Original Article

Properties of Neural Crest-Like Cells Differentiated from Human Embryonic Stem Cells

(stem cell differentiation / neural crest / odontogenesis / cell therapies)

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Abstract. Neural crest cells (NCCs) derive early in vertebrate ontogenesis from neural tube as a population of migratory cells with exquisite differentiation potential. Abnormalities in NCC behaviour are cause of debilitating diseases including cancers and a spectrum of neurocristopathies. Thanks to their multilineage differentiation capacity NCCs offer a cell source for regenerative medicine. Both these aspects make NCC biology an important issue to study, which can currently be addressed using methodologies based on pluripotent stem cells. Here we contributed to understanding the biology of human NCCs by refining the protocol for differentiation/propagation of NCC-like cells from human embryonic stem cells and by characterizing the molecular and functional phenotype of such cells. Most importantly, we improved formulation of media for NCC culture, we found that poly-L-ornithine combined with fibronectin provide good support for NCC growth, we unravelled the tendency of cultured NCCs to maintain heterogeneity of CD271 expression, and we showed that NCCs derived here possess the capacity to react to BMP4 signals by dramatically up-regulating *MSX1*, which is linked to odontogenesis.

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Abbreviations: EGF – epidermal growth factor, hESCs – human embryonic stem cells, hiPSCs – human induced pluripotent stem cells, MFI – median fluorescence index, NCCs – neural crest cells, PBS – phosphate-buffered saline, SCs – stem cells, TBP – TATA box-binding protein.

Introduction

Stem cells (SCs) include a spectrum of cells defined by two key phenomena: (i) pluripotency – the ability to differentiate into a wide variety of adult organism cell types, and (ii) self-renewal – the ability to indefinitely divide and produce cells with unchanged differentiation potential. In normal ontogenesis, these unique cells enable embryonal/foetal development of multicellular organisms and provide a cell source for life-long maintenance of tissues and organs. Based on the intriguing properties of SCs, innovative approaches and methodologies are being designed and verified that will enable exploitation of SCs and their differentiated progeny in drug development, disease modelling, regenerative medicine, and cell therapies. Still, to realize the full potential of SCs, it is critical to elaborate protocols for highly controlled differentiation of SCs towards particular somatic phenotypes, which are based on understanding the underlying signalling pathways.

Classically viewed, ontogenesis is characterized by the process of progressive restriction of cell differentiation potential. The population of cells that during neurulation arise at the neural plate border between presumptive neuroepithelium and epidermis contradicts this general scenario. These cells, so called neural crest cells (NCCs), in fact possess much broader differentiation potential than the cells of ectodermal germ layer from which they originate. Somatic derivatives of NCCs include such diverse cell types as neurons and glia, melanocytes, endocrine cells, smooth muscle cells, osteoblasts, chondrocytes, fibroblasts, pericytes, and odontoblasts (reviewed in Crane and Trainor, 2006; Le Douarin et al., 2008). Given the differentiation multipotency of NCCs and their ability to self-renew, demonstrated on rat NCCs by Stemple and Anderson (1992), NCCs are currently mostly viewed as a population of stem/progenitor cells (Hall, 2000). Still, the extent of homogeneity of NCCs with respect to their developmental potential re-

quires further investigation since both less and more restricted cells have been found in the early-stage NCC population (reviewed in Dupin and Sommer, 2012). Besides having a wide differentiation repertoire, NCCs are equipped by exquisite migratory capacity (Tucker, 2004). Thanks to this motility NCCs can be navigated during embryogenesis to enormously distant locations in the body such as the entire body surface, intestine, and/or peripheral nerves.

Given the above-mentioned properties of NCCs, it is not unexpected that NCCs are of particular clinical significance. There is a series of human pathologies all originating from misbehaviour of NCCs and/or their progeny, including (i) a large group of developmental disorders collectively known as neurocristopathies (reviewed in Etchevers et al., 2006) and (ii) several malignancies such as neuroblastoma, melanoma, and pheochromocytoma (Manelli et al., 2007; Cheung and Dyer, 2013; Shakhova, 2014). Furthermore, epithelial-to-mesenchymal transition, a phenomenon that is typical of NCCs, possibly represents a model for analysing one of the critical steps in transition of cancers from localized to metastatic form (Acloque et al., 2009). Finally, besides their relevance to a plethora of pathologies, NCCs and particularly NCC-like cells residing in adult tissues offer a potential cell source for future cell replacement therapies. In conclusion, unravelling the mechanisms that drive formation, migration and differentiation of NCCs will impact our understanding of serious pathologies and may set grounds for novel therapeutic interventions.

Unfortunately, characterization of human NCCs and elucidation of their biology is still in infancy because of ethical and technical restrictions in accessing primary NCCs from human embryonic tissues (Thomas et al., 2008; Betters et al., 2010). As introduced above, in the last years the methodologies based on using human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) are being developed to bypass various limitations including those linked to working with human embryos. *In vitro* differentiating hESCs and hiPSCs offer an alternative renewable source of cells at various stages of their specification including cells of the neural crest lineage. Until now, the neural induction of hESCs and the generation of neural crest derivatives has been achieved by several groups using various more or less controlled protocols (Pomp et al., 2005; Lee et al., 2007; Brokhman et al., 2008; Pomp et al., 2008; Chambers et al., 2009; Jiang et al., 2009; Curchoe et al., 2010; Cimadamore et al., 2011; Kreitzer et al., 2013; Menendez et al., 2013; Zeltner et al., 2014).

Our study aims at improving the existing protocols for differentiation of hESCs into cells with neural-crest phenotype and for their propagation in culture by modifying culture media and substrata. Here we also address the issue of heterogeneity of such putative NCCs and their capacity to *in vitro* differentiate towards the odontogenic lineage.

Material and Methods

Cell culture

Undifferentiated hESCs were grown in colonies on gelatin-coated dishes in the presence of mitotically inactivated mouse embryonic fibroblasts in HES medium. Cells of the CCTL14 line of hESCs were used throughout this study. HES medium consists of DMEM/F12 (Life Technologies, Carlsbad, CA) with 15% knockout serum replacement (Life Technologies), 1% L-glutamine (GE Healthcare, Little Chalfont, UK), 1% MEM non-essential amino acids (GE Healthcare), 0.5% penicillin/streptomycin (GE Healthcare), 100 μM β-mercaptoethanol (Carl Roth, Karlsruhe, Germany) and 4 ng/ml FGF-2 (PeproTech, Rocky Hill, NJ). Medium was changed in daily intervals.

Four different media (KSR, MEC1, MEC2, and MEC3) were used for differentiation of hESCs towards the neural crest lineage. The composition of KSR medium was as follows: DMEM (Life Technologies) with 15% knockout serum replacement (Life Technologies), 1% L-glutamine (GE Healthcare), 1% MEM non-essential amino acids (GE Healthcare), 0.5% penicillin/streptomycin (GE Healthcare), 100 μM β-mercaptoethanol (Carl Roth). The compositions of MEC1, MEC2 and MEC3 media are shown in Table 1. The putative NCC cells produced by differentiation were passaged in 4- to 5-day intervals and they were seeded at a density of 30,000 cells per cm^2.

Culture dishes were coated as follows. Coating with Matrigel (BD Biosciences, San Jose, CA) was achieved by incubating dishes for 2 h at 37 °C with Matrigel diluted 78 times in culture medium. For coating with combinations of proteins of extracellular matrix, the dishes were exposed to the protein solutions for 2 h at 37 °C and then washed twice with distilled water before seeding the cells. The proteins were dissolved in distilled water to final concentrations of 20 ng/ml for poly-L-ornithine, 10 ng/ml for fibronectin, and 10 ng/ml for laminin (all from Sigma-Aldrich, St. Louis, MO). Coating with MaxGel™ ECM (Sigma-Aldrich) was achieved by incubating dishes for 2 h at 37 °C with MaxGel diluted 100 times in culture medium.

Immunocytochemistry

For detection of intracellular antigens cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized for 3 min with 0.5% Triton-X, blocked for 1 h with 1% bovine serum albumin in PBS (pH 7.4) (further referred to as blocking solution), and incubated for 1 h at room temperature with primary antibody diluted in blocking solution. For detection of cell surface antigen CD271 cells were fixed as above and then directly incubated overnight at 4 °C with primary antibody diluted in blocking solution. Primary antibodies used were as follows: mouse monoclonal antibody against human CD57 conjugated with PE/Cy5® (ab25445; Abcam, Cambridge, UK), mouse monoclonal antibody

Table 1. Compositions of MEC media

100 ml	MEC1	MEC2	MEC3	Manufacturer	Catalogue Number
DMEM/F12 medium (with HEPES and L-glutamine)	**98 ml**	---	---	Life Technologies	11330-0321330-032
Neurobasal medium	---	**95 ml**	**94 ml**	Life Technologies	21103-049
Glucose	**155 mg**	---	---	Sigma-Aldrich	G6152
Insulin	**2 mg**	---	---	Sigma-Aldrich	I9278
$NaHCO_3$	**200 mg**	---	---	Sigma-Aldrich	S5761
L-glutamine	---	**1 ml**	**1 ml**	GE Healthcare	M11-004
Nonessential AA	---	---	**1 ml**	GE Healthcare	M11-003
Pen/Strep	**1 ml**	**1 ml**	**1 ml**	GE Healthcare	P11-010
β-ME	---	---	**20 µM**	Carl Roth	4227.1
B27	---	**2 ml**	**2 ml**	Life Technologies	12587-010
N2	**1 ml**	**1 ml**	**1 ml**	Life Technologies	17502-048
FGF-2	**10 ng/ml**	**10 ng/ml**	**20 ng/ml**	PeproTech	100-18B
EGF	**10 ng/ml**	**10 ng/ml**	**20 ng/ml**	PeproTech	AF100-15

against SOX9 (ab76997, Abcam), rabbit monoclonal antibody against SOX10 (ab155279, Abcam), and mouse monoclonal antibody against human CD271 conjugated with FITC (345104; Biolegend, San Diego, CA). Cells were washed with PBS (pH 7.4) and incubated for 1 h at room temperature with appropriate secondary antibody where required: Alexa Fluor 594-conjugated secondary antibody against rabbit immunoglobulins (A11012; Life Technologies) or mouse immunoglobulins (A11005, Life Technologies). Cell nuclei were counterstained with DAPI (Sigma-Aldrich), and cells were mounted in Mowiol containing 1,4-diazobicyclo[2.2.2.]-octane to prevent fading. Microscopy was performed using a DM5000B Leica microscope (Leica, Wetzlar, Germany).

Flow cytometry and cell sorting

For analysis of cell surface antigens and cell sorting, cells were harvested using Accutase as described above, washed in blocking solution, and incubated for 30 min on ice with primary antibodies diluted in blocking solution without preceding fixation. Antibodies against CD57 and CD271 described above were used. Cell pellets were then resuspended and dead cells were visualized using a LIVE/DEATH® stain kit (Life Technologies) according to the manufacturer's instructions. Before flow cytometric analysis the cells were washed once in blocking solution. Measurement of antigen expression was performed using a FACSCanto II cytometer (BD Biosciences, San Jose, CA) and cells were sorted using a FACSARIA II cytometer (BD Biosciences). Cell debris, doublets, and aggregates were excluded from analysis based on FSC-Area/FSC-High and SSC dot plot. Dead cells were excluded from the analysis based on LIVE/DEATH® staining. We analysed 10,000 cells for each sample. The level of antigen expression was expressed as the ratio of the median fluorescence index (MFI) of specific antibody to the MFI of isotype-stained controls: FITC-conjugated IgG1 (400108, Biolegend) for CD271 and PE/Cy5®-conjugated IgM (ab151937, Abcam) for CD57.

For analysis of intracellular antigens, cells were harvested as above, fixed with 4% paraformaldehyde for 20 min at room temperature, and further processed using Stem Cell Transcription Factor Analysis Kit (BD Biosciences) according to the manufacturer's instructions. Measurement was performed using a FACSVerse cytometer (BD Biosciences). Cell debris, doublets, aggregates, and dead cells were excluded as described above. We analysed 30,000 cells for each sample. All flow cytometry data were analysed using the FlowJo software (http://www.flowjo.com).

RNA isolation and quantitative real-time polymerase chain reaction

Total RNA was isolated using RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. The quality and quantity of RNA was determined using NanoDrop 1000 (Thermo Scientific, Waltham, MA). Synthesis of cDNA from isolated RNA was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche, Madison, WI). Each sample was analysed in triplicate using TaqMan gene expression assay Hs00427183_m1 for *MSX1* (4331182, Life Technologies). DNA amplification was detected using the LightCycler® 480 System (Roche). The relative gene expression was calculated by normalization to the TATA box-binding protein (TBP) expression (TaqMan gene expression assay Hs99999910_m1).

Adipogenic differentiation

For adipogenic differentiation, NCCs were cultured in mesenchymal medium containing DMEM/F12 (Life Technologies) supplemented with 10% foetal bovine serum (GE Healthcare), 1% L-glutamine, and 1% penicillin/streptomycin for one week, followed by three weeks of culture in StemPro Adipogenesis Differentiation Kit

(Life Technologies) according to the manufacturer's instructions. Medium was changed every 2–3 days. Differentiation was assessed by staining with Oil Red O (Sigma-Aldrich).

Chondrogenic differentiation

For chondrogenic differentiation, NCCs were cultured in mesenchymal medium for one week similarly as for adipogenic differentiation. To achieve chondrogenesis, spheres produced by pelleting 250,000 cells each were then cultured for three weeks in StemPro Chondrogenesis Differentiation Kit (Life Technologies) according to the manufacturer's instructions. Medium was changed every 2–3 days. Differentiation was assessed in sections made from paraffin-embedded spheres by staining with Alcian Blue (Serva, Heidelberg, Germany).

Results

Overall, the initial 11-day long differentiation step was inspired by the dual SMAD inhibition strategy published by Lee and al. (2010), but it was accomplished in parallel in three variants differing since day 5 in the media composition. For all three media, MEC1, MEC2, and MEC3, Matrigel was used as a culture substrate. As depicted in Fig. 1A, the initial differentiation conditions included KSR medium with 10 μM TGFβ inhibitor (SB431542) and 500 ng/ml of noggin. Since day 5 of differentiation, an increasing amount (25 %, 50 %, and 75 %) of MEC1, MEC2, and MEC3 media, respectively, was added to KSR medium in two-day intervals without changing the concentration of TGFβ inhibitor and noggin. The differentiating cells remained unpassaged until day 11. On day 11 both TGFβ inhibitor and noggin were withdrawn and the cells were passaged on Matrigel-coated dishes into 100% MEC1, MEC2, and MEC3, respectively. The first observation that we made was a significant difference in plating efficiency in the given MEC medium with MEC3 providing the best condition to the cells (Fig. 1B).

Based on this simple but repeated observation, MEC3 medium was chosen for all the following experiments. Although in the original protocol by Lee and colleagues the differentiated cells were grown on the coating composed of poly-L-ornithine, fibronectin, and laminin, our unpublished observations indicated that such a complex surface is not in fact needed for the maintenance of the NCC phenotype. Therefore, we tested three different coatings that were less complex but still represented a more defined environment than Matrigel. Specifically, these were: (i) commercially available matrix Maxgel™ declared to efficiently support neural stem cells, (ii) combination of poly-L-ornithine and laminin, and (iii) combination of poly-L-ornithine and fibronectin. Similarly to the evaluation of the effects of MEC media, we followed the cells for several passages and based upon their survival rate and morphological homogeneity we selected poly-L-ornithine and fibronectin as the most

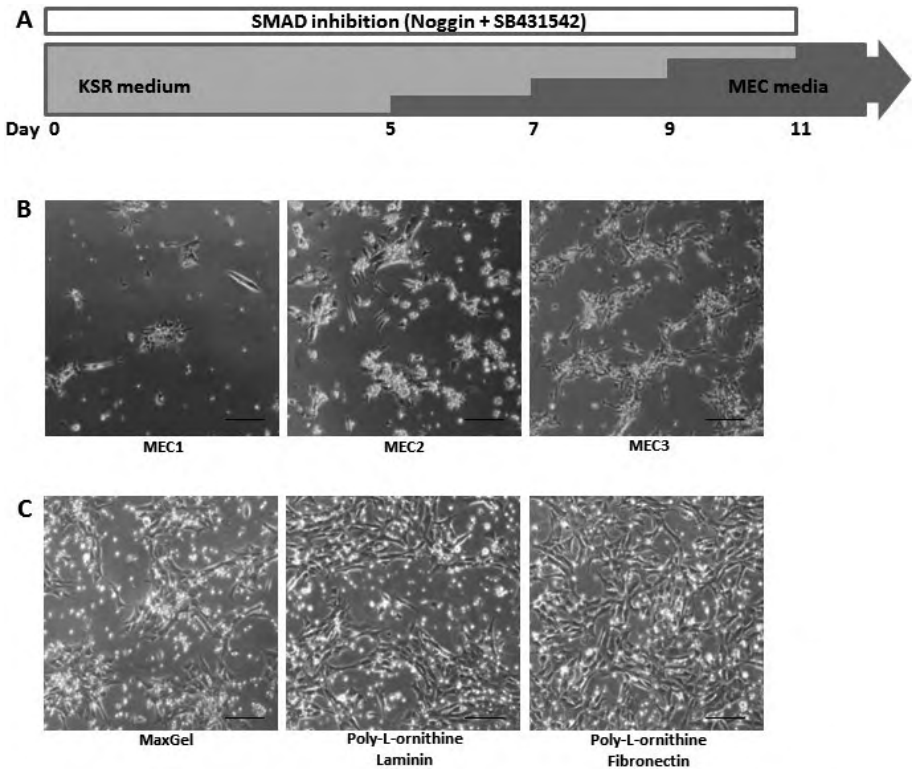

Fig. 1. Schematic depicting the key elements of differentiation of hESCs towards neural crest phenotype (**A**), and morphology of putative NCCs growing in different media (**B**) and on different culture substrates (**C**), as determined by light microscopy. All experiments shown in panels B and C were performed using an unsorted population of cells. Scale bars in panels B and C represent 50 mm.

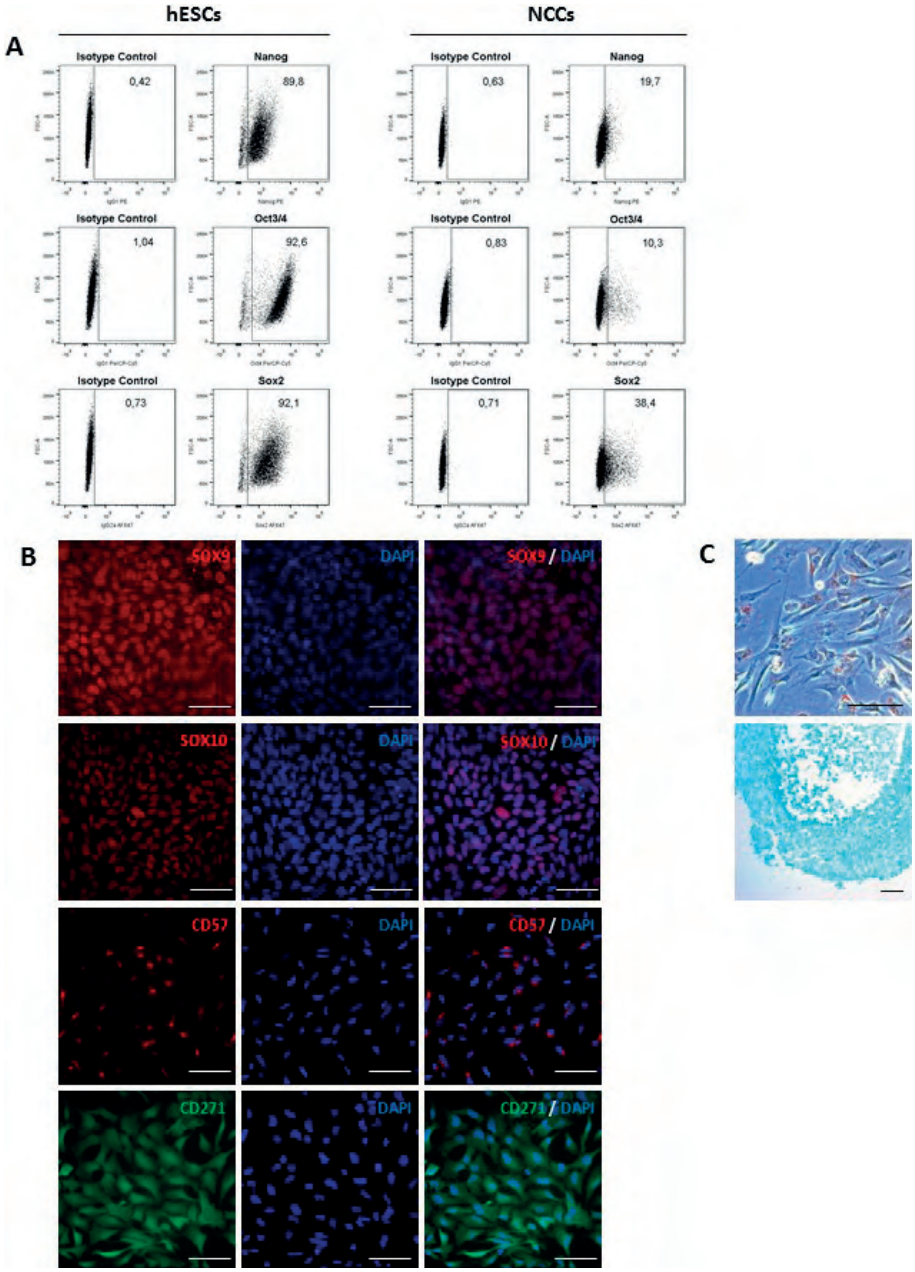

Fig. 2. Phenotype of putative NCCs as determined by flow cytometric analysis of the expression of markers of undifferentiated state – Nanog, Oct3/4, and Sox2 (**A**), by visualization of molecular markers of neural crest lineage, Sox9, Sox10, CD57, and CD271, using immunofluorescence (**B**), and by capability to differentiate into adipogenic lineage (**C** – top picture) and chondrogenic lineage (**C** – bottom picture). All experiments shown in panels A, B, and C were performed using an unsorted population of cells. Scale bars in panels B and C represent 50 μm.

convenient coating (Fig. 1C). Taken together, cells propagated on poly-L-ornithine/fibronectin-coated dishes in MEC3 medium were used throughout the rest of this study.

As stated, the conditions described above robustly supported growth of differentiated cells – putative NCCs. At this quality point, we finally decided for more complex characterization of the cell phenotype. Specifically, we: (i) applied flow cytometry to evaluate the expression of stemness-associated transcription factors – Nanog, Oct3/4, and Sox2; (ii) employed indirect immunofluorescence to determine the presence of proteins typically linked to NCCs; and (iii) functionally assayed the differentiation capacity. As shown in Fig. 2A, the percentage of Nanog-, Oct3/4-, and Sox2-positive cells in the differentiated cell population was only 19.7, 10.3, and 38.4 %, respectively, compared to 89.9, 92.7, and 92.1 % found in non-differentiated hESCs. As typical of NCCs, the cells differentiated and propagated here were positive for Sox9, Sox10, CD57, and CD271, with all the analysed proteins having proper localization to the nuclei (Sox9, Sox10), cell surface (CD57) and Golgi (CD271), respectively (Fig. 2B). Finally, *in vitro* differentiation of our putative NCCs had proved their capaci-

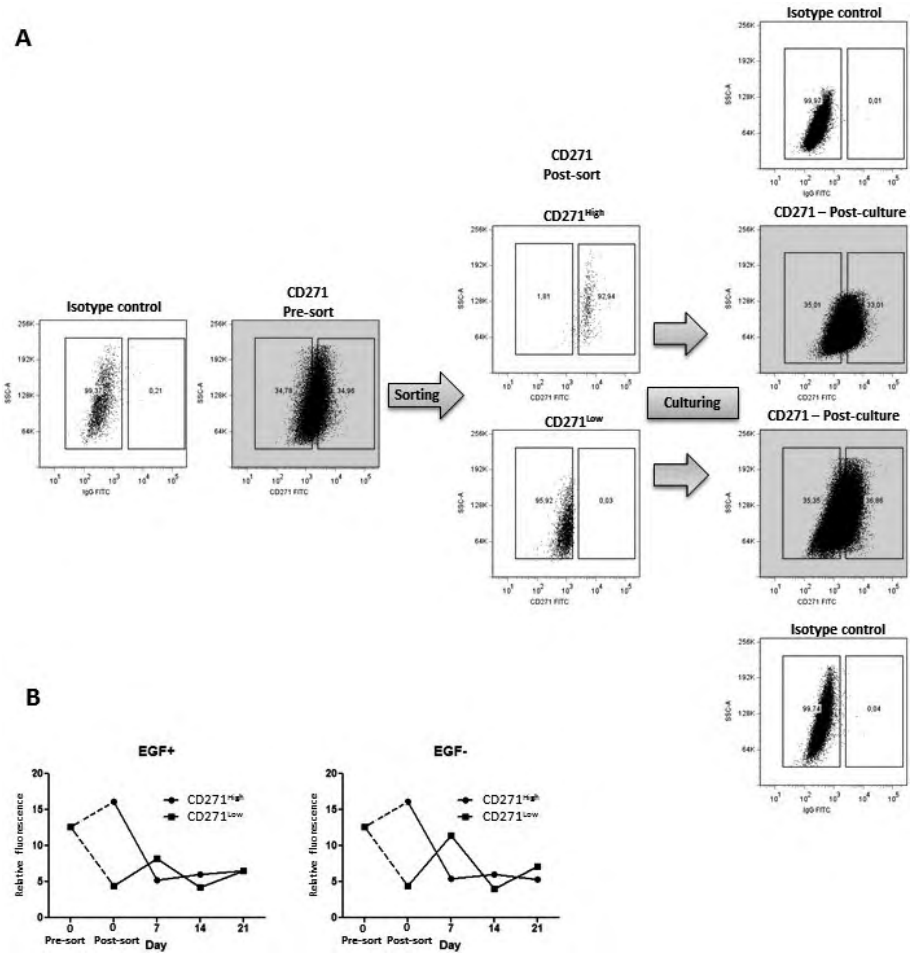

Fig. 3. Stability of expression of CD271 in the population of putative NCCs as determined by flow cytometry combined with cell sorting. Panel **A**: Both CD271^{High} and CD271^{Low} populations (Post-sort) develop the original spectrum of CD271 expression (Pre-sort) after 10-day culture (Post-culture). Panel **B**: The presence (left hand graph) or absence (right hand graph) of EGF does not influence re-establishment of the original spectrum of CD271 expression in both CD271^{High} and CD271^{Low} cell populations. The graph represents folds of fluorescence intensities of isotype controls.

ty to give rise to cells with biochemical signs of adipocytes and chondrocytes (Fig. 2C).

Although morphologically the cells appeared rather as a homogenous population, the immunofluorescence visualization of molecular markers shown in Fig. 2B still indicated a certain degree of heterogeneity. To address this issue more quantitatively, we had chosen the CD271 cell surface molecule that in normal development distinguishes neural progenitors from the cells of neural crest lineage and analysed its expression by flow cytometry. Repeated flow cytometric analyses showed that the population of cultured cells was invariably composed of cells differing for about two orders of magnitude in the level of expression of CD271 (data not shown and Fig. 3A). Sorting for CD271 was previously used to enrich cell populations for the cells of the neural crest lineage (Lee et al., 2010), so we decided to also employ this approach. We divided the original unsorted population of cells into two groups according to the level of expression of CD271, which we further refer to as CD271^{low} and CD271^{high} cells (Fig. 3A; post-sorting). Both CD271^{low} and CD271^{high} groups of cells were then propagated separately in culture under the same conditions as above and the expression of CD271 was determined again for both groups on day 10 after sorting. Unexpectedly, flow cytometric analysis unravelled establishment of the whole spectrum of intensities of CD271 in both groups (CD271^{low} and CD271^{high}) that was indistinguishable from that of the original unsorted cell population (Fig. 3A; post-culture). In other words, the group of cells having low expression of CD271 at the beginning (after sorting) gave rise to a high proportion of cells with high expression of CD271 and vice versa. It is of note that this phenomenon occurred under unchanged culture conditions without any external stimulus.

Media for propagation of cells of neuroectodermal origin typically contain epidermal growth factor (EGF), and it was also the case for the media used here with the concentration of EGF being as high as 20 ng/ml. We were curious whether or not EGF may be the key driving force that underlies development of CD271-positive cells inside the population of cells that do not express this NCC-associated molecule. Therefore, we cultured

the CD271^{low} and CD271^{high} groups of cells in media with and without EGF and monitored the level of expression of CD271 by flow cytometry for three weeks in weekly intervals. As shown in Fig. 3B, the CD271 heterogeneity became re-established, in only one week, in all four groups irrespectively of the presence or absence of EGF in culture media.

Corresponding to the above-described behaviour of the population of our putative NCCs, it needs to be said that we have also experienced their noticeable long-term phenotypic stability. We build this understanding on repeated flow cytometric evaluation of the expression of CD271 and also CD57 in the NCCs, which were not only maintained in culture for a minimum of 50 passages, but which also went through freezing/thawing steps (data not shown).

As mentioned above, NCCs are precursors of many highly specific cell types, including cells of odontogenic lineage. Here we used the odontogenic potential as another measure to assess the differentiation potential of our putative NCCs. In normal development NCCs give rise to the mesenchyme which, via intricate interactions with odontogenic epithelium, ultimately produces odontoblasts. Among the signalling axes involved in odontogenesis, BMP4-MSX1 is one of the most critical (Bei and Maas, 1998). Therefore, here we evaluated the capacity of our putative NCCs to up-regulate *MSX1* upon the BMP4 signal. The unsorted cells were cultured for 14 days in three different media, (i) complete MEC3 medium, (ii) MEC3 medium without FGF-2 and EGF, and (iii) MEC3 medium without FGF-2 and EGF supplemented with 50 ng/ml of BMP4, and the expression of *MSX1* was determined by quantitative PCR in two-day intervals. As expected, the level of *MSX1* message increased dramatically in the cells treated by BMP4, reaching about 160-fold its original level on day 14, but it changed only very little in the two other groups (only about 5-fold increase in complete MEC3 medium and 12-fold increase in MEC3 medium without FGF-2 and EGF) (Fig. 4).

Discussion

Recently, neural crest-like cells have been obtained in several laboratories by *in vitro* differentiation of human pluripotent stem cells (Pomp et al., 2005; Lee et al., 2007; Brokhman et al., 2008; Pomp et al., 2008; Chambers et al., 2009; Jiang et al., 2009; Curchoe et al., 2010; Cimadamore et al., 2011; Kreitzer et al., 2013; Menendez et al., 2013; Zeltner et al., 2014) with the aim to provide an *in vitro* growing population of cells that would help to unravel the exact principles of neural crest development and to understand the aetiology of neurocristopathies, and possibly become useful in regenerative medicine. In principle, the strategies that have been used to achieve the NCC phenotype included co-culture of adhered SCs with other (inductive) cell types, formation of neurospheres from SCs, and inhibition of SMAD signalling pathways in SCs with optional cell sorting by flow cytometry. Here we built on the recent protocol developed by the lab of Studer (Lee et al., 2010), with the aim to evaluate the effects of modifications introduced to the media formulation and to the culture substratum on NCC behaviour.

The key specifics of the media tested here were inspired by the previously published experiments combined with our experiences with culturing other cell lineages originating from the neural tube. Specifically, the MEC1 medium is adopted from the original work by Lee and colleagues (Lee et al., 2010) with the only difference made by us being an addition of antibiotics. Medium MEC2 is a formulation introduced in 2012 by the group of Studer (unpublished), which is based on Neurobasal medium and N2/B27 supplements contained at recommended concentrations. Finally, the MEC3 medium represents modification of the MEC2 medium designed in our lab by adding nonessential amino acids and 20 mM β-mercaptoethanol, and by increasing the concentrations of FGF-2 and EGF two-fold (to 20 ng/ml for both), since others have observed good functionality of cultured NCCs with such higher concentrations of the given growth factors (Jiang et al., 2009; Curchoe et al., 2010; Ramogolam et al., 2011). In concordance with our assumption, the richest medium, MEC3, performed the best for the putative NCCs produced here by the double SMAD inhibition approach. Although we now cannot provide information on whether or not all the extra components contribute to such better performance, we can still identify the tetrad of factors that deserve further optimization. Similarly, a still unresolved question is the

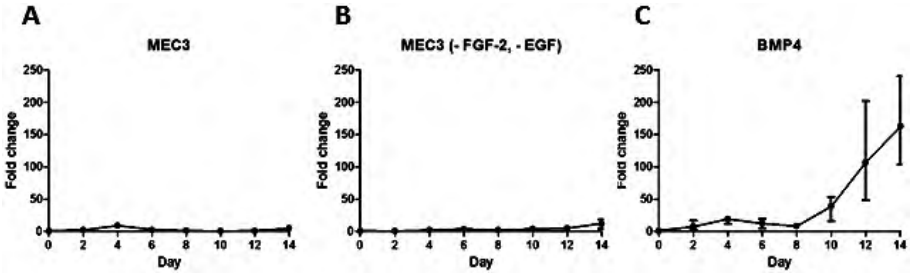

Fig. 4. Expression of *MSX1* in putative NCCs upon their exposure to BMP4 as determined by quantitative PCR. Panels **A**, **B**, and **C** represent NCCs cultured up to 14 days under three different types of conditions, in complete MEC3 medium, in MEC3 medium without FGF-2 and EGF, and in MEC3 medium without FGF-2 and EGF supplemented with 50 ng/ml of BMP4, respectively. This experiment was performed using an unsorted population of cells.

substratum that is optimal for obtaining and maintenance of the NCC phenotype. Indeed, various coatings, such as Matrigel, laminin alone, fibronectin alone, and/or combination of poly-L-ornithine/fibronectin/laminin were used for this purpose (Curchoe et al., 2010; Lee et al., 2010; Dupin and Coelho-Agular, 2013; Menedez et al., 2013). Here we evaluated three different coatings that we previously found as performing well for some other differentiated cell types. Of those three tested, a simple combination of poly-L-ornithine with fibronectin provided sufficiently solid support to our putative NCCs, making the culture conditions defined as well as economic.

In normal development, neural crest cells represent a transitional population of migratory cells, which differentiate into various cell types in their final destinations. With such properties, vertebrate NCCs indeed tend to be phenotypically non-homogenous when they are explanted and placed into culture (Sieber-Blum and Cohen, 1980; Pfaltzgraff et al., 2012). Our study further supports this notion by showing two facts that were typical of putative NCCs obtained and propagated here. First, although we did not quantify this phenomenon, there was observable (by immunofluorescence) variability among individual cells in the expression of several markers (Sox9, Sox10, CD57, and CD271). Second and more importantly, there was a strong tendency of the cells to re-establish and maintain a gradient in the expression of CD271, as we have repeatedly proved by experiments involving cell sorting for this molecule. It is of note that this phenomenon does not require external signals from EGF. Indeed, it is not unexpected that cultured cells maintain a certain population hierarchy due to the signal gradients established in the "dish", but it is important to appreciate this fact and to unravel its future functional consequences, primarily in terms of the differentiation propensity.

It has been shown by others that neural crest-like cells obtained by differentiation of human pluripotent SCs are capable of differentiation into various relevant cell types, such as neurons, chondrocytes, adipocytes, osteoblasts, melanocytes, and Schwann cells (reviewed in Crane and Trainor, 2006; Le Douarin et al., 2008). One of the motivations for us to work out methodologies for differentiation and propagation of NCCs is to establish the cellular grounds for bioengineering components of dentition. Therefore, even at this initial stage we have included into our study evaluation of the odontogenic potential of our putative NCCs, above only showing the "routine" capacity to produce cells of adipogenic and chondrogenic lineages. Thus, here we demonstrate for the first time that NCCs can be stimulated by an external factor, specifically by BMP4, to activate expression of the gene (*MSX1*) that is typical of odontogenic mesenchyme. We envision this fact as a highly specific measure of the differentiation propensity of the cells produced here, which sets grounds for more complex *in vitro* and *in vivo* studies addressing odontogenesis from NCCs.

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