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Human- and mouse-derived neurons can be simultaneously obtained by co-cultures of human oral mucosal stem cells and mouse neural stem cells

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Objective: To observe simultaneous differentiation and analyse possible interactions between co-cultured human oral mucosal stem cells (hOMSC) and mouse neural stem cells (mNSC).

Materials and Methods: hOMSC and mNSC were co-cultured in mouse and in human medium, and their immunocytochemical characterization to detect survival rate and differentiation pattern was performed. Co-cultures in different media were compared to hOMSC in human medium and mNSC in mouse medium as controls.

Results: Co-culture of hOMSC and mNSC in medium for human cells led to normal differentiation pattern of human cells, while mNSC were directed towards astrocytes. When the same cells were cultivated in the mouse medium, both cell types succeeded to form neurons, although mNSC showed a tendency to overgrow hOMSC. hOMSC alone in the human-specific medium differentiated towards ectodermal (Oct4, Map2) and mesodermal (Osterix) cell populations. mNSC in the mouse-specific medium differentiated towards Map2-, β3-tubulin- and NeuN-positive neurons.

Conclusions: hOMSC and mNSC can form co-cultures. Different media considerably affected the differentiation pattern of co-cultures, whereas one cell population itself modestly influenced differentiation of the other cell type. The in vitro differentiation pattern of hOMSC in the mouse neural tissue environment suggested that hOMSC could be beneficial in the brain tissue affected by ischaemia.

KEYWORDS
differentiation, mouse neural stem cells, neural crest stem cells, neuroectoderm, oral mucosa

1 | INTRODUCTION

Neurological diseases bring a huge burden for humankind. Due to the lack of easy access to the central nervous system and its inherent scarce capacity for regeneration, options for treatment of neurological diseases are limited. Therapeutic approaches based on stem cells (SC) bring promise that preclinical achievements observed in the last decades could evolve into the new treatment modalities for neurological diseases (Bohl et al., 2016; Mitrecić, Nicaise, Gajović, & Pochet, 2010; Mitrecic et al., 2012). Mouse neural stem cells (mNSC) are multipotent cell population, which can differentiate in neurons and astrocytes. Intravenously administered rodent NSC successfully migrated and differentiated in the rat model of amyotrophic lateral sclerosis (Mitrecić, Gajović, & Pochet, 2009), and also, when transplanted in the brain of a murine ischaemic stroke model, mNSC successfully differentiated into mature neurons (Alić et al., 2016; Mitrečić, Alić, &

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Gorup, 2017; Nicaise, Mitrecic, Falnikar, & Lepore, 2015). Although stem cells have been proven as very useful in preclinical trials, applications in human patients require obtaining cells from easy accessible sources within patients. Obtaining of human oral mucosa stem cells (hOMSC) does not result in irreparable tissue damage, which makes oral mucosa an excellent source of any cells (Mitrečić, 2011; Mitrečić, Mavrić, & Gajović, 2008), including SC, compared to any other oral tissues (Arthur et al., 2008; Young, Sloan, & Song, 2013). Moreover, the excellent potential of hOMSC has been shown by the fact that they can differentiate towards ectodermal, mesodermal and endodermal cell lineages (Marynka-Kalmani et al., 2010). hOMSC transplanted into various neural damage mouse models resulted in clinical improvement and possible regeneration of the neural tissue (Cho et al., 2014; Ganz, Arie, Buch, et al.s, 2014). As hOMSC are very promising candidates for transplantation in the mouse model of brain ischaemia, in which regenerative process includes reactivation of neurogenesis, the main aim of this study was to perform the analyses by observing interactions between hOMSC and mNSC cell populations in vitro. For this purpose, we performed their co-cultivation in various combinations of media and here we describe the success rate of their survival and differentiation.

2 | MATERIALS AND METHODS

2.1 | Donors

hOMSC were isolated from buccal punch biopsies of six healthy 20- to 44-year-old volunteers who signed informed consent. The study and hOMSC sampling were in accordance with Declaration of Helsinki and approved by the Independent Ethical Committee of the University of Zagreb School of Dental Medicine.

2.2 | Animal cells

In this study, a GFP mouse strain, kept on an albino C57Bl6 background, has been used. The animals were kept in the animal facility of the Croatian Institute for Brain Research. All experiments on animals were approved by the Internal Review Board of the Ethical Committee of the School of Medicine University of Zagreb (04-77/2010-238) and Faculty of Veterinary Medicine (251/61-01/139-13-4), and they were carried out in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes.

2.3 | Cell culture

Buccal hOMSC were isolated as described previously (Liu et al., 1997; Marynka-Kalmani et al., 2010) and expanded in low-glucose DMEM medium (Gibco) with 10% foetal calf serum (FCS). After proliferation (2–3 passages), cells were plated on fibronectin (Gibco)-coated coverslips and differentiated towards ectodermal and mesodermal cell population. For ectodermal differentiation, cells were cultured in DMEM medium supplemented with 10 ng/ml β-NGF, 50 ng/ml BDNF, N2-supplement and antibiotics, all provided by Gibco, while, for the mesodermal differentiation, cells were cultured in αMEM medium (Gibco) supplemented with 12% FCS, 50 μg/ml vitamin C (Sigma), 10^{-7} M dexamethasone and 10 mM glycerol-2-phosphate. Cells were fixed on days 1, 3, 5, 7 and 14 of differentiation, and immunocytochemistry was performed.

2.4 | Co-culture of hOMSC and mouse NSC

Mouse neural stem cells were isolated and proliferated as described previously (Alić et al., 2016; Kapuralin et al., 2015). For this purpose, we cultured cells in four different condition: (i) hOMSC in human-specific DMEM medium supplemented with ectodermal factors, (ii) hOMSC + mNSC in the same medium, (iii) hOMSC + mNSC in mouse-specific DMEM/F12 medium supplemented with ectodermal factors and (iv) mNSC in mouse DMEM/F12 medium.

2.5 | Live/dead assay

A live/dead cell viability assay (Thermo Fisher Scientific) was performed as a single experiment (thus limiting significance of this result) to show percentage of live and dead cells in the cell cultures.

2.6 | Immunocytochemistry

Immunocytochemistry was performed as described previously (Kapuralin et al., 2015; Kosi et al., 2015). Primary and secondary antibodies used in this study are shown in Table S1. Fluorescence analysis was made with a Zeiss LSM510 Meta confocal microscope.

3 | RESULTS AND DISCUSSION

3.1 | Differentiation of hOMSC

After isolation of cells according to the original protocol (Marynka-Kalmani et al., 2010), we analysed cellular fate by markers of differentiation (multipotency—Sox2, Mash1, Oct4, Nestin; ectodermal fate—Map2, β3-tubulin, NeuN; and mesodermal—Osterix, Collagen 1 and CD 44) (see, e.g. Figure 1). In addition, cell viability was measured using live/dead assay (Thermo Fisher Scientific). On the first day of differentiation, we detected 90% live and 10% dead cells. On the seventh day of differentiation, the total number of dead cells reached 27% (Figure 2d). This is consistent with data published in our previous publications (Nicaise, Mitrecic, & Pochet, 2011).

When differentiated in the medium that promotes ectodermal derivatives, hOMSC expressed Nestin, Oct4 and Sox2. Interestingly, Nestin and Sox2 were positive during the whole studied period (14 days). Cells expressed Nestin in the cytoplasm, especially in the perinuclear part of cells (Figure 1a). As we observed in our previous studies using mouse cells, Sox2 was well expressed in cytoplasm of hOMSC (Figure 1a and c). Mash1 and Oct4 were also present in cytoplasm, and they co-localized during the first week of differentiation. This might suggest that hOMSC retain their multipotent potential much longer than mNSC, which lose the presence of these markers

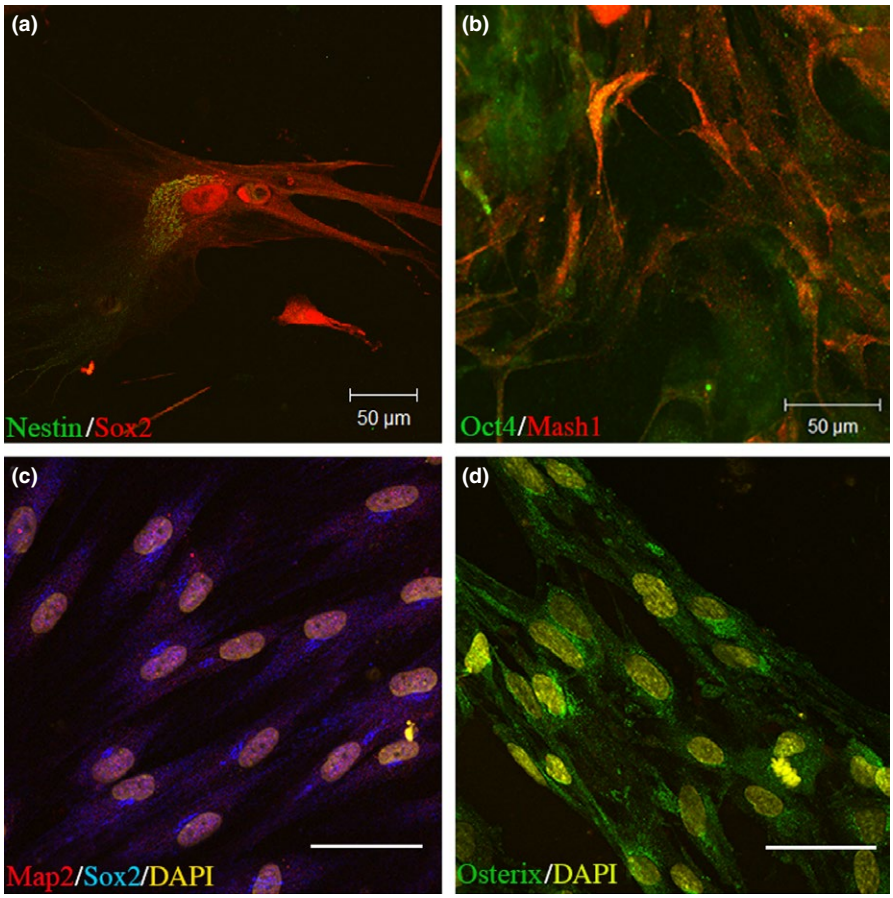

FIGURE 1 *In vitro* differentiation of hOMSC towards ectodermal (a, b and c) and mesodermal lineage (d). On the 7th day (a, b) of differentiation, hOMSC express cytoplasmic embryonic stem cells markers (Nestin, Oct4 and Mash1) and nuclear marker (Sox2). On the 14th day, most of ectodermal cells express Map2 and retain Sox2 expression (c). Mesodermal cells highly express Osterix on the 14th day of differentiation (d). Scale bar 50 μm

already after a few days. Another possible explanation could be that mouse cells, which originated from embryonic tissue, are highly predestined for complete differentiation. The human cell population used in this study is a specific one, and it represents a small isolated population that keeps its multipotent niche throughout the life of an adult person. With maturation, these cells express Map2 and β3-tubulin markers. After the 7th day of differentiation, around 90% of these cells express Map2 (Figure 1c).

When we cultivated cells in the medium that supports a mesodermal direction of differentiation, more than 90% of cells highly expressed Osterix after the 7th day of differentiation (Figure 1d). Osterix is one of the crucial transcription factors needed for the development and differentiation of osteoblasts, one of the major cellular derivatives of mesoderm. The same cells were collagen 1 and CD 44 negative (not shown). This might be linked to the time of cultivation being limited to 2 weeks in this experiment. Some publications suggest that onset of collagen 1 production requires longer incubation times.

3.2 | Co-culture of hOMSC and mNSC

To analyse survival and differentiation of hOMSC in different conditions, we have grown hOMSC in co-culture with mNSC in the media primarily designed for either human or mouse stem cells. The ratio of live and dead cells in co-culture in both tested media (human and mouse) was 70%–85% live cells and 15%–30% dead cells during the seventh day of differentiation. While the mNSC in the mouse-specific medium survived similarly to hOMSC in human medium (Figure 2d), hOMSC appeared as small round cells (Figure 2a) and they changed morphology soon after plating on coverslips in differentiation medium (Figure 2b). In the co-culture, we easily distinguished hOMSC and mNSC (Figure 2c). After observing their separate behaviour, we co-cultured and differentiated them towards ectodermal cell populations. To distinguish mouse and human cells, we used a mouse strain, which expresses GFP (green fluorescent protein) in all cells. When we co-cultured hOMSC and mNSC in the medium suitable for human cells, more than 70% of both cell types survived and differentiated. hOMSC showed the same differentiation pattern as we described above (Figure 2e). Interestingly, in human-specific medium, more than 90% of mNSC expressed GFAP (glial fibrillary acid protein), while the only few cells were Map2, β3-tubulin and NeuN positive. This massive induction of GFAP-positive astrocytes was probably caused by FCS present in human medium. Moreover, those GFAP-positive cells were Nestin positive during all studied periods, which suggests that human-specific medium caused a delay in differentiation of mNSC. On the other hand, survival of co-cultured hOMSC and mNSC in the mouse-specific medium was the same, but we noted that in the mouse-specific medium, more hOMSC died while the mNSC survived better (Figure 2f). In the mouse-specific medium, as we

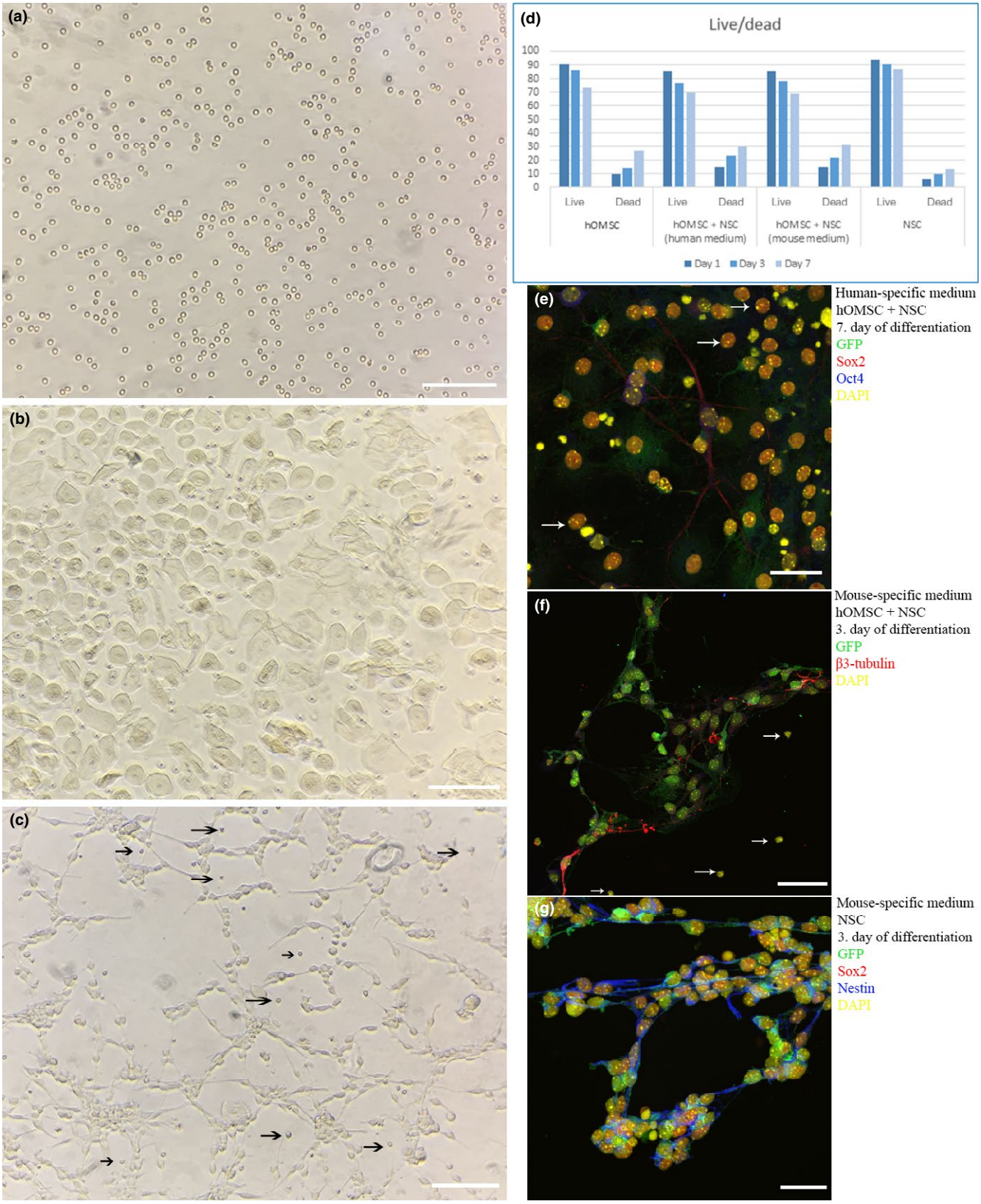

FIGURE 2 hOMSC on the day 0 in proliferation medium (a), hOMSC on the day 2 of differentiation in human-specific medium (b) and co-culture hOMSC and NSC on day 1 of differentiation in mouse-specific medium (c). Analysis of survival during differentiation period (d). Fluorescence analysis of hOMSC and mNSC in different conditions: co-culture in human-specific medium on 7th day of differentiation shows Sox2 expression, intranuclear and cytoplasmic in both hOMSC and mNSC, while the Oct 4 expression is very weak at this time point; GFP was expressed only in mouse cells (e). Co-culture in mouse-specific medium shows neuronal differentiation of mNSC stained with β3-tubulin, while the hOMSC were small and round on the 3rd day of differentiation (f). Differentiation of mNSC in mouse-specific medium on the 3rd day in culture (g). Scale bar 200 μm (a, b and c) and 100 μm (e, f, and g). Arrows show hOMSC in co-culture

usually differentiate mNSC (Figure 2g), most of cells were neurons and they overgrew hOMSC, making them sometimes very hard to find between mature and branched neurons. In the mouse-specific medium, only 3% of cells expressed GFAP, which has been already reported in our own publications (Alić et al., 2016).

There is an increasing interest in utilizing SC derived from the oral region tissues in various models of neurological diseases. Most of published work relates to dental pulp stem cells (DPSC) (Arthur et al., 2008; Young et al., 2013). However, mature functional neurons could not be differentiated from DPSC (Aanismaa et al., 2012). Dental pulp was considered as a convenient source of SC, yet in order to harvest SC from the pulp, tooth vitality is irreparably damaged or the tooth needs to be extracted. The regenerative ability of nasal mucosa stem cells has been recently well proven: in a parkinsonian rat model (Müller et al., 2015), in an hemi-parkinsonian rat model (Ganz, Arie, Buch, et al.s, 2014) and in a spinal cord injury model (Cho et al., 2014). Moreover, hOMSC in an experimental rat model of sciatic nerve injury revealed a neuroprotective effect, improving the motor function following transplantation (Ganz, Arie, Ben-Zur, et al., 2014). The major idea of the current work was to test co-cultivation of hOMSC and mNSC. As we plan to use hOMSC in experiments in which those cells will be transplanted into the mouse model of brain ischaemia (MCAO), here we analysed processes occurring during in vitro cultivation of hOMSC and mNSC. In our hands, this was proven as a useful in vitro model of neurogenesis following ischaemic brain stroke. Here, we showed that co-culturing of hOMSC and mNSC yielded better results in the mouse medium. In this condition, mNSC grow and differentiate normally, while hOMSC exhibit similar parameters as when grown alone in their own medium. The most obvious difference was in the rate of expansion of hOMSC, which was slower in the mouse-specific medium.

The results of this study suggest that hOMSC exhibit a rather high level of robustness and that their transplantation into the mouse brain affected by ischaemia could be a feasible step forward towards testing the potential of this population for future clinical trials and application in treatment of human stroke.

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CONFLICT OF INTERESTS

None to declare.

AUTHOR CONTRIBUTION

Ivan Alajbeg initiated the idea, co-developed research concept, performed collection of tissue samples, participated in work with cultures, drafted the article and approved the submitted version of manuscript. Ivan Alić participated in experiment design, collection of tissue samples, has lead the work with cultures, provided mouse neural stem cells and co-culture formation, labelling and analysis, drafted the article and approved the submitted version of manuscript. Ana Andabak Rogulj participated in tissue sampling, substantially participated in work with cultures, labelling and analysis, and approved the submitted version. Vlaho Brailo substantially participated in tissue sampling and approved the submitted version of manuscript. Dinko Mitrečić devised the idea of co-culturing human stem cells with mouse neural stem cells, elaborated and performed isolation of human oral mucosal cells and designed consecutive experiments, advised in interpretation of results, performed substantial critical revision of the article and approved the submitted version of manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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