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# Interaction of adult human neural crest-derived stem cells with a nanoporous titanium surface is sufficient to induce their osteogenic differentiation

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**Abstract** Osteogenic differentiation of various adult stem cell populations such as neural crest-derived stem cells is of great interest in the context of bone regeneration. Ideally, exogenous differentiation should mimic an endogenous differentiation process, which is partly mediated by topological cues. To elucidate the osteoinductive potential of porous substrates with different pore diameters (30 nm, 100 nm), human neural crest-derived stem cells isolated from the inferior nasal turbinate were cultivated on the surface of nanoporous titanium covered membranes without additional chemical or biological osteoinductive cues. As controls, flat titanium without any topological features and osteogenic medium was used. Cultivation of human neural crest-derived stem cells on 30 nm pores resulted in osteogenic differentiation as demonstrated by alkaline phosphatase activity after seven days as well as by calcium deposition after 3 weeks of cultivation. In contrast, cultivation on flat titanium and on membranes equipped with 100 nm pores was not sufficient to induce osteogenic differentiation. Moreover, we demonstrate an increase of osteogenic transcripts including Osterix, Osteocalcin and up-regulation of Integrin β1 and α2 in the 30 nm pore approach only. Thus, transplantation of stem cells pre-cultivated on nanostructured implants might improve the clinical outcome by support of the graft adherence and acceleration of the regeneration process.
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*Abbreviations:* NCSCs, neural crest-derived stem cells; MSCs, mesenchymal stem cells; FGF-2, fibroblast growth factor-2; EGF, epidermal growth factor; ITSCs, inferior turbinate stem cells; RT, room temperature; PFA, paraformaldehyde; qRT-PCR, quantitative reverse transcription polymerase chain reaction; SEM, scanning electron microscope; ALP, alkaline phosphatase; $ddH_2O$, double distilled water; FCS, fetal calf serum; PBS, phosphate buffered saline; DAI, days after induction; FAK, Focal-Adhesion-Kinase.

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## Introduction

Cranial neural crest-derived stem cells (NCSCs) represent an adult dormant stem cell population able to give rise to ectodermal and mesenchymal derivatives (reviewed in (Kaltschmidt et al., 2012)). It should be mentioned, that in the head region bones are generated by neural crest cells. We and others demonstrated *in vitro* an efficient mesodermal differentiation of NCSCs derived from different craniofacial regions making this cell population a promising candidate for future use in regenerative medicine (Widera et al., 2009) (Delorme et al., 2010; Dong et al., 2010; Hauser et al., 2012; Kawanabe et al., 2010; Marynka-Kalmani et al., 2010). Especially in context of bone regeneration under contribution of autologously transplanted stem cells, directed differentiation of human NCSCs into an osteogenic lineage has gained great interest. Usually, the *in vitro*-osteogenesis by cranial NCSCs is induced by supplementation of the cultivation medium with a cocktail of (bio-) chemical agents including the synthetic glucocorticoid dexamethasone (Baek et al., 2013; Calloni et al., 2009). Importantly, *in vivo*, dexamethasone exhibits severe side effects common to other glucocorticoids including immunosuppressant action, which could increase the risk of infection after autologous transplantation of NCSCs. Moreover, dexamethasone was reported to transform human NCSCs into tumorigenic phenotype in the SCID mice transplantation model (Marynka-Kalmani et al., 2010). Thus, osteogenic differentiation protocols for future clinical use ideally should avoid harsh chemical cues.

Since physiological differentiation of neural crest cells towards osteogenic lineage is at least partly mediated by the topology (e.g. the porous surface of the present bone) (Dangaria et al., 2011), the use of physical and topological cues as an osteogenic trigger has gained great interest. Indeed appropriate nanoscale topology has been reported to induce osteogenic differentiation of a variety of cell types including osteogenic cell lines, immature human osteoblasts and mesenchymal stem cells (MSCs) (Dalby et al., 2006, 2007; Lavenus et al., 2011). However, to our knowledge, there are no reports on topological induction of osteogenic differentiation for multipotent human NCSCs.

In the last couple of years several craniofacial NCSC-populations within the head were described. Such cells were identified e.g. within the dental pulp and the periodontal ligament (Arnold et al., 2010; Waddington et al., 2009; Widera et al., 2007). Importantly, adult stem cells tend to transform towards tumorigenic phenotype during long-term *in vitro* expansion (Kaus et al., 2010; Nguyen et al., 2013). Thus, due to the limited source material dental pulp-derived and periodontal stem cells are not ideal candidates for up-scaling and their future clinical use. Noteworthy, as recommended by the Food and Drug Administration (FDA), the cultivation time of human stem cells for transplantation purposes should not exceed five weeks (Fink, 2009). NCSCs derived from the human inferior turbinate (inferior turbinate stem cells / ITSCs) have the advantage of easy accessibility and relatively high frequency within the tissue of origin. Moreover, large amounts of inferior turbinate tissue are routinely removed during surgeries against snoring without severe side effects (Fairbanks, 1985). In a previous study, we demonstrated that human inferior turbinates contain 16.55 + 5.1% p75 expressing ITSCs independent from the age and the gender of the donor (Hauser et al., 2012). In addition, ITSCs could be efficiently cultivated and rapidly expanded in a FCS-free medium in a closed cGMP-grade cell culture system without changing their stemness characteristics (Greiner et al., 2011, 2014).

Titanium is a common and broadly used material in orthopedic devices (reviewed in (Wennerberg and Albrektsson, 2009)). Besides its well-known biocompatibility, it has mechanical properties similar to bone, making titanium an ideal candidate for the design of stem cell-supported prostheses, which may improve the osseointegration into the surrounding bone. Moreover, titanium surfaces can be topographically manipulated at the nanometer scale, thereby improving their biocompatibility and potential to integrate into the bone (for review see (Lavenus, Ricquier, Louarn and Layrolle, 2010; Sjostrom et al., 2013; Wennerberg and Albrektsson, 2009). Indeed Lavenus et al. reported in 2011 that nanofeatured titanium surfaces are well biocompatible and induce osteogenic differentiation of human MSCs (Lavenus et al., 2011).

In this study, we investigated the biocompatibility and influence of anisotropically arranged nanopores with 30 nm and 100 nm diameter on human neural crest-derived ITSCs. Polycarbonate membranes with 30 nm as well as 100 nm nanopores were covered with a 5 nm titanium layer and used in cell culture as a substrate. Here, we demonstrate that a nanoporous titanium surface is biocompatible with adult ITSCs. We further show that a titanium surface with a pore size of 30 nm and not of 100 nm is able to induce osteogenic differentiation of ITSCs without biochemical cues, as demonstrated by alkaline phosphatase (ALP) activity and calcium depositions visualized using Alizarin Red S and von Kossa staining. Moreover, using qRT-PCR, we demonstrate a significantly elevated transcript level for osteogenic marker genes including Osterix and Osteonectin in ITSCs cultivated on titanium with 30 nm pores compared to flat titanium and titanium surface with a 100 nm pore size. Future studies will investigate the potential replacement of FSC as a component of the differentiation medium by human blood plasma, which can be applied autologously and supports growth of ITSCs without affecting their stem cell characteristics (Greiner et al., 2011, 2014).

In sum, we show that titanium is highly biocompatible with adult human NCSCs and that nanofeaturing of such a surface with anisotropic 30 nm pores is sufficient to induce their osteogenic differentiation.

## Materials and methods

### Generation of a nanoporous titanium surface

To make the substrates suitable for further experiments single discs with diameters of 10 mm and 20 mm were blanked out of the track etched polycarbonate membrane (Whatman). Following this the discs and the flat glass control substrate were washed with 100% EtOH, sputtered with a 5 nm titanium layer, washed with $ddH_2O$, affixed in 12 well cell culture plate (TPP) and sterilized with UV irradiation.

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