expression of Sox1, often only referred to as a neural differentiation marker [43] but also known to play a role in the development of the lens placode in the chicken [44]. Sox1 expression was clearly detected in the two p75+lo/HNK-1+ populations (3 and 4) and was almost undetectable in the two p75+hi/HNK-1+ populations (1 and 2; Fig. 3F). Therefore, the p75+lo/CD15+ fraction was enriched in cells with CNS and anterior placodal identity.

Further confirmation of the heterogeneity of the p75+/ HNK-1+ population was given by the expression of genes involved in paraxial mesoderm formation and skeletal muscle specification in the p75+lo/HNK-1+ fractions (supplemental online Fig. 3). Moreover, skeletal muscle cells were generated (although inefficiently) from the p75+/HNK-1+ and p75−/ HNK-1+ subpopulations (supplemental online Fig. 3).

Detection, Isolation, and Characterization of Lens Cells

As previously shown (Fig. 2B, 2C), hESCs differentiating in high-density cultures, feeder-free conditions, and ITS medium robustly expressed markers of anterior neuroectoderm and anterior placodes/anterior neural ridge. We therefore speculated that tissues formed during the first 4 weeks of differentiation would include the most anterior placodal domain that eventually segregates into lens, olfactory, and adenosynprophal placies.

In order to isolate putative lens cells by FACS, we selected multiple surface markers with the following criteria. For positive selection, we chose the hepatocyte growth factor receptor c-Met/FGFR, here referred to as c-Met [12]. This molecule has been reported to be expressed in the fetal human lens epithelial cell line FHL-124 and in the native human lens epithelium [13]. Considering that c-Met is also expressed in cells of neural origin [45], we used the previously studied markers p75, HNK-1, and CD15 for a negative selection of the neural component. Putative lens cells were isolated by FACS from hESCs differentiating in ITS medium at different time frames, from day 19 to day 23 or from day 29 to day 32. The use of c-Met allowed an early detection and isolation of cells expressing lens-cell markers (day 16 of hESC differentiation was the earliest time point tested). The longer differentiation time frame enabled us to identify other cell populations with lens fate potential by using an additional surface marker, hyaluronan receptor CD44. This receptor is expressed in the cortical lens fiber cells of the mouse lens, but not in the lens epithelium [14]. In cataractous human lens specimens, CD44 is expressed in lens epithelial cells [15], whereas in normal, noncataractous human lens, its expression pattern is unclear. In our

![Figure 2](image-url)
differentiation conditions, the CD44 receptor was more strongly detected by FACS analysis after 27 days of hESC differentiation. Multiple populations exhibiting different combinations of surface markers were isolated by FACS in each of the two time frames, days 19–23 and 29–32 (Fig. 4A, 4B). The sorting strategy followed to select the populations indicated in Figure 4B is shown in Figure 4C. Statistical analysis of the sorted populations revealed that the candidate lens populations (Fig. 4B, P1, P2, and P4) represented a very small percentage of the differentiating hESC (Fig. 4A, 4B), as would be expected for eye lens cells forming in an embryo. In support of the neural selection potential of p75, HNK-1, and CD15, we observed the formation of neural
Derivation of Cranial Tissues and Lens From hESCs

**A** Populations sorted after 20-23 days of hESC differentiation

| p75 | c-Met | HNK-1 | CD15 | % sorted cells ± S.D. |
|-----|-------|-------|------|-----------------------|
| P1  | -     | +     | -    | 0.382 ±0.001          |
| P2  | +     | +     | -    | 0.028 ±0.001          |
| P3  | +     | -     | +    | 0.399 ±0.228          |
| P4  | -     | -     | +    | 1.830 ±0.246          |

**B** Populations sorted after 29-31 days of hESC differentiation

| p75 | c-Met | HNK-1 | CD44 | CD15 | % sorted cells ± S.D. |
|-----|-------|-------|------|------|-----------------------|
| P1  | -     | +     | -    | -    | 0.964 ±0.168          |
| P2  | -     | +     | -    | +    | 0.212 ±0.016          |
| P3  | -     | +     | +    | -    | 1.127 ±0.140          |
| P4  | -     | -     | -    | +    | 1.478 ±0.815          |
| P5  | -     | +     | -    | +    | 3.803 ±0.494          |
| P6  | -     | -     | +    | -    |                      |

**C**

LENTOID BODIES d13-d16 post-sort

**D**

**E**

**F**

**G**

**H**

**I**

**J**

**K**

**L**

**M**

**N**

**O**

**P**

**Q**

**R**
tissue (Fig. 4D) in postsorting cultures from HNK-1+/CD15+ cells isolated at day 23, as well as days 29–32 of in vitro hESC differentiation (Fig. 4A, P4; Fig. 4B, P5) and in the p75+/HNK-1+/CD15+ population sorted at day 23 (Fig. 4A, P3). Cell populations that showed expression of either p75 or HNK-1 surface markers in addition to the c-Met or CD44 markers (such as populations 2 [Fig. 4A] and 3 [Fig. 4B]) also included some neurons in postsorting cultures (Fig. 4E, 4F). The CD44 single positive population (Fig. 4B, P4) was composed of cells with diverse morphology (Fig. 4G–4L), suggesting a heterogeneous origin. Additionally, postsorting cell survival and proliferation was poor in our culture conditions (Material and Methods), although a few cells expressing the proliferation marker Ki-67 were detected (Fig. 5I). The CD44+ population also had small cell clusters expressing the lens cell markers Prox1 (nuclear), FoxE3 (peripheral cytoplasm), and CRYAA (Fig. 5J). Only evident after 10 days of in vitro culture, expression of these markers demonstrated that the CD44 receptor was capable of selecting lens cells in addition to other non-lens cell types. No cells with neuronal morphology were observed in populations 1 and 2 (Fig. 4B) during extensive (up to 20 days) postsorting cultures, an important feature for the future use of these cells for drug screenings or therapeutic applications. The small fraction of p75−/c-Met+/HNK-1−/CD44+/CD15− cells (P2) possessed proliferative ability, as indicated by the expression of the proliferation marker Ki-67 (Fig. 5A), and lentoid body formation potential within 10 days of postsorting culture (Fig. 4M–4O). The same population also exhibited expression of specific lens cells markers (Fig. 5B, SC). The population with the best growth and survival potential was the p75−/c-Met+/HNK-1−/CD44−/CD15− population (P1), which besides expressing the proliferation marker Ki-67 (Fig. 5D) also formed lentoid bodies by days 7–8 of postsorting culture (Fig. 4Q). These cells showed early nuclear localization of the lens cell marker Prox1 (Fig. 5E) and abundant expression of CRYAA and CRYAB (Fig. 5G, 5H), as well as expression of the cell-cycle inhibitor p57, which is known to be upregulated by Prox1 [46] (Fig. 5F). These cells were also characterized by abundant secretion of collagen IV (Fig. 5F), an extracellular matrix component produced by lens cells [47]. Additional staining and a comparison between p75−/c-Met+/HNK-1−/CD44−/CD15− lens epithelium, p75−/c-Met+/HNK-1−/CD44−/CD15− derived lentoid bodies, and mouse lens is shown in supplemental Figure 5.

Quantitative PCR Analysis of Fate-Specific Genes in the Sorted Cells Confirms the Identity of the Selected Populations

Genes playing important roles in the formation and maintenance of lens placode, lens vesicle, and lens epithelium, such as Pax6, AP2α, and FoxE3 [48, 49], exhibited high levels of expression in all populations able to form lentoid bodies within days of postsorting culture (populations 1–4; Fig. 6A). This was in contrast to populations 5 (which generated uncharacterized neurons; Fig. 4D) and 6 (mature lentoid bodies). The CD44 single-positive population (population 4; Figs. 4B, 6A) included a subpopulation of lens forming cells (Fig. 5J), as part of a heterogeneous population (Fig. 4G–4L) that requires further characterization. To confirm that population 1, which was more abundant and exhibited higher survival and growth potential in postsorting cultures than population 2, was capable of forming lentoid bodies composed of differentiating lens fibers, we analyzed the expression of genes involved in lens fiber differentiation. The Maf and Prox1 genes are known to be activators of expression of several crystallin genes in differentiating lens fibers [46, 50]. Additionally, CRYAA, Mip26, and Filensin [51, 52] are genes highly expressed in lens fibers. As shown in Figure 6B, the lens differentiation genes tested here were expressed at a much higher level in population 6 (differentiating lentoid bodies) than in any other postsorting population. Furthermore, expression of genes often associated with the neuronal fate of CNS or placodal origin, such as FoxG1 and Dlx5, was stronger in the HNK-1+ and HNK-1+/CD15+ populations (populations 3 and 5), validating the use of these surface markers to exclude the neural component. The Sox1 gene, known to play a role in lens fiber maturation [53] and in neural cell determination [43], was expressed at higher levels in differentiating lentoid bodies (population 6) and in the populations with neural components (populations 3 and 5).

**DISCUSSION**

The correct specification and segregation of NC and CP progenitors from the NPB requires the concerted action of surrounding tissues, including neural plate, non-neural epithelium, and underlying mesoderm, as sources of multiple signals [3]. Therefore,

![Figure 4. Fluorescence-activated cell sorting-based isolation of lens candidate cells.](https://example.com/figure4)

**Figure 4.** Fluorescence-activated cell sorting-based isolation of lens candidate cells. (A): Four populations (P1–P4) were sorted after 20–23 days of hESC (H9) differentiation in insulin-transferrin-selenium (ITS) minimal media. The percentage of sorted cells represents the average of three independent sorting experiments ±SD. (B): Five populations (P1–P5) were sorted after 29–31 days of hESC (H9) differentiation. The percentages of single cells sorted represent the average of three independent experiments. (C): Sorting strategy for lens cells populations. Shown is a representative experiment in which hESCs differentiating for 29 days in ITS minimal media were sorted using the p75, c-Met, HNK-1, CD44, and CD15 surface markers. The populations collected from sorting were as follows: p75−/c-Met+/HNK-1−/CD44−/CD15− (1.2%), p75−/c-Met+/HNK-1−/CD44−/CD15− (0.2%), p75−/c-Met−/HNK-1−/CD44−/CD15− (0.9%), p75−/c-Met−/HNK-1−/CD44+/CD15− (0.8%), and p75−/c-Met−/HNK-1−/CD44−/CD15+ (3.2%). The numbers indicate what percentage of the total cells sorted is represented by that population. The arrows indicate the path of selection followed for each of the four populations in the central dot plot; these populations were selected based on the c-Met and CD44 surface profiles. The gates in each dot plot indicate the analyzed population for the next step. (D): Neurons derived from the HNK-1+/CD15+ populations (P4 in [A] and P5 in [B]) upon 7 days of postsorting culture. Population p75+/HNK-1+/CD15+ (P3 in [A]) also generated a similar neuronal growth (data not shown). (E): P2 in (A) p75+/c-Met+/HNK-1−/CD15−, grown postsorting for 18 days, exhibited the presence of cells with neuron morphology (arrows), as well as the formation of lentoid bodies (arrowhead). (F): P3 in (B) p75−/c-Met−/HNK-1−/CD44−/CD15−), grown postsorting for 6 days, also exhibited cells with neuron morphology (white arrows). (G–L): P4 in (B) p75−/c-Met−/HNK-1−/CD44−/CD15−, grown postsorting for 10 days, exhibited cells with different morphologies; morphological subtypes are shown in (H–L). A small group of cells that had acquired a lentoid body-like shape is shown in (J), and those with a different neuronal morphology are shown in (K) and (L). (M–O): P2 in (B) p75−/c-Met−/HNK-1−/CD44−/CD15− at day 5 (M), day 8 (N), and day 10 (O) of postsorting culture exhibited the ability to start forming lentoid bodies around days 9–10 postsorting (arrow in [O]). (P–R): P1 in (B) p75−/c-Met−/HNK-1−/CD44−/CD15− at day 5 (P), day 8 (Q), and day 10 (R) of postsorting culture exhibited the best survival and proliferation ability, and it exhibited lentoid body-like structures (arrows) from day 8 postsorting (Q, R). Scale bars = 50 μm (D–R). Abbreviations: d, day; hESC, human embryonic stem cells; P, population.
we applied differentiation conditions (Materials and Methods) to hESCs that are permissive for the formation of neural and non-neural ectoderm, both of which are required for the in vitro specification of NPB cells in a more comparable manner to a developing embryo. Furthermore, this system allows the identification of cell types, such as placode-derived tissues, that physiologically form at cranial level but have not been studied in humans. The demonstrated ability of p75 and HNK-1 surface markers to detect subpopulations with neural, NC, and mesodermal fates indicates that the heterogeneity and complexity of p75+/HNK-1+ populations generated in our differentiating conditions are reflective of those existing in the embryo. The p75 surface marker is in fact known to be expressed in neural [24], NC [54, 55], and mesoderm-derived cells [56, 57]. Thus, our findings emphasize (a) the extreme caution needed in selecting surface markers for the isolation of specific cell
types, and (b) the requirement for using an array of markers to further refine the specificity of the selected populations. We applied these conditions to set up a sorting strategy for isolation of lens cells.

Based on the early and strong expression of forebrain and midbrain markers, such as FoxG1 and Otx2, as well as the expression of anterior hindbrain markers, such as Pax2 and Krox20, we can infer that in our differentiation conditions hESCs are capable of generating cells with a position identity up to the anterior hindbrain (Fig. 2C). The absence of HoxB4 transcript, whose expression starts at rhombomere 7, or protein (unless cells were treated with retinoic acid, a posteriorizing agent; data not shown) indicates the inability of the cells to generate tissues with a more caudal fate (although time frames longer than 40 days were not tested). In addition, transcription factors known to pattern the antero-posterior (A-P) axis of the CNS (such as Pax6, Otx2, and HoxB4) were not expressed in our lentoid bodies, suggesting a more anterior fate for these cells.

Figure 6. Quantitative polymerase chain reaction (qPCR) analysis of transcripts levels in the sorted populations. (A) Human embryonic stem cells (hESCs) were differentiated in insulin-transferrin-selenium medium for 29 and 30 days, and the specific populations shown (1–6) were sorted on the basis of the indicated surface marker profiles and analyzed immediately postsorting. Population 6 represents lentoid bodies derived from population 1 isolated by fluorescence-activated cell sorting upon 23 or 29 days of hESC differentiation and collected/analyzed after 13 or 16 days of postsorting differentiation. (B) qPCR analysis. The relative expression level of each gene is calibrated to its expression in population 1. Ct values for each target gene are normalized to Ct values of Gapdh as a reference gene. Values represent mean ± SEM of two independent experiments. * = off-scale expression of those genes in lentoid bodies, with the following specific values: CryAA, 622 ± 46; Mip26, 11,519 ± 0.086; Filensin, 56 ± 32. The high variability in differentiation gene expression in lentoid bodies reflects the different maturation/differentiation level of the lentoid bodies tested (day 13 vs. day 16). Abbreviation: Pop., population.
FoxG1, Six3, Otx2, Gbx2, and Pax2) are also expressed in the PPR and developing cranial placodes with a pattern similar to that reported for the CNS [4]. Therefore, our observations also provide useful directions on the A-P position of the potential placodal tissues that can form in this system.

To our knowledge, this is the first report of FACS-based isolation of hESC-derived lens cells, which defines a methodology aimed at the identification and isolation of this population rather than at the enrichment for lens cells. Although we found that cells sorted using different antigen combinations displayed lens cell potential, the c-Met receptor has proven to be central to our purification strategy. When used together with a negative neural selection antigen panel (p75, HNK-1, CD15), c-Met allows the isolation of lens epithelial cells with lentoid body-forming potential, without detectable neural contamination. The hyaluronan receptor CD44 was also valuable for the isolation of lens cells even in the absence of c-Met selection. We speculate that CD44/H11001 cells could represent a different part of the developing lens. Interestingly, to support speculation that our sorting strategy is capable of selecting different types of lens cells, we applied a slightly modified published protocol aimed at enriching for differentiated lentoid bodies from hESCs [8]. Notably, we detected a decrease in the c-Met single-positive population (early lens epithelium) and an increase in the CD44 single-positive population (supplemental online Fig. 4). The CD44 population may include more advanced lens cells as described in the mouse [14].

The schematic illustration in Figure 7 summarizes the strategy we used to investigate the nature of multiple tissues formed from hESCs. The p75+/HNK-1+ population not only is inclusive of NCs but also contains neural cells. This information was used to improve the purification of the c-Met+ lens cell population from neural cells. Furthermore, the addition of the later detected marker, CD44, allowed us to identify multiple populations with lens cell characteristics. The presence of a variety of such populations suggests that the lens cells formed in this in vitro system may reflect the diversity of cells identified within the entire lens (e.g., lens epithelium cells of the germinative zone or differentiating lens cells in the transition zone or lens fibers in the cortical region). Future studies will need to clarify whether different subtypes of lens cells are selected by the diverse combination of surface markers.

These findings hold a great potential for further investigation of human lens development and regeneration, as well as for the use of hESC-derived lens cells for drug screening and future therapeutic applications. When working with pluripotent cells such as hESCs, it is an essential prerequisite to purify and isolate the cells of interest. Our method for the isolation of proliferative lens epithelium able to differentiate into mature lens cells represent an important step toward these goals. Posterior capsule opacification remains an important post-cataract surgery complication that could be relieved if an entire natural (not artificial) lens, composed of its proliferative anterior lens epithelium, could be generated in vitro.

Notably, immunostaining analysis of the Foxe3 transcription factor revealed a peripheral cytoplasm localization in all lens cells capable of forming lentoid bodies (Fig. 5B, 5E, 5J), in agreement with reported observations in regenerating rat lens tissue [58]. Interestingly, FoxE3 is known to play an important function in lens vesicle separation from overlying ectoderm with a possible role in

Figure 7. Schematic summary of the sorting strategy and cell populations isolated from differentiating hESCs. Light blue indicates surface markers used to identify a heterogeneous population composed of a neural, non-neural/placodal, neural crest, and to a lesser extent mesodermal fraction. Magenta indicates surface markers used for positive selection of populations with lens-forming potential. The expression of the genes shown in black was tested by reverse transcription-polymerase chain reaction in the indicated populations immediately postsorting. Expression of lens-characteristic genes shown in green was detected by immunostaining in postsorting cultures. Abbreviations: hESC, human embryonic stem cell; ITS, insulin-transferrin-selenium.
HNK-1 detection of lens epithelium. Isolation of multiple cell populations with lens forming ability, and finding a combination of surface markers that allows the FACS with skeletal muscle potential. More importantly, we have defined a combination of surface markers that allows the FACS isolation of multiple cell populations with lens forming ability, and we identified c-Met/HGF receptor as a principal marker for early detection of lens epithelium.

Conclusion
We have evaluated the fate potential of FACS-purified p75+/HNK-1+ cell populations derived from hESCs differentiating in conditions permissive for formation of neural ectoderm, non-neural ectoderm, and anterior cranial tissues. We have found that these surface markers identify a heterogeneous population of neural crest progenitors from human embryonic stem cells. J Exp Zool B Mol Dev Evol 2010;332:189 –195.

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