Non-proliferative neurogenesis in human periodontal ligament stem cells

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Understanding the sequence of events from undifferentiated stem cells to neuron is not only important for the basic knowledge of stem cell biology, but also for therapeutic applications. In this study we examined the sequence of biological events during neural differentiation of human periodontal ligament stem cells (hPDLSCs). Here, we show that hPDLSCs-derived neural-like cells display a sequence of morphologic development highly similar to those reported before in primary neuronal cultures derived from rodent brains. We observed that cell proliferation is not present through neurogenesis from hPDLSCs. Furthermore, we may have discovered micronuclei movement and transient cell nuclei lobulation coincident to in vitro neurogenesis. Morphological analysis also reveals that neurogenic niches in the adult mouse brain contain cells with nuclear shapes highly similar to those observed during in vitro neurogenesis from hPDLSCs. Our results provide additional evidence that it is possible to differentiate hPDLSCs to neuron-like cells and suggest the possibility that the sequence of events from stem cell to neuron does not necessarily requires cell division from stem cell.

Neurons are highly polarized cells exhibiting functionally and structurally distinct processes called axons and dendrites1-2. Understanding the mechanisms that underlie the temporal and spatial control of neuronal polarization is not only important for neurobiology3, but also for nerve regeneration in injured nervous systems4.

The process of neuronal polarization has been studied for decades using dissociated rodent postnatal cerebellar granule neurons and embryonic hippocampal pyramidal neurons in culture1-2. The establishment of polarity in cultured rodent neurons are divided into different stages5-6.

Upon isolation, dissociated pyramidal neurons retract their processes, so their neuronal development in vitro start as rounded spheres that spread lamellipodia (stage 1). These spheres appear symmetrical, extending and retracting several immature neurites of a similar length (stage 2). Elongation of a single process, that which presumably becomes the axon, breaks this symmetry (stage 3). The next step involves the remaining short neurites morphologically developing into dendrites (stage 4). The last step (stage 5) in neuronal polarization from dissociated pyramidal neurons in culture is the functional polarization of axon and dendrites, including dendritic spine formation and axon branch formation5. Dissociated granule neurons also present a lamellipodia after attaching to the substratum (stage 1). These spheres extend a unipolar process at a single site on the plasma membrane (stage 2) followed by extension of a second process from the opposite side of the cell body, resulting in a bipolar morphology (stage 3). One of the two axon elongates further and start branching (stage 4), and shorter dendritic processes develop around the cell body (stage 5)5.

Although much progress has been made in the knowledge of how rodent neurons establish their polarity1-3,5,6, less is known about the process of neuronal polarization in human cells3,5.

The major barrier to studying human neurons is the inaccessibility of living tissue, therefore an enormous effort has been made in this study to derive neurons from human stem cells7-11.

Neural crest stem cells (NCSCs) are a migratory cell population that generate numerous cell lineages during development, including neurons and glia12,13. NCSCs can be isolated not only from embryonic neural crest, but also from fetal and adult neural crest-derived tissues14.

The periodontal ligament (PDL) is a connective tissue surrounding the tooth root that contains a source of human NCSCs which can be accessed with minimal technical requirements and little inconvenience to the donor15. Isolation and characterization of multipotent stem cells from the human PDL have been previously described16,17.

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In previous publication\textsuperscript{18}, we showed that human adult periodontal ligament (hPDL) tissue and hPDL-derived cells express marker genes of stem cells and neural crest cells. \textit{In vitro}, hPDL-derived cells differentiate into neural-like cells based on cellular morphology and neural marker expression. \textit{In vivo}, hPDL-derived cells survive, migrate and expressed neural markers after being grafted to the adult mouse brain. Furthermore, some hPDL-derived cells graft into neural stem cell (NSC) niches such as the ventricular-subventricular zone (V-SVZ) of the anterolateral ventricle wall and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus. It is important to mention that the hPDLSCs located in the NSC niches show neural stem morphology. Moreover, hPDLSCs expressed ion channel receptors\textsuperscript{19} and displayed inward currents conducted through voltage-gated sodium (Na\textsuperscript{+}) channels and spontaneous electrical activities after neurogenic differentiation\textsuperscript{20,21}. Therefore, the neural crest origin\textsuperscript{14,15} and neural differentiation potential both \textit{in vitro} and \textit{in vivo}\textsuperscript{13-24} make human periodontal ligament stem cells (hPDLSCs) interesting for investigating stem cell to neuron differentiation mechanisms.

Here, we show that hPDLSCs-derived neural-like cells display a sequence of morphologic development highly similar to those reported before in primary neuronal cultures derived from rodent brains during neurogenesis, providing additional evidence that it is possible to differentiate hPDLSCs to neuron-like cells.

We observed that cell proliferation is not present through neurogenesis from hPDLSCs. In fact, the cell shape of hPDLSCs is reset and start their neuronal development as round shapes. Furthermore, we may have discovered a transient cell nuclei lobulation coincident to \textit{in vitro} neurogenesis, without being related to cell proliferation. We observed that small DNA containing structures may move within the cell to specific directions and temporarily form lobed nuclei.

Morphological analysis also reveals that the V-SVZ of the anterolateral ventricle wall and the SGZ of the hippocampal dentate gyrus in the adult mouse brain contains cells with nuclear shapes highly similar to those observed during \textit{in vitro} neurogenesis from hPDLSCs. We suggest the possibility that neuronal differentiation from NSCs may also occur during \textit{in vivo} adult mammalian neurogenesis without being related to cell proliferation.

**Results**

**hPDLSCs cultured in basic media.** Under proliferation conditions, hPDLSCs displayed a fibroblast-like morphology with low-density microvilli on the cell surface (Fig. 1a) and actin microfilaments and \(\beta\)-III tubulin microtubules oriented parallel to the longitudinal axis of the cell (Fig. 1b). The cytoskeletal protein class III beta-tubulin isotype is widely regarded as a neuronal marker in developmental neurobiology and stem cell research\textsuperscript{25}. Dental and oral-derived stem cells displayed spontaneous expression of neural marker \(\beta\)-III tubulin, even without having been subjected to neural induction\textsuperscript{26}. Western blot analysis verified the expression of \(\beta\)-III tubulin in hPDLSCs (Fig. 1c). During interphase, undifferentiated hPDLSCs displayed a flattened, ellipsoidal nucleus, often located in the center of the cell and with a nuclear volume around 925\(\pm\)356 \(\mu\)m\(^3\) (Fig. 1d).

During mitosis, \(\beta\)-III tubulin is present in the mitotic spindle and it is detectable in all phases of mitosis (Fig. 1e). The cytoskeletal protein class III beta-tubulin isotype is a component of the mitotic spindle in multiple cell types\textsuperscript{27}. At the end of mitosis, division of the cytoplasm by cytokinesis is observed (Fig. 1f). Mitosis and cytokinesis last no more than 2 hours (Fig. 1g).

**hPDLSCs cultured in neural induction media.** After 14 days of neural differentiation conditions, the hPDLSCs displayed different morphologies, including round cells with small phase-bright cell bodies and short processes; highly irregular-shaped cells; and, also, unipolar, bipolar and multipolar-shaped cells with small phase-bright cell bodies and multiple branched processes (Fig. 2a). In addition, cells of different size were also observed (Fig. 2b). Furthermore, microscopic analysis revealed that some hPDLSCs have different nuclear shapes, including lobed nuclei connected by an internuclear bridge (Fig. 2c).

We examined the sequence of developmental events during the first day in culture. We observed that different-shaped cells are stages of development of hPDLSCs during neural differentiation (Fig. 2d). The results may indicate that the cell culture simultaneously contains hPDLSCs at different stages of neurogenesis and neuronal polarization. We acknowledge that the definitive sequence of \textit{in vitro} neurogenesis and neuronal polarization from hPDLSCs will be provided only by time-lapse microscopy of a single cell, but in our experimental conditions, several pieces of data suggest how these steps may occur.

**\textit{In vitro} neurogenesis from hPDLSCs.** After neural induction, hPDLSCs undergo a dramatic change in shape and size, first adopting highly irregular forms and then gradually contracting into round cells with small phase-bright cell bodies (Fig. 3a). Cytoskeletal remodeling is observed during the morphological changes that occurred when the hPDLSCs round up to a near-spherical shape. Actin microfilaments no longer surround the nucleus and became cortical. Unlike actin, \(\beta\)-III tubulin seems to accumulate around the nucleus (Fig. 3b). Actin microfilament and \(\beta\)-III tubulin microtubule network are almost lost in the rounded cells (Fig. 3c).

Scanning electron micrographs show that hPDLSCs also experience dramatic changes in cell surface features. Under proliferation conditions, hPDLSCs remain very flat, presenting low-density microvilli on their surface (Fig. 1a), but there is a marked increase in the number of microvilli as the cells round up to near-spherical shape (Fig. 3d). We also observed that the surface of the rounded cells is almost devoid of microvilli (Fig. 3e).

These results are consistent with previous studies reporting that microvilli increase in number during mitosis but become greatly diminished in number after cytokinesis is completed and the cells enter interphase\textsuperscript{28,29}. Our results may indicate that the dramatic change in shape and size observed during neurogenesis from hPDLSCs is completed.

Morphological and immunocytochemical analysis revealed that rounded cells do not represent dividing cells due to the absence of cytokinesis, mitotic chromosomes and mitotic spindle during the described of \textit{in vitro
neurogenesis processes from hPDLSCs (Figs. 2, 3), contrary to what happens during mitosis (Fig. 1e–g). In addition, the duration of the morphological changes that occurred when the hPDLSCs round up to a near-spherical shape (Fig. 2d) last more than mitosis and cytokinesis (Fig. 1g).
Neuronal polarization of hPDLSCs-derived neural-like cells. Morphological analysis revealed that hPDLSCs-derived neural-like cells display a sequence of morphologic development highly similar to those reported before in dissociated-cell cultures prepared from rodent brain\textsuperscript{1,2,5}\textsuperscript{a} (Figs. 4–6).
neural-like cells also start their development as rounded spheres that initiated neurite outgrowth at a single site on the plasma membrane, first becoming unipolar, stages 1–2 (Fig. 4a). We did not observe the development of lamellipodia around the circumference of the cell body. These unipolar cells, later transformed into cells containing several short neurites, developed around the cell body, stage 3 (Fig. 4b). An analysis of the cytoskeletal organization during spherical stages of hPDLSCs-derived neural-like cells showed that the β-III tubulin microtubules and actin microfilament network is reorganized. Cytoskeletal protein β-III tubulin was densely accumulated under the cellular membrane of the cell body and in cell neurites (Fig. 4a,b) while actin microfilaments were mainly found in cell neurites (Fig. 4c).

We observed that hPDLSCs-derived neural-like cells produce neurites that showed growth cone formations at their tips (Fig. 4c–e). The central domain of the growth cone contains β-III tubulin microtubules and the
Figure 4. Neuronal polarization of hPDLSCs-derived neural-like cells. (a) hPDLSCs-derived neural-like cells start their development as rounded spheres that initiate neurite outgrowth at a single site on the plasma membrane. (b) These later transform into cells containing several short neurites developed around the cell body. (c) The cytoskeletal network is reorganized. β-III tubulin accumulates densely under the cellular membrane of the cell body and in cell neurites while actin microfilaments are mainly found in cell neurites. (d) The peripheral domain in the growth cone of hPDLSCs-derived neural-like cells is composed of radial F-actin bundles and the central domain contains β-III tubulin microtubules. (e) Micrographs showing that the growth cone also contains filopodia and vesicles on the cell surface. (f) Microtubule-associated proteins Tau and MAP2 were also found in hPDLSCs-derived neural-like cells. At later stages of development, hPDLSCs-derived neural-like cells gradually adopt a complex morphology (g) giving rise to a variety of neuron-like forms (h). (i) Cytoskeletal protein β-III tubulin and F-actin staining shown that hPDLSCs-derived neural-like cells develop distinct axon-like and dendrite-like processes (numbers locate the areas shown in higher power). The scale bars are 25 μm in the light microscope images, and 10 μm in the scanning electron micrographs. b, actin bundles; f, filopodia; LM, light microscopy; SEM, scanning electron microscopy; v, vesicles.
Figure 5. hPDLSCs-derived neural-like cells have developed well-differentiated axonal-like and dendritic-like domains. (a) Scanning electron micrographs show that hPDLSCs-derived neural-like cells are composed of multiple branched processes with different spine-like protrusions highly similar to filopodium, mushroom, thin, stubby, and branched dendritic spines shapes. hPDLSCs-derived neural-like cells also display different types of axonal branch-like structures, including bifurcation (b), terminal arborization (c), and collateral formation (d) (inserts and numbers locate the areas showed in higher power). The scale bars are 25 μm in light microscope images and 5 μm in the scanning electron micrographs. a, arborization; B, bifurcation; b, branched; c, collateral formation; f, filopodium; LM, light microscopy; m, mushroom; S, spine-like protusions; s, stubby; SEM, scanning electron microscopy; t, thin.
The peripheral domain is composed of radial F-actin bundles (Fig. 4d), similar to the typical spatial organization described in neurons\textsuperscript{30,31}. Scanning electron micrographs also showed that the growth cone of hPDLSCs-derived neural-like cells contained filopodia and vesicles on the cell surface (Fig. 4e). These findings are consistent with a

**Figure 6.** hPDLSCs-derived neural-like cells are connected by synapse-like interactions. hPDLSCs-derived neural-like cells connect to one another (a) through different types of synapses-like interactions, including dendrodendritic-like, axoaxonic-like and axodendritic-like synapses (b). (c) Synapse-associated proteins Cx43, Synaptophysin and Synapsin1 are found in the cell membrane of hPDLSCs-derived neural-like cells at the neurite contact areas. Scale bar: 25 μm. AA, axoaxonic-like synapse; AD, axodendritic-like synapse; DD, dendrodendritic-like synapse; LM, light microscopy; SEM, scanning electron microscopy.
Nuclear remodeling. Nuclear morphology was examined in hPDLSCs under proliferation and neural differentiation conditions. The dynamic localization of the nucleoli was analyzed by immunostaining for fibrillarin, the main component of the active transcription centers\(^\text{37}\) and the dynamic localization of the nuclear lamina was analyzed by immunostaining for lamin A/C, a nuclear lamina component\(^\text{38}\).

First, we analyzed the nuclear morphology in proliferative hPDLSCs. As noted above, during interphase, hPDLSCs displayed a flattened, ellipsoidal nucleus, often located in the center of the cell, and with a nuclear volume around 925.356 ± 52.6184\(\mu\)m\(^3\) (Fig. 1d). The nuclei of hPDLSCs contained two or more nucleoli and the inside surface of the nuclear envelope is lined with the nuclear lamina (Fig. 1a). Previous studies have shown that the nuclear lamina and nucleolus are reversibly disassembled during mitosis\(^\text{39,40}\). Microscopic analysis of hPDLSCs revealed that the dynamic localization of fibrillarin and lamin A/C proteins during mitosis are similar to those observed in previous studies (Fig. S1b). Futhermore, hPDLSCs-derived neural-like cells also displayed different types of axonal branch-like structures, including bifurcation (Fig. 5b), arborization (Fig. 5c), and collateral formation (Fig. 5d).

The last step in neuronal polarization from rodent neurons in culture is synapse formation\(^\text{1,2,5,6}\). The most frequent types of synaptic communication include axodendritic, axosomatic, axoaxonic and dendrodendritic synapses. Morphological analysis revealed that the hPDLSCs-derived neural-like cells connected to one another (Fig. 6a) through different types of synapse-like interactions, including dendrodendritic-like, axoaxonic-like and axodendritic-like synapses (Fig. 6b). Synapse-associated proteins Cx43, Synaptophysin and Synapsin1 were found accumulated in the cell surface of neurites (Fig. 6c).

During neuronal polarization, no lobed nuclei were observed as hPDLSCs-derived neural-like cells gradually adopted a complex morphology by forming several processes, stage 4 (Fig. 4g) that grew and arborized, acquiring dendritic-like and axonal-like identities, giving rise to a variety of neuron-like morphologies (Fig. 4h).

Our morphological analysis revealed that hPDLSCs-derived neural-like cells developed well-differentiated axonal-like and dendritic-like domains. These types of processes differ from each other in morphology (Figs. 4i and 5). Cytoskeletal protein β-III tubulin and F-actin staining showed that the hPDLSCs-derived neural-like cells comprised multiple branched dendrite-like processes with dendritic spines-like structures (Fig. 4i). Scanning electron micrographs showed that the hPDLSCs-derived neural-like cells also contained multiple branched dendrite-like processes with variously shaped spine-like protrusions, highly similar to filopodium, mushroom, thin, stubby, and branched spines\(^\text{34}\). Dendritic spines are actin-rich compartments that protrude from the microtubule-rich dendritic shafts of principal neurons\(^\text{35}\). Based on morphology, complexity, and function, axon branching is grouped into different categories, including arborization, bifurcation, and collateral formation\(^\text{36}\).

In addition, ultrastructural morphological characteristics of hPDLSCs were examined under neural differentiations conditions. Transmission electron microscopy (TEM) is considered the gold standard to confirm apoptosis\(^\text{41}\). Apoptotic cell contains certain ultrastructural morphological characteristics, including electron-dense nucleus, disorganized cytoplasmic organelles, large clear vacuoles, nuclear fragmentation and apoptotic bodies. TEM analysis revealed that hPDLSCs under neural differentiations conditions do not meet the criteria described above (Fig. S4). Therefore, hPDLSCs with lobed nuclei do not represent apoptotic cells.

During neuronal polarization, no lobed nuclei were observed as hPDLSCs-derived neural-like cells gradually acquired a more mature neuronal-like morphology (Fig. S5a). We also found that the cells round up to a near-spherical shape the nuclear volume of the hPDLSCs decreases to an approximate volume of 279.589 ± 38.8905\(\mu\)m\(^3\) (Fig. S5b).
Interestingly, the morphological analysis revealed that the adult rodent V-SVZ of the anterolateral ventricle wall (Fig. 8a) and the SGZ of the hippocampal dentate gyrus (Fig. 8b), where adult neurogenesis has been clearly demonstrated, contained cells with nuclear shapes highly similar to those observed during in vitro neurogenesis from hPDLSCs.

It is important to mention that nuclear morphology of hPDLSCs observed during in vitro neurogenesis from hPDLSCs (Fig. 7) are highly similar to nuclear morphology of V-SVZ B cells in the human fetal brain and V-SVZ B cells in adult mice brain. Although it has been suggested that lobed nuclei connected by an internuclear bridge are associated with quiescence in aNSCs, we observed that this kind of nuclei may be associated to nuclear movement within the cell during initial phases of neurogenesis, without being related to cell proliferation.

Discussion

In this study we show that hPDLSCs-derived neural-like cells display stages of development highly similar to those reported before in primary neuronal cultures derived from rodent brains. The hPDLSCs-derived neural-like cells gradually adopted a complex morphology by forming several processes, that grew and arborized, acquiring dendritic-like and axonal-like identities, giving rise to a variety of neuron-like morphologies.

During neuronal polarization in vivo, the stages of development observed in mouse immature neurons are not necessarily the same as those observed in vitro, since they depend on the stage of development and the brain region. In a previous publication, we showed that hPDLSCs-derived neural-like cells integrate and differentiate after implantation into the adult mammalian brain. It is important to mention that the hPDLSCs-derived neural-like cells located in the NSC niches show neural stem morphology, which suggests hPDLSCs-derived neural-like cells may achieve a correct polarization in vivo.
Therefore, our results provide additional evidence that it is possible to differentiate hPDLSCs to neuron-like cells, as suggested by their neural-crest origin, stem cell characteristics and neural differentiation potential both in vitro and in vivo. However, it is important to mention that future research is required to resolve the potential limitations in their medical application, and also to optimize the diversity of in vitro neural induction protocols that have been designed for dental stem cells. In addition, future analysis will be necessary to study neural lineages derived from hPDLSCs including neuron-like, astrocyte-like, and oligodendrocyte-like cells.

In this study, we also show that cell proliferation is not present through neurogenesis from hPDLSCs. The undifferentiated polygonal and fusiform cell shapes are reset and start their neuronal development as round spheres. As noted above, morphological characteristics of the hPDLSCs, including cytoskeleton and nuclear morphology were examined in cells under proliferation and neural differentiation conditions.

During mitosis, the nucleolus and the nuclear lamina are reversibly disassembled and β-III tubulin protein is present in the mitotic spindle and it is detectable in all phases of mitosis. Moreover, mitotic chromosomes and cytokinesis were observed. Mitosis and cytokinesis last no more than 2 hours. During neurogenesis, the nucleolus and the nuclear lamina are not disassembled, contrary to what happens during mitosis. Moreover, mitotic chromosomes, mitotic spindle and cytokinesis were not observed. In addition, the duration of the morphological changes that occurred when the hPDLSCs round up to a near-spherical shape lasts much longer than mitosis and cytokinesis. Collectively, these results revealed that round cells do not represent dividing cells.

In this study, we may have discovered a transient cell nuclei lobulation coincident to in vitro neurogenesis. Ultrastructural analysis with transmission electron microscopy revealed that hPDLSCs with lobed nuclei do not represent apoptotic cells due to the absence of features of cells undergoing apoptosis. Interestingly, the morphological analysis revealed that the adult rodent V-SVZ of the anterolateral ventricle wall and the SGZ of the hippocampal dentate gyrus contained cells with nuclear shapes highly similar to those observed in during in vitro neurogenesis from hPDLSCs. Scale bar: 10 μm. GLC, granule cell layer; LV, lateral ventricle.

Figure 8. Neurogenic niches in the adult mammalian brain also contains cells with irregular nuclei. Morphological analysis reveals that the adult rodent V-SVZ of the anterolateral ventricle wall (a), as well as the SGZ of the hippocampal dentate gyrus (b), contain cells with nuclear shapes highly similar to those observed in during in vitro neurogenesis from hPDLSCs. Scale bar: 10 μm. GLC, granule cell layer; LV, lateral ventricle.
proliferating cells, suggest that neurogenesis occurs progressively through sequential phases of proliferation and the neuronal differentiation of aNSCs.

In the V-SVZ, putative aNSCs (type B cells) divide to give rise to intermediate progenitor cells (type C cells), which divide a few times before becoming neuroblasts (type A cells). The neuroblast then migrate into the olfactory bulb and differentiate into distinct types of neurons.14,48,49

In the SGZ, putative aNSCs divide to give rise to intermediate progenitor cells which exhibit limited rounds of proliferation before generating polarized neuroblast49-53. Neuroblast, as polarized cells, then migrate, guided by the leading process, along SGZ and differentiate into dentate granule neurons.14,54

Previous ultrastructure and immunocytochemistry studies show that the V-SVZ stem cell niche contains cells with different morphologies and irregular nuclei.42-45,47,48,56-58. Type-B cells have irregular nuclei that frequently contain invaginations and irregular contours of the plasma membrane. Type-C cells nuclei contained deep invaginations and these cells are more spherical. Type-A cells have elongated cell body with one or two processes and the nuclei are occasionally invaginated.45. Importantly, some studies have shown that murine and human V-SVZ have segmented nuclei connected by an internuclear bridge.42,44,45. Although it has been suggested that lobed nuclei connected by an internuclear bridge are associated with quiescence in aNSCs,55 we observed that this kind of nuclei may be associated to nuclear movement within the cell during initial phases of neurogenesis, without being related to cell proliferation.

In addition, previous reports also shown irregular shaped nuclei in the adult SGZ48-66. Adult SGZ NSCs (type 1 cells) have irregular contours of the plasma membrane, and differences in heterochromatin aggregation has been also observed.53. Furthermore, adult SGZ NSCs (type 2 cells) had an irregularly shaped nucleus.53-55. Importantly, one study also found that many cultured hippocampal neurons have irregular nuclei or even consisted of two or more lobes connected by an internuclear bridge.46

It has commonly been assumed that adult neurogenesis occurs progressively through sequential phases of proliferation.54,55. Despite the advantages for the detection of adult neurogenesis using exogenous nucleotide analog administration or endogenous cell cycle markers, in addition to retroviral transduction, cell stage and lineage commitment markers, recent findings indicate that some observations interpreted as cell division could be false-positive signals.59-72. The main method used to labeled new neurons has been the incorporation of the thymidine analogs into the genome of dividing cells during S-phase of the cell cycle, but nevertheless thymidine analogs such as tritiated thymidine, BrdU, CldU and IdU may also be incorporated during DNA turnover or DNA repair.48-72. Infection with retrovirus is another method used to label new neurons however, retoviral vectors not specifically infect dividing cells.73 and also, it has even been observed that postmitotic pyramidal neurons can also be labeled by fused infected microglia.74. Although the expression of endogenous cell cycle proteins is also used to label new neurons, recent findings indicate that cell cycle proteins expression is not necessarily related to cell division.55. Proliferating cell nuclear antigen is also involved in DNA repair.74. Positivity of the proliferation marker Ki-67 in noncycling cells has also been observed.65

These findings indicate that there is a lack of a reliable definitive method to label new neurons. In addition, it is important to note that almost none of these labeled new born neurons show mitotic chromosomes or mitotic spindle to really confirm that adult neurogenesis occurs progressively through sequential phases of proliferation.43,47-53. Importantly, the existence of non-proliferative neuronal precursors in several brain areas has also been observed.64. Moreover, the self-renewal and multipotent properties demonstrated by NSC in vitro14 have not been clearly demonstrated in vivo.54,47,78

Collectively, these results suggest the possibility that the sequence of events from aNSCs to neuron may also occur without being related to cell proliferation. It would therefore be interesting to examine whether SVZ and SGZ intermediate progenitor cells represent different stages of neurogenesis without being related to cell proliferation.

It is known for many decades that adult cells can change their indentity through spontaneous dedifferentiation, differentiation and transdifferentiation in vitro and in vivo.60-82. Future analysis will be necessary to study if hPDLSCs convert directly to neural-like cells or if they go through neural progenitor stage first. Our results suggest the possibility that hPDLSCs could be use to advance knowledge of the cellular plasticity.

Beyond the central nervous system, the presence of lobed nuclei has been reported in most blood and immune cells, but the functional significance of multilobed nuclear structures is not yet known.63-86. We observed that the nuclei of hPDLSCs during initial phases of neurogenesis are highly similar to those reported in immune cells. Thus, we suggest the possibility that multilobed nuclear structures may be associated to nuclear movement within the cell. Therefore, it would also be interesting to examine whether these putative madague cells also represent different stages of haematopoietic stem cell differentiation without being related to cell proliferation.

One of the most important discoveries in this work is the observation that small DNA containing structures may move within the cell to specific directions and temporarily form lobed nuclei. These small DNA containing structures displayed a spherical or oviod shape, and it seems that some of them are connected to the main body of the nucleus by thin strands of nuclear material. It is important to note that some DNA containing structures are highly similar to nuclear envelope-limited chromatin sheets (ELCS).45,87,88. Fibrillarin and lamin A/C proteins were detected in these small DNA containing structures.

It is known for many decades that chromatin particles can appear in the cellular cytoplasm and they are referred to as micronuclei, nucleoplasmic bridge and nuclear bud.14-89. Although these nuclear anomalies have been associated with chromosomal instability events during mitosis,90-92. recent reports showed generation of micronuclei during interphase.93-95. These findings call into question that micronuclei, nucleoplasmic bridge and nuclear bud does necessarily generated during mitosis.96. Moreover, a high frequency of human mesenchymal stem cells with nuclear bud, micronuclei and nucleoplasmic bridge was detected under normal in vitro culture.97. Therefore, the mechanisms that lead to extra-nuclear bodies formation and their biological relevance are still far from been understood.

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In this study, we show that there can be a relationship in the formation of the nuclear bud, micronuclei, nucleoplasmic bridge and nuclear envelope-limited chromatin sheets. Collectively, these results suggest the possibility that the interphase cell nucleus may reversibly disassemble into functional subunits that may move independently within the cell, if necessary.

Multinuclear cells are commonly found in various human organs including heart, liver, salivary glands, muscle and endometrium, but their functional advantage remains unknown. In addition, alterations in nuclear morphologies are closely associated with a wide range of human diseases, including cancer. hPDLSCs could facilitate an understanding of the mechanisms regulating nuclear morphology in response to cell shape changes and their functional relevance.

Methods

Ethical conduct of research. Methods were carried out in accordance with the relevant guidelines and regulations. The experimental protocols were approved by the Institutional Review Board of the Miguel Hernández University of Elche (No. UMH.IN.SM.03.16) and the signed informed consent was obtained from all patients before the study. The authors declare that all experiments on human subjects were conducted in accordance with the Declaration of Helsinki. All protocols and care of the mice were carried out according to the guidelines of the European Communities Council Directive of 24 November 1986 (86/609/EEC). The authors further attest that all efforts were made to minimize the number of animals used and their suffering.

Cell culture. Dissociated cell cultures of hPDL tissue were prepared as previously described. Human pre-molars were extracted and collected from three different healthy adult donors undergoing orthodontic therapy in Murcia dental hospital (Spain). hPDL was scraped from the middle third region of the root surface. After washing the extracted PDL with Ca and Mg-free Hank’s balance salt solution (HBSS; Gibco), hPDL was digested with 3 mg/ml type I collagenase (Worthington Biochemical Corporation) and 4 mg/ml dispase II (Gibco) in alpha modification minimum essential medium eagle (α-MEM) (α-MEM; Sigma-Aldrich) for 1 h at 37 °C. The reaction was stopped by the addition of α-MEM. PDL derived from different subjects were pooled together to obtain single cell suspensions by passing the cells through a 70 μm strainer (BD Falcon). Cells were centrifuged, and the pellet was resuspended in in serum-containing media (designated as the basal media), composed of α-MEM supplemented with 15% calf serum (Sigma), 100 units/ml penicillin-streptomycin (Sigma) and 2 mM L-glutamine (Sigma). The cell suspension was plated into six-well multiwell plates (BD Falcon) and incubated at 37 °C in 5% CO₂. The cells at passage 3–4 were then used for the following experiments. We used μ-Dish 35 mm, high Grid-500 (Ibidi) for live cell imaging. Numeric marks on the bottom of each dish allow users to identify the location of cells.

To induce neural differentiation, cells were cultured in serum-free media (designated as the neural induction media), consisting in Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12, Gibco) supplemented with bFGF (20 ng/ml, R&D Systems), EGF (20 ng/ml, R&D Systems), glucose (0.8 mg/ml, Sigma), N2-supplement (Gibco), 2 mM L-glutamine (Sigma), and 100 units/ml penicillin-streptomycin (Sigma). Cells at passage 3–4 were allowed to adhere to the plates overnight. Media was removed the following day and neural induction media was added directly to the cells. Neural induction media were changed every 3 days until the end of the experiment (2 weeks).

In previous publication, we showed that several stem cell and neural crest cell markers are expressed in human adult periodontal ligament (hPDL) tissue and hPDL-derived cells.

Immunocytochemistry. A standard immunocytochemical protocol was used as previously described. Cells were plated onto collagen IV (Sigma) coated plastic or glass coverslips, and maintained in basal media or neural induction media. Cells were rinsed with PBS and fixed in freshly prepared 4% paraformaldehyde (PFA; Sigma). Fixed cells were blocked for 1 h in PBS containing 10% normal horse serum (Gibco) and 0.25% Triton X-100 (Sigma) and incubated overnight at 4 °C with antibodies against: β-III-tubulin (TUJ1; 1:500, Covance), Tau (GTX49353; 1:300, GeneTex), MAP2 (840601; 1:300, Biolegend), Connexin-43 (3512; 1:300, Cell Signalling), Synaptophysin (18-0130; 1:300, Zymed), Synapsin1 (NB300-104; 1:300, Novus), Fibrillarin (ab5821; 1:300, Abcam) and Lamin A/C (GTX101127; 1/300, GeneTex) in PBS containing 1% normal horse serum and 0.25% Triton X-100. On the next day, cells were rinsed and incubated with the corresponding secondary antibodies: Alexa Fluor® 488 (anti-mouse or anti-rabbit; 1:500, Molecular Probes), Alexa Fluor® 594 (anti-mouse or anti-rabbit; 1:500, Molecular Probes), biotinylated anti-rabbit (BA1000, 1:250; Vector Laboratories), biotinylated anti-chicken (BA9010, 1:250, Vector Laboratories), CY3-streptavidin (1:500, GE Healthcare). Cell nuclei were counterstained with DAPI (0.2 mg/ml in PBS, Molecular Probes). Alexa Fluor 488® phalloidin was used to selectively stain F-actin (Molecular Probes). Data are representative of ten independent experiments per condition.

Western blotting. A standard Western Blott protocol was used as previously described. hPDL-derived cells (in basal media) were harvested using trypsin/EDTA (Gibco), washed twice with PBS, resuspended in RIPA lysis buffer (Millipore) in the presence of protease inhibitors (Pierce™ protease inhibitor Mini Tables, Pierce Biotechnology Inc) and PMSF 1 M (Abcam) for 30 min at 4 °C. Protein concentration was determined using the Bradford protein assay (Sigma-Aldrich). Proteins were separated in 8% SDS-polyacrylamide gel (PAGE-SDS) and transferred to a nitrocellulose membrane (Whatman). PageRuler™ Prestained Protein Ladder (Thermo Scientific) has been used as size standards in protein electrophoresis (SDS-PAGE) and Western-Blotting. After transfer, nitrocellulose membranes were stained with Ponceau S solution (Sigma-Aldrich) to visualize protein bands. Blots were then incubated overnight at 4 °C with rabbit antibody against β-III-tubulin (TUJ1; 1:1000, Covance). Secondary antibody was used at 1:7000 for peroxidase anti-mouse Ab (PI-2000, Vector Laboratories). Immunoreactivity was detected using the enhanced chemiluminescence (ECL) Western blot detection system.
Immunohistochemistry. A standard immunohistochemistry protocol was used as previously described. Experiments were carried out according to the guidelines of the European Community (Directive 86/609/EC) and in accordance with the Society for Neuroscience recommendations. Animals used in this study were 12-week-old immune-suppressed mouse (Hsd:Athymic Nude-Foxn1 nu/nu; Harlan Laboratories Models, S.L.), housed in a temperature and humidity controlled room, under a 12 h light/dark cycles, with ad libitum access to food and water. The animals were anesthetized and intracardially perfused with freshly prepared, buffered 4% PFA (in 0.1 M PB, pH 7.4). Brains were removed, post-fixed for 12 h in the same fixative at 4°C and dehydrated in 30% sucrose solution at 4°C until sunk. 30 µm thick coronal sections were collected using a freezing microtome. Serial sections were used for DAPI staining. Free-floating sections were incubated and mounted onto Superfrost Plus glass slides (Thermo Scientific). The slides were dried O/N and coverslipped with mowiol-NPG (Calbiochem).

Images and data analyses. Analyses and photography of visible and fluorescent stained samples were carried out in an inverted Leica DM IRB microscope equipped with a digital camera Leica DFC350FX (Nussloch) or in confocal laser scanning microscope Leica TCS-SP8. Digitized images were analyzed using LASX Leica confocal software. Z-stacks of confocal fluorescent images were also analyzed to calculate the nuclear volume by using ImageJ software. We used Photoshop software to improve the visibility of fluorescence images without altering the underlying data. The fluorescent photographs presented in this study are original fluorescence images and inverted images (negatives) that are produced from the original fluorescence images.

Scanning electron microscopy. Cells were plated onto collagen IV (Sigma) coated glass coverslips and maintained in basal media or neural induction media. Cells were treated with fixative for 20 minutes. Coverslips were postfixed in 1% osmium tetroxide for 1 hour and dehydrated in graded ethanol washes. The coverslips were allowed to dry at a conventional critical point and were then coated with gold-palladium sputter coated. Coverslips were view on a Jeol 6100 scanning electron microscope.

Transmission electron microscopy. hPDL-derived cells were harvested using trypsin/EDTA (Gibco) and were treated with fixative for 60 minutes. Cells were postfixed in osmium tetroxide solution, dehydrated embedded in resin. Ultrathin sections (70–90 nm) were cut, stained with lead citrate, and examined under Jeol 1011 and Philips Tecnai 12 transmission electron microscopes.

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References
1. Tahirovic, S. & Bradke, F. Neuronal polarity. Cold Spring Harb. Perspect. Biol. 1, a001644, https://doi.org/10.1101/cshperspect.a001644 (2009).
2. Takano, T., Xu, C., Funahashi, Y., Namba, T. & Kaibuchi, K. Neuronal polarization. Development. 142, 2088–2093 (2015).
3. Schelki, M. & Bradke, F. Neuronal polarization: From spatiotemporal signaling to cytoskeletal dynamics. Mol. Cell Neurosci. 84, 11–28 (2017).
4. Kaplan, A., Bueno, M., Hua, L. & Fournier, A. E. Maximizing functional axon repair in the injured central nervous system: Lessons from neuronal development. Dev. Dyn. 247, 18–23 (2018).
5. Dotti, C. G., Sullivan, C. A. & Banker, G. A. The establishment of polarity by hippocampal neurons in culture. J. Neurosci. 8, 1454–1468 (1988).
6. Powell, S. K., Rivas, R. J., Rodriguez-Boulan, E. & Hatten, M. E. Development of polarity in cerebellar granule neurons. J. Neurobiol. 32, 223–236 (1997).
7. Ohara, Y. et al. Early-stage development of human induced pluripotent stem-cell derived neurons. J. Neurosci. Res. 93, 1804–1813 (2015).
8. Azari, H. & Reynolds, B. A. In vitro models for neurogenesis. Cold Spring Harb. Perspect. Biol. 8, a021279, https://doi.org/10.1101/cshperspect.a021279 (2016).
9. Conti, L. & Cattaneo, E. Neural stem cell systems: physiological players or in vitro entities? Nat. Rev. Neurosci. 11, 176–187 (2010).
10. Casarosa, S., Bozzi, Y. & Conti, L. Neural stem cells: ready for therapeutic applications? Mol. Cell Ther. 2, https://doi.org/10.18696/2052-8426-2-31 (2014).
11. Goldman, S. A. Stem and progenitor cell-based therapy of the central nervous system: hopes, hype, and wishful thinking. Cell Stem Cell. 18, 174–188 (2016).
12. Bronner-Fraser, M. Origins and developmental potential of the neural crest. Exp. Cell Res. 218, 405–417 (1995).
13. Crane, J. F. & Trainor, P. A. Neural crest stem and progenitor cells. Annu. Rev. Cell Dev. Biol. 22, 267–286 (2006).
14. Achilleos, A. & Trainor, P. A. Neural crest stem cells: discovery, properties and potential for therapy. Cell Res. 22, 288–304 (2012).
15. Liu, J. A. & Cheung, M. Neural crest stem cell and their potential therapeutic applications. Dev. Biol. 419, 199–216 (2016).
16. Seo, B. M. et al. Investigation of multipotent postnatal stem cells from human periodontal ligament. Lancet 364, 149–155 (2004).
17. Tomokiy, A. et al. Detection, Characterization, and Clinical Application of Mesenchymal Stem Cells in Periodontal Ligament Tissue. Stem Cells Int. 26, 5450768, https://doi.org/10.1155/2015/5450768 (2018).
18. Bueno, C. et al. Human adult periodontal ligament-derived cells integrate and differentiate after implantation into the adult mammalian brain. Cell Transplant. 22, 2017–2028 (2013).
19. Citricosta, L., Diomedes, F., Trubiani, O., Bramanti, P. & Mazzone, P. Physiological Expression of Ion Channel Receptors in Human Periodontal Ligament Stem Cells. Cells. 8, E219, https://doi.org/10.3390/cells8030219 (2019).
20. Fortino, V. R., Chen, R. S., Pelaiz, D. & Cheung, H. S. Neurogenesis of neural crest-derived periodontal ligament stem cells by EGF and bFGF. J. Cell Physiol. 229, 479–488 (2014).
21. Ng, T. K. et al. Transdifferentiation of periodontal ligament-derived stem cells into retinal ganglion-like cells and its microRNA signature. Sci. Rep. 5, 16429, https://doi.org/10.1038/srep16429 (2015).
66. Urbach, A., Redecker, C. & Witte, O. W. Induction of neurogenesis in the adult dentate gyrus by cortical spreading depression. Stroke. 39, 3064–3072 (2008).
67. Gallay, L., Lowell, S., Rubin, L. L. & Anderson, D. J. Deregressivization of dimetarrual patterning by FGF confers trilineage differentiation capacity on CNS stem cells in vitro. Neuron. 40, 485–499 (2003).
68. Wittmann, M. et al. Synaptic activity induces dramatic changes in the geometry of the cell nucleus: interplay between nuclear structure, histone H3 phosphorylation, and nuclear calcium signaling. J. Neurosci. 29, 14687–14700 (2009).
69. Rakic, P. Adult neurogenesis in mammals: an identity crisis. J. Neurosci. 22, 614–618 (2002).
70. Cooper-Kuhn, C. M. & Kuhn, H. G. Is it all DNA repair? Methodological considerations for detecting neurogenesis in the adult brain. Brain Res. Dev. 134, 13–21 (2002).
71. Breunig, J. J., Arellano, J. I., Mackidis, J. D. & Rakic, P. Everything that glitters isn’t gold: a critical review of postnatal neural precursor analyses. Cell Stem Cell. 1, 612–627 (2007).
72. Kuhn, H. G., Eisch, A. J., Spalding, K. & Peterson, D. A. Detection and Phenotypic Characterization of Adult. Neurogenesis. Cold Spring Harb. Perspect. Biol. 8, a025981. https://doi.org/10.1101/cshperspect.a025981 (2016).
73. Ackman, J. B., Siddiqi, F., Walkonis, R. S. & LoTurco, J. J. Fusion of Microglia with Pyramidal Neurons after Retroviral Infection. J. Neurosci. 26, 11413–11422 (2006).
74. Uberti, D., Ferrar-Toninelli, G. & Memo, M. Involvement of DNA damage and repair systems in neurodegenerative process. Oxid. L. Lett. 139, 99–105 (2003).
75. Van Oijen, M. G., Medema, R. H., Slootweg, P. J. & Rijkse, G. Positivity of the proliferation marker Ki-67 in noncycling cells. Am. J. Clin. Pathol. 110, 24–31 (1998).
76. König, R. et al. Distribution and fate of DCX/PSA-NCAM expressing cells in the adult mammalian cortex: a local reservoir for adult cortical neuroplasticity? Front. Biol. 11, 193–213 (2016).
77. Rotheneichner, P. et al. Cellulat Plasticity in the Adult Murine Piriform Cortex: Continuous Maturation of Dormant Precursors Into Exacitatory Neurons. Cereb. Cortex. 28, 2610–2621 (2018).
78. Reynolds, B. A. & Weiss, S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science. 255, 1707–1710 (1992).
79. Suh, H. et al. In vivo fate analysis reveals the multipotent and self-renewal capacities of Sox2+ neural stem cells in the adult hippocampus. Cell Stem Cell. 1, 515–528 (2007).
80. Raff, M. Adult stem cell plasticity: fact or artifact? Science. 255, 379–384 (1992).
81. Tata, P. R. & Rajagopal, J. Cellular plasticity: 1712 to the present day. Nat. Rev. Mol. Cell. Biol. 17, 413–425 (2016).
82. Merrell, A. J. & Stanger, B. Z. Adult cell plasticity in vivo: de-differentiation and transdifferentiation are back in style. Nat. Rev. Mol. Cell Biol. 17, 413–425 (2016).
83. Hoffmann, K., Sperling, K., Olins, A. L. & Olins, D. E. The granulocyte nucleus and lamin B receptor: avoiding the ovid. Chromosoma. 116, 227–235 (2007).
84. Carvalho, L. O., Aquino, E. N., Neves, A. C. & Fontes, W. The neutrophil nucleus and its role in neutrophilic function. J. Cell Biochem. 116, 1831–1836 (2015).
85. Skinner, B. M. & Johnson, E. E. Nuclear morphologies: their diversity and functional relevance. Chromosoma. 126, 195–212 (2017).
86. Georgopoulos, K. In search of the mechanism that shapes the neutrophil’s nucleus. Genes Dev. 31, 85–87 (2017).
87. Olins, D. E. & Olins, A. L. Nuclear envelope-limited chromatin sheets (ELCS) and heterochromatin higher order structure. Chromosoma. 118, 537–548 (2009).
88. Etskov, M., Sosnovski, S., Olins, A. L. & Olins, D. E. ELCS in ice: cryo-electron microscopy of nuclear envelope-limited chromatin sheets. Chromosoma. 123, 303–312 (2014).
89. Fenech, M. et al. Molecular mechanisms of micronucleus, nucleoplasmic bridge and nuclear bud formation in mammalian and human cells. Mutagenesis. 26, 125–132 (2011).
90. Hutchinson, H. E. & Ferguson-Smith, M. A. The significance of Howell-Jolly bodies in red cell precursors. J. Clin. Pathol. 12, 451–53 (1959).
91. Dawson, D. W. & Bury, H. P. The significance of Howell-Jolly bodies and giant metamyelocytes in marrow smears. J. Clin. Pathol. 14, 374–380 (1961).
92. Heddle, J. A. & Carrano, A. V. The DNA content of micronuclei induced in mouse bone marrow by gamma-irradiation: evidence that micronuclei arise from acenticate chromosomal fragments. Mutat. Res. 44, 63–69 (1977).
93. Shimizu, N., Itoh, N., Utiyama, H. & Wahl, G. M. Selective entrapment of extrachromosomally amplified DNA by nuclear budding and micronucleation during S phase. J. Cell Biol. 140, 1307–1320 (1998).
94. Haaf, T. et al. Sequestration of mammalian Rad51-recombination protein into micronuclei. J. Cell Biol. 144, 11–20 (1999).
95. Utani, K., Okamoto, A. & Shimizu, N. Generation of micronuclei during interphase by coupling between cytoplasmic membrane blebbing and nuclear budding. PLoS One. 6, e27233, https://doi.org/10.1371/journal.pone.0027233 (2011).
96. Guo, X. et al. The molecular origins and pathophysiological consequences of micronuclei: New insights into an age-old problem. Mutat. Res. 779, 1–35 (2019).
97. Cornélio, D. A., Tavares, J. C., Pimentel, T. V., Cavalcanti, G. B. & Battistuzzo de Medeiros, S. R. Cytokinesis-block micronucleus assay adapted for analyzing genomic instability of human mesenchimal stem cells. Stem Cells Dev. 23, 823–838 (2014).
98. Hintsche, H. et al. Fate of micronuclei and micronucleated cells. Mutat. Res. 771, 85–98 (2017).
99. Norppa, H. & Falck, G. C. What do human micronuclei contain? Mutagenesis. 18, 221–233 (2003).
100. Milko, M. et al. Two nuclei inside a single cardiac muscle cell. More questions than answers about the binucleation of cardiomyocytes. Biologia. 72, 825–830 (2017).
101. Manhart, A., Windser, S., Baylies, M. & Mogilner, A. Mechanical positioning of multiple nuclei in muscle cells. PLoS Comput. Biol. 14, e1006208, https://doi.org/10.1371/journal.pcbi.1006208, eCollection (2018).
102. Zink, D., Fischer, A. H. & Nickerson, J. A. Nuclear structure in cancer cells. Nat. Rev. Cancer. 4, 677–687 (2004).
103. Gundersen, G. G. & Worman, H. J. Nuclear positioning. Cell. 152, 1376–1389 (2013).
104. Dupin, I. & Etienne-Manneville, S. Nuclear positioning: mechanisms and functions. Int. J. Biochem. Cell Biol. 43, 1698–1707 (2011).

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Author contributions
C.B. conceived of the study, designed the study, carried out the molecular lab work and drafted the manuscript. M.M. carried out the molecular lab work and participated in data analysis. S.M. conceived of the study, helped draft the manuscript and financial support. All authors discussed and commented on the manuscript.
Competing interests
The authors declare no competing interests.

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