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Ma, Ming San

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CHAPTER 2

Generation of induced pluripotent stem cells from adult neural crest stem cells

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INTRODUCTION

Mammalian somatic cells can be reprogrammed towards a pluripotent state by means of transduction with (combinations of) specific transcription factors such as Oct4, Sox2, Klf4, c-Myc (Yamanaka factors), Nanog, or Lin28 (Takahashi and Yamanaka, 2006; Takahashi, et al., 2007; Yu, et al., 2007; Liao, et al., 2008). These so-called induced pluripotent stem (iPS) cells are in many aspects similar to embryonic stem (ES) cells and provide a potential autologous source of stem cells for application in tissue engineering and regenerative medicine (Chin, et al., 2009; Wernig, et al., 2008; Hanna, et al., 2007). In order to develop safe and reproducible iPS technology for future clinical use, focus over the past few years has been to find strategies 1. to minimize the number of reprogramming factors needed (Kim, et al., 2008); 2. to avoid potentially oncogenic factors like c-Myc (Nakagawa, et al., 2007); 3. to replace integrative viruses with other nonintegrative transgene carriers to avoid potentially mutational insertions (Okita, et al., 2008; Zhou and Freed, 2009); 4. to simplify and standardize reprogramming using defined small molecules instead of transcription factors (Desponts and Ding, 2010; Jia, et al., 2010; Yu, et al., 2009); and 5. to find cell types which are both easily accessible and more prone to reprogramming (Kim, et al., 2009; Emini, et al., 2008; Tsai, et al., 2010). In the present study we focus on the latter strategy.

The neural crest is a population of migratory multipotent progenitor cells in vertebrates that emerges and delaminates from the dorsal neural tube during neurulation. It contributes significantly to the development of mammalian craniofacial tissues (reviewed in Santagati and Rijli, 2003). As a result, neural crest stem cells (NCSCs) persist in several craniofacial structures in the adult organism, and therefore are a relatively accessible source of stem cells for transplantation studies and tissue engineering (reviewed in Shakhova and Sommer, 2010; Kalt Schmidt, et al., 2012). The mammalian hair follicle contains several compartments of stem cells which are responsible for regeneration of these structures during the anagen phase of the hair as well as after injury (reviewed in Fuchs and Horsley, 2008; Tiede, et al., 2007). A cell population of neural crest stem cells (NCSCs) which are responsible for regeneration of these structures during the anagen phase of the hair as well as after injury (reviewed in Fuchs and Horsley, 2008; Tiede, et al., 2007). A cell population of neural crest stem cells (NCSCs) which are responsible for regeneration of these structures during the anagen phase of the hair as well as after injury (reviewed in Fuchs and Horsley, 2008; Tiede, et al., 2007). A cell population of neural crest stem cells (NCSCs) which are responsible for regeneration of these structures during the anagen phase of the hair as well as after injury (reviewed in Fuchs and Horsley, 2008; Tiede, et al., 2007). A cell population of neural crest stem cells (NCSCs) which are responsible for regeneration of these structures during the anagen phase of the hair as well as after injury (reviewed in Fuchs and Horsley, 2008; Tiede, et al., 2007).

In the present study we isolated nestin-expressing NCSCs from the adult mouse whisker follicle (Sieber-Blum, et al., 2004; Sieber-Blum and Hu, 2008; Amoh, et al., 2005; Amoh, et al., 2009), as well as adult mouse ENCSCs from the myenteric plexus (Shakhova and Sommer, 2010), and analyzed the expression of pluripotency genes in these NCSC populations. We hypothesized that (high) endogenous expression of one or more pluripotency factors might facilitate pluripotency induction compared to, for instance, skin fibroblasts and/or that less transcription factors would be necessary to induce pluripotency. We could confirm the pluripotent expression profile of adult NCSCs, but also show that this does not facilitate their reprogramming into iPS cells, and that a full set of Yamanaka factors is still required.

MATERIAL AND METHODS

Isolation of HFNCSCs

Neural crest stem cells were isolated from mouse whisker follicles as previously described (Sieber-Blum et al., 2004). In short, whiskers of adult C57BL/6 mice and nestin-GFP transgenic mice were dissected under a binocular microscope. Hair follicle bulges were cultured on PDL-laminin-coated culture plates (one per well) in NCSCS medium consisting of Neuralbasal (Gibco Invitrogen 21103-049) with 1% Glutamax (Gibco Invitrogen 35050-038), 1% L-glutamine (PAA M11-006), 5 μg/ml Heparin (Sigma H4784), 2% B27 serum free supplement (Gibco Invitrogen 17504-044), 20 μg/ml epidermal growth factor (EGF) (Gibco Invitrogen PHG0311), 20 μg/ml basic fibroblast growth factor (bFGF) (Gibco Invitrogen PHG0021), and 0.2% Primocin (Amaxa VZA-1022), under standard culture conditions. The medium was changed every 2-3 days.

Isolation of ENCSCs

Enteric neural crest stem cells were isolated from adult mouse myenteric plexus (MP). In short, the gastrointestinal tract was isolated and the colon dissected longitudinally. The muscular layers were separated under a binocular microscope and enzymatically digested using Liberase TH (Roche 05401135001) in Hank’s Balanced Salt Solution (HBSS) for 4.5 hours at 37°C. The MP were then separated from the remaining tissue by gentle shaking, transferred to MEM-Hepes (PAN-Biotech), and allowed to sediment. After centrifugation the MP were plated out and cultured in NCSC medium supplemented with 100 ng/ml glial cell-derived neurotrophic factor (GDNF) (Tebu-bio 450-10-B) under standard culture conditions. The medium was changed every 2-3 days.

like bodies (NLB) in the presence of EGF, bFGF, and GDNF. They can differentiate into glia and neurons in vitro (although it remains a question whether NCSCs actually undergo neurogenesis or gliogenesis in vivo (Kruger, et al., 2002); they have the ability to colonize the colon after transplantation in animal models (Almond, et al., 2007; Natarajan, et al., 1999; Kruger, et al., 2002; Bondurand, et al., 2003; Burns, 2005). In the present study we isolated nestin-expressing NCSCs from the adult mouse whisker follicle (Sieber-Blum, et al., 2004; Sieber-Blum and Hu, 2008; Amoh, et al., 2005; Amoh, et al., 2009), as well as adult mouse ENCSCs from the myenteric plexus (Shakhova and Sommer, 2010), and analyzed the expression of pluripotency genes in these NCSC populations. We hypothesized that (high) endogenous expression of one or more pluripotency factors might facilitate pluripotency induction compared to, for instance, skin fibroblasts and/or that less transcription factors would be necessary to induce pluripotency. We could confirm the pluripotent expression profile of adult NCSCs, but also show that this does not facilitate their reprogramming into iPS cells, and that a full set of Yamanaka factors is still required.
IPS Induction

For reprogramming of the mouse HFNCSCs we have used both the retroviral and lentiviral transduction approach. For retroviral transduction, retroviral particle containing supernatants were produced using Phoenix Eco packaging cells which were transfected using Fugene with separate pMX-based vectors encoding murine Oct4, Sox2, Klf4 and cMyc plasmids (Addgene) as described previously (Czepiel, et al., 2011). For lentiviral transduction, HEK293T cells were transfected with a lentiviral self-inactivating pRRL.PPT.SF plasmid containing murine Oct4, Klf4, Sox2, c-Myc, and dTomato complimentary DNAs and co-transfected with pMD2-VSV-G and pCMV-Δ8.91 packaging vectors. Lentiviral supernatant was collected 48 hours post-transfection and filtered through a 0.45 µm filter (Whatman). Around 20,000 HFNCSCs were transduced with viral supernatant supplemented with 8 µg/ml Polybrene (Sigma-Aldrich), which was replaced by fibroblast medium after 24 hours. Transfection efficiency was evaluated based on fluorescent dTomato expression. Four days post-transduction cells were dissociated using 0.05% trypsin-EDTA and seeded onto irradiated mouse embryonic fibroblasts (iMEFs) feeder layers on a 1% sodium pyruvate for 5 days.

EB formation

For embryoid body (EB) formation HFNCSC-derived IPS cells (HF-IPScs) were dissociated using 0.25% trypsin/EDTA and grown for 8 days as suspension cultures in 10 cm low attachment petri dishes in EB medium consisting of KO-DMEM supplemented with 15% fetal calf serum, 2 mM L-glutamin, 100 U/ml penicillin/streptomycin, 100 µM β-mercaptoethanol, 2 mM nonessential amino acids. EBs were replated with 1% gelatin coated 6-wells plate and cultured in ES medium. Colonies were counted after 7 days and four clones were isolated using a binocular microscope and subcultured by passaging with trypsin-EDTA and growing for 8 days as suspension cultures in 10 cm low attachment petri dishes in EB medium. Previously generated fibroblast-derived iPS cells (F-iPSCs) and IB10 embryonic stem cells (ESCs) were also cultured on iMEFs in ES medium.

Immunocytochemistry

Immunocytochemistry was performed as previously described (Czepiel, et al., 2011). The following antibodies were used: mouse anti-mouse nestin (1:200; Chemicon, MAB 353), mouse anti-mouse Oct4 (1:100; Santa Cruz, 5279), rabbit anti-mouse Sox2 (1:200; Abcam, 15830), mouse anti-mouse Sox2 (1:200; Cell Signaling, 49005), rabbit anti-Nanog (1:200; Abcam, 80892), mouse anti-mouse SSEA-1 IgM (1:200; Santa Cruz, 21702), mouse anti-mouse desmin (1:200; Dako M0760), mouse anti-mouse GATA (1:200; Santa Cruz, 25310), and mouse anti-mouse β-III tubulin (1:400; Abcam 7751). The following fluorescently labeled secondary antibodies were used: Alexa Fluor 488-conjugated donkey anti-rabbit (1:300; Molecular Probes, A21206), Alexa Fluor 488-conjugated goat anti-mouse IgM (1:300; Invitrogen, M31601), DyLight 488-conjugated goat anti-rabbit (1:100; Jackson ImmunoResearch), and Cy3-conjugated donkey anti-goat (1:1300; Jackson ImmunoResearch). For nuclear visualization, cells were counterstained with Hoechst 33342 (1:1000; Jackson Immunoresearch) in PBS. Alkaline phosphatase activity of IPS colonies was detected using an alkaline phosphatase detection kit (Sigma).

qRT-PCR

Total RNA was extracted (Chomczynski and Sacchi, 1987) and transcribed into cDNA using random hexamers and M-MLV reverse transcriptase (Fermentas). Purity of the RNA was confirmed by means of gel electrophoresis. Quantitative real-time PCR was performed in 384-wells plates using the IQ SYBR Green Supermix (Bio-Rad) on an AB17900HT system (Applied Biosystems) with the following cycling parameters: 95°C for 3 minutes, 39 cycles with 95°C for 10 seconds, and 58°C for 30 seconds. qRT-PCR primers were designed with PeriPrimer for the transcripts listed in Table 1. Housekeeping genes hydroxymethylbilane synthase (HMBS) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as internal controls for normalization. The data were processed with SDS version 2.3 analysis software (Applied Biosystems). The relative expression ratio of target genes was quantified using the 2^-ΔΔCt calculation method (Schmittgen and Livak, 2008).

Western Blot

Proteins were extracted from cell samples using lysis buffer supplemented with protease inhibitors. Cell lysates were sonicated and quantitated by DC Protein Assay (Bio-Rad, Hercules, CA) and proteins were separated on 15% sodium dodecyl sulfate-polyacrylamide gels and then transferred onto polyvinylidene fluoride membranes. Membranes were incubated in Odyssey blocking buffer and incubated O/N in primary antibodies diluted in PBS + 0.1% Tween-20. The following primary antibodies were used: mouse anti-mouse Oct4 (1:1,000; Santa Cruz, 5279; non cross-reactive with Oct-3/4 isoform B), mouse anti-mouse Sox2 (1:1,000; Cell Signaling, 49005), rabbit anti-mouse Klf4 (1:1,000; Abcam 34814), rabbit anti-mouse Nanog (1:1,000; Abcam, 80892), mouse anti-mouse β-actin (1:10,000; Abcam 6276), and rabbit anti-mouse β-actin (1:10,000; AbD Serotec AHP1387). The following day, membranes were washed and
incubated for 1 hour in fluorescent conjugated secondary antibodies: donkey anti-mouse IRDye 680CW (1:10,000; LI-COR Biosciences), and donkey anti-rabbit IRDye 800CW (1:10,000; LI-COR Biosciences). Blots were washed, scanned with the LI-COR Odyssey infrared imaging system (LI-COR Biosciences), and analyzed with Odyssey 2.0 software.

RESULTS

Isolation and culturing of NCSCs

We isolated hair follicle-derived neural crest stem cells from the adult mouse whisker follicle of nestin-GFP transgenic mice as well as from C57Bl/6 mice (Figure 1A). Explants were placed in laminin-coated 6-wells plates and NCSCs were allowed to migrate out of the follicle bulges (Figure 1B, C). For assessment of pluripotency gene expression with immunohistochemistry, C57Bl/6 cells were replated in small badges of 10-100 cells on laminin and allowed to clonally expand in selective proliferation medium. In this way pure populations of HFNCSCs could be obtained which proliferated in monolayers as described previously (Sieber-Blum et al. 2004). To compare the pluripotency gene expression profile with HFNCSCs, ENCSCs were isolated from adult mouse myenteric plexus and grown as neurospheres (Figure 1D-F).

Expression of pluripotency factors in NCSCs

Monolayers of nestin-GFP HFNCSCs (p0) were FACS-sorted for GFP after 1 week in vitro and analyzed for mRNA expression of pluripotency genes using qRT-PCR. Similarly, after 5 days in vitro, the ENCSC-neurospheres were harvested and analyzed by means of qRT-PCR (Figure 2A). Pluripotency gene expression was compared with that in mouse embryonic fibroblasts (MEFs) and embryonic stem cells (ESCs). Compared to MEFs, Oct4, Sox2, and Nanog are expressed up to 500-fold higher in HFNCSCs (Oct4: p > 0.05; Sox2 and Nanog: p < 0.05). HFNCSCs expressed Klf4, Sox2, c-Myc, and Nanog at levels that did not significantly differ from those in ES cells (p > 0.05 for all factors); only Oct4 is expressed a 100-fold higher in ESCs (p < 0.05). Similar to HFNCSCs, ENCSCs expressed Oct4, Sox2, and Nanog at considerably higher levels (around 50 times) than MEFs, although the expression in ENCSCs was lower than in ESCs and HFNCSCs. To further characterize HFNCSCs as potential candidate for iPSC induction, after 1 week in vitro HFNCSCs were analyzed by means of immunocytochemistry. All cells were nestin-positive while >95% strongly expressed Sox2 (Figure 2B). Also pluripotency markers Oct4 and Nanog were expressed although weaker in comparison to Sox2. Oct4 expression was seen mainly in the cytoplasm but not in the nucleus (in accordance with previous findings (Atlasi 2008).

Reprogramming of HFNCSCs into iPS cells

In view of the specific endogenous pluripotency expression profiles of the HFNCSCs, we tested whether we could reprogram these cells into iPSCs while omitting the transcription factors already

Figure 1: A: Schematic depiction of the location of the bulge (B) region within the hair follicle; others structures depicted are blood sinus (BS), capsula (C), dermal papilla (DP), outer root shaft (ORS), and sebaceous gland (SG). B: Brightfield images showing isolation of the bulge of a mouse whisker follicle. Sequentially: isolated hair follicle; transversal cut is made just below the ring sinus and above the papilla (dotted lines); a longitudinal incision is made through the middle section of the follicle (dotted line); the capsula is opened and the bulge region with the attached hair is isolated (arrow indicates bulge). Scale bar: 200 µm. C: Bright field images of adult mouse hair follicle neural crest stem cells (HFNCSCs) migrating out of the bulge after 5 days on laminin (left panel), and 2 weeks after isolation (and after removal of bulge) (middle panel, magnification in right panel). D: Brightfield image of isolated murine gastrointestinal tract including stomach (ST), duodenum (D), and small intestine (SI). E: Schematic depiction of the location of the myenteric plexus (MP) between muscularis interna (MI) and muscularis externa (ME). Other structures are: lumen (L), mucosa (M), submucosa (SM), submucous plexus (SP), and serosa (S). F: Bright field image showing enteric spheres with neural processes after 1 week of culturing.
Chapter 2

Neural crest stem cell-derived iPS cells

Figure 2: A: qRT-PCR analysis for mRNA expression levels of Oct4, Sox2, Klf4, c-Myc, and Nanog in ESCs, HFNCSCs, and ENCSCs, showing relative values normalized to HMBS and compared to expression levels of MEFs (logarithmic scale). Error bars represent S.D. (n = 3); *p < 0.05, #p = 0.045 compared to ESCs, One-way ANOVA, Bonferroni post-hoc multiple comparisons test. B: Characterization of HFNCSCs with immunocytochemistry. HFNCSCs strongly express nestin and weakly express Oct4; Sox 2 and Nanog are also expressed; nuclei are counterstained with Hoechst (blue). Scale bar: 200 µm.

Figure 3: Characterization of HF-iPSCs. A: HF-iPSC colonies in culture and stained for alkaline phosphatase (AP). B: Colonies stained for pluripotency markers showing Oct4, Sox2, SSEA-1, and Nanog expression. C: Default differentiation of dissociated EBs plated on Matrigel™-coated culture dishes gives rise to cells expressing Desmin (mesoderm), GATA-4 (endoderm), and βIII-Tubulin (ectoderm). Nuclei were counterstained with Hoechst (blue). Scale bar = 100 µM. D: qRT-PCR analysis for mRNA expression levels of Oct4, Sox2, Klf4, c-Myc, and Nanog in F-iPSCs and HF-iPSCs, showing relative values normalized to HMBS and compared to expression levels of MEFs (logarithmic scale). Error bars represent S.D. (n = 3); *p < 0.05, one-way ANOVA, Bonferroni post-hoc multiple comparisons test. E: Western Blot analysis of cell culture samples confirming presence of pluripotency factors in ESCs, F-iPSCs, and HF-iPSCs.
highly expressed by the HFNCSCs (Sox2, Klf4, and/or c-Myc were left out in different combinations as well as altogether; Oct4 was always present). After 4 weeks, no colonies were observed in any of the conditions. Only lentiviral transduction of all four Yamanaka factors in a polycistronic vector resulted in the appearance of iPS colonies within 5-7 days post-transduction (Figure 3A). Of 2 \times 10^4 transduced cells, we counted an average of 5 colonies per field of view at 100x magnification, or a total of 1125 colonies; efficiency was calculated at 0.056 % which is slightly higher than efficiencies with fibroblasts and other cell types. Colonies displayed a proper ESC-like morphology with a large nuclear/cytoplasmic ratio and prominent nucleoli. HFNCSC-derived iPS cells (HF-iPSCs) were characterized as to their reprogramming and pluripotent characteristics. Like ESCs, HF-iPSCs showed positive alkaline phosphatase activity and expression of ESC–associated antigens Oct3/4, Sox2, Nanog, SSEA-1, and SSEA-4 (Figure 3A, B; results shown for one clone). HF-iPSCs were further characterized by means of quantitative real time-PCR analysis for pluripotency markers and reprogramming factors. High expression levels of Oct4, Sox2, Klf4, and Nanog mRNA were observed. Expression levels of Sox2 and c-Myc were lower (both \( p < 0.05 \)), while Oct4, Klf4, and Nanog were higher in HF-iPSCs in comparison to previously generated fibroblast-derived iPS cells (F-iPSCs) (Nanog \( p < 0.05 \), Oct4 and Klf4 \( p > 0.05 \)) (Figure 3D). Expression of pluripotency factors was confirmed on the protein level by means of Western Blot analysis; expression levels were comparable to those in F-iPSCs, and comparable to or higher (Sox2, Nanog) than levels in ESCs (Figure 2E). Embryoid bodies (EBs) were generated using RA according to a -4/+4 protocol, default differentiated in vitro, and stained for markers representative of the three germ layers: desmin, GATA and β-III tubulin (Figure 3C). Positive staining indicated proper default differentiation into the three germ layers.

**DISCUSSION**

Several authors have reported the expression of pluripotency-related genes in adult NCSCs populations. Yu described the presence of nestin-positive cells within the human hair follicle bulge, which in addition were positive for pluripotency factors Nanog and Oct4 (Yu, et al., 2006). Sieber-Blum also showed the expression of different pluripotency factors in bulge NCSCs; c-Myc, Klf4, and Sox2 were expressed at similar levels compared to ESCs while Oct4 and Nanog were significantly lower expressed than in ESCs (Sieber-Blum and Hu, 2008). We confirmed that the nestin-expressing NCSCs from the bulge region of the adult mouse whisker follicle (HFNCSCs) co-express pluripotency genes Oct4, Sox2, Klf4 and Nanog. Expression of Oct4 was lower than in ESCs, but in contrast to Sieber-Blum, we showed Nanog expression levels similar to ESCs. Like Yu, we were able to detect expression of both Oct4 and Nanog on the protein level. In addition we demonstrated a similar expression pattern in another adult NCSC type, namely enteric neural crest stem cells (ENSCS), suggesting that the presence of the pluripotency transcription factors was neural crest stem cell specific.

**Oct4, Sox2, Klf4, c-Myc, and Nanog and regulation of pluripotency**

Interestingly, expression of Nanog, Oct4, and Sox2 is considered to be restricted to pluripotent cells (Chambers and Tomlinson, 2009); in mouse ESCs these genes form a core transcriptional circuitry regulating pluripotency (Boyer, et al., 2005). Oct4, Sox2 and Nanog bind to and autoregulate their own as well each other’s promoters; this way the autoregulatory network may enhance the stability of pluripotency and reflects the ‘master regulatory’ function of these factors in determining the stem cell state (reviewed in Jaenisch and Young, 2008). This same network is thought to play a crucial role in establishing and maintaining pluripotency upon reprogramming of somatic cells (reviewed in Jaenisch and Young, 2008). Nichols et al. showed by means of target gene deletion that Oct4 is essential for the pluripotent identity of the inner cell mass (Nichols, et al., 1998). Upon differentiation, Oct4 determines the developmental fate of ESCs, as critical levels of Oct4 cause cells to differentiate into either endoderm or mesoderm, or trophectoderm, while overexpression of Oct4 triggers differentiation into endoderm and mesoderm (Niwa, et al., 2000; Ivanova, et al., 2006). Sox2 and Nanog are required for maintenance of epiblast and ESC pluripotency; Sox2-deficient ESCs differentiate into trophectoderm while Nanog-deficient ESCs differentiate into extra-embryonic endoderm (Mitsui, et al., 2003; Masui, et al., 2007). Nanog downregulation also induces expression of markers for trophectoderm and epiblast-derived lineages such as mesoderm, ectoderm and neural crest cells. Wang et al. however showed that pluripotency is differently regulated in human ESCs (Wang, et al., 2012); here Oct4 together with the BMP4 pathway regulates the specification of major cell fates, while NANOG acts as a specific repressor of neuroectoderm and neural crest lineages (Wang, et al., 2012).

**Function of pluripotency factors in neural crest stem cells**

The expression of pluripotency factors in non-pluripotent stem cells has been described before. Some authors have shown Oct4 and Nanog expression in mesenchymal stem cells (MSCs) and neural stem cells (NSCs) (Tsai, et al., 2012; Massa, et al., 2012). Recently, the group of Morshhead reported the presence of periventricular primitive NSCs in the adult mouse forebrain, which are LIF-responsive and Oct4-positive, but not pluripotent (they give rise to adult GFAP-positive/Oct4-negative NSCs)(Sachewsky, et al., 2014). The studies by Tsai et al. and Massa et al. indicate that Oct4 and Nanog are essential for the maintenance of multipotency and proliferation in MSCs and NSCs (Tsai, et al., 2012; Massa, et al., 2012). Knockdown of Oct4 and Nanog in MSCs leads to reduced proliferation and upregulation of differentiation markers (Tsai, et al., 2012); Oct4 and Nanog were strongly downregulated after differentiation of neurosphere-derived NSCs. Little is known about the mechanistic function of these factors in adult stem cells. The expression of Oct4, Sox2, and Nanog has recently been described in HFNCSCs as well as ENCSCs from postnatal rats and postnatal humans (Yu, et al., 2006; Hagl, et al., 2013)(Table 2). We confirmed the expression of Oct4, Sox2, and Nanog in murine HFNCSCs and ENCSCs. In neural crest stem cells, intermediate level expression of Oct4 is thought to regulate the repression of mesoderm and endoderm differentiation while Sox2 expression may repress trophectoderm differentiation (Niwa, et al., 2000; Adachi, et al., 2010). Both in mouse and human ESCs, especially Nanog appears to repress differentiation of neural
Neural crest stem cell-derived iPS cells

Crest lineages. High levels in NCSCs might be necessary to hold back differentiation towards neural crest lineages. Factors from the neural crest niche (e.g., the dermal papilla) might induce changes in Oct4 and Nanog expression and thus allow neural crest differentiation. Factors like Sox10 may also play a crucial role in maintenance or differentiation permission (Kim, et al., 2003). It must be noted that alternatively spliced transcripts or alternative translation products of the Oct4 gene can lead to diverse expression patterns and functions; splice variants of Oct4 (Oct4B) might wrongfully suggest characteristics of pluripotency (Wang and Dai, 2010). Also, reports of Oct4 and Nanog expression in adult somatic cells have been challenged by the proposed possibility of detection of pseudogene transcripts (Liedtke, et al., 2007); some of these transcripts might be functionally important, others might not (Lin, et al., 2007).

Table 2: Studies describing expression of pluripotency genes in mammalian NCSC populations.

<table>
<thead>
<tr>
<th>Cell population</th>
<th>Origin</th>
<th>Defined by expression of</th>
<th>Pluripotency genes</th>
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</thead>
<tbody>
<tr>
<td>Sieber-Blum (2008)</td>
<td>Mousewhisker follicle bulge</td>
<td>Neural crest</td>
<td>Sox10, nestin</td>
</tr>
<tr>
<td>Yu (2006)</td>
<td>Human scalp hair follicle, precise region not described</td>
<td>Neural crest</td>
<td>Nestin, neural crest markers</td>
</tr>
<tr>
<td>Tsai (2009)</td>
<td>Mouse back skin dermal papilla</td>
<td>Mesenchyme</td>
<td>Lef1/integrin α-9</td>
</tr>
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</table>

iPS induction from HFNCSCs

As an accessible cell source, HFNCSCs are a clinically relevant cell type for iPS induction. We showed that, despite their high expression of pluripotency factors, HFNCSCs can only be reprogrammed using a full set of Yamanaka factors, with an efficiency comparable or only slightly higher than generally found for fibroblasts. Our data suggest that the levels and activities of the endogenous factors were inadequate to significantly facilitate iPS reprogramming of HFNCSCs. This implies that endogenous expression might not be necessarily sufficient for substitution of transgenes, and that the ability and disposition for reprogramming still depends on the cell type and its epigenetic signature. Cell type appears to be a major factor in determining reprogrammability (Maherali and Hochedlinger, 2008). Unlike e.g. neural progenitor cells, HFNCSCs or neural crest stem cells in general might be too specified to overcome certain epigenetic barriers restricting reprogramming, unless a full set of factors is expressed. Characterization of four-factor derived HF-iPSCs did show ESC-like colony morphology, EB formation and differentiation patterns, as well as pluripotency marker expression, confirming proper pluripotency induction. The expression of transgene mRNAs was higher than in fibroblast-derived iPSCs, especially for Oct4 and Nanog, potentially reflecting the high expression levels in the original cells. Assessment of the methylation profiles of the promotors of key pluripotency genes would be insightful for determining whether these high expression levels in HF-iPSCs in comparison to F-iPSCs are due to epigenetic differences.
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