Correction

DEVELOPMENTAL BIOLOGY


The authors note that on page 14862, left column, first full paragraph, all unit concentrations in “μM” should instead appear as “nM.”

The authors also note that on page 14865, right column, second paragraph, line 15 “20 nM human recombinant BMP4” should instead appear as “0.02 nM human recombinant BMP4.”

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Functional melanocytes derived from human pluripotent stem cells engraft into pluristratified epidermis

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Melanocytes are essential for skin homeostasis and protection, and their defects in humans lead to a wide array of diseases that are potentially extremely severe. To date, the analysis of molecular mechanisms and the function of human melanocytes have been limited because of the difficulties in accessing large numbers of cells with the specific phenotypes. This issue can now be addressed via a differentiation protocol that allows melanocytes to be obtained from pluripotent stem cell lines, either induced or of embryonic origin, based on the use of high concentrations of a single cytokine, bone morphogenic protein 4. Human melanocytes derived from pluripotent stem cells exhibit all the characteristic features of their adult counterparts. This includes the enzymatic machinery required for the production and functional delivery of melanin to keratinocytes. Melanocytes also integrate appropriately into organotypic epidermis reconstructed in vitro. The availability of human cells committed to the melanocytic lineage in vitro will enable the investigation of those mechanisms that guide the developmental processes and will facilitate analysis of the molecular mechanisms responsible for genetic diseases. Access to an unlimited resource may also prove a vital tool for the treatment of hypopigmentation disorders when donors with matching haplotypes become available in clinically relevant banks of pluripotent stem cell lines.

ES cells | melanogenesis | cell therapy | BMP4

Molecular and cellular mechanisms of melanocyte development have been explored in a variety of species using experimental grafting and transgenesis, but the lack of appropriate technologies has greatly limited our knowledge of these mechanisms in humans to date. In vertebrates, melanocytes originate from migratory neural crest cells that emerge from the neural plate during embryogenesis (1, 2). Neural crest specification from the primitive ectoderm is regulated by bone morphogenic protein (BMP) and FGF signaling pathways (3, 4). Once committed, neural crest cells undergo a complex process of differentiation, proliferation, and migration out of the neural tube along defined pathways through the entire body to differentiate into melanocytes or neuroectodermal derivatives (5). However, this process is still not fully understood and the molecular mechanisms involved have not been fully elucidated.

Pluripotent stem cells, either of embryonic origin or following genetic reprogramming, have already been widely used to model early stages of differentiation along a variety of lineages (14–16). In the case of melanocytes, a pioneering study was carried out using cocultures of mouse ES cells with the stromal cell line ST2 (17). This study confirmed that application of the known melanocyte activators stem cell factor (SCF), EDN3, 12-O-tetradecanoyl phorbol acetate (TPA), and dexamethasone (DEX) promotes cell differentiation. More recently, these studies have been extended to our species (18), showing a facilitating effect of WNT3a, EDN3, and SCF on the differentiation of human ES cells (hESCs). However, that study, which, to our knowledge, remains the only demonstration of hESC-derived melanocytes, was based on a first stage of differentiation requiring the formation of embryoid bodies and the secondary selection of pigmented cells. This has precluded any specific analysis of the hESC-to-melanocyte differentiation process itself, which is not accessible during the formation of the embryoid bodies.

We have reconsidered this issue here by taking the advantage of a finding made in a previous study that aimed at differentiating hESCs into another ectodermal derivative, namely, keratinocytes (19). Indeed, although keratinocytes formed 50–60% of the cells differentiated for 40 d from hESCs with high concentrations of BMP4 and ascorbic acid, the remaining cells comprised clusters of pigmented cells. Because most, if not all, of the cells in those cultures were ectodermal derivatives, it was hypothesized that they may be either neural crest-derived melanocytes or neuroectodermally derived retinal pigment epithelium cells (RPEs) (20). Analysis of morphological and molecular phenotypes has allowed us to separate two subpopulations of cells, with the specific characteristics of each phenotype. Melanocytes derived from pluripotent stem cells, both of embryonic origin and following genetic reprogramming of adult cells, were then further characterized phenotypically and functionally.

Results

Two different hESC lines (H9 and SA01) and one human induced pluripotent stem cell (iPSC) line were used in this study (cell line characterization is shown in Fig. S1). For the sake of clarity, the H9 cell line has been taken as a representative case in all following figures because results with the SA01 line were similar. Results obtained for the analyzed iPSC line, which were also altogether similar, are presented as supplementary data (SI Methods).


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BMP4-Mediated Control of Pluripotent Stem Cell Differentiation Along the Ectodermal Lineage. Undifferentiated pluripotent stem cells were seeded on mitomycin-treated 3T3 feeder cells in FAD medium and then supplemented with BMP4 and ascorbic acid for more than 40 d. The concentrations of BMP4 were gradually decreased to identify the optimal conditions for the derivation of neural crest progenitors (Fig. S2). The engagement of pluripotent stem cells at the earliest stage of cell commitment (10 d of differentiation) after treatment with different concentrations of BMP4 was evaluated using quantitative PCR. At that time point, hESCs were mostly engaged in epithelial commitment (KRT18, p63) at the highest concentrations of BMP4 tested (5–0.5 μM) (Fig. S2A). Neural crest cells (HNK1+ and p75+) were mostly generated when intermediate concentrations of BMP4 were used (0.02–0.004 μM). Neural cells (SOX1+2) were observed in large numbers at the lowest concentrations tested (0.004–0.0008 μM) (Fig. S2B). Results obtained with hESCs were similar for iPSCs. Morphological observations confirmed these results by showing the typical morphology of neural crest cells at an intermediate concentration of BMP4 in parallel with the loss of the typical epithelial morphology observed at a high concentration of BMP4 (Fig. S2C). Flow cytometry analysis confirmed the key role of an intermediate concentration of BMP4 for neural crest induction by revealing that a concentration of 0.02 μM increased the proportion of HNK1+ p75+ positive cells up to 36% (Fig. S2D). These data were confirmed by immunofluorescence using PAX3 antibodies (Fig. S2E). Moreover, inhibition of BMP signaling delayed differentiation of pigmented cells at the end of the process (Fig. S2F). Accordingly, BMP4 at a concentration of 0.02 μM was used in subsequent experiments on the neural crest-derived melanocytic lineage. Under these experimental procedures, pigmented cells appeared and progressively increased in number during the differentiation process. Morphological (Fig. 1A and molecular (Fig. 1B–D) characterization of pluripotent stem cell-derived pigmented cells was performed along the differentiation process. Quantitative PCR analysis showed a decrease in the pluripotency marker genes OCT4, NANOG, and SOX2 (Fig. 1B and Fig. S3A) in parallel to an increase in the genes that encode the regulators of melanin synthesis: TYRP1, TYROSINASE (TYR), and MITF (Fig. 1C and Fig. S3B). The increase in the neural crest-derived cell markers SOX10 and PAX3 and the neural derivative marker PAX6 was paralleled by enrichment of the cultures in the pigmented cells (Fig. 1D, Figs. S3C, and Table S1).

Derivation of a Pure Population of Melanocytes from Pluripotent Stem Cells. After 50 d of differentiation, the pigmented cells were mechanically isolated and subcultured in M254-CF medium (Invitrogen) suited to the survival of melanocytes (Fig. 1E). At this stage, the pigmented cells displayed two different morphologies. A first subpopulation exhibited epithelium-like structures, whereas a second one exhibited bipolar processes extending from a small ovoid cell body (Fig. 1E). In the first subpopulation, TYRP1 immunostaining coregistered with the typical markers of the RPEs, RPE 65 and PAX6, indicating its RPE-like phenotype (Fig. S4A). Accordingly, quantitative PCR showed an increase in both genes controlling melanin synthesis, TYRP1 and TYR, and RPE markers, namely, OTX2, BEST, RPE 65, and the RPE-specific isoform of MITF (MITF-D) (Fig. S4B). In contrast, among the enriched pigmented cell population, 5–10% of the cells, depending on the cell line analyzed, expressed the neural crest lineage-specific marker PAX3, which was not expressed in hESC-derived RPEs (RPE-hESCs) (Fig. S4C). These cells corresponded to the non-pavimentous cell subpopulation, as shown after isolation by differential trypsinization and subculture in M254-CF medium. After 4 passages, cells exhibited a morphology similar to that of human epidermal melanocytes (HEMs) (Fig. 2A and Fig. S4A). Immunostaining analysis of these cells, subsequently called mel-hESCs and mel-iPSCs for “melanocytes derived from hESCs and iPSCs,” revealed an appropriate cytoplasmic localization of TYRP1, TYR, S100, SILV, and Rab27 associated with a subcellular localization of PAX3, SLUG, and MITF in the nucleus and an absence of OCT4, TRA1-81, and PAX6 (Fig. 2B and Fig. S5B). These cells also demonstrated gene expression profiles similar to HEMS, as illustrated by the expression of SOX10, PAX3, the melanocyte-specific isoform of MITF (MITF-M), TYRP1, and TYR, associated with a background level of PAX6, OTX2, and MITF-D (Fig. 2C and Fig. S5C). FACS analysis after 4 passages confirmed the absence of SSEA4 and TRA1-81 expression (Fig. 2D and Fig. S5D). Conversely, more than 90% of these cells expressed TYRP1 and MITF (Fig. 2D and Fig. S5D). TaqMan array gene expression profiling, using a selected panel of 96 genes related to melanocyte biology, confirmed similar expression patterns of mel-hESCs, mel-iPSCs, and HEMS for a majority of genes (Fig. S5 E and F and Table S2). Mel-hESCs and mel-iPSCs could be propagated for up to 12 passages, frozen, and thawed without apparent changes in morphology (Fig. S6A), main phenotypic markers (Fig. S6B), cell proliferation rate (Fig. S6C), or mortality (Fig. S6D). Proliferation curves of HEMS, mel-hESCs, and mel-iPSCs were performed, revealing no differences in terms of proliferation rates between the source of melanocytes after 4 and 8 passages of cultures. Based on the flow cytometry cell quantification and subsequent calculation of their proliferation rate, we estimate the total number of melanocytes produced with this method at 2 × 10^4 to 10^5 at the first
passage, which allows the total production of $10^8$ melanocytes at passage 4 and at $2 \times 10^{12}$ melanocytes at passage 8. Spinning-disk confocal microscopy and time-lapse recording revealed the presence of dark-containing organelles (Fig. 3C) that moved along the main axis of cell processes (Movies S1, S2, and S3). EM confirmed that the organelles present in mel-hESCs and mel-iPSCs were bona fide melanosomes at all stages of maturation, ranging from immature unpigmented (types I and II) up to mature melanin-containing (types III and IV) melanosomes (Fig. 3B and Fig. S7A).

**Functional Characterization of Melanocytes Derived from Pluripotent Stem Cells.** The functional status of melanocytes derived from pluripotent stem cells was first assessed by coculturing them with human adult keratinocytes. After 3 d of coculture, TYRP1/Keratin 14 (keratinocyte marker) immunostaining revealed TYRP1$^+$ organelles in keratinocytes when they had been cocultured with melanocytes and not in controls that were grown separately (Fig. 3C and Fig. S7B). TYRP1$^+$ organelles were localized in the perinuclear compartment of keratinocytes cocultured with mel-hESCs or mel-iPSCs, as well as in keratinocytes cocultured with HEMs. Automated quantification using the ArrayScan system (Cellomics) revealed about 20% TYRP1$^+$ keratinocytes in cocultures with either mel-hESCs or mel-iPSCs and up to 40% with HEMs (Fig. 3D and Fig. S7C). EM confirmed the presence of pigmented organelles in keratinocytes cocultured with either mel-hESCs or mel-iPSCs (Fig. 3E and Fig. S7D and E). Further functional evaluation of mel-hESCs was sought using the 3D reconstruction of a pluristratified epidermis in vitro, by mixing melanocytes with adult basal keratinoctyes seeded as a monolayer on a matrix at the medium-air interface (Fig. 4). After the development of a fully pluristratified epidermis, macroscopic observation revealed pigmentation in the reconstructed tissue that contained melanocytes. Fontana–Masson staining and TYRP1 immunostaining confirmed the presence of melanin-containing cells in the basal layer of the epidermis (Fig. 4A). In upper layers of the reconstructed epidermis, melanin-containing processes intermingled with keratinocytes. Treatment of the melanized epidermis with α-melanocyte stimulating hormone enhanced the production of melanin in the reconstructed tissue (Fig. 4B).
Discussion

The main result of this study is the in vitro generation of functional melanocytes from pluripotent stem cells, both hESCs and iPSCs, capable of producing melanin that can be taken up by keratinocytes in cocultures. The availability of human cells committed to the melanocytic lineage in vitro, at all stages of differentiation, will enable investigation of those mechanisms that guide the developmental processes and may provide evidence of the human equivalent of melanoblasts, which has remained elusive up to now. In parallel, our results will facilitate the in vitro analysis of those molecular mechanisms that underlie the melanocytic defects observed in genetic diseases. Lastly, the demonstrated functional integration of melanocytes derived from hESCs and iPSCs lines into reconstituted pluristratified epidermis potentially paves the way for promoting cell therapy in hypopigmentation disorders, such as vitiligo. Human melanocytes were derived here in vitro from undifferentiated pluripotent stem cells using a moderate concentration of BMP4. This protocol, in contrast to previous ones that relied on embryoid bodies, enables the engagement of the cells into the neural crest lineage to be followed step by step and, subsequently, the specification of the terminally differentiated cells. This offers a convenient model for the study of the molecular mechanisms underlying melanocyte development. Many reports support the idea that a gradient of BMP activity is the crucial point for division of the ectoderm into the neural plate, neural crest, and epidermis (21). It has been shown in Xenopus and zebrafish models that a gradient of BMP is able to specify the neural plate border, including neural crest cells (21). More precisely, Tribulo et al. (22) demonstrated that a specific level of BMP4 activity leads to the SLUG expression implicated in melanocytic development. Lower or higher amounts of BMP4 failed to induce SLUG expression, confirming previous reports of induction of the neural crest by a gradient of BMP (22). Molecularly, BMP4 signaling activates SLUG promoter activity in the neural crest through direct binding of the phosphorylated BMP signal transducer Smad1 (23). This Smad activation induces apoptotic cell death of neuroectodermal precursor cells already engaged in the neural cell fate. BMP4 assayed on mouse ES cell differentiation induced a dramatic apoptotic death of Sox-1+ neural precursors with a concomitant epidermal engagement (24). In 2007, a pioneer study demonstrated that activation of BMP signaling could permit the differentiation of human pluripotent stem cells into neural crest stem cells through the generation of neural rosettes (25). BMP provoked a significant increase in HNK1+/p75+ neural crest precursors, which could be reversed using a BMP antagonist (25). Altogether, these findings indicate that neural crest precursors can emerge from...
hESC-derived neural rosettes through a BMP treatment. In our study, we optimized the concentration of BMP4 to potentize the neural crest commitment of human pluripotent stem cells. The molecular mechanisms by which different levels of BMP activate a diversity of pathways of differentiation are yet to be defined, and our in vitro model may offer a path for such a study. Molecular mechanisms and genetic pathways involved in melanocyte development were originally described in chicken models using chimeras (26, 27). These pioneer studies demonstrated that melanocytes initially derive from a group of migratory embryonic cells referred to as the neural crest, which secondarily mature under the control of BMPs, Wnt/β-catenin, and FGFs into pigm- ented progenitors (28). Several studies, mainly performed on the mouse, have shown that melanocytic development depends on the activity of the Wnt pathway, namely, the β-catenin and its coregulat ors Wnt3a and β-catenin, and FGFs into pigment- ed progenitors (28). The observation that melanocytes are largely identical to embryonic melanoblasts and that the development of melanocytes in the cases of albinism, Pudlak syndrome, which ex- hibits defects in melanin production and transfer (32, 33). Direct analysis of pathological mechanisms in human cells has, in contrast, remained hampered by difficulties in accessing a relevant biological resource. The derivation of melanocytes from human pluripotent stem cells meets that need insofar as these cell lines can be obtained from donors with specified genotypes of interest and, as such, may provide cellular models of genetic diseases. Human pluripotent stem cell lines have been considered as potentially providing unique models for human diseases since the initial deriv- ation of an hESC line (34). Preimplantation genetic diagnosis (PGD) opened up the possibility of prospectively identifying em- bryos with particular genetic disorders and then deriving “disease-specific” hESCs [e.g., for Huntington disease (35), fragile X syn- drome (36), myotonic dystrophy type 1 (37)]. More recently, hu- man iPSCs have broadened the pathological modeling of pluripotent stem cells to virtually all other monogenic diseases for which PGD was not available (38, 39). There have already been attempts to establish disease models through patient-specific reprogramming in a search for molecular mechanisms associated with diseases [e.g., Parkinson disease (40), spinal muscular atrophy (41), amyotrophic lateral sclerosis (42)]. Our results open the way to extending those studies to pigmentary disorders by providing an accurate protocol for differentiating iPSCs from patients as well as hESCs from PGD embryos into functional melanocytes. The analysis of defective molecular mechanisms is able to target cell- autonomous defects in the specific machinery associated with melanocyte differentiation and function. Indeed, the melanocytes derived here from human pluripotent stem cells expressed all the tested genes that regulate key processes involved in pigmentation as well as in neural crest development, melamn synthesis, and melanosome transfer. As in all other types of differentiation from pluripotent stem cell lines, however, it remains to be established how the “theoretical age” of the cells, taking the undifferentiated stage as equivalent to a first-week blastocyst, influences their phenotype, compared with cells directly obtained from adult donors. It is thought that the melanocytes transfected, although present, may be quantitatively less efficient for hESC- and iPSC-derived melanocytes may relate to such a difference in overall maturation. In addition to cell-autonomous mechanisms, the results obtained in the present study open the way to analyses of cell-to-cell interaction in an organotypic epidermal context. Reconstructing epidermis in vitro with melanocytes and kerati- nocyes derived from individuals with unique genotypes of in- terest may also be of major importance in the analysis of those mechanisms underlying differential sensitivity to UV stress or chemical toxicity (43). Cell therapy utilizing melanocytes has been used experimentally for years as an adjunct treatment for the hypopigmentary disorder vitiligo (44). Vitiligo affects 1% of the world’s population and is associated with high morbidity, particularly psychiatric complications. It is an autoimmune dis- ease characterized by a progressive loss of skin pigmentation caused by the infiltration of CD4+ and CD8+ T lymphocytes into the skin and loss of melanocytes (45). This forces patients to apply UV protection continuously to the affected areas. Over the past 20 y, progress has been made in understanding these diseases, largely as a result of parallel studies of human patients and inbred mice with similar phenotypes, allowing, in particular, an indication of the functional alteration of melanocytes in the cases of albinism, Griscelli syndrome, or Hermansky–Pudlak syndrome, which ex- hibits defects in melanin production and transfer (32, 33). Direct analysis of pathological mechanisms in human cells has, in contrast, remained hampered by difficulties in accessing a relevant biological resource. 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ascorbic acid (Sigma-Aldrich). Cells were grown in the same medium until clones of pigmented populations were observed and isolated. After selection, pigmented cells were dissociated using 0.5% trypsin (Invitrogen) and seeded as single cells in M254-CF medium (Invitrogen) supplemented with human melanocyte growth factor supplements (Invitrogen) suitable for melanocyte culture. After 2 wk of culture in these conditions, cells presenting a morphology similar to that of melanocytes were mechanically isolated based on their morphology and amplified separately in the same medium for up to 12 passages using rapid differential trypsinization. All the molecular characterization of mel-hESCs and mel-iPSCs was systematically performed during 4 passages after isolation.

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