Establishing an efficient protocol for the generation of donor specific iPS cells

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Introduction

Human induced pluripotent stem cells (iPS cells) are considered to be a valuable tool for studying in vitro embryonic development, cell differentiation and tissue formation. Additionally, they provide the possibility to study various pathogenetic pathways and may be used for approaches in regenerative medicine. In this context, somatic cells are reprogrammed by forced expression of several factors, known as the Yamanaka factors. The arising iPS cells share all abilities of embryonic stem cells, such as self renewal, differentiation into cells of all three germ layers and the ability of teratoma formation.

We focused our work on establishing a noninvasive protocol to obtain adult somatic cells and reprogram them into iPS cells with a high efficiency leading to an improved success rate of iPS generation. Therefore we chose keratinocytes from plucked human hair as a starting population and reprogrammed them by lentiviral transduction of an excisable construct containing all reprogramming factors as a single transcript. Subsequently, infected keratinocytes are transferred to a feeder cell layer of fibroblasts that are known to support the viability and metabolism of the reprogramming cells. When testing various culture conditions and feeder cell types during the reprogramming we found that the use of rat embryonic fibroblasts (REF) is superior to the commonly used mouse embryonic fibroblasts (MEF) in the reprogramming process leading to a significant increase of iPS colonies, displaying all hallmarks of pluripotency. Together, the presented method of keratinocyte derived human iPS cell (k-hiPSC) generation, the utilized cell source and the newly found feeder cell type proved to have a very high success rate and was used to generate numerous lines from healthy and diseased donors.

Figure Legends

1A: Schematic procedures of keratinocyte reprogramming. Plucked human hair was used as a source for keratinocytes. After the initial outgrowth, the keratinocytes were infected with a lentivirus carrying the reprogramming factors Oct4, Sox2, Klf4 and c-Myc. Two days later they were transferred onto a feeder layer of MEFs or REFs until the arising colonies were mechanically passaged.

1B: DIC microscopy images of keratinocyte outgrowth from a plucked hair, confluent cultured keratinocytes, emerging k-hiPSC colonies on REFs and an k-hiPSC colony growing on Matrigel in mTeSR1 medium. All scale bars are 100 µm.

1C: Two weeks after seeding the same amount of keratinocytes on different feeder cells, an AP staining or SSEA4 flow cytometry was performed. The number of emerging AP-positive k-hiPSC colonies is approximately three-fold higher on REFs compared to MEFs. The ratio of SSEA4-positive k-hiPS cells to total cell counts is about two-fold higher on REFs.
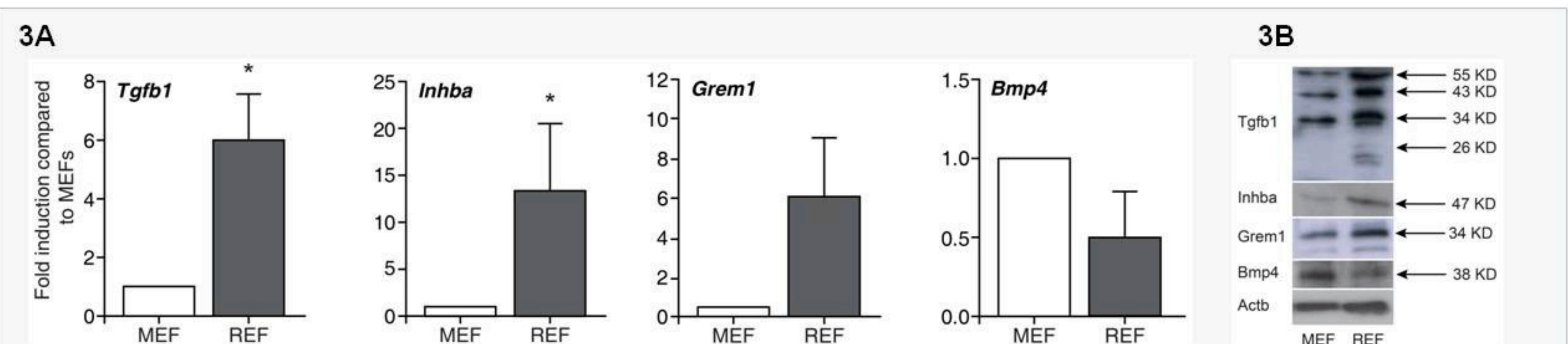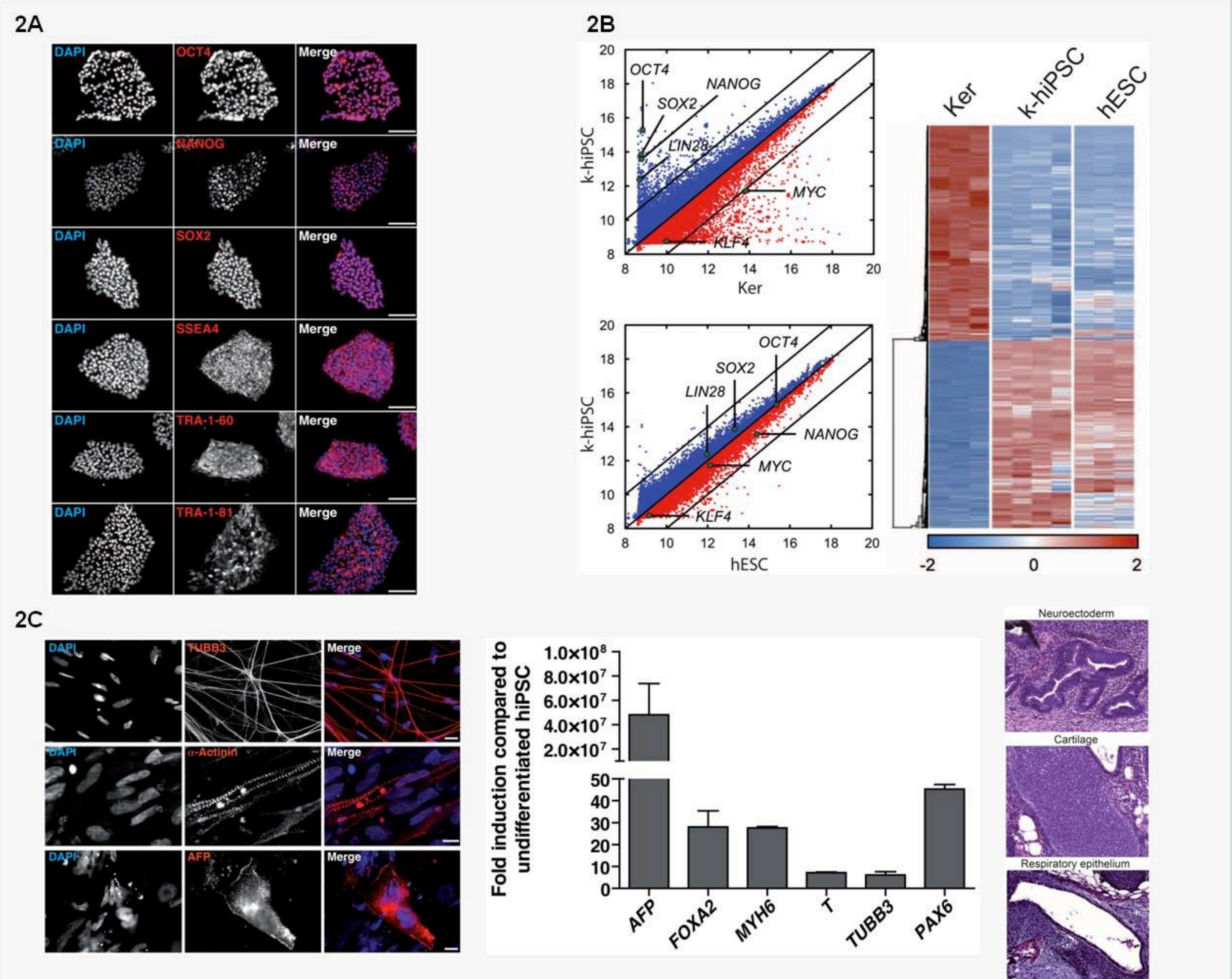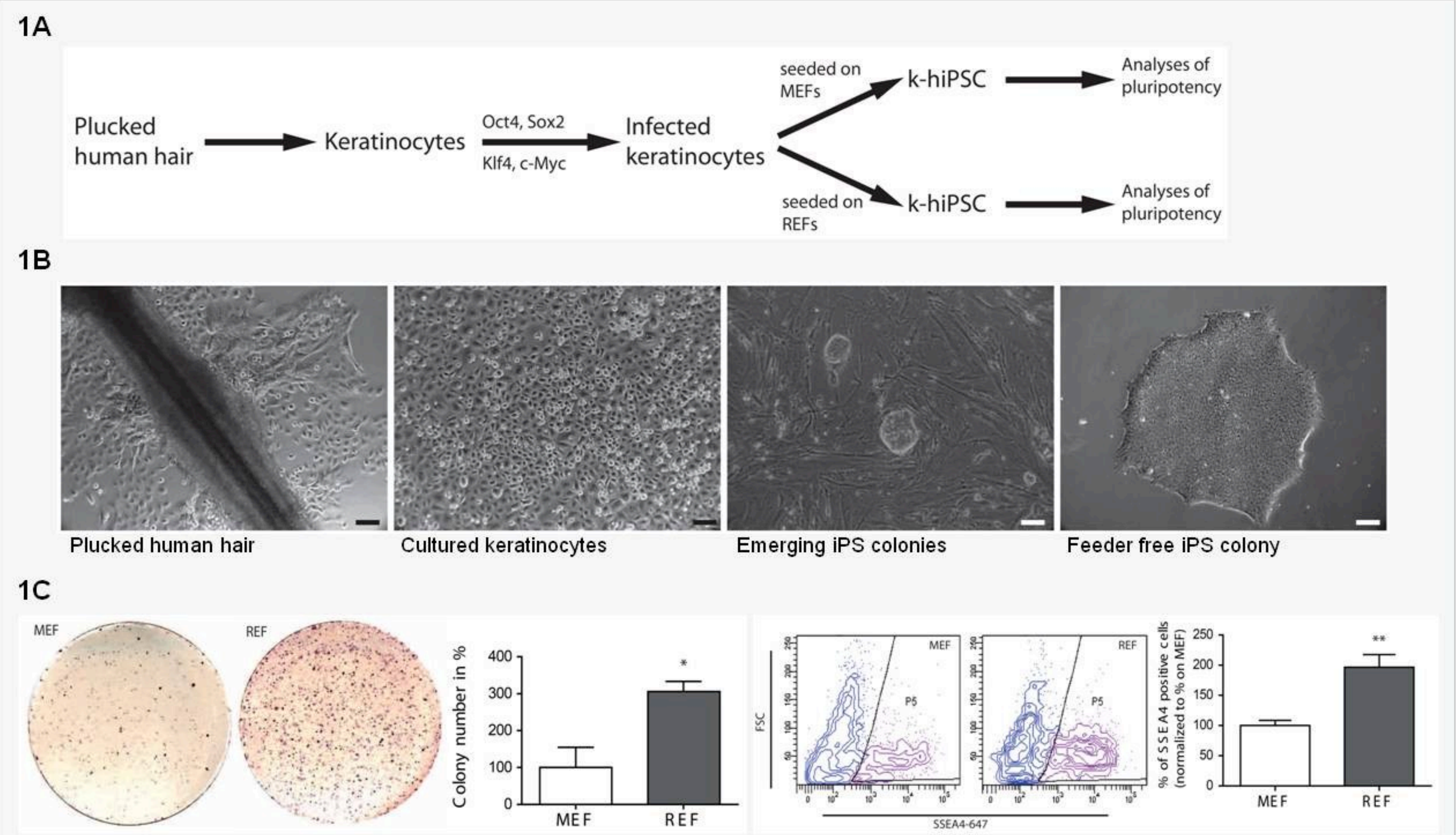
2A: Immunofluorescence stainings of the nuclear factors OCT4, NANOG and SOX2 and surface markers SSEA4, TRA-1-60 and TRA-1-81. All scale bars are 100 µm.

2B: Scatter plot of k-hiPSC gene expression compared to keratinocytes or hESC. Typical pluripotency markers are expressed similarly in k-hiPSC and hESC while differing in keratinocytes. The heatmap of human transcriptome microarrays compares keratinocytes, k-hiPSC and hESC. The differently regulated genes between keratinocytes and k-hiPSC are selected. Downregulation is shown in blue and upregulation is shown in red.

2C: Immunofluorescence stainings show cells positive for ectodermal (TUBB3: Tubulin beta-III), mesodermal (a-Actinin) and endodermal (AFP: Alpha fetoprotein) marker proteins. Scale bars, 20 µm. Up-regulation of several marker genes was measured by qRT-PCR. The endodermal markers AFP and FOXA2, the mesodermal markers MYH6 and T as well as the ectodermal markers TUBB3 and PAX6 are up-regulated compared to undifferentiated k-hiPS cells. Teratomas formed by k-hiPS cells after injection into SCID mice contain ectodermal (neuroectoderm), mesodermal (cartilage) and endodermal (respiratory epithelium) tissue showing that k-hiPS cells can differentiate into all three germ layers in vivo.

3A: Analyses of candidate genes mediating the improvement of reprogramming by REF feeder layers. qRT-PCR show higher mRNA levels for Tgfb1, Inhba and Grem1 in REFs compared to MEFs, while Bmp4 mRNA levels were non-significantly lower in REFs compared to MEFs.

3B: Western blot results confirm an upregulation of protein levels of Tgfb1, Inhba and Grem1 and a downregulation of Bmp4 in REFs compared to MEFs. Equal loading was confirmed by Actin detection and by amido black protein staining and subsequent concentration measurement.

1A

1B
Plucked human hair — Cultured keratinocytes — Emerging iPS colonies — Feeder free iPS colony

1C

2A

2B

2C

3A

3B

Conclusions

The use of the noninvasive and easy to get human keratinocytes as well as the use of REFs as feeder layer for reprogramming of keratinocytes into k-hiPS cells leads to cells which pass all pluripotency tests and arise with a significantly improved efficiency compared to earlier protocols. This is crucial for the generation of iPS cells from people carrying rare genetic mutations and may make the iPS technology interesting for new fields of research. Our group has used this improved protocol to generate several lines from autism spectrum disorder patients carrying rare mutations as well as control lines from several healthy individuals.

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