Neurogenesis similarities in different human adult stem cells

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Human neurological disorders and spinal cord injuries are caused by a loss of neurons and glial cells in the brain or spinal cord. The proof-of-principle of cell therapy is that replacing damaged cells with new healthy ones will restore the lost function. Isolating and manipulating autologous neural stem cells (NSCs) could provide an ideal source of cells for use in cell replacement and the transfer of genes to a diseased central nervous system. NSCs are immature cells present, not only during embryonic development, but also in the adult brain of all mammalian species, including humans. The presence of NSCs in the adult mammalian brain has been described in two neurogenic niches, the ventricular-subventricular zone (V-SVZ) of the anterolateral ventricle wall and the subgranular zone of the hippocampal dentate gyrus. Typically, NSCs are defined as self-renewing multipotent cells that can generate neurons, astrocytes and oligodendrocytes. The major barrier to isolating adult NSCs in humans is the inaccessibility of living tissue. For this reason, an enormous effort has been made to derive neurons from human adult stem cells isolated from various tissue sources.

During the last two decades, it has been reported that human mesenchymal stem cells present in various tissues, including bone marrow, can be induced to overcome their mesenchymal fate and differentiate into neural cells (Munoz-Elias et al., 2003; Jeong et al., 2004; Krabbe, 2005; Azedi et al., 2017; Radhakrishnan et al., 2019), a phenomenon known as transdifferentiation (Krabbe, 2005; Tata and Rajagopal, 2016). However with the accumulation of studies emphasizing the neural transdifferentiation of human bone marrow-derived stem cells (hBMSCs), the level of criticism towards these observations has also grown, suggesting that the change in cell morphology observed in hBMSCs cultures during neural transdifferentiation could be an artefact rather than a differentiation trail (Bernani et al., 2005; Krabbe et al., 2005).

The purpose of this study is to find neurogenesis similarities in distinct cell types isolated from adult tissues, including tissue-specific human stem cells, NSCs in the adult mammalian brain and primary neuronal cultures derived from rodent brain.

In our laboratory we focus on differentiating human periodontal ligament stem cells (hPDLSCs) to generate a neuronal lineage. In a previous publication (Bueno et al., 2013), we showed that hPDLSCs differentiate *in vitro* into neuron-like cells, based on cellular morphology and neural marker expression. *In vivo*, hPDLSCs-derived neuron like cells integrate and differentiate after being grafted to the adult mouse brain. Furthermore, some hPDLSCs-derived neuron-like cells located in NSC niches, such as the V-SVZ of the anterolateral ventricle wall and the subgranular zone of the hippocampal dentate gyrus, display neural stem morphology. Moreover, other research groups have reported that hPDLSCs-derived neuron-like cells express ion channel receptors and display inward currents and spontaneous electrical activities after neurogenic differentiation (Fortino et al., 2014). Therefore, the neural differentiation potential both *in vitro* and *in vivo*, make hPDLSCs interesting for investigating stem cell to neuron differentiation mechanisms.

In a recent study (Bueno et al., 2019), we showed that when hPDLSCs are exposed to the neural induction medium, they rapidly undergo a dramatic change in shape and size; they first adopt highly irregular forms and then gradually contract into round cells. Subsequently, these rounded cells gradually adopt a complex morphology, acquiring dendrite-like and axon-like identities, giving rise to a variety of neuron-like morphologies. We observed that cell proliferation is not present during neurogenesis from hPDLSCs. In fact, the cell shape of hPDLSCs is reset and they start their neuronal development as round spheres. Morphological analysis also revealed that nuclear remodeling occur when hPDLSCs become near-spherical in shape. Small DNA-containing structures arise from the main nuclei and start to move towards specific positions in the cell, temporarily forming lobed nuclei. Interestingly, we also observed that adult rodent neurogenic niches contain cells with nuclear shapes that are very similar to those observed when hPDLSCs become near-spherical. Importantly, if we compares the morphological changes observed in hPDLSC cultures during neural induction (Bueno et al., 2019) with previous reports (Munoz-Elias et al., 2003; Jeong et al., 2004; Bertani et al., 2005; Krabbe et al., 2005; Wittmann et al., 2009; Azedi et al., 2017; Cebrián-Silla et al., 2017; Radhakrishnan et al., 2019), several similarities are apparent.

Firstly, we observed that hPDLSCs-derived neural-like cells produce neurites that show growth cone formations at their tip, with a similar spatial organisation as that described in neurons. The central domain of the growth cone contains β-III tubulin microtubules and the peripheral domain comprises radial F-actin bundles (Bueno et al., 2019).

Secondly, hPDLSCs-derived neural-like cells develop well-differentiated dendrite-like and axon-like domains. We also observed that hPDLSCs-derived neural-like cells produce micron-sized membrane protrusions with F-actin compartments that extend outwards from the β-III tubulin dendritic shafts, making these resembles the typical dendritic spine structures described in neurons. In addition, hPDLSCs-derived neural-like cells also display different types of axonal branch-like structures that are very similar to those described in neurons (Bueno et al., 2019).

Thirdly, hPDLSCs-derived neural-like cells establish their polarity in a very similar way to that previously reported for cultured rodent neurons during neurogenesis (Bueno et al., 2019).

Fourthly, the nuclear morphology of hPDLSCs observed during the initial phases of neurogenesis *in vitro* bears great similarities to the nuclear morphology of V-SVZ NSCs in the human foetal brain, as well as the adult mouse brain (Cebrián-Silla et al., 2017), and is also very similar to the nuclear morphology of many cultured hippocampal neurons (Wittmann et al., 2009).

Lastly, previous reports also showed that when mesenchymal stem cells isolated from various sources in the human body (including bone marrow-derived stem cells, menstrual blood-derived stem cells, umbilical cord-derived stem cells and adipose-derived stem cells), are exposed to the neural induction medium, they also rapidly undergo a dramatic change in shape and size, gradually contracting into round cells with small phase-bright cell bodies (Munoz-Elias et al., 2003; Jeong et al., 2004; Azedi et al., 2017; Radhakrishnan et al., 2019).

To test whether nuclear remodelling also occurs when hBMSCs become near-spherical in shape, we differentiated hBMSCs with the same neural induction medium we used for hPDLSCs. As illustrated in **Figure 1**, nuclear remodelling also occurs when hBMSCs become near-spherical. Although we acknowledge that the definitive nuclear remodelling sequence, when hBMSCs become near-spherical, can only be determined through time-lapse microscopy, we are able to suggests how these steps may occur. Small DNA-containing structures arise from the main nuclei (**Figure 1A**) and start to move towards specific positions within the cell (**Figure 1B**), temporarily forming lobed nuclei (**Figure 1C**). Next, these lobed nuclei connect to one another, forming nucleoplasmic bridges (**Figure 1D**). Finally, these lobed nuclei connected by an internuclear bridge (**Figure 1E**) merge into a single nucleus with an eccentric position within the hBMSCs (**Figure 1F**).

It is important to mention that the nuclear morphology of the hBMSCs observed during neural differentiation is very similar to the nuclear morphology of the hPDLSCs during the initial phases of neurogenesis (Bueno et al., 2019). Therefore, these preliminary findings may indicate that hBMSCs and hPDLSCs have similar morphological neural development sequences *in vitro*. As noted adove, it is important to mention that some authors suggest that the change in cell morphology observed in hBMSCs cultures could be an artefact rather than a differentiation trail (Bertani et al., 2005). However, more recent research has demonstrated successful hBMSCs neuronal differentiation corroborated by multiple techniques both *in vitro* and *in vivo*. In addition, we and others have observed that when adult stem cells isolated from different sources in the human body are exposed to various neural induction protocols, they also rapidly undergo a dramatic change in shape and size, gradually contracting into round cells with small phase-bright cell bodies (Munoz-Elias et al., 2003; Jeong et al., 2004; Krabbe et al., 2005; Bueno et al., 2013, 2019; Azedi et al., 2017; Radhakrishnan et al., 2019). It would, therefore, be interesting to examine whether mesenchymal-like stem cells and neural crest-derived stem cells isolated from various sources in the human body also display similar morphological neural development sequences to those observed in hPDLSCs.

As noted above, the nuclear morphology of hPDLSCs and hBMSCs observed during the initial phases of neurogenesis *in vitro* are very similar to the nuclear morphology of V-SVZ NSCs in the human foetal brain as well as in the adult mouse brain (Cebrián-Silla et al., 2017); their nuclear morphology is also very similar to that of many cultured hippocampal neurons (Wittmann et al., 2009). Although it has been suggested that these nuclear morphologies are associated with quiescence in adult NSCs (Cebrián-Silla et al., 2017), we have observed that this kind of nuclei may be associated with nuclear movement within the cell during the initial phases of neurogenesis, without being related to cell proliferation. These findings may call into question whether adult neurogenesis always occurs progressively, through sequential phases of division, differentiation, and elimination. It is important to note that recent findings indicate that some observations interpreted as cell division could be false-positive signals (Breunig et al., 2007). In addition, almost no reports describing newborn neurons in the adult brain show mitotic chromosomes or mitotic spindles, which would confirm that adult neurogenesis occurs progressively through

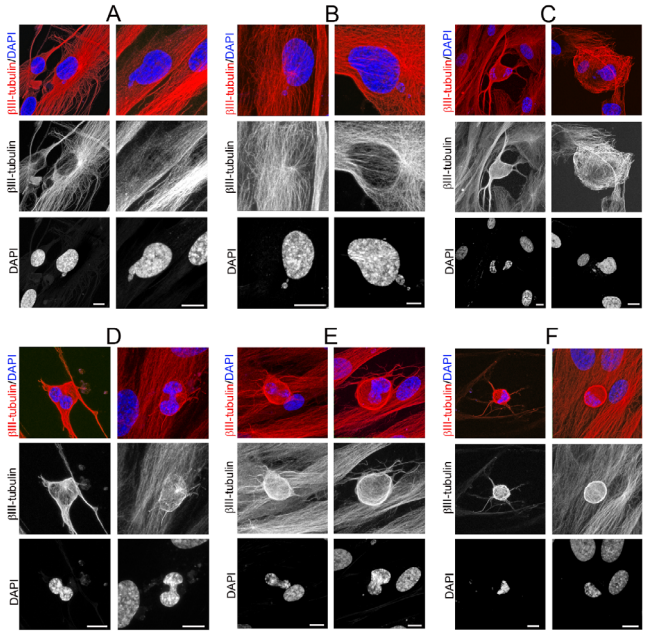

Figure 1 | **Nuclear remodeling occurs when hBMSCs round up to near-spherical shape.**
Small DNA-containing structures arise from the main nuclei (A) and start to move towards specific positions within the cell (B), temporarily forming lobed nuclei (C). Next, these lobed nuclei connect to one another, forming nucleoplasmic bridges (D). Finally, these lobed nuclei connected by an internuclear bridge (E) merge into a single nucleus with an eccentric position within the hBMSCs (F). Scale bars: 10 µm. hBMSCs have been stained for β-III tubulin (red) and DAPI (blue). hBMSCs: Human bone marrow-derived stem cells. Figure 1 is our unpublished data.

sequential proliferation phases.

It has been known for many decades that adult cells can change their identity throught spontaneous dedifferentiation, transdetermination and transdifferentiation, both *in vitro* and *in vivo*, a phenomenon known as cellular plasticity (Tata and Rajagopal, 2016). In addition, previous reports call into question the standar model in which stem cells divide to yield multipotential progenitors that subsequently divide and undergo neuronal differentiation (Munoz-Elias et al., 2003). Our results provide additional evidence that the sequence of events from stem cell to neuron does not necessarily requires cell division from stem cell (Bueno et al., 2019). Nowadays, the formal definitions of "stem cell", "lineage-restricted stem cell" and "terminally differentiated" are being questioned.

One of the most important discoveries made during the neural differentiation of hPDLSCs was the observation that small DNA-containing structures arise from the main nuclei and may move in specific directions within the cell to temporarily form lobed nuclei (Bueno et al., 2019). It has been known for some time that many cells exhibit unusual nuclear structures and extra-nuclear bodies in the cellular cytoplasm. They are referred to as, nuclear buds, nuclear pockets, nuclear envelope-limited chromatin sheets, micronuclei and nucleoplasmic bridges. These unusual nuclear structures and extra-nuclear bodies were described independently within all the main classes of tissue in mammals, as well as in a broad spectrum of vertebrate and invertebrate animals, plants and protozoa. The exact role of these extra-nuclear bodies and their possible correlation with unusual nuclear structures is not yet known (Guo et al., 2019). As noted above, we observed these unusual nuclear structures and extra-nuclear bodies in both hPDLSCs (Bueno et al., 2019) and hBMSCs (**Figure 1**) during neural differentiation.

Although future analysis involving time-lapse microscopy is necessary for elucidating whether DNA-containing structures move within the cell, and therefore if a correlation between the formation of these extra-nuclear bodies and unusual nuclear structures can be established, a recent study has reported that micronuclei arise from the main nuclei and move within the cell, ultimately being loaded into exosomes (Yokoi et al., 2019). It is important to note that nuclear morphology alterations, including extra-nuclear bodies, are closely associated with a wide range of human diseases, including cancer (Guo et al., 2019). Indeed, the possible role of aberrant cellular plasticity in cancer development it being actively researched (Merrell et al., 2016).

In summary, although further research is required to confirm the successful differentiation of human adult-derived stem cells to generate a neural lineage, and finally enable autologous cells to be obtained for cell replacement in a diseased central nervous system, we have observed that these cells could also help increase our understanding of the mechanisms regulating nuclear morphology in response to cell shape changes and their functional relevance.

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