Human neural crest cells contribute to coat pigmentation in interspecies chimeras after in utero injection into mouse embryos

Malkiel A. Cohen[a], Katherine J. Wert[a], Johanna Goldmann[a], Styliani Markoulaki[a], Yosef Buganim[a,1], Dongdong Fu[a], and Rudolf Jaenisch[a,b,2]

[a]Whitehead Institute for Biomedical Research, Cambridge, MA 02142; and [b]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142

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The neural crest (NC) represents multipotent cells that arise at the interphase between ectoderm and prospective epidermis of the neurulating embryo. The NC has major clinical relevance because it is involved in both inherited and acquired developmental abnormalities. The aim of this study was to establish an experimental platform that would allow for the integration of human NC cells (hNCCs) into the gastrulating mouse embryo. NCCs were derived from pluripotent mouse, rat, and human cells and microinjected into embryonic-day-8.5 embryos. To facilitate integration of the NCCs, we used recipient embryos that carried a *c-Kit* mutation (W^{sh}/W^{sh}), which leads to a loss of melanoblasts and thus eliminates competition from the endogenous host cells. The donor NCCs migrated along the dorsolateral migration routes in the recipient embryos. Postnatal mice derived from injected embryos displayed pigmented hair, demonstrating differentiation of the NCCs into functional melanocytes. Although the contribution of human cells to pigmentation in the host was lower than that of mouse or rat donor cells, our results indicate that hNCCs, injected in utero, can integrate into the embryo and form mature functional cells in the animal. This mouse–human chimeric platform allows for a new approach to study NC development and diseases.

human neural crest cells | chimera | embryonic stem cells | melanocytes

Genetically engineered mice have been highly informative in studying the developmental origin of many inherited diseases (1–3). However, mouse models often fail to reproduce the pathophysiology of human disorders due to interspecies divergence, such as metabolic differences between mouse and human (4), or differences in genetic background (5). To overcome some of the limitations of transgenic mouse models, transplantation of disease-relevant human cells into mice has been informative and is frequently used in cancer research. However, this approach is primarily restricted to the study of end-stage-disease cell types and provides only limited insight into tumor initiation and early progression of the disease under in vivo conditions, with the exception of the hematopoietic lineages, where human hematopoietic stem cells were found to successfully engraft into immune-deficient mice and provided a powerful approach for studying blood diseases (6).

Somatic cell reprogramming provides patient-specific induced pluripotent stem cells (iPSCs) that carry all genetic alterations contributing to the disease pathophysiology and thus allows for generating the disease-relevant cell types in culture (7). However, many complex diseases involve progressive cellular or genetic alterations that occur before the manifestation of a clinical phenotype. Therefore, it is not clear whether a disease-relevant phenotype can be observed in short-term cultures of cells derived from patients with long-latency diseases, such as Parkinson's or Alzheimer's disease or cancers like melanoma. A major challenge is establishing model systems that, using human embryonic stem cells (hESCs) or hiPSCs, will allow for the investigation of human disease under appropriate in vivo conditions.

Transplantation of hiPSCs or hiPSC-derived cells into mouse embryos would present an attractive solution to many of the aforementioned limitations. The main advantage of such an approach is that the transplanted cells would integrate into the embryo and participate in normal embryonic development, and consequently could be studied over the lifetime of the mouse. Currently, it is controversial whether the injection of hESCs/hiPSCs into preimplantation mouse blastocysts can generate even low-grade chimeric embryos (8–11). As an alternative approach, we explored whether multipotent somatic cells would be able to functionally integrate into postgastrulation mouse embryos and allow for the generation of mouse–human chimeras. We investigated the potential of human neural crest cells (hNCCs), derived from hESCs/hiPSCs, to integrate into the mouse embryo and contribute to the NC-associated melanocyte lineage. The NC, a multipotent cell population, arises at the boundary between the neuroepithelium and the prospective epidermis of the developing embryo. Trunk NCCs migrate over long distances, with the lateral migrating NCCs generating all of the melanocytic cells of the animal's skin (12).

NCC migration, development, and differentiation into various tissues have been studied in vivo by generating quail–chick NC chimeras. In this model, donor quail tissues were grafted into similar regions of developing chicken embryos (13). The experimental approach of our present study was based on the generation of mouse–mouse NC chimeras that had been created by injection of primary mouse NCCs into the amniotic cavity of embryonic-day (E) 8.5 embryos (14, 15). The donor mouse NCCs

Significance

We generated mouse–human neural crest chimeras by introducing neural crest cells derived from human embryonic stem cells or induced pluripotent stem cells (iPSCs) in utero into the gastrulating mouse embryo. The cells migrated in the embryo along normal migration routes and contributed to functional pigment cells in the postnatal animal, as demonstrated by coat color contribution. This experimental system represents a novel paradigm that allows studying the developmental potential of human cells under in vivo conditions. Importantly, this platform will allow for the investigation of human diseases in the animal by using patient-derived iPSCs.

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[1]Present address: Department of Developmental Biology and Cancer Research, Institute for Medical Research Israel-Canada, Hebrew University-Hadassah Medical School, Jerusalem 91120, Israel.

[2]To whom correspondence should be addressed. Email: jaenisch@wi.mit.edu.

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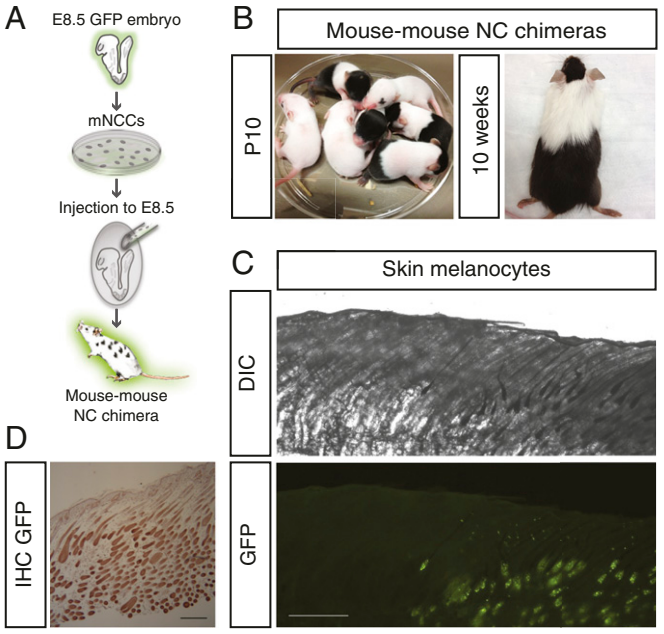

Fig. 1. Primary mNCCs contribute to host pigmentation. (*A*) Primary mNCCs were isolated from E8.5 embryos of *Rosa26*$^{CAGGS::GFP}$ C57BL/6 mice and injected into E8.5 host W^{sh}/W^{sh} mouse embryos. (*B*) Significant coat color contribution in P10 (*Left*) and adult chimeras (*Right*). (*C*) Differential interference contrast (DIC) imaging of the skin and hairs of a chimeric mouse shows strong pigmentation to the host (*Upper*) and the presence of GFP-positive cells in the skin and hair follicles (*Lower*). (*D*) Contribution was further confirmed by immunohistochemistry (IHC) with anti-GFP staining of skin sections. (Scale bars: 100 μm.)

(mNCCs), having been placed outside of the embryo, enter into the neural tube, presumably through the still-open neural pores, and transverse the epidermis. The donor mNCCs used in this previous study were collected from pigmented C57BL/6 mice, whereas the host embryos were derived from BALB/c albino mice. Thus, contribution of the donor mNCCs to the host embryo could be determined by the presence of pigmentation in the coats of the injected mice. The injected primary mNCCs contributed to coat color formation in the head and hind limb regions only, but not in the midtrunk area, likely reflecting the entry point of the cells through the neural pores with the anterior–posterior movement of the cells being hindered by endogenous melanoblasts (15). Indeed, when embryos carrying the white-spotted *c-Kit* mutation (W^{sh}/W^{sh}), which lack melanoblasts, were used as a host, extensive coat color contribution revealing anterior–posterior cell migration was observed, presumably because the donor NCCs could spread into the empty niches (14).

Here, we differentiated mouse, rat, and human ESCs or iPSCs into NCCs that were injected in utero into E8.5 albino wild-type and *c-Kit*–mutant W^{sh}/W^{sh} embryos. Both the mouse and human NCCs migrated laterally under the epidermis and ventrally into deeper regions of the embryo. Importantly, analysis of postnatal animals derived from mouse, rat, or human NCC-injected embryos displayed coat color pigmentation from the donor cells. Our results demonstrate that NCCs from different species can integrate into the developing mouse embryo, migrate through the dermis, and differentiate into functional pigment cells in postnatal mice. The generation of postnatal mouse–human chimeras carrying differentiated and functional human cells allows for a novel experimental system in which to study human diseases in an in vivo, developmentally relevant environment.

Results

Primary mNCCs Functionally Integrate into the Developing Embryo After in Utero Microinjection. To determine the potential of NCCs to incorporate into the developing embryo, primary mNCCs were dissected from E8.5 *Rosa26*$^{CAGGS::GFP}$ C57BL/6 gastrulation embryos and injected into E8.5 host W^{sh}/W^{sh} mouse embryos (Fig. 1*A*). Because the donor mNCCs were from a pigmented mouse strain, but the host mice, lacking melanoblasts, were white in appearance, contribution of the injected mNCCs would be detectable by coat color pigmentation in postnatal mice. Postnatal mice that were injected with primary mNCCs as embryos displayed substantial coat color pigmentation, indicating both the survival of the donor cells as well as the ability of the donor mNCCs to migrate and generate functional melanocytes (Fig. 1*B* and Table 1). Approximately 33% of the injected mice had detectable pigment contribution from the donor mNCCs, a frequency that is similar to published data (Table 1; refs. 14 and 15).

Because the injected mNCCs were labeled with GFP, fluorescence imaging was used to further analyze donor-cell contribution to the W^{sh}/W^{sh} hosts. The presence of a GFP signal was detected in the skin and hair follicles in a pigmented mouse–mouse NC chimera, confirming that these pigmented hair follicles were comprised of melanocytes derived from the microinjected mNCCs (Fig. 1 *C* and *D*). These data show that

Table 1. Chimeric contribution of donor NCCs after microinjection into E8.5 gastrulation mouse embryos

Mice	Chimeric embryos or postnatal mice out of total (%)	Extent of donor cell contribution*
Embryos		
mESC-derived mNCCs	35/91 (38)	+++
hESC/hiPSC-derived hNCCs	77/285 (27)	+
Postnatal		
Primary mNCCs	9/27 (33)	++++++
mESC-derived mNCCs	13/32 (40)	+++
riPSC-derived rNCCs	5/16 (31)	++
hESC/hiPSC-derived hNCCs	15/41 (36)	+
hESC/hiPSC-derived hNCCs + *c-MYC*	20/62 (32)	+

Embryos: CD1 and W^{sh}/W^{sh} embryos were injected at E8.5 with mESC- or hESC/hiPSC-derived NCCs and isolated a few days later. Chimeric contribution was estimated by the presence of fluorescent cells. No significant differences in the fraction of chimeric embryos were detected between mNCCs and hNCCs. The extent of chimeric contribution (arbitrarily represented by + marks) shows a higher contribution for chimeric embryos injected with mNCCs than with hNCCs. Postnatal mice: W^{sh}/W^{sh} mice that have been injected at E8.5 with primary mNCCs, mESC-derived mNCCs, riPSC-derived rNCCs, hESC/hiPSC-derived hNCCs, or hNCCs overexpressing *c-MYC* were examined postnatally. The fraction of mice with coat color pigmentation are presented. No significant differences in the fraction of chimeric mice were seen. The efficiency of the donor cells to contribute to coat pigmentation, represented by + marks, is high for primary mNCCs, lower using mESC-derived NCCs and rNCCs, and limited to a few single hairs using hNCCs.

*Estimation range of donor contribution to hosts embryo or coat color: ++++++, 10–50%; +++, 1–5%; ++, 0.1–0.5%; +, 0.01–0.1%.

primary mNCCs can provide extensive contribution to host coat pigmentation.

In Vitro-Derived mNCCs Functionally Integrate into the Developing Embryo. We tested whether in vitro-derived mNCCs, like primary mNCCs, could incorporate into the developing embryo and contribute to the NC-associated melanocyte lineage after microinjection. *Rosa26*$^{CAGGS::tdTomato}$ C57BL/6 mouse ESCs (mESCs) were differentiated into NCCs following standard protocols (16) and microinjected into E8.5 CD1 mouse embryos (Fig. 2*A*). Embryos injected with mESC-derived NCCs were analyzed from E10.5 to E18.5. Fig. 2*B* shows that mESC-derived NCCs were detected in both the anterior and the posterior regions of the embryo, highlighting their ability to incorporate into the embryo. The clusters of mESC-derived NCCs appear to disperse into single cells, consistent with the cells migrating through the dermis and under the epidermis in the developing embryo (Fig. 2*B*, boxed regions).

While the pigmented mNCC donor cells can be detected in the CD1 albino host, the donor cells have to compete with the amelanotic host melanoblasts. Analysis of postnatal CD1 mice injected with mESC-derived NCCs displayed pigmentation that was mainly restricted to the cranial region (Fig. 2*C*). When *c-Kit*-mutant W^{sh}/W^{sh} mice, which are more permissive because they lack melanoblasts, were injected with mESC-derived NCCs, pigmentation was detected in both the cranial and trunk regions (Fig. 2*D*).

Approximately 40% of the W^{sh}/W^{sh} host mice injected with in vitro-derived donor mNCCs displayed pigmentation in the coat, a frequency similar to the number of chimeras produced by injection with primary mNCCs (Table 1). However, contribution from the in vitro-derived mNCCs to coat pigmentation was restricted to smaller patches of black hairs in the cranial and lower trunk regions, suggesting that ESC-derived mNCCs have a reduced capacity to proliferate and/or migrate compared with primary mNCCs. In summary, our experiments show that in vitro ESC-derived mNCCs can contribute to the developing mouse embryo and differentiate into functional cells in postnatal NC chimeras, although the extent of pigmentation was lower than when primary mNCCs were used as donor cells.

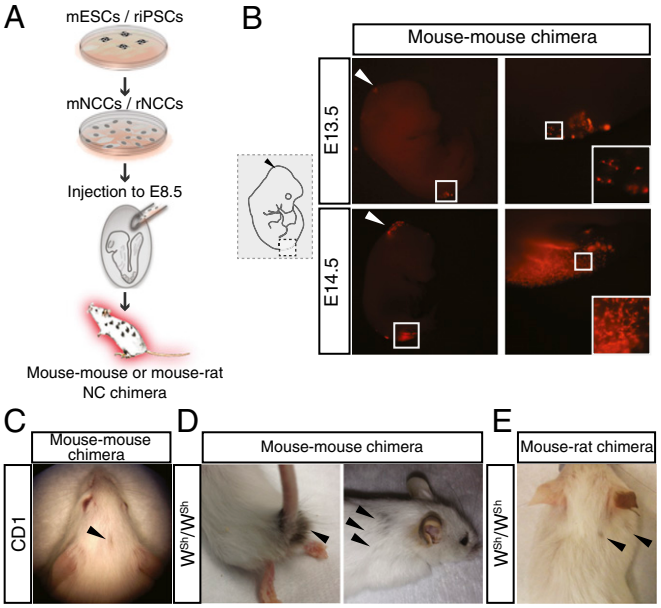

Fig. 2. mESC- and riPSC-derived NCCs participate in normal embryonic development after microinjection into postimplantation mouse embryos. (*A*) *Rosa26*$^{CAGGS::tdTomato}$ C57BL/6 mESCs were differentiated in vitro into NCCs and then microinjected into E8.5 mouse embryos to generate mouse–mouse NC chimeras. In addition, dark agouti riPSCs were differentiated to NCCs and microinjected into E8.5 mouse embryos to generate mouse–rat chimeras. (*B*) At 5–6 days after injection, tdTomato-positive donor mNCCs are visible along the dorsal side of the embryo, both in the cranial (arrowheads) and trunk (boxes) regions of the embryo. Higher magnification of an E13.5 embryo shows the presence of tdTomato-positive mNCCs migrating with spindle-like morphology, suggesting differentiation into melanocytes (*B*, *Upper*, boxed region). Higher magnification of the tdTomato-labeled mNCCs at E14.5 shows cell migration and proliferation (*B*, *Lower*, boxed region). (*C*) When C57BL/6 mNCCs were injected to CD1 albino mice, mNCCs were found to contribute to coat color pigmentation in the cranial area. (*D*) Postnatal W^{sh}/W^{sh} mice show contribution of the mNCCs to coat color pigmentation in both the caudal (*Left*) and the trunk (*Right*) regions. (*E*) Rat iPSCs were differentiated in vitro into rNCCs and microinjected into E8.5 W^{sh}/W^{sh} mouse embryos to generate mouse–rat NC chimeras. Postnatal mice show contribution of the donor rNCCs to coat color pigmentation in the trunk region (arrowheads).

NCCs Derived from Rat iPSCs Contribute to NC Interspecies Chimeras. We investigated whether rat-derived NCCs would generate interspecies chimeras when injected into mouse embryos. Hence, we derived rat iPSCs (riPSCs) from a pigmented dark agouti rat. The cells expressed pluripotency markers (Fig. S1*A*) and, when injected into mouse blastocysts, generated high-grade mouse–rat chimeras, in agreement with previous results (Fig. S1*B*; ref. 17). The riPSCs were differentiated to rat NCCs (rNCCs) and injected into E8.5 W^{sh}/W^{sh} mouse embryos. Fig. 2*E* shows coat color contribution of the donor rNCCs to the trunk of postnatal mice. Although the extent of pigmentation derived from the rNCCs was lower than that of mNCCs, the overall efficiency of chimera formation was similar (31%; Table 1). Our results indicate that in vitro-derived rNCCs are able to migrate within the developing embryo and contribute to melanocytes in adult interspecies NC chimeras.

NCCs Derived from Human ES and iPS Cells. We investigated whether hNCCs derived from hESCs or hiPSCs could integrate into the developing mouse embryo and form mouse–human interspecies chimeras. To ensure strong pigmentation of the human cells, we used skin fibroblasts from an African-American donor to derive hiPSCs (AA-hiPSCs). The reprogrammed AA-hiPSCs displayed pluripotent cell morphology, retained a normal karyotype, and expressed the key pluripotency markers NANOG, OCT3/4, and SOX2 (Fig. S2 *A* and *B*). To facilitate tracing of the cells after microinjection, we introduced a GFP reporter by targeting a *CAGGS::GFP* construct to the *AAVS1* locus (18). Correct targeting was confirmed by the presence of GFP-positive AA-hiPSC colonies, as well as by Southern blot analysis (Fig. S2 *C* and *D*).

The hESC (WIBR#3) and the AA-hiPSC lines, carrying the ubiquitously expressed GFP reporter, were differentiated into NCCs following an established protocol (19). The hESC- and hiPSC-derived NCCs expressed known NC markers, such as transcription factor AP-2 alpha (TFAP2-α) and human natural killer 1 (HNK-1; Fig. S3*A*). Similarly, as expected for NCCs, key transcription factors such as *SLUG, SNAIL*, *SOX9*, *SOX10*, and *PAX3* were expressed, whereas *SOX2* and *PAX6*, two transcription factors strongly associated with neural precursor (NP) cells, were down-regulated (Fig. S3*B*; refs. 20 and 21).

To test whether the hNCCs were multipotent, we used various protocols to induce differentiation into NC-associated lineages. hNCCs were differentiated toward melanocytic, neuronal, or mesenchymal fates. The melanocyte-like cells were pigmented, displaying melanocyte cell morphology and expressing a key melanocyte transcription factor, MITF (Fig. S3*C*). The NC-derived neuronal cultures displayed typical neuronal morphology and stained for known markers of the peripheral nervous system (PNS), such as PERIPHERIN, HNK-1, and TRKA (Fig. S3*D*). Mesenchymal differentiation was characterized by changes in cell morphology compared with hNCCs (Fig. S3*E*), as well as the expression of mesenchymal-specific genes, such as *ENG* (*Endoglin*), *CD44*, and *THY1* (*CD90*; Fig. S3*F*). Our results indicate robust differentiation of the hESCs and hiPSCs to cells with NC identity and with the potential to generate different NC-derived lineages.

hESC- or hiPSC-Derived NCCs Integrate into Mouse Embryos. GFP-positive hESC- or hiPSC-derived hNCCs were microinjected into E8.5 mouse embryos (Fig. 3*A*). When analyzed between E10.5 and E13.5, GFP-positive cells were detected in the anterior and posterior regions of both CD1 (Fig. 3*B*, *Left*) and *c-Kit*-mutant W^{sh}/W^{sh} (Fig. 3*B*, *Right*) host embryos. The GFP-positive hNCCs were seen migrating as single cells under the epidermis (Fig. 3*B*). Approximately 27% of the embryos injected with hNCCs retained detectable GFP-positive cells during embryogenesis, showing that the number of interspecies mouse–human chimeric embryos was similar to that of mouse–mouse NC chimeras (Table 1). However, based on scoring for the number of GFP-positive migrating cells (*Materials and Methods*), the efficiency of hESC/hiPSC-derived NCCs to incorporate and contribute to the embryo was significantly lower than that of mESC-derived NCCs (Table 1). Our results suggest a species-specific limitation in the ability of the hNCCs to survive and integrate into the host mouse embryos.

As controls, we injected undifferentiated hESCs, hESC-derived NPs, and human fibroblasts into E8.5 mouse embryos. As shown in Fig. 3 *C–E*, these cells did not disperse and migrate, but instead aggregated and formed tumor-like structures at the surface of the embryo. In summary, our results indicate that hESC/hiPSC-derived NCCs, although at a reduced efficiency in comparison with mouse primary and mESC-derived mNCCs, can transverse the epithelial layer of the embryo and move along the dorsolateral and ventral migration routes in contrast to undifferentiated hESCs, hNPs, or human fibroblasts.

hESC- and hiPSC-Derived NCCs Contribute to Pigmentation in Postnatal Mice. To assess whether hNCCs could survive through embryogenesis and produce mature and functional cells, we analyzed postnatal mice from injected embryos (Fig. 4*A*). Because bone morphogenetic protein 4 (BMP4) has been shown to enhance melanoblast differentiation from NC cultures (22, 23), we added BMP4 to the final steps of our in vitro differentiation protocol to promote the hNCC contribution to the pigmentation lineage. Fig. 4*B* demonstrates that donor hNCCs injected in utero into W^{sh}/W^{sh} mice formed single and dispersed pigmented hair, mainly in the head region. The number of chimeric mice was similar to that found for both primary and in vitro-derived mNCCs (~35%). However, the efficiency of the hESC/hiPSC-derived hNCCs to contribute to coat pigmentation was reduced in the interspecies mouse–human chimeras (Table 1) to single black hairs in the cranial region, suggesting a limitation in the ability of the hNCCs to survive and/or proliferate within the host mouse embryo.

To independently confirm the presence of human cells, we subjected follicles of pigmented hairs to immunostaining and

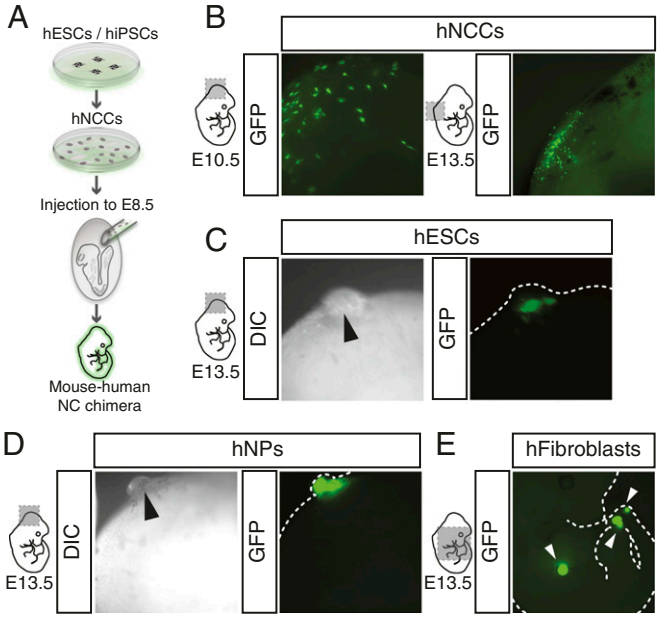

Fig. 3. hESC- and hiPSC-derived hNCCs survive and contribute to host embryos. (*A*) *AAVS1-CAGGS::GFP* hESCs/hiPSCs were differentiated to hNCCs and microinjected into E8.5 mouse embryos to generate mouse–human NC chimeric embryos. (*B*) hNCCs survive and contribute to embryonic NC development of E10.5–E13.5 mouse embryos. GFP-positive hNCCs were visible in cranial (*B*, *Left*) and lower trunk (*B*, *Right*) regions of the injected embryos. (*C–E*) *AAVS1-CAGGS::GFP*-labeled undifferentiated hESCs (*C*), hNPs (*D*), and GFP-positive human fibroblasts (*E*) were injected into E8.5 host mouse embryos, and their contribution was examined 5–6 days after injection. In all three cases, clusters of cells were found on the surface of the embryo (black or white arrowheads).

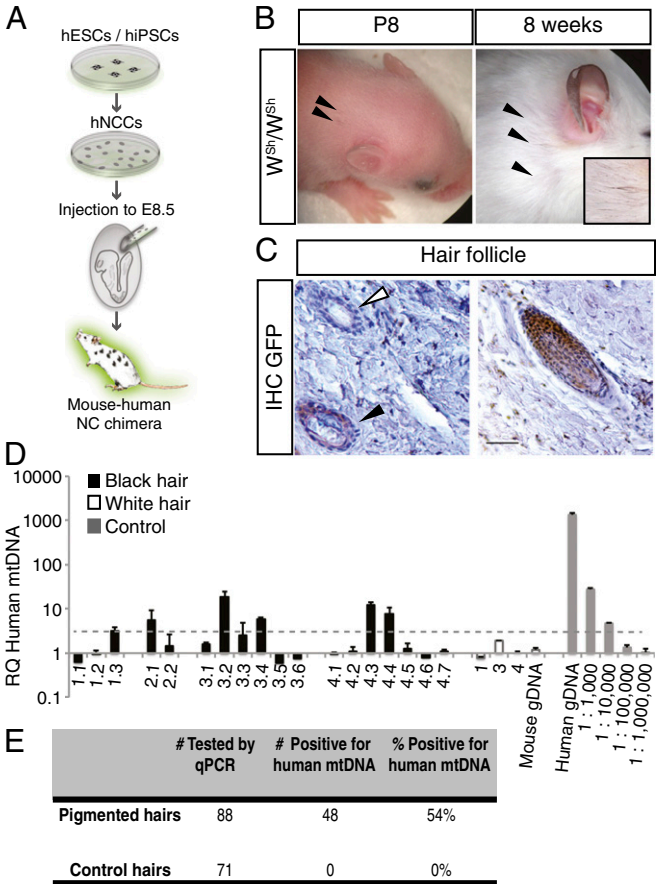

Fig. 4. hESC- and hiPSC-derived hNCCs survive and contribute to host coat pigmentation. (*A*) hNCCs were microinjected into E8.5 W^{sh}/W^{sh} mouse embryos to generate postnatal mouse–human NC chimeras. (*B*) Coat color contribution in adult W^{sh}/W^{sh} mice, injected as embryos with hNCCs at E8.5, is seen as single, dispersed, pigmented hairs (arrowheads). (*C*) The skin of a chimeric mouse was immunostained for GFP to confirm human contribution to coat pigmentation. GFP-positive cells were found to contribute to some hair follicles, indicating the presence of donor human cells. Black arrowhead, GFP-positive hair follicle; white arrowhead, GFP-negative hair follicle. (Scale bar: 50 μm.) (*D*) A sensitive qPCR assay, designed for specific detection of human cells, displayed contribution of hNCCs in the bulge of single pigmented hairs collected from four different chimeric mice (black bars). Mouse DNA samples from white hairs (white bars) and positive human DNA samples, as well as a series of human–mouse cell dilution samples (gray bars), were run as negative and positive controls. The dashed line represents the lower sensitivity threshold of the assay. (*E*) Summary of qPCR assays for pigmented hairs (n = 88) and control white hairs (n = 71) from several mouse–human NC chimera experiments. The analysis indicates that human mtDNA was detected in 54% of pigmented hairs and in none of the control samples. Error bars represent SD. gDNA, genomic DNA; mtDNA, mitochondrial DNA; RQ, relative quantification.

DNA analyses. Fig. 4*C* shows a significant number of GFP-positive human-derived cells in some of the hair follicles (black arrowhead). To quantitatively detect even low contributions of human cells, we developed a highly sensitive quantitative PCR (qPCR) assay that is based on the presence of human mitochondrial DNA (mtDNA; Fig. S4). The sensitivity of the assay allows for the detection of as few as one human cell in a population of 10,000 mouse cells. As shown in Fig. 4*D*, the assay revealed the presence of human DNA in numerous follicles of pigmented hair from multiple mouse–human NC chimeras (black bars). More than 50% of pigmented hairs collected for DNA analysis were positive for human mtDNA signals, whereas no human mtDNA was detected in white hairs (Fig. 4*E*). The failure to detect human mtDNA in all pigmented hairs could be (*i*) due to pigmented hairs having been removed without the full hair follicle, thus lacking cells; or (*ii*) because the contribution of human melanocytes sufficient for some pigmentation may be lower than the threshold for mtDNA detection.

The contributions of mESC- or hESC-derived NCCs to pigmentation were much lower than that of primary mNCCs (compare Fig. 1*B*), possibly because in vitro-derived NCCs are less proliferative. To assess whether oncogene-mediated stimulation of proliferation would promote chimera contribution, we introduced a Doxycycline (Dox)-inducible lentiviral construct overexpressing the human *c-MYC* oncogene into the hNCCs (Fig. S5). The cells were injected in utero into E8.5 W^{sh}/W^{sh} mice, and pregnant females were treated with Dox in drinking water. Pigmented hairs were found at approximately the same frequency in the postnatal mice (32%), but the extent of pigmentation was not significantly higher than that seen with hNCCs without *c-MYC* expression (Table 1). Our results suggest that forced expression of *c-MYC* did not significantly enhance contribution to the host melanocytes. In summary, our results indicate that hNCCs, when introduced into gastrulating mouse embryos, are able to integrate into the embryo and migrate along the lateral routes. Importantly, the cells mature to functional melanocytes and contribute to visible hair pigmentation in the adult mouse.

Discussion

In this study, we introduced ESC- or iPSC-derived mouse, rat, and human NCCs in utero into mouse embryos and examined their ability to integrate and migrate in the embryo as well as contribute to the pigment lineage in postnatal mice. The cells were injected during the stage of development when the endogenous NCCs delaminate from the neural epithelium and migrate dorsolaterally along defined routes generating all pigment cells of the animal's skin (24). Using a fluorescence reporter, we detected the survival and migration of the donor NCCs in the embryo, through the dermis, and along the dorsolateral route at different days after injection. Thus, the hNCCs, similar to mNCCs, followed normal NC migration patterns. Based on previous results, we used *c-Kit* receptor (W^{sh}/W^{sh}) mutant embryos as hosts to give the donor cells a selective advantage by providing "empty niches" for the NCC-derived melanoblasts (14, 15). Confirming the previous results, primary mNCCs produced widespread coat color pigmentation, whereas NCCs derived in vitro from mESCs contributed to pigmentation only in the cranial and lower trunk region. Similarly, when NCCs derived from rat or human ESCs/iPSCs, instead of mNCCs, were used as donor cells, only small pigment patches or single, dispersed, pigmented hairs, respectively, were detected in the cranial and trunk region. The contribution of rNCCs was lower than that of mouse, and contribution of hNCCs was lower than that of rat (compare Figs. 1*B*, 2 *D* and *E*, and 4*B* and Table 1). Thus, our findings suggest that evolutionary distance between mouse and rat vs. mouse and human may influence chimeric contribution to the murine host. Alternatively, culture conditions between mouse, rat, and human NCCs may yield cells of different proliferative capacity. Our results indicate that NCCs, which are differentiated in vitro from pluripotent cells, have a significantly lower capacity to migrate and/or to proliferate in the embryo than primary NCCs, possibly because the ESC-/iPSC-derived cells are more mature and thus may have a lower potential for self-renewal. To overcome this restriction, it will be necessary to optimize both the culture conditions and differentiation protocols. We attempted to stimulate proliferation of NCCs by ectopic expression of *c-MYC,* but overexpressing this gene failed to significantly enhance donor cell contribution.

Although mESC-derived NCCs were able to provide functional melanocytes in both the *c-Kit*–mutant W^{sh}/W^{sh} mice, as well as the CD1 mice, which are less permissive hosts (Fig. 2 *C* and *D*), the hESC-/hiPSC-derived NCCs were only able to provide pigmentation to W^{sh}/W^{sh} mice. They lacked the ability to contribute to visible pigmentation in CD1 mice, suggesting limited capacity to compete with endogenous melanoblasts. Although the contribution of hNCCs to postnatal W^{sh}/W^{sh} mice was limited, the frequency for generating NC chimeras in W^{sh}/W^{sh} mice was similar for all donor cell groups, i.e., primary mNCCs, as well as mouse, rat, and human ESC-/iPSC-derived NCCs (Table 1). Thus, our results suggest that the frequency of chimera formation depends on the success rate of hitting the amnion cavity during in utero injection. The significantly lower extent of pigmentation in the interspecies chimeras, compared with mouse–mouse chimeras, suggests that the host environment might limit the maturation and differentiation of the injected rat and human NCCs. We consider several possibilities that may restrict contribution or survival of hNCCs to tissues in the chimeric embryos and mice. (*i*) It is possible that the human cells elicited an immune reaction, leading to elimination of the human cells after injection. Previous observations using primary mNCCs as donor cells detected fading of the widespread coat color pigmentation when the donor cells were H-2 mismatched, whereas no fading of coat color pigmentation was visible when donor mNCCs had the same H-2 haplotype as the host (14). The use of immune-compromised embryos as hosts for mouse–human NC chimera formation would test this possibility. (*ii*) hNCCs may be unable to compete with the fast proliferation and migration of the endogenous mouse NCCs. (*iii*) Factors important for supporting NCC proliferation and migration may be species-specific, causing the mouse environment to be suboptimal for hNCCs. The use of humanized host embryos that express human growth factors may enhance chimera formation from the human donor cells (25).

The generation of interspecies chimeras is of great interest because it represents an experimental tool for regenerative medicine, allowing, in combination with patient-derived hiPSCs, for studying human disease (26). In a previous study, viable postnatal interspecies mouse–rat chimeras have been generated after injection of naïve rat ESCs/iPSCs into mouse blastocysts or of naïve mouse ESCs/iPSCs into rat blastocysts (17). Similarly, the generation of mouse–human chimeras by injection of human naïve pluripotent cells into mouse blastocysts has been reported, but remains controversial (8–10). In a different approach, primed hESCs were introduced into postimplantation mouse embryos cultured in vitro (11) and shown to contribute to different parts of the host epiblast, depending on exposure of the donor cells to different culture conditions. The results described in the present study indicate that, as an alternative approach, multipotent somatic cells are able to functionally integrate into postgastrulation mouse embryos and allow for the generation of postnatal mouse–human chimeras carrying differentiated and functional human cells. The NC donor cells were injected into gastrulating embryos at the stage of host development when the NCCs delaminate from the neuroepithelium. Matching the developmental stage of the host with that of the donor cells may be crucial to allow proper integration into the embryo and functional contribution to pigmentation in the postnatal mice.

We focused on NC chimeras, because the NC is involved in both inherited and acquired developmental abnormalities, termed neurocristopathies (27). NC disorders include syndromes such as familial dysautonomia, Hirschsprung's disease, and Treacher

Collins syndrome, which are caused by impaired NCC migration or degeneration of cells in NC-derived tissues. Thus, the creation of mouse–human NC chimeras represents a novel experimental platform to investigate NC-associated human diseases under in vivo conditions. It will be particularly attractive to use this experimental platform to study NC-derived tumors (28, 29). Because tumor formation is clonal, it should be possible to study initiation, progression, and manifestation of the tumors under in vivo conditions, even if donor cell contribution is low. However, to obtain more efficient contribution to other NC-derived lineages, such as the sensory neurons or craniofacial bones, it may be necessary to genetically disable the lineage so that the developmental niches become "empty" to give easier access to the donor cells (26). Indeed, the use of mutant *Pdx1* blastocysts as recipients for rat ESCs resulted in chimeric mice with a functional pancreas composed exclusively of rat ESC-derived cells (17).

Materials and Methods

Mice. C57BL/6 and W^{sh}/W^{sh} mice were obtained from the Jackson Laboratory. CD1 mice were obtained from Charles River Laboratories. Mice were maintained in the Whitehead Institute animal facility. All experiments were approved by the Committee on Animal Care at the Massachusetts Institute of Technology and animal procedures were performed following the National Institute of Health guidelines.

mNCCs and rNCCs. mESCs were cultured on MEFs in serum/LIF, as described (30), and riPSCs were cultured on MEFs in 2i/LIF (31). Differentiation into NCCs was modified from a published protocol (*SI Materials and Methods*; ref. 16).

hNCCs. The hESC line WIBR#3 (32) and hiPSC line AA#1 were cultured as described (10). Cell differentiation to NCCs was modified from published protocol (19). Briefly, neural induction was obtained by using 10 μM SB431542 and 500 nM LDN193189 for 3 days. Further NC differentiation was acquired by using 3 μM CHIR99021 (all from Stemgent) for 8 days (*SI Materials and Methods*). hNCCs were cultured in N2B27 supplemented with bFGF and EGF (both 20 ng/mL).

NCCs Injection and Contribution. Microinjections of NCCs were performed as described (refs. 14 and 15; *SI Materials and Methods*). Injected pregnant female mice were killed between E9.5 and E15.5 of gestation. Embryos were harvested, and NCC contribution to the embryos was determined by the presence of a fluorescent protein signal. Embryos containing a fluorescent signal were imaged by using a stereomicroscope (Nikon SMZ18).

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