FACULTAD DE MEDICINA Y ENFERMERIA

Departamento de Biología Celular e Histología

ROLE OF NOTCH AND WNT SIGNALING IN METABOLIC AND EPIGENETIC REPROGRAMMING OF HUMAN DENTAL PULP STEM CELLS (DPSCs)

Verónica Uribe-Echevarria Zubizarreta

Diciembre, 2018

ACKNOWLEDGEMENTS/AGRADECIMIENTOS

The present work has been done thanks to the financial support of the research projects given by the Basque Country (IT-831/13), the University of the Basque Country (UPV/EHU; GIU16/66), and Foundation Jesus Gangoiti Barrera. This research would have not be possible also by the financial support of Children with Cancer UK (project grant 15-193) to Alice Agliano and by Cancer Research UK and EPSRC Cancer Imaging Centre in association with the MRC and Department of Health (England) (C1060/A10334, C1060/A16464) to the Division of Radiotherapy and Imaging at the Institute of Cancer Research. Verónica Uribe-Echevarria received a PhD fellowship from the Basque Government and a Global Training grant from the Basque Government.

Después de estos cuatro años de Doctorado, bastante intensos, que para mi sorpresa se me han hecho cortos. Pero, mi experiencia en la universidad y en el grupo de investigación comenzó bastante antes, con una alumna de tercer año emocionada con la idea de formar parte de una investigación. Ahí comenzó una oportunidad, un sueño, y no hubiera sido posible sin la ayuda y el apoyo de un gran número de personas que me han acompañado en todo este proceso.

Lo primero de todo, quiero dar las gracias a Gaskon Ibarretxe, mi director de tesis, por darme la oportunidad de unirme a este fenomenal grupo, por su supervisión y su guía. Por haberme considerado una más del equipo nada más empezar y por ser capaz de mantener la motivación y la pasión por la investigación tanto en los buenos como no en los tan buenos momentos y de transmitirlo a todos y cada uno de los miembros del grupo. Recuerdo mi primer día de laboratorio, como si fuera ayer. Recuerdo la entrevista, llena de motivación y de ideas. Recuerdo la presentación al resto de miembros del grupo, como si fuera una más. Todo fue clave para continuar con vosotros mi doctorado.

En segundo lugar, pero no por ello menos importante, tengo que dar las gracias a mi tutor y codirector Fernando Unda, por su supervisión y por su apoyo. Por ser capaz de transmitirme calma y seguridad cuando has visto que lo he necesitado. Tanto tus consejos, tanto tu experiencia como tu guía han sido muy importantes durante todo este proceso de aprendizaje.

I would like also to express my gratitude to Dr. Nada Al-Saffar, from The Institute of Cancer Research, in London, for giving me the opportunity to work with her wonderful group for six months.

Pero sobre todo también a ti Ali, sin ti mi doctorado hubiera sido totalmente diferente, has sido capaz de aportarme templanza, organización y efectividad cuando lo necesitaba. Además de brindarme un gran apoyo y guía. Siempre recordaré el "brainstorming" que tenía contigo a la hora plantear un experimento o un proyecto contigo. Magníficas ideas nos quedaron pendientes, quién sabe igual algún día juntas o separadas las llevábamos a cabo.

Me gustaría también dedicar una parte importante de mis agradecimientos a mis compañeros de laboratorio. A las ya doctoras, por su cercanía a Maitane, y a Patri, por su apoyo y ayuda, por esos momentos de risas, aunque también de lloros, y porque todo ello casi hace que te considere de mi familia (podría ser también que me recuerdas a mi hermana, que conozcas a mi familia….). A mis compañeros Igor y Lutxu, por hacerme sentirme literalmente, como en casa. Recuerdo mi primer día de laboratorio cuando me enseñaron a hacer mi primera inmunocitoquíca, pendientes de explicarme todo y que lo entendiera todo lo mejor posible. Y ya sea por sus acertadas explicaciones o sea por la suerte del principiante, mi primer experimento con resultados, adjuntado como portada de mi tesis. Gracias también a Iker y a Olatz, los "expertise" juveniles del laboratorio, por vuestra ayuda cuándo lo he necesitado y por los buenos momentos de risas. Hacéis que crea en la vida científica después de la tesis.

También quisiera agradecer al resto de compañeros de BioCell, compañeros de escolares y extraescolares durante el doctorado. A Noelia y Xandra, que han sabido escucharme y aconsejarme. A Cristina, Arantxa, Andrea, Carlos, José y Maddalen, que siempre nos prestan fungible cuando les pedimos. A Patri, María y Vero, Maider, Irene y Alba, que me transmiten siempre positivismo. Y a Miguel, compañero de parte de la carrera, de doctorado, de batallas y amigo, por sucomprensión y por compartir esa loca motivación científica basada en la entropía. A todos vosotros os agradezco esos momentos claves tanto dentro como fuera del departamento en forma de aventura, de convivencia, y de sobre todo momentos de risas y complicidad, súper importantes durante estos años.

Gracias a los chicos de microscopía Alex, Jon y Ricardo, a la secretaria del departamento Mari Jose, y también a las técnicos Txus y Cristina.

También quiero mencionar a mis amigas del colegio Olaia, Irune, Irene, Paloma y Agurtzane, por escucharme, por motivarme, por vuestro apoyo y por comprenderme en esos momentos de altibajos. A la gente maravillosa que conocí durante la carrera que después de compartir momentos increíbles, qué siguen estando ahí y con los que comparto experiencias y apoyo Sergio, Jorge, Ariane y Mario, espero que sigamos compartiendo batallitas. A mis amigas de Bizi Bizitza, por escucharme y apoyarme en las etapas difíciles, y especialmente a María, que aunque empezamos como compañeras de trabajo, terminamos como grandes amigas y lo que nos queda por delante Mary!!

Por supuesto no puedo olvidar a mi familia, especialmente a Aita y Ama, por su apoyo incondicional, por transmitirme esa perseverancia y constancia, y alentarme a no rendirme nunca. A mi abuela Pili, que siempre me decía que estudiaba mucho, ahora seguiré estudiando más. A mis sobrinos Oier y Ane, aunque muy pequeños y leerán esta tesis dentro de muchos años, siempre me dan alegría pase lo que pase. Agradecer también a Edu por su ayuda y al resto de la familia por darme energías y motivación durante esta etapa. Y a mi hermana Laura, siempre de apoyo y aguantando mis quejas y lloros, santa paciencia que tienes, muchas gracias, nunca te lo podréagradecer lo suficiente.

¡De corazón gracias a todos!!

INDEX

RESUMEN

Las células stem de la pulpa dental (DPSCs; en inglés) son células extraídas de la pulpa dental de los terceros molares (cordales). Durante el desarrollo embrionario, estas células migran desde la cresta neural como una población de células ectomesenquimales, tras experimentar una transición epitelio-mesenquimal (EMT), para formar la mayor parte de los tejidos craniofaciales.

Las DPSCs presentan unas propiedades que las convierten en un recurso especialmente útil para la terapia celular. Entre estas propiedades destaca el potencial de diferenciación hacia distintos tipos de linajes celulares y tejidos, incluido hueso, cartílago, nervioso, músculo y tejido adiposo, entre otros. Por otro lado, a diferencia de otros tipos de células stem adultas mesenquimales, las DPSC son fáciles de obtener de pacientes con un procedimiento de extracción sencillo, mínimamente invasivo. Además, estas células se cultivan fácilmente en forma de cultivo primario y se amplifican rápidamente gracias a su alta tasa de renovación celular. En cuanto al mantenimiento y crioconservación de esta población de células, requieren una metodología relativamente sencilla. Además, podría considerarse la obtención de células de pacientes para terapias futuras de trasplante celular autólogo, evitando así los posibles problemas derivados de otros tipos de trasplante (alogénico), como son los efectos secundarios derivados de los tratamientos de inmunosupresión y el rechazo inmunológico. También, presentan propiedades inmunosupresoras y buena tolerancia a los biomateriales, lo que las hace especialmente atractivas para su uso en clínica *(ANEXO 1)*.

En cuanto al potencial de reparación de las DPSC destaca su troncalidad, reflejada tanto por la alta tasa de renovación celular, como por la expresión de marcadores de células stem pluripotentes como Oct4, Nanog, Sox2, cMyc, Lin28, Rex1, Stella y Ssea1. Esta troncalidad puede ser potenciada mediante la reprogramación celular. Numerosas metodologías de reprogramación han sido desarrolladas durante los últimos años, como la incorporación mediante la transfección de factores de pluripotencia con vectores Yamanaka y con virus Sendai, retrovirus o lentivirus. Recientemente, se ha desarrollado una nueva tecnología de edición genética, CRISPR, que permitiría la adición de genes de pluripotencia concretos. Sin embargo, todas estas metodologías pueden dar lugar a un acumulo de errores y de mutaciones que convierte a las células madre, derivadas por estas metodologías, en potencialmente arriesgadas y, por tanto, no aptas para su uso en terapia

celular en humanos. Es por todo ello que el conocimiento de las vías de señalización Notch y Wnt/β-Catenina, por ejemplo, y de su intervención en la troncalidad y renovación de las células stem sería de utilidad en el campo de la reprogramación celular. *(ANEXO 2).*

Las vías de señalización Notch y Wnt/β-Catenina son cruciales en el mantenimiento y la diferenciación de distintos tipos de células stem en células maduras. El uso de proteínas recombinantes para la modulación de estas vías a diferentes tiempos y concentraciones podría ayudar en el control sobre el mantenimiento de estas poblaciones de células stem, sin que ello suponga una alteración definitiva o la acumulación de errores en el DNA de las células de interés. Además, la modulación en la troncalidad o pluripotencia viene acompañada de una remodelación del metabolismo y de la epigenética, todo ello necesario para garantizar la eficiencia de la reprogramación. El estudio de todos estos cambios en la fisiología de las DPSCs es necesario para asegurar una terapia celular efectiva, precisa y segura, que garantice la obtención de células sanas y sin errores *(ANEXO 2).*

Las vías de señalización Notch y Wnt/β-Catenina tienen un papel importante en el mantenimiento y diferenciación de las poblaciones de células madre. En el caso de las DPSCs, las vías Notch y Wnt/β-Catenina son indispensables en la expresión de marcadores de pluripotencia (Oct4, Nanog, Sox2, cMyc, Lin28, Rex1, Stella y Ssea1) y también en el mantenimiento de la proliferación de células stem. Además, ambas vías funcionan conjuntamente para el mantenimiento de estas condiciones de pluripotencia y renovación celular *(ANEXO 3).*

Cuando tratamos con el fármaco DAPT (inhibidor de la γ-secretasa) se inhibe la vía de señalización Notch y disminuyen los marcadores de pluripotencia (Oct4, Nanog, Sox2, cMyc, Lin28, Rex1, Stella y Ssea1), además de la proliferación de las DPSCs. Por el contrario, tanto cuando usamos el fármaco BIO (inhibidor de GSK3β) como la proteína recombinante WNT-3A para sobreactivar la vía de señalización canónica Wnt/β-Catenina, observamos un aumento en la expresión de factores de pluripotencia mencionados anteriormente, así como en la proliferación celular en las DPSC. Además, los tratamientos previos con BIO y WNT-3A mejoraron la eficiencia de la diferenciación de las DPSCs hacia adipocitos y osteocitos. Estas alternativas, podrían usarse, por tanto,

con el objetivo de mejorar tratamientos de diferenciación in vitro e incluso para acelerar procesos de regeneración *in vivo (ANEXO 3).*

La manipulación experimental de las vías de señalización Notch y Wnt/β-Catenina en las DPSC, desencadena una remodelación del metabolismo, que se traduce en cambios fisiológicos importantes tanto a nivel celular como a nivel molecular. En la inhibición de la vía Notch, los cambios a nivel molecular se traducen en una disminución o paralización parcial de la vía glucolítica y una ralentización del metabolismo en las vías catabólicas primarias, lo cual se evidencia por una acumulación de glucosa y una disminución de la cantidad de especies reductoras a nivel celular. La actividad mitocondrial y la expresión génica de las principales subunidades mitocondriales no parecen verse afectadas, pero sí que observamos una disminución en el contenido total de lípidos presentes en la célula y una disminución en la expresión de los enzimas responsables de su degradación. En la activación de Wnt/β-Catenina, el aumento del consumo de glucosa por las DPSCs es evidente, así como de otras especies de aminoácidos como glutamina y glutamato, lo cual parece indicarnos una alta actividad glucolítica en estas condiciones. La incrementada producción de especies reductoras y la alta actividad mitocondrial, puede deberse a una hiperexcitación e hiperactividad del metabolismo de las DPSCs. La expresión génica de las subunidades mitocondriales parece mantenerse, sin sufrir grandes cambios, interesante desde el punto de vista funcional de la mitocondria. La hiperexcitación mitocondrial parece desembocar en el aumento en la generación de lípidos, los cuales se almacenan en el citoplasma en forma de gotas lipídicas, además de un aumento en la degradación de ácidos grasos durante la fosforilación oxidativa. Los efectos observados en el tratamiento de la vía de señalización Wnt/β-Catenina se corresponden con una remodelación del metabolismo que podría estar directamente ligada con el aumento de la troncalidad, que tiene lugar de manera simultánea en las DPSCs (*ANEXO 4*).

A nivel epigenético, también se produce una remodelación de los factores epigenéticos en condiciones de tratamiento activador de Wnt/β-Catenina en las DPSCs. La remodelación de la cromatina, acompañada con cambios en los perfiles de metilación y acetilación tanto del DNA como de las histonas parece crucial para sobrepasar la barrera epigenética que determina las capacidades de troncalidad y diferenciación celular. Es así como se podría conseguir una reprogramación celular más efectiva. La troncalidad de las DPSCS queda evidenciada por la expresión de los marcadores de pluripotencia Oct4, cMyc, Sox2 y Nanog y por el aumento del número de células dentro del ciclo celular. En

primer lugar, los niveles de metilación de DNA son menores cuando las DPSCs son tratadas con los activadores de Wnt/β-Catenina, especialmente tras la exposición con la proteína recombinante WNT-3A, que parece ser una activación más efectiva desde el punto de vista epigenético. Por el contrario, el patrón de acetilación es mayor en las DPSCs con la vía de señalización Wnt/β-Catenina sobreactivada, tanto con el fármaco BIO como con la proteína recombinante WNT-3A. En cuanto a la metilación de histonas observamos cambios en H3K27me, H3K4me3 y H3K9me3, lo que sugiere que en condiciones de activación de la via Wnt/β-Catenina, las histonas H3 sufren una mayor metilación. En cuanto a la inactivación de Notch, no observamos cambios a nivel epigenético en las células DPSC. Los cambios epigenéticos se encuentran muy ligados a los cambios en el metabolismo, por ejemplo, a nivel de acetilación de histonas a través del metabolito acetyl-Coa, tanto derivado de la glucolisis como de la oxidación de ácidos grasos. Por tanto, todos estos cambios a nivel epigenético podrían darnos alguna explicación sobre cómo cooperan el metabolismo y la epigenética para hacer efectiva la reprogramación celular (*ANEXO 5*).

En conclusión, las vías de señalización Notch y Wnt/β-Catenina, son dos vías de señalización indispensables para la expresión de factores pluripotentes y para el mantenimiento de la población de células stem. Ambas vías de señalización celular podrían ser moduladas con el fin de facilitar una reprogramación controlada y medida de las células stem dentales. Esto podría garantizar la utilización más segura de las células stem reprogramadas para su uso en clínica. De esta manera, además podrían realizarse seguimientos del estado fisiológico tanto a nivel metabólico como epigénetico de las células que hayan sufrido una reprogramación celular y determinar qué patrones podrían comprometer su fisiología y funcionalidad.

Por último, la reprogramación parcial y controlada de estas células podría utilizarse para la mejora de protocolos de diferenciación existentes en cuanto a rapidez y efectividad de obtención de poblaciones celulares concretas y además diseñar nuevos protocolos no contemplados anteriormente, bien por su complejidad o por no ser totalmente eficientes en la obtención de distintos tipos celulares.

Es por ello, que el estudio de las vías de señalización Notch y Wnt/β-Catenina, y el efecto de la modulación de ambas vías de señalización en la reprogramación genética,

metabólica y epigenética podría suponer grandes avances en la mejora de los actuales métodos de reprogramación celular.

ABSTRACT

Dental Pulp Stem Cells (DPSCs) are cells extracted from the dental pulp of third molars (wisdom teeth). During embryonic development, these stem cells migrate from neural crest as an ectomesenchymal population after undergoing an epithelial-mesenchymal transition (EMT), to form most of craniofacial tissues.

Stem cells from dental pulp present some interesting properties, which make them a unique resource of stem cells for cell therapy. Among these features, they present a high differentiation potential to different cell lineage types and tissues including bone, cartilage, muscle and adipose tissues. Moreover, these types of cells are easily obtained from patients, cultured in primary cultures and amplified long-term due to their high self-renewal capacity. As for their maintenance and cryopreservation, these stem cells require a simple methodology. Indeed, stem cells from dental pulp present immunosuppressive properties and a good adaptation to biomaterials, all of which makes them especially attractive for their use in tissue engineering *(ANEX 1)*.

Regarding DPSCs regeneration potential, it is to highlight their stemness, characterized by their high self-renewal capacity and their expression of pluripotency core factors Oct4, Nanog, Sox2, cMyc, Lin28, Rex1, Stella y Ssea1. This stemness could be enhanced by cell reprogramming. During the last few years, several methodologies have been developed like transcription factors transfection with Yamanaka vectors and Sendai virus, retrovirus or lentivirus, and the recent technology CRISPR. However, all these methodologies lead to mistakes and mutations converting transfected stem cells into potential risk to use in humans. Taking all together, the comprehensive knowledge of Notch and Wnt/β-Catenin signaling pathways would be of great interest, because they are a fundamental part of the signaling network, which regulates stemnnes and differentiation. Notch and Wnt/β-Catenin signaling pathways play a crucial role in the maintenance and differentiation of different types of stem cells. The use of recombinant proteins for the modulation of these pathways at different times and concentrations would enable a control over the maintenance of these populations of stem cells, avoiding permanent DNA alteration and mistakes in stem cells. Indeed, the modulation of stemness and pluripotency comes together with metabolism and epigenetic remodelling to maximize reprogramming efficiency. The study of all these changes in DSPCs physiology could be necessary to develop an effective, acute and safe cell therapy *(ANEX 2)*.

Notch and Wnt/β-Catenin signaling pathways play a significant role in stem cells maintenance and differentiation. Notch and Wnt/β-Catenin pathways are crucial for the expression of pluripotency stem cell factors (Oct4, Nanog, Sox2, cMyc, Lin28, Rex1, Stella y Ssea1) and the self-renewal of stem cells. Both pathways work together to maintain these conditions. When we used the treatments DAPT (γ-secretase inhibitor) for Notch inactivation, pluripotency stem cell factors were reduced and this came along with a lower self-renewal capacity. Conversely, when we treated cells with BIO (GSK3β inhibitor) and the recombinant protein WNT-3A for Wnt overactivation, both the expression of pluripotency stem cell factors and self-renewal were enhanced. Interestingly, the pre-treatment of DPSCs with BIO and WNT-3A also improved the efficiency of two differentiation media protocols to obtain osteocytes and adipocytes, respectively. This strategy could be used to enhance differentiation media protocols *in vitro* and even to accelerate regeneration processes *in vivo (ANEX 3)*.

Notch and Wnt/β-Catenin modulation result in a metabolic remodelling that includes important physiological changes. In Notch inhibition, changes at molecular level are translated into a reduction or partial blocking of glycolysis and a slower activity of the primary catabolic pathways, which was evidenced by a cellular accumulation of glucose and a decrease in cellular reducing power. Mitochondria activity was not affected, but there was a reduction in total cellular lipid content. On the contrary, during Wnt/β-Catenin overactivation, there was a clear increase in the consumption of glucose, together with other metabolites such as glutamine and glutamate. Mitochondrial metabolism was largely stimulated and the cellular lipid content was increased as well. These effects correspond to a metabolic remodelling, which is intimately related to stemness in DPSCs (*ANEX 4*).

As for the epigenetic map, we also found a clear chromatin remodelling, characterized by changes in methylation and acetylation patterns in DNA and histones after Wnt/β-Catenin activation. This seems to be crucial to overcome the epigenetic barrier, which determines cell stemness and differentiation capacities, and to achieve an effective cell reprogramming. Firstly, DNA methylation level was lower when cells were treated with Wnt/β-Catenin coactivators. Conversely, the acetylation profile is higher in Wnt/β-Catenin overactivation. Regarding histone methylation, we observed an effect in H3K27me, H3K4me3 and H3K9me3, so after these treatments HistoneH3 is more highly methylated. In Notch inhibition, we did not see any major epigenetic changes. Epigenetic

changes are linked to remodelling in metabolism and both could be affected together and regulating each other (*ANEX 5*).

Overall, Notch and Wnt/β-Catenin signaling pathways are indispensable for the expression of pluripotency stem cell factors and the self-renewal of DPSCs, and the activity of these pathways could be manipulated to control reprogramming safety of DPSCs with regard to their use in cell therapy. Moreover, a comprehensive knowledge of this pluripotency network with metabolism and epigenetics could help to find out new effective ways of reprogramming by avoiding cell damage at cellular and molecular level. A partial and controlled reprogramming of DPSCs may be also used to improve the efficiency of already existent differentiation protocols to obtain specific differentiated cell populations. This could be also interesting to facilitate the design of new cell differentiation protocols, which are presently impossible to carry out due to their complexity.

1. INTRODUCTION

1.1 DENTAL PULP STEM CELLS: CHARACTHERISTICS AND ORIGIN

Dental Pulp Stem Cells are multipotent stem cells described as a unique population of precursor cells isolated from the postnatal human dental pulp capable of regenerating a reparative dentin-like complex (Ledesma-Martinez et al., 2016).

Further, immune-suppressive and present non-tumorigenic properties of DPSCs convert them into suitable stem cells well tolerated upon grafting (Pierdomenico et al., 2005; Wilson et al., 2015). One interesting feature is that DPSCs retain the ability to differentiate into different cell types including myocytes, odontoblasts, osteoblasts, chondrocytes, adipocytes, neural progenitors, hepatocytes, cells of blood vessels and smooth muscle cells (Arthur et al., 2008; Aurrekoetxea et al., 2015; d'Aquino et al., 2009; La Noce et al., 2014; Stevens et al., 2008) (Figure 1 A).

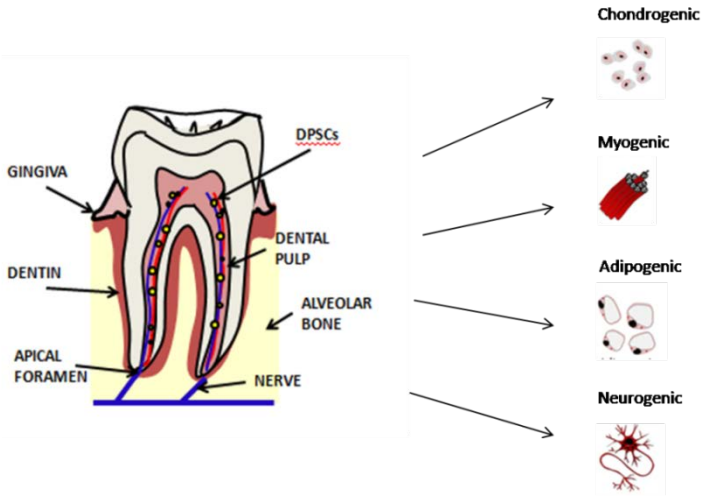


Figure 1A. Schematic representation of adult tooth. A) The tooth is placed in the gingiva and the alveolar foramen. The population of Dental Pulp Stem Cells (DPSCs) are located in areas adjacent to the nerves and blood vessels inside the dental pulp. These nerves are linked to the roots in the apical foramen. Dentin, which is secreted by odontoblasts, is placed covering the tooth and protecting it from external agents. DPSCs also remain potential to differentiate in chondrocytes, muscle cells, adipocytes and neurons.

Other stem cell types with similar properties could be obtained from gingival and periodontal tissues (Abe et al., 2012; Huang et al., 2009; Ibarretxe et al., 2012; Lima et al., 2017; Liu et al., 2015; Petrovic and Stefanovic, 2009) (Figure 1 B).

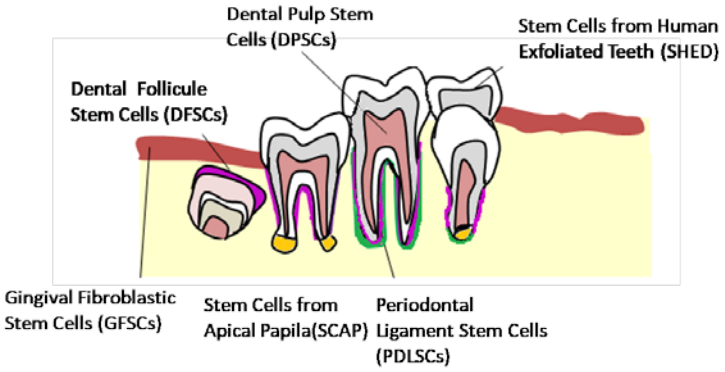


Figure 1B. Different stem cells extracted from dental tissues. Dental tissues remain different stem cells with potential for cell therapy: Dental Follicle Stem Cells (DFSCs), Dental Pulp Stem Cells (DPSCs), Stem Cells from Human Exfoliated Teeth (SHED), Gingival Fibroblastic Stem Cells (GFSCs), Stem Cells from Apical Papilla (SCAP) and Periodontal Ligament Stem Cells (PDLSCs).

As for *in vitro* characteristics, DPSCs express fibroblastic (Collagen I, Vimentin and α-SMA) and neurogenic markers (β-3Tubulin, and Nestin) and their shape reminds to neurons with long neuritis. One explanation to this would be their ectomesenchymal origin (Figure 2 A,B,C, D).Moreover, DPSCs show stem cell (Oct4 and cMyc) and cell proliferation markers (ki67 and PCNA) providing huge quantity of stem cells for transplantation (Atari et al., 2012; Ferro et al., 2012; Kerkis et al., 2006; Rosa et al., 2016)(Figure 2 E, F, G, H).

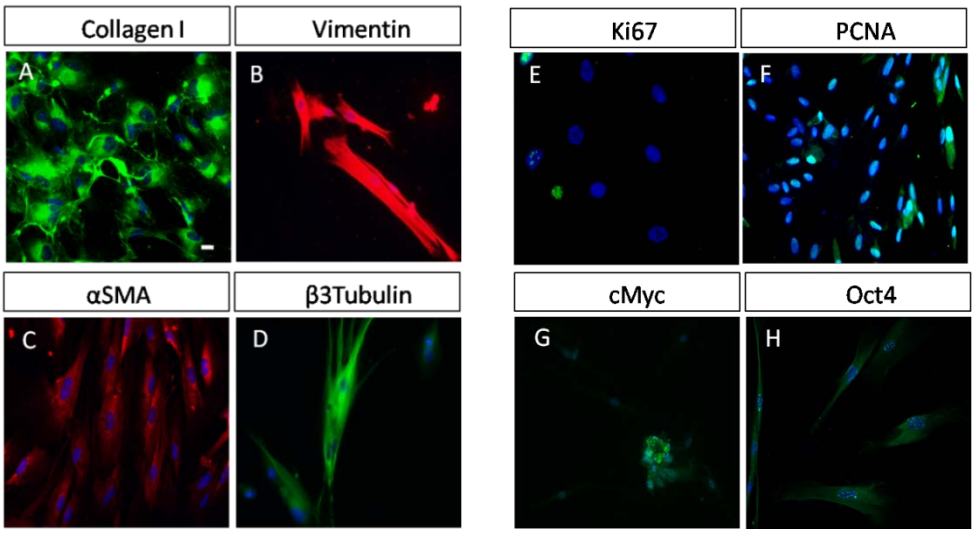


Figure 2. Characterization of DPSCs *in vitro*. A-D) DPSCs expressed mesenchymal (Collagen I, Vimentin, α-SMA) and neural markers (β-3Tubulin, Nestin) marker in basal conditions, due to their have ectomesenchymal origin. Scale bar=20 µm. **E-H)** DPSCs as proliferative cells express Ki67 and PCNA and also present pluripotency stem cells factors such as Oct4 and cMyc. Scale bar=20 µm.

Microenvironment described as extracellular scaffold, cell interactions and growth factors, is crucial during differentiation of DPSCs because these signals control stem cells orientation, polarity and type of cell division (symmetric or asymmetric) (Gattazzo et al., 2014). During embryonic development and, after the three germ layer formation (ectoderm, mesoderm and endoderm), pluripotent stem cells from embryo or Embryonic Stem Cells (EMSC), suffer several divisions and receive different signals leading to genetic determination, which occurs in humans between the 8 and 10 week of the embryo development. Then, multipotent stem cells, have all the genetic information and depend on surrounding signals to differentiate into the different type of cells that form the adult organism (Wagers et al., 2002).After neural tube fusion, a population of stem cells migrate from the neural crest and suffers the phenomena known as epithelial-mesenchymal transition (EMT). In the head, these cells migrate to generate craniofacial tissues (dental pulp and dental follicle of the teeth, muscles, bones, cartilages and even ganglion of peripheral nerves) (Aurrekoetxea et al., 2015; Gronthos et al., 2002). Consequently and because of the origin of these cells, DPSCs have a huge potential to generate dental and connective tissues (Gronthos et al., 2002).Interestingly, DPSCs formed fully differentiated adipocytes, chondrocytes and osteocytes *in vitro* after several weeks of differentiation (Grottkau et al., 2010). However, these protocols take long time of differentiation and usually are low efficient, leading to lost cells or death. Find a strategy to enhance more ratios of cell differentiation could be of suitable importance in cell therapy.

DPSCs come from the cranial neural crest (NC) and have some characteristics of neural crest progenitors(Abe et al., 2012; Janebodin et al., 2011; Miletich and Sharpe, 2004; Simoes-Costa and Bronner, 2015).In the same way that NC-derived cells, dental stem cells are characterized by the expression of neural crest factors such as Snail/Snai1, Slug/Snai2, Twist1, Hnk1, Pax3, Neurogenin2 and Sox10 (Kiraly et al., 2009; Schiraldi et al., 2012), and core factors including Oct4a, cMyc, Sox2, Klf-4, Lin28, Rex1, SSEA1 and Nanog (Atari et al., 2012; Ferro et al., 2012; Janebodin et al., 2011; Kerkis et al., 2006; Rosa et al., 2016; Uribe-Etxebarria et al., 2017). These factors could be crucial to the maintenance and self-renewal of dental stem cell populations.

1.2 PLURIPOTENCY NETWORK OF EMBRYONIC STEM CELL FACTORS WORKS THROUGH NOTCH AND WNT-βCATENIN SIGNALING.

Wnt and Notch pathway are involved in the preservation of pluripotency of both ES cells and adult stem cells, and are critical for the maintenance of the stem cell phenotype (Androutsellis-Theotokis et al., 2006; Borggrefe et al., 2016; Clevers et al., 2014; Perdigoto and Bardin, 2013; Reya and Clevers, 2005). Wnt signalling is endogenously activated in ES cells and downregulated upon differentiation (Sato et al., 2004). Wnt and Notch pathways also play an important role in the emergence of the neural crest and some of these cells differentiate into dental pulp cells(Garcia-Castro et al., 2002; Hari et al., 2012; Leung et al., 2016; Rogers et al., 2012; Stuhlmiller and Garcia-Castro, 2012). It is known that dental stem cells present higher levels of core factors and Wnt/Notch activity than do other mesenchymal stem cells in the adult body (Atari et al., 2012; Huang et al., 2009; Janebodin et al., 2011; Vasanthan et al., 2015). However, the role of these pathways in the maintenance of stemness and self-renewal in DPSCs is still unclear.

Activation of Notch signaling through ligand binding triggers proteolytic cleavage of Notch receptors, first by A-Disintegrin-And-Metalloproteases (ADAM) followed by γ-secretases, which results in the cleavage and release of the Notch intracellular domain (NICD) from the membrane. The NICD translocates to the nucleus where it directly interacts with CSL/RBPj/CBF-1 transcription factors to turn on the expression of Notch target genes such as the Hairy Enhancer of Split (Hes) family (D'Souza et al., 2010). As for Wnt signaling, three different pathways Canonical Wnt signaling Non-Canonical Wnt signaling, and Wnt signaling Ca2+ dependent form this pathway.

In Canonical Wnt signaling, the interactions between Wnt protein ligands and the proteins Frizzled/LRP receptors lead to the recruitment of AXIN, APC and GSKβ3 to the membrane, thus preventing phosphorylation and degradation of β-CATENIN protein. As a result, β-CATENIN accumulates in the cytoplasm and translocates into the nucleus, where it interacts with TCF/LEF family factors and leads to the expression of Wnt signaling target genes (Clevers, 2006). Both pathways regulate each other at multiple points (Borggrefe et al., 2016; Fukunaga-Kalabis et al., 2015)and promote the maintenance of self-renewal and inhibition of differentiation in many stem cell types, including DPSCs (Mizutani et al., 2007; Scheller et al., 2008; Yiew et al., 2017).However, Non-Canonical Wnt signaling acts independent from β-CATENIN, especially in adhesion

proteins such integrins, cadherines, among other(Adler and Taylor, 2001; Boutros et al., 1998). The third singalong is Ca2+ dependent and activates Protein Kinase C (Kuhl et al., 2000; Sheldahl et al., 1999).

Pharmacological manipulation of the Notch and Wntβ-Catenin Canonical pathways is relatively simple via the use of well-known drugs such as DAPT (γ-secretase inhibitor; Notch signalling blocker), and BIO (GSK3-β inhibitor; Wnt signaling activator), and by recombinant activator proteins such as WNT-3A. The use of these drugs BIO and DAPT has already proven to be a valuable complementary strategy to induce either cellular reprogramming or cellular differentiation (Ichida et al., 2014; Kitajima et al., 2016). Therefore, a thorough understanding of the stemness and differentiation potential of DPSCs and their modulation by cell signalling pathways would be highly desirable to apply these cells more efficiently in areas such as regenerative medicine, tissue engineering and drug screening. In this work, we used DAPT and BIO, as well as human recombinant WNT-3A as pharmacological modulators to investigate the role of Notch/Wnt in maintaining stemness and the expression of pluripotency core factors in DPSCs with the goal of optimizing existing protocols of somatic cell differentiation using DPSCs.

Some authors have attributed pluripotent markers expression to the crosstalk between Notch and Wnt signaling(Kwon et al., 2011; Uribe-Etxebarria et al., 2017). During Wnt signaling, β-Catenin goes to the nucleus, activates transcription and Wnt target genes such as Sox2, Oct4, Nanog and Jagged1, which activates Notch signaling, leading to cell proliferation. Wnt pathway is regulated by the GSK3β, which leads to β-Catenin degradation. When GSK3β is inhibited, this pathway overactivates, and as a result, β-Catenin goes permanently to the nucleus and activates Notch signaling through Jagged1overtranscription.

As for Notch signaling, cell-contact is indispensable due to that the convertases are the responsible for the two cleavages for NICD translocation to the nucleus and Notch target genes transcription. γ-secretase inhibitor DAPT prevents NICD active form from the second cleavage and therefore Notch signaling is inhibited. Moreover, β-Catenin aggregates to Notch receptor in the membrane and consequently is degraded in the proteasome. Finally, Wnt signaling activity would also be affected by Notch inhibition (Andersen et al., 2012; Hori et al., 2013) (Figure 3).

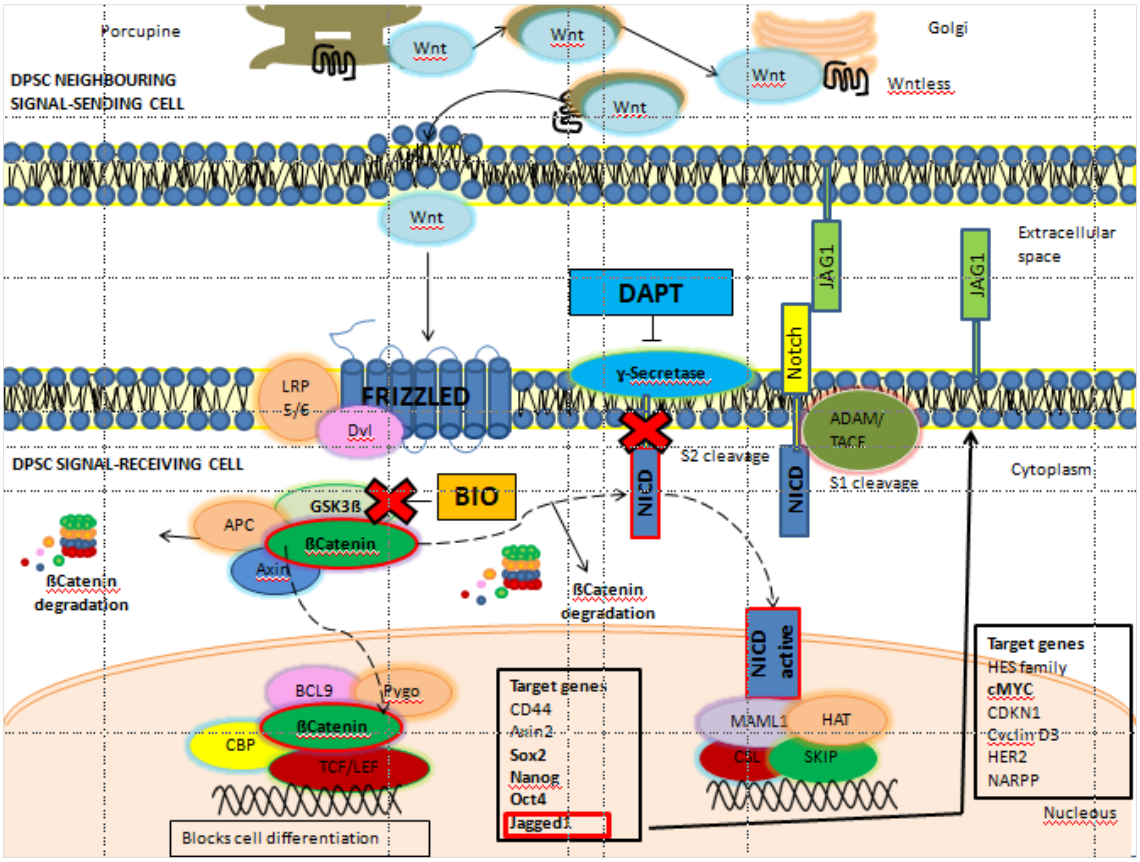


Figure 3. Notch and Wnt signaling crosstalk are responsible for pluripotency markers expression in mesenchymal stem cells (Kwon et al., 2011; Uribe-Etxebarria et al., 2017, Andersen et al., 2012; Hori et al., 2013). During Wnt signaling, β-Catenin goes to the nucleus, activates transcription factors such as Sox2, Oct4, Nanog. Indeed, express Jagged1, that activates Notch signaling and cMyc transcription. When GSK3β is inhibited, β-Catenin is continuously translocated to the nucleus and upregulates all transcription factors mentioned before. Consequently, Notch and Wnt signaling are overactivated. As for Notch signaling, the γ-secretase inhibitor DAPT prevents Notch receptor cleavage, NICD active fragment transport to the nucleus and stem cell transcription factors expression. In this inhibition Wnt signaling is also affected because Notch receptor joins to β-Catenin causing its degradation in the proteasome and therefore, Wnt inhibition. Consequently, pluripotent markers expression has been assigned to the crosstalk between Notch and Wnt signaling.

1.3 NOTCH AND WNT-βCATENIN CONTROL CELL CYCLE AND SELF-RENEWAL OF PLURIPOTENT STEM CELLS

Pluripotent stem cells (PSCs) are defined by an unlimited self-renewal capacity and an undifferentiated state. The cell cycle of PSCs is defined by a minimal time spent in G1/G0 (Coronado et al., 2013; Ghule et al., 2011; Li and Kirschner, 2014). The fundamental understanding of how the cell cycle and stemness are linked has become of crucial importance between broad disciplines including regenerative medicine, cancer biology, and aging.

As mentioned before, the pluripotency network consists of a core set of embryonic stem transcription factors (Oct4 (Pou5f1), Sox2, Nanog, Lin28, among others) that serve to establish the undifferentiated state and the self-renewing capacity of PSCs cells (Hackett and Surani, 2014; Young, 2011). It has been identified also a crosstalk of cMyc with the cell cycle genes (Kim et al., 2008; Kim et al., 2010). cMyc activity increases the rate of proliferation and reprogramming by helping to establish a pluripotent cell cycle in reprogrammed cells (Huskey et al., 2015; Kim et al., 2008; Kim et al., 2010; Lavagnolli et al., 2015; Rahl et al., 2010). cMyc work also as a target with the key pluripotency factors Oct4 and Nanog (Cartwright et al., 2005; Hishida et al., 2011; Loh et al., 2006). Other authors have described the implication between the expression of cMyc and Wnt signalling (Cartwright et al., 2005) and the relationship with pluripotency (Hishida et al., 2015; Sun et al., 1999). Because of augmentation of cell proliferation is the potentially increased accumulation of genetic mutations due to error-prone DNA synthesis. Oct4 binds to and inhibit Cdk1 resulting in an extension of G2 phase, which allows more time for the DNA repair machinery to correct mutations *de novo* (Zhao et al., 2014). Apparently, the core pluripotency network can control the cell cycle, but cell cycle regulators also control pluripotency (Abe et al., 2012; Huskey et al., 2015; Kallas et al., 2014; Neganova et al., 2014). Some studies have shown that cells under proliferation are bound to suffer reprogramming (Guo et al., 2014; Roccio et al., 2013). The understanding of the relationship between pluripotency network and the cell cycle machinery would be of great interest in regenerative medicine to develop safety cell therapies and in the study of treatments to control proliferative cancer cells.

1.4 METABOLISM REPROGRAMMING IN STEM CELLS

Cell metabolism is known to play a significant role in the production of energy and maybe be involved in the regulation of cell fate. In fact, some authors have described metabolism to be implicated in cell-fate determination and stem cell activity in different contexts (Buck et al., 2016; Gascon et al., 2016; Zhang et al., 2016a; Zhang et al., 2016b; Zheng et al., 2016). Moreover, metabolic remodelling seems to mediate the process of reprogramming. Firstly, cells that enter in a pluripotent state through reprogramming require an early metabolic switch to take place. One explanation to this is that the metabolic requirements of differentiated cells are different from highly proliferative pluripotent stem cells. The first tandem of activated genes during reprogramming corresponds to genes involved in increased proliferation and metabolic change (Cacchiarelli et al., 2015).

Classically, metabolism has been divided in oxidative, completed in the mitochondria, or non-oxidative or glycolytic, completed in the cytoplasm (Chandel et al., 2016; Ryall et al., 2015b). The expression of glycolysis-related genes and lactate production in PSCs is described to be higher than in differentiated cells, while oxygen consumption seem to decline (Armstrong et al., 2010; Folmes et al., 2011; Mathieu et al., 2014; Prigione et al., 2010; Varum et al., 2011). Moreover, it has been shown that high glucose culture medium reprograms mouse somatic cells into iPSCs more efficiently than low-glucose medium (Jang et al., 2012; Zhu et al., 2010). Thus, these findings show that high glycolysis is crucial for pluripotency; but how PSCs maintain and acquire high glycolysis levels or to what extent this metabolic behaviour affects pluripotency is still unknown. Mitochondrial activity seems to be crucial since work as a tool of Oxidative Phosphorylation or OXPHOS metabolism for energy production in cell reprogramming (Sperber et al., 2015). Finally, it has been described the formation of lipid accumulations in lipid droplets as source of energy during this remodelling (Sperber et al., 2015).

PSCs have a very specific metabolic pattern that probably reflects their rapid proliferation and the specific microenvironment from which they are derived. Although distinct metabolic properties are critical for the generation and maintenance of PSCs, it is unknown how PSCs maintain and acquire this metabolic signature. Oxidative phosphorylation or OXPHOS is used by somatic cells for energy production, but upon reprogramming, the transition to pluripotency is accompanied with a shift to a glycolytic

metabolism (Burk and Schade, 1956; Folmes et al., 2011; Panopoulos et al., 2012; Prigione et al., 2010; Varum et al., 2011). PSCs also rely on one-carbon metabolism for histone methylation and maintenance of pluripotency (Shiraki et al., 2014; Shyh-Chang et al., 2013).

Interestingly, the well-studied core pluripotency factors Oct4 and cMyc regulate glycolysis directly by targeting key glycolytic enzymes in embryonic stem cells, such as Hk2 and Pkm2 which affect Lactate, Acetate and Glucose metabolites (Folmes et al., 2013; Kim et al., 2015). Apparently, one of the keys regulators is the core pluripotent factor cMyc, which directly binds and regulates metabolic gene targets, influencing glycolysis(Kim et al., 2004; Osthus et al., 2000; Shim et al., 1997), mitochondrial biogenesis (Li et al., 2005; Zhang et al., 2007), glutamine (Gao et al., 2009) and proline catabolism (Liu et al., 2012). Lin 28 gene has been reported to play a relevant role in PSC metabolism by maintaining the low mitochondrial function and regulating one-carbon metabolism, nucleotide metabolism, and histone methylation (Zhang et al., 2016b). Interestingly, several authors have described that stem cell factors Oct4 and Lin28 regulate stem cell metabolism and conversion to pluripotency, and form a complex core pluripotent factor network with other core factors such as Sox2 and Nanog(Matoba et al., 2006; Zhang et al., 2016b). Further research is necessary to understand all these process that governs metabolism remodelling through pluripotency core factors.

Research over the last years has identified an essential role for metabolites in the regulation of epigenetics and transcription, including S-adenosyl methionine (SAM) produced via the one-carbon cycle, acetyl-CoA from glycolysis, α–ketoglutarate (α-KG) and flavin adenine dinucleotide (FAD) from the NAD+/TCA cycle, and also from the integration of glycolysis and OXPHOS(Ryall et al., 2015a; Ryall et al., 2015b). SAM leads to histone methylation whereas ascorbate and a-ketoglutarate are the responsible for histone demethylation (Carey et al., 2015; Shyh-Chang et al., 2013).

It has been shown that the lack of acetyl-CoA causes a loss of pluripotency and delays cell differentiation (Moussaieff et al., 2015). Apparently, the reason besides is that acetyl-Coa is crucial to regulate and maintain histone acetylation in stem cells allowing chromatin remodelling during differentiation (Moussaieff et al., 2015).

The mechanism by which cellular metabolism can influence stem cell fate is mostly unknown, but it is clear that it influences the epigenetic landscape, which in turn affects

gene expression (Harvey et al., 2016). According to these findings, some metabolic intermediates may contribute in the regulation of open chromatin, which is crucial for pluripotent potential of stem cells. Therefore, it would be of great interest understanding how metabolism contribute to pluripotency state and how is linked to epigenetics.

1.5 CELL REPGROGRAMMING NEEDS EPIGENETIC REMODELING OF STEM CELLS

It is known that at early stages during embryonic development, some genes remain active, but when differentiation goes on (later stages) gradually become silenced and subsets of cell type-specific genes are turned on. This is the result of selectivity active expression of transcription factors in association with chromatin modification and remodelling, which includes covalent histone modification and DNA methylation of CpG dinucleotides, among other epigenetic modifications.

Gene activity is mostly determined by chromatin structure and interactions of chromatin-binding proteins. Histone deacetylase inhibitors (HDACi) play a significant role in homeostatic balance between histone acetylation and deacetylation, increasing the transcriptional rate and influencing cell physiology. That suggests that inhibition of HDACs has a great potential in restorative dentistry through elevating the expression of NANOG and SOX2 (Gu et al., 2013; Jin et al., 2013). Regarding histone methylation , histone demethylases or HMTs inactivation associates with reduced mineralization of DPSCs and genes involved in odontoblast differentiation (Rodas-Junco et al., 2017). Therefore, epigenetic changes play a crucial role in the terminal differentiation of odontogenic lineages in DPSCs. As an example, the inhibition of eZH2, enzyme which catalyses H3K27me3 methylation (Hui et al., 2014), impedes DPSCs cell proliferation by decreasing cell number, arresting the cell cycle, and increasing apoptosis (Bayarsaihan, 2016). Accordingly, adipogenic induction of DPSCs leads to the suppression of eZH2, which in turn diminishes transcriptional activity of PPAR-r and CEBP/a differentiation markers (Bayarsaihan, 2016). As for global DNA methylation, the odontogenic-specific DNA hypomethylation was suggested to be a typical feature of DPSCs, and the spatial distribution of 5mC and 5hmC in DPSCs is still unknown (Ai et al., 2018). In concordance to previous studies, DNA demethylation have been shown to increase the capability of odontogenic differentiation in DPSCs(Bayarsaihan, 2016). During DPSCs differentiation, the main key is the DNA methyltransferase, DNMT1, whereas expression

changes of DNMT3A, DNMT3B and members of the TET family were relatively low. Taking all before into consideration, it seems that DNA demethylation may play a fundamental role in reparative dentinogenesis in human dental pulp (Bayarsaihan, 2016).

Since Yamanaka findings about key transcription factors of pluripotency, increasing knowledge has been recruited in several studies to understand the cooperation between pluripotency stem cells factors and the remodeling of epigenetics during cell reprogramming.

The most studied embryonic stem cell factors Oct4, cMyc, Nanog, Sox2, Klf4 and Lin28 have been widen in a complex network form by other factors such as Rex1, Ssea1, Stella, which interact with epigenetic landscape(Boyer et al., 2005; Ivanova et al., 2006; Loh et al., 2006; Rao and Orkin, 2006).cMyc may be associated with histone acetyltransferase (HAT) complex, and induces global histone acetylation. Indeed, in ESCs chromatin architecture Oct4 acts directly regulating downstream target genes encoding H3K9 HDACs which modulate the H3K9 methylation status of the pluripotency factors, Nanog among others, respectively, to maintain stem cell identity (Loh et al., 2007).

Moreover, pluripotency factors interact between each other building a complex network. Nanog acts as a transcription repressor for genes that are important for cell differentiation (Chambers et al., 2003; Mitsui et al., 2003). Klf4 might contribute to activation of Nanog and other ES cells-specific genes (Takahashi and Yamanaka, 2006). Oct4 also interacts as part of the Nanog interactome (Wang et al., 2006) and with Sox2 (Navarro et al., 2008)to maintain pluripotency (Endoh et al., 2008).

These all factors induce somatic cell de-differentiation thanks to the chromatin remodelling. Therefore, we could attribute chromatin modification because of these transcription factors, which may play an important role in the reprogramming progress.

1

frontiers in Physiology REVIEW

Front Physiol. 2015 Oct 16;6:289. doi: 10.3389/fphys.2015.00289. eCollection 2015.

Dental pulp stem cells as a multifaceted tool for bioengineering and the regeneration of craniomaxillofacial tissues

Maitane Aurrekoetxea*, Patricia García-Gallastegui*, Igor Irastorza, Jon Luzuriaga, Verónica Uribe-Etxebarria, Fernando Unda and Gaskon Ibarretxe*.

Department of Cell Biology and Histology, Faculty of Medicine and Dentistry, University of the Basque Country UPV/EHU, Leioa, Spain

ABSTRACT

Dental pulp stem cells, or DPSC, are neural crest-derived cells with an outstanding capacity to differentiate along multiple cell lineages of interest for cell therapy. In particular, highly efficient osteo/dentinogenic differentiation of DPSC can be achieved using simple in vitro protocols, making these cells a very attractive and promising tool for the future treatment of dental and periodontal diseases. Among craniomaxillofacial organs, the tooth and salivary gland are two such cases in which complete regeneration by tissue engineering using DPSC appears to be possible, as research over the last decade has made substantial progress in experimental models of partial or total regeneration of both organs, by cell recombination technology. Moreover, DPSC seem to be a particularly good choice for the regeneration of nerve tissues, including injured or transected cranial nerves. In this context, the oral cavity appears to be an excellent testing ground for new regenerative therapies using DPSC. However, many issues and challenges need yet to be addressed before these cells can be

employed in clinical therapy. In this review, we point out some important aspects on the biology of DPSC with regard to their use for the reconstruction of different craniomaxillofacial tissues and organs, with special emphasis on cranial bones, nerves, teeth, and salivary glands. We suggest new ideas and strategies to fully exploit the capacities of DPSC for bioengineering of the aforementioned tissues.

Keywords: DPSC, differentiation, tooth, bone, salivary gland, nerve, cell therapy

INTRODUCTION: DPSC AND TISSUE ENGINEERING OF THE ORAL CAVITY

The oral cavity is a complex multi-organic structure. Because oral tissues and organs are functionally connected at many levels, irreversible damage to any of the mislikely to eventually affect the others, causing extensive malfunction. Tooth decay, periodontal disease, alveolar bone resorption, orthodontic problems, orofacial neuropathic pain, and impaired salivary gland function are conditions that seriously affect oral health of a large part of the world population. Owing to their functional connectivity, once damage is diagnosed tone organ of the oral cavity it is important to intervene rapidly and efficiently, to repair or replace the injured or lost tissues, to avoid severe degradation of oral health. Synthetic replacement materials and prostheses (fillings, bridges, implants, etc.) have traditionally been the treatment of choice to treat dental decay. However, all functions of the original biological tooth are not fully restored by this kind of replacement therapies. Other organs of the oral cavity (e.g., nerves, salivary glands) are simply not amenable to mechanical substitution approaches. Thus, tissue engineering represents a new collection of treatment options for the complete biological regeneration of craniomaxillofacial tissues and organs. The development of this field requires three essential components: (i) stem cells, (ii) biomaterial scaffolds, and (iii) stimulating factors or inductive signals. Tissue engineering is now fully considered as an alternative to the conventional treatments for dental injury and disease, offering substantial advantages over traditional dental restoration techniques (Nör, 2006; Wang et al., 2012).

Stem cells are the cornerstone of regenerative cell therapy. An enormous variety of multipotent stem cells have been isolated and studied from different human tissues, such as the bone marrow (Ding and Morrison, 2013), adipose tissue (Kapur et al., 2015), skin (Blanpain and Fuchs, 2009), and the umbilical cord (Yan et al., 2013; Kalaszczynska and Ferdin, 2015). Among them, mesenchymal stem cells (MSC) are the most promising for clinical purposes (Rastegar et al., 2010; Ménardand Tarte, 2013).In the oral cavity, adult tooth tissues also contain different active populations of stem cells with mesenchymal phenotype (Huang et al., 2009). Unlike other types of MSC, dental stem

cells originate from the neural crest (Janebodinetal.,2011) and are lineage related with peripheral nerve glial progenitor cells(Kaukuaetal.,2014), which places them in a privileged position to mediate regeneration of both connective and nerve tissues (Ibarretxe et al., 2012; Martens et al., 2013). Several types of human dental stem cells have been identified. Among them; DFPC, Dental Follicle progenitor cells; PDLSC, Periodontal ligament stem cell; SCAP, Stem cells from apical papilla; SHED, Stem cells from primary exfoliated deciduous teeth; DPSC, Dental pulp stem cells, which are without a doubt the most used for research purposes (Figure 1). Although it still remains a considerable challenge to obtain all the different types of neuronal and glial cells from DPSC, their differentiation to specialized connective tissue cells has proven to be relatively simple. Efficient protocols for obtaining adipocytes, chondroblasts and osteo/odontoblasts are firmly established in the literature (Gronthos et al., 2002; Huang et al., 2009; Kawashima, 2012).

In this review, we summarize the current understanding of DPSC and their capacity to regenerate injured orofacial tissues, with special focus on the teeth and associated periodontal tissues, peripheral cranial nerves, and the salivary glands. We present the different strategies used in experimental restorative models with potential applications in dentistry and oral medicine, together with the different scaffold biomaterials and stimulating factor combinations employed to elicit an optimal cellular response required to regenerate damaged tissues. We critically comment on some crucial aspects to be considered for bioengineering and the regeneration of each of these tissues, based on our own research experience.

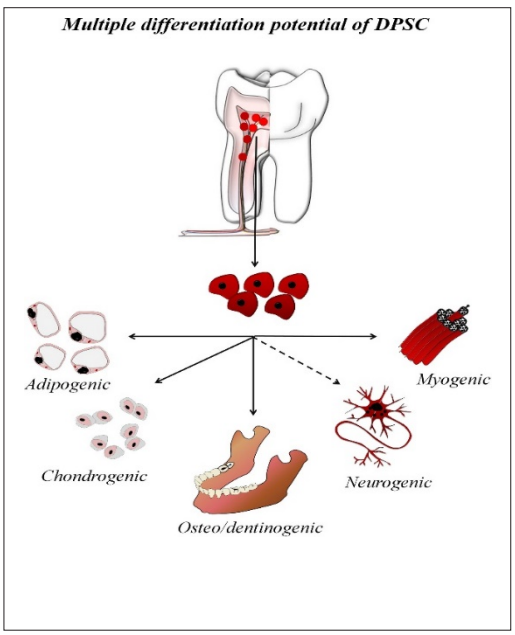


Figure 1. Multiple differentiation potential of DPSC. DPSC are isolated from the adult dental pulp and can be in vitro expanded and differentiated to multiple cell lineages, including all connective tissue-lineages and some neural cell lineages.

DPSC FOR BONE REGENERATION

Regeneration of maxillary and mandibular bone is fundamental in the field of implantology (Lee et al., 2014). Osteogenic differentiation of DPSC is easily induced in vitro by adding ascorbic acid (Asc), dexamethasone (Dexa), and β-glycerophosphate (β-gly) to the culture medium, along with fetal bovine serum (Laino et al., 2005; Langenbach and Handschel, 2013). Asc is an essential enzyme cofactor to generate pro- collagen (Vater et al., 2011), which is necessary for the correct synthesis of Collagen type I, the main organic component of the bone matrix. Dexa is required for osteoblast differentiation, working as Runx2 inductor by activating WNT/β-catenin signaling in MSC (Hamidouche et al., 2008). Runx2 transcription factor expression is fundamental in driving cellular commitment to osteogenic lineages. Finally, β-gly is a donor of inorganic phosphate, which is essential for creating hydroxyapatite mineral, and as a signaling molecule to induce DPSC osteo- differentiation (Foster et al., 2006;

Fatherazi et al., 2009; Tada et al., 2011).

The application of this differentiation cocktail is highly effective in inducing DPSC to generate a mineralized bone-like extracellular matrix in vitro. When DPSC are exposed to this differentiation cocktail for 7–21 days, they start to strongly express different osteoblast differentiation markers, such as Osterix, Runx2, Osteocalcin, and Bone Sialoprotein. Alkaline phosphatase (ALP), and Alizarin red staining are positive under these conditions, and are used to demonstrate effective differentiation of DPSC to functional osteoblasts, and the generation of a calcified hydroxyapatite (HA)-containing extracellular matrix (Mangano et al., 2010; Yu et al., 2010; Li et al., 2011). DPSC-derived osteoblasts are phenotypically and functionally similar to normal primary osteoblasts, although some differences remain in terms of their gene expression profiles (Carinci et al., 2008). Naïve undifferentiated DPSC are negative for the adult osteo/odontoblast differentiation marker Osterix, but exhibit detectable levels of Runx2, suggesting they are primed or pre-committed to differentiate to osteo/odontoblasts (Yu et al., 2010). When the osteogenic differentiation cocktail is added to DPSC, Runx2, and Osterix expression increase and these cells generate extracellular calcified bone-like matrix nodules, that stain distinctively and positively with Alizarin red (Laino et al., 2005; Figure 2).

In the last few years, the use of DPSC for in vivo bone regeneration by tissue engineering has been extensively studied by relying on these protocols and using different experimental models and types of scaffolds with different results (Morad et al., 2013). Woven bone chips obtained by human DPSC (hDPSC) osteoinduction are able to induce the generation of adult bone tissue with an integral blood supply, when they are heterotopically transplanted in immune-compromised (IC) animals (d'Aquino et al., 2007). However, the use of scaffolding materials is often necessary to optimize the 3D structure of the formed bone tissue and enhance the osteoblastic differentiation of hDPSC. To this end, different materials have been successfully employed as a vehicle of

hDPSC, including 3D gelatin scaffolds (Li et al., 2011), self-assembling biodegradable peptide nanofiber hydrogels (Chan et al., 2011), CaP/PLGA (calcium phosphate/polylactic-co-glycolic acid) scaffolds (Graziano et al., 2008a,b), particulate hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic scaffolds (Zhang et al., 2008), and natural scaffolds like biocoral (Mangano et al., 2011) and chitosan/collagen (Yang et al., 2011).Finally, alginate and PuraMatrix™ hydrogels also provide a three dimensional (3D) biodegradable carrier of stem cells for bone tissue engineering purposes, with the additional large advantage that these formulations are injectable, forming 3D hydrogels upon contact with the physiological environment and thus completely filling the tissue lesion, with minimally invasive dental and orthopedic surgeries. Dental stem cells encapsulated in either alginate or PuraMatrix™ exhibited high capacities for osteo-differentiation both in vitro and in vivo (Misawa et al., 2006; Cavalcanti et al., 2013; Moshaverinia et al., 2013).

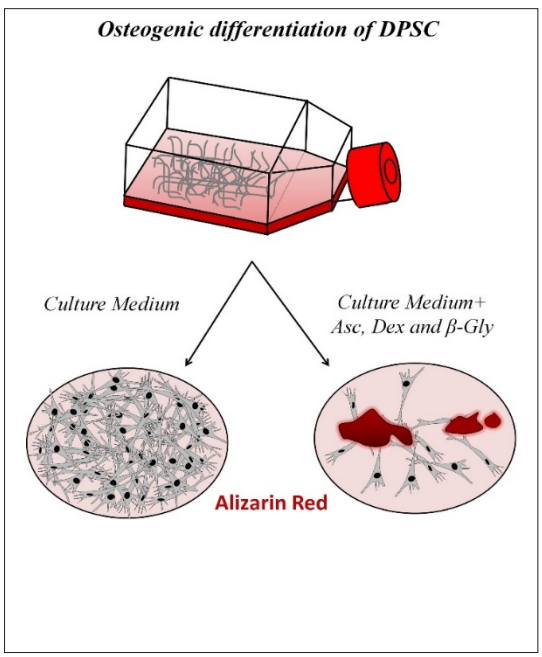


Figure 2. Osteogenic differentiation of DPSC. In the presence of a three-ingredient cocktail containing dexamethasone, β-glycerophosphate and Ascorbate added to the culture medium for 7days, DPSC differentiate to osteoblast-like cells that generate a mineralized bone tissue matrix that stains positively for Alizarin red.

Inductive signals also play a critical role to stimulate development of new bone tissue. The development of a well- defined, safe, and controlled technique to obtain and locally deliver growth factors derived from autologous plasma has provided a powerful tool to enhance bone tissue regeneration, which has been successfully implemented in the clinical practice (Anitua et al., 2008, 2012). Formulations such as Platelet-rich plasma (PRP), Platelet-rich fibrin (PRF), and Plasma rich in growth factors (PRGF) provide a biologically enriched culture medium for the stimulation and functional differentiation of primary cells with latent regenerative potential. Several studies have shown that PRGF in particular provides additional benefits for bone regeneration (Paknejad et al., 2012; Rivera et al., 2013). It has been reported that PRP and PRGF induce proliferation and enhance osteogenic differentiation in SHEDs, DPSCs, and PDLSCs (Lee et al., 2011). Other studies have used recombinant human Bone morphogenetic protein 2 (rhBMP-2) to enhance osteogenic differentiation of DPSC in animal models of alveolar bone reconstruction (Liu et al., 2011). The use of rhBMP-2 is approved and widely extended to perform maxillary sinus floor augmentation in dental practice (Nazirkar et al., 2014; Freitas et al., 2015).

Despite a large body of preclinical evidence, very few clinical trials have still been performed to evaluate new advanced therapeutic medicinal products (ATMP) for bone regeneration based on DPSC (La Noce et al., 2014). A notable exception is one clinical trial performed with seven patients to repair mandibular bone defects by transplant of DPSC in a collagen scaffold (d'Aquino et al., 2009). After 3 years of follow-up, the clinical outcomes were positive (Giuliani et al., 2013). However, several obstacles remain, including a changing regulatory framework, a shortage of technology for automation of cell production by Good manufacturing practice (GMP) standards, a non-alignment

between academic and industrial research, and a need for long- term product follow-up (La Noce et al., 2014; Leijten et al., 2015). These limitations have hampered the emergence of DPSC as a tool to enhance bone regeneration at clinical level.

DPSC FOR DENTAL PULP REGENERATION

Caries and root fracture are the cause of approximately fifty percent of all tooth extractions. Tooth injuries such as pulp exposure, deep caries and irreversible pulpitis are currently treated by endodontics and dental refilling/reconstruction. This procedure usually entails the amputation or entire removal of the dental pulp and replacement of the tissue with an inert material. Root fracture and tooth loss are closely related to this loss of pulp vitality because innervation and vasculature affect pulp homeostasis and root dentin regeneration. The regeneration of pulp injuries has become a goal for functional tooth restoration in dentistry (Nakashima and Akamine, 2005; Nakashima et al., 2009).

The first research reports on DPSC showed that these cells present optimal characteristics to attain functional regeneration of the dental-pulp chamber. Xenogenic transplant of HA/TCP scaffolds with hDPSC into the dermis of IC mice resulted in the formation of pulp-like tissue and polarized odontoblasts that produced tubular dentin, showing that dentinogenic differentiation was one of the prime or default programs established in the DPSC phenotype (Gronthos et al., 2000, 2002). Recently, more refined studies combining SHED with injectable scaffolds (Puramatrix™ and rhCollagen type I) have demonstrated that dental stem cells are not only kept alive when injected into human premolar root canals in vivo, but are also able to fully reconstruct vascularized pulp tissue throughout the root canal, and to differentiate to DSPP and DMP-expressing odontoblasts that generate new dentin (Rosa et al., 2013).In a similar experimental design, other authors employed combinations of DPSC and umbilical vein endothelial cells embedded in Parametric™ scaffold, to improve

vascularization and angiogenesis of de novo formed dental pulp-like tissue (Dissanayaka et al., 2015).

Other studies have focused on the regenerative potential of different cytokines and growth factors, with the goal to improve cellular chemotaxis and cell homing into the emptied dental pulp space in vivo. Different combinations of cytokines and growth factors which included basic Fibroblast growth factor (bFGF), Platelet derived growth factor (PDGF), or Vascular endothelial growth factor (VEGF) in the presence of basal levels of Nerve growth factor (NGF) and BMP-7 were effective in regenerating a revascularized and recellularized dental pulp-like tissue integrated into endodontically-treated root canal dentinal walls in vivo (Kim et al., 2010).

In addition, it has been shown that bFGF and Stromal-derived factor (SDF1) also exert a potent chemotactic recruitment on DPSC, while BMP-7 induces their differentiation (Suzuki et al., 2011). In a recent study, SDF1 loaded onto silk-fibroin scaffolds promoted the complete regeneration and revascularization of the dental pulp of mature dog teeth that had been subjected to endodontic treatment in situ (Yang et al., 2015), suggesting a promising future for the functional reconstruction of dental pulp tissue in next generation dentistry.

DPSC FOR COMPLETE DENTAL REGENERATION

In 2009, a ground-breaking report was published by the group of T. Tsuji on the generation of complete and fully functional teeth by combining isolated mouse dental epithelial and MSC (Ikeda et al., 2009). More recently, in 2011, the same research group developed a complete tooth unit consisting of a mature tooth, periodontal ligament and alveolar bone, using a similar recombination technique (Oshima et al., 2011). These tooth units were transplanted into toothless mouse jaw regions in vivo, and erupted correctly and in occlusion. They also presented an adequate dental structure, an adequate hardness of enamel and dentin, a proper function of the periodontal ligament, a positive alveolar bone remodeling

response to orthodontic forces, and a positive response to noxious stimuli such as mechanical stress and pain.

Although precise replication of these results has not yet been reported on the literature, the aforementioned work represents a substantial advance in stem cell combination technology, with the potential to develop completely new bioengineered replacement organs for next generation regenerative therapy. One of the main limitations to date is probably the lack of consistent sources of epithelial cells that can be combined with dental mesenchymal cells (DPSC or others) to generate a complete bioengineered tooth germ. The team of T. Tsuji employed embryonic mouse tooth germ epithelia as precursors of the enamel organ, a structure that fully disappears in adult teeth. In the search for dental-competent sources of epithelial stem cells, some authors have reported success using adult gingival epithelial cells, which upon recombination with mesenchymal cells generate mature teeth and form enamel and dentin (Angelova Volponi et al., 2013). Other authors have employed iPSCs-derived cells to generate mature bioengineered teeth by similar recombination methods (Wen et al., 2012; Cai et al., 2013; Otsu et al., 2014). The substantial progress made in the field of dental bioengineering warrants further research on this line for the next years.

DPSC FOR REGENERATION OF NERVE TISSUE AND DAMAGED CRANIALNERVES

DSPC originate from the neural crest (Janebodin et al., 2011) and share a common origin with peripheral nerve glial progenitor cells (Kaukua et al., 2014). These features make DPSC a very interesting choice for regeneration of the peripheral nervous system, including nerves of the oral cavity. Some reports claim that DPSC can even differentiate to functionally active adult neurons (Arthur et al., 2008; Kiraly et al., 2009; Gervois et al., 2015). These conclusions are based on the acquisition of neuron- specific gene and protein markers by

DPSC, and also on their capacity to generate neuronal-like voltage-dependent sodium and calcium currents, and action potential-like membrane voltage oscillations. However, it should be noted that to date there has been no definite proof of differentiation of DPSC to genuine neurons that can exhibit repetitive firing of action potentials upon electrical stimulation, and establish synapses showing functional plasticity as identified by transmission electron microscopy (TEM).

However, it is undeniable that DPSC present some striking similarities with neural stem cells. When DPSC are grown in culture conditions lacking fetal serum, they reorganize morphologically and switch from a uniform cell monolayer to a more quiescent state characterized by the appearance of prominent spheroid structures that resemble CNS-derived neurospheres which stain positively for the neural stem cell marker Nestin (Ibarretxe et al., 2012; Bonnamain et al., 2013; Xiao and Tsutsui, 2013; Gervois et al., 2015; Figure 3). Migratory cells are occasionally observed to leave the DPSC spheroids and virtually all these cells express the neuron-lineage marker β3-tubulin. Moreover, their morphology is in some cases surprisingly similar to migratory neuroblasts, with a long and thin leading process terminated by a prominent growth cone, and a trailing cell body displaying a few short cytoplasmic processes (Marín et al., 2006). However, a note of caution should be added: another cell type of the dental pulp, the odontoblast, is also characterized by the expression of Nestin, the generation of voltage-dependent Na+ currents, and a similar morphology (Fujita et al., 2006; Ichikawa et al., 2012). The DPSC population grown in these conditions is fairly heterogeneous, both morphologically and physiologically, with a range of different morphologies from fibroblast-like to neuroblast-like cells, and also a variable rate of response to stimulation with neurotransmitter receptor agonists.

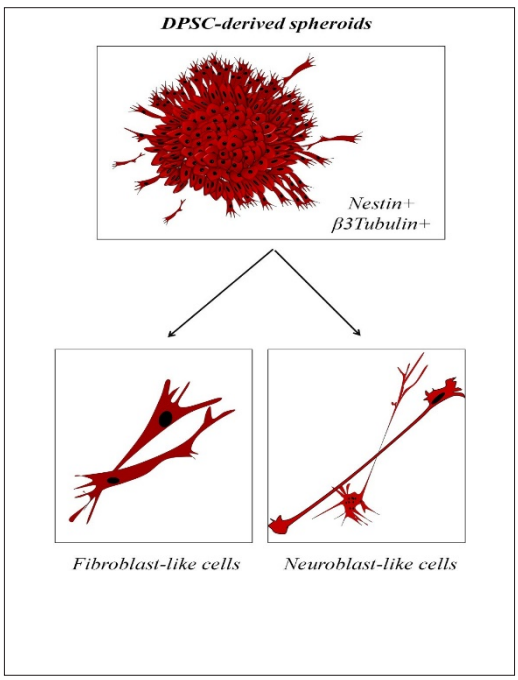


Figure 3. Neurosphere-like properties of DPSC-derived spheroids. DPSC grown in serum-devoid conditions rearrange to form characteristic spheroids that stain positive for neural stem cell markers. Migratory cells outside of the spheroids express some neuronal markers and present a variable morphology, with either fibroblast-like or neuroblast-like features.

Regardless of the debate on the true capacity of DPSC to differentiate to neurons and other neural cell lineages, there is little doubt that these cells could be a very interesting choice to repair injured nerve tissues as a result of their active secretion of neurotrophic and immune-modulatory factors (Nosrat et al., 2004; Pierdomenico et al., 2005). The high expression of certain neural markers and neurotransmitter receptors by DPSC also suggests that these cells may actively respond to signals of the neural environment and effectively integrate into injured nerve tissues, promoting the reestablishment of functional nerve connectivity (Arthur et al., 2009; Kadar et al., 2009; Kiraly et al., 2009). For instance, some recent reports show that transplanted DPSC are able to differentiate in vivo to myelinating cells in the spinal cord, replacing lost cells and promoting regeneration of transected axons in acute models of spinal cord injury (Sakai et

al., 2012).

In the case of the peripheral nerve system, treatment of nerve injuries represents a clinical challenge because of the difficulties of regenerating transected nerves. Though numerous surgical successes have been reported with a short nerve gap, there is still no satisfactory technique for long nerve defects, which often require a complex clinical reconstruction. Autologous nerve grafting has been considered the gold standard for repairing peripheral nerve gaps caused by traffic accidents or tumor resectioning (Kumar and Hassan, 2002; Hayashi and Maruyama, 2005; Bae et al., 2006). However, this technique has inevitable disadvantages, such as a limited supply of available nerve grafts and permanent loss of the sacrificed donor nerve function. Brain- derived neural progenitor cells also promote regeneration of transected nerves (Murakami et al., 2003). However, the use of cells from other neural tissues involves potentially serious clinical complications along with ethical considerations.

Taking all these arguments into account, there is an active search for new sources of cells to be used in craniofacial nerve bridging and regeneration. Considerable advances have been made in this field using DPSC for the treatment of facial nerve injuries. Particularly, patients with facial paralysis, especially younger ones, may experience tremendous psychosocial distress about their condition (Chan and Byrne, 2011). Recent studies have used DPSC transplanted in PLGA tube scaffolds to achieve a complete functional regeneration of the facial nerve in rats to recovery levels comparable to those obtained with peripheral nerve autografts (Sasaki et al., 2011, 2014). Interestingly, recent research also indicates that hDPSC can be differentiated to Schwann-like cells that efficiently myelinate DRG neuron axons in vitro, a finding confirmed by ultrastructural TEM analysis (Martens et al., 2014). Considering the important role that Schwann cells play in axonal protection and regeneration of peripheral nerves (Walsh and Midha, 2009), and the difficulty of their harvesting and maintenance, the generation of DPSC- derived autologous Schwann cells may represent a milestone in the design

of new treatments for conditions of peripheral nerve injury, including facial paralysis.

Finally, another important property of DPSC is their active secretion of neurotrophic factors (Nosrat et al., 2004; Bray et al., 2014), which may be exploited to treat neuropathic pain states associated with peripheral nerve injury. In the case of orofacial pain, some of the most distressing and painful conditions that can be experienced by a human being are neuralgias affecting the trigeminal nerve, or CN V. These are characterized by intense stabbing pain and spasms, usually associated with a mechanical injury, compression, demyelination and inflammation of trigeminal sensory fibers (Love and Coakham, 2001; Sabalys et al., 2013). It is known that local application of Glial derived neurotrophic factor (GDNF) exerts a potent analgesic effect and reverses the symptoms associated with neuropathic pain (Boucher et al., 2000). Because DPSC secrete important amounts of GDNF, it is conceivable that autologous DPSC transplantation to the injured trigeminal nerve may also potentially provide important benefits to treat severe orofacial pain disorders, alone or in combination with pharmacological enhancers of GDNF signaling (Hedstrom et al., 2014), some of which are even formulated to permit topical application.

DPSC FOR SALIVARY GLAND REGENERATION

Salivary gland regeneration is an area of intense research because the most common treatment for head and neck cancer (i.e., radiotherapy) irreversibly affects salivary gland function, causing hyposalivation and xerostomia. In consequence, difficulty in mastication, swallowing, taste, speech, rampant dental caries and mucosa infections appear, leading to high discomfort in patients and a reduced quality of life (Vissink et al., 2010). Palliative treatments such as mechanical stimulation of salivary gland activity (chewing gum), or pharmacological agents such as pilocarpine (Salagen™), as well as saliva substitutes, show a limited efficacy in relieving the symptoms (Taylor, 2003).

Tissue engineering of a salivary gland replacement organ would be a conclusive way to

treat these patients. To accomplish this complex task, there are some issues that must be taken into account: (i) optimization of the 3D scaffold material properties (including non-toxicity, permeability and biodegradability, among others), (ii) identification of an epithelial stem cell population capable of appropriate salivary differentiation (iii) definition of ideal culture conditions and extracellular matrix components (ECM) with laminin, integrins, MMPs to mimic the microenvironment of the gland, and (iv) selection of a population of MSC to be combined with salivary epithelial cells to generate a full bioengineered salivary gland germ. Finally, in comparison with dental bioengineering, salivary gland bioengineering presents an important additional requirement: apart from the communication between the epithelium and the mesenchyme, epithelial-axonal interactions are also required from the very beginning of salivary gland morphogenesis, because innervation plays an important role in regulating the activity of epithelial progenitor cells (Knox et al., 2010) and salivary gland branching and tubulogenesis (Nedvetsky et al., 2014). Therefore, salivary gland bioengineering strategies must also take into account that this is an organ that heavily relies on the establishment of a functional innervation to properly complete its development (Lombaert et al., 2011; Knosp et al., 2012).

Some studies indicate that isolated mouse salivary gland stem cells (cKit+, CK14+, Msi-1+, and Sca-1+) can grow and form salispheres that produce amylase (Lombaert et al., 2008). Moreover, injection of a liquid suspension containing only 300–400 cKit+ cells into the mouse SMG was shown to partially rescue the loss of morphology and function induced by irradiation insults (Lombaert et al., 2008; Coppes and Stokman, 2011; Nanduri et al., 2013). In recent years, the technology to generate salivary gland organoids in vitro has progressed remarkably. Recent major advances in the field include the advent of bioengineered glands constructed from dissociated cells from adult and fetal salivary glands (Ogawa et al., 2013; Nanduri et al., 2014). An important study by the group of T. Tsuji showed that the bioengineered salivary glands generated by a cell recombination protocol contained epithelium, mesenchyme, endothelium, and nerve cells. Most importantly, the entire organ could be transplanted into adult mice, reconnect with the existing ductal system and function properly (Ogawa et al., 2013; Ogawa and

Tsuji, 2015). Thus, the salivary gland joins the tooth in the list of organs that can be fully generated ex vivo by tissue engineering. As in the case of the tooth, the main challenge to translate these findings to clinical practice is still the need for sources of stem cells that are sufficiently abundant and competent for salivary epithelial differentiation, and that do not involve destruction of donor salivary gland tissue.

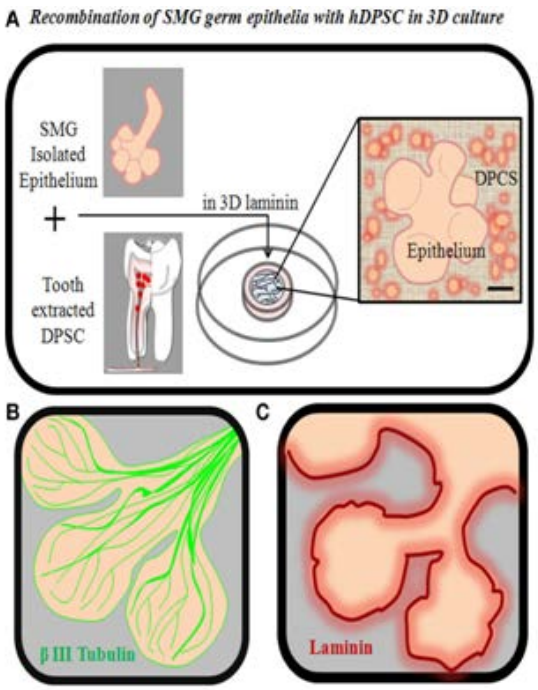


Figure 4. Recombination of salivary gland germ epithelia with DPSC in 3D culture.(A) SMG epithelia can be mechanically and enzymatically isolated from SMG mesenchyme, and recombined with hDPSC in 3D laminin scaffolds to generate a bioengineered salivary gland germ (B) SMG acini from E14.5mouse, showing the extensive natural innervations of the SMG by β3-tubulin + nerve fiber sat this developmental stage. (C) SMG acini from E14.5mouse, showing the natural localization of lamina in the basal lamina that separates the salivary epithelium and mesenchyme.

REFERENCES

Angelova Volponi, A., Kawasaki, M., and Sharpe, P. T. (2013). Adult Human Gingival Epithelial Cells as a Source for Whole-tooth Bioengineering. J. Dent. Res. 92, 329–334. doi: 10.1177/0022034513481041

Anitua, E., Alkhraisat, M. H., and Orive, G. (2012). Perspectives and challenges in regenerative medicine using plasma rich in growth factors. J. Control. Release 157, 29–38. doi: 10.1016/j.jconrel.2011.07.004

Anitua, E., Sánchez, M., Orive, G., and Andia, I. (2008). Delivering growth factors for therapeutics. Trends Pharmacol. Sci. 29, 37–41. doi: 10.1016/j.tips.2007.10.010

Arthur, A., Rychkov, G., Shi, S., Koblar, S. A., and Gronthos, S. (2008). Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues. Stem Cells 26, 1787–1795. doi: 10.1634/stemcells.2007-0979

Arthur, A., Shi, S., Zannettino, A. C., Fujii, N., Gronthos, S., and Koblar, S. A. (2009). Implanted adult human dental pulp stem cells induce endogenous axon guidance. Stem Cells 27, 2229–2237. doi: 10.1002/stem.138

Bae, Y. C., Zuker, R. M., Manktelow, R. T., and Wade, S. (2006). A comparison of commissure excursion following gracilis muscle transplantation for facial paralysis using a cross-face nerve graft versus the motor nerve to the masseter nerve. Plast. Reconstr. Surg. 117, 2407–2413. doi: 10.1097/01.prs.0000218798.95027.21

Blanpain, C., and Fuchs, E. (2009). Epidermal homeostasis: a balancing act of stem cells in the skin. Nat. Rev. Mol. Cell Biol. 10, 207–217. doi: 10.1038/ nrm2636

Bonnamain, V., Thinard, R., Sergent-Tanguy, S., Huet, P., Bienvenu, G., Naveilhan, P., et al. (2013). Human dental pulp stem cells cultured in serum- free supplemented medium. Front. Physiol. 4:357. doi: 10.3389/fphys.2013. 00357Boucher, T. J., Okuse, K., Bennett, D. L., Munson, J. B., Wood, J. N., and McMahon, S. B. (2000). Potent analgesic effects of GDNF in neuropathic pain states.

Science 290, 124–127. doi: 10.1126/science.290.5489.124

Bray, A. F., Cevallos, R. R., Gazarian, K., and Lamas, M. (2014). Human dental pulp stem cells respond to cues from the rat retina and differentiate to express the retinal neuronal marker rhodopsin. Neuroscience 280, 142–155. doi: 10.1016/j.neuroscience.2014.09.023

Cai, J., Zhang, Y., Liu, P., Chen, S., Wu, X., Sun, Y., et al. (2013). Generation of tooth-like structures from integration-free human urine induced pluripotent stem cells. Cell Regen. 2:6. doi: 10.1186/2045-9769-2-6

Carinci, F., Papaccio, G., Laino, G., Palmieri, A., Brunelli, G., D'Aquino, R., et al. (2008). Comparison between genetic portraits of osteoblasts derived from primary cultures and osteoblasts obtained from human pulpar stem cells. J. Craniofac. Surg. 19, 616–625. doi: 10.1097/SCS.0b013e31816aabc8

Cavalcanti, B. N., Zeitlin, B. D., and Nör, J. E. (2013). A hydrogel scaffold that maintains viability and supports differentiation of dental pulp stem cells. Dent. Mater. 29, 97–102. doi: 10.1016/j.dental.2012.08.002

Chan, B., Wong, R. W., and Rabie, B. (2011). In vivo production of mineralised tissue pieces for clinical use: a qualitative pilot study using human dental pulp cell. Int. J. Oral Maxillofac. Surg. 40, 612–620. doi: 10.1016/j.ijom.2011.01.008

Chan, J. Y., and Byrne, P. J. (2011). Management of facial paralysis in the 21st century. Facial Plast. Surg. 27, 346–357. doi: 10.1055/s-0031-1283053

Coppes, R. P., and Stokman, M. A. (2011). Stem cells and the repair of radiation-induced salivary gland damage. Oral Dis. 17, 143–153. doi: 10.1111/j.1601-0825.2010.01723.x

d'Aquino, R., De Rosa, A., Lanza, V., Tirino, V., Laino, L., Graziano, A., et al. (2009). Human mandible bone defect repair by the grafting of dental pulp stem/progenitor cells and collagen sponge biocomplexes. Eur. Cell. Mater. 18, 75–83.

d'Aquino, R., Graziano, A., Sampaolesi, M., Laino, G., Pirozzi, G., De Rosa, A., et al. (2007). Human postnatal dental pulp cells co-differentiate into osteoblasts and endotheliocytes: a pivotal synergy leading to adult bone tissue formation. Cell Death Differ. 14, 1162–1171. doi: 10.1038/sj.cdd.4402121

Ding, L., and Morrison, S. J. (2013). Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. Nature 495, 231–235. doi: 10.1038/nature11885

Dissanayaka, W. L., Hargreaves, K. M., Jin, L., Samaranayake, L. P., and Zhang,

C. (2015). The interplay of dental pulp stem cells and endothelial cells in an injectable peptide hydrogel on angiogenesis and pulp regeneration in vivo. Tissue Eng. Part A. 21, 550–563. doi: 10.1089/ten.tea.2014.0154

Fatherazi, S., Matsa-Dunn, D., Foster, B. L., Rutherford, R. B., Somerman, M. J., and Presland, R. B. (2009). Phosphate regulates osteopontin gene transcription. J. Dent. Res. 88, 39–44. doi: 10.1177/0022034508328072

Foster, B. L., Nociti, F. H. Jr., Swanson, E. C., Matsa-Dunn, D., Berry, J. E., Cupp,

C. J., et al. (2006). Regulation of cementoblast gene expression by inorganic phosphate in vitro. Calcif. Tissue Int. 78, 103–112. doi: 10.1007/s00223-005-0184-7

Freitas, R. M., Spin-Neto, R., Marcantonio Junior, E., Pereira, L. A., Wikesjö, U. M., and Susin, C. (2015). Alveolar ridge and maxillary sinus augmentation using rhBMP-2: a systematic review. Clin. Implant Dent. Relat. Res. 17, e192–e201. doi: 10.1111/cid.12156

Fujita, S., Hideshima, K., and Ikeda, T. (2006). Nestin expression in odontoblasts and odontogenic ectomesenchymal tissue of odontogenic tumours. J. Clin. Pathol. 59, 240–245. doi: 10.1136/jcp.2004.025403

Gervois, P., Struys, T., Hilkens, P., Bronckaers, A., Ratajczak, J., Politis, C., et al. (2015). Neurogenic maturation of human dental pulp stem cells following neurosphere generation induces morphological and electrophysiological characteristics of functional neurons. Stem Cells Dev. 24, 296–311. doi: 10.1089/scd.2014.0117

Giuliani, A., Manescu, A., Langer, M., Rustichelli, F., Desiderio, V., Paino, F., et al. (2013). Three years after transplants in human mandibles, histological and in-line holotomography revealed that stem cells regenerated a compact rather than a spongy bone: biological and clinical implications. Stem Cells Transl. Med. 2, 316–324. doi: 10.5966/sctm.2012-0136

Graziano, A., d'Aquino, R., Laino, G., and Papaccio, G. (2008a). Dental pulp stem cells: a promising tool for bone regeneration. Stem Cell Rev. 4, 21–26. doi: 10.1007/s12015-008-9015-3

Graziano, A., d'Aquino, R., Laino, G., Proto, A., Giuliano, M. T., Pirozzi, G., et al. (2008b). Human CD34+ stem cells produce bone nodules in vivo. Cell Prolif. 41, 1–11. doi: 10.1111/j.1365-2184.2007.00497.x

Gronthos, S., Brahim, J., Li, W., Fisher, L. W., Cherman, N., Boyde, A., et al. (2002). Stem cell properties of human dental pulp stem cells. J. Dent. Res. 81, 531–535. doi: 10.1177/154405910208100806

Gronthos, S., Mankani, M., Brahim, J., Robey, P. G., and Shi, S. (2000). Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc. Natl. Acad. Sci. U.S.A. 97, 13625–13630. doi: 10.1073/pnas.240309797

Hamidouche, Z., Haÿ, E., Vaudin, P., Charbord, P., Schüle, R., Marie, P. J., et al. (2008). FHL2 mediates dexamethasone-induced mesenchymal cell differentiation into osteoblasts by activating Wnt/beta-catenin signaling-dependent Runx2 expression. FASEB J. 22, 3813–3822. doi: 10.1096/fj.08-106302

Hayashi, A., and Maruyama, Y. (2005). Neurovascularized free short head of the biceps femoris muscle transfer for one-stage reanimation of facial paralysis.

Plast. Reconstr. Surg. 115, 394–405. doi: 10.1097/01.PRS.0000149405.89201.9E

Hedstrom, K. L., Murtie, J. C., Albers, K., Calcutt, N. A., and Corfas, G. (2014). Treating small fiber neuropathy by topical application of a small molecule modulator of ligand-induced GFRalpha/RET receptor signaling. Proc. Natl. Acad. Sci. U.S.A. 111, 2325–2330. doi: 10.1073/pnas.1308889111

Huang, G. T., Gronthos, S., and Shi, S. (2009). Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. J. Dent. Res. 88, 792–806. doi: 10.1177/0022034509340867

Ibarretxe, G., Crende, O., Aurrekoetxea, M., García-Murga, V., Etxaniz, J., and Unda, F. (2012). Neural crest stem cells from dental tissues: a new hope for dental and neural regeneration. Stem Cells Int. 2012:103503. doi: 10.1155/2012/ 103503

Ichikawa, H., Kim, H. J., Shuprisha, A., Shikano, T., Tsumura, M., Shibukawa, Y., et al. (2012). Voltage-dependent sodium channels and calcium-activated potassium channels in human odontoblasts in vitro. J. Endod. 38, 1355–1362. doi: 10.1016/j.joen.2012.06.015

Ikeda, R., Morita, K., Nakao, K., Ishida, K., Nakamura, T., Takano-Yamamoto, T., et al. (2009). Fully functional bioengineered tooth replacement as an organ replacement therapy. Proc. Natl. Acad. Sci. U.S.A. 106, 13475–13480. doi: 10.1073/pnas.0902944106

Janebodin, K., Horst, O. V., Ieronimakis, N., Balasundaram, G., Reesukumal, K., Pratumvinit, B., et al. (2011). Isolation and characterization of neural crest-derived stem cells from dental pulp of neonatal mice. PLoSONE 6:e27526. doi: 10.1371/journal.pone.0027526

Janebodin, K., and Reyes, M. (2012). Neural crest-derived dental pulp stem cells function as ectomesenchyme to support salivary gland tissue formation. Dentistry S13:001. doi: 10.4172/2161-1122S13-001

Kadar, K., Kiraly, M., Porcsalmy, B., Molnar, B., Racz, G. Z., Blazsek, J., et al. (2009). Differentiation potential of stem cells from human dental origin - promise for tissue engineering. J. Physiol. Pharmacol. 60, 167–175.

Kalaszczynska, I., and Ferdin, K. (2015). Wharton's jelly derived mesenchymalstem cells: future of regenerative medicine?recent findings and clinical significance. Biomed. Res. Int. 2015:430847. doi: 10.1155/2015/430847

Kapur, S. K., Dos-AnjosVilaboa, S., Llull, R., and Katz, A. J. (2015). Adipose tissue and stem/progenitor cells: discovery and development. Clin. Plast. Surg. 42, 155–167. doi: 10.1016/j.cps.2014.12.010

Kaukua, N., Shahidi, M. K., Konstantinidou, C., Dyachuk, V., Kaucka, M., Furlan, A., et al. (2014). Glial origin of mesenchymal stem cells in a tooth model system. Nature 513, 551–554. doi: 10.1038/nature13536

Kawashima, N. (2012). Characterisation of dental pulp stem cells: a new horizon for tissue regeneration? Arch. Oral Biol. 57, 1439–1458. doi: 10.1016/j.archoralbio.2012.08.010

Kim, J. Y., Xin, X., Moioli, E. K., Chung, J., Lee, C. H., Chen, M., et al. (2010). Regeneration of dental-pulp-like tissue by chemotaxis-induced cell homing. Tissue Eng. Part A 16, 3023–3031. doi: 10.1089/ten.tea. 2010.0181

Kiraly, M., Porcsalmy, B., Pataki, A., Kadar, K., Jelitai, M., Molnar, B., et al. (2009). Simultaneous PKC and cAMP activation induces differentiation of human dental pulp stem cells into functionally active neurons. Neurochem. Int. 55, 323–332. doi: 10.1016/j.neuint.2009.03.017

Knosp, W. M., Knox, S. M., and Hoffman, M. P. (2012). Salivary gland organogenesis. Wiley interdisciplinary reviews. Dev. Biol. 1, 69–82. doi: 10.1002/wdev.4

Knox, S. M., Lombaert, I. M., Reed, X., Vitale-Cross, L., Gutkind, J. S., and Hoffman, M. P. (2010). Parasympathetic innervation maintains epithelial progenitor cells during salivary organogenesis. Science 329, 1645–1647. doi: 10.1126/science.1192046

Kumar, P. A., and Hassan, K. M. (2002). Cross-face nerve graft with free-muscle transfer for reanimation of the paralyzed face: a comparative study of the single-stage and two-stage procedures. Plast. Reconstr. Surg. 109, 451–462. doi: 10.1097/00006534-200202000-00006

La Noce, M., Paino, F., Spina, A., Naddeo, P., Montella, R., Desiderio, V., et al. (2014). Dental pulp stem cells: state of the art and suggestions for a true translation of research into therapy. J. Dent. 42, 761–768. doi: 10.1016/j.jdent.2014.02.018

Laino, G., d'Aquino, R., Graziano, A., Lanza, V., Carinci, F., Naro, F., et al. (2005). A new population of human adult dental pulp stem cells: a useful source of living autologous fibrous bone tissue (LAB). J. Bone Miner. Res. 20, 1394–1402. doi: 10.1359/JBMR.050325

Langenbach, F., and Handschel, J. (2013). Effects of dexamethasone, ascorbic acid and β-glycerophosphate on the osteogenic differentiation of stem cells in vitro. Stem Cell Res. Ther. 4, 117. doi: 10.1186/scrt328

Lee, J. E., Jin, S. H., Ko, Y., and Park, J. B. (2014). Evaluation of anatomical considerations in the posterior maxillae for sinus augmentation. World J. Clin. Cases 2, 683–688. doi: 10.12998/wjcc.v2.i11.683

Lee, J. Y., Nam, H., Park, Y. J., Lee, S. J., Chung, C. P., Han, S. B., et al. (2011). The effects of platelet-rich plasma derived from human umbilical cord blood on the osteogenic differentiation of human dental stem cells. In vitro Cell. Dev. Biol. 47, 157–164. doi: 10.1007/s11626-010-9364-5

Leijten, J., Chai, Y. C., Papantoniou, I., Geris, L., Schrooten, J., and Luyten, F. P. (2015). Cell based advanced therapeutic medicinal products for bone repair: keep it simple? Adv. Drug Deliv. Rev. 84, 30–44. doi: 10.1016/j.addr.2014.10.025

Li, J. H., Liu, D. Y., Zhang, F. M., Wang, F., Zhang, W. K., and Zhang, Z. T. (2011). Human dental pulp stem cell is a promising autologous seed cell for bone tissue engineering. Chin. Med. J. 124, 4022–4028.

Liu, H. C., E, L. L., Wang, D. S., Su, F., Wu, X., Shi, Z. P., et al.

(2011). Reconstruction of alveolar bone defects using bone morphogenetic protein 2 mediated rabbit dental pulp stem cells seeded on nano-hydroxyapatite/collagen/poly(L-lactide). Tissue Eng. Part A 17, 2417–2433. doi: 10.1089/ten.tea.2010.0620

Lombaert, I. M., Brunsting, J. F., Wierenga, P. K., Faber, H., Stokman, M. A., Kok, T., et al. (2008). Rescue of salivary gland function after stem cell transplantation in irradiated glands. PLoSONE 3:e2063. doi: 10.1371/journal.pone. 0002063

Lombaert, I. M., Knox, S. M., and Hoffman, M. P. (2011). Salivary gland progenitor cell biology provides a rationale for therapeutic salivary gland regeneration. Oral Dis. 17, 445–449. doi: 10.1111/j.1601-0825.2010.01783.x

Love, S., and Coakham, H. B. (2001). Trigeminal neuralgia: pathology and pathogenesis. Brain 124, 2347–2360. doi: 10.1093/brain/124.12.2347

Mangano, C., De Rosa, A., Desiderio, V., d'Aquino, R., Piattelli, A., De Francesco, F., et al. (2010). The osteoblastic differentiation of dental pulp stem cells and bone formation on different titanium surface textures. Biomaterials 31, 3543–3551. doi: 10.1016/j.biomaterials.2010.01.056

Mangano, C., Paino, F., d'Aquino, R., De Rosa, A., Iezzi, G., Piattelli, A., et al. (2011). Human dental pulp stem cells hook into biocoral scaffold forming an engineered biocomplex. PLoSONE 6:e18721. doi: 10.1371/journal.pone.0018721

Marín, O., Valdeolmillos, M., and Moya, F. (2006). Neurons in motion: same principles for different shapes? Trends Neurosci. 29, 655–661. doi: 10.1016/j.tins.2006.10.001

Martens, W., Bronckaers, A., Politis, C., Jacobs, R., and Lambrichts, I. (2013). Dental stem cells and their promising role in neural regeneration: an update. Clin. Oral Invest. 17, 1969–1983. doi: 10.1007/s00784-013-1030-3

Martens, W., Sanen, K., Georgiou, M., Struys, T., Bronckaers, A., Ameloot, M., et al. (2014). Human dental pulp stem cells can differentiate into Schwann cells and promote and guide neurite outgrowth in an aligned tissue-engineered collagen construct in vitro. FASEB J. 28, 1634–1643. doi: 10.1096/fj.13-243980

Ménard, C., and Tarte, K. (2013). Immunoregulatory properties of clinical grade mesenchymal stromal cells: evidence, uncertainties, and clinical application. Stem Cell Res. Ther. 4, 64. doi: 10.1186/scrt214

Misawa, H., Kobayashi, N., Soto-Gutierrez, A., Chen, Y., Yoshida, A., Rivas-Carrillo, J. D., et al. (2006). Pura Matrix facilitates bone regeneration in bone defects of calvaria in mice. Cell Transplant. 15, 903–910. doi: 10.3727/000000006783981369

Morad, G., Kheiri, L., and Khojasteh, A. (2013). Dental pulp stem cells for in vivo bone regeneration: a systematic review of literature. Arch. Oral Biol. 58, 1818–1827. doi: 10.1016/j.archoralbio.2013.08.011

Moshaverinia, A., Chen, C., Akiyama, K., Xu, X., Chee, W. W., Schricker, S. R., et al. (2013). Encapsulated dental-derived mesenchymal stem cells in an injectable and biodegradable scaffold for applications in bone tissue engineering. J. Biomed. Mater. Res. Part A 101, 3285–3294. doi: 10.1002/jbm.a.34546

Murakami, T., Fujimoto, Y., Yasunaga, Y., Ishida, O., Tanaka, N., Ikuta, Y., et al. (2003). Transplanted neuronal progenitor cells in a peripheral nerve gap promote nerve repair. Brain Res. 974, 17–24. doi: 10.1016/s0006-8993(03)02539-3

Nakashima, M., and Akamine, A. (2005). The application of tissue engineering to regeneration of pulp and dentin in endodontics. J. Endod. 31, 711–718. doi: 10.1097/01.don.0000164138.49923.e5

Nakashima, M., Iohara, K., and Sugiyama, M. (2009). Human dental pulp stem cells with highly angiogenic and neurogenic potential for possible use in pulp regeneration. Cytokine Growth Factor Rev. 20, 435–440. doi: 10.1016/j.cytogfr.2009.10.012

Nanduri, L. S., Baanstra, M., Faber, H., Rocchi, C., Zwart, E., de Haan, G., et al. (2014). Purification and ex vivo expansion of fully functional salivary gland stem cells. Stem Cell Rep. 3, 957–964. doi: 10.1016/j.stemcr.2014.09.015

Nanduri, L. S., Lombaert, I. M., van der Zwaag, M., Faber, H., Brunsting, J. F., van Os, R. P., et al. (2013). Salisphere derived c-Kit+ cell transplantation restores tissue homeostasis in irradiated salivary gland. Radiother. Oncol. 108, 458–463. doi: 10.1016/j.radonc.2013.05.020

Nazirkar, G., Singh, S., Dole, V., and Nikam, A. (2014). Effortless effort in bone regeneration: a review. J. Int. Oral Health 6, 120–124.

Nedvetsky, P. I., Emmerson, E., Finley, J. K., Ettinger, A., Cruz-Pacheco, N., Prochazka, J., et al. (2014). Parasympathetic innervation regulates tubulogenesis in the developing salivary gland. Dev. Cell 30, 449–462. doi: 10.1016/j.devcel.2014.06.012

Nör, J. E. (2006). Tooth regeneration in operative dentistry. Oper. Dent. 31, 633–642. doi: 10.2341/06-000

Nosrat, I. V., Smith, C. A., Mullally, P., Olson, L., and Nosrat, C. A. (2004). Dental pulp cells provide neurotrophic supportfor dopaminergic neurons and differentiate into neurons in vitro; implications for tissue engineering and repair in the nervous system. Eur. J. Neurosci. 19, 2388–2398. doi: 10.1111/j.0953-816x.2004.03314.x

Ogawa, M., Oshima, M., Imamura, A., Sekine, Y., Ishida, K., Yamashita, K., et al. (2013). Functional salivary gland regeneration by transplantation of a bioengineered organ germ. Nat. Commun. 4, 2498. doi: 10.1038/ncomms3498

Ogawa, M., and Tsuji, T. (2015). Reconstitution of a bioengineered salivary gland using a three-dimensional cell manipulation method. Curr. Protoc. Cell Biol. 66, 19.17.1–19.17.13. doi: 10.1002/0471143030.cb1917s66

Oshima, M., Mizuno, M., Imamura, A., Ogawa, M., Yasukawa, M., Yamazaki, H., et al. (2011). Functional tooth regeneration using a bioengineered tooth unit as a mature organ replacement regenerative therapy. PLoS ONE 6:e21531. doi: 10.1371/journal.pone.0021531

Otsu, K., Kumakami-Sakano, M., Fujiwara, N., Kikuchi, K., Keller, L., Lesot, H., et al. (2014). Stem cell sources for tooth regeneration: current status and future prospects. Front. Physiol. 5:36. doi: 10.3389/fphys.2014.00036

Paknejad, M., Shayesteh, Y. S., Yaghobee, S., Shariat, S., Dehghan, M., and Motahari, P. (2012). Evaluation of the effect of plasma rich in growth factors (PRGF) on bone regeneration. J. Dent. 9, 59–67.

Pierdomenico, L., Bonsi, L., Calvitti, M., Rondelli, D., Arpinati, M., Chirumbolo, G., et al. (2005). Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp. Transplantation 80, 836–842. doi: 10.1097/01.tp.0000173794.72151.88

Rastegar, F., Shenaq, D., Huang, J., Zhang, W., Zhang, B. Q., He, B. C., et al. (2010). Mesenchymal stem cells: molecular characteristics and clinical applications. World J. Stem Cells 2, 67–80. doi: 10.4252/wjsc.v2.i4.67

Rivera, C., Monsalve, F., Salas, J., Morán, A., and Suazo, I. (2013). Platelet-rich plasma, plasma rich in growth factors and simvastatin in the regeneration and

repair of alveolar bone. Exp. Ther. Med. 6, 1543–1549. doi: 10.3892/etm.2013.1327

Rosa, V., Zhang, Z., Grande, R. H., and Nör, J. E. (2013). Dental pulp tissue engineering in full-length human root canals. J. Dent. Res. 92, 970–975. doi: 10.1177/0022034513505772

Sabalys, G., Juodzbalys, G., and Wang, H. L. (2013). Aetiology and pathogenesis of trigeminal neuralgia: a comprehensive review. J. Oral Maxillofac. Res. 3, e2. doi: 10.5037/jomr.2012.3402

Sakai, K., Yamamoto, A., Matsubara, K., Nakamura, S., Naruse, M., Yamagata, M., et al. (2012). Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms. J. Clin. Invest. 122, 80–90. doi: 10.1172/JCI 59251

Sasaki, R., Aoki, S., Yamato, M., Uchiyama, H., Wada, K., Ogiuchi, H., et al. (2011). PLGA artificial nerve conduits with dental pulp cells promote facial nerve regeneration. J. Tissue Eng. Regen. Med. 5, 823–830. doi: 10.1002/term.387

Sasaki, R., Matsumine, H., Watanabe, Y., Takeuchi, Y., Yamato, M., Okano, T., et al. (2014). Electrophysiologic and functional evaluations of regenerated facial nerve defects with a tube containing dental pulp cells in rats. Plast. Reconstr. Surg. 134, 970–978. doi: 10.1097/PRS.0000000000000602

Sequeira, S. J., Gervais, E. M., Ray, S., and Larsen, M. (2013). Genetic modification and recombination of salivary gland organ cultures. J. Vis. Exp. 28:e50060. doi: 10.3791/50060

Shirasuna, K., Sato, M., and Miyazaki, T. (1981). A neoplastic epithelial duct cell line established from an irradiated human salivary gland. Cancer 48, 745–752. Suzuki, T., Lee, C. H., Chen, M., Zhao, W., Fu, S. Y., Qi, J. J., et al. (2011). Induced migration of dental pulp stem cells for in vivo pulp regeneration. J. Dent. Res.

90, 1013–1018. doi: 10.1177/0022034511408426

Tada, H., Nemoto, E., Foster, B. L., Somerman, M. J., and Shimauchi, H. (2011). Phosphate increases bone morphogenetic protein-2 expression through cAMP- dependent protein kinase and ERK1/2 pathways in human dental pulp cells. Bone 48, 1409–1416. doi: 10.1016/j.bone.2011.03.675

Taylor, S. E. (2003). Efficacy and economic evaluation of pilocarpine in treating radiation-induced xerostomia. Expert Opin. Pharmacother. 4, 1489–1497. doi: 10.1517/14656566.4.9.1489

Vater, C., Kasten, P., and Stiehler, M. (2011). Culture media for the differentiation of mesenchymal stromal cells. ActaBiomater. 7, 463–477. doi: 10.1016/j.actbio.2010.07.037

Vissink, A., Mitchell, J. B., Baum, B. J., Limesand, K. H., Jensen, S. B., Fox, P. C., et al. (2010). Clinical management of salivary gland hypofunction and xerostomia in head-and-neck cancer patients: successes and barriers. Int. J. Radiat. Oncol. Biol. Phys. 78, 983–991. doi: 10.1016/j.ijrobp.2010.06.052

Walsh, S., and Midha, R. (2009). Practical considerations concerning the use of stem cells for peripheral nerve repair. Neurosurg. Focus 26:E2. doi: 10.3171/FOC.2009.26.2.E2

Wang, Y., Preston, B., and Guan, G. (2012). Tooth bioengineering leads the next generation of dentistry. Int. J. Paediatr. Dent. 22, 406–418. doi: 10.1111/j.1365- 263X.2011.01206.x

Wen, Y., Wang, F., Zhang, W., Li, Y., Yu, M., Nan, X., et al. (2012). Application of induced pluripotent stem cells in generation of a tissue-engineered tooth-like structure. Tissue Eng. Part A 18, 1677–1685. doi: 10.1089/ten.tea. 2011.0220

Xiao, L., and Tsutsui, T. (2013). Characterization of human dental pulp cells- derived spheroids in serum-free medium: stem cells in the core. J. Cell. Biochem. 114, 2624–2636. doi: 10.1002/jcb.24610

Yan, M., Sun, M., Zhou, Y., Wang, W., He, Z., Tang, D., et al. (2013). Conversion of human umbilical cord mesenchymal stem cells in Wharton's jelly to dopamine neurons mediated by the Lmx1a and neurturin in vitro: potential therapeutic application for Parkinson's disease in a rhesus monkey model. PLoS ONE 8:e64000. doi: 10.1371/journal.pone.0064000

Yang, J. W., Zhang, Y. F., Wan, C. Y., Sun, Z. Y., Nie, S., Jian, S. J., et al. (2015).
Autophagy in SDF-1α-mediated DPSC migration and pulp regeneration.
Biomaterials 44, 11–23. doi: 10.1016/j.biomaterials.2014.12.006

Yang, X., Han, G., Pang, X., and Fan, M. (2011). Chitosan/collagen scaffold containing bone morphogenetic protein-7 DNA supports dental pulp stem cell differentiation in vitro and in vivo. J. Biomed. Mater. Res. Part A 2011. doi: 10.1002/jbm.a.34064. [Epub ahead of print].

Yu, J., He, H., Tang, C., Zhang, G., Li, Y., Wang, R., et al. (2010). Differentiation potential of STRO-1+ dental pulp stem cells changes during cell passaging. BMC Cell Biol. 11:32. doi: 10.1186/1471-2121-11-32

Zhang, W., Walboomers, X. F., van Osch, G. J., van den Dolder, J., and Jansen, J.
A. (2008). Hard tissue formation in a porous HA/TCP ceramic scaffold loaded with stromal cells derived from dental pulp and bone marrow. Tissue Eng. Part A 14, 285–294. doi: 10.1089/tea.2007.0146

2

Pluripotency network with metabolism and epigenetics in Human Dental Pulp Stem Cells

Verónica Uribe-Etxebarria[1]., Alice Agliano.[2], NereaSubirán.[3], Fernando Unda[1]. And Gaskon Ibarretxe[1].

[1]: Cell Biology and Histology Department. University of the Basque Country (UPV/EHU).Barrio Sarriena.n. 48940Leioa, Spain

[2]: Cancer Research UK Cancer Imaging Centre, Division of Radiotherapy and Imaging, The Institute of Cancer Research and The Royal Marsden NHS Foundation Trust, 123 Old Brompton Road, London, SW7 3RP, United Kingdom.

[3]: Physiology Department. University of the Basque Country (UPV/EHU).Barrio Sarriena.n. 48940Leioa, Spain

ABSTRACT

Dental Pulp Stem Cells (DPSCs) are stem cells extracted from the dental pulp of third molars and deciduous teeth. Stem cells from dental pulp present some interesting properties that convert them in unique resource of stem cells for cell therapy; present high differentiation potential and they are easily obtained from patients, amplified and cryopreserve. Interestingly, stem cells from dental pulp present inmunosupresory properties and are well tolerated by biomaterials, which make them especially attractive for their use in clinics.

As for DPSCs regeneration potential, it is to highlight their stemness, given by high self-renewal and the expression of pluripotency core factors Oct4, Nanog, Sox2, cMyc, Lin28, Rex1, Stella y Ssea1. This stemness could be enhanced by cell reprogramming with transcription factors transfection and the recent technology CRISPR. However, this methodology could lead to aberrant mutations in DNA converting stem cells in potentially dangerous to use in humans. Notch and Wnt/β-Catenin signaling pathways play a crucial role in the maintenance and differentiation of different type of stem cells. To understand how Notch and Wnt/β-Catenin signaling pathways work with stemness and self-renewal would be of great interest in order to create controlled cell reprogramming strategies.

Indeed, the modulation of stemness and pluripotency is coming together with metabolism and epigenetic remodelling for ensure reprogramming efficiency.

Metabolism remodelling includes important physiological molecular and cellular changes in glycolysis, mitochondrial activity and lipid precursors, which could be necessary for ensuring a safety cell therapy with DPSCs. In epigenetic landscape, chromatin remodelling is accompanied with changes in methylation and acetylation patterns of DNA and histones. These changes are crucial for defeating epigenetic barrier and creating an effective cell reprogramming. Epigenetic changes are linked to remodelling in metabolism and could be affected together affecting each other. The knowledge of the link between pluripotency network with metabolism and epigenetic in Notch and Wnt signaling modulation could help to determine an effective manner of reprogramming avoiding cell physiology damage.

INTRODUCTION

Dental Pulp Stem Cells (DPSCs) are stem cells extracted from the dental pulp of third molars and deciduous teeth. These stem cells are neural crest-derived cells with an outstanding capacity to differentiate along multiple cell lineages of interest for cell therapy (Aurrekoetxea et al., 2015). Despite cell therapy improvements there are some limitations due to the obtainment, maintenance and cryopreservation of adult stem cells(Hunt, 2011).DPSCs present some characteristics such as relative abundance, low morbidity, easy accessibility, differentiation potential and tolerance to biomaterials and this together make DPSCs highly promising for tooth tissue engineering and craniomaxillofacial regeneration (Aly, 2015; Aurrekoetxea et al., 2015; Ibarretxe et al., 2012; Kellner et al., 2014; Wu et al., 2015; Yang et al., 2016). Moreover, the capacity of DPSCs to differentiate into specialized cells makes them attractive candidates for clinical applications (Bray et al., 2014; Syed-Picard et al., 2015).Nevertheless, it is known that DPSCs, as other adult human stem cells, have a limited potential to differentiate into some type of cells due to their genetic determination. It is known that DSPCs lost their differentiation potential and stem cells markers along aging (Alraies et al., 2017). Therefore, it is crucial to study different methodology for improving their differentiation potential (Gronthos et al., 2000).Development of methodology to convert adult stem cells into Induced Pluripotent Stem Cells or iPSCs has risen during the last few years. As an example, one of the most employed methods is the transfection through Yamanaka factors (cMyc, Oct4, Nanog, Sox2 and Klf4) to produce pluripotent stem cells with potential to generate almost all cell type (Takahashi et al., 2007; Takahashi and Yamanaka, 2006b). Nevertheless, an excess of reprogramming factors may trigger cell-cycle arrest, death, mutations or inappropriate differentiation in transfected cells(Banito et al., 2009; Kawamura et al., 2009; Li et al., 2009).Therefore, achieving the right balance of reprogramming factors appears to be critical. Different forms of genomic impairment such as elimination p53 and Ink4/Arf locus allows more efficient reprogramming, but also leads to chromosomal instability(Kawamura et al., 2009; Li et al., 2009; Marion et al., 2009). The use of microRNA has also been reported successful results in improving DPSCs differentiation potential (Li et al., 2015). Taking all together, understanding pathways mechanism may be interesting alternative to modulate reprogramming under control. It is still unclear how pluripotency networks operate with metabolism and epigenetics, which are essential to establish more efficient and safety differentiation

protocols. It is known that dental stem cells present higher levels of core factors (cMyc, Sox2, Nanog, Oct4, Rex1, Lin 28, Rex1, Stella and Ssea1) (Atari et al., 2012; Huang et al., 2009; Janebodin et al., 2011; Uribe-Etxebarria et al., 2017; Vasanthan et al., 2015). Indeed, both pathways have been demonstrated to enhance stemness and different differentiation media efficiency (Uribe-Etxebarria et al., 2017).However, the role of these pathways in the maintenance of stemness and self-renewal in DPSCs is still unclear. Interestingly, cellular metabolism might also play a crucial role being involved in the regulation of cell fate and stem cell activity (Buck et al., 2016; Gascon et al., 2016; Zhang et al., 2016b; Zheng et al., 2016).Furthermore, the mechanism by which cellular metabolism can influence stem cell fate has only recently begun to be explored; however, it is clear that it does so, at least in part, by influencing the epigenetic landscape, which in turn affects gene expression (Harvey et al., 2016).

Overall, Notch and Wnt/β-Catenin signalling pathways are indispensable for pluripotency stem cell factors expression and self-renewal and could be altered to control reprogramming safety and measured of DPSCS that guarantees their safety use in cell therapy. Moreover, the knowledge of this pluripotency network with metabolism and epigenetic could help to determine an effective way of reprogramming avoiding cell physiology damage at cellular and molecular level. Partial reprogramming and controlled of these cells may be used for existent differentiation protocols enhancement in terms of rapidness and effectiveness of specific cell populations. It could be interesting also the design of new protocols difficult to carry out due to their complexity. Stemness, metabolism and epigenetic seems to form a complex network and here we review some of the last important findings in this network with insights into our previous research.

DENTAL PULP STEM CELLS CHARACTHERISTICS AND ORIGIN

Dental Pulp Stem Cells are described as a unique population of precursor cells isolated from the postnatal human dental pulp capable of regenerating a reparative dentin-like complex (Ledesma-Martinez et al., 2016). Indeed, DPSCs are easily accessible for extraction, retained as a population of the dental pulp; these stem cells are also easily isolated from the pulp cavity of teeth (Figure 1 A).Further, immune-suppressive and non-tumorigenic properties of DPSCs convert them into suitable stem cells well tolerated upon grafting (Pierdomenico et al., 2005; Wilson et al., 2015). One interesting feature is that DPSCs retain the ability to differentiate into different cell types including myocytes,

odontoblasts, osteoblasts, chondrocytes, adipocytes, neural progenitors, hepatocytes, cells of blood vessels and smooth muscle cells (Arthur et al., 2008; Aurrekoetxea et al., 2015; d'Aquino et al., 2009; La Noce et al., 2014; Stevens et al., 2008)(Figure 1 A).

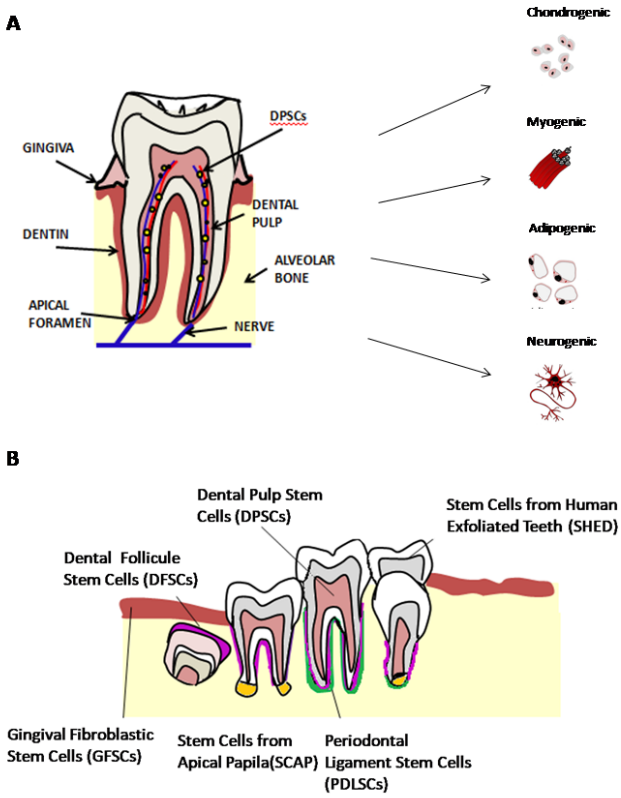


Figure 1.Schematic representation of adult tooth. A) The tooth is placed in the gingiva and the alveolar foramen. The population of Dental Pulp Stem Cells (DPSCs) are located in areas adjacent to the nerves and blood vessels inside the dental pulp. These nerves are linked to the roots in the apical foramen. Dentin, which is secreted by odontoblasts, is placed covering the tooth and protecting it from external agents. DPSCs also remain potential to differentiate in chondrocytes, muscle cells, adipocytes and neurons. **B) Different stem cells extracted from dental tissues.** Dental tissues remain different stem cells with potential for cell therapy: Dental Follicle Stem Cells (DFSCs), Dental Pulp Stem Cells (DPSCs), Stem Cells from Human Exfoliated Teeth (SHED), Gingival Fibroblastic Stem Cells (GFSCs), Stem Cells from Apical Papilla (SCAP) and Periodontal Ligament Stem Cells (PDLSCs).

Other stem cell types with similar properties could be obtained from gingival and periodontal tissues (Abe et al., 2012; Huang et al., 2009; Ibarretxe et al., 2012; Lima et al., 2017; Liu et al., 2015a; Petrovic and Stefanovic, 2009) (Figure 1. B). As for *in vitro* characteristics, DPSCs express fibroblastic (Collagen I, Vimentin and α-SMA) and neurogenic markers (β-3Tubulin, and Nestin) and their shape reminds to neurons with long neuritis. One explanation to this would be their ectomesenchymal origin (Figure 2 A, B, C, D). Moreover, DPSCs show stem cell markers(Oct4 and cMyc) and cell proliferation markers (KI67 and PCNA) providing huge quantity of stem cells for transplantation (Atari et al., 2012; Ferro et al., 2012; Kerkis et al., 2006; Rosa et al., 2016)(Figure 2 E, F, G, H).

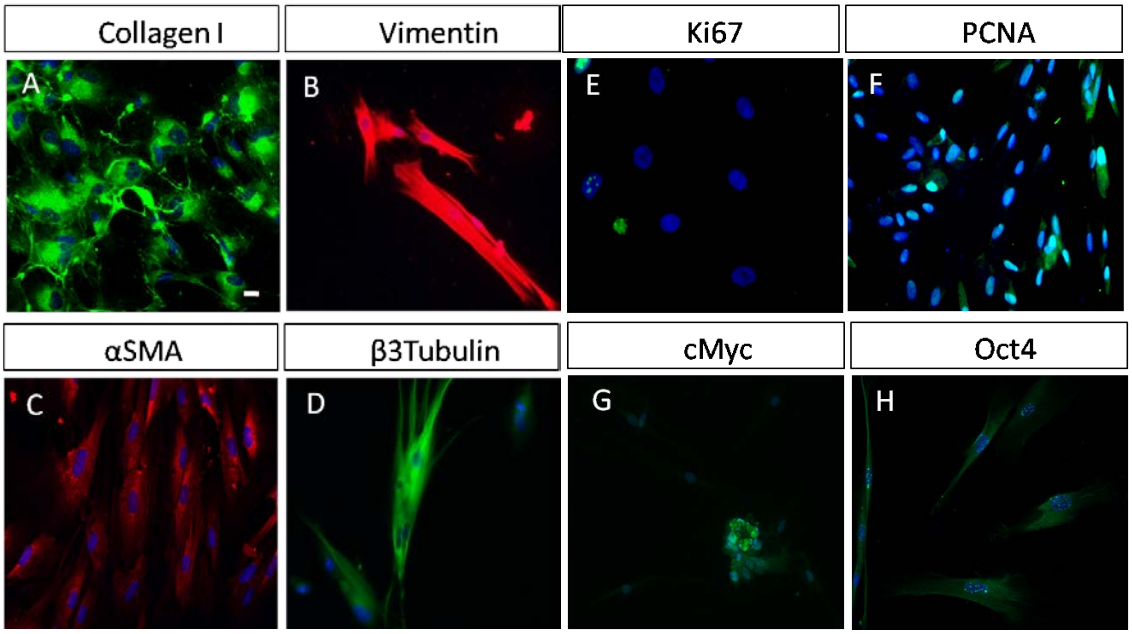


Figure 2.Characterization of DPSCs *in vitro*. A-D) DPSCs expressed mesenchymal (Collagen I, Vimentin, α-SMA) and neural markers (β-3Tubulin, Nestin) marker in basal conditions, due to their have ectomesenchymal origin. Scale bar=20 µm. E-H) DPSCs as proliferative cells express Ki67 and PCNA and also present pluripotency stem cells factors such as Oct4 and cMyc. Scale bar=20 µm.

Microenvironment described as extracellular scaffold, cell interactions and growth factors, is crucial during differentiation of DPSCs because these signals control stem cells orientation, polarity and type of cell division (symmetric or asymmetric) (Gattazzo et al., 2014). During embryonic development and, after the three germ layer formation (ectoderm, mesoderm and endoderm), pluripotent stem cells from embryo or Embryonic Stem Cells (EMSC), suffer several divisions and receive different signals leading to

genetic determination. This genetic determination occurs in humans between the 8 and 10 week of the embryo development. Then, multipotent stem cells, have all the genetic information and depend on surrounding signals to differentiate into the different type of cells that form the adult organism (Wagers et al., 2002).After neural tube fusion, a population of stem cells migrate from the neural crest and suffers the phenomena known as epithelial-mesenchymal transition (EMT). In the head, these cells migrate to generate craniofacial tissues (dental pulp and dental follicle of the teeth, muscles, bones, cartilages and even ganglion of peripheral nerves) (Aurrekoetxea et al., 2015; Gronthos et al., 2002). Consequently and because of the source of these cells, DPSCs have a huge potential to generate dental and connective tissues (Gronthos et al., 2002).

DPSCs come from the cranial neural crest (NC) have some characteristics of neural crest progenitors (Abe et al., 2012; Janebodin et al., 2011; Miletich and Sharpe, 2004; Simoes-Costa and Bronner, 2015).It has been proposed that evolution of the NC-specification program has enabled cells at the neural plate border to acquire multipotency and migratory ability (Green et al., 2015). A subgroup of transcription factors termed neural plate border specifiers is required for the establishment of the neural plate border, as well as regulation of the downstream target factors known as neural crest specifiers, which mediate the induction of neural crest lineage(Simoes-Costa and Bronner, 2015). In the same way that NC-derived cells, dental stem cells are characterized by the expression of neural crest factors such as Snail/Snai1, Slug/Snai2, Twist1, Hnk1, Pax3, Neurogenin2 and Sox10 (Kiraly et al., 2009; Schiraldi et al., 2012), and core factors including Oct4a, cMyc, Sox2, Klf-4, Lin28, Rex1, SSEA1 and Nanog (Atari et al., 2012; Ferro et al., 2012; Janebodin et al., 2011; Kerkis et al., 2006; Rosa et al., 2016; Uribe-Etxebarria et al., 2017). These pluripotency factors are fundamental to maintaining stem cell pluripotency (Chambers and Tomlinson, 2009; Hishida et al., 2015; Takahashi et al., 2007), therefore DPSCs may present some superior features with respect to other multipotent stem cell populations of the adult human body (Atari et al., 2011; Atari et al., 2012; Rosa et al., 2016).

PLURIPOTENCY NETWORK OF EMBRYONIC STEM CELL FACTORS WORKS THROUGH NOTCH AND WNT-βCATENIN SIGNALING.

Wnt and Notch pathway are involved in the preservation of pluripotency of both ES cells and adult stem cells, and are critical for the maintenance of the stem cell phenotype (Androutsellis-Theotokis et al., 2006; Borggrefe et al., 2016; Clevers et al., 2014; Perdigoto and Bardin, 2013; Reya and Clevers, 2005). Wnt signalling is endogenously activated in ES cells and downregulated upon differentiation (Sato et al., 2004). Wnt and Notch pathways also play an important role in the emergence of the neural crest and some of these cells differentiate into dental pulp cells (Garcia-Castro et al., 2002; Hari et al., 2012; Leung et al., 2016; Rogers et al., 2012; Stuhlmiller and Garcia-Castro, 2012). It is known that dental stem cells present higher levels of core factors and Wnt/Notch activity than do other mesenchymal stem cells in the adult body (Atari et al., 2012; Huang et al., 2009; Janebodin et al., 2011; Vasanthan et al., 2015). However, the role of these pathways in the maintenance of stemness and self-renewal in DPSCs is still unclear.

Activation of Notch signaling through ligand binding triggers proteolytic cleavage of Notch receptors, first by A-Disintegrin-And-Metalloproteases (ADAM) followed by γ-secretases, which results in the cleavage and release of the Notch intracellular domain (NICD) from the membrane. The NICD translocates to the nucleus where it directly interacts with CSL/RBPj/CBF-1 transcription factors to turn on the expression of Notch target genes such as the Hairy Enhancer of Split (Hes) family (D'Souza et al., 2010). As for Wnt signaling, three different pathways Canonical Wnt signaling, Non-Canonical Wnt signaling, and Wnt signaling Ca2+ dependent form this pathway.

In Canonical Wnt signaling, the interactions between Wnt protein ligands and the proteins Frizzled/LRP receptors lead to the recruitment of AXIN, APC and GSKβ3 to the membrane, thus preventing phosphorylation and degradation of β-CATENIN protein. As a result, β-CATENIN accumulates in the cytoplasm and translocates into the nucleus, where it interacts with TCF/LEF family factors and leads to the expression of Wnt signaling target genes (Clevers, 2006). Both pathways regulate each other at multiple points (Borggrefe et al., 2016; Fukunaga-Kalabis et al., 2015)and promote the maintenance of self-renewal and inhibition of differentiation in many stem cell types, including DPSCs (Mizutani et al., 2007; Scheller et al., 2008; Yiew et al., 2017).However, Non-Canonical Wnt signaling acts independent from β-CATENIN, especially in adhesion

proteins such integrins, cadherines, among other (Adler and Taylor, 2001; Boutros et al., 1998). The third singaling is Ca2+ dependent and activates Protein Kinase C (Kuhl et al., 2000; Sheldahl et al., 1999).

Pharmacological manipulation of the Notch and Wnt β-Catenin Canonical pathways is relatively simple via the use of well-known drugs such as DAPT (γ-secretase inhibitor; Notch signaling blocker), and BIO (GSK3-β inhibitor; Wnt signaling activator), and by recombinant activator proteins such as WNT-3A. The use of these drugs BIO and DAPT has already proven to be a valuable complementary strategy to induce either cellular reprogramming or cellular differentiation (Ichida et al., 2014; Kitajima et al., 2016). Therefore, a thorough understanding of the stemness and differentiation potential of DPSCs and their modulation by cell signaling pathways would be highly desirable to apply these cells more efficiently in areas such as regenerative medicine, tissue engineering and drug screening. In this work, we used DAPT and BIO, as well as human recombinant WNT-3A as pharmacological modulators to investigate the role of Notch/Wnt in maintaining stemness and the expression of pluripotency core factors in DPSCs with the goal of optimizing existing protocols of somatic cell differentiation using DPSCs.

Some authors have attributed pluripotent markers expression to the crosstalk between Notch and Wnt signaling (Kwon et al., 2011; Uribe-Etxebarria et al., 2017). During Wnt signaling, β-Catenin goes to the nucleus, activates transcription and Wnt target genes such as Sox2, Oct4, Nanog and Jagged1, which is expressed in the membrane and activates Notch signaling, leading to cell proliferation. Wnt pathway is regulated by the GSK3β, which leads to β-Catenin degradation. When GSK3β is inhibited, this pathway is overactivated, and as a result β-Catenin goes permanently to the nucleus and Notch signalling is activated through Jagged1 overtranscription.

As for Notch signalling cell-contact is indispensable due to that, the convertases are the responsible for the two cleavages for NICD translocation to the nucleus and Notch target genes transcription. γ-secretase inhibitor DAPT, prevents NICD active form from the second cleavage and therefore Notch signalling is inhibited. Moreover, β-Catenin aggregates to Notch receptor in the membrane and consequently is degraded in the proteasome. Finally, Wnt signalling activity would also be affected by Notch inhibition (Andersen et al., 2012; Hori et al., 2013) (Figure 3).

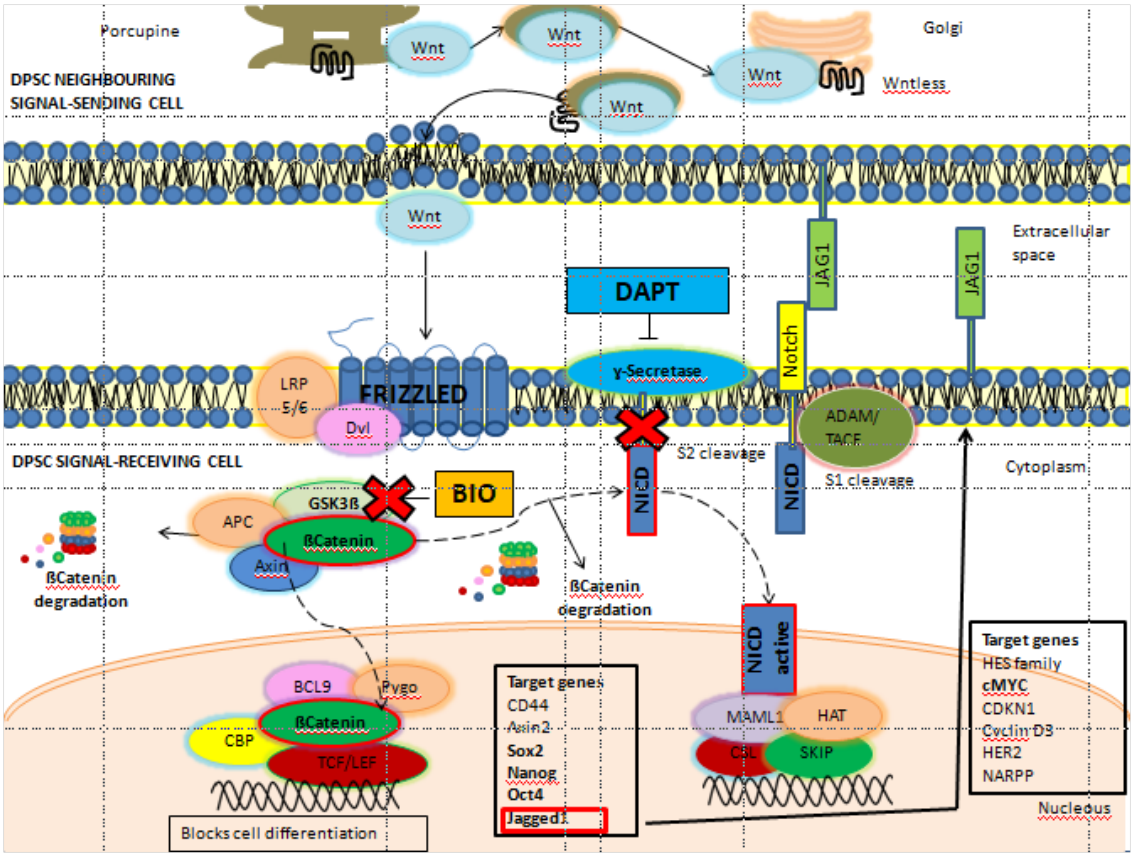


Figure 3. Notch and Wnt signaling crosstalk are responsible for pluripotency markers expression in mesenchymal stem cells (Kwon et al., 2011; Uribe-Etxebarria et al., 2017, Andersen et al., 2012; Hori et al., 2013). During Wnt signaling, β-Catenin goes to the nucleus, activates transcription factors such as Sox2, Oct4, Nanog. Indeed express Jagged1, that activates Notch signaling and cMyc transcription. When GSK3β is inhibited, β-Catenin is continuously translocated to the nucleus and upregulates all trasncription factors mentioned before. Consequently, Notch and Wnt signaling are overactivated. As for Notch signaling, the γ-secretase inhibitor DAPT prevents Notch receptor cleavage, NICD active fragment transport to the nucleus and stem cell transcription factors expression. In this inhibition Wnt signaling is also affected because Notch receptor joins to β-Catenin causing its degradation in the proteasome and therefore, Wnt inhibition. Consequently, pluripotent markers expression has been assigned to the crosstalk between Notch and Wnt signaling.

NOTCH AND WNY SIGNALING CONTROL CELL CYCLE AND SELF-RENEWAL OF PLURIPOTENT STEM CELLS

Pluripotent stem cells are defined by an unlimited self-renewal capacity and an undifferentiated state. These cells have cell cycle is defined by minimal time spent in G1 (Coronado et al., 2013; Ghule et al., 2011; Li and Kirschner, 2014). The fundamental understanding of how the cell cycle and stemness are linked has become of crucial importance between broad disciplines including regenerative medicine, cancer biology, and aging. As mentioned before, the pluripotency network consists of a core set of embryonic stem transcription factors, including Oct4 (Pou5f1), Sox2, and Nanog, among others. All of them serve to establish the undifferentiated state and the self-renewing capacity of ESC cells (Hackett and Surani, 2014; Young, 2011).

When Yamanaka and colleagues first successfully reprogrammed somatic cells to a pluripotent state, they observed that Induced Pluripotent Stem Cells (iPSCs) cells grew at a rate similar than ESC cells (Takahashi et al., 2007), with minimal spent in G1/G0 phase (Ghule et al., 2011; Ruiz et al., 2011). Mitotic nuclei are reprogrammed at rates significantly higher than interphase nuclei and that this is due in part to the de-ubiquitination of histones during mitosis resulting in gene derepression (Halley-Stott et al., 2014). Similar studies have shown that enriching for proliferating cells enhances reprogramming (Guo et al., 2014; Roccio et al., 2013).The mechanisms that underlie this increase in proliferation as cells reprogram have been the focus of multiple studies that we have mentioned before. The process of reprogramming stresses the cells, e. g. through DNA damage, induction of apoptotic programs, and oncogene expression (Marion et al., 2009), and reducing the checkpoint activity of p53 and Cdk inhibitors promotes successful reprogramming (Lin et al., 2014). Along with CDK regulation, modulating the proper levels Cyclins is fundamental to proper reprogramming (Abe et al., 2012; Edel et al., 2010; McLenachan et al., 2012). Studies in core factors, cell cycle phase and CDKS and Cyclins could give more information about how reprogramming works through pluripotency and cell cycle.

Control of cell cycle by embryonic stem cell markers

Although it is known that these transcription factors play a crucial role in the activation of the greater pluripotency network (Boyer et al., 2005), a crosstalk with the cell cycle machinery has been also identified. Early studies of the core pluripotency network have

identified cMyc, a key regulator of many cell cycle genes (Kim et al., 2008; Kim et al., 2010), also as a target of Oct4 and Nanog, being therefore crucial for the maintenance of pluripotency (Cartwright et al., 2005; Hishida et al., 2011; Loh et al., 2006). Other authors have described also the dependency of pluripotency on Wnt signaling(Hishida et al., 2015; Sun et al., 1999). The Wnt signaling pathway has been shown to elevate the level of cMyc (Cartwright et al., 2005). cMyc activity increases the rate of proliferation and reprogramming by helping to establish a pluripotent cell cycle in reprogrammed cells (Huskey et al., 2015; Kim et al., 2008; Kim et al., 2010; Lavagnolli et al., 2015; Rahl et al., 2010).

cMyc and Oct4 can directly modulateE2F activity (Huskey et al., 2015; Kim et al., 2010; Zhang et al., 2009), one of the major regulators of the cell cycle, which can affect the activity of Cyclin /CDK complexes. In addition, Nanog can upregulate CDKs and the CDK activator, Cdc25a (Zhang et al., 2009). To further enhance high CDK activity, several CDK inhibitors are repressed in part by core pluripotency factors (Lee et al., 2010; Singh and Dalton, 2009). Not surprisingly, activating some pathways such as E2Fs, Wnt and FGF pathways are implicated in the regulation of the core pluripotency network genes Oct4, Sox2, and Nanog (O'Connor et al., 2011; Uribe-Etxebarria et al., 2017; Yeo et al., 2011).

One complication of fast cell proliferation is the potentially increased accumulation of genetic mutations due to error-prone DNA synthesis. Oct4 binds to and inhibits Cdk1 resulting in an extension of G2 phase which allows more time for the DNA repair machinery to correct mutations *de novo* (Zhao et al., 2014). Apparently, the core pluripotency network can control the cell cycle, but cell cycle regulators also control pluripotency (Abe et al., 2012; Huskey et al., 2015; Kallas et al., 2014; Neganova et al., 2014).

METABOLISMREPROGRAMMINGIN PLURIPOTENT STEM CELLS

Cellular metabolism could be implicated in cell-fate determination and stem cell activity as well as is crucial in the production of energy(Buck et al., 2016; Gascon et al., 2016; Zhang et al., 2016a; Zhang et al., 2016c; Zheng et al., 2016). In fact, metabolic remodelling appears to mediate the process of reprogramming. When cells enter in a pluripotent state through reprogramming, requires an early metabolic switch to take place, because the metabolic requirements of differentiated cells are different from highly

proliferative pluripotent stem cells. The first tandem of activated genes during reprogramming is enriched for genes important for increased proliferation and metabolic change (Cacchiarelli et al., 2015).

Mitochondrial changes during metabolism remodelling

During reprogramming of human somatic cells into iPSCs, mitochondria need to undergo significant remodelling, a process known as mitochondria rejuvenation, during which mitochondrial activity seems to be lower (Folmes et al., 2012; Folmes et al., 2011; Gu et al., 2016; Ma et al., 2015; Prigione et al., 2010; Suhr et al., 2010; Varum et al., 2011; Zhou et al., 2012). These findings could explain the temporary upregulation of mitochondrial proteins in cells undergoing reprogramming, which has previously been observed via proteomic analyses (Hansson et al., 2012). This transient “hyper-energetic” state seems to be required for reprogramming of differentiated cells into pluripotent stem cells, which is accompanied with an increment in reactive oxygen species (ROS) production and enhancement of the glycolytic rate.

Glycolytic and Oxidative Phosphorylation (OXPHOS) activity prevalence during metabolism remodelling

The expression of glycolysis-related genes and lactate production in human PSCs is higher than in differentiated cells and oxygen consumption declines(Armstrong et al., 2010; Folmes et al., 2011; Mathieu et al., 2014; Prigione et al., 2010; Varum et al., 2011). High glucose culture medium reprograms mouse somatic cells into iPSCs more efficiently than low-glucose medium (Jang et al., 2012; Zhu et al., 2010). Thus, these findings show that high glycolysis is crucial for pluripotency; but how PSCs maintain and acquire high glycolysis levels or to what extent this metabolic behaviour affects pluripotency is still unknown. Mitochondrial activity seems to be crucial since work as a tool of Oxidative Phosphorylation or OXPHOS metabolism for energy production in cell reprogramming as well as (Sperber et al., 2015). Finally, it has been described the formation of lipid accumulations in lipid droplets as source of energy during this remodelling (Sperber et al., 2015).

Until few years, metabolism profile of stem cells that suffered reprogramming described Warburg Effect, but more studies in this field demonstrated that not all reprogrammed stem cells followed this pattern. The Warburg effect has been described over the years to

operate in highly proliferative cells to rapidly build up anabolic intermediates for proliferation while minimizing ROS-induced damage. Therefore, the Warburg effect is a specific mode of glycolytic metabolism, defined as unusually rapid glycolysis followed by lactate synthesis even under aerobic conditions. These intermediates feed into, but are not limited to, glycolytic shunts into the pentose phosphate pathway for nucleotide and NADPH synthesis, shunts into amino acid synthesis via 3-phosphoglycerate and pyruvate, and shunts into lipid synthesis via dihydroxyacetone phosphate. The pyruvate-lactate step in the Warburg effect is necessary to recycle the rate-limiting NAD+ coenzyme and keep the redox reactions in glycolysis running rapidly. Apparently, the pyruvate-acetyl-CoA step is important for pluripotency, but not the pyruvate-lactate step. This is a critical distinction to make, because it suggests that the Warburg effect is not rate limiting for pluripotency (Moussaieff et al., 2015b). The upregulation of glycolysis occurs early during iPSC reprogramming, well before a small fraction of cells acquires pluripotency (Folmes et al., 2011; Shyh-Chang et al., 2013).Moreover, acetyl-coA metabolite suffers a rapid decrease during differentiation of ESC suggesting the relevant role of this metabolite in the balance between pluripotency and differentiation (Mathieu et al., 2014). Many authors have described two different stem cells states in reprogramming primed and naive stem cells which follows distinct metabolism(Weinberger et al., 2016). We represented a schematic model showing principal differences between the metabolism of primed and naive stem cells between according to Weinberger model (Figure 4). Primed stem cells principally depend on glycolysis instead of OXPHOS whereas naive stem cells retain high levels of OXPHOS during high glycolysis (Mathieu et al., 2014; Sperber et al., 2015; Wanet et al., 2015; Zhou et al., 2012).According to our data, we hypothesize that DPSCs treated with Wnt activators, remains some primed and naive stem cells characteristics. We have studied some other features that can help to better understand more about metabolism how it works with epigenetics in reprogramming (Figure 5).

Pluripotent cell property	Naive pluripotent cell	Primed pluripotent cell	Human DPSCs FBS + BIO	Human DPSCs FBS + WNT-3A
MERK-ERK dependence	No	Yes	Unknown	Unknown
Long-term dependence on FGF signalling	No	Yes	Unknown	Unknown
Long-term dependence on TGB or Activin A signalling	No	Yes	Unknown	Unknown
Dominant OCT4 enhancer	Distal	Proximal	Unknown	Unknown
H3K27me3 on developmental regulators	Low	High	Unknown	Unknown
Global DNA hypomethylation	Yes	No	No	Yes
X chromosome inactivation	No	Yes	Don´t know	Don´t know
Dependence on DNMT1, DICER, METTL3, MBD3	No	Yes	Yes	Yes
Priming markers (OTX2, ZIC2)	Low	High	Unknown	Unknown
Pluripotency markers (NANOG, KLFs ESRRB)	High	Low	High	High
TFE nuclear localization	High	Low	Unknown	Unknown
CD24/MHC class 1	Low/low	High/low	Unknown	Unknown
Expressed adhesion molecules	E-cadherin	N-cadherin	Unknown	Unknown
Promotion of pluripotency maintenace by NANOG or PRDM14	Yes	No	Yes	Yes
Metabolism	OxPhos/Glycolytic	Glycolytic	OxPhos/Glycolytic	OxPhos/Glycolytic
Mitochondrial membrane activity and depolarization	High	Low	High	High
Hypomethylation of promoter and enhancer regions	Yes	No	Unknown	Unknown
Competence as initial starting cells for PGCLC induction	High	Low	Unknown	Unknown
Capacity for colonization of host pre-implantation ICM and contribution to advanced embryonic chiemeras	High	Low	Unknown	Unknown
KIT	Yes	No	Unknown	Unknown
Tolerance for abscence of exogenous L-glutamine	Yes	No	Unknown	Unknown
Competence as initial starting cell for TSC induction	High	Low	Unknown	Unknown

Figure 4.Characteristics of naïve and primed stem cells according to gene expression, metabolism and epigenetics. Pluripotent Stem cells could be divided according to some features showed in the following table in naïve and primed stem cells. Weinberger model explained the differences between these two populations of pluripotent stem cells studying gene patter expression, behavior in metabolism and epigenetics (Weinberger et al., 2016). We observed some of these features in our treated DSPCs, but some of them observed in Weinberger model are still unknown in our system model.

Pluripotent cell property	Naive pluripotent cell	Primed pluripotent cell	Human DPSCs FBS + BIO	Human DPSCs FBS + WNT-3A
Long-term dependence on Notch dependence	Yes*	Yes*	Yes	Yes
Cell proliferation	Low	High	Yes	High
Pluripotency markers (NANOG, KLFs ESRRB, OCT4, CMYC, LIN28, SOX2)	High	High	High	High
Hystone acetylation	Yes	No	Yes	Yes
Global DNA hypomethylation	Yes	No	No	Yes
Hystone methylation H3K4me3, H3K37me3, H3K9me3.	High	Low	High	High
Dependence on DNMT1, DNMT3A, DNMT3B	Yes/Yes/yes	Yes/Yes/Yes/Yes	Yes/No/No	Yes/No/No
Metabolism	OxPhos/Glycolytic	Glycolytic	OxPhos/Glycolytic	OxPhos/Glycolytic
Production of NADH, FADH2	Yes	Yes	Yes	Yes
ATPase activity	Yes	Yes	Yes	Yes
Mitochondrial membrane activity and depolarization	High	Low	High	High
Perinuclear mitochondria	Yes	Yes	Yes	Yes
Promotion of pluripotency maintenace by NANOG or PRDM14	Yes	No	Yes	Yes
Lipid droplets	High	Low	Unknown	Unknown
Oxidation of fatty acids	High	Low	High	High

Figure 5.Comparative between Weinberger model in naïve and primed stem cells and other features of pluripotent stem cells observed in our DPSCs model. We observed some features in our Wnt overactivated DSPCs that are attributed to naïve stem cells, such as cell proliferation, pluripotency markers (NANOG, KLFs ESRRB, OCT4, CMYC, LIN28, SOX2), histone acetylation, global DNA hypo methylation, histone methylation H3K4me3, H3K37me3, H3K9me3, dependence on DNMT1, DNMT3A, DNMT3B, glycolytic and oxidative metabolism , production of NADH, FADH2, ATPase activity, mitochondrial membrane activity and depolarization, perinuclear mitochondria, promotion of pluripotency maintenance by NANOG or PRDM14, lipid droplets and oxidation of fatty acids.

Control of metabolism by embryonic stem cell markers

It has been described that somatic stem cells can be reprogrammed to pluripotency by Oct4 and Sox2 together with Klf4 and cMyc (OSKM) or Nanog and Lin28 (OSNA) (Burk and Schade, 1956; Park et al., 2008; Takahashi et al., 2007; Takahashi and Yamanaka, 2006a; Yu et al., 2007). Pluripotent Stem Cells (PSCs) have a very specific metabolic pattern that probably reflects their rapid proliferation and the specific microenvironment from which they are derived. Although distinct metabolic properties are critical for the generation and maintenance of Pluripotent Stem Cells (PSCs), it is unknown how PSCs maintain and acquire this metabolic signature. OXPHOS is used by somatic cells for energy production, but upon reprogramming, the transition to pluripotency is accompanied with a shift to a glycolytic metabolism (Burk and Schade, 1956; Folmes et al., 2011; Panopoulos et al., 2012; Prigione et al., 2010; Varum et al., 2011). PSCs also rely on one-carbon metabolism for histone methylation and maintenance of pluripotency (Shiraki et al., 2014; Shyh-Chang et al., 2013). Interestingly, the well-studied core pluripotency factors Oct4, cMyc, Sox2 and Nanog regulate glycolysis directly by targeting key glycolytic enzymes in embryonic stem cells, such as Hk2 and Pkm2, which affect Lactate, Acetate and Glucose metabolites (Folmes et al., 2013; Kim et al., 2015). Apparently, one of the keys regulators is the core pluripotent factor cMyc, which directly binds and regulates metabolic gene targets, influencing glycolysis(Kim et al., 2004; Osthus et al., 2000; Shim et al., 1997), mitochondrial biogenesis (Li et al., 2005; Zhang et al., 2007), glutamine (Gao et al., 2009) and proline catabolism (Liu et al., 2012). Furthermore, the sustainment of high glycolytic flux delays ESC differentiation and enables certain populations of ESCs to retain the capacity for self-renewal and differentiation potential. Lin 28 gene has been reported to play a relevant role in PSC metabolism by maintaining the low mitochondrial function associated with primed pluripotency and in regulating one-carbon metabolism, nucleotide metabolism, and histone methylation (Zhang et al., 2016c).Interestingly, several authors have described that stem cell factors Oct4 and Lin28 regulate stem cell metabolism and conversion to pluripotency, and forma complex core pluripotent factor network with other core factors such as Sox2 and Nanog(Matoba et al., 2006; Zhang et al., 2016c).It has been recently reported that at a very early stage of reprogramming known as naive state, the increase in glycolysis is accompanied by a transient burst of OXPHOS activity or Oxidative Phosphorylation activity (Burk and Schade, 1956; Hawkins et al., 2016; Kida

et al., 2015). These findings could explain the temporary increase of mitochondrial proteins in cells undergoing reprogramming (Hansson et al., 2012).

CELL REPGROGRAMMING NEEDS EPIGENETIC REMODELING OF STEM CELLS

It is known that at early stages during embryonic development, some genes remain active, but when differentiation goes on (later stages) gradually become silenced and subsets of cell type-specific genes are turned on. This is the result of selectivity active expression of transcription factors in association with chromatin modification and remodelling, which includes covalent histone modification and DNA methylation of CpG dinucleotides, among other epigenetic modifications.

Histone modifications during epigenetic remodelling

Gene activity is mostly determined by chromatin structure and interactions of chromatin-binding proteins. To search for the elusive keys for DPSC cells pluripotency, many researchers have turned to the study of ES cells chromatin. It is to take into consideration that some features of chromatin in ES cells are different from the somatic cells, including nuclear architecture, chromatin structure, chromatin dynamics, and histone modifications. For example, ES cells chromatin displays characteristics of loosely euchromatin, such as an abundance of acetylated histone modifications and increased accessibility to nucleases (Boyer et al., 2006; Meshorer and Misteli, 2006). Consistent with the fact that the chromatins of pluripotent nuclei is in an open conformation, recent studies have shown that lineage-specific genes replicated earlier in pluripotent cells than differentiated cells, and had unexpectedly high levels of acetylated H3K9 and methylated H3K4. This modification is also combined with H3K27 tri-methylation. H3K27 methylation is functionally important for preventing differentiation genes expression in ES cells (Azuara et al., 2006; Bernstein et al., 2006).

Regarding DPSCs, p300, a well-known histone acetylase (HAT) play an important role in maintaining stemness of DPSCs, increasing transcript levels of NANOG and SOX2 and maintaining lower expression levels of odontoblastic differentiation markers (Wang et al., 2014). Histone deacetylase inhibitors (HDACi) alter the homeostatic balance between histone acetylation and deacetylation, increase the transcriptional rate and influence cell physiology suggesting that inhibition of HDACs has a great potential in

restorative dentistry through elevating the expression of NANOG and SOX2(Gu et al., 2013; Jin et al., 2013). Moreover, different types of histone deacetylases (HDACs) such as HDAC1, HDAC2, HDAC3, HDAC4 and HDAC9 act as important accelerator of odontoblast differentiation (Jin et al., 2013; Klinz et al., 2012; Paino et al., 2010). Histone marks in DPSCs revealed that H3K4me3 and H3K9me3 are the most prominent in DPSCs (Gopinathan et al., 2013). During differentiation, early mineralization genes are enriched with H3K4me3 whereas H3K27me3 and H3K9me3 are higher in late mineralization genes (Gopinathan et al., 2013). Interestingly, one demethylase is responsible for H3K27me3 removing, which contributes to odontogenic/osteogenic differentiation of DPSCs (Hoang et al., 2016). All of these results indicate an important regulatory circuit involving histone modifications that are crucial in determining cell fate in DPSCs. The potential application of eZH2, enzyme which catalyses H3K27me3 methylation in tissue regeneration, including nervous system, muscle, pancreas, and dental pulp has been recently discussed (Hui et al., 2014). Inhibition of the enzymatic activity of eZH2 impedes DPSCs cell proliferation by decreasing cell number, arresting the cell cycle, and increasing apoptosis (Bayarsaihan, 2016). Adipogenic induction of DPSCs leads to the suppression of eZH2, which in turn diminishes transcriptional activity of PPAR-r and CEBP/a (Bayarsaihan, 2016). According to recent research, the expression of pluripotency genes OCT4, NANOG, and SOX2 is higher in DPSCs than in Dental Follicle Stem Cells or DFSCs and it has been observed a substantial increase in H3K27me3. The inactivation of histone demethylase KDM6B associates with reduced mineralization of DPSCs and genes involved in odontoblast differentiation (Rodas-Junco et al., 2017). All of these results indicate an important regulatory circuit involving histone modifications that are crucial in determining cell fate in DPSCs.

Global DNA Methylation at CpG islands

DNA methylation at CpG islands is another gene silence mechanism of epigenetics crucial for gene expression. Silencing of certain genes by DNA methylation is required for induction of differentiation of ES cells whereas DNA hypomethylation is required for stemness condition maintenance (Jackson et al., 2004). Recent research focus on hypermethylation of promoter/enhancer regions of key pluripotency stem cells factors such as Oct4 that correlates with correlates with gene silencing in differentiated cells. Interestingly, hypomethylation in ES cells allows cells to maintain high level of Oct4 expression, thus keeping them in pluripotent state (Taranger et al., 2005).

As for DPSCs epigenetic alterations, it has been described that an inhibitor of DNA methyltransferase diminishes global DNA methylation in Periodontal Ligament Stem Cells (PDLSCs), interestingly involving Wnt/β-Catenin activation (Liu et al., 2016).This experiment at same concentration was performed in DPSCs and it was shown to increment of odontogenic differentiation potential (Zhang et al., 2015).

The odontogenic-specific DNA hypomethylation was suggested to be a typical feature of DPSCs (Ai et al., 2018). Some researchers tried to understand the spatial distribution of 5mC and 5hmC in odontoblasts and DPSCs. According to them, during differentiation the DNA methyltransferase, DNMT1 was vigorously transcribed, while expression changes of DNMT3A, DNMT3B and members of the TET family were relatively modest. In concordance to previous studies, DNA demethylation have been shown to increase the capability of odontogenic differentiation in DPSCs (Bayarsaihan, 2016).Taking all before into consideration, it seems that DNA demethylation may play a fundamental role in reparative dentinogenesis in human dental pulp. Despite some progress in this field, relatively little is known about the epigenetic pattern and consequent pleiotropic effects that drive DPSCs into both stemness and specialized lineages.

Control of epigenetic remodelling by embryonic stem cells factors

Since Yamanaka findings about key transcription factors of pluripotency, increasing knowledge has been recruited in several research to understand how pluripotency stem cells factors and the remodeling of epigenetics during reprogramming cooperates.

The most studied embryonic stem cell factors Oct4, cMyc, Nanog, Sox2, Klf4 and Lin28 have been widen in a complex network form by other factors such as Rex1, Ssea1, Stella, which interact with epigenetic landscape (Boyer et al., 2005; Ivanova et al., 2006; Loh et al., 2006; Rao and Orkin, 2006).cMyc may be associated with histone acetyltransferase (HAT) complex, and induces global histone acetylation, thus allowing Oct4 and Sox2 to bind to their specific target loci. Nanog regulates pluripotency as a transcription repressor for downstream genes that are important for cell differentiation such as Gata4 and Gata6 (Chambers et al., 2003; Mitsui et al., 2003). Klf4 might contribute to activation of Nanog and other ES cells-specific genes through p53 repression (Takahashi and Yamanaka, 2006b). Apparently, Oct4 cooperates with Nanog and Sox2 to repress Xist (Navarro et al., 2008). Oct4 also interacts with several polycomb group proteins (e.g., Ring1B, Rybp) as part of the Nanog interactome (Wang et al., 2006) to maintain pluripotency (Endoh et

al., 2008). Indeed, in ESCs chromatin architecture Oct4 acts directly the directly regulating downstream target genes encoding the H3K9 demethylases Jmjd1a and Jmjd2c, which modulate the H3K9 methylation status of the pluripotency factors Tcl1 and Nanog, respectively, to maintain stem cell identity (Loh et al., 2007).

These all factors induce somatic cell de-differentiation thanks to the chromatin remodelling. Therefore, we could attribute chromatin modification because of these transcription factors, which may play an important role in the reprogramming progress.

REPROGRAMMING NETWORK BETWEEN EPIGENETICS AND METABOLISM

As mentioned before, metabolism can be broadly divided in oxidative, completed in the mitochondria or non-oxidative, completed in the cytoplasm (Chandel et al., 2016; Ryall et al., 2015b). Nevertheless, research over the last years has identified an essential role for metabolites in the regulation of epigenetics and transcription, including S-adenosyl methionine (SAM) produced via the one-carbon cycle, acetyl-CoA from glycolysis, α–ketoglutarate (α-KG) and flavin adenine dinucleotide (FAD) from the NAD+/TCA cycle, and also from the integration of glycolysis and OXPHOS(Ryall et al., 2015a; Ryall et al., 2015b).

Acetyl-Coa interaction with epigenetics

Several studies place glycolysis, as a crucial producer of glycolytic intermediates necessary for the production of new biomass and metabolites required for epigenetic changes in transcription (Wellen et al., 2009).Acetyl-CoA can contribute to several cellular processes including the initiation of the TCA cycle, the de novo synthesis of lipids, and acetylation of specific amino-acid residues (predominantly lysine) on both histone and non-histone proteins (Koopman et al., 2014; Lunt and Vander Heiden, 2011).In fact, acetyl-CoA from glycolysis was found to serve as the substrate for histone acetylation. However, mitochondrial acetyl-CoA from fatty acid oxidation does not contribute to histone acetylation in absence of glucose (Wellen et al., 2009). These links between glycolysis and histone acetylation described before, have been observed in ESCs (Moussaieff et al., 2015b), skeletal muscle stem cells (Ryall et al., 2015b), tumour cells (Liu et al., 2015b) and yeast (Friis et al., 2009).Lack of acetyl-CoA production has been shown to cause a loss of pluripotency and delays cell differentiation (Moussaieff et al.,

2015b).Apparently, the reason besides is that acetyl-Coa is crucial to regulate and maintain histone acetylation in stem cells allowing chromatin remodelling during differentiation (Moussaieff et al. 2015). During ESC differentiation: pyruvate becomes fully oxidized in mitochondria, leading to acetyl-coA depravation, loss of histone acetylation and subsequent loss of pluripotency markers expression (Moussaieff et al., 2015a; Moussaieff et al., 2015b). The activity of the Histone Deacetylase Sirtuin (SIRT1) has also been shown to be crucial for reprogramming (Tang and Rando, 2014) Moreover, acetate, an acetyl-CoA precursor, prevented histone deacetylation and blocked the differentiation of ESCs (Moussaieff et al., 2015b). Nevertheless, others have attributed glycolysis-derived acetyl-CoA, through ATP citrate lyase (ACLY), the enhancer of the histone acetylation during adipogenesis (Wellen et al., 2009). These findings suggest that cytosolic acetyl-CoA does not always correlate with stemness and in this case, switch might exist to modulate histone acetylation and chromatin plasticity during cellular differentiation.

SAM contribution to epigenetic landscape

Several are the metabolites that contribute to the regulation of histone modifications and pluripotency in ESC metabolism. One of these is S-adenosylmethionine or SAM, which leads to histone methylation whereas ascorbate and a-ketoglutarate are the responsible for histone demethylation (Carey et al., 2015; Shyh-Chang et al., 2013). As SAM acts as the substrate for methyl transferases, is therefore a key regulator for maintaining the undifferentiated state of ESC and is highly expressed in iPSCs. SAM levels are controlled by nicotinamide N-methyl transferase (NNMT) by increasing H3K27me3 repressive marks (Sperber et al., 2015). NNMT role in pluripotency is here hypothesized to control SAM levels, SIRT1 and acetylation in DPSCs.

The mechanism by which cellular metabolism can influence stem cell fate is mostly unknown, but it is clear that it influences the epigenetic landscape, which in turn affects gene expression (Harvey et al., 2016).According to these findings, some metabolic intermediates may contribute in the regulation of open chromatin that is crucial for pluripotent potential of stem cells. Therefore, it would be of great interest understanding how metabolism contribute to pluripotency state and how is linked to epigenetics.

The epigenetic changes that occur during somatic cell reprogramming are also distinct from the epigenetic changes required to maintain pluripotency. Whereas somatic cell

reprogramming involves complex global remodelling of histone modifications and DNA methylation, pluripotency maintenance requires an enrichment of histone acetylation to maintain chromatin plasticity, relative to differentiated cells(Mattout et al., 2015).We incorporate in the following figure how specific metabolism pathways relevant for DPSCs integrate with epigenetics signalling (Figure6).

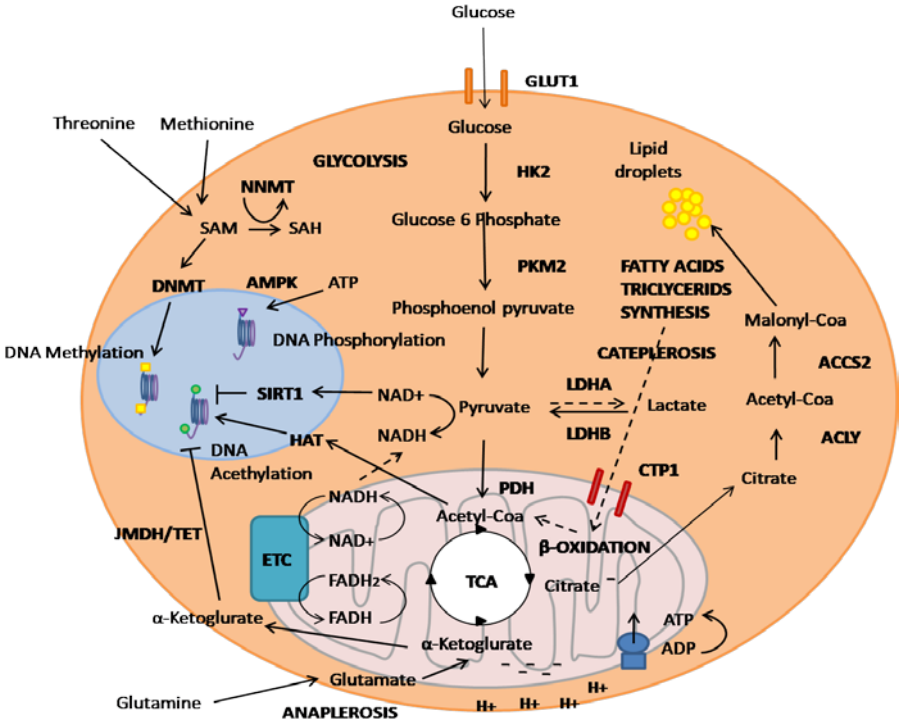


Figure 6. Representation of epigenetic and metabolic changes during reprogramming in DPSCs. Wnt signaling activation by either BIO or WNT-3A increases glucose consumption by overexpression of glycolytic enzymes HK2 and/or PKM2. LDHA and LDHB contribute to lactate-pyruvate conversion. LDHB is overexpressed while LHDA is downregulated in Wnt activated DPSCs. Pyruvate dehydrogenase complex subunits are also upregulated in BIO/WNT-3A treated DPSCs and this leads to fuel the mitochondrial TCA cycle. These “hyper-energized” DPSCs show a net accumulation of lipids and a mitochondrial hyperpolarization. Overexpression of cytosolic ACLY and ACSS2 enzymes suggests cataplerosis leading to cytosolic accumulation of acetyl-coA, which can be then used for lipid biosynthesis. This excess of acetyl-CoA is used for histone acetylation. The total amount of NAD^+ is used by SIRT 1 to quit acetylation marks. Contrarily, SAM from the One Carbon Metabolism acts as contributor for DNA methylation and NNMT convert SAM to SAH. This NNMT indirectly contributes to DNA de-methylation whereas then level of acetylation is higher in these conditions thanks to HAT and SIRT1 activities fueled by NAD+/NADH. Interestingly, ATP can contribute to DNA phosphorylation, but high levels can contribute to block AMPK, blocking DNA phosphorylation. Meanwhile, mitochondria consume amino acids such as glutamine and glutamate to replenish TCA metabolites in a coordinated cycle of cataplerosis and anaplerosis. Cytosolic fatty acids also appear to participate in this process, as suggested by the overexpression of CPT1 and β-oxidation enzymes at mRNA level. DPSCs reprogrammed with BIO or

WNT-3A thus show a boost in glycolysis without the characteristic lactate accumulation observed in the classic Warburg effect.

PLURIPOTENCY STEM CELLS FACTORS NETWORK

Recent studies have enabled the construction of transcriptional regulatory networks in ESCs that provide clues about how these factors control pluripotency and undergo subsequent differentiation events. These crucial transcription factors have also been described to form an intrinsic core-regulatory circuit that maintains ESCs in the pluripotent state during *in vitro* experiments. Using different methodology such as RNA interference method, microarray analysis, and genome wide chromatin immunoprecipitation experiments, numerous target genes bound by Nanog, Oct4, and Sox2 have been identified. These factors appear to form a tight transcriptional regulatory circuit that maintains ES cells in a pluripotent state (Boyer et al., 2005; Ivanova et al., 2006; Loh et al., 2006; Rao and Orkin, 2006).

These transcription factors, playing important roles in ES cells, form a regulatory pluripotency network that maintains ES cells self-renewal and stemness pluripotency by sustaining transcription factors expression at precise level(Cowan et al., 2005). Further studies found that forcing the expression of particularly transcription factors (Oct4, Sox2, c-Myc, Klf4), in somatic cells might reprogram them (Takahashi and Yamanaka, 2006b).

Oct4 is a POU domain-containing transcription factor that binds to an octamer sequence, ATGCAAAT(Zhao, 2013). Oct4 expression is activated at the four-cell stage and is expressed in most of stem cells, from ESCs and iPSCs, to some adult stem cells such as Dental Pulp Stem Cells(Zeineddine et al., 2014). Oct4 is highly expressed in human ESCs, and its expression diminishes when these cells differentiate into adult stem cells(Shi and Jin, 2010). Several target genes of Oct4 have been described until now, and these include Fgf4, Utf1, Opn, Rex1/ Zfp42, Fbx15, and Sox2 (Nishimoto et al., 1999; Tomioka et al., 2002; Zeng et al., 2004).Oct4 promoter contains conserved distal and proximal enhancers that can either repress or activate its expression depending on the binding factors occupying these sites (Pan et al., 2002). Its expression can be regulated by itself (Chew et al., 2005; Okumura-Nakanishi et al., 2005). This network regulation may contribute to a stable maintenance of the steady levels of Oct4 in ESCs. Furthermore, Nanog and FoxD3 also can activate Oct4 expression (Pan et al., 2006). Oct4 activity is modulated by interactions with other transcription factors that include Sox2, FoxD3, and

ESG1 which are highly expressed in ES cells. Sox2 (SRY-relatedHMGbox2), a member of the HMG-domain DNA-binding protein family, forms a complex with Oct4 protein. The complex Oct4/Sox2 can regulate Oct4 and Sox2 expression and further research indicated that also Nano could be modulated by this complex (Kuroda et al., 2005; Rodda et al., 2005), due to an Oct4/Sox2-binding site in the Nanog promoter. Therefore, Oct4/Sox2 complex plays a crucial role in maintaining the expression of embryonic stem cell factors in ESCs through autoregulatory and multicomponent loop network motifs. Therefore, Oct4 can regulate Nanog expression and maintains its activity by directly activating Nanog promoter when expressed below steady state, yet represses it at par with or above steady state concentration in ES cells. This can explain the evidence that overexpression of Oct4 induces differentiation (Pan et al., 2006).

Nanog is another homeobox-containing transcription factor with an essential role in maintaining the stemness, being a major regulator of the pluripotent state (Chambers et al., 2003; Mitsui et al., 2003). The mechanism through which Nanog regulates stem cell pluripotency remains entirely unknown. Nanog regulates pluripotency as a transcription repressor for downstream genes that are important for cell differentiation such as Gata4 and Gata6 (Chambers et al., 2003; Mitsui et al., 2003). However, Nanog can also activate the genes necessary for self-renewal such as Rex1 (Pan and Pei, 2005; Shi et al., 2006). Nanog can also activate Oct4 promoter (Pan et al., 2006) and may interact with Wnt and BMP4 signaling pathways (Suzuki et al., 2006). Network between these factors Oct4, Sox2, and Nanog may be the main transcriptional factors in regulating ESCs pluripotency.

Nanog has been identified as a major driver of pluripotency (Rao and Orkin, 2006; Silva et al., 2009).Rex1 is a target gene of Oct4/Sox2 complex. Rex1 is also directly regulated by Nanog, suggesting that Rex1 may be an intersection of Nanog and Oct4/Sox2 signaling (Shi et al., 2006).

The cMyc may be associated with histone acetyltransferase (HAT) complex, and induces global histone acetylation, thus allowing Oct4 and Sox2 to bind to their specific target loci.

Klf4 might contribute to activation of Nanog and other ES cells-specific genes through p53 repression (Takahashi and Yamanaka, 2006b).

CONCLUSIONS

In this review, we have presented an extensive discussion of how metabolism and substrate utilization can regulate transcription, through metabolites acting as co-factors for epigenetic regulators in DPSCs compare to ESCs. Moreover, we have described the current state of knowledge regarding the changes in metabolism that occur during stem cell differentiation and reprogramming. However, it is our hope that this review has also highlighted the gaps in our understanding of the process of metabolic and epigenetic reprogramming in stem cells. Future research investigating epigenetic changes in stem cell populations will likely include analyses of intracellular metabolites and cellular metabolism as a complex network. The understanding of the process of metabolic and epigenetic reprogramming in stem cells will lead to significant advances in the fields of stem cell transplantation and regenerative medicine.

REFERENCES

Abe S, Hamada K, Miura M, Yamaguchi S. 2012. Neural crest stem cell property of apical pulp cells derived from human developing tooth. Cell biology international 36(10):927-936.

Adler PN, Taylor J. 2001. Asymmetric cell division: plane but not simple. Current biology : CB 11(6):R233-236.

Ai T, Zhang J, Wang X, Zheng X, Qin X, Zhang Q, Li W, Hu W, Lin J, Chen F. 2018. DNA methylation profile is associated with the osteogenic potential of three distinct human odontogenic stem cells. Signal transduction and targeted therapy 3:1.

Alraies A, Alaidaroos NY, Waddington RJ, Moseley R, Sloan AJ. 2017. Variation in human dental pulp stem cell ageing profiles reflect contrasting proliferative and regenerative capabilities. BMC Cell Biol 18(1):12.

Aly LA. 2015. Stem cells: Sources, and regenerative therapies in dental research and practice. World journal of stem cells 7(7):1047-1053.

Andersen P, Uosaki H, Shenje LT, Kwon C. 2012. Non-canonical Notch signaling: emerging role and mechanism. Trends in cell biology 22(5):257-265.

Androutsellis-Theotokis A, Leker RR, Soldner F, Hoeppner DJ, Ravin R, Poser SW, Rueger MA, Bae SK, Kittappa R, McKay RD. 2006. Notch signalling regulates stem cell numbers in vitro and in vivo. Nature 442(7104):823-826.

Armstrong L, Tilgner K, Saretzki G, Atkinson SP, Stojkovic M, Moreno R, Przyborski S, Lako M. 2010. Human induced pluripotent stem cell lines show stress defense mechanisms and mitochondrial regulation similar to those of human embryonic stem cells. Stem cells 28(4):661-673.

Arthur A, Rychkov G, Shi S, Koblar SA, Gronthos S. 2008. Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues. Stem cells 26(7):1787-1795.

Atari M, Barajas M, Hernandez-Alfaro F, Gil C, Fabregat M, Ferres Padro E, Giner L, Casals N. 2011. Isolation of pluripotent stem cells from human third molar dental pulp. Histology and histopathology 26(8):1057-1070.

Atari M, Gil-Recio C, Fabregat M, Garcia-Fernandez D, Barajas M, Carrasco MA, Jung HS, Alfaro FH, Casals N, Prosper F, Ferres-Padro E, Giner L. 2012. Dental pulp of the third molar: a new source of pluripotent-like stem cells. Journal of cell science 125(Pt 14):3343-3356.

Aurrekoetxea M, Garcia-Gallastegui P, Irastorza I, Luzuriaga J, Uribe-Etxebarria V, Unda F, Ibarretxe G. 2015. Dental pulp stem cells as a multifaceted tool for bioengineering and the regeneration of craniomaxillofacial tissues. Frontiers in physiology 6:289.

Azuara V, Perry P, Sauer S, Spivakov M, Jorgensen HF, John RM, Gouti M, Casanova M, Warnes G, Merkenschlager M, Fisher AG. 2006. Chromatin signatures of pluripotent cell lines. Nat Cell Biol 8(5):532-538.

Banito A, Rashid ST, Acosta JC, Li S, Pereira CF, Geti I, Pinho S, Silva JC, Azuara V, Walsh M, Vallier L, Gil J. 2009. Senescence impairs successful reprogramming to pluripotent stem cells. Genes Dev 23(18):2134-2139.

Bayarsaihan D. 2016. Deciphering the Epigenetic Code in Embryonic and Dental Pulp Stem Cells. The Yale journal of biology and medicine 89(4):539-563.

Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, Jaenisch R, Wagschal A, Feil R, Schreiber SL, Lander ES. 2006. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 125(2):315-326.

Borggrefe T, Lauth M, Zwijsen A, Huylebroeck D, Oswald F, Giaimo BD. 2016. The Notch intracellular domain integrates signals from Wnt, Hedgehog, TGFbeta/BMP and hypoxia pathways. Biochimica et biophysica acta 1863(2):303-313.

Boutros M, Paricio N, Strutt DI, Mlodzik M. 1998. Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. Cell 94(1):109-118.

Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG, Gifford DK, Melton DA, Jaenisch R, Young RA. 2005. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 122(6):947-956.

Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, Lee TI, Levine SS, Wernig M, Tajonar A, Ray MK, Bell GW, Otte AP, Vidal M, Gifford DK, Young RA, Jaenisch R. 2006. Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature 441(7091):349-353.

Bray AF, Cevallos RR, Gazarian K, Lamas M. 2014. Human dental pulp stem cells respond to cues from the rat retina and differentiate to express the retinal neuronal marker rhodopsin. Neuroscience 280:142-155.

Buck MD, O'Sullivan D, Klein Geltink RI, Curtis JD, Chang CH, Sanin DE, Qiu J, Kretz O, Braas D, van der Windt GJ, Chen Q, Huang SC, O'Neill CM, Edelson BT, Pearce EJ, Sesaki H, Huber TB, Rambold AS, Pearce EL. 2016. Mitochondrial Dynamics Controls T Cell Fate through Metabolic Programming. Cell 166(1):63-76.

Burk D, Schade AL. 1956. On respiratory impairment in cancer cells. Science 124(3215):270-272.

Cacchiarelli D, Trapnell C, Ziller MJ, Soumillon M, Cesana M, Karnik R, Donaghey J, Smith ZD, Ratanasirintrawoot S, Zhang X, Ho Sui SJ, Wu Z, Akopian V, Gifford CA, Doench J, Rinn JL, Daley GQ, Meissner A, Lander ES, Mikkelsen TS. 2015. Integrative Analyses of Human Reprogramming Reveal Dynamic Nature of Induced Pluripotency. Cell 162(2):412-424.

Carey BW, Finley LW, Cross JR, Allis CD, Thompson CB. 2015. Intracellular alpha-ketoglutarate maintains the pluripotency of embryonic stem cells. Nature 518(7539):413-416.

Cartwright P, McLean C, Sheppard A, Rivett D, Jones K, Dalton S. 2005. LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. Development 132(5):885-896.

Clevers H. 2006. Wnt/beta-catenin signaling in development and disease. Cell 127(3):469-480.

Clevers H, Loh KM, Nusse R. 2014. Stem cell signaling. An integral program for tissue renewal and regeneration: Wnt signaling and stem cell control. Science (New York, NY) 346(6205):1248012.

Coronado D, Godet M, Bourillot PY, Tapponnier Y, Bernat A, Petit M, Afanassieff M, Markossian S, Malashicheva A, Iacone R, Anastassiadis K, Savatier P. 2013. A short G1 phase is an intrinsic determinant of naive embryonic stem cell pluripotency. Stem Cell Res 10(1):118-131.

Cowan CA, Atienza J, Melton DA, Eggan K. 2005. Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. Science 309(5739):1369-1373.

Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A. 2003. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 113(5):643-655.

Chambers I, Tomlinson SR. 2009. The transcriptional foundation of pluripotency. Development (Cambridge, England) 136(14):2311-2322.

Chandel NS, Jasper H, Ho TT, Passegue E. 2016. Metabolic regulation of stem cell function in tissue homeostasis and organismal ageing. Nat Cell Biol 18(8):823-832.

Chew JL, Loh YH, Zhang W, Chen X, Tam WL, Yeap LS, Li P, Ang YS, Lim B, Robson P, Ng HH. 2005. Reciprocal transcriptional regulation of Pou5f1 and Sox2 via the Oct4/Sox2 complex in embryonic stem cells. Molecular and cellular biology 25(14):6031-6046.

d'Aquino R, De Rosa A, Laino G, Caruso F, Guida L, Rullo R, Checchi V, Laino L, Tirino V, Papaccio G. 2009. Human dental pulp stem cells: from biology to clinical applications. Journal of experimental zoology Part B, Molecular and developmental evolution 312B(5):408-415.

D'Souza B, Meloty-Kapella L, Weinmaster G. 2010. Canonical and non-canonical Notch ligands. Current topics in developmental biology 92:73-129.

Edel MJ, Menchon C, Menendez S, Consiglio A, Raya A, Izpisua Belmonte JC. 2010. Rem2 GTPase maintains survival of human embryonic stem cells as well as enhancing reprogramming by regulating p53 and cyclin D1. Genes Dev 24(6):561-573.

Endoh M, Endo TA, Endoh T, Fujimura Y, Ohara O, Toyoda T, Otte AP, Okano M, Brockdorff N, Vidal M, Koseki H. 2008. Polycomb group proteins Ring1A/B are functionally linked to the core transcriptional regulatory circuitry to maintain ES cell identity. Development 135(8):1513-1524.

Ferro F, Spelat R, D'Aurizio F, Puppato E, Pandolfi M, Beltrami AP, Cesselli D, Falini G, Beltrami CA, Curcio F. 2012. Dental pulp stem cells differentiation reveals new insights in Oct4A dynamics. PloS one 7(7):e41774.

Folmes CD, Dzeja PP, Nelson TJ, Terzic A. 2012. Mitochondria in control of cell fate. Circulation research 110(4):526-529.

Folmes CD, Martinez-Fernandez A, Faustino RS, Yamada S, Perez-Terzic C, Nelson TJ, Terzic A. 2013. Nuclear reprogramming with c-Myc potentiates glycolytic capacity of derived induced pluripotent stem cells. Journal of cardiovascular translational research 6(1):10-21.

Folmes CD, Nelson TJ, Martinez-Fernandez A, Arrell DK, Lindor JZ, Dzeja PP, Ikeda Y, Perez-Terzic C, Terzic A. 2011. Somatic oxidative bioenergetics transitions into pluripotency-dependent glycolysis to facilitate nuclear reprogramming. Cell metabolism 14(2):264-271.

Friis RM, Wu BP, Reinke SN, Hockman DJ, Sykes BD, Schultz MC. 2009. A glycolytic burst drives glucose induction of global histone acetylation by picNuA4 and SAGA. Nucleic acids research 37(12):3969-3980.

Fukunaga-Kalabis M, Hristova DM, Wang JX, Li L, Heppt MV, Wei Z, Gyurdieva A, Webster MR, Oka M, Weeraratna AT, Herlyn M. 2015. UV-Induced Wnt7a in the Human Skin Microenvironment Specifies the Fate of Neural Crest-Like Cells via Suppression of Notch. The Journal of investigative dermatology 135(6):1521-1532.

Gao P, Tchernyshyov I, Chang TC, Lee YS, Kita K, Ochi T, Zeller KI, De Marzo AM, Van Eyk JE, Mendell JT, Dang CV. 2009. c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. Nature 458(7239):762-765.

Garcia-Castro MI, Marcelle C, Bronner-Fraser M. 2002. Ectodermal Wnt function as a neural crest inducer. Science (New York, NY) 297(5582):848-851.

Gascon S, Murenu E, Masserdotti G, Ortega F, Russo GL, Petrik D, Deshpande A, Heinrich C, Karow M, Robertson SP, Schroeder T, Beckers J, Irmler M, Berndt C, Angeli JP, Conrad M, Berninger B, Gotz M. 2016. Identification and Successful Negotiation of a Metabolic Checkpoint in Direct Neuronal Reprogramming. Cell Stem Cell 18(3):396-409.

Gattazzo F, Urciuolo A, Bonaldo P. 2014. Extracellular matrix: a dynamic microenvironment for stem cell niche. Biochimica et biophysica acta 1840(8):2506-2519.

Ghule PN, Medina R, Lengner CJ, Mandeville M, Qiao M, Dominski Z, Lian JB, Stein JL, van Wijnen AJ, Stein GS. 2011. Reprogramming the pluripotent cell cycle: restoration of an abbreviated G1 phase in human induced pluripotent stem (iPS) cells. J Cell Physiol 226(5):1149-1156.

Gopinathan G, Kolokythas A, Luan X, Diekwisch TG. 2013. Epigenetic marks define the lineage and differentiation potential of two distinct neural crest-derived intermediate odontogenic progenitor populations. Stem Cells Dev 22(12):1763-1778.

Green SA, Simoes-Costa M, Bronner ME. 2015. Evolution of vertebrates as viewed from the crest. Nature 520(7548):474-482.

Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, DenBesten P, Robey PG, Shi S. 2002. Stem cell properties of human dental pulp stem cells. J Dent Res 81(8):531-535.

Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. 2000. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proceedings of the National Academy of Sciences of the United States of America 97(25):13625-13630.

Gu S, Liang J, Wang J, Liu B. 2013. Histone acetylation regulates osteodifferentiation of human dental pulp stem cells via DSPP. Frontiers in bioscience 18:1072-1079.

Gu W, Gaeta X, Sahakyan A, Chan AB, Hong CS, Kim R, Braas D, Plath K, Lowry WE, Christofk HR. 2016. Glycolytic Metabolism Plays a Functional Role in Regulating Human Pluripotent Stem Cell State. Cell Stem Cell 19(4):476-490.

Guo S, Zi X, Schulz VP, Cheng J, Zhong M, Koochaki SH, Megyola CM, Pan X, Heydari K, Weissman SM, Gallagher PG, Krause DS, Fan R, Lu J. 2014. Nonstochastic reprogramming from a privileged somatic cell state. Cell 156(4):649-662.

Hackett JA, Surani MA. 2014. Regulatory principles of pluripotency: from the ground state up. Cell Stem Cell 15(4):416-430.

Halley-Stott RP, Jullien J, Pasque V, Gurdon J. 2014. Mitosis gives a brief window of opportunity for a change in gene transcription. PLoS Biol 12(7):e1001914.

Hansson J, Rafiee MR, Reiland S, Polo JM, Gehring J, Okawa S, Huber W, Hochedlinger K, Krijgsveld J. 2012. Highly coordinated proteome dynamics during reprogramming of somatic cells to pluripotency. Cell reports 2(6):1579-1592.

Hari L, Miescher I, Shakhova O, Suter U, Chin L, Taketo M, Richardson WD, Kessaris N, Sommer L. 2012. Temporal control of neural crest lineage generation by Wnt/beta-catenin signaling. Development (Cambridge, England) 139(12):2107-2117.

Harvey AJ, Rathjen J, Gardner DK. 2016. Metaboloepigenetic Regulation of Pluripotent Stem Cells. Stem Cells Int 2016:1816525.

Hawkins KE, Joy S, Delhove JM, Kotiadis VN, Fernandez E, Fitzpatrick LM, Whiteford JR, King PJ, Bolanos JP, Duchen MR, Waddington SN, McKay TR. 2016. NRF2 Orchestrates the Metabolic Shift during Induced Pluripotent Stem Cell Reprogramming. Cell reports 14(8):1883-1891.

Hishida T, Nakachi Y, Mizuno Y, Katano M, Okazaki Y, Ema M, Takahashi S, Hirasaki M, Suzuki A, Ueda A, Nishimoto M, Hishida-Nozaki Y, Vazquez-Ferrer E, Sancho-Martinez I, Izpisua Belmonte JC, Okuda A. 2015. Functional compensation between Myc and PI3K signaling supports self-renewal of embryonic stem cells. Stem cells 33(3):713-725.

Hishida T, Nozaki Y, Nakachi Y, Mizuno Y, Okazaki Y, Ema M, Takahashi S, Nishimoto M, Okuda A. 2011. Indefinite self-renewal of ESCs through Myc/Max transcriptional complex-independent mechanisms. Cell Stem Cell 9(1):37-49.

Hoang M, Kim JJ, Kim Y, Tong E, Trammell B, Liu Y, Shi S, Lee CR, Hong C, Wang CY, Kim Y. 2016. Alcohol-induced suppression of KDM6B dysregulates the mineralization potential in dental pulp stem cells. Stem Cell Res 17(1):111-121.

Hori K, Sen A, Artavanis-Tsakonas S. 2013. Notch signaling at a glance. J Cell Sci 126(Pt 10):2135-2140.

Huang GT, Gronthos S, Shi S. 2009. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. Journal of dental research 88(9):792-806.

Hui T, A P, Zhao Y, Wang C, Gao B, Zhang P, Wang J, Zhou X, Ye L. 2014. EZH2, a potential regulator of dental pulp inflammation and regeneration. J Endod 40(8):1132-1138.

Hunt CJ. 2011. Cryopreservation of Human Stem Cells for Clinical Application: A Review. Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie 38(2):107-123.

Huskey NE, Guo T, Evason KJ, Momcilovic O, Pardo D, Creasman KJ, Judson RL, Blelloch R, Oakes SA, Hebrok M, Goga A. 2015. CDK1 inhibition targets the p53-NOXA-MCL1 axis, selectively kills embryonic stem cells, and prevents teratoma formation. Stem Cell Reports 4(3):374-389.

Ibarretxe G, Crende O, Aurrekoetxea M, Garcia-Murga V, Etxaniz J, Unda F. 2012. Neural crest stem cells from dental tissues: a new hope for dental and neural regeneration. Stem cells international 2012:103503.

Ichida JK, Tcw J, Williams LA, Carter AC, Shi Y, Moura MT, Ziller M, Singh S, Amabile G, Bock C, Umezawa A, Rubin LL, Bradner JE, Akutsu H, Meissner A, Eggan K. 2014. Notch inhibition allows oncogene-independent generation of iPS cells. Nature chemical biology 10(8):632-639.

Ivanova N, Dobrin R, Lu R, Kotenko I, Levorse J, DeCoste C, Schafer X, Lun Y, Lemischka IR. 2006. Dissecting self-renewal in stem cells with RNA interference. Nature 442(7102):533-538.

Jackson M, Krassowska A, Gilbert N, Chevassut T, Forrester L, Ansell J, Ramsahoye B. 2004. Severe global DNA hypomethylation blocks differentiation and induces histone hyperacetylation in embryonic stem cells. Molecular and cellular biology 24(20):8862-8871.

Janebodin K, Horst OV, Ieronimakis N, Balasundaram G, Reesukumal K, Pratumvinit B, Reyes M. 2011. Isolation and characterization of neural crest-derived stem cells from dental pulp of neonatal mice. PloS one 6(11):e27526.

Jang H, Kim TW, Yoon S, Choi SY, Kang TW, Kim SY, Kwon YW, Cho EJ, Youn HD. 2012. O-GlcNAc regulates pluripotency and reprogramming by directly acting on core components of the pluripotency network. Cell Stem Cell 11(1):62-74.

Jin H, Park JY, Choi H, Choung PH. 2013. HDAC inhibitor trichostatin A promotes proliferation and odontoblast differentiation of human dental pulp stem cells. Tissue engineering Part A 19(5-6):613-624.

Kallas A, Pook M, Trei A, Maimets T. 2014. Assessment of the Potential of CDK2 Inhibitor NU6140 to Influence the Expression of Pluripotency Markers NANOG, OCT4, and SOX2 in 2102Ep and H9 Cells. International journal of cell biology 2014:280638.

Kawamura T, Suzuki J, Wang YV, Menendez S, Morera LB, Raya A, Wahl GM, Izpisua Belmonte JC. 2009. Linking the p53 tumour suppressor pathway to somatic cell reprogramming. Nature 460(7259):1140-1144.

Kellner M, Steindorff MM, Strempel JF, Winkel A, Kuhnel MP, Stiesch M. 2014. Differences of isolated dental stem cells dependent on donor age and consequences for autologous tooth replacement. Archives of Oral Biology 59(6):559-567.

Kerkis I, Kerkis A, Dozortsev D, Stukart-Parsons GC, Gomes Massironi SM, Pereira LV, Caplan AI, Cerruti HF. 2006. Isolation and characterization of a population of immature dental pulp stem cells expressing OCT-4 and other embryonic stem cell markers. Cells, tissues, organs 184(3-4):105-116.

Kida YS, Kawamura T, Wei Z, Sogo T, Jacinto S, Shigeno A, Kushige H, Yoshihara E, Liddle C, Ecker JR, Yu RT, Atkins AR, Downes M, Evans RM. 2015. ERRs Mediate a Metabolic Switch Required for Somatic Cell Reprogramming to Pluripotency. Cell Stem Cell 16(5):547-555.

Kim H, Jang H, Kim TW, Kang BH, Lee SE, Jeon YK, Chung DH, Choi J, Shin J, Cho EJ, Youn HD. 2015. Core Pluripotency Factors Directly Regulate Metabolism in Embryonic Stem Cell to Maintain Pluripotency. Stem cells 33(9):2699-2711.

Kim J, Chu J, Shen X, Wang J, Orkin SH. 2008. An extended transcriptional network for pluripotency of embryonic stem cells. Cell 132(6):1049-1061.

Kim J, Woo AJ, Chu J, Snow JW, Fujiwara Y, Kim CG, Cantor AB, Orkin SH. 2010. A Myc network accounts for similarities between embryonic stem and cancer cell transcription programs. Cell 143(2):313-324.

Kim JW, Zeller KI, Wang Y, Jegga AG, Aronow BJ, O'Donnell KA, Dang CV. 2004. Evaluation of myc E-box phylogenetic footprints in glycolytic genes by chromatin immunoprecipitation assays. Molecular and cellular biology 24(13):5923-5936.

Kiraly M, Porcsalmy B, Pataki A, Kadar K, Jelitai M, Molnar B, Hermann P, Gera I, Grimm WD, Ganss B, Zsembery A, Varga G. 2009. Simultaneous PKC and cAMP activation induces differentiation of human dental pulp stem cells into functionally active neurons. Neurochem Int 55(5):323-332.

Kitajima K, Nakajima M, Kanokoda M, Kyba M, Dandapat A, Tolar J, Saito MK, Toyoda M, Umezawa A, Hara T. 2016. GSK3beta inhibition activates the CDX/HOX pathway and promotes hemogenic endothelial progenitor differentiation from human pluripotent stem cells. Experimental hematology 44(1):68-74.e61-10.

Klinz FJ, Korkmaz Y, Bloch W, Raab WH, Addicks K. 2012. Histone deacetylases 2 and 9 are coexpressed and nuclear localized in human molar odontoblasts in vivo. Histochemistry and cell biology 137(5):697-702.

Koopman R, Ly CH, Ryall JG. 2014. A metabolic link to skeletal muscle wasting and regeneration. Front Physiol 5:32.

Kuhl M, Sheldahl LC, Malbon CC, Moon RT. 2000. Ca(2+)/calmodulin-dependent protein kinase II is stimulated by Wnt and Frizzled homologs and promotes ventral cell fates in Xenopus. J Biol Chem 275(17):12701-12711.

Kuroda T, Tada M, Kubota H, Kimura H, Hatano SY, Suemori H, Nakatsuji N, Tada T. 2005. Octamer and Sox elements are required for transcriptional cis regulation of Nanog gene expression. Molecular and cellular biology 25(6):2475-2485.

Kwon C, Cheng P, King IN, Andersen P, Shenje L, Nigam V, Srivastava D. 2011. Notch post-translationally regulates beta-catenin protein in stem and progenitor cells. Nature cell biology 13(10):1244-1251.

La Noce M, Mele L, Tirino V, Paino F, De Rosa A, Naddeo P, Papagerakis P, Papaccio G, Desiderio V. 2014. Neural crest stem cell population in craniomaxillofacial development and tissue repair. Eur Cell Mater 28:348-357.

Lavagnolli T, Gupta P, Hormanseder E, Mira-Bontenbal H, Dharmalingam G, Carroll T, Gurdon JB, Fisher AG, Merkenschlager M. 2015. Initiation and maintenance of pluripotency gene expression in the absence of cohesin. Genes Dev 29(1):23-38.

Ledesma-Martinez E, Mendoza-Nunez VM, Santiago-Osorio E. 2016. Mesenchymal Stem Cells Derived from Dental Pulp: A Review. Stem Cells Int 2016:4709572.

Lee J, Go Y, Kang I, Han YM, Kim J. 2010. Oct-4 controls cell-cycle progression of embryonic stem cells. The Biochemical journal 426(2):171-181.

Leung AW, Murdoch B, Salem AF, Prasad MS, Gomez GA, Garcia-Castro MI. 2016. WNT/beta-catenin signaling mediates human neural crest induction via a pre-neural border intermediate. Development (Cambridge, England) 143(3):398-410.

Li F, Wang Y, Zeller KI, Potter JJ, Wonsey DR, O'Donnell KA, Kim JW, Yustein JT, Lee LA, Dang CV. 2005. Myc stimulates nuclearly encoded mitochondrial genes and mitochondrial biogenesis. Molecular and cellular biology 25(14):6225-6234.

Li H, Collado M, Villasante A, Strati K, Ortega S, Canamero M, Blasco MA, Serrano M. 2009. The Ink4/Arf locus is a barrier for iPS cell reprogramming. Nature 460(7259):1136-1139.

Li VC, Kirschner MW. 2014. Molecular ties between the cell cycle and differentiation in embryonic stem cells. Proc Natl Acad Sci U S A 111(26):9503-9508.

Li X, Wang D, Huang H, Zhang J, Lian J, Yuan X, Yang J, Zhang G. 2015. Linking benthic community structure to terrestrial runoff and upwelling in the coral reefs of northeastern Hainan Island. Estuarine, Coastal and Shelf Science 156(1):92-102.

Lima RL, Holanda-Afonso RC, Moura-Neto V, Bolognese AM, DosSantos MF, Souza MM. 2017. Human dental follicle cells express embryonic, mesenchymal and neural stem cells markers. Arch Oral Biol 73:121-128.

Lin YC, Murayama Y, Hashimoto K, Nakamura Y, Lin CS, Yokoyama KK, Saito S. 2014. Role of tumor suppressor genes in the cancer-associated reprogramming of human induced pluripotent stem cells. Stem Cell Res Ther 5(2):58.

Liu J, Yu F, Sun Y, Jiang B, Zhang W, Yang J, Xu GT, Liang A, Liu S. 2015a. Concise reviews: Characteristics and potential applications of human dental tissue-derived mesenchymal stem cells. Stem cells (Dayton, Ohio) 33(3):627-638.

Liu W, Le A, Hancock C, Lane AN, Dang CV, Fan TW, Phang JM. 2012. Reprogramming of proline and glutamine metabolism contributes to the proliferative and metabolic responses regulated by oncogenic transcription factor c-MYC. Proc Natl Acad Sci U S A 109(23):8983-8988.

Liu XS, Little JB, Yuan ZM. 2015b. Glycolytic metabolism influences global chromatin structure. Oncotarget 6(6):4214-4225.

Liu Z, Chen T, Sun W, Yuan Z, Yu M, Chen G, Guo W, Xiao J, Tian W. 2016. DNA Demethylation Rescues the Impaired Osteogenic Differentiation Ability of Human Periodontal Ligament Stem Cells in High Glucose. Scientific reports 6:27447.

Loh YH, Wu Q, Chew JL, Vega VB, Zhang W, Chen X, Bourque G, George J, Leong B, Liu J, Wong KY, Sung KW, Lee CW, Zhao XD, Chiu KP, Lipovich L, Kuznetsov VA, Robson P, Stanton LW, Wei CL, Ruan Y, Lim B, Ng HH. 2006. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nature genetics 38(4):431-440.

Loh YH, Zhang W, Chen X, George J, Ng HH. 2007. Jmjd1a and Jmjd2c histone H3 Lys 9 demethylases regulate self-renewal in embryonic stem cells. Genes Dev 21(20):2545-2557.

Lunt SY, Vander Heiden MG. 2011. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. Annu Rev Cell Dev Biol 27:441-464.

Ma T, Li J, Xu Y, Yu C, Xu T, Wang H, Liu K, Cao N, Nie BM, Zhu SY, Xu S, Li K, Wei WG, Wu Y, Guan KL, Ding S. 2015. Atg5-independent autophagy regulates mitochondrial clearance and is essential for iPSC reprogramming. Nat Cell Biol 17(11):1379-1387.

Marion RM, Strati K, Li H, Murga M, Blanco R, Ortega S, Fernandez-Capetillo O, Serrano M, Blasco MA. 2009. A p53-mediated DNA damage response limits reprogramming to ensure iPS cell genomic integrity. Nature 460(7259):1149-1153.

Mathieu J, Zhou W, Xing Y, Sperber H, Ferreccio A, Agoston Z, Kuppusamy KT, Moon RT, Ruohola-Baker H. 2014. Hypoxia-inducible factors have distinct and stage-specific roles during reprogramming of human cells to pluripotency. Cell Stem Cell 14(5):592-605.

Matoba R, Niwa H, Masui S, Ohtsuka S, Carter MG, Sharov AA, Ko MS. 2006. Dissecting Oct3/4-regulated gene networks in embryonic stem cells by expression profiling. PLoS One 1:e26.

Mattout A, Aaronson Y, Sailaja BS, Raghu Ram EV, Harikumar A, Mallm JP, Sim KH, Nissim-Rafinia M, Supper E, Singh PB, Sze SK, Gasser SM, Rippe K, Meshorer E. 2015. Heterochromatin Protein 1beta (HP1beta) has distinct functions and distinct nuclear distribution in pluripotent versus differentiated cells. Genome biology 16:213.

McLenachan S, Menchon C, Raya A, Consiglio A, Edel MJ. 2012. Cyclin A1 is essential for setting the pluripotent state and reducing tumorigenicity of induced pluripotent stem cells. Stem Cells Dev 21(15):2891-2899.

Meshorer E, Misteli T. 2006. Chromatin in pluripotent embryonic stem cells and differentiation. Nature reviews Molecular cell biology 7(7):540-546.

Miletich I, Sharpe PT. 2004. Neural crest contribution to mammalian tooth formation. Birth defects research Part C, Embryo today : reviews 72(2):200-212.

Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S. 2003. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113(5):631-642.

Mizutani K, Yoon K, Dang L, Tokunaga A, Gaiano N. 2007. Differential Notch signalling distinguishes neural stem cells from intermediate progenitors. Nature 449(7160):351-355.

Moussaieff A, Kogan NM, Aberdam D. 2015a. Concise Review: Energy Metabolites: Key Mediators of the Epigenetic State of Pluripotency. Stem cells 33(8):2374-2380.

Moussaieff A, Rouleau M, Kitsberg D, Cohen M, Levy G, Barasch D, Nemirovski A, Shen-Orr S, Laevsky I, Amit M, Bomze D, Elena-Herrmann B, Scherf T, Nissim-Rafinia M, Kempa S, Itskovitz-Eldor J, Meshorer E, Aberdam D, Nahmias Y. 2015b. Glycolysis-mediated changes in acetyl-CoA and histone acetylation control the early differentiation of embryonic stem cells. Cell metabolism 21(3):392-402.

Navarro P, Chambers I, Karwacki-Neisius V, Chureau C, Morey C, Rougeulle C, Avner P. 2008. Molecular coupling of Xist regulation and pluripotency. Science 321(5896):1693-1695.

Neganova I, Tilgner K, Buskin A, Paraskevopoulou I, Atkinson SP, Peberdy D, Passos JF, Lako M. 2014. CDK1 plays an important role in the maintenance of pluripotency and genomic stability in human pluripotent stem cells. Cell Death Dis 5:e1508.

Nishimoto M, Fukushima A, Okuda A, Muramatsu M. 1999. The gene for the embryonic stem cell coactivator UTF1 carries a regulatory element which selectively interacts with a complex composed of Oct-3/4 and Sox-2. Molecular and cellular biology 19(8):5453-5465.

O'Connor MD, Wederell E, Robertson G, Delaney A, Morozova O, Poon SS, Yap D, Fee J, Zhao Y, McDonald H, Zeng T, Hirst M, Marra MA, Aparicio SA, Eaves CJ. 2011. Retinoblastoma-binding proteins 4 and 9 are important for human pluripotent stem cell maintenance. Exp Hematol 39(8):866-879 e861.

Okumura-Nakanishi S, Saito M, Niwa H, Ishikawa F. 2005. Oct-3/4 and Sox2 regulate Oct-3/4 gene in embryonic stem cells. J Biol Chem 280(7):5307-5317.

Osthus RC, Shim H, Kim S, Li Q, Reddy R, Mukherjee M, Xu Y, Wonsey D, Lee LA, Dang CV. 2000. Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. J Biol Chem 275(29):21797-21800.

Paino F, Ricci G, De Rosa A, D'Aquino R, Laino L, Pirozzi G, Tirino V, Papaccio G. 2010. Ecto-mesenchymal stem cells from dental pulp are committed to differentiate into active melanocytes. Eur Cell Mater 20:295-305.

Pan G, Li J, Zhou Y, Zheng H, Pei D. 2006. A negative feedback loop of transcription factors that controls stem cell pluripotency and self-renewal. FASEB J 20(10):1730-1732.

Pan G, Pei D. 2005. The stem cell pluripotency factor NANOG activates transcription with two unusually potent subdomains at its C terminus. J Biol Chem 280(2):1401-1407.

Pan GJ, Chang ZY, Scholer HR, Pei D. 2002. Stem cell pluripotency and transcription factor Oct4. Cell Res 12(5-6):321-329.

Panopoulos AD, Yanes O, Ruiz S, Kida YS, Diep D, Tautenhahn R, Herrerias A, Batchelder EM, Plongthongkum N, Lutz M, Berggren WT, Zhang K, Evans RM, Siuzdak G, Izpisua Belmonte JC. 2012. The metabolome of induced pluripotent stem cells reveals metabolic changes occurring in somatic cell reprogramming. Cell Res 22(1):168-177.

Park IH, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW, Daley GQ. 2008. Reprogramming of human somatic cells to pluripotency with defined factors. Nature 451(7175):141-146.

Perdigoto CN, Bardin AJ. 2013. Sending the right signal: Notch and stem cells. Biochimica et biophysica acta 1830(2):2307-2322.

Petrovic V, Stefanovic V. 2009. Dental tissue--new source for stem cells. TheScientificWorldJournal 9:1167-1177.

Pierdomenico L, Bonsi L, Calvitti M, Rondelli D, Arpinati M, Chirumbolo G, Becchetti E, Marchionni C, Alviano F, Fossati V, Staffolani N, Franchina M, Grossi A, Bagnara GP. 2005. Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp. Transplantation 80(6):836-842.

Prigione A, Fauler B, Lurz R, Lehrach H, Adjaye J. 2010. The senescence-related mitochondrial/oxidative stress pathway is repressed in human induced pluripotent stem cells. Stem cells 28(4):721-733.

Rahl PB, Lin CY, Seila AC, Flynn RA, McCuine S, Burge CB, Sharp PA, Young RA. 2010. c-Myc regulates transcriptional pause release. Cell 141(3):432-445.

Rao S, Orkin SH. 2006. Unraveling the transcriptional network controlling ES cell pluripotency. Genome biology 7(8):230.

Reya T, Clevers H. 2005. Wnt signalling in stem cells and cancer. Nature 434(7035):843-850.

Roccio M, Schmitter D, Knobloch M, Okawa Y, Sage D, Lutolf MP. 2013. Predicting stem cell fate changes by differential cell cycle progression patterns. Development 140(2):459-470.

Rodas-Junco BA, Canul-Chan M, Rojas-Herrera RA, De-la-Pena C, Nic-Can GI. 2017. Stem Cells from Dental Pulp: What Epigenetics Can Do with Your Tooth. Front Physiol 8:999.

Rodda DJ, Chew JL, Lim LH, Loh YH, Wang B, Ng HH, Robson P. 2005. Transcriptional regulation of nanog by OCT4 and SOX2. J Biol Chem 280(26):24731-24737.

Rogers CD, Jayasena CS, Nie S, Bronner ME. 2012. Neural crest specification: tissues, signals, and transcription factors. Wiley interdisciplinary reviewsDevelopmental biology 1(1):52-68.

Rosa V, Dubey N, Islam I, Min KS, Nor JE. 2016. Pluripotency of Stem Cells from Human Exfoliated Deciduous Teeth for Tissue Engineering. Stem cells international 2016:5957806.

Ruiz S, Panopoulos AD, Herrerias A, Bissig KD, Lutz M, Berggren WT, Verma IM, Izpisua Belmonte JC. 2011. A high proliferation rate is required for cell reprogramming and maintenance of human embryonic stem cell identity. Current biology : CB 21(1):45-52.

Ryall JG, Cliff T, Dalton S, Sartorelli V. 2015a. Metabolic Reprogramming of Stem Cell Epigenetics. Cell Stem Cell 17(6):651-662.

Ryall JG, Dell'Orso S, Derfoul A, Juan A, Zare H, Feng X, Clermont D, Koulnis M, Gutierrez-Cruz G, Fulco M, Sartorelli V. 2015b. The NAD(+)-dependent SIRT1 deacetylase translates a metabolic switch into regulatory epigenetics in skeletal muscle stem cells. Cell Stem Cell 16(2):171-183.

Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. 2004. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nature medicine 10(1):55-63.

Scheller EL, Chang J, Wang CY. 2008. Wnt/beta-catenin inhibits dental pulp stem cell differentiation. Journal of dental research 87(2):126-130.

Schiraldi C, Stellavato A, D'Agostino A, Tirino V, d'Aquino R, Woloszyk A, De Rosa A, Laino L, Papaccio G, Mitsiadis TA. 2012. Fighting for territories: time-lapse analysis of dental pulp and dental follicle stem cells in co-culture reveals specific migratory capabilities. Eur Cell Mater 24:426-440.

Sheldahl LC, Park M, Malbon CC, Moon RT. 1999. Protein kinase C is differentially stimulated by Wnt and Frizzled homologs in a G-protein-dependent manner. Current biology : CB 9(13):695-698.

Shi G, Jin Y. 2010. Role of Oct4 in maintaining and regaining stem cell pluripotency. Stem Cell Res Ther 1(5):39.

Shi W, Wang H, Pan G, Geng Y, Guo Y, Pei D. 2006. Regulation of the pluripotency marker Rex-1 by Nanog and Sox2. J Biol Chem 281(33):23319-23325.

Shim H, Dolde C, Lewis BC, Wu CS, Dang G, Jungmann RA, Dalla-Favera R, Dang CV. 1997. c-Myc transactivation of LDH-A: implications for tumor metabolism and growth. Proc Natl Acad Sci U S A 94(13):6658-6663.

Shiraki N, Shiraki Y, Tsuyama T, Obata F, Miura M, Nagae G, Aburatani H, Kume K, Endo F, Kume S. 2014. Methionine metabolism regulates maintenance and differentiation of human pluripotent stem cells. Cell metabolism 19(5):780-794.

Shyh-Chang N, Locasale JW, Lyssiotis CA, Zheng Y, Teo RY, Ratanasirintrawoot S, Zhang J, Onder T, Unternaehrer JJ, Zhu H, Asara JM, Daley GQ, Cantley LC. 2013. Influence of threonine metabolism on S-adenosylmethionine and histone methylation. Science 339(6116):222-226.

Silva J, Nichols J, Theunissen TW, Guo G, van Oosten AL, Barrandon O, Wray J, Yamanaka S, Chambers I, Smith A. 2009. Nanog is the gateway to the pluripotent ground state. Cell 138(4):722-737.

Simoes-Costa M, Bronner ME. 2015. Establishing neural crest identity: a gene regulatory recipe. Development 142(2):242-257.

Singh AM, Dalton S. 2009. The cell cycle and Myc intersect with mechanisms that regulate pluripotency and reprogramming. Cell Stem Cell 5(2):141-149.

Sperber H, Mathieu J, Wang Y, Ferreccio A, Hesson J, Xu Z, Fischer KA, Devi A, Detraux D, Gu H, Battle SL, Showalter M, Valensisi C, Bielas JH, Ericson NG, Margaretha L, Robitaille AM, Margineantu D, Fiehn O, Hockenbery D, Blau CA, Raftery D, Margolin AA, Hawkins RD, Moon RT, Ware CB, Ruohola-Baker H.

2015. The metabolome regulates the epigenetic landscape during naive-to-primed human embryonic stem cell transition. Nat Cell Biol 17(12):1523-1535.

Stevens A, Zuliani T, Olejnik C, LeRoy H, Obriot H, Kerr-Conte J, Formstecher P, Bailliez Y, Polakowska RR. 2008. Human dental pulp stem cells differentiate into neural crest-derived melanocytes and have label-retaining and sphere-forming abilities. Stem Cells Dev 17(6):1175-1184.

Stuhlmiller TJ, Garcia-Castro MI. 2012. Current perspectives of the signaling pathways directing neural crest induction. Cellular and molecular life sciences : CMLS 69(22):3715-3737.

Suhr ST, Chang EA, Tjong J, Alcasid N, Perkins GA, Goissis MD, Ellisman MH, Perez GI, Cibelli JB. 2010. Mitochondrial rejuvenation after induced pluripotency. PLoS One 5(11):e14095.

Sun H, Lesche R, Li DM, Liliental J, Zhang H, Gao J, Gavrilova N, Mueller B, Liu X, Wu H. 1999. PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. Proc Natl Acad Sci U S A 96(11):6199-6204.

Suzuki A, Raya A, Kawakami Y, Morita M, Matsui T, Nakashima K, Gage FH, Rodriguez-Esteban C, Izpisua Belmonte JC. 2006. Nanog binds to Smad1 and blocks bone morphogenetic protein-induced differentiation of embryonic stem cells. Proc Natl Acad Sci U S A 103(27):10294-10299.

Syed-Picard FN, Du Y, Lathrop KL, Mann MM, Funderburgh ML, Funderburgh JL. 2015. Dental pulp stem cells: a new cellular resource for corneal stromal regeneration. Stem Cells Transl Med 4(3):276-285.

Takahashi K, Okita K, Nakagawa M, Yamanaka S. 2007. Induction of pluripotent stem cells from fibroblast cultures. Nature protocols 2(12):3081-3089.

Takahashi K, Yamanaka S. 2006a. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663-676.

Takahashi K, Yamanaka S. 2006b. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663-676.

Tang AH, Rando TA. 2014. Induction of autophagy supports the bioenergetic demands of quiescent muscle stem cell activation. EMBO J 33(23):2782-2797.

Taranger CK, Noer A, Sorensen AL, Hakelien AM, Boquest AC, Collas P. 2005. Induction of dedifferentiation, genomewide transcriptional programming, and epigenetic reprogramming by extracts of carcinoma and embryonic stem cells. Molecular biology of the cell 16(12):5719-5735.

Tomioka M, Nishimoto M, Miyagi S, Katayanagi T, Fukui N, Niwa H, Muramatsu M, Okuda A. 2002. Identification of Sox-2 regulatory region which is under the control of Oct-3/4-Sox-2 complex. Nucleic acids research 30(14):3202-3213.

Uribe-Etxebarria V, Luzuriaga J, Garcia-Gallastegui P, Agliano A, Unda F, Ibarretxe G. 2017. Notch/Wnt cross-signalling regulates stemness of dental pulp stem cells through expression of neural crest and core pluripotency factors. Eur Cell Mater 34:249-270.

Varum S, Rodrigues AS, Moura MB, Momcilovic O, Easley CAt, Ramalho-Santos J, Van Houten B, Schatten G. 2011. Energy metabolism in human pluripotent stem cells and their differentiated counterparts. PLoS One 6(6):e20914.

Vasanthan P, Govindasamy V, Gnanasegaran N, Kunasekaran W, Musa S, Abu Kasim NH. 2015. Differential expression of basal microRNAs' patterns in human dental pulp stem cells. Journal of cellular and molecular medicine 19(3):566-580.

Wagers AJ, Christensen JL, Weissman IL. 2002. Cell fate determination from stem cells. Gene therapy 9(10):606-612.

Wanet A, Arnould T, Najimi M, Renard P. 2015. Connecting Mitochondria, Metabolism, and Stem Cell Fate. Stem Cells Dev 24(17):1957-1971.

Wang J, Rao S, Chu J, Shen X, Levasseur DN, Theunissen TW, Orkin SH. 2006. A protein interaction network for pluripotency of embryonic stem cells. Nature 444(7117):364-368.

Wang T, Liu H, Ning Y, Xu Q. 2014. The histone acetyltransferase p300 regulates the expression of pluripotency factors and odontogenic differentiation of human dental pulp cells. PLoS One 9(7):e102117.

Weinberger L, Ayyash M, Novershtern N, Hanna JH. 2016. Dynamic stem cell states: naive to primed pluripotency in rodents and humans. Nature reviews Molecular cell biology 17(3):155-169.

Wellen KE, Hatzivassiliou G, Sachdeva UM, Bui TV, Cross JR, Thompson CB. 2009. ATP-citrate lyase links cellular metabolism to histone acetylation. Science 324(5930):1076-1080.

Wilson R, Urraca N, Skobowiat C, Hope KA, Miravalle L, Chamberlin R, Donaldson M, Seagroves TN, Reiter LT. 2015. Assessment of the Tumorigenic Potential of Spontaneously Immortalized and hTERT-Immortalized Cultured Dental Pulp Stem Cells. Stem cells translational medicine 4(8):905-912.

Wu W, Zhou J, Xu CT, Zhang J, Jin YJ, Sun GL. 2015. Derivation and growth characteristics of dental pulp stem cells from patients of different ages. Molecular medicine reports 12(4):5127-5134.

Yang J, Yuan G, Chen Z. 2016. Pulp Regeneration: Current Approaches and Future Challenges. Front Physiol 7:58.

Yeo HC, Beh TT, Quek JJ, Koh G, Chan KK, Lee DY. 2011. Integrated transcriptome and binding sites analysis implicates E2F in the regulation of self-renewal in human pluripotent stem cells. PLoS One 6(11):e27231.

Yiew NK, Chatterjee TK, Tang YL, Pellenberg R, Stansfield BK, Bagi Z, Fulton DJ, Stepp DW, Chen W, Patel V, Kamath VM, Litwin SE, Hui DY, Rudich SM, Kim HW, Weintraub NL. 2017. Novel role for Wnt inhibitor APCDD1 in adipocyte differentiation: implications for diet-induced obesity. The Journal of biological chemistry.

Young RA. 2011. Control of the embryonic stem cell state. Cell 144(6):940-954.

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. 2007. Induced pluripotent stem cell lines derived from human somatic cells. Science (New York, NY) 318(5858):1917-1920.

Zeineddine D, Hammoud AA, Mortada M, Boeuf H. 2014. The Oct4 protein: more than a magic stemness marker. American journal of stem cells 3(2):74-82.

Zeng X, Miura T, Luo Y, Bhattacharya B, Condie B, Chen J, Ginis I, Lyons I, Mejido J, Puri RK, Rao MS, Freed WJ. 2004. Properties of pluripotent human embryonic stem cells BG01 and BG02. Stem cells 22(3):292-312.

Zhang D, Li Q, Rao L, Yi B, Xu Q. 2015. Effect of 5-Aza-2'-deoxycytidine on odontogenic differentiation of human dental pulp cells. J Endod 41(5):640-645.

Zhang H, Gao P, Fukuda R, Kumar G, Krishnamachary B, Zeller KI, Dang CV, Semenza GL. 2007. HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity. Cancer cell 11(5):407-420.

Zhang H, Ryu D, Wu Y, Gariani K, Wang X, Luan P, D'Amico D, Ropelle ER, Lutolf MP, Aebersold R, Schoonjans K, Menzies KJ, Auwerx J. 2016a. NAD(+) repletion improves mitochondrial and stem cell function and enhances life span in mice. Science 352(6292):1436-1443.

Zhang J, Khvorostov I, Hong JS, Oktay Y, Vergnes L, Nuebel E, Wahjudi PN, Setoguchi K, Wang G, Do A, Jung HJ, McCaffery JM, Kurland IJ, Reue K, Lee WN, Koehler CM, Teitell MA. 2016b. UCP2 regulates energy metabolism and differentiation potential of human pluripotent stem cells. EMBO J 35(8):899.

Zhang J, Ratanasirintrawoot S, Chandrasekaran S, Wu Z, Ficarro SB, Yu C, Ross CA, Cacchiarelli D, Xia Q, Seligson M, Shinoda G, Xie W, Cahan P, Wang L, Ng SC, Tintara S, Trapnell C, Onder T, Loh YH, Mikkelsen T, Sliz P, Teitell MA, Asara JM, Marto JA, Li H, Collins JJ, Daley GQ. 2016c. LIN28 Regulates Stem Cell Metabolism and Conversion to Primed Pluripotency. Cell Stem Cell 19(1):66-80.

Zhang X, Neganova I, Przyborski S, Yang C, Cooke M, Atkinson SP, Anyfantis G, Fenyk S, Keith WN, Hoare SF, Hughes O, Strachan T, Stojkovic M, Hinds PW, Armstrong L, Lako M. 2009. A role for NANOG in G1 to S transition in human embryonic stem cells through direct binding of CDK6 and CDC25A. J Cell Biol 184(1):67-82.

Zhao FQ. 2013. Octamer-binding transcription factors: genomics and functions. Frontiers in bioscience 18:1051-1071.

Zhao R, Deibler RW, Lerou PH, Ballabeni A, Heffner GC, Cahan P, Unternaehrer JJ, Kirschner MW, Daley GQ. 2014. A nontranscriptional role for Oct4 in the regulation of mitotic entry. Proc Natl Acad Sci U S A 111(44):15768-15773.

Zheng X, Boyer L, Jin M, Mertens J, Kim Y, Ma L, Ma L, Hamm M, Gage FH, Hunter T. 2016. Metabolic reprogramming during neuronal differentiation from aerobic glycolysis to neuronal oxidative phosphorylation. eLife 5.

Zhou W, Choi M, Margineantu D, Margaretha L, Hesson J, Cavanaugh C, Blau CA, Horwitz MS, Hockenbery D, Ware C, Ruohola-Baker H. 2012. HIF1alpha induced switch from bivalent to exclusively glycolytic metabolism during ESC-to-EpiSC/hESC transition. EMBO J 31(9):2103-2116.

Zhu S, Li W, Zhou H, Wei W, Ambasudhan R, Lin T, Kim J, Zhang K, Ding S. 2010. Reprogramming of human primary somatic cells by OCT4 and chemical compounds. Cell Stem Cell 7(6):651-655.

2. HYPOTHESIS AND OBJECTIVES

This PhD Thesis Work thesis purpose is to study the role of Notch and Wnt/β-Catenin signaling pathways in the reprogramming of Human Dental Pulp Stem Cells. Overexpression of pluripotency stem cells markers leads to cell reprogramming in somatic cells and the metabolism and epigenetic remodelling is necessary to ensure its effectiveness. Reprogramming is a well-known strategy used to obtain different type of cells for using them in cell therapy. Because of all this mentioned before we have studied the molecular effect of Notch and Wnt/β-Cateninsignaling modulation in pluripotency markers expression, self-renewal, metabolism and epigenetic, knowing that this could be translated into an approach to obtain different cell types in a controlled, safety and efficient methodology of cell reprogramming.

Having all these points before into consideration and the hypotheses described, in this PhD Thesis Work we have reached one major objective:

To study the molecular and physiological implications of Notch and Wnt/β-Catenin signaling pathways in cell reprogramming.

To reach conclusions in this general objective we have developed these other minor objectives:

1- To study the effect of Notch and Wnt/β-Cateninsignaling pathways in stemness pluripotency stem cell factors and self-renewal in Human Dental Pulp Stem Cells population.

2- To verify if Notch and Wnt/β-Catenin signaling pathways modulation could be used as a pre-treatment of Human Dental Pulp Stem Cells to induce reprogramming and enhance differentiation media protocols efficiency.

3- To analyze Notch and Wnt/β-Cateninsignaling modulation in metabolism remodelling through glycolysis, mitochondrial activity and lipid turn over.

4- To observe the implication of Notch and Wnt/β-Catenin signaling modulation in methylation and acetylation pattern of DNA and histones that allows epigenetic remodelling of chromatin in Human Dental Pulp Stem Cells.

3. RESULTS

3

Eur Cell Mater.2017 Nov 1; 34:249-270. doi: 10.22203/eCM.v034a16.

Notch Wnt cross-signaling regulates stemness of Dental Pulp Stem Cells through expression of neural crest and core pluripotency factors

Verónica Uribe-Etxebarria[1], Jon Luzuriaga[1], Patricia García-Gallastegui[1], Alice Agliano[2], Fernando Unda[1] and Gaskon Ibarretxe[1].

1 Cell Biology and Histology Department, Faculty of Medicine and Nursing, University of the Basque Country (UPV/EHU), Barrio Sarriena S/N, 48940 Leioa, Spain

2 Cancer Research UK Cancer Imaging Centre, Division of Radiotherapy and Imaging, The Institute of Cancer Research and The Royal Marsden NHS Foundation Trust,123 Old Brompton Road, London, SW7 3RP, United Kingdom

ABSTRACT

Dental Pulp Stem Cells (DPSCs) from adult teeth express neural crest (NC) markers together with core transcription factors associated with stem cell pluripotency, such as Oct4a, Sox2, cMyc, Rex1, Stella/Dppa3, SSEA1/Fut4, Lin28 and Nanog. The possibility to boost the natural stemness features of DPSCs by mild methods that do not involve gene and/or chromatin modification is highly desirable for cell therapy. Canonical Wnt and Notch are two highly conserved developmental signaling pathways that are involved in NC emergence and stem cell self-renewal. We determined that both pathways operate coordinately to regulate the expression of core pluripotency and NC factors in DPSCs. Pharmacological inhibition of the Notch pathway by the γ-secretase inhibitor DAPT for 48 hours abolished the expression of NC and core factors together with a silencing of canonical Wnt signaling and a clear reduction in the stemness potential of DPSCs, as shown by a lower ability to generate mature, fully differentiated osteoblasts and adipocytes. Conversely, pharmacological activation of the Wnt pathway, by either the

GSK3-β inhibitor BIO or human recombinant protein WNT-3A for 48 hours, largely increased the expression of NC and core factors together with an increased efficiency of DPSCs to differentiate into mature osteoblasts and adipocytes. These results show that a short preconditioning activation of Wnt/Notch signaling by small molecules and/or recombinant proteins enhances the stemness and potency of DPSCs in culture, which could be useful to optimize the therapeutic use of these and other tissue-specific stem cells.

Keywords: dental pulp stem cells, multipotency, self-renewal, pluripotency core factors, neural crest, stemness and differentiation, osteogenesis, adipogenesis, Notch, Wnt, BIO, DAPT, WNT3A.

INTRODUCTION

During embryogenesis, neural crest (NC) cells generate most craniomaxillofacial tissues, including all major tissues of the teeth except the enamel. The remarkable abilities of the NC earned it the designation of "the fourth embryonic layer" (Shyamala *et al.*, 2015; Thomas *et al.*, 2008). Importantly, some NC stem cells with a non-differentiated phenotype remain in the dental pulp of mature teeth and are known as Dental Pulp Stem Cells (DPSCs) (Aurrekoetxea *et al.*, 2015; Gronthos *et al.*, 2002; Gronthos *et al.*, 2000; Ibarretxe *et al.*, 2012; Janebodin *et al.*, 2011; Kaukua *et al.*, 2014; Liu *et al.*, 2015). Other stem cell types with similar properties may be obtained from gingival and periodontal tissues (Abe *et al.*, 2012; Huang *et al.*, 2009; Ibarretxe et al., 2012; Lima *et al.*, 2017; Liu et al., 2015; Petrovic and Stefanovic, 2009), and even from periodontal inflammatory lesions like human periapical cysts (Marrelli *et al.*, 2013; Marrelli *et al.*, 2015; Tatullo *et al.*, 2015). As NC-derived cells, dental stem cells are characterized by the expression of neural crest factors such as Snail/Snai1, Slug/Snai2, Twist1, Hnk1, Pax3, Neurogenin2 and Sox10 (Kiraly *et al.*, 2009; Schiraldi *et al.*, 2012), and core factors including Oct4a, Sox2, Klf4, Lin28, SSEA1 and Nanog(Atari *et al.*, 2012; Ferro *et al.*, 2012; Janebodin et al., 2011; Kerkis *et al.*, 2006; Rosa *et al.*, 2016). Core factors are fundamental to maintaining stem cell pluripotency (Chambers and Tomlinson, 2009; Takahashi *et al.*, 2007; Yu *et al.*, 2007), thus suggesting that dental stem cells may present some superior features with respect to other multipotent stem cell populations of the adult human body (Atari *et al.*, 2011; Atari et al., 2012; Rosa et al., 2016) . This could be very relevant to cell therapy because stem cells from dental tissues are known to be easily accessible for extraction and well-tolerated upon grafting due to their immune-suppressive properties (Pierdomenico *et al.*, 2005). Furthermore, DPSCs are non-tumorigenic even after their immortalization by telomerase overexpression (Wilson *et al.*, 2015). Finally, given that the dental pulp is rather well preserved in mid-to-advanced age patients, DPSCs are also suitable for autologous therapy (Ibarretxe et al., 2012; Kellner *et al.*, 2014; Wu *et al.*, 2015)**.**

The canonical Notch and Wnt signaling pathways are critical for the maintenance of the stem cell phenotype (Androutsellis-Theotokis *et al.*, 2006; Borghese *et al.*, 2010; Clevers *et al.*, 2014; Perdigoto and Bardin, 2013; Reya and Clevers, 2005). Both pathways also play an important role in the emergence of the neural crest (Hari *et al.*, 2012; Leung *et*

al., 2016; Rogers *et al.*, 2012; Stuhlmiller and Garcia-Castro, 2012). It is known that dental stem cells present higher levels of core factors and Wnt/Notch activity than do other mesenchymal stem cells in the adult body (Atari et al., 2012; Huang et al., 2009; Janebodin et al., 2011; Vasanthan *et al.*, 2015). However, the role of these pathways in the maintenance of stemness and self-renewal in DPSCs is still unclear.

Activation of Notch signaling through ligand binding triggers proteolytic cleavage of Notch receptors, first by A-Disintegrin-And-Metalloproteases (ADAM) followed by γ-secretases, which results in the cleavage and release of the Notch intracellular domain (NICD) from the membrane. The NICD translocates to the nucleus where it directly interacts with CSL/RBPj/CBF-1 transcription factors to turn on the expression of Notch target genes such as the Hairy Enhancer of Split (*Hes*) family (D'Souza *et al.*, 2010)**.** In Canonical Wnt signaling, the interactions between Wnt protein ligands and Frizzled/LRP receptors lead to the recruitment of AXIN, APC and GSK3β to the membrane, thus preventing phosphorylation and degradation of β-CATENIN protein. As a result, β-CATENIN accumulates in the cytoplasm and translocates into the nucleus, where it interacts with TCF/LEF family factors and leads to the expression of Wnt signaling target genes (Clevers, 2006). Both pathways regulate each other at multiple points (Borggrefe *et al.*, 2016; Fukunaga-Kalabis *et al.*, 2015) and promote the maintenance of self-renewal and inhibition of differentiation in many stem cell types, including DPSCs (Mizutani *et al.*, 2007; Scheller *et al.*, 2008; Yiew *et al.*, 2017).

Pharmacological manipulation of the Notch and Wnt pathways is relatively simple via the use of well-known drugs such as DAPT (γ-secretase inhibitor; Notch signaling blocker), and BIO (GSK3-β inhibitor; Wnt signaling activator), and by recombinant activator proteins such as WNT-3A. The use of BIO and DAPT has already proven to be a valuable complementary strategy to induce either cellular reprogramming or cellular differentiation (Ichida *et al.*, 2014; Kitajima *et al.*, 2016). Therefore, a thorough understanding of the stemness and differentiation potential of DPSCs and their modulation by cell signaling pathways would be highly desirable to apply these cells more efficiently in areas such as regenerative medicine, tissue engineering and drug screening. In this work, we used DAPT and BIO, as well as human recombinant WNT-3A as pharmacological modulators to investigate the role of Notch/Wnt in maintaining stemness and the expression of pluripotency core factors in DPSCs with the goal of optimizing

existing protocols of somatic cell differentiation using DPSCs.

MATERIALS AND METHODS

DPSC culture

DPSCs were isolated from human third molars obtained from healthy donors between 15 and 30 years of age by fracture and enzymatic digestion of the pulp tissue for 1 h at 37 °C with 3 mg/ml collagenase (17018-029, Thermo Fisher Scientific, Boston, Massachusetts, USA) and 4 mg/ml dispase (17105-041, Thermo Fisher Scientific) followed by mechanical dissociation. The DPSCs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), L-glutamine (1 mM) and the antibiotics penicillin (100 U/ml) and streptomycin (150 µg/ml). The DPSCs could be amplified and maintained in these conditions for very long periods (> 6 months). However, to avoid cell aging issues, we only employed DPSCs that had been grown in culture for less than 3 months and had accumulated no more than 6 total passages. Comparative experiments between control and treatment conditions were always and without exception performed in parallel using DPSCs from the same donor.

Notch and Wnt pathway pharmacological modulation

To block Notch signaling, we employed DAPT ((N-[N (3, 5-diflorophenacetyl-L-alanyl)] 5-phnylglycine t-butyl ester), a γ-secretase inhibitor, (565784, Calbiochem, San Diego, California, USA), at a concentration of 2.5 µM. DAPT was added to the culture medium for 48 hours prior to the assays where DAPT-treated DPSCs were compared with DPSCs treated only with the control vehicle, 2.5 µM DMSO. To overactivate Wnt signaling, we used 2.5 µM BIO (6-bromoindirubin-3´-oxine), a GSK3β inhibitor (361550, Calbiochem), which was added to the medium for 48 hours prior to the assays. BIO-treated cells were compared with DPSCs exposed to the inactive analog MBIO (methyl-6-bromoindirubin-3´-oxine) at 2.5 µM as a corresponding control (361556, Calbiochem). WNT-3A recombinant protein (5036-WN-010, R&D Systems, Minneapolis, USA) was added to the DPSCs cultures to overactivate Wnt signaling in two concentrations: 2.5 µM and 5 µM.

Osteogenic differentiation of DPSCs

We used the following protocol to induce DPSC differentiation to mature osteoblasts: 6 µM β-glicerophosphate (G9422, Sigma-Aldrich, St. Louis, Massachusetts, USA), 10 nM dexamethasone (D4902, Sigma), and 52 nM ascorbic acid (127. 0250, Merck, Darmstadt, Germany) were added to the cell cultures in DMEM + 10 % FBS for three weeks. The DPSCs had been previously subjected to preconditioning treatment with DMSO, DAPT, MBIO or BIO for 48 h, as described. Terminal osteoblast differentiation was assessed by detection of extracellular calcified bone matrix deposits via Alizarin Red staining using 2 g/100 ml Alizarin Red S (400480250, Across Organics, Geel, Belgium) at pH 4.3. The DPSCs were fixed with 10 % formalin (F7503, Sigma) for 30 min. We then incubated the cells with Alizarin S Red for 45 min before washing the cells four times with PBS to remove any background staining. The Alizarin Red absorbance at 450 nm was quantified using a Synergy HT Multi-Mode Microplate Reader (Biotek, Winooski, Vermont, USA). Alkaline Phosphatase (ALP) staining was also used to assess osteoblast differentiation, as this enzymatic activity is present in mature bone matrix-secreting cells. We dissolved one BCIP/NBT tablet (B5655-5ATB, Sigma) in 10 ml miliQ water and added this solution to DPSCs fixed for 1 min with 10 % formalin. We then washed the cells with PBS containing 0.05 % Tween 20 (STBB3609, Sigma). ALP activity was quantified by absorbance at 405 nm with a Synergy HT Multi-Mode Microplate Reader.

Adipogenic differentiation of DPSCs

To induce adipogenic differentiation, we treated DPSC cultures with 0.5 mM IBMX (I5879, Sigma), 1 µg/ml insulin (91077C, SAFC Biosciences, St. Louis, Massachusetts, USA) and 1 µM dexamethasone (D4902, Sigma) for four weeks after a preconditioning treatment with DMSO, DAPT, MBIO or BIO for 48 h. The DPSCs were exposed to this differentiation cocktail and subsequently fixed with 10 % formalin for 10 min and then washed with PBS containing 60 % Isopropanol. Lipid droplets in mature adipocytes were detected using Oil Red staining solution, which contained 5.14 µM Oil Red Stock (O-0625, Sigma) in miliQ water. The cells were stained for 10 min, and Oil Red absorbance was measured at 490 nm using a Synergy HT Multi-Mode Microplate Reader (Biotek).

RNA extraction, conventional RT-PCR and quantitative Real-Time PCR (qPCR)

Cell pellets were frozen and stored at -80 °C. Total RNA was extracted from the cells

using the RNeasy Kit (74104, Qiagen, Hilden, Germany) and checked for purity by calculating the 260/280 ratio via the Nanodrop Synergy HT (Biotek). cDNA (50 ng/µl) was obtained by reverse transcription of total extracted RNA using the iScript cDNA Kit (1708890, BioRad, Hercules, CA, USA) with the following reagents: iScript reverse Transcriptase (1 µl), 5x iScript Reaction Mix (4 µl) and Nuclease Free water (variable) to a final volume of 20 µl. We analyzed gene expression using 1 µl of cDNA (5 ng/µl) diluted in 4 µl of My TaqTM Red Mix (BIO-25043, Bioline, St. Petersburg, Russia), 1 µl of primers (0.625 µM) and Nuclease Free Water for a total volume reaction of 10 µl, for conventional RT-PCR. Amplification products were separated by electrophoresis in a 2 % agarose gel. Quantitative Real-Time PCR experiments were conducted in an iCyclerMyiQ^{TM} Single-Color Real-Time PCR Detection System (BioRad, USA), using 4.5 µl of Power SYBR® Green PCR Master Mix 2x (4367659, Applied Biosystems^{TM,} Applied Biosystems, Carlsbad, CA, USA), 0.5 µl of primers (0.3125 µM), 0.3 µl of cDNA (1.5 ng/µl), and Nuclease Free water for a total volume reaction of 10 µl. All primers were obtained from public databases and checked for optimal efficiency (> 90 %) in the qPCR reaction under our experimental conditions. The relative expression of each gene was calculated using the standard $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001) normalized with respect to the average between *β-Actin* and *Gapdh* as internal controls. All reactions were performed in triplicate. qPCR was run on ABI PRISM® 7000 (Thermo Fisher Scientific, Thermo Fisher Scientific, Boston, MA, USA). Data were processed by CFX Manager™ Software (BioRad, USA). We assessed that all qPCR reactions yielded only one amplification product by the melting curve method. We used the following primer pairs for different genes obtained via the Primer-Blast method (Primer Bank):

Primers		**Sequence 5´ - 3´**	**Annealing (ºC)**	**Amplicon (bp)**
β-Actin	Upstream	GTTGTCGACGACGAGCG	58.5	93
	Downstream	GCACAGAGCCTCGCCTT	59.7	
GAPDH	Upstream	CTTTTGCGTCGCCAG	60.3	131
	Downstream	TTGATGGCAACAATATCCAC	60.8	
Notch1	Upstream	ATAGTCTGCCACGCC	54	149
	Downstream	AGTGTGAAGCGGCCA	54.9	
Notch2	Upstream	AAGCCCAGACATTCTTGCAGCTTG	64.1	107
	Downstream	TCCAGGGCATAATTCCCAACAGGA	63.7	
Notch3	Upstream	ACCCCCAAGAGGCAAGTGT	61.1	125
	Downstream	AGGATGAAAAAGACTAAAAGGAAGGAA	59	
Notch4	Upstream	GCGATAATGCGAGGAAGATACG	59.4	118
	Downstream	TCGGAATGTTGGAGGCAGAAC	60.6	
Hes1	Upstream	GGTACTTCCCCAGCACACTT	59	138
	Downstream	TGAAGAAAGATAGCTCGCGG	57.7	
β-Catenin	Upstream	GAAGCTGGTGGAATGCAAGC	60.1	279
	Downstream	GACAGTACGCACAAGAGCCT	60	
Nestin	Upstream	GGTCCTAGGGAATTGCAGC	57.9	144
	Downstream	CTCAAGATGTCCCTCAGCCT	58.8	
Jagged1	Upstream	AGATCTCAATTACTGTGGGAC	57.1	88
	Downstream	GCAGGAACACTGATATTTGTC	58.7	
Jagged2	Upstream	TCTTGCAAAAACCTGATTGG	62.6	86
	Downstream	CAGTCGTTGACGTTGATATG	59.2	
Lef1	Upstream	TGCCAAATATGAATTAACGACCCA	59	151
	Downstream	GAGAAAAGTGCTCGTCACTGT	58.5	
Oct4 *	Upstream	CGTGAAGCTGGAGAAGGAGA	60.7	137

	Downstream	CATCGGCCTGTGTATATCCC	60.1	
cMyc	Upstream	GTCAAGAGGCGAACACACAAC	60	162
	Downstream	TTGGACGGACAGGATGTATGC	60.1	
Sox2	Upstream	ATAATAACAATCATCGGCGG	61.1	90
	Downstream	AAAAAGAGAGAGGCAAACTG	57.8	
Klf4	Upstream	TCTTGAGGAAGTGCTGAG	56.5	147
	Downstream	ATGAGCTCTTGGTAATGGAG	58.3	
Nanog	Upstream	GTCAAGAAACAGAAGACCAG	56.4	184
	Downstream	GCCACCTCTTAGATTTCATTC	59.2	
Lin28	Upstream	CTGGTGGAGTATTCTGTATTG	56.2	81
	Downstream	ACCTGTCTCCTTTTGATCTG	58.3	
Rex1	Upstream	TATCTCAACCTGTTCATCGAG	59.3	130
	Downstream	CCACATTCAGGTAGATGTTC	56.9	
Ssea1/Fut4	Upstream	ACAAAATCATCTGTTGGGAC	58.9	85
	Downstream	AGCAGATAAGCACTTTCAAC	56.2	
Stella/Dppa3	Upstream	GAGGAGTAAGAACATTGCTG	56.5	133
	Downstream	CTTGATTCCTTCTTAACTCCC	58.3	
Snail/Snai1	Upstream	AACAATGTCTGAAAAGGGAC	58.1	94
	Downstream	ATAGTTCTGGGAGACACATC	55.4	
Slug/Snai2	Upstream	AAACAACCTGAAGACTTGTG	56.8	157
	Downstream	TTCTTTGTACAGTGGTTTGG	57.7	
Sox10	Upstream	ACTTAGTGGAGTTCTCATCC	54.7	106
	Downstream	AAGAATGAGGTTATTGGCAC	58.1	
Pax3	Upstream	ATCAACTGATGGCTTTCAAC	59.2	120
	Downstream	CAGCTTGTGGAATAGATGTG	58.3	
Pax7	Upstream	AGGAGTACAAGAGGGAAAAC	56.4	108
	Downstream	TAATCGAACTCACTGAGGG	57.8	
Neurogenin2	Upstream	AGGGAAGAGGACGTGTTAGTGC	61.9	225

	Downstream	GCAATCGTGTACCAGACCCAG	61	
Twist1	Upstream	CTAGATGTCATTGTTTCCAGAG	57.9	136
	Downstream	CCCTGTTTCTTTGAATTTGG	60.9	
Wnt3	Upstream	CTGTGACTCGCATCATAAG	56.8	186
	Downstream	ATGTGGTCCAGGATAGTC	54.3	
Wnt1	Upstream	CTATTTATTGTGCTGGGTCC	58.5	125
	Downstream	AGAAACTGAGGAGAGAAGAG	54.2	
Hnk1	Upstream	TGTGAGTGCTGGTAATGAG	57.2	169
	Downstream	ACTGCCCTCATCCTTATG	57.5	

*** transcript variants *(Pou5f1; Oct4a)* and *(Pou5f1p1; Oct4pg1)***

Protein extraction

The cells were washed with 0.9 % NaCl several times, and the proteins were extracted with 100 µl Lysis Buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.5 % sodium deoxycholate, 0.1 % SDS, 1 % IGEPAL® CA-630 in dH_2O, and Proteinase Inhibition Cocktail Set III 1:100, 539134, Calbiochem). Protein quantification was performed in each Western Blot using the DC™ Protein Assay (Bio Rad, 5000112), including Reagent A (#500-0113), Reagent B (#500-0114) and Reagent S (#500-0115).

Western blot (WB)

The samples were diluted in NuPAGE sample buffer (NP0007, Novex, Life technologies, Carlsbad California, USA) and loaded onto a 4-12 % Invitrogen NuPAGE BisTris Gel (1 mm x 10 well; NP032180X, Novex, Life Technologies) followed by transfer onto 0.45 µm-pore nitrocellulose membranes (Inmmobilon® Transfer Membranes; EMD Millipore, USA) and run in an XCell Sure Lock Electrophoresis machine (NP0007, Novex, Life Technologies). For Western Blot analyses, we used anti β- ACTIN (1:1000, 4967, Cell Signaling), anti-GAPDH (1:10000, MAB374, Millipore, Missouri, USA), anti-PARP antibody (1:2000, 9542S, Cell Signaling, Massachusetts, USA), anti-SOX2 (1:500, GTX101506, GeneTex, California, USA), anti- NANOG1,2 (1:1000, D73G4, #4903, Cell Signaling), anti-N1ICD antibody (1:1000, ab8925, Abcam, Cambridge, UK), anti-total β-CATENIN antibody (1:4000, ab6302, Abcam) and anti-active β-CATENIN

antibody (1:500, 05-665, Millipore). The secondary antibodies (P0260, DAKO, Hovedstaden, Denmark; NA9340, GE Healthcare, UK) were added at a 1:2000 dilution. The membranes were stripped using Red Blot (M2504, Inmmobilon® EMD Millipore).

Immunofluorescence (IF)

DPSCs cultured over glass coverslips were fixed with 4 % paraformaldehyde for 10 min and washed with PBS. Blocking was performed by 10 min incubation with normal goat serum (501972, Thermo Fisher Scientific). The DPSCs were then incubated overnight with primary antibodies diluted in PBS + 2 % BSA + 0.1 % Tx-100 at 4 °C. The rabbit anti-N1ICD (ab8925, Abcam), anti-OCT4 (ab19857, Abcam), anti-CMYC (ab32 [9E10], Abcam), anti-KI67 (ab15580, Abcam) and anti-total βCATENIN (ab6302, Abcam) antibodies were used at 1:200, , 1:100, 1:1000, 1:100 and 1:3000 dilutions, respectively. Goat anti-rabbit Alexa Fluor (488:A11012; 594: A11012, Invitrogen, Carlsbad, CA, USA) were employed as secondary antibodies at a 1:200 dilution, followed by DAPI which was used to counterstain cell nuclei. Images were captured with an epifluorescence Axioskop microscope (Zeiss, Germany) operated with Nikon NIS-Elements and an Apotome Confocal Microscope (Zeiss, Germany) operated with Nikon DS-Qi1Mc software (Tokyo, Japan). The fluorescence intensities in the samples were quantified by Fiji-ImageJ (*Schindelin et al., 2012)* after background subtraction.

Cell proliferation and death assays

We used propidium iodide (PI, 20 µg/ml, Sigma) to detect cell death and Calcein-AM (5 µM, Life Technologies) to detect cell viability. We incubated both fluorescent dyes for 30 min at 37 °C in culture medium and washed the cells 3 times with PBS. Fluorescence quantification was accomplished using microfluorimetry by measuring light emission at 495 nm (Calcein-AM; green fluorescence) and 630 nm (PI; red fluorescence) in a Fluoroskan Ascent plate reader (Thermo Scientific).

Statistical analyses

Statistical analyses were performed with Excel, IBM SPSS Statistics v.9 (SPSS, Chicago, IL, USA) and Graph Pad v.6 software (Graph Pad Inc., USA). All data sets were subjected to a Kolmogorov-Smirnov normality test prior to analysis. For small sample sizes, non-parametric tests were chosen by default. Comparisons between only two groups were made using U-Mann Whitney test. Comparisons between multiple groups were made using Kruskal-Wallis followed by Dunn´s post hoc test. $P \leq 0.05$ was considered to be statistically significant.

RESULTS

Notch activity was required for the expression of core pluripotency factors and self-renewal of DPSCs

Control DPSCs showed detectable amounts of core pluripotency factors such as *Oct4 (Pou5f1 and Pou5f1p1), Sox2, Nanog, Lin28, Rex1, Stella, SSEA1* and *cMyc* transcripts by RT-PCR amplicon bands. To assess whether DAPT treatment would affect the expression of core factors in DPSCs, we exposed the DPSC cultures to transient applications of 2.5 µM DAPT for 48 h. Interestingly, for many factors that were expressed, the RT-PCR bands were significantly reduced or virtually lost when the DPSCs were pre-treated with DAPT (Fig. 1a). As an internal control to assess Notch activity, we examined the expression of the Notch target gene *Hes1*. We consistently observed a clear decrease in *Hes1* expression following DAPT treatment. We next wanted to corroborate these changes by qPCR, and we determined that all core factors had significantly decreased levels of expression when the DPSCs were exposed to DAPT. In some cases, such as that of *Rex1* or *SSEA1* expression, it was decreased by more than 90 % with respect to the control conditions (Fig. 1b). Finally, these changes were also corroborated by IF and WB, where OCT4 (nuclear active form OCT4-A; (Atlasi *et al.*, 2008; Ferro et al., 2012; Liedtke *et al.*, 2007), SOX2, NANOG (canonical isoform 1 (Saunders *et al.*, 2013; Wang *et al.*, 2008), CMYC and Notch activity markers, such as Notch 1-Receptor Intracellular Domain (N1ICD), had also a consistently reduced expression at the protein level (Fig. 1c-l).

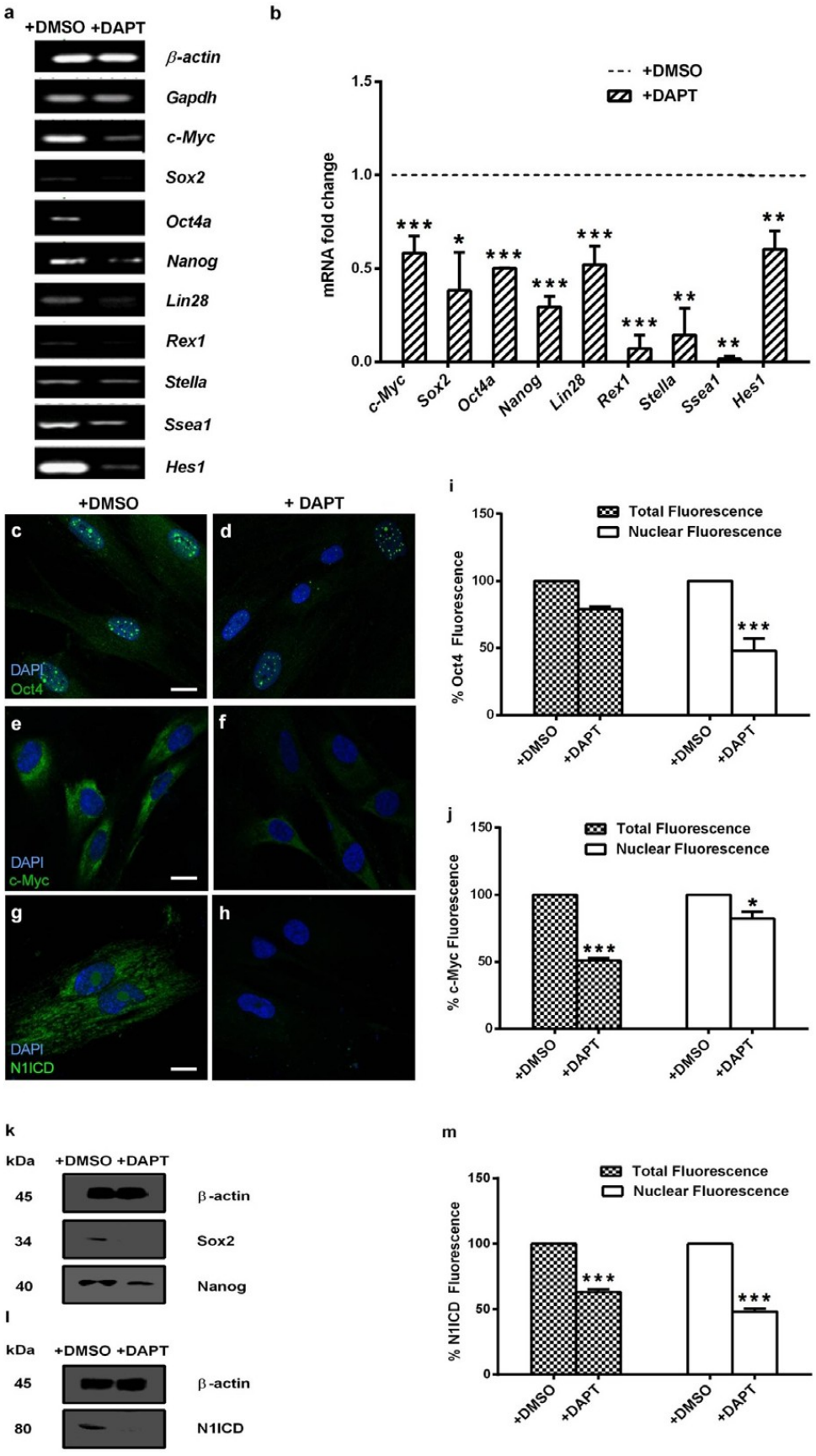

a
+DMSO +DAPT
β-actin
Gapdh
c-Myc
Sox2
Oct4a
Nanog
Lin28
Rex1
Stella
Ssea1
Hes1
b
+DMSO
+DAPT
mRNA fold change
1.5
1.0
0.5
0.0
c-Myc
Sox2
Oct4a
Nanog
Lin28
Rex1
Stella
Ssea1
Hes1
+DMSO
+ DAPT
c
d
DAPI
Oct4
e
f
DAPI
c-Myc
g
h
DAPI
N1ICD
i
Total Fluorescence
Nuclear Fluorescence
% Oct4 Fluorescence
150
100
50
0
+DMSO
+DAPT
j
Total Fluorescence
Nuclear Fluorescence
% c-Myc Fluorescence
k
kDa
+DMSO +DAPT
45
β-actin
34
Sox2
40
Nanog
l
kDa
+DMSO +DAPT
45
β-actin
80
N1ICD
m
Total Fluorescence
Nuclear Fluorescence
% N1ICD Fluorescence

Figure 1. Notch inhibition by DAPT reduces the expression of core pluripotency factors in DPSCs. (a): RT-PCR revealed differences in the expression of *cMyc*, *Sox2*, *Nanog*, *Oct4*, and *Hes1* expression following DAPT exposure. **(b):** Q-PCR analysis confirmed a decrease in core factors *cMyc*, *Sox2*, *Nanog*, *Oct4*, *Rex1*, *Stella*, *SSEA1* and Hes1 transcripts between the control (DMSO) and DAPT conditions. Data are normalized to reference *β-Actin* and *GAPDH* levels and presented as the mean +SEM (n=3). The dashed line represents normalized gene expression in control conditions. **(c-h):** IF images of DPSCs grown for 48 h in presence or absence of DAPT and stained for OCT4 **(c, d)**, CMYC **(e, f)** and N1ICD **(g, h)** in green. DAPI labels cell nuclei in blue. Scale bar=20 µm. **(i, j, m):** Bar charts showing relative total and nuclear OCT4 **(i)**, CMYC **(j)**, and N1ICD **(m)** fluorescence in control and DAPT-treated DPSCs. The data are presented as mean +SEM (n=3). **(k, l):** Representative WB showing SOX2 **(k)**, NANOG and active cleaved N1ICD **(l)**. β-ACTIN was used as protein loading control. *: $p<0.05$;**: $p<0.01$;***: $p<0.001$. U-Mann Whitney test.

The proliferation rates of DPSCs in the presence or absence of DAPT were compared, to verify if the decreased expression of core factors in DPSCs also reflected a decreased ability for self-renewal and proliferation. DPSCs were incubated with a mixture of calcein-AM/PI, after being cultivated for 48 h in the presence or absence of DAPT. No PI fluorescence was detectable in either condition, indicating that cell viability was not compromised by DAPT treatment. However, calcein fluorescence was reduced by approximately 40 % in the DAPT conditions compared to control (Fig. 2a-c). To corroborate this result, the number of proliferative cells positive for Ki67,amarkerofcellproliferationpresentduringtheG1, S, G2 and M phases of the cell cycle, but absent from non-dividing cells (G0), was evaluated. Ki67 labelling was significantly lower in the DAPT-treated DPSCs with respect to controls, thus indicating a reduction in the amount of cycling proliferative cells (Fig. 2e-g). Finally, it was confirmed that, despite inducing a reduction in DPSC proliferation, DAPT treatment did not cause any genomic damage or apoptotic cell death. The cleaved poly-ADP ribose polymerase (PARP) levels were assessed by WB and resulted negative in all conditions (Fig. 2d). Taken together, these results suggested that Notch inhibition caused a decrease in the expression of core pluripotency factors in DPSCs, which resulted in decreased self-renewal and proliferation capacities, without affecting cell viability.

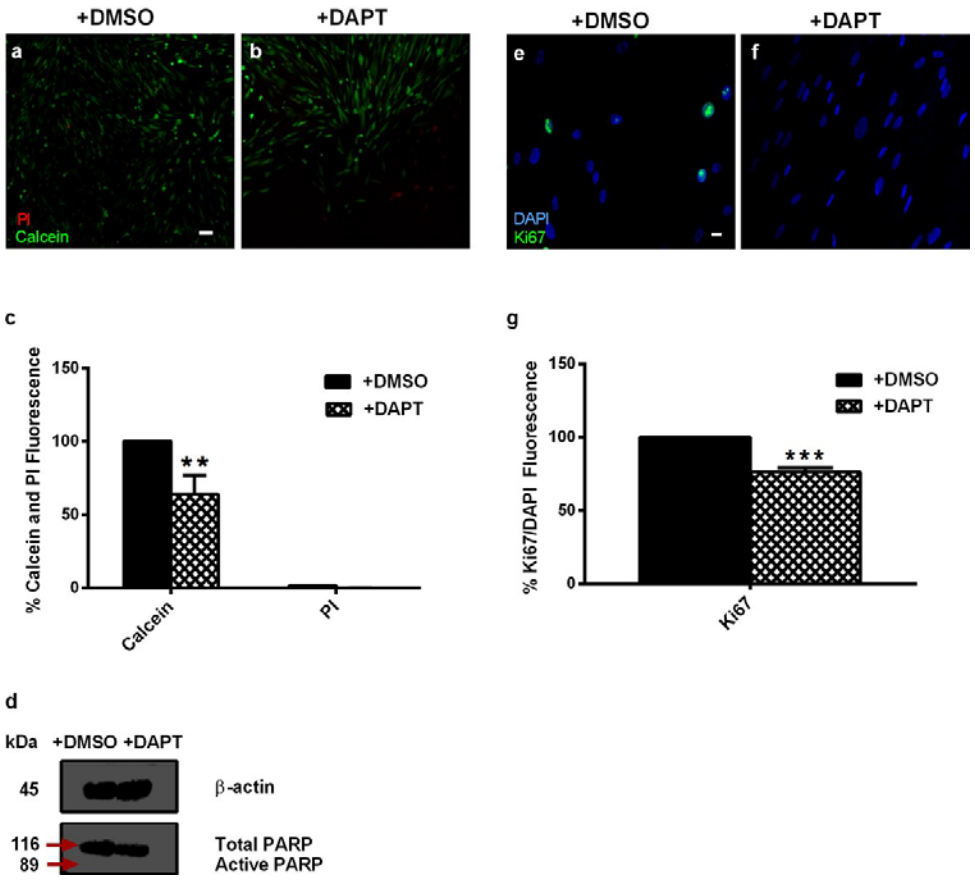


Figure 2. Cell proliferation and cell death analysis in DPSC exposed to DAPT. (**a, b):** Calcein-AM (green) and Propidium Iodide (PI; red) DPSC grown in control and DAPT conditions for 48 h. Scale bar: 200 µm. **(c):** Quantification of relative Calcein and PI fluorescence. Data are presented as mean + SEM (n =7). **(d):** Western Blot showing absence of cleaved PARP protein (89kDa; red arrow) in both DMSO and DAPT conditions, in comparison with total inactive non-cleaved PARP (116kDa) which was well detected (**e, f):** IF for KI67 in DMSO and DAPT conditions. Scale bar: 20µm. **(g):** Quantification of KI67 labeling in DAPT-treated and control DPSCs. Data are presented as mean +SEM (n =7). **: $p < 0.01$. ***: $p < 0.001$. U-Mann Whitney test.

Notch and Wnt/β-Catenin signaling interact and positively regulate each other in DPSCs

To assess whether Notch signaling interacted with Wnt/β-Catenin signaling in DPSCs, we studied the effect of DAPT treatment on the expression of Wnt signaling targets by qPCR. *Notch1-4* receptor expression was negatively affected by the treatment, whereas the expression of the Notch ligand *Jagged1* was unaffected (Fig. 3a, b). These results showed an overall downregulation of Notch signaling in DPSCs as induced by DAPT treatment. Intriguingly, we also determined that DAPT treatment reduced the expression of the canonical Wnt mediators *Wnt3*a and *β-Catenin,* and also that of the Wnt target gene

Lef1 by ~50-70 % of their respective control values (Fig. 3a, b). We wanted to confirm this result by assessing β-CATENIN protein levels both by WB and IF. A significant decrease in the amount of both total and nuclear active β-CATENIN was found in DPSCs exposed to DAPT for 48 h (Fig. 3c-f). Therefore, Notch inhibition results in a parallel Wnt/β-Catenin signaling inhibition in DPSCs.

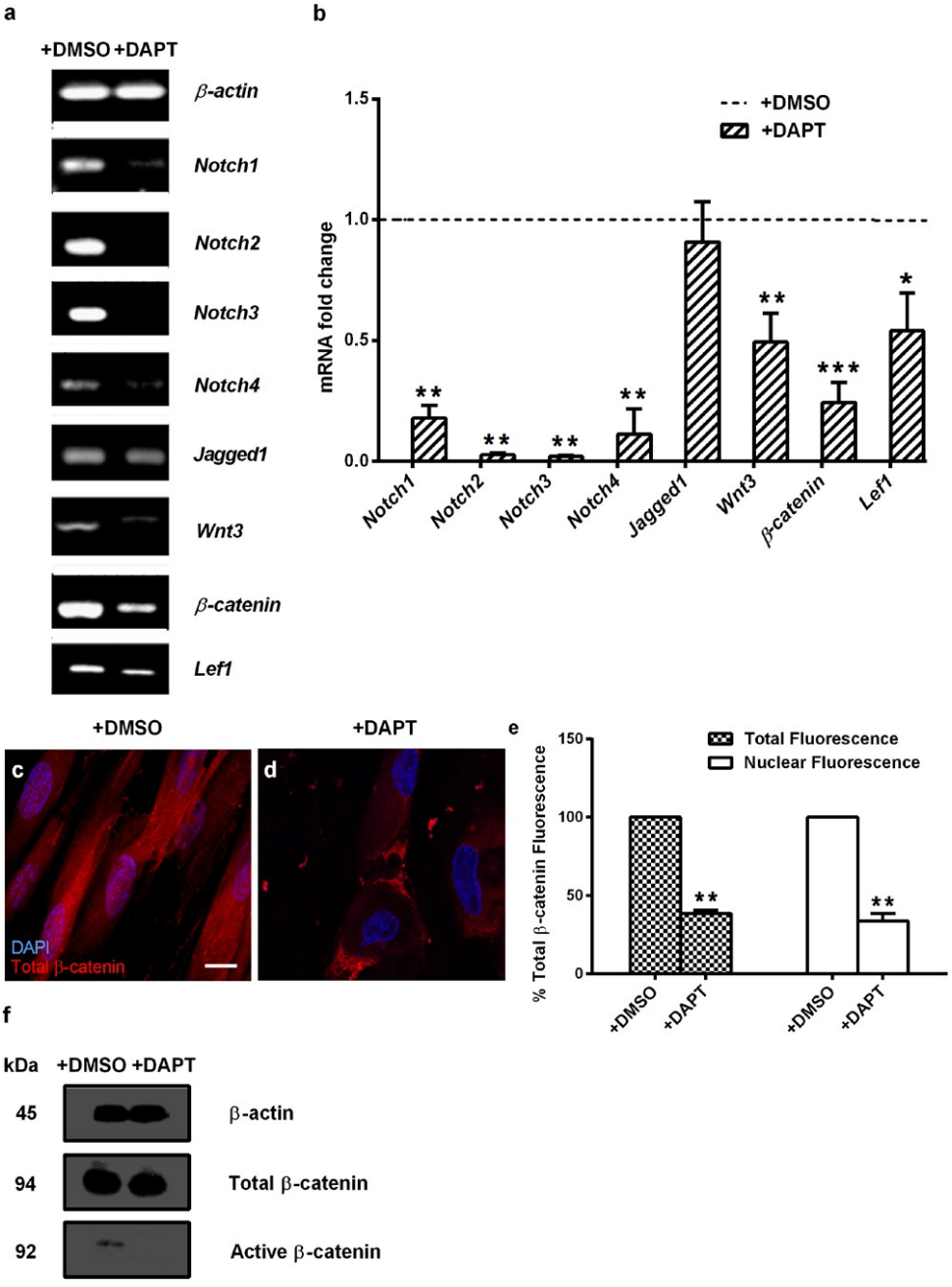


Figure 3. Notch inhibition by DAPT negatively regulates Wnt/β-Catenin signaling in DPSCs. (a): RT-PCR revealed differences in the expression of key Wnt signaling mediators and targets *Wnt3a*, *β-Catenin* and *Lef1* in DPSCs following DAPT exposure. **(b):** QPCR confirmed a decrease in both Notch receptors and Wnt factors between control (DMSO) and DAPT conditions. Data are normalized to reference *β-Actin* and *GAPDH* levels and presented as the mean +SEM (n=4). The dashed line represents normalized gene expression in control conditions. **(c, d):** IF images of total β-CATENIN in DAPT-treated DPSCs compared with controls. Scale bar=20 µm **(e):** Bar chart showing relative total and nuclear β-CATENIN fluorescence in control and DAPT conditions **(f):** WB of total and nuclear active β-CATENIN in control and DAPT-treated DPSCs. β-ACTIN was used as a protein loading control.*: $p<0.05$;**: $p<0.01$;***: $p<0.001$. U-Mann Whitney test.

BIO-induced Wnt activation enhanced the expression of pluripotency core factors in DPSCs.

To investigate whether Wnt/β-Catenin activation would enhance the expression of pluripotency core factors in DPSCs, we used BIO treatment for 48 h to hyperactivate Wnt signaling by inhibiting β-CATENIN degradation. As a control for treatment efficacy, BIO-treated DPSCs showed increased levels of both total and activeβ-CATENIN protein, whereas other Wnt targets such as *Lef1* also demonstrated consistently increased expression (Fig. 4a, b, g, h, k-l). Therefore, BIO induced a strong hyperactivation of Wnt signaling in DPSCs. We then assessed the expression of pluripotency core factors in BIO-treated DPSCs by RT-PCR and qPCR. We determined that BIO also induced overexpression of pluripotency factors at both the transcript (Fig. 4a, b), and protein levels (Fig. 4c-k).

In addition to boosting the expression of core pluripotency factors, BIO also increased DPSC proliferation ability. As shown by the Calcein/PI assay, the number of viable cells in culture significantly increased after BIO treatment with respect to controls (Fig. 5a-c). Consistently, the KI67/DAPI fluorescence ratio increased in response to BIO treatment, which indicated a greater proportion of active dividing cells in these conditions (Fig. 5e-g). We detected neither PI-positive cells nor an active PARP signal in BIO-treated cells (Fig. 5d). Together, these experiments demonstrated that Wnt activation by BIO treatment increased the proliferation and self-renewal capacities of DPSCs without compromising their viability and genomic integrity.

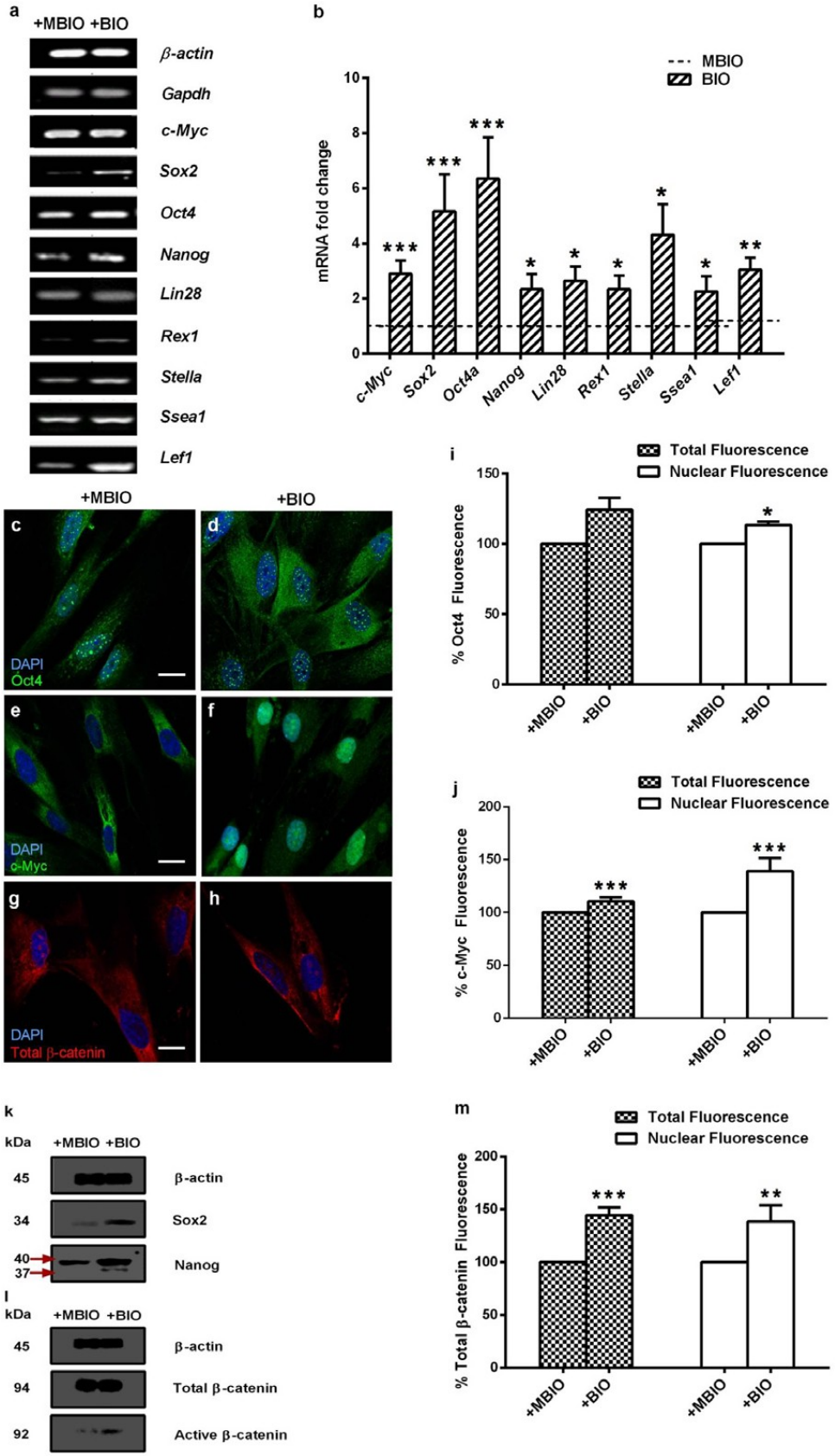

a
+MBIO +BIO
β-actin
Gapdh
c-Myc
Sox2
Oct4
Nanog
Lin28
Rex1
Stella
Ssea1
Lef1
b
MBIO
BIO
mRNA fold change
c-Myc
Sox2
Oct4a
Nanog
Lin28
Rex1
Stella
Ssea1
Lef1
+MBIO
+BIO
c
d
DAPI
Oct4
e
f
DAPI
c-Myc
g
h
DAPI
Total β-catenin
i
Total Fluorescence
Nuclear Fluorescence
% Oct4 Fluorescence
j
% c-Myc Fluorescence
k
kDa
45
β-actin
34
Sox2
40
37
Nanog
l
94
Total β-catenin
92
Active β-catenin
m
% Total β-catenin Fluorescence

Figure 4. Wnt activation by BIO leads to increased expression of core pluripotency factors in DPSCs. **(a):** RT-PCR revealed differences in the expression of *cMyc*, *Sox2*, *Nanog*, *Oct4* and *Lef1* following BIO exposure. **(b):** Q-PCR analysis confirmed an increase in *cMyc*, *Sox2*, *Nanog*, *Oct4*, *Rex1*, *Stella*, *SSEA1*and *Lef1* expression between control (MBIO) and BIO conditions. Data are normalized to reference *β-Actin* and *GAPDH* levels, and presented as the mean + SEM (n=3). The dashed line represents normalized gene expression in control conditions. **(c-h):** IF images of DPSCs grown in the presence of MBIO or BIO for 48 h and stained for OCT4 **(c, d)**, CMYC **(e, f)** and total β-CATENIN **(g, h)** in green. DAPI labels nuclei in blue. Scale bar: 20 µm. **(i, j, m):** Bar charts showing relative total and nuclear OCT4 **(i)** CMYC **(j)** and total β-CATENIN fluorescence in MBIO and BIO-treated DPSCs. The data are presented as the mean +SEM (n=3). **(k, l):** Representative WB showing an increase in SOX2 **(k)**, native NANOG1 and NANOG2 isoforms, with slightly different MWs **(k),** as well as in both total β-CATENIN and activeβ-CATENIN **(l)** protein expression. β-ACTIN was used as a protein loading control. *: p<0.05;**: p<0.01;***: p<0.001. U-Mann Whitney test.

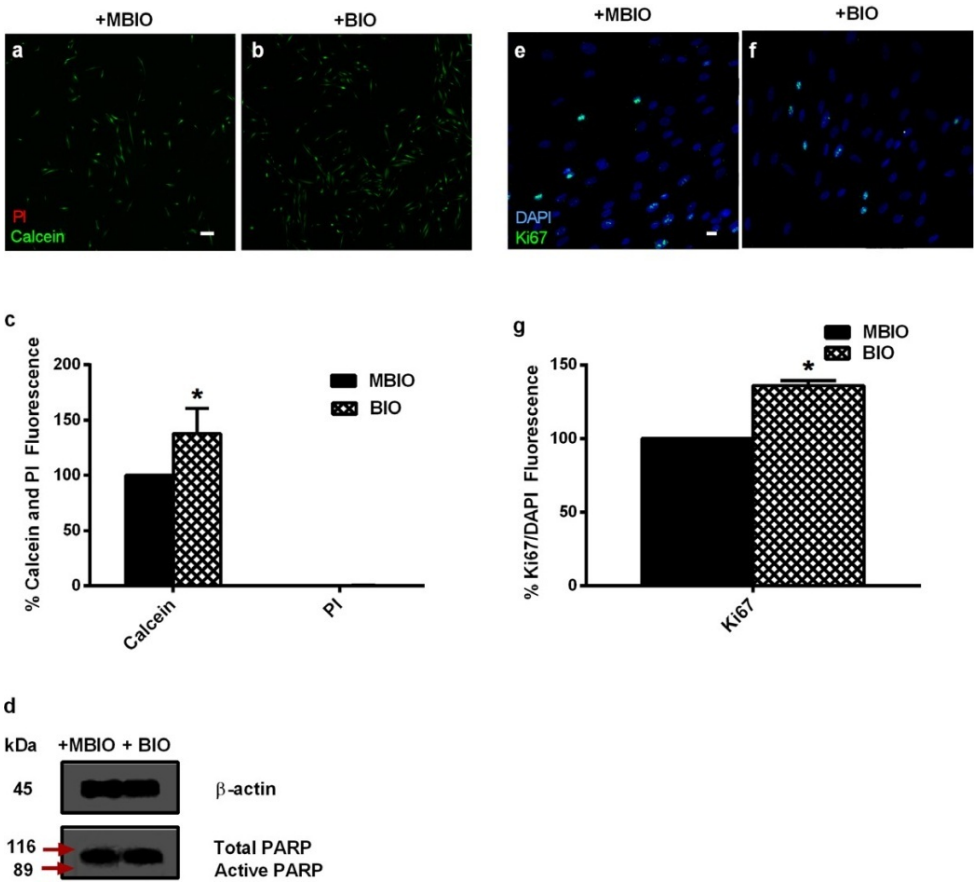


Figure 5. Cell proliferation and cell death analysis in DPSC exposed to BIO. (**a, b):** Calcein-AM (green) and Propidium Iodide (PI; red) DPSC grown in MBIO and BIO conditions for 48 h. Scale bar: 200 µm. **(c):** Quantification of relative Calcein and PI fluorescence. Data are presented as mean + SEM (n =7). **(d):** Western Blot showing absence of cleaved PARP protein (89kDa; red arrow) in both MBIO and BIO conditions, in comparison with total inactive non-cleaved PARP (116kDa) which was well detected. (**e, f):** IF forKI67 in MBIO and BIO conditions. Scale bar: 20µm. **(g):** Quantification of KI67 labeling in MBIO- and BIO-treated DPSC. Data are presented as mean +SEM (n =7). *: p < 0.05. U-Mann Whitney test.

Wnt activation by BIO induced Notch upregulation in DPSCs

Given that we found that DAPT treatment affects Wnt signaling in DPSCs, we wondered whether Notch activity would also be affected in BIO-treated DPSCs. We found that in BIO-treated DPSCs, most Notch receptors did not undergo any changes in expression, except the *Notch2* receptor, which was found to be slightly upregulated at the transcript level (Fig. 6a, b).

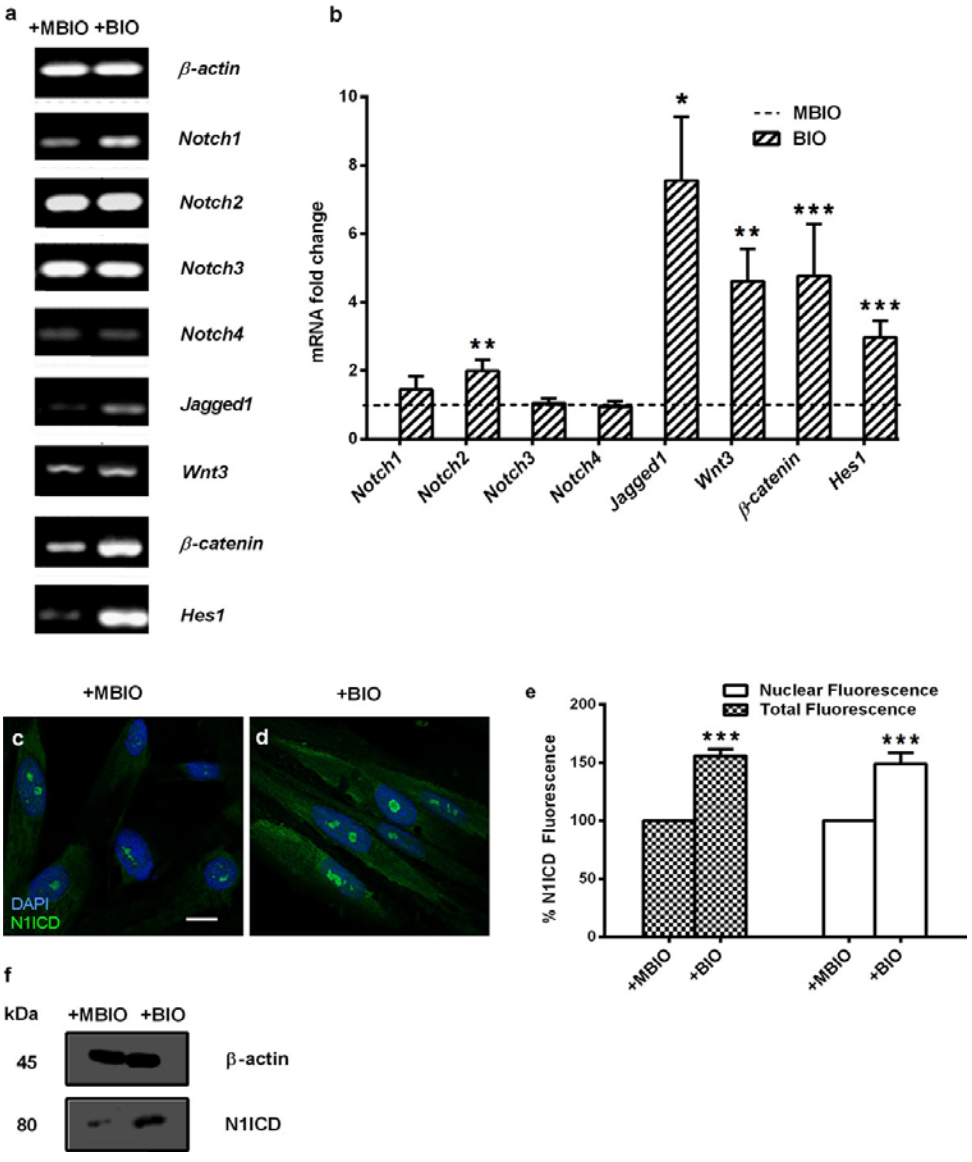


Figure 6. Wnt activation by BIO positively regulates Notch signaling in DPSCs. (a): RT-PCR revealed

differences in the expression of Notch mediators, ligands and receptors under BIO exposure. **(b):** Q-PCR analysis confirmed an increase in *Notch 2*, *Jagged1*, *Wnt3, β-Catenin* and *Hes1* expression between control (MBIO) and BIO conditions. The data are normalized to reference *β-Actin* and *GAPDH* levels and presented as the mean + SEM (n=4). The dashed line represents normalized gene expression in control conditions. **(c, d)** IF images of active N1ICD, in cells treated with BIO and MBIO. Scale bar=20 µm **(e)** Bar chart showing relative total and nuclear N1ICD fluorescence in MBIO- and BIO-treated cells. **(f)** WB showing an increase in N1ICD expression in BIO-treated DPSCs. β-ACTIN was used as a protein loading control. *: $p<0.05$;**: $p<0.01$;***: $p<0.001$. U-Mann Whitney test.

However, the Notch canonical ligand *Jagged1* underwent a much larger 8-fold increase in expression when the DPSCs were exposed to BIO, followed by *Wnt3a* and *β-catenin* with a ~5-fold increased expression each (Fig. 6b). Moreover, *Hes1* was also prominently upregulated in these conditions, which constituted solid evidence that Notch signaling was being hyperactivated by BIO exposure (Fig. 6b). We also tested Notch signaling upregulation at the protein level using an antibody for active cleaved N1ICD. Increased levels of N1ICD were detected for the BIO-treated DPSCs by both WB and IF (Fig. 6c-f).

Wnt/Notch activation enhanced expression of neural crest markers in DPSCs

As Notch/Wnt are crucial in NC induction and their activation enhanced core factor expression in DPSCs, we tested whether NC markers would also be affected by Notch/Wnt pharmacological modulation. We found that both *Snai1* and *Snai2* markers are constitutively expressed by DPSCs, especially *Snail/Snai1*, and we did not find a reliable detectable *Sox10* expression in DPSCs in our control conditions (Fig. 7a, b). Other NC gene markers such as *Pax3, Neurogenin2, Twist1* and *HNK1*were also expressed in control DPSCs. However, when we cultured DSPCs with DAPT, the expression of all these markers was downregulated to less than half of basal levels as assessed by qPCR, with some cases such as *Pax3* were downregulation accounted to more than 90 % of basal expression (Fig. 7a, b). Conversely, when we cultured cells with BIO, most markers increased expression to about twice of control levels, with the notable exception of *Pax3* which increased more than 10-fold (Fig. 7c, d).Some NC gene markers which presented a very low expression in control DPSCs, such as the case of *Sox10*, yielded consistent amplicon bands in conventional RT-PCR following BIO treatment. All these changes were corroborated by qPCR (Fig. 7c, d).

Thus, Notch/Wnt activation upregulated the expression of both pluripotency and NC

markers in DPSCs.

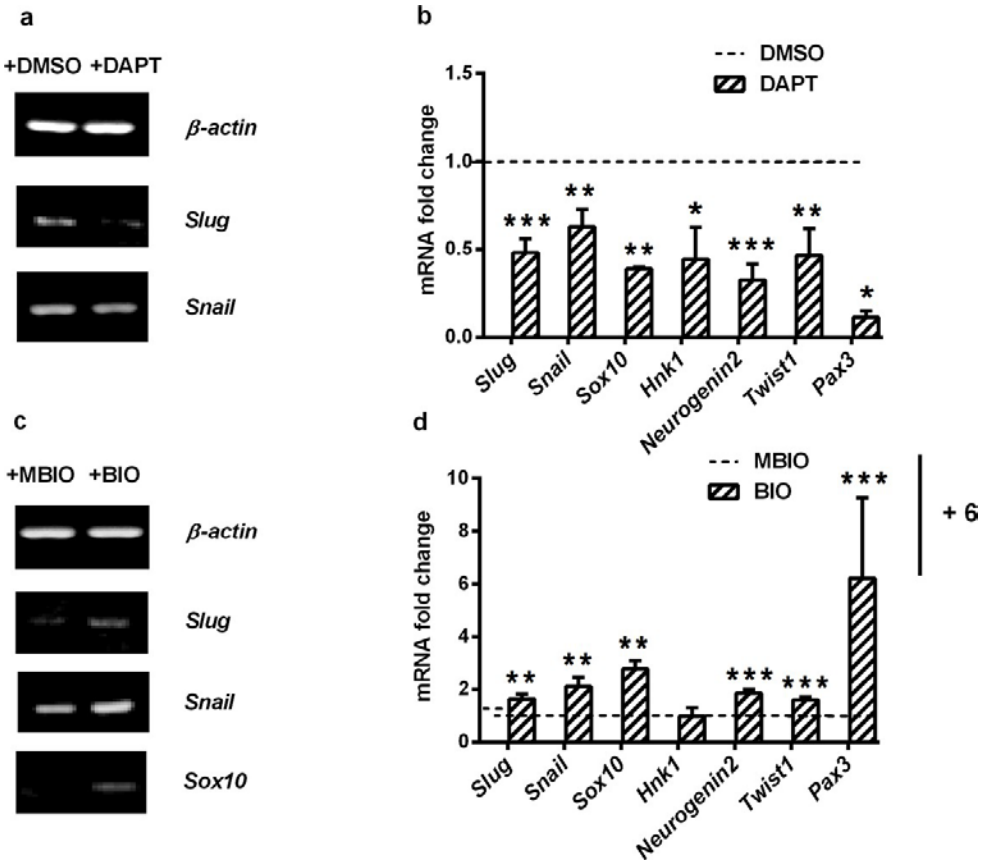


Figure 7. BIO and DAPT antagonistically regulate the expression of neural crest markers Snail/Snai1, Slug/Snai2, Sox10, HNK1, Neurogenin2, Twist and Pax3 in DPSCs. (a) RT-PCR showing NC marker gene expression in DMSO and DAPT-treated DPSCs. **(b)** Q-PCR showing relative differences on *Snai1*, *Snai2*, *Sox10*, *HNK1*, *Neurogenin2*, *Twist* and*Pax3* expression. (**c)** RT-PCR showing NC marker gene expression in MBIO and BIO-treated DPSCs. **(d)** Q-PCR showing relative differences in *Snai1*, *Snai2*, *Sox10*, *Neurogenin2*, *Twist* and*Pax3* expression. Data are normalized to reference *β-Actin* and *GAPDH* levels and represented as the mean + SEM (n=3). The dashed line represents normalized gene expression in control conditions. *: $p<0.05$;**: $p<0.01$;***: $p<0.001$. U-Mann Whitney test.

Wnt/Notch pre-activation enhances differentiation of DPSCs to osteoblasts and adipocytes

Established *in vitro* protocols can be used to induce terminal differentiation of DPSCs to mature osteoblasts and adipocytes (Gronthos et al., 2002; Langenbach and Handschel, 2013). Thus, we hypothesized that a short (48 h) pre-activation of the Notch/Wnt signaling pathway would render DPSCs more responsive to differentiation signals. Conversely, Notch/Wnt pre-inhibition would result in a reduced DPSC differentiation capacity. To test our hypothesis, we exposed DPSCs to BIO or DAPT for 48 h as before. Untreated DPSCs were used as controls. Then, control and treated cells were exposed to osteoblastic differentiation medium in the absence of BIO or DAPT. As evidence of terminal osteoblastic differentiation, we used ALP and Alizarin Red assays. Interestingly, after 3 weeks of osteogenic treatment, Alizarin Red+ deposit formation as assessed by light absorbance was between two- and three-fold higher in the DPSCs pretreated with BIO, and significantly lower in the DPSCs pretreated with DAPT when compared with controls (Fig. 8 a-d, m; a´-d´). ALP enzymatic activity was also found to be significantly higher (i.e., osteoblastic differentiation more efficient) when the DPSCs were pretreated with BIO (Fig. 8e-h, m; e´-h´). To verify whether Notch/Wnt modulation could also affect DPSC differentiation into adipocytes, we exposed DPSCs to BIO or DAPT for 48 h. Control and treated cells were then exposed to adipogenic differentiation medium for 4 weeks. We performed Oil Red staining to assess adipocyte generation. Interestingly, we found that BIO pretreatment also enhanced DPSC conversion to adipocytes as assessed by Oil Red after the differentiation treatment period. Oil Red staining was significantly higher in the BIO-pretreated cells, near twice as much as controls (Fig. 8i-l, n; i´-l´).

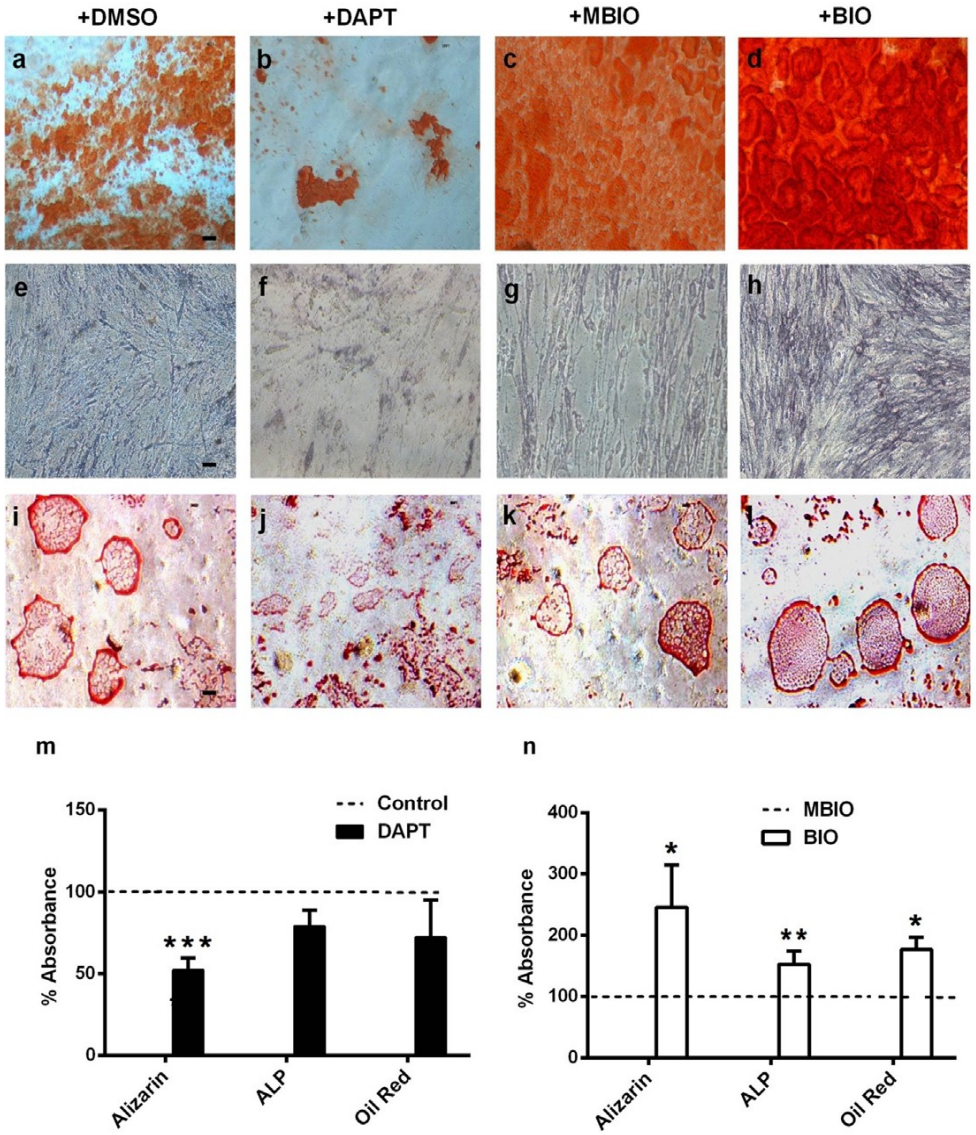
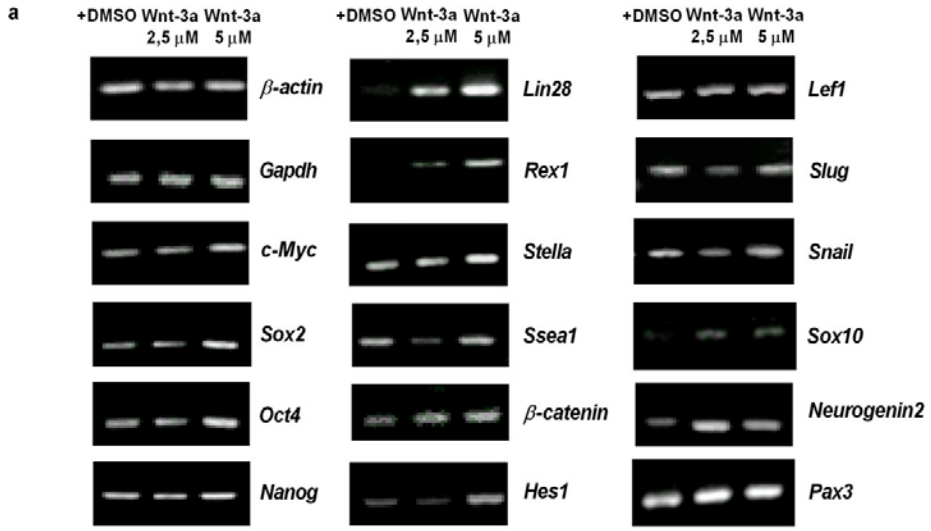


Figure 8. A preconditioning exposure to BIO for 48 h enhances DPSC differentiation efficiency into osteoblasts and adipocytes. (a-d): Alizarin S Red staining to assess calcified matrix formation and osteoblast differentiation in control (DMSO, MBIO), BIO and DAPT conditions. **(e-h):** Alkaline phosphatase (ALP) staining images at high magnification to detect osteoblastic commitment and differentiation in control (DMSO, MBIO), BIO and DAPT conditions. Scale bar=100 µm. **(i-l):** Oil Red staining to assess lipid droplet formation and adipocyte differentiation in control (DMSO, MBIO), BIO and DAPT conditions. Scale bar=200µm **(m):** Relative absorbance quantification at 450 nm, 405 nm and 409nm for Alizarin Red, ALP and Oil Red, respectively for DMSO and DAPT. **(n):** Relative absorbance quantification at 450 nm, 405 nm and 409nm for Alizarin Red, ALP and Oil Red, respectively for MBIO and BIO conditions. The data are normalized to DMSO and MBIO as internal controls and presented as the mean +SEM (n=6) *: $p<0.05$;**: $p<0.01$;***: $p<0.001$. U-Mann Whitney test.

Wnt activation by exposure to human recombinant Wnt-3A protein for 48 h also enhances expression of NC and pluripotency core factors in DPSCs

Since BIO is known to be a general GSK-3β inhibitor (Meijer *et al*., 2003) this treatment could affect other signaling pathways apart from Wnt. In order to prove that the observed effects on core factor and NC factor expression and DPSC stemness depended specifically on the canonical Wnt signaling pathway we used WNT-3A protein, a well-described prototypical canonical Wnt ligand (Famili *et al.*, 2015; Zhang *et al.*, 2009) to stimulate DPSCs for 48 h in similar conditions to BIO. We used two concentrations of human recombinant WNT-3A: 2.5 µM and 5 µM, to verify any possible dose-dependent effects.



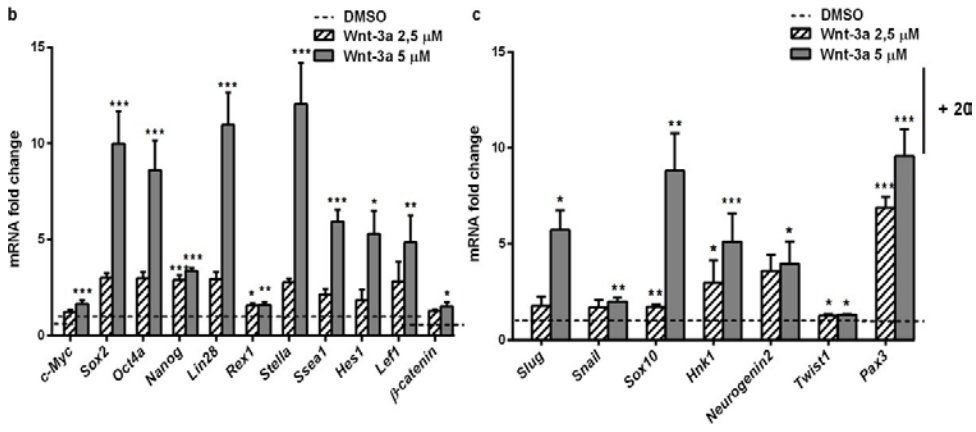


Figure 9. Wnt activation by WNT-3A also increases the expression of pluripotency core factors, Notch markers and neural crest markers in DPSCs. (a) RT-PCR showing core factors (*cMyc*, *Sox2*, *Nanog*, *Oct4*, *Rex1*, *Stella*, *SSEA1*), *Hes1* and NC markers (*Snai1*, *Snai2*, *Sox10*,*HNK1*, *Neurogenin2*, *Twist* and*Pax3)* expression in DMSO and WNT-3A at 2.5 µM and 5 µM concentration in DPSCs. **(b)** QPCR showing relative differences on expression of core factors *cMyc*, *Sox2*, *Nanog*, *Oct4*, *Rex1*, *Stella*, *SSEA1*, *Hes1, Lef1 and β-Catenin* expression.**(c)** QPCR showing relative differences on expression of NC markers *Snai1*, *Snai2*, *Sox10*,*HNK1*, *Neurogenin2*, *Twist* and*Pax3* expression. Data are normalized to reference *β-Actin* and *GAPDH* levels and represented as the mean + SEM (n=3). The dashed line represents normalized gene expression in control conditions. *: p<0.05;**: p<0.01;***: p<0.005. Dunn´s Test, Kruskall Wallis H Test.

Interestingly, we found that all the tested pluripotency and NC factors increased their expression when exposed to WNT-3A, being the effect more pronounced at the concentration of 5 µM. Among the markers that were most strongly affected we detected Sox2, Oct4 (Pou5f1 and Pou5f1p1), Lin28, Stella, SEEA1, Slug, HNK1 and Sox10 (all within a ~5-10 fold increase in expression) and Pax3 (~30x increase with 5 µM WNT-3A). As a control for Wnt activation, Lef1 expression was also solidly upregulated after WNT-3A treatment. All expression changes were verified by RT-PCR and qPCR (Fig. 9a-c).

Exposure to WNT-3A also upregulates Notch/Wnt crosstalk signaling in DPSCs

We also wanted to verify whether the Notch/Wnt interaction that was observed after DAPT/BIO treatments was also observed after treating cells with WNT-3A. QPCR experiments had already showed a clear upregulation of *Hes1* expression in DPSCs exposed to WNT-3A (Fig. 9b). We corroborated this result by performing IF and found a significantly increased N1ICD nuclear fluorescence, especially at a concentration of 5 µM WNT-3A (Fig. 10a-c, k). Similarly, nuclear β-CATENIN protein labeling was also found to be increased as well (Fig. 10d-f, k). Finally, so could be said about KI67 labeling levels (Fig. 10g-i; k). Thus, 5 µM WNT-3A induced a coordinated upregulation of Notch/Wnt cross-signaling and an increased proliferation capacity in DPSCs.

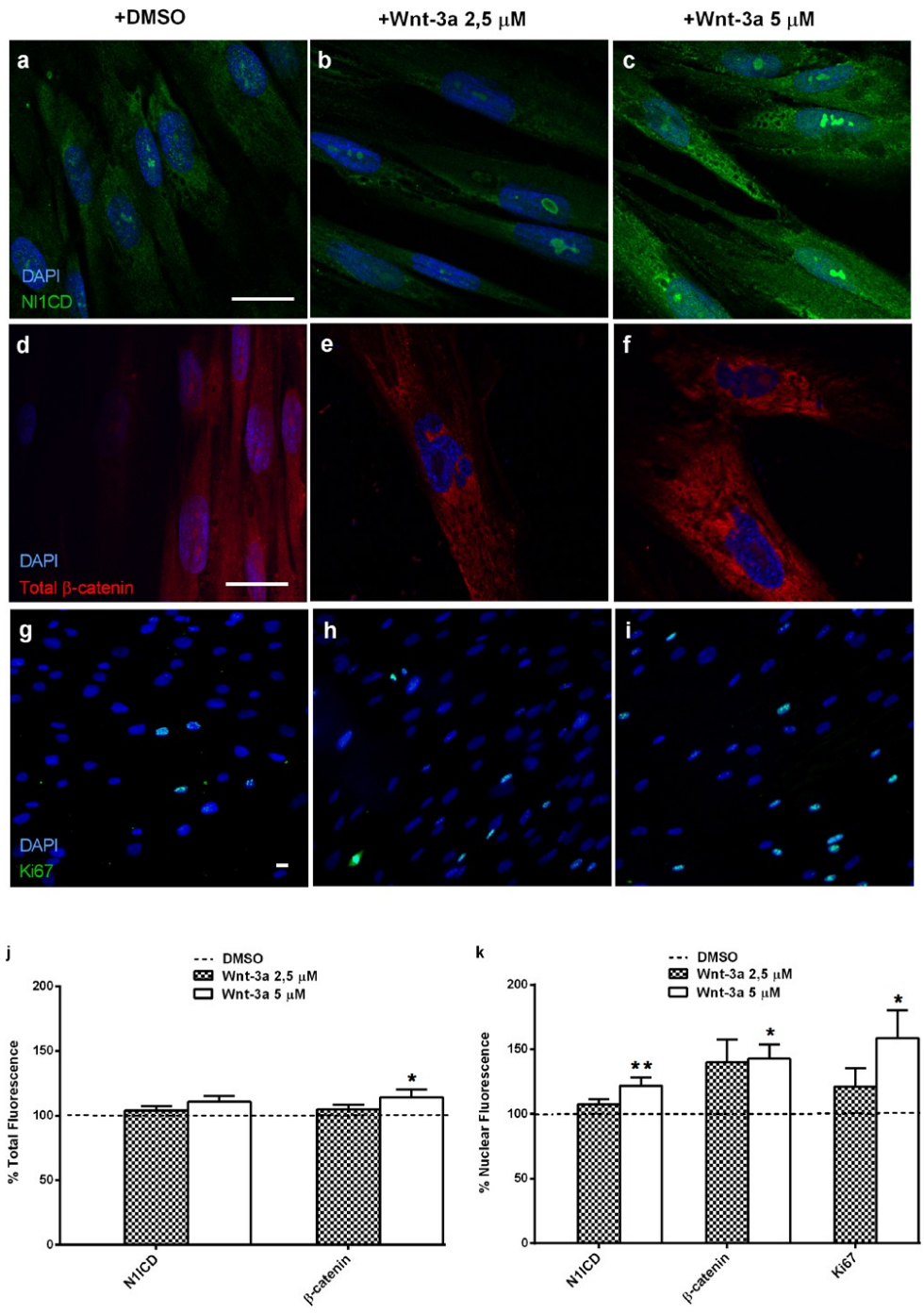


Figure 10. Wnt activation by WNT-3A also upregulates Notch signaling in DPSCs. (a-i): IF images of DPSCs grown for 48 h in presence or absence of WNT-3A at two different concentrations 2.5 and 5 µM and immunolabeled for N1ICD **(a-c),** total β-CATENIN **(d-f)** and KI67 **(g-i).** DAPI labels cell nuclei in blue. Scale bar= 20 µm. **(j):** Bar charts showing relative total N1ICD and total β-CATENIN fluorescence in control and WNT-3A-treated DPSCs. **(k):** Bar charts showing relative nuclear N1ICD, β-CATENIN and KI67 labeling fluorescence in control and WNT-3A-treated DPSCs. Data are presented as mean + SEM (n=3). *: p<0.05;**: p<0.01;***: *p*<0.001. Dunn´s Test, Kruskall Wallis H Test.

A preconditioning treatment with human recombinant WNT-3A also enhances differentiation capacity of DPSCs to osteoblasts and adipocytes

Finally and in view of the last results, we wanted to verify whether a preconditioning treatment with WNT-3A would also render DPSCs more responsive to osteogenic and adipogenic differentiation treatments.

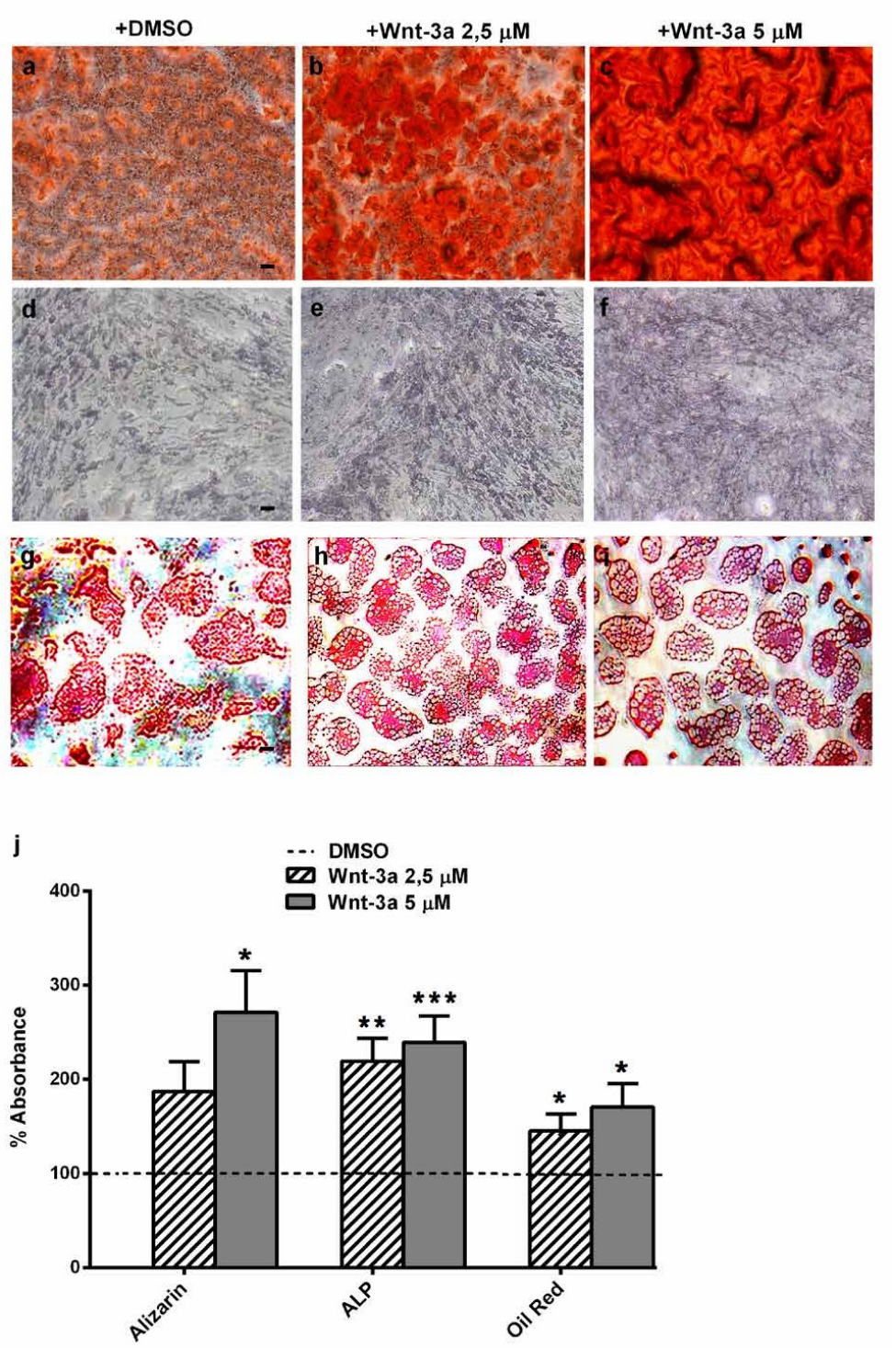


Figure 11. A preconditioning treatment with WNT-3A for 48 h also enhances DPSC differentiation efficiency into osteoblasts and adipocytes. (a-c): Alizarin S Red staining to assess calcified matrix formation and osteoblast differentiation in control (DMSO) and WNT-3A 2.5 and 5 µM conditions. **(d-f):** Alkaline phosphatase (ALP) staining images at high magnification to detect osteoblastic lineage commitment and differentiation in control (DMSO) and WNT-3A 2.5 and 5 µM conditions. **(g-i):** Oil Red staining to assess lipid droplet formation and adipocyte differentiation in control (DMSO) and WNT-3A 2.5 and 5 µM treatment. Scale bar=200µm **(j):** Relative absorbance quantification at 450 nm, 405 nm and 409nm for Alizarin Red, ALP and Oil Red, respectively. Data are normalized to DMSO as internal control and presented as mean + SEM (n=4) *: $p<0.05$. Dunn´s Test, Kruskall Wallis H Test.

Thus, we exposed DPSCs to a preconditioning treatment with WNT-3A for 48 h and subsequently placed cells in osteogenic and adipogenic culture media respectively, in similar conditions as shown before with DAPT/BIO. As predicted, pretreatment with WNT-3A enhanced DPSC capacity to differentiate to these two cell lineages (Fig. 11).

DISCUSSION

We report for the first time that Notch and Wnt signaling are required for the expression of neural crest and pluripotency core factors in DPSCs. Importantly, both pathways positively regulate each other to maintain DPSC stemness, and specific activation or inhibition of one pathway invariably affects the other in the same way. These findings shed light on the control mechanisms of DPSC self-renewal and maintenance and have important potential implications regarding the use of DPSCs for cell therapy.

There are several reasons for research interests regarding DPSCs and other dental stem cells. These cells are easy accessible in both young and aged patients, they possess a significant capacity for *in vitro* expansion, and they have non-tumorigenic phenotypes. Furthermore, a strong body of research evidence shows that DPSCs have a greater multi-lineage differentiation potential than do other tissue-specific stem cells (Atari et al., 2012; Kerkis et al., 2006; Rosa et al., 2016). It is illustrative of the growing acknowledgement of the stemness potential of DPSC that some authors have referred to some subpopulations of these cells as Dental Pulp Pluripotent Stem Cells, or DPPSCs (Atari et al., 2012).

Arguably, induced Pluripotent Stem Cells (iPSCs) derived from autologous somatic cells could be regarded as the ideal source of stem cells for *in vivo* cell therapy; these cells are truly pluripotent (i.e., have no restriction to differentiate to any type of adult cell), can be

extracted from any donor's tissue and have the donor's same genetic background. However, traditional cell reprogramming procedures rely on permanent gene transfection with Yamanaka or related core pluripotency factors (Takahashi *et al.*, 2007), and this is not yet acceptable for safety reasons. Hence, while these cell reprogramming technologies are not mature enough for clinical use, the interest to search for alternative sources of autologous pluripotent-like cells, including DPSCs, is growing very fast. The possibility to boost the natural pluripotency-like features of DPSCs by methods that do not involve permanent chromatin modification or gene transfection is highly desirable for cell therapy.

Canonical Notch and Wnt signaling pathways have been described as pivotal regulators of stemness and pluripotency (Clevers et al., 2014; Dravid *et al.*, 2005; Fox *et al.*, 2008; Li and Chen, 2012; Lowell *et al.*, 2006; Lluis *et al.*, 2008; Park *et al.*, 2008; Simandi *et al.*, 2016; Yan *et al.*, 2010), and their pharmacological manipulation has already been tested as a strategy to enhance either cell differentiation (Kitajima et al., 2016) or cell reprogramming (Ichida et al., 2014). Notch inhibition by γ-secretase blockers has been associated with an increased efficiency of keratinocyte reprogramming (Ichida et al., 2014). In our model system, however, we found a very different effect: Notch inhibition by DAPT decreased the expression of core factors in DPSCs and diminished their capacity to generate fully differentiated osteoblast and adipocyte cells. In this regard, it is worth remarking that Notch effects have long been known to be extremely context-dependent; the same Notch signal can have very different outcomes depending on the cell type, physiological state, and extracellular environment. Regarding Wnt signaling, its pharmacological activation by GSK-3β inhibitors in mouse iPSCs has been associated with increased cell differentiation (Kitajima et al., 2016). However, in our DPSC model, BIO application significantly increased the expression of core factors and enhanced DPSC differentiation potential to generate adult osteoblasts and adipocytes. Again, we attribute these differences to the various cell culture systems that were tested in this and other studies.

One important difference between our study and the aforementioned studies is the presence/absence of serum in the culture medium. The use of medium containing FBS has long been considered the gold standard for manipulation and *in vitro* expansion of DPSCs (Gronthos et al., 2002; Gronthos et al., 2000). *In vitro* expansion of DPSCs is

often inevitable due to the low amounts of tissue material and total cells that can be collected from the human dental pulp, as compared to other stem cell sources. In this regard, despite ongoing progress in culture media formulations that do not contain fetal serum for maintenance of DPSCs (Bonnamain *et al.*, 2013; Eubanks *et al.*, 2014; Jung *et al.*, 2016; Xiao and Tsutsui, 2013), currently the addition of FBS or related agents to the culture media permits to easily overcome the issue of initial cell expansion. However, it is becoming increasingly apparent that FBS-containing media also induce DPSCs to differentiate into a default osteo/odontogenic pathway (Pisciotta *et al.*, 2012; Yu *et al.*, 2010) and this may not be the best choice to generate certain cell lineages, particularly neural cells, from DPSCs (Jung et al., 2016). Moreover, upon continual expansion induced by the presence of 10 % FBS, DPSC cultures also tend to generate populations of committed/differentiated cells (Mokry *et al.*, 2010), and therefore, progressive stem cell exhaustion was also a concern in this study. We confirmed that the proliferation rates and core factor expression decreased steadily in DPSCs upon serial passaging (data not shown). This correlation between sustained stem cell aging and loss of multipotency has also been reported elsewhere (Bose and Shenoy, 2016).

One important finding of this study is that the Notch and Wnt pathways operate coordinately as part of a common network to maintain DPSC stemness. We found that the expression of core factors in DPSCs is completely dependent upon Notch/Wnt signaling. Notch inhibition by DAPT applications virtually abolishes the expression of core factors and decreases DPSC stemness, and this is accompanied by parallel Wnt inhibition. Conversely, Wnt activation by BIO or WNT-3A significantly increases the expression of core factors and DPSC stemness. In view of this evidence, the question becomes what connection these pathways have at the molecular level. Many positive and negative interactions have been reported between both pathways in a myriad of model systems (Borggrefe et al., 2016; Fukunaga-Kalabis et al., 2015; Kwon *et al.*, 2011; Nicolas *et al.*, 2003; Shi *et al.*, 2015). In colorectal cancer cells, it was shown that Wnt signaling affects Notch through β-CATENIN-mediated transcriptional activation of the gene *Jagged1* (Rodilla *et al.*, 2009). JAGGED1 is a Notch ligand which can activate Notch receptors and consequently, Notch signaling. Consistent with this, BIO-induced Wnt activation in DPSCs elicited a sharp increase in the transcriptional levels of *Jagged1*. It is likely that such an increased *Jagged1* transcription would eventually activate Notch receptors in DPSC cultures. In addition, β-CATENIN has also been reported to physically

bind to and interact with reprogramming factors, in particular OCT-4A (Simandi *et al.*, 2016), and *Oct4a* transcription levels are also positively regulated by Wnt signaling in pluripotent stem cells (Li and Chen, 2012; Simandi et al., 2016). Finally, Wnt/β-Catenin is also known to increase *cMyc* gene transcription, which promotes cell proliferation (Barker *et al.*, 2000). Consistently, DPSCs treated with BIO or WNT-3A expressed an increased number of *cMyc* transcripts, which relates to an increased cell proliferation in these conditions.

Together with enhanced core factor expression, activation of the Notch/Wnt signaling pathways also increased the expression of NC markers by DPSCs. Both Notch and Wnt signals have long been associated with NC induction(Gazarian and Ramirez-Garcia, 2017; Hari et al., 2012; Leung et al., 2016; Rogers et al., 2012; Stuhlmiller and Garcia-Castro, 2012). Indeed, NC cells also express relatively high levels of core factors, which possibly relates to their ample capacity to generate very diverse cell lineages (Thomas et al., 2008). We found that NC markers *Pax3, Hnk1, Twist, Neurogenin2, Snail/Snai1, Slug/Snai2 and Sox10* had increased expression levels when the DPSCs were exposed to WNT-3A or BIO, and their expression decreased in the presence of DAPT, thus showing a similar relationship to the one found with pluripotency core factors.

Our results demonstrate that Wnt/Notch activation induces DPSCs to increase their stemness. This could have important implications to optimize the clinical use of these cells. A short preconditioning treatment with WNT-3A or BIO for 48 hours was enough to improve DPSC potential to differentiate to at least two different adult cell lineages, osteoblasts and adipocytes, which are both of mesenchymal origin. This is a very important finding because it proves that DPSC exhaustion by continuous exposure to FBS could be at least partially prevented by transiently boosting the activity of Notch/Wnt pathways, by means of small molecules and/or recombinant proteins, which would enhance stemness traits of DPSC cultures just 48h before use for differentiation or cell transplant. It would also be very interesting to verify whether this preconditioning effect holds true also for other non-mesenchymal cell lineages of interest, such as Schwann cells (Martens *et al.*, 2014), Neuron-like cells (Gervois *et al.*, 2015) and Hepatocyte-like cells (Atari et al., 2012). Finally, the adoption of this preconditioning strategy could also be applicable to differentiation protocols that do not rely on FBS (Eubanks et al., 2014; Xiao and Tsutsui, 2013), allowing the fastest translation to clinical therapy.

CONCLUSION

Our data show that Notch and Wnt signaling are required for maintenance of neural crest and core pluripotency factor expression in DPSCs**.** Furthermore, both pathways operate coordinately as part of the same signaling network to maintain DPSC stemness. Thus, a Notch/Wnt signaling activation preconditioning step by BIO or WNT-3A for 48 h significantly enhanced the capacity of DPSCs to respond to established *in vitro* protocols for osteoblastic and adipogenic differentiation. Conversely, Notch/Wnt inhibition decreased stemness of DPSCs. These preconditioning changes involve levels of expression of core pluripotency and neural crest factors, which correlated with an enhanced differentiation potential of DPSC. This strategy could be used alone or in conjunction with other methods of cell reprogramming that do not involve permanent gene transduction or modifications at genome level to generate DPSCs and other tissue-specific adult stem cells with increased self-renewal and cell differentiation potential for cell therapy.

REFERENCES

Abe S, Hamada K, Miura M, Yamaguchi S (2012) Neural crest stem cell property of apical pulp cells derived from human developing tooth. Cell biology international, 36:927-936.

Androutsellis-Theotokis A, Leker RR, Soldner F, et al. (2006) Notch signalling regulates stem cell numbers in vitro and in vivo. Nature, 442:823-826.

Atari M, Barajas M, Hernandez-Alfaro F, et al. (2011) Isolation of pluripotent stem cells from human third molar dental pulp. Histology and histopathology, 26:1057-1070.

Atari M, Gil-Recio C, Fabregat M, et al. (2012) Dental pulp of the third molar: a new source of pluripotent-like stem cells. Journal of cell science, 125:3343-3356.

Atlasi Y, Mowla SJ, Ziaee SA, Gokhale PJ, Andrews PW (2008) OCT4 spliced variants are differentially expressed in human pluripotent and nonpluripotent cells. Stem cells, 26:3068-3074.

Aurrekoetxea M, Garcia-Gallastegui P, Irastorza I, et al. (2015) Dental pulp stem cells as a multifaceted tool for bioengineering and the regeneration of craniomaxillofacial tissues. Frontiers in physiology, 6:289.

Barker N, Morin PJ, Clevers H (2000) The Yin-Yang of TCF/beta-catenin signaling. Advances in cancer research, 77:1-24.

Bonnamain V, Thinard R, Sergent-Tanguy S, et al. (2013) Human dental pulp stem cells cultured in serum-free supplemented medium. Front Physiol, 4:357.

Borggrefe T, Lauth M, Zwijsen A, Huylebroeck D, Oswald F, Giaimo BD (2016) The Notch intracellular domain integrates signals from Wnt, Hedgehog, TGFbeta/BMP and hypoxia pathways. Biochimica et biophysica acta, 1863:303-313.

Borghese L, Dolezalova D, Opitz T, et al. (2010) Inhibition of notch signaling in human embryonic stem cell-derived neural stem cells delays G1/S phase transition and accelerates neuronal differentiation in vitro and in vivo. Stem cells, 28:955-964.

Bose B, Shenoy PS (2016) Pluripotent Conversion of Muscle Stem Cells Without Reprogramming Factors or Small Molecules. Stem cell reviews, 12:73-89.

Clevers H (2006) Wnt/beta-catenin signaling in development and disease. Cell, 127:469-480.

Clevers H, Loh KM, Nusse R (2014) Stem cell signaling. An integral program for tissue renewal and regeneration: Wnt signaling and stem cell control. Science (New York, N.Y.), 346:1248012.

Chambers I, Tomlinson SR (2009) The transcriptional foundation of pluripotency. Development (Cambridge, England), 136:2311-2322.

D'Souza B, Meloty-Kapella L, Weinmaster G (2010) Canonical and non-canonical Notch ligands. Current topics in developmental biology, 92:73-129.

Dravid G, Ye Z, Hammond H, et al. (2005) Defining the role of Wnt/beta-catenin signaling in the survival, proliferation, and self-renewal of human embryonic stem cells. Stem cells, 23:1489-1501.

Eubanks EJ, Tarle SA, Kaigler D (2014) Tooth storage, dental pulp stem cell isolation, and clinical scale expansion without animal serum. Journal of endodontics, 40:652-657.

Famili F, Naber BA, Vloemans S, de Haas EF, Tiemessen MM, Staal FJ (2015) Discrete roles of canonical and non-canonical Wnt signaling in hematopoiesis and lymphopoiesis. Cell Death Dis, 6:e1981.

Ferro F, Spelat R, D'Aurizio F, et al. (2012) Dental pulp stem cells differentiation reveals new insights in Oct4A dynamics. PloS one, 7:e41774.

Fox V, Gokhale PJ, Walsh JR, Matin M, Jones M, Andrews PW (2008) Cell-cell signaling through NOTCH regulates human embryonic stem cell proliferation. Stem cells, 26:715-723.

Fukunaga-Kalabis M, Hristova DM, Wang JX, et al. (2015) UV-Induced Wnt7a in the Human Skin Microenvironment Specifies the Fate of Neural Crest-Like Cells via Suppression of Notch. The Journal of investigative dermatology, 135:1521-1532.

Gazarian KG, Ramirez-Garcia LR (2017) Human Deciduous Teeth Stem Cells (SHED) Display Neural Crest Signature Characters. PLoS One, 12:e0170321.

Gervois P, Struys T, Hilkens P, et al. (2015) Neurogenic maturation of human dental pulp stem cells following neurosphere generation induces morphological and electrophysiological characteristics of functional neurons. Stem Cells Dev, 24:296-311.

Gronthos S, Brahim J, Li W, et al. (2002) Stem cell properties of human dental pulp stem cells. J Dent Res, 81:531-535.

Gronthos S, Mankani M, Brahim J, Robey PG, Shi S (2000) Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proceedings of the National Academy of Sciences of the United States of America, 97:13625-13630.

Hari L, Miescher I, Shakhova O, et al. (2012) Temporal control of neural crest lineage generation by Wnt/beta-catenin signaling. Development (Cambridge, England), 139:2107-2117.

Huang GT, Gronthos S, Shi S (2009) Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. Journal of dental research, 88:792-806.

Ibarretxe G, Crende O, Aurrekoetxea M, Garcia-Murga V, Etxaniz J, Unda F (2012) Neural crest stem cells from dental tissues: a new hope for dental and neural regeneration. Stem cells international, 2012:103503.

Ichida JK, Tcw J, Williams LA, et al. (2014) Notch inhibition allows oncogene-independent generation of iPS cells. Nature chemical biology, 10:632-639.

Janebodin K, Horst OV, Ieronimakis N, et al. (2011) Isolation and characterization of neural crest-derived stem cells from dental pulp of neonatal mice. PloS one, 6:e27526.

Jung J, Kim JW, Moon HJ, Hong JY, Hyun JK (2016) Characterization of Neurogenic Potential of Dental Pulp Stem Cells Cultured in Xeno/Serum-Free Condition: In Vitro and In Vivo Assessment. Stem cells international, 2016:6921097.

Kaukua N, Shahidi MK, Konstantinidou C, et al. (2014) Glial origin of mesenchymal stem cells in a tooth model system. Nature, 513:551-554.

Kellner M, Steindorff MM, Strempel JF, Winkel A, Kuhnel MP, Stiesch M (2014) Differences of isolated dental stem cells dependent on donor age and consequences for autologous tooth replacement. Archives of Oral Biology, 59:559-567.

Kerkis I, Kerkis A, Dozortsev D, et al. (2006) Isolation and characterization of a population of immature dental pulp stem cells expressing OCT-4 and other embryonic stem cell markers. Cells, tissues, organs, 184:105-116.

Kiraly M, Porcsalmy B, Pataki A, et al. (2009) Simultaneous PKC and cAMP activation induces differentiation of human dental pulp stem cells into functionally active neurons. Neurochem Int, 55:323-332.

Kitajima K, Nakajima M, Kanokoda M, et al. (2016) GSK3beta inhibition activates the CDX/HOX pathway and promotes hemogenic endothelial progenitor differentiation from human pluripotent stem cells. Experimental hematology, 44:68-74.e61-10.

Kwon C, Cheng P, King IN, et al. (2011) Notch post-translationally regulates beta-catenin protein in stem and progenitor cells. Nature cell biology, 13:1244-1251.

Langenbach F, Handschel J (2013) Effects of dexamethasone, ascorbic acid and beta-glycerophosphate on the osteogenic differentiation of stem cells in vitro. Stem Cell Res Ther, 4:117.

Leung AW, Murdoch B, Salem AF, Prasad MS, Gomez GA, Garcia-Castro MI (2016) WNT/beta-catenin signaling mediates human neural crest induction via a pre-neural border intermediate. Development (Cambridge, England), 143:398-410.

Li J, Chen B (2012) Oct4 was a novel target of Wnt signaling pathway. Molecular and cellular biochemistry, 362:233-240.

Liedtke S, Enczmann J, Waclawczyk S, Wernet P, Kogler G (2007) Oct4 and its pseudogenes confuse stem cell research. Cell Stem Cell, 1:364-366.

Lima RL, Holanda-Afonso RC, Moura-Neto V, Bolognese AM, DosSantos MF, Souza MM (2017) Human dental follicle cells express embryonic, mesenchymal and neural stem cells markers. Arch Oral Biol, 73:121-128.

Liu J, Yu F, Sun Y, et al. (2015) Concise reviews: Characteristics and potential applications of human dental tissue-derived mesenchymal stem cells. Stem cells (Dayton, Ohio), 33:627-638.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif.), 25:402-408.

Lowell S, Benchoua A, Heavey B, Smith AG (2006) Notch promotes neural lineage entry by pluripotent embryonic stem cells. PLoS biology, 4:e121.

Lluis F, Pedone E, Pepe S, Cosma MP (2008) Periodic activation of Wnt/beta-catenin signaling enhances somatic cell reprogramming mediated by cell fusion. Cell stem cell, 3:493-507.

Marrelli M, Paduano F, Tatullo M (2013) Cells isolated from human periapical cysts express mesenchymal stem cell-like properties. Int J Biol Sci, 9:1070-1078.

Marrelli M, Paduano F, Tatullo M (2015) Human periapical cyst-mesenchymal stem cells differentiate into neuronal cells. J Dent Res, 94:843-852.

Martens W, Sanen K, Georgiou M, et al. (2014) Human dental pulp stem cells can differentiate into Schwann cells and promote and guide neurite outgrowth in an aligned tissue-engineered collagen construct in vitro. FASEB journal : official publication of the Federation of American Societies for Experimental Biology, 28:1634-1643.

Mizutani K, Yoon K, Dang L, Tokunaga A, Gaiano N (2007) Differential Notch signalling distinguishes neural stem cells from intermediate progenitors. Nature, 449:351-355.

Mokry J, Soukup T, Micuda S, et al. (2010) Telomere attrition occurs during ex vivo expansion of human dental pulp stem cells. Journal of biomedicine & biotechnology, 2010:673513.

Nicolas M, Wolfer A, Raj K, et al. (2003) Notch1 functions as a tumor suppressor in mouse skin. Nature genetics, 33:416-421.

Park IH, Zhao R, West JA, et al. (2008) Reprogramming of human somatic cells to pluripotency with defined factors. Nature, 451:141-146.

Perdigoto CN, Bardin AJ (2013) Sending the right signal: Notch and stem cells. Biochimica et biophysica acta, 1830:2307-2322.

Petrovic V, Stefanovic V (2009) Dental tissue--new source for stem cells. TheScientificWorldJournal, 9:1167-1177.

Pierdomenico L, Bonsi L, Calvitti M, et al. (2005) Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp. Transplantation, 80:836-842.

Pisciotta A, Riccio M, Carnevale G, et al. (2012) Human serum promotes osteogenic differentiation of human dental pulp stem cells in vitro and in vivo. PloS one, 7:e50542.

Reya T, Clevers H (2005) Wnt signalling in stem cells and cancer. Nature, 434:843-850.

Rodilla V, Villanueva A, Obrador-Hevia A, et al. (2009) Jagged1 is the pathological link between Wnt and Notch pathways in colorectal cancer. Proceedings of the National Academy of Sciences of the United States of America, 106:6315-6320.

Rogers CD, Jayasena CS, Nie S, Bronner ME (2012) Neural crest specification: tissues, signals, and transcription factors. Wiley interdisciplinary reviews.Developmental biology, 1:52-68.

Rosa V, Dubey N, Islam I, Min KS, Nor JE (2016) Pluripotency of Stem Cells from Human Exfoliated Deciduous Teeth for Tissue Engineering. Stem cells international, 2016:5957806.

Saunders A, Faiola F, Wang J (2013) Concise review: pursuing self-renewal and pluripotency with the stem cell factor Nanog. Stem cells, 31:1227-1236.

Scheller EL, Chang J, Wang CY (2008) Wnt/beta-catenin inhibits dental pulp stem cell differentiation. Journal of dental research, 87:126-130.

Schiraldi C, Stellavato A, D'Agostino A, et al. (2012) Fighting for territories: time-lapse analysis of dental pulp and dental follicle stem cells in co-culture reveals specific migratory capabilities. Eur Cell Mater, 24:426-440.

Shi Y, Shu B, Yang R, et al. (2015) Wnt and Notch signaling pathway involved in wound healing by targeting c-Myc and Hes1 separately. Stem cell research & therapy, 6:120-015-0103-0104.

Shyamala K, Yanduri S, Girish HC, Murgod S (2015) Neural crest: The fourth germ layer. Journal of oral and maxillofacial pathology : JOMFP, 19:221-229.

Simandi Z, Horvath A, Wright LC, et al. (2016) OCT4 Acts as an Integrator of Pluripotency and Signal-Induced Differentiation. Molecular cell, 63:647-661.

Stuhlmiller TJ, Garcia-Castro MI (2012) Current perspectives of the signaling pathways directing neural crest induction. Cellular and molecular life sciences : CMLS, 69:3715-3737.

Takahashi K, Okita K, Nakagawa M, Yamanaka S (2007) Induction of pluripotent stem cells from fibroblast cultures. Nature protocols, 2:3081-3089.

Tatullo M, Falisi G, Amantea M, Rastelli C, Paduano F, Marrelli M (2015) Dental Pulp Stem Cells and Human Periapical Cyst Mesenchymal Stem Cells in Bone Tissue Regeneration: Comparison of Basal and Osteogenic Differentiated Gene Expression of a Newly Discovered Mesenchymal Stem Cell Lineage. J Biol Regul Homeost Agents, 29:713-718.

Thomas S, Thomas M, Wincker P, et al. (2008) Human neural crest cells display molecular and phenotypic hallmarks of stem cells. Human molecular genetics, 17:3411-3425.

Vasanthan P, Govindasamy V, Gnanasegaran N, Kunasekaran W, Musa S, Abu Kasim NH (2015) Differential expression of basal microRNAs' patterns in human dental pulp stem cells. Journal of cellular and molecular medicine, 19:566-580.

Wang J, Levasseur DN, Orkin SH (2008) Requirement of Nanog dimerization for stem cell self-renewal and pluripotency. Proc Natl Acad Sci U S A, 105:6326-6331.

Wilson R, Urraca N, Skobowiat C, et al. (2015) Assessment of the Tumorigenic Potential of Spontaneously Immortalized and hTERT-Immortalized Cultured Dental Pulp Stem Cells. Stem cells translational medicine, 4:905-912.

Wu W, Zhou J, Xu CT, Zhang J, Jin YJ, Sun GL (2015) Derivation and growth characteristics of dental pulp stem cells from patients of different ages. Molecular medicine reports, 12:5127-5134.

Xiao L, Tsutsui T (2013) Characterization of human dental pulp cells-derived spheroids in serum-free medium: stem cells in the core. J Cell Biochem, 114:2624-2636.

Yan X, Qin H, Qu C, Tuan RS, Shi S, Huang GT (2010) iPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin. Stem cells and development, 19:469-480.

Yiew NK, Chatterjee TK, Tang YL, et al. (2017) Novel role for Wnt inhibitor APCDD1 in adipocyte differentiation: implications for diet-induced obesity. The Journal of biological chemistry.

Yu J, He H, Tang C, et al. (2010) Differentiation potential of STRO-1+ dental pulp stem cells changes during cell passaging. BMC cell biology, 11:32.

Yu J, Vodyanik MA, Smuga-Otto K, et al. (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science (New York, N.Y.), 318:1917-1920.

Zhang Z, Deb A, Zhang Z, et al. (2009) Secreted frizzled related protein 2 protects cells from apoptosis by blocking the effect of canonical Wnt3a. J Mol Cell Cardiol, 46:370-377.

4

WILEY Journal of Cellular Physiology

Wnt signaling reprograms metabolism in Dental Pulp Stem Cells

Véronica Uribe-Etxebarria[1], Alice Agliano[2], Fernando Unda[1], Gaskon Ibarretxe [1].

[1]: Cell Biology and Histology Department. University of the Basque Country (UPV/EHU). Barrio Sarriena.s.n.48940 Leioa, Spain.

[2]: Cancer Research UK Cancer Imaging Centre, Division of Radiotherapy and Imaging, The Institute of Cancer Research and The Royal Marsden NHS Foundation Trust, 123 Old Brompton Road, London, SW7 3RP, United Kingdom.

ABSTRACT

Human Dental Pulp Stem Cells (DPSCs) can differentiate to a wide range of different cell lineages, and share some gene expression and functional similarities with pluripotent stem cells. The stemness of DPSCs can also be pharmacologically enhanced by the activation of canonical Wnt signaling. Here we examined the metabolic profile of DPSCs during reprogramming linked to Wnt activation, by a short (48 h) exposure to either the GSK3-β inhibitor BIO or human recombinant protein WNT-3A. Both treatments largely increased glucose consumption, and induced a gene overexpression of pyruvate and mitochondrial acetyl-coA producing enzymes, thus activating mitochondrial TCA metabolism in DPSCs. This ultimately led to an accumulation of reducing power and a mitochondrial hyperpolarization in DPSCs. Interestingly, Nile Red staining showed that lipid fuel reserves were being stored in Wnt-activated DPSCs. We associate this metabolic reprogramming with an energy-priming state allowing DPSCs to better respond to subsequent high demands of energy and biosynthesis metabolites for cellular growth. These results show that enhancement of the stemness of DPSCs by Wnt activation comes along with a profound metabolic remodeling, which is distinctly characterized by a crucial participation of mitochondrial metabolism.

Keywords: dental pulp stem cells, pluripotency, metabolism, Notch, Wnt, cataplerosis.

Abbreviations: Dental Pulp Stem Cells (DPSCs), Neural Crest (NC), Induced Pluripotent Stem Cells (iPSCs), Pluripotent Stem Cells (PSCs), Embryonic Stem Cells (ESC), Tricarboxylic Acid Cycle (TCA), Electron Transport Chain (ETC), Reactive Oxigen Species (ROS), Hypoxic Inducible Factor (HIF), Glycerophosphocoline (GPC), Phosphocoline (PC), Dimethylsulfoxide (DMSO), (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), Wingless-Type MMTV Integration Site Family Member 3A (WNT-3A), methyl-6-bromoindirubin-3´-oxine (MBIO), 6-bromoindirubin-3´-oxine (BIO), Nuclear Magnetic Resonance (NMR), Tetra Methyl Rhodamine Ethyl-Ester (TMRE).

INTRODUCTION

Dental Pulp Stem Cells (DPSCs) constitute a very promising tool for regenerative medicine procedures. These stem cells can differentiate to very diverse cell lineages, and have been successfully employed in different experimental animal models of pathology(Hollands et al., 2018), as well as in several clinical trials to regenerate oral and dental tissues in humans (Giuliani et al., 2013; Nakashima et al., 2017). DPSCs have a high clonogenic potential with a distinct ectomesenchymal neural crest (NC) phenotype (Gronthos et al., 2002; Gronthos et al., 2000), which endows them with the capacity to form not only mesenchymal lineage cells like adipocytes, osteoblasts, odontoblasts and chondrocytes, but also neurons, Schwann cells (Gervois et al., 2015; Martens et al., 2014), smooth muscle and vascular endothelial cells, among others (Karbanova et al., 2011).

Reprogramming and generating pluripotent stem cells out of somatic cells remains a promising alternative to obtain autologous differentiated cells for graft therapies. However, current reprogramming methods often rely on permanent genetic modification, precluding their use for human medical therapy. Interestingly, DPSCs have also been described to show a pluripotent-like phenotype (Atari et al., 2012) and a high plasticity for cell reprogramming, even using mild methods which do not involve gene transfection, making them a promising alternative source of pluripotent-like cells (Atari et al., 2011; Pisal et al., 2018; Uribe-Etxebarria et al., 2017; Yan et al., 2010).

Stem cell differentiation and/or somatic cell reprogramming are characterized by profound changes in cell metabolism. Over the last years, increasing experimental evidence pictures metabolism as a key regulator of both stem cell potency and differentiation (Hanahan and Weinberg, 2011; Pavlova and Thompson, 2016; Zhang et al., 2016b). This metabolic priming is suited to respond to the demands of cell growth and proliferation (Lunt and Vander Heiden, 2011). Thus, important evidence links metabolism, mitochondrial dynamics, and protein homeostasis with stemness (Garcia-Prat et al., 2017). There exist two major metabolic states of the cell: aerobic/oxidative (occurring in the mitochondria) and anaerobic/glycolytic (occurring in the cytosol), which perform at least three essential functions: 1) the generation of the energy (ATP) and reducing power (NADH, $FADH_2$, NADPH) necessary for biosynthesis processes; 2) the production of glycolytic intermediates essential for anabolic reactions during cell division and, 3) the release of metabolites employed in enzymatic reactions, including those involved in epigenetic modification (Teslaa and Teitell, 2015).

Compelling evidence shows that the specific metabolic requirements of pluripotent stem cells tip the balance towards a higher utilization of anaerobic pathways, at the expense of a reduced utilization of aerobic oxidative phosphorylation, which in turn associates with the process of cell differentiation (Chandel et al., 2016; Mathieu and Ruohola-Baker, 2017). Inducing the transition from oxidative into glycolytic metabolism promotes somatic cell reprogramming to iPSCs (Folmes et al., 2011; Gu et al., 2016). Stemness is promoted, and differentiation is prevented by glycolysis induction or oxidative metabolism inhibition (Mathieu and Ruohola-Baker, 2017; Varum et al., 2011) whereas differentiation of iPSCs occurs through oxidative metabolism, which is characterized by a high ATP and low lactate content (Cho et al., 2006; Folmes et al., 2011; Varum et al., 2011). However, despite these findings controversy remains about the precise role of mitochondrial oxidative metabolism in the early onset of pluripotency. There is also evidence for a transient mitochondrial oxidative phosphorylation burst during the initial stages after nuclear reprogramming (Hawkins et al., 2016; Kida et al., 2015).

Here we report that a deep metabolic remodeling occurs in DPSCs during the first 48 h of reprogramming under Notch and Wnt signaling modulation conditions, which were previously described to regulate the expression of pluripotency core factors and self-renewal in these cells (Uribe-Etxebarria et al., 2017). This work gives evidence of a metabolic switch, distinctly characterized by a mitochondrial involvement in the generation of large amounts of reducing power, and a cytoplasmic accumulation of lipid fuel reserves, associated with an enhanced DPSC stemness. Combinatorial modulation of signaling pathways reveals cell-type-specific requirements for a highly efficient and synchronous reprogramming to iPSCs (Vidal et al., 2014). Therefore, the present study provides very interesting new data with regard to approachable future research to design new protocols for a safe DPSC reprogramming.

MATERIALS AND METHODS

DPSC culture

DPSCs were isolated from human third molars obtained from healthy donor patients between 15 and 30 years of age by fracture and enzymatic digestion of the pulp tissue for 1 h at 37°C with 3 mg/ml collagenase (Thermo Fisher Scientific Cat# 17018-029, Boston, Massachusetts, USA) and 4 mg/ml dispase (Thermo Fisher Scientific Cat# 17105-041) followed by mechanical dissociation. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), L-glutamine (1 mM) and the antibiotics penicillin (100 U/ml) and streptomycin (150 µg/ml). The DPSCs could be amplified and maintained in these conditions for very long periods (> 6 months). However, to avoid cell aging issues, we only employed DPSCs that had been grown in culture for less than 3 months and had accumulated no more than 6 total passages. Comparative experiments between control and treatment conditions were always and without exception performed in parallel using DPSCs from the same donor.

Notch and Wnt pathway pharmacological modulation

To inhibit Notch signaling pathway, we employed DAPT (N-[N-(3, 5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester), a γ-secretase inhibitor, (Calbiochem Cat#565784, San Diego, California, USA), at a concentration of 2.5 µM. DAPT was added to the culture medium for 48 h prior to the assays where DAPT-treated DPSCs were compared with DPSCs treated only with the control vehicle, 2.5 µM DMSO. To over-activate Wnt signaling pathway, we used 2.5 µM BIO (6-bromoindirubin-3´-oxine), a GSK3β inhibitor (Calbiochem Cat#361550), which was added to the medium for 48 h prior to the assays. BIO-treated cells were compared with DPSCs exposed to the inactive analog MBIO (methyl-6-bromoindirubin-3´-oxine) at 2.5 µM as a corresponding control (Calbiochem Cat#361556). WNT-3A recombinant protein (R&D Systems Cat#5036-WN-010, Minneapolis, USA) was also added to the DPSC cultures to over-activate Wnt signaling, at a concentration of 2.5 µM during a total incubation time of 48 h, as in the rest of the treatments.

^{1}H-NMR of DPSCs

To obtain an NMR spectrum, an average of $6x10^6$ cells was extracted using the dual phase extraction method (Al-Saffar et al., 2006). Lyophilized samples of the water soluble fraction were reconstituted in deuterium oxide (D_2O). ^{1}H-NMR spectra were acquired as previously described (Al-Saffar et al., 2006). Metabolite concentration were determined by integration and normalized relative to the peak integral of an internal reference (TSP 0.15%) and corrected for the number of cells extracted per sample.

RNA extraction, conventional RT-PCR and quantitative Real-Time PCR (qPCR)

Total RNA was extracted from the cells using the RNeasy Kit (Qiagen Cat#74104, Hilden, Germany) and checked for purity by calculating the 260/280 ratio via the Nanodrop Synergy HT (Biotek). cDNA (50 ng/µl) was obtained by reverse transcription of total extracted RNA using the iScript cDNA Kit (BioRad Cat#1708890, CA, USA) with the following reagents: iScript reverse Transcriptase (1 µl), 5x iScript Reaction Mix (4 µl) and Nuclease Free water (variable) to a final volume of 20 µl. We analyzed gene expression using 1 µl of cDNA (5 ng/µl) diluted in 4 µl of My TaqTM Red Mix (Bioline Cat#BIO-25043, St. Petersburg, Russia), 1 µl of primers (0.625 µM) and Nuclease Free Water for a total volume reaction of 10 µl, for conventional RT-PCR. Amplification products were separated by electrophoresis in a 2 % agarose gel. Quantitative Real-Time PCR experiments were conducted in an iCyclerMyiQ^{TM} Single-Color Real-Time PCR Detection System (BioRad, USA), using4.5 µl of Power SYBR® Green PCR Master Mix 2x (Applied Biosystems^{TM,}Cat# 4367659, Carlsbad, CA, USA), 0.5 µl of primers (0.3125 µM), 0.3 µl of cDNA (1.5 ng/µl), and Nuclease Free water for a total volume reaction of 10 µl. All primers were obtained from public databases and checked for optimal efficiency (> 90 %) in the qPCR reaction under our experimental conditions. The relative expression of each gene was calculated using the standard $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001) normalized with respect to the average between *β-ACTIN* and *GAPDH* as internal controls. All reactions were performed in triplicate. qPCR was run on ABI PRISM® 7000 (Thermo Fisher Scientific, Thermo Fisher Scientific, Boston, MA, USA). Data were processed by CFX Manager™ Software (BioRad, USA). We assessed that all qPCR reactions yielded only one amplification product by the melting curve method. The primer pairs for different genes obtained via the Primer-Blast method (Primer Bank) are listed in Table 1.

Primers		**Sequence 5´ - 3´**	**Annealing (ºC)**	**Amplicon (bp)**
β-ACTIN	F	GACGACATGGAGAAAATCTG	59.7	131
	R	ATGATCTGGGTCATCTTCTC	58	
GAPDH	F	GTTTTGCGTCGCCAG	60.3	139
	R	TTGATGGCAACAATATCCAC	60.8	
Hexokinase2 (HK2)	F	GAAAGCAACTGTTTGAGAAG	56.7	162
	R	CAATGTCTGAGATGTCTTTGG	59.8	
Pyruvate kinase isoenzyme M2 (PKM2)	F	ATGTTGATATGGTGTTTGCG	60.9	142
	R	ATTTCATCAAACCTCCGAAC	60.4	
Lactate dehydrogenase A (LDHA)	F	CACCATGATTAAGGGTCTTTAC	58.9	87
	R	AGGTCTGAGATTCCATTCTG	58.2	
Lactate dehydrogenase B (LDHB)	F	TTGAAAGTGCCTATGAAGTC	56.9	152
	R	ATTCTCAATGCCATACATCC	58.8	
Pyruvate dehydrogenase A1 (PDHA1)	F	CAGCACTGATTACTACAAGAG	54.2	120
	R	CCCTTCCCAGATCTACAATAG	59.1	
Pyruvate deshidrogenase B (PDHB)	F	GAGGTGATAAATATGCGTACC	57.6	92
	R	CCTTCCACAGTTACAAGATG	57.2	
Pyruvate dehydrogenase X (PDHX)	F	GCAAATGCCAGATGTTAATG	60.6	147
	R	GCAATTTCCTGGATACCTTTAG	60.4	
ATP citrate lyase (ACLY)	F	TGTAGTGACCAAAGATGGAG	57.7	81
	R	TTCACTTTGCAGATGTAGTC	55.3	
Acyl-Coa synthetase short chain family member 2 (ACSS2)	F	GCTCAAGAAGCAGATTAGAG	56	137
	R	CATGGTCATTCTGAGCAATC	60.7	

Carnitine palmitoyltransferase 1A (CPT1A)	F	AAGTTTTATCTGAGCCTTGG	57.5	195
	R	AGAACTTGGAAGAAATGTGG	58.2	
Monocarboxylate transporter 1 (MCT1/SLC16A1)	F	TATTGGAGTCATTGGAGGTC	59	194
	R	TTAGAAAGCTTCCTCTCCATC	59.1	
Hes family bHLH transcription factor 1 (Hes1)	F	GGTACTTCCCCAGCACACTT	59	137
	R	GAAGAAAGATAGCTCGCGG	57.7	
Lymphoid enhancer binding factor 1 (Lef1)	F	TGCCAAATATGAATTAACGACCCA	59	151
	R	GAGAAAAGTGCTCGTCACTGT	58.5	
Cytochrome c oxidase subunit 6C (COX6C)	F	TTGTATAAGTTTCGTGTGGC	57.7	117
	R	TACACTCTGAAAGATACCAGC	56.1	
Cytochrome c oxidase subunit 7A2 (COX7A2)	F	AAATAAAGTTCCGGAGAAGC	58.9	123
	R	GTTCCACCAACTGTAAGAATC	57.6	
Cytochrome c oxidase subunit 7C (COX7C)	F	GAATTTGCCATTTTCAGTGG	61.9	77
	R	TAGCAAATGCAGATCCAAAG	60.4	
Cytochrome c oxidase subunit 4I1(COX4I1)	F	ATTGAAGGAGAAGGAGAAGG	58.9	82
	R	CTCCTTGAACTTAATGCGATAC	59.3	
Cytochrome c oxidase subunit 6B1 (COX6B1)	F	AAGACATGGAGACCAAAATC	58.9	149
	R	AGAGATATCGCCTCCTTTAG	57.6	
ATP synthase F1 complex	F	ACGTTTCAATGATGGATCTG	60	111
	R	TCTGCATCTGTAAGTCTCTTC	56.2	

subunit alpha (ATP5A1)				
ATP synthase F1 complex, beta subunit (ATP5B)	F	TACCACCAATTCTAAATGCC	58.9	85
	R	GTGCTCTCACCCAAATG	57.5	
ATP synthase F1 complex subunit epsilon (ATP5E)	F	CTCAGCTACATCCGATACTC	56.4	154
	R	CATTTCAAGCTTTAGTCAGGG	60.3	
NADH ubiquinone oxidoreductase core subunit S1 (NDUFS1)	F	TTACTTCCAGCAAGCAAATG	60.5	123
	R	GAGGCTCTGCTAATTGAATC	58.1	
NADH ubiquinone oxidoreductase core subunit S2 (NDUFS2)	F	GATGTTTGAGTTCTACGAGC	56.5	185
	R	GATTTCGCCAGATCCTATTG	61	
Acyl-CoA dehydrogenase medium chain (ACADM)	F	TACTTGTAGAGCACCAAGC	55.5	118
	R	GTATTTCGACGACCAGAATC	58.6	
Hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex subunit alpha (HADHA)	F	ACTAAAACCTCCAGAGGAAC	56.4	123
	R	GTCAATTTTTCCACCAATCC	60.6	

Immunoblotting

The cells were washed with 0.9% NaCl several times and the proteins were extracted with 100 µl of Lysis Buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% IGEPAL® CA-630 in dH_2O, and Proteinase Inhibition Cocktail Set III 1:100, Calbiochem Cat#539134,). Protein quantification was performed in each Western Blot using the DC™ Protein Assay (Bio Rad Cat#5000112), including Reagent A (#500-0113), Reagent B (#500-0114) and Reagent S (#500-0115).

The samples were diluted in NuPAGE sample buffer (Novex, Life technologies, Cat#NP0007, Carlsbad, California, USA) and loaded onto a 4-12% Invitrogen NuPAGEB is Tris Gel (1 mm x 10 well; Novex, Cat#NP032180X, Life Technologies) followed by transfer onto 0.45 µm-pore nitrocellulose membranes (Inmmobilon® Transfer Membranes; EMD Millipore, USA) and run in an XCell Sure Lock Electrophoresis machine (Novex, Cat#NP0007, Life Technologies).For Western Blot analyses, we used anti β-ACTIN antibody (1:1000, Cell Signaling Technology Cat# 4967, RRID:AB_330288), anti-GAPDH antibody (1:10000, Millipore Cat# MAB374, RRID:AB_2107445, Missouri, USA), anti-lactate dehydrogenase A (LDH-A) antibody (1:10000, Santa Cruz Biotechnology Cat# sc-27230, RRID:AB_672142, Texas, USA), anti-lactate dehydrogenase B (LDH-B) antibody (1:1000, Thermo Fisher Scientific Cat# PA5-43141, RRID:AB_2609663) and anti-Hexokinase 2 (HK2) antibody (1:1000, Cell Signaling Technology Cat# 2106S, RRID:AB_823520). The secondary antibodies anti-rabbit and anti-mouse (GE Healthcare Cat# NA9340-1ml, RRID: AB_772191, UK; Dako Cat# P0260, RRID: AB_2636929, Hovedstaden, Denmark) were added at a 1:2000 dilution. The membranes were stripped using Red Blot (Inmmobilon® EMD Millipore M Cat# 2504).

Nile Red Assay of cellular lipid content

DPSCs cultured over glass coverslips were fixed with 4% paraformaldehyde for 10 min and washed with PBS. The DPSCs were then incubated for 15 min with 1µg/ml Nile Red (Thermo Fisher Scientific, Cat#N1142, Waltham, Massachusetts, USA) diluted in PBS, followed by DAPI which was used to counterstain cell nuclei. Images were captured with an epifluorescence Axioskop microscope (Zeiss, Germany) with a Nikon NIS-Elements and an Apotome Confocal Microscope (Zeiss, Germany) operated with Nikon DS-Qi1Mc

software (Tokyo, Japan). The fluorescence intensities in the samples were quantified by Fiji-ImageJ(Schindelin et al., 2012) after background subtraction.

Cell viability and Mitochondrial Membrane Potential Assays (TMRE) and ROS determination

We used Calcein-AM (5 µM, Thermo Scientific Cat#C3100MP) to detect cell presence and viability and Tetra Methyl Rhodamine Ethyl-ester, or TMRE (200 nM, Thermo Fisher Scientific Cat#T669) to provide an estimation of the mitochondrial membrane potential of DPSCs. In addition, we used 2, 7-dichlorofluorescein diacetate or DC-FDA (100µM, Thermo Fisher Scientific Cat#D399) to evaluate the production of reactive oxygen species (ROS) by DPSCs. As a positive control for ROS production, we used 0.1%, 0.5% and 1.5% of H_2O_2. In these experiments, DAPI was also included as a nuclear counterstain. We incubated DPSCs with fluorescent dyes for 30 min at 37°C in culture medium and washed the cells 3 times with PBS. Fluorescence quantification was accomplished using microfluorimetry by measuring light emission at 495 nm (Calcein-AM; green fluorescence), 630 nm (PI; red fluorescence), 527 nm (DCF-DA; green fluorescence) and 359 nm (DAPI; blue fluorescence) in a Fluoroskan Ascent plate reader (Thermo Scientific). Data are plotted as normalized mean ± SEM of TMRE/Calcein and mean ± SEM of DCF-DA/DAPI fluorescence to compensate for differences in cell density in the reading field.

Alamar Blue detection

Alamar Blue (Thermo Fisher Scientific Cat#DAL1025) was used to detect cell viability and estimate the cellular reducing power of DPSCs. Alamar Blue was diluted 1:10 and absorbance was read at 600 nm (reduced state) in a Fluoroskan Ascent plate reader or in a Nanodrop Synergy HT (Biotek) run with Microplate Software: Biotek Gen5 Data Analysis Software(Biotek).

NAD+/NADH Detection Kit

This assay was performed using a NAD+/NADH Kit (Sciencell Research Laboratories, Cat#8368). Previously, non-specific proteins of the DPSC samples were eliminated by an Amicon Ultra-0.5 Centrifugal Filter Unit with Ultracel-10 membrane (Millipore Cat#UFC501008). Absorbance was measured at 490 nm with an ELISA plate reader in Nanodrop Synergy HT (Biotek) with Microplate Software: Biotek Gen5 Data Analysis

Software (Biotek). The NAD/NADH ratio was calculated following the manufacturer's instructions.

Statistical analyses

Statistical analyses were performed with Microsoft Excel, IBM SPSS Statistics v.9 (SPSS, Chicago, IL, USA) and Graph Pad v.6 software (Graph Pad Inc., USA). All data sets were subjected to a Kolmogorov-Smirnov normality test prior to analysis. For small sample sizes, non-parametric tests were chosen by default. Comparisons between only two groups were made using U-Mann Whitney test. Comparisons between multiple groups were made using Kruskal-Wallis followed by Dunn´s post hoc test. $P \leq 0.05$ was considered to be statistically significant.

RESULTS

Notch activity is required for the maintenance of glycolytic metabolism of DPSCs

In a previous report (Uribe-Etxebarria et al., 2017), it was characterized that Notch inhibition by DAPT treatment (2.5 µM for 48 h) decreased stemness of DPSCs. Here we wanted to assess whether such inhibition induced also changes at metabolic level by assessing the presence of cellular metabolites using nuclear magnetic resonance (NMR). Thus, it was found that DAPT significantly affected the levels of intracellular lactate (54.02% ± 14.90%; p= 0.002), glucose (163.96% ± 13.04%; p=0.037) and glycerophosphocoline or GPC (57.45% ± 12.63%; p=0.04) in DPSCs (Figure 1. A). Following DAPT treatment, glucose was more accumulated in DPSCs, whereas the levels of lactate were found to be significantly lower than in control samples. The levels of aminoacids (glutamate, glutamine), and metabolites involved in membrane phospholipid turnover (choline, phosphocholine PC, GPC) were not either not affected or decreased in DPSCs after DAPT treatment (Figure 1. A). In addition, transcript mRNA expression analysis for some key protein enzymes of the glycolytic and mitochondrial metabolism Hexokinase 2 (HK2) and Pyruvate Dehydrogenase B and X (PDHB, PDHX) were all found to be negatively affected by the exposition to DAPT (Figure 1. B). The expression of the mitochondrial fatty acid carrier CPT1A (Carnitine palmitoyl transferase) and the plasma membrane monocarboxylate transporter SLC16A1/MCT1 was downregulated at mRNA level when the DPSCs were treated with DAPT (Figure 1. B). Lactate Dehydrogenase A (LDHA) and Lactate Dehydrogenase B (LDHB) gene expression levels also underwent a decrease of more than 50% with respect to the control conditions (Figure 1. B). Finally, WB also confirmed these changes, where LDHA, LDHB and HK2 had a consistently reduced expression also at the protein level in DPSCs (Figure 1. C).

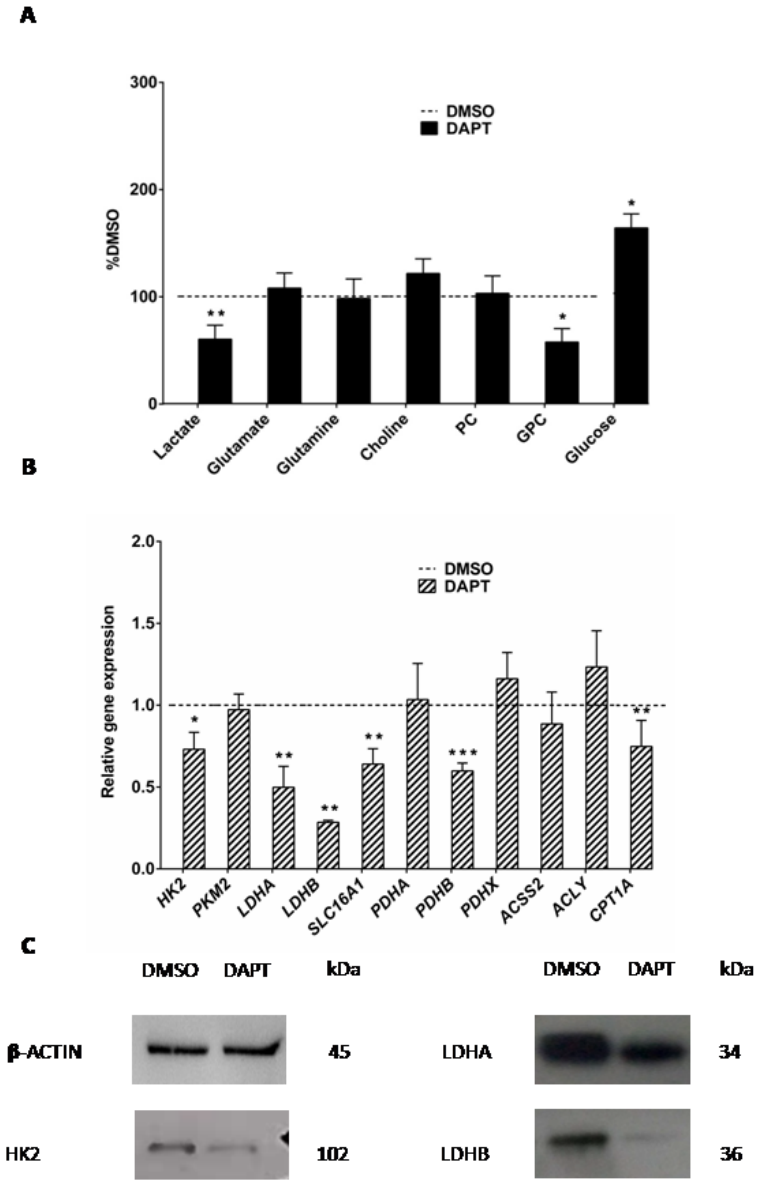


Figure 1. Notch inhibition by DAPT affects glycolytic metabolism in DPSCs. (A): NMR analysis revealed differences in the levels of Lactate, GPC and Glucose following DAPT exposure. **(B):** Q-PCR analysis confirmed a decrease in *HK2, LDHA, LDHB, SLG16A1, PDHB* and *CPT1A* expression between the control (DMSO) and DAPT conditions. Data are normalized to reference *β-ACTIN* and *GAPDH* levels and presented as the mean +SEM (n=6). The dashed line represents normalized gene expression to control conditions. **(C):** Representative WB showing LDHA, LDHB and HK2. β-ACTIN was used as protein loading control. *: p<0.05;**: p<0.01;***: *p*<0.001. U-Mann Whitney test.

BIO-induced Wnt activation increases glucose utilization and the expression of genes promoting mitochondrial TCA activity and lipid biosynthesis in DPSCs

To investigate whether Wnt/β-catenin activation would affect metabolism in DPSCs, we used a 2.5 µM BIO treatment for 48 h to overactivate Wnt signaling by inhibiting β-catenin degradation, as this had been previously associated to an increase in DPSC stemness(Uribe-Etxebarria et al., 2017). By NMR we observed that treatment with BIO induced a significant reduction in the levels of glucose (14.2% ± 2.2%; p=0.037), and glutamine (41.4% ± 9.1%; p =0.002) and increased the cellular amount of GPC (240.7% ± 66.9%; p =0.037) (Figure 2A). In addition, Wnt activation also induced the overexpression of several genes involved in: (i) glycolysis, pyruvate kinase isozyme M2 (*PKM2*); (ii) mitochondrial acetyl-coA biosynthesis, pyruvate dehydrogenase B (*PDHB*); (iii) cytosolic acetyl-coA biosynthesis, ATP-citrate lyase (*ACLY*); and (iv) cytosolic fatty acid synthesis, Acyl-coA synthetase short-chain family member (*ACSS2*). It was noteworthy that while ACLY was overexpressed, the expression of the mitochondrial fatty acid transporter CPT1 was also significantly upregulated at transcript level as well (Figure 2B). There was no cellular accumulation of lactate in BIO-treated DPSCs (Figure 2A). However, in these conditions there was a clear upregulation of *LDHB* and *PDHB*, and a downregulation of *LDHA* transcript expression (Figure 2B). The expression of *SLC16A1/MCT1*was also significantly enhanced at mRNA level in DPSCs following BIO treatment (Figure 2B). We also tested the expression of enzymes LDHA, LDHB and HK2 by WB. We confirmed previous findings by detecting increased levels of LDHB and decreased levels of LDHA, whereas no significant changes were observed in HK2 in BIO-treated DPSCs (Figure 2C).

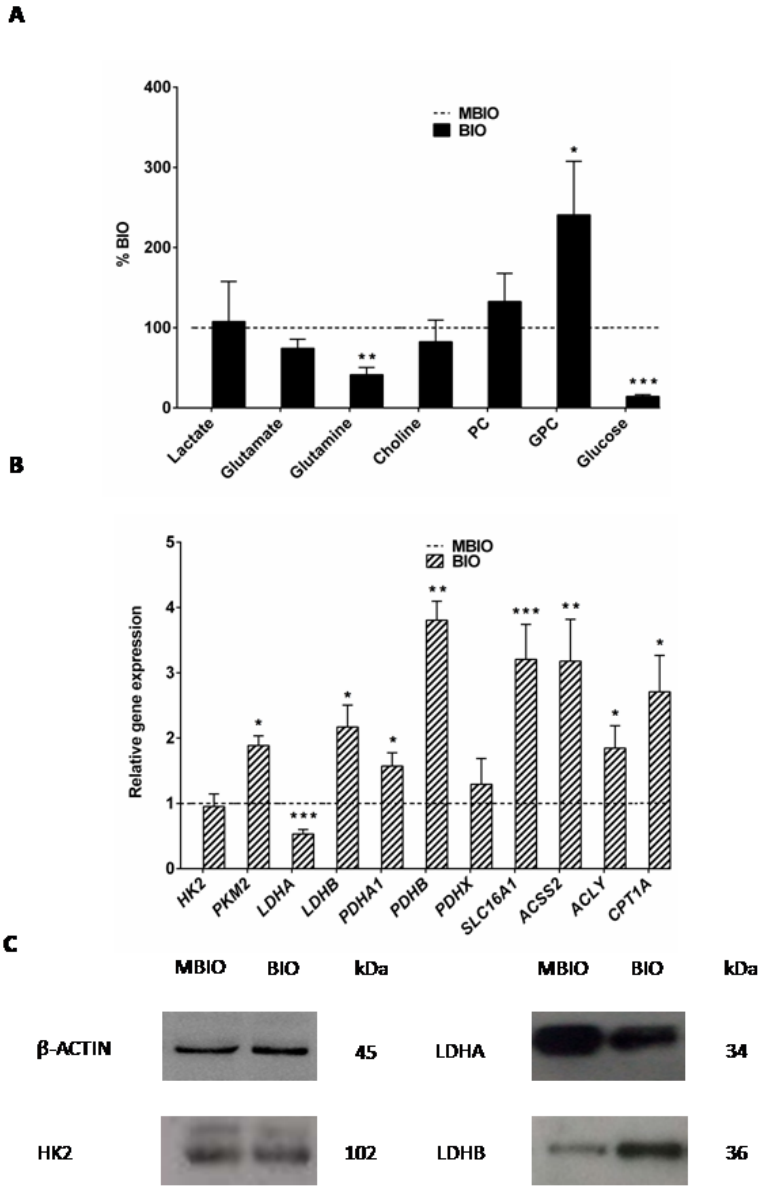


Figure 2. Wnt activation by BIO increases glucose utilization and the expression of enzymes involved in mitochondrial TCA metabolism and lipid biosynthesis in DPSCs. (A): NMR analysis revealed differences in the levels of Lactate, Glutamine, GPC and Glucose following BIO exposure. **(B):** Q-PCR analysis confirmed an increase in *PKM2, LDHA, LDHB, PDHA, PDHB, SLG16A, ACSS2, ACLY* and *CPT1A* expression between control (MBIO) and BIO conditions. Data are normalized to reference *β-ACTIN* and *GAPDH* levels, and presented as the mean +SEM (n=6). The dashed line represents normalized gene expression to control conditions. **(C):** Representative WB showing an increase in LDHB, while no changes were observed in LDHA or in HK2 protein expression. β-ACTIN was used as a protein loading control. *: $p<0.05$;**: $p<0.01$;***: $p<0.001$. U-Mann Whitney test.

Exposure to human recombinant WNT-3A for 48 h increases glucose utilization and the expression of genes promoting mitochondrial TCA activity and lipid biosynthesis in DPSCs

To ensure that the effects induced by BIO could be specifically attributed to the activation of canonical Wnt signaling pathway (Famili et al., 2015; Zhang et al., 2009) we also used WNT-3A, a well-described prototypical canonical Wnt activator ligand. After treatment of DPSCs with WNT-3A, we observed some of the same effects found following BIO treatment, with a higher consumption of glucose (64.8% ± 11.7%; p =0.004) and glutamate (68.2% ± 3.1%; p =0.003, respectively) with respect to control DPSC levels. We also found an increase in choline consumption (22.4% ± 15.2%; p =0.036), although the levels of PC and GPC were not significantly affected (Figure 3A). By qPCR we detected an upregulation in the expression of some key gene markers for glycolysis (*HK2*), TCA cycle (*PDHB*), lactate transporter (*SLC16A1/MCT1*), pyruvate synthesis (*LDHB*), and acetyl-coA and fatty acid biosynthesis (*ACLY; ACCSS2* respectively) in DPSCs (Figure 3B). Most of these changes were consistent with what was observed in BIO conditions. *CPT1A* gene expression was again found to be significantly increased. Assessment of protein expression levels for LDHA, LDHB and HK2 by WB also confirmed a clear upregulation of HK2 and LHDB enzymes, in WNT-3A treated DPSCs (Figure 3C).

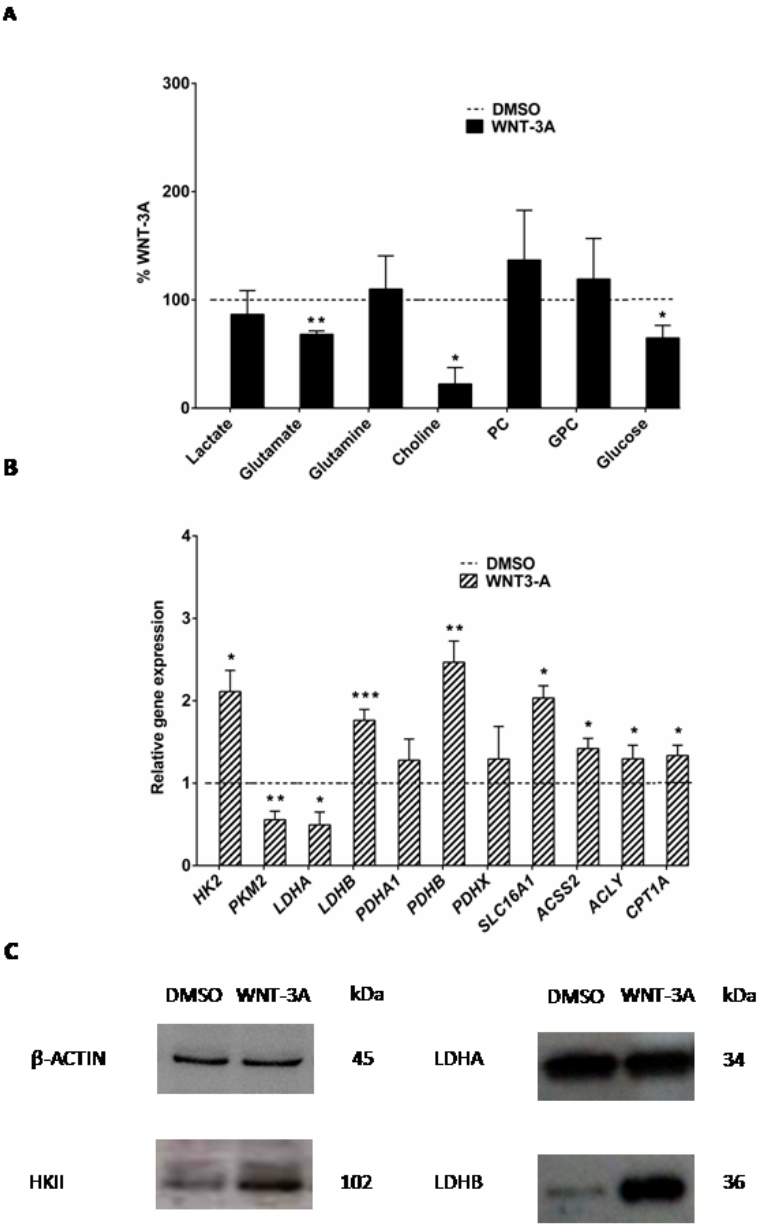


Figure 3. Wnt activation by WNT-3A increases glucose utilization, and the expression of enzymes involved in TCA metabolism and lipid biosynthesis in DPSCs. (A) NMR analysis revealed differences in the levels of Glutamate, Choline and Glucose following WNT-3A exposure. **(B)** Q-PCR showing relative differences on expression of *HK2, PKM2, LDHA, LDHB, PDHB, SLC16A1*, *ACSS2, ACLY* and *CPT1*. Data are normalized to reference *β-ACTIN* and *GAPDH* levels and represented as the mean +SEM (n=6). The dashed line represents normalized gene expression to control conditions. **(C)** Representative WB showing an increase in LDHB and HK2, while no changes were observed in LDHA. β-ACTIN was used as a protein loading control. *: $p<0.05$;**: $p<0.01$;***: $p<0.001$. U-Mann Whitney test.

Wnt activation increases cellular reducing power and the amount of NAD+ and NADH in DPSCs

As the metabolic and gene expression profile of DPSCs was altered after Wnt/Notch modulation, we tested whether the overall reducing power of DPSCs would be affected in these conditions as well. To asses this hypothesis, firstly, we measured the levels of reduced NADH and oxidized NAD+ in DPSC cultures subjected to DAPT/BIO/WNT-3A treatments. We found that reduced NADH levels increased significantly when DPSCs were exposed to the recombinant protein WNT-3A, compared to controls (2.3 µM ± 0.1 µM; p =0.018). The levels of the oxidized NAD+ form were also found to be increased, suggesting that this reducing power was being actively used by the cells (Figure 4A). NADH levels were not affected in DAPT-treated cells, and they were found to be increased, although not significantly (p =0.105), in BIO-treated cells (Figure 4A). Then, as a confirmation for these results, we used Alamar Blue to assess the overall reducing power of DPSCs subjected to different treatments (Figure 4. B). Alamar Blue is a general indicator of cellular reducing power because it can react with different reduced nucleotide species such as NADPH, NADH and $FADH_2$. Quantification of Alamar Blue absorbance determined that BIO (193.2% ± 22.7%; p =0.046) and WNT-3A-treated DPSCs (276.9% ± 24.3%; p =0.024) presented an increased reducing power respect to control conditions, whereas the reducing power was significantly lower in DPSCs treated with DAPT (30.7% ± 14.3%; p = 0.001).

Wnt activation induces hyperpolarization of mitochondria of DPSCs

To study whether Notch and Wnt signaling could also affect the energetic state of mitochondria, we first assessed the mitochondrial potential by measuring the uptake of the fluorescent cationic lipid species tetramethyl-rhodamine ethyl ester (TMRE) by live DPSCs that were treated with DAPT, BIO and WNT-3A. Incubation with 200 nM TMRE revealed the mitochondrial morphology and localization in DPSCs in red fluorescence. TMRE fluorescence was increased in BIO and WNT-3A treated DPSCs (Figure 5A, B, C). The increased TMRE uptake was confirmed by fluorescence quantification (Figure 5D). Thus, WNT-3A-treated DPSCs increased significantly their mitochondrial membrane potential with respect to control DPSCs (124.7% ± 1.1%; p =0.022) (Figure 5D). However, results of DC-FDA assays did not show any significant differences in ROS production after these treatments. Normalized DC-FDA fluorescence levels in treated

DPSCs with respect to controls were 83.3% ± 14.3% for BIO and 91.5% ± 15.1% for WNT-3A, respectively (n = 20). Instead, following treatment with 0.5%, 1% and 1.5% H_2O_2 (30 min) the DC-FDA signal in DPSCs was more than four-fold higher for 0.5% H_2O_2 (430% ± 29%), and was more than two orders of magnitude increased in the other two conditions (23092% ± 161.7% ; 26070% ± 126%).

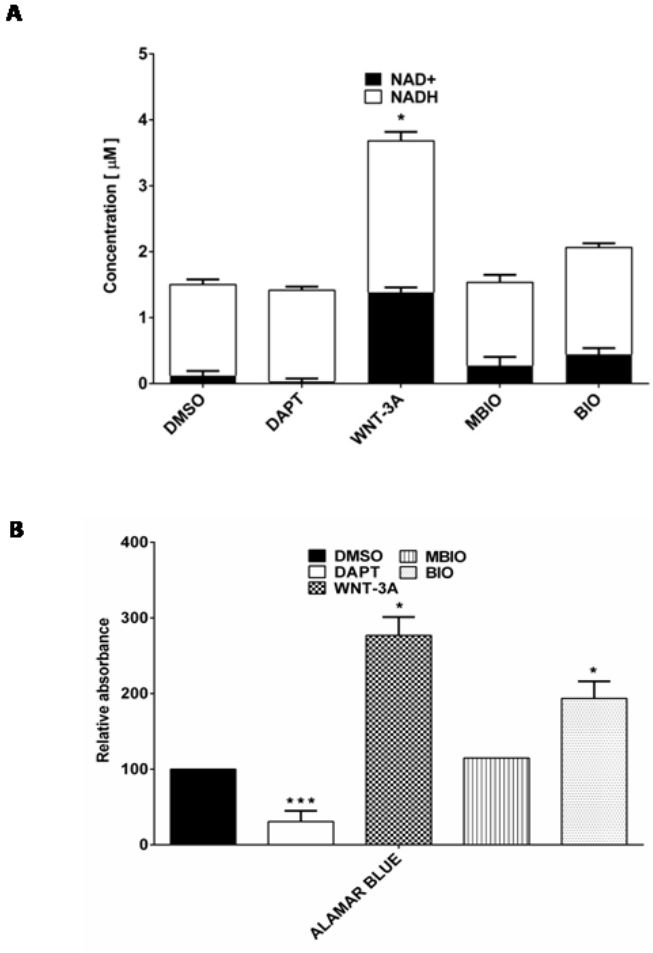


Figure 4. Wnt activation increases reducing power in DPSCs. (A): NADH and NAD+ assay revealed differences in the cellular concentration of NADH and NAD+ in DPSCs following DAPT, BIO and WNT-3A exposure. **(B):** Alamar Blue detection confirmed that reducing power in DPSCs was higher in BIO and WNT-3A conditions. **(C):** Quantification of Alamar Blue detection at 600 nm in DMSO, DAPT, MBIO, BIO and WNT3-A-treated DPSCs. Data are presented as the mean +SEM (n=6). *: $p<0.05$;**: $p<0.01$;***: $p<0.001$. U-Mann Whitney test.

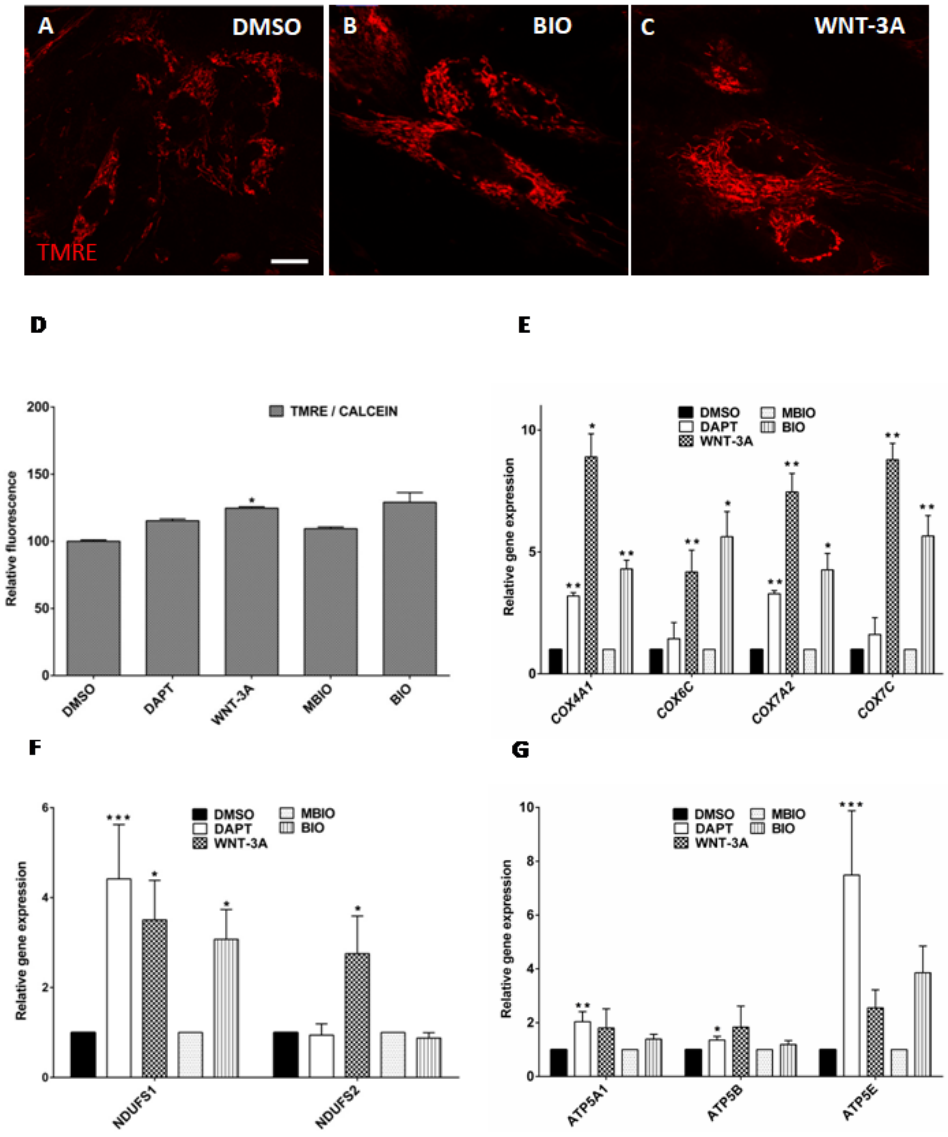


Figure 5. Wnt activation hyperpolarizes mitochondria and increases the levels of mRNA expression of ETC subunits, but not ATP synthase subunits, in DPSCs (A, B, C): TMRE-loaded DPSCs grown with DMSO, BIO and WNT-3A treatment for 48 h. Scale bar: 20 µm. **(D):** Quantification of relative TMRE/Calcein fluorescence. Data are presented as mean+ SEM (n =12). **(E, F, G):** Q-PCR showing relative differences in Cytochrome c Oxidase subunits (*COX4A1, COX6C, COX7A2 AND COX7C*), ATP synthase subunits (*ATP5A1, ATP5B and ATP5E),* and NADH-Ubiquinone Oxidoreductase subunits (*NDUFS1* and *NDUFS2)*. Data are normalized to reference *β-ACTIN* and *GAPDH* levels and represented as the mean +SEM (n=6). *: p<0.05;**: p<0.01;***: p<0.001. U-Mann Whitney test.

Wnt activation induces the overexpression of mitochondrial electron transport chain (ETC) genes, but not ATP synthase genes in DPSCs

In order to estimate ETC activity in Wnt-activated DPSCs we tested the expression of some genes coding for some mitochondrial ETC complex subunits by qPCR. We observed increased levels of transcript expression in most of the cytochrome C oxidase (complex IV) subunits tested: COX4A.i1, COX6c, COX7a and COX7c, and also in the NADH-Ubiquinone oxidoreductase (complex I) subunits NDUFS1 and NDUFS2 in DPSCs treated with BIO and/or WNT-3A (Figure 5E, F). Interestingly, some of these subunits were also upregulated in the case of DAPT treatment (Figure 5E, F). Finally, the assessment of expression levels of mitochondrial ATP synthase subunits ATP5a, ATP5e and ATP5b provided us with information about the utilization of the mitochondrial proton gradient to produce ATP. Interestingly, there was no increase in the expression of ATP synthase subunits in either BIO or WNT-3A treated DPSCs (Figure 5G), despite a clear upregulation of the expression of ETC complexes and an increased mitochondrial membrane potential in these conditions. A significant increase in ATP synthase subunit expression was only found in cells treated with DAPT (Figure 5G).

Wnt activation promotes lipid biosynthesis and accumulation in DPSCs

In order to assess whether the treatments with DAPT, BIO and WNT-3A were inducing changes in the cytoplasmic lipid content in DPSCs, we performed a Nile Red staining. The treatment with WNT-3A significantly increased lipid accumulation in DPSCs, almost twice as much comparing to control (180 % ±19.4%; p =0.005) (Figure 6C, G). As a positive control, we used sister cultures of DPSCs which underwent an adipogenic pharmacological treatment, which presented a roughly three-fold increase in Nile Red fluorescence compared to the non-treated cells (Figure 6F, G). Cells treated with DAPT showed a non-significant reduction in Nile Red staining (Figure 6B, G).

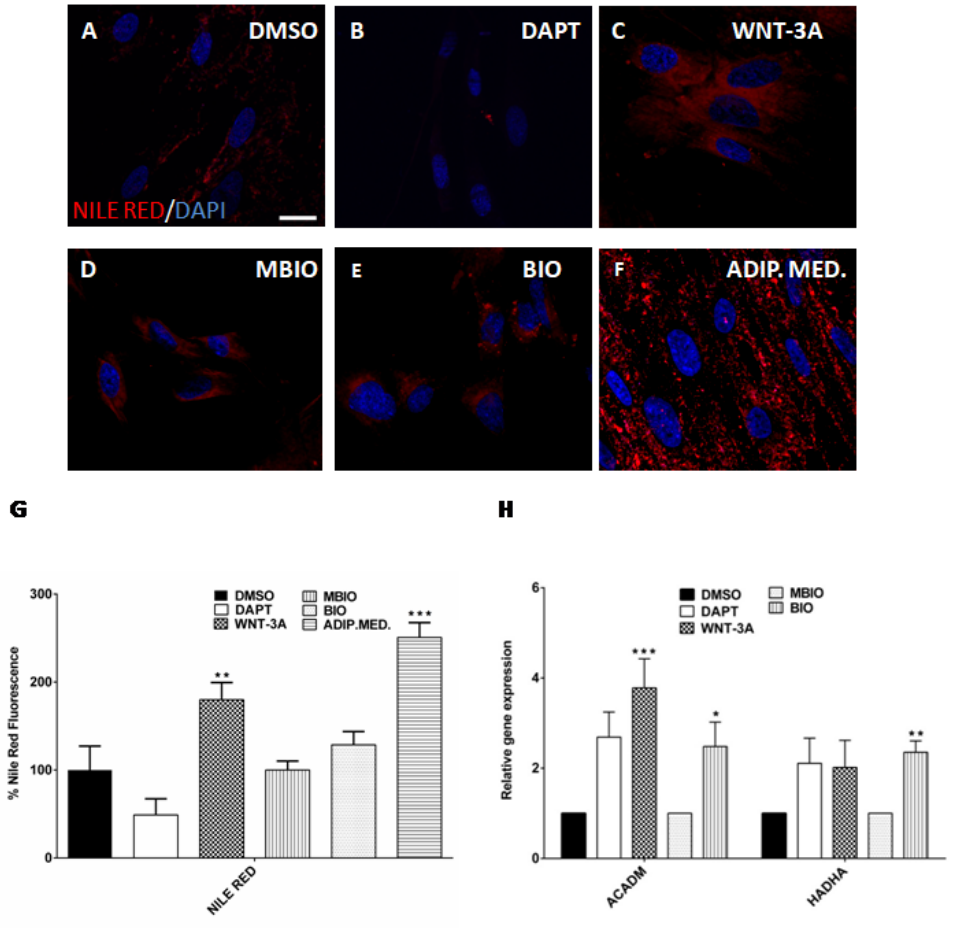


Figure 6. Wnt activation promotes both lipid biosynthesis and oxidation in DPSCs. (A, F): Nile Red Staining revealed differences in the accumulation of lipid droplets under BIO and WNT-3A exposure. Scale bar=20 µm. **(G):**Bar chart showing relative fluorescence of Nile Red lipid droplets in DMSO, DAPT, MBIO, BIO and WNT-3A conditions. **(H):** Q-PCR analysis showed an increase in *ACADM* and *HADHA* expression in WNT-3A and BIO–treated cells. The data are normalized to reference *β-ACTIN* and *GAPDH* levels and presented as the mean +SEM (n=6). *: $p<0.05$;**: $p<0.01$;***: $p<0.001$. U-Mann Whitney test.

Wnt-activated DPSCs overexpress β-oxidation enzymes at mRNA level

Mitochondrial β-oxidation of fatty acids provides for molecules of acetyl-coA ready to enter the TCA cycle, while simultaneously generating reducing power (Giudetti et al., 2016). A limiting step in this process is the transport of fatty acids to mitochondria, which is catalyzed by the carnitine shuttle, in which CPT1 critically participates. Since we had demonstrated that DPSCs treated with BIO or WNT-3A accumulated cellular lipids while also significantly overexpressed CPT1 at mRNA level, we examined whether transcript expression for β-oxidation enzymes would also be somehow affected in these conditions. Interestingly, an upregulation of expression was found for Acyl-coA dehydrogenase medium chain (*ACADM)* and Hydroxyacyl-CoA dehydrogenase (*HADHA)* genes in BIO and WNT-3A-treated DPSCs (Figure 6H).

DISCUSSION

DPSCs are a promising source of pluripotent-like stem cells for cell therapy, which apart from being easily accessible, possess a significant capacity for *in vitro* expansion, have non-tumorigenic phenotypes and a greater multi-lineage differentiation potential than other tissue-specific stem cells (Atari et al., 2012; Kerkis et al., 2006; Rosa et al., 2016). Compared to Pluripotent Stem Cells (PSCs), Embryonic Stem Cells (ESCs) and induced Pluripotent Stem Cells (iPSCs), DPSCs do not pose ethical or safety issues. In a previous report, we demonstrated that the stemness of DPSCs could be enhanced by a controlled activation of Notch/Wnt signaling pathways, which are functionally interconnected in these cells. Thus, a short (48 h) pharmacological Wnt activation by BIO or WNT-3A caused an important increase in the expression of pluripotency core factors in DPSCs (Uribe-Etxebarria et al., 2017). This kind of gentle approach to enhance stemness without relying on traditional nuclear reprogramming methods may prove beneficial to fully exploit the capabilities of DPSCs and other tissue-specific stem cells.

Over the last few years, metabolism has entered the stage as a fundamental regulator of pluripotency (Folmes et al., 2011; Mathieu and Ruohola-Baker, 2017). In fact, activation of glycolysis pathways is known to enhance somatic cell reprogramming efficiency (Folmes et al., 2012) and pharmacological activation of glycolysis, in combination with other small compounds, allows for a reduction of the traditional recipe of Yamanaka factors for nuclear reprogramming to a simplified version containing just OCT-4 (Zhu et al., 2010). Consistently, pharmacological inhibition of glycolysis results in a reduced

reprogramming efficiency (Folmes et al., 2011). This evidence illustrates how metabolic plasticity can facilitate (or impair) stemness. In fact, the differentiation of pluripotent cells and the reprogramming of somatic cells often involve metabolic switches at very early stages, before changes in phenotype and/or the expression of pluripotency core factors can be yet observed (Folmes et al., 2011).

The current prevailing view acknowledges that fully differentiated cells rely mainly on mitochondrial oxidative phosphorylation to meet their energy demands, while non-committed PSCs primarily use glycolysis and production of lactate. This metabolic signature was first described in cancer cells as “The Warburg Effect” (Vander Heiden et al., 2009; Warburg, 1956), and it was later consistently observed in both ESCs and iPSCs(Mathieu and Ruohola-Baker, 2017; Varum et al., 2011). Following DAPT treatment, glucose was accumulated in DPSCs, suggesting a lower utilization of this primary fuel for glycolysis. Consistently, also the levels of lactate were found to be significantly lower than in control samples. Interestingly, despite being the glycolysis inhibited, glutamine and glutamate were not being used as alternative source of energy following DAPT treatment, since the levels of these aminoacids were not changed, with respect to control DPSCs.

In Wnt-activated DPSCs, we observed an upregulation of glycolysis after 48 h. This was characterized by a decrease in cellular glucose levels, assessed by NMR, and an increased expression of glycolytic enzymes at both mRNA and protein level. However, WNT-3A treated DPSCs did not show the portrait of a classic Warburg effect, since most of the glucose appeared to be directed to pyruvate and mitochondrial acetyl-coA synthesis, rather than towards the production of lactate. In fact, lactate levels were not increased at all in Wnt-activated DPSCs. Furthermore, we found an overexpression of the membrane transporter SLC16A1/MCT1, suggesting that lactate might even be taken up by DPSCs as an alternative source of pyruvate. The overexpression of the lactate-to-pyruvate converter enzyme LDHB and the downregulation of expression of its antagonistic enzyme LDHA, at both gene and protein levels, during both BIO and WNT-3A treatment seem to add strong support to this view.

If Wnt-activated DPSCs were driving an enhanced glycolytic input to feed the mitochondrial TCA cycle, as also suggested by the overexpression of mitochondrial PDH complex subunits, and the accumulation of cellular reducing power (NADH), then the question became: with what purpose? It seemed that at least some of this reducing power

was being used by the mitochondrial ETC, as suggested by the increased expression of some key Complex I and Complex IV subunits. Consistently, mitochondria were hyperpolarized in DPSCs exposed to BIO and WNT-3A, with an about 25% increased uptake of TMRE. However, this mitochondrial hyperpolarization did not came along with a concomitant increase in the expression of ATP synthase subunits, which suggested that the accumulation of reducing power in these conditions was not being primarily used to synthesize more ATP. The observation of mitochondrial hyperpolarization is interesting, since this has also been observed in other PSCs (Folmes et al., 2011). The mitochondrial potential magnitude has been reported to be predictive of the stemness of ESCs, where populations of high-mitochondrial potential ESCs were more prone to generating teratomas after transplantation, whereas low-mitochondrial potential ESCs were found to be pre-committed for somatic differentiation (Schieke et al., 2008).

Recent evidence showed that maintenance of a high mitochondrial membrane potential is required for a burst in reactive oxygen species (ROS) generation which regulates cell proliferation by hypoxia inducible factor (HIF) expression (Martinez-Reyes et al., 2016). However, in our DPSC cultures we could not detect any significant increase in ROS production by DC-FDA fluorimetric assays after treatment with BIO or WNT-3A. Interestingly, experiments of somatic cell reprogramming to PSCs at very early stages showed that the increase in glycolysis associated with nuclear reprogramming was at first accompanied by a burst in ETC activity, before switching to a classic Warburg-like glycolytic metabolism (Hawkins et al., 2016; Kida et al., 2015). In our experimental model, DPSCs were exposed to Wnt activators and the cell phenotype was assessed short-term, after only 48 h, a time frame which could easily correspond with such early reprogrammed cells. Other studies have reported that a functional ETC is essential for maintaining pluripotency (Zhang et al., 2016a), and that disruption of mitochondrial dynamics could directly impact reprogramming efficiency (Facucho-Oliveira et al., 2007; Vazquez-Martin et al., 2012). Altogether, mitochondrial changes in our model of DPSCs seem to correspond with a stemness-associated metabolic plasticity, although a possible involvement of ROS signaling in this context should be further clarified.

Accumulation of reducing power in DPSCs renders cells “hyper-energetic”, with a high capacity for cellular biosynthesis. TCA cycle intermediaries such as citrate and oxaloacetate are used for the *de novo* synthesis of lipids and nucleotides, which could provide cells with a source of energy and metabolites when differentiation signals begin

(Chandel et al., 2016). In fact, another of the most interesting findings of the present work is that DPSCs were accumulating cytoplasmic lipid droplets after Wnt activation, as assessed by Nile Red staining. Lipid biosynthesis requires cytosolic acetyl-coA, which is primarily derived from a cytosolic export of mitochondrial citrate, to provide for an increase in cellular reducing power and release of biosynthesis metabolites in a process known as cataplerosis (Owen et al., 2002). Then, a critical metabolic reaction is catalyzed by the ACLY enzyme, which transforms this citrate to acetyl-coA, thus linking carbohydrate and lipid metabolism (Wellen et al., 2009). Importantly, ACLY expression was found to be clearly upregulated in DPSCs after BIO and WNT-3A treatment. In this context, the accumulation of cytosolic acetyl-coA could serve two main purposes: first (i), to provide a primary substrate for lipid biosynthesis, and second (ii) to provide a substrate for histone acetylation, which has been shown necessary to maintain pluripotency (Moussaieff et al., 2015b). In fact, compelling evidence indicates that during early stages of differentiation of human ESCs, cytosolic acetyl-coA levels drop and this was associated with histone deacetylation and spontaneous cell differentiation. In contrast, a rise of cellular acetyl-coA levels was linked with maintenance of pluripotency (Moussaieff et al., 2015b).

Thus, in view of our results and despite the fact that we could not actually measure acetyl-coA levels in our model, in all likelihood cataplerosis was occurring in DPSCs after Wnt-activation. This would clearly support lipid biosynthesis as storage of fuel reserves to prepare for subsequent differentiation stimuli demanding a fast production of ATP and metabolites, but additionally, it could also mediate histone acetylation related to an enhanced stemness(Martinez-Reyes et al., 2016; Moussaieff et al., 2015a). Interestingly, together with an increased lipid biosynthesis, we detected reduced levels of glutamate and glutamine in Wnt-activated DPSCs, indicating compensatory anaplerosis to sustain high TCA cycle activity levels. Finally, some of the fatty acids generated by DPSCs after Wnt activation were also being transported to mitochondria for β-oxidation, as suggested by the increased expression of *CPT-1* and other genes (*ACADM, HADHA*) coding for β-oxidation enzymes. These results feature a complex and coordinated cycle of cataplerosis and anaplerosis to support the activity of the mitochondrial TCA cycle, and thus generate a large reducing power and a mitochondrial hyperpolarization in DPSCs. The main findings of the present work are summarized in Figures 7 and 8.

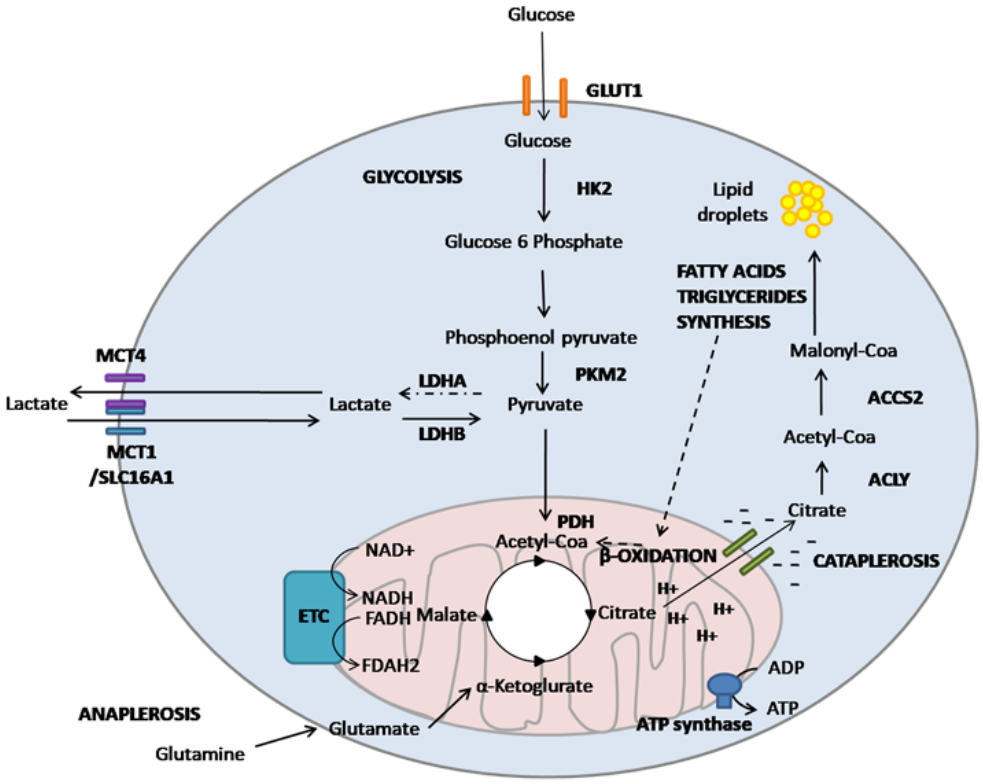


Figure 7. Representation of metabolic pathways and steps affected during Wnt activation in DPSCs. Wnt signaling activation by either BIO or WNT-3A increases glucose consumption by overexpression of glycolytic enzymes HK2 and/or PKM2. LDHA and LDHB participate in lactate to pyruvate conversion. LDHB is overexpressed while LHDA is downregulated in Wnt activated DPSCs. Pyruvate dehydrogenase complex subunits are also upregulated in BIO/WNT-3A treated DPSCs, thus fueling the mitochondrial TCA cycle. These "hyper-energized" DPSCs show a net accumulation of lipids and a mitochondrial hyperpolarization. Overexpression of cytosolic ACLY and ACSS2 enzymes suggests cataplerosis leading to cytosolic accumulation of acetyl-coA, which can be then used for lipid biosynthesis. Meanwhile, mitochondria consume amino acids such as glutamine and glutamate to replenish TCA metabolites in a coordinated cycle of cataplerosis and anaplerosis. Cytosolic fatty acids also appear to participate in this process, as suggested by the overexpression of CPT1 and β-oxidation enzymes at mRNA level. DPSCs reprogrammed with BIO or WNT-3A thus show a boost in glycolysis without the characteristic lactate accumulation observed in the classic Warburg effect.

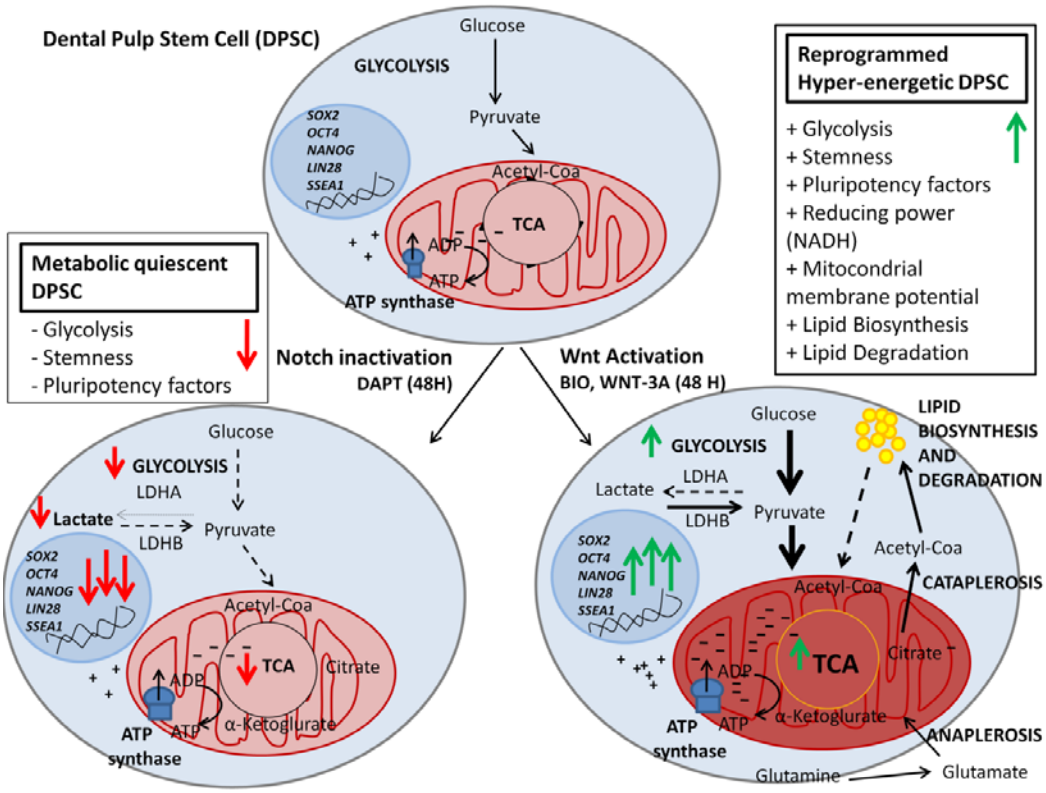


Figure 8. The modulation of DPSC stemness by Notch and Wnt signaling comes associated with a deep metabolic remodeling. Wnt-activated DPSCs show an atypical Warburg effect, boosting glycolysis but also mitochondrial TCA activity. DPSCs in these conditions accumulate large amounts of reducing power, and cytoplasmic lipids. Understanding metabolic changes linked to DPSC reprogramming could be of great interest to make the best use of DPSCs for cell therapy.

CONCLUSIONS

In conclusion our data show that enhancement of DPSC stemness by short-term Wnt signaling activation for 48 h comes along with a profound metabolic remodeling, featuring a boost of glycolysis but also of mitochondrial TCA activity. This metabolic plasticity bears some resemblance with other models of somatic cell reprogramming at very early stages, where a distinct involvement of mitochondrial potential and oxidative phosphorylation have also been reported, in contrast to the classically portrayed Warburg effect featured by lactate accumulation. We associate these changes to a transient hyper-energetic priming stage, where DPSCs accumulate a large reducing power for biosynthesis. Interestingly, DPSCs also generated cytoplasmic lipid reserves, a process likely associated with cataplerosis. Altogether, we showed that the increase in pluripotency core factor expression observed after Wnt activation in DPSCs was mirrored by important changes in both glycolytic and oxidative metabolism, suggesting that stemness and metabolic plasticity are intimately related. A characterization and modulation of these metabolic changes could be of great interest to make the best use of DPSCs and their stemness/differentiation capabilities regarding cell therapy.

REFERENCES

Al-Saffar NM, Troy H, Ramirez de Molina A, Jackson LE, Madhu B, Griffiths JR, Leach MO, Workman P, Lacal JC, Judson IR, Chung YL. 2006. Noninvasive magnetic resonance spectroscopic pharmacodynamic markers of the choline kinase inhibitor MN58b in human carcinoma models. Cancer Res 66(1):427-434.

Atari M, Barajas M, Hernandez-Alfaro F, Gil C, Fabregat M, Ferres Padro E, Giner L, Casals N. 2011. Isolation of pluripotent stem cells from human third molar dental pulp. Histology and histopathology 26(8):1057-1070.

Atari M, Gil-Recio C, Fabregat M, Garcia-Fernandez D, Barajas M, Carrasco MA, Jung HS, Alfaro FH, Casals N, Prosper F, Ferres-Padro E, Giner L. 2012. Dental pulp of the third molar: a new source of pluripotent-like stem cells. Journal of cell science 125(Pt 14):3343-3356.

Chandel NS, Jasper H, Ho TT, Passegue E. 2016. Metabolic regulation of stem cell function in tissue homeostasis and organismal ageing. Nat Cell Biol 18(8):823-832.

Cho YM, Kwon S, Pak YK, Seol HW, Choi YM, Park DJ, Park KS, Lee HK. 2006. Dynamic changes in mitochondrial biogenesis and antioxidant enzymes during the spontaneous differentiation of human embryonic stem cells. Biochemical and biophysical research communications 348(4):1472-1478.

Facucho-Oliveira JM, Alderson J, Spikings EC, Egginton S, St John JC. 2007. Mitochondrial DNA replication during differentiation of murine embryonic stem cells. J Cell Sci 120(Pt 22):4025-4034.

Famili F, Naber BA, Vloemans S, de Haas EF, Tiemessen MM, Staal FJ. 2015. Discrete roles of canonical and non-canonical Wnt signaling in hematopoiesis and lymphopoiesis. Cell Death Dis 6:e1981.

Folmes CD, Dzeja PP, Nelson TJ, Terzic A. 2012. Mitochondria in control of cell fate. Circulation research 110(4):526-529.

Folmes CD, Nelson TJ, Martinez-Fernandez A, Arrell DK, Lindor JZ, Dzeja PP, Ikeda Y, Perez-Terzic C, Terzic A. 2011. Somatic oxidative bioenergetics transitions into pluripotency-dependent glycolysis to facilitate nuclear reprogramming. Cell metabolism 14(2):264-271.

Garcia-Prat L, Sousa-Victor P, Munoz-Canoves P. 2017. Proteostatic and Metabolic Control of Stemness. Cell Stem Cell 20(5):593-608.

Gervois P, Struys T, Hilkens P, Bronckaers A, Ratajczak J, Politis C, Brone B, Lambrichts I, Martens W. 2015. Neurogenic maturation of human dental pulp stem cells following neurosphere generation induces morphological and electrophysiological characteristics of functional neurons. Stem Cells Dev 24(3):296-311.

Giudetti AM, Stanca E, Siculella L, Gnoni GV, Damiano F. 2016. Nutritional and Hormonal Regulation of Citrate and Carnitine/Acylcarnitine Transporters: Two Mitochondrial Carriers Involved in Fatty Acid Metabolism. International journal of molecular sciences 17(6).

Giuliani A, Manescu A, Langer M, Rustichelli F, Desiderio V, Paino F, De Rosa A, Laino L, d'Aquino R, Tirino V, Papaccio G. 2013. Three years after transplants in human mandibles, histological and in-line holotomography revealed that stem cells regenerated a compact rather than a spongy bone: biological and clinical implications. Stem Cells Transl Med 2(4):316-324.

Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, DenBesten P, Robey PG, Shi S. 2002. Stem cell properties of human dental pulp stem cells. J Dent Res 81(8):531-535.

Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. 2000. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proceedings of the National Academy of Sciences of the United States of America 97(25):13625-13630.

Gu W, Gaeta X, Sahakyan A, Chan AB, Hong CS, Kim R, Braas D, Plath K, Lowry WE, Christofk HR. 2016. Glycolytic Metabolism Plays a Functional Role in Regulating Human Pluripotent Stem Cell State. Cell Stem Cell 19(4):476-490.

Hanahan D, Weinberg RA. 2011. Hallmarks of cancer: the next generation. Cell 144(5):646-674.

Hawkins KE, Joy S, Delhove JM, Kotiadis VN, Fernandez E, Fitzpatrick LM, Whiteford JR, King PJ, Bolanos JP, Duchen MR, Waddington SN, McKay TR. 2016. NRF2 Orchestrates the Metabolic Shift during Induced Pluripotent Stem Cell Reprogramming. Cell reports 14(8):1883-1891.

Hollands P, Aboyeji D, Orcharton M. 2018. Dental pulp stem cells in regenerative medicine. British dental journal.

Karbanova J, Soukup T, Suchanek J, Pytlik R, Corbeil D, Mokry J. 2011. Characterization of dental pulp stem cells from impacted third molars cultured in low serum-containing medium. Cells Tissues Organs 193(6):344-365.

Kerkis I, Kerkis A, Dozortsev D, Stukart-Parsons GC, Gomes Massironi SM, Pereira LV, Caplan AI, Cerruti HF. 2006. Isolation and characterization of a population of immature dental pulp stem cells expressing OCT-4 and other embryonic stem cell markers. Cells, tissues, organs 184(3-4):105-116.

Kida YS, Kawamura T, Wei Z, Sogo T, Jacinto S, Shigeno A, Kushige H, Yoshihara E, Liddle C, Ecker JR, Yu RT, Atkins AR, Downes M, Evans RM. 2015. ERRs Mediate a Metabolic Switch Required for Somatic Cell Reprogramming to Pluripotency. Cell Stem Cell 16(5):547-555.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif) 25(4):402-408.

Lunt SY, Vander Heiden MG. 2011. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. Annu Rev Cell Dev Biol 27:441-464.

Martens W, Sanen K, Georgiou M, Struys T, Bronckaers A, Ameloot M, Phillips J, Lambrichts I. 2014. Human dental pulp stem cells can differentiate into Schwann cells and promote and guide neurite outgrowth in an aligned tissue-engineered collagen construct in vitro. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 28(4):1634-1643.

Martinez-Reyes I, Diebold LP, Kong H, Schieber M, Huang H, Hensley CT, Mehta MM, Wang T, Santos JH, Woychik R, Dufour E, Spelbrink JN, Weinberg SE, Zhao Y, DeBerardinis RJ, Chandel NS. 2016. TCA Cycle and Mitochondrial Membrane Potential Are Necessary for Diverse Biological Functions. Mol Cell 61(2):199-209.

Mathieu J, Ruohola-Baker H. 2017. Metabolic remodeling during the loss and acquisition of pluripotency. Development 144(4):541-551.

Moussaieff A, Kogan NM, Aberdam D. 2015a. Concise Review: Energy Metabolites: Key Mediators of the Epigenetic State of Pluripotency. Stem cells 33(8):2374-2380.

Moussaieff A, Rouleau M, Kitsberg D, Cohen M, Levy G, Barasch D, Nemirovski A, Shen-Orr S, Laevsky I, Amit M, Bomze D, Elena-Herrmann B, Scherf T, Nissim-Rafinia M, Kempa S, Itskovitz-Eldor J, Meshorer E, Aberdam D, Nahmias Y. 2015b. Glycolysis-mediated changes in acetyl-CoA and histone acetylation control the early differentiation of embryonic stem cells. Cell metabolism 21(3):392-402.

Nakashima M, Iohara K, Murakami M, Nakamura H, Sato Y, Ariji Y, Matsushita K. 2017. Pulp regeneration by transplantation of dental pulp stem cells in pulpitis: a pilot clinical study. Stem Cell Res Ther 8(1):61.

Owen OE, Kalhan SC, Hanson RW. 2002. The key role of anaplerosis and cataplerosis for citric acid cycle function. J Biol Chem 277(34):30409-30412.

Pavlova NN, Thompson CB. 2016. The Emerging Hallmarks of Cancer Metabolism. Cell metabolism 23(1):27-47.

Pisal RV, Suchanek J, Siller R, Soukup T, Hrebikova H, Bezrouk A, Kunke D, Micuda S, Filip S, Sullivan G, Mokry J. 2018. Directed reprogramming of comprehensively characterized dental pulp stem cells extracted from natal tooth. Scientific reports 8(1):6168.

Rosa V, Dubey N, Islam I, Min KS, Nor JE. 2016. Pluripotency of Stem Cells from Human Exfoliated Deciduous Teeth for Tissue Engineering. Stem cells international 2016:5957806.

Schieke SM, Ma M, Cao L, McCoy JP, Jr., Liu C, Hensel NF, Barrett AJ, Boehm M, Finkel T. 2008. Mitochondrial metabolism modulates differentiation and teratoma formation capacity in mouse embryonic stem cells. J Biol Chem 283(42):28506-28512.

Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, Preibisch S, Rueden C, Saalfeld S, Schmid B, Tinevez JY, White DJ, Hartenstein V, Eliceiri K, Tomancak P, Cardona A. 2012. Fiji: an open-source platform for biological-image analysis. Nature methods 9(7):676-682.

Teslaa T, Teitell MA. 2015. Pluripotent stem cell energy metabolism: an update. EMBO J 34(2):138-153.

Uribe-Etxebarria V, Luzuriaga J, Garcia-Gallastegui P, Agliano A, Unda F, Ibarretxe G. 2017. Notch/Wnt cross-signalling regulates stemness of dental pulp stem cells through expression of neural crest and core pluripotency factors. Eur Cell Mater 34:249-270.

Vander Heiden MG, Cantley LC, Thompson CB. 2009. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 324(5930):1029-1033.

Varum S, Rodrigues AS, Moura MB, Momcilovic O, Easley CAt, Ramalho-Santos J, Van Houten B, Schatten G. 2011. Energy metabolism in human pluripotent stem cells and their differentiated counterparts. PLoS One 6(6):e20914.

Vazquez-Martin A, Cufi S, Corominas-Faja B, Oliveras-Ferraros C, Vellon L, Menendez JA. 2012. Mitochondrial fusion by pharmacological manipulation impedes somatic cell reprogramming to pluripotency: new insight into the role of mitophagy in cell stemness. Aging 4(6):393-401.

Vidal SE, Amlani B, Chen T, Tsirigos A, Stadtfeld M. 2014. Combinatorial modulation of signaling pathways reveals cell-type-specific requirements for highly efficient and synchronous iPSC reprogramming. Stem Cell Reports 3(4):574-584.

Warburg O. 1956. On the origin of cancer cells. Science 123(3191):309-314.

Wellen KE, Hatzivassiliou G, Sachdeva UM, Bui TV, Cross JR, Thompson CB. 2009. ATP-citrate lyase links cellular metabolism to histone acetylation. Science 324(5930):1076-1080.

Yan X, Qin H, Qu C, Tuan RS, Shi S, Huang GT. 2010. iPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin. Stem cells and development 19(4):469-480.

Zhang J, Khvorostov I, Hong JS, Oktay Y, Vergnes L, Nuebel E, Wahjudi PN, Setoguchi K, Wang G, Do A, Jung HJ, McCaffery JM, Kurland IJ, Reue K, Lee WN, Koehler CM, Teitell MA. 2016a. UCP2 regulates energy metabolism and differentiation potential of human pluripotent stem cells. EMBO J 35(8):899.

Zhang J, Ratanasirintrawoot S, Chandrasekaran S, Wu Z, Ficarro SB, Yu C, Ross CA, Cacchiarelli D, Xia Q, Seligson M, Shinoda G, Xie W, Cahan P, Wang L, Ng SC, Tintara S, Trapnell C, Onder T, Loh YH, Mikkelsen T, Sliz P, Teitell MA, Asara JM, Marto JA, Li H, Collins JJ, Daley GQ. 2016b. LIN28 Regulates Stem Cell Metabolism and Conversion to Primed Pluripotency. Cell Stem Cell 19(1):66-80.

Zhang Z, Deb A, Zhang Z, Pachori A, He W, Guo J, Pratt R, Dzau VJ. 2009. Secreted frizzled related protein 2 protects cells from apoptosis by blocking the effect of canonical Wnt3a. J Mol Cell Cardiol 46(3):370-377.

Zhu S, Li W, Zhou H, Wei W, Ambasudhan R, Lin T, Kim J, Zhang K, Ding S. 2010. Reprogramming of human primary somatic cells by OCT4 and chemical compounds. Cell Stem Cell 7(6):651-655.

5

Wnt-3a recombinant protein mediates self-renewal and epigenetic reprogramming through embryonic stem cell core transcriptional network in Human Dental Pulp Stem Cells.

Verónica Uribe-Etxebarria.[1], Nerea Subirán.[2], Patricia García-Gallastegui.[1], Miguel Pérez .[1], Fernando Unda.[1], Gaskon Ibarretxe [1].

[1]: Cell Biology and Histology Department. University of the Basque Country (UPV/EHU). Barrio Sarriena.n. 48940 Leioa, Spain

[2]: Physiology Department. University of the Basque Country (UPV/EHU). Barrio Sarriena.n. 48940 Leioa, Spain

ABSTRACT

Dental Pulp Stem Cells (DPSCs) from adult teeth express core transcription factors, such as cMyc, Oct4a, Nanog and Sox2. The possibility to boost the natural stemness features of DPSCs and be able to determine pluripotent state by fast methods like Mass Spectroscopy, might convert actual reprogramming methods more suitable and safety for cell therapy. Canonical Wnt and Notch are two highly conserved developmental signaling pathways that are involved in pluripotent markers expression. We determine that both pathways regulate the expression of core factors but only Wnt signaling alter significantly cell cycle progression. The enhanced expression of pluripotent markers is also linked with epigenetic remodeling during reprogramming. Pharmacological inhibition of the Notch pathway by the γ-secretase inhibitor DAPT for 48 hours abolished the expression of core factors together. Conversely, pharmacological activation of the Wnt pathway, by either the GSK3-β inhibitor BIO or human

recombinant protein WNT-3A for 48 hours, largely increased the expression of core factors together with an increased cell cycle progression, DNA hipomethylation and histones acetylation and methylation. Efficiency of DPSCs to be reprogrammed could rely on epigenetic barrier, and these epigenetic studies give more information about how epigenetic barrier could be defeat for an optimal reprogramming. These results show that activation of Wnt signaling by small molecule WNT-3A enhances the stemness, cell proliferation and plastic of epigenetic barrier allowing safety methods and more efficient than precious ones and Mass Spectroscopy could be useful to optimize the therapeutic use of these and other tissue-specific stem cells.

Keywords: dental pulp stem cells, epigenetics, pluripotency core factors, methylation, acetylation, histones, reprogramming, stemness and differentiation, Notch, Wnt, BIO, DAPT, WNT-3A.

INTRODUCTION

Dental Pulp Stem Cells or DPSCs arises from neural crest (NC) to generate most craniomaxillofacial tissues (Aurrekoetxea *et al.*, 2015; Gronthos *et al.*, 2002; Gronthos *et al.*, 2000; Ibarretxe *et al.*, 2012; Janebodin *et al.*, 2011; Kaukua *et al.*, 2014; Liu *et al.*, 2015). Interestingly, DPSCs present some advantages with respect to other multipotent stem cell populations of the adult human body (Atari *et al.*, 2011; Atari *et al.*, 2012; Rosa *et al.*, 2016) such as the expression of the core pluripotent factors Oct4a, Sox2, Klf4, Lin28, SSEA1 and Nanog (Atari et al., 2012; Ferro *et al.*, 2012; Janebodin et al., 2011; Kerkis *et al.*, 2006; Rosa et al., 2016; Uribe-Etxebarria *et al.*, 2017). Those all factors are fundamental to maintaining stem cell pluripotency (Chambers and Tomlinson, 2009; Hackett and Surani, 2014; Takahashi *et al.*, 2007; Young, 2011; Yu *et al.*, 2007). The use of DPSC could also be very relevant to cell therapy because stem cells from dental tissues are known to be easily accessible for extraction, well-tolerated upon grafting due to their immune-suppressive properties (Pierdomenico *et al.*, 2005), non-tumorigenic (Wilson *et al.*, 2015) and are also suitable for autologous therapy (Ibarretxe et al., 2012; Kellner *et al.*, 2014; Wu *et al.*, 2015)**.**

Somatic cells can be reprogrammed into a pluripotent state by ectopic expression of Oct4, Sox2, Klf4 and cMyc. This suggests promising treatments for many human diseases and regeneration issues. However, this reprogramming method is not efficient at all because of the restrictions of epigenetic barrier (Polo *et al.*, 2012; Takahashi and Yamanaka, 2006). Therefore, the study of these multiple epigenetic modifications during the differentiation of stem cells could be of suitable importance. Moreover, some authors have demonstrated the higher efficiency of DPSCs compared to other stem cells for reprogramming due to the similar methylation pattern to Pluripotent Stem Cells or PSCs (Thekkeparambil Chandrabose *et al.*, 2018).

The epigenome of stem cells is slightly different from the genome of differenciated stem cells. Stem cells present a genome in euchromatic conformation whereas the genome of somatic stem cells is enriched in the heterochromatic conformation (Boyer *et al.*, 2005; David *et al.*, 2011; Wang *et al.*, 2013). Currently, it is known that DNA methylation levels are low in pluripotent stem cells both *in vitro* and *in vivo* (Nashun *et al.*, 2015; Polo et al., 2012). In PSCs acetylation weakened DNA interaction with lysines (K) of histones leading to the unfolding of chromatin and activation of gene

transcription (Tessarz and Kouzarides, 2014). Contrarily, loss of acetylation, catalyzed by histone deacteylases (HDACs), leads to heterochromatin conformation, favouring the repression of transcriptional activity (Marmorstein and Zhou, 2014). As for histone modifications, general description attributes H3K4me3 as an activator mark whereas H3K9me3 and H3K27me3 to repressive mark associated with transcriptional silencing (Zhou *et al.*, 2011).

DNA methylation profile of Dental Pulp Stem Cells is remarkably stable in human tissues. Interestingly, it has been described that an inhibitor of DNA methyltransferase diminishes global DNA methylation in Periodontal Ligament Stem Cells (PDLSCs), interestingly involving Wnt-βCatenin activation (Liu *et al.*, 2016). This experiment at same concentration was performed in DPSCs and it was shown to increment of odontogenic differentiation potential (Zhang *et al.*, 2015). Regarding other epigenetic marks, such as acetylation, it has been shown that p300, a well-known histone acetylase or HAT, seems to play an important role in maintaining stemness of DPSCs. P300 increased transcript levels of NANOG and SOX2 and maintained lower expression levels of odontoblastic differentiation markers (Wang *et al.*, 2014). Moreover, different types of histone deacetylases or HDACs such as HDAC1, HDAC2, HDAC3, HDAC4 and HDAC9 act as important accelerator of odontoblast differentiation (Jin *et al.*, 2013; Klinz *et al.*, 2012; Paino *et al.*, 2010). Histone marks in DPSCs revealed that H3K4me3 and H3K9me3 are the most prominent in DPSCs (Gopinathan *et al.*, 2013). During differentiation, early mineralization genes are enriched with H3K4me3 whereas H3K27me3 and H3K9me3 are higher in late mineralization genes (Gopinathan et al., 2013). Interestingly, one demethylase, is responsible for H3K27me3 removing which contributes to odontogenic/osteogenic differentiation of DPSCs (Hoang *et al.*, 2016). All of these results indicate an important regulatory circuit involving histone modifications that are crucial in determining cell fate in DPSCs. Despite some progress, the epigenetic code and consequent pleiotropic effects that drive DPSCs into specialized cell lineages is something little known.

The canonical Notch and Wnt signaling pathways are critical for the maintenance of the stem cell phenotype (Androutsellis-Theotokis *et al.*, 2006; Borghese *et al.*, 2010; Clevers *et al.*, 2014; Perdigoto and Bardin, 2013; Reya and Clevers, 2005) and the inhibition of differentiation in many stem cell types, including DPSCs (Mizutani *et al.*, 2007; Scheller *et al.*, 2008; Yiew *et al.*, 2017). It is known that dental stem cells present

higher levels of core factors and Wnt/Notch cross-linked activity than do other mesenchymal stem cells in the adult body (Atari et al., 2012; Huang *et al.*, 2009; Janebodin et al., 2011; Uribe-Etxebarria et al., 2017; Vasanthan *et al.*, 2015). However, the role of these pathways in the maintenance of stemness, self-renewal and epigenetics in DPSCs is still unclear. A better understanding of the chromatin marks and gene expression would lead to significant improvements in nuclear reprogramming of Induced Pluripotent Stem Cells or iPSCs, transdiferentiation, and *ex vivo* expansion of stem cells for transplant therapies. Moreover, it is important to understand how the regulation of transcription and self-renewal capacity cooperates to enable stem cells reprogramming. For this purpose, we have studied epigenetic profile of DPSCs treated with Notch and Wnt signaling modulators and identified global and histone methylation and acetylation pattern comparing to DPSCs treated with osteogenic and adipogenic differentiation media protocols.

MATERIALS AND METHODS

DPSC culture

DPSCs were isolated from human third molars obtained from healthy donors between 15 and 30 years of age by fracture and enzymatic digestion of the pulp tissue for 1h at 37 °C with 3 mg/ml collagenase (17018-029, Thermo Fisher Scientific, Boston, Massachusetts, USA) and 4 mg/ml dispase (17105-041, Thermo Fisher Scientific) followed by mechanical dissociation. The DPSCs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), L-glutamine (1 mM) and the antibiotics penicillin (100 U/ml) and streptomycin (150 µg/ml). The DPSCs could be amplified and maintained in these conditions for very long periods (> 6 months). However, to avoid cell aging issues, we only employed DPSCs that had been grown in culture for less than 3 months and had accumulated no more than 6 total passages. Comparative experiments between control and treatment conditions were always and without exception performed in parallel using DPSCs from the same donor.

Notch and Wnt pathway pharmacological modulation

To block Notch signaling, we employed DAPT ((N-[N (3, 5-diflorophenacetyl-L-alanyl)] 5-phnylglycine t-butyl ester), a γ-secretase inhibitor, (565784, Calbiochem, San Diego, California, USA), at a concentration of 2.5 µM. DAPT was added to the culture medium for 48 hours prior to the assays where DAPT-treated DPSCs were compared with DPSCs treated only with the control vehicle, 2.5 µM DMSO. To overactivate Wnt signaling, we used 2.5 µM BIO (6-bromoindirubin-3´-oxine), a GSK3β inhibitor (361550, Calbiochem), which was added to the medium for 48 hours prior to the assays. BIO-treated cells were compared with DPSCs exposed to the inactive analog MBIO (methyl-6-bromoindirubin-3´-oxine) at 2.5 µM as a corresponding control (361556, Calbiochem). WNT-3A recombinant protein (5036-WN-010, R&D Systems, Minneapolis, USA) was added to the DPSCs cultures to overactivate Wnt signaling in two concentrations: 2.5 µM and 5 µM.

Osteogenic differentiation of DPSCs

We used the following protocol to induce DPSC differentiation to mature osteoblasts: 6 µM β-glicerophosphate (G9422, Sigma-Aldrich, St. Louis, Massachusetts, USA), 10 nM dexamethasone (D4902, Sigma), and 52 nM ascorbic acid (127. 0250, Merck, Darmstadt, Germany) were added to the cell cultures in DMEM + 10 % FBS for three weeks. The DPSCs had been previously subjected to preconditioning treatment with DMSO, DAPT, MBIO or BIO for 48 h, as described. Terminal osteoblast differentiation was assessed by detection of extracellular calcified bone matrix deposits via Alizarin Red staining using 2 g/100 ml Alizarin Red S (400480250, Across Organics, Geel, Belgium) at pH 4.3. The DPSCs were fixed with 10 %formalin (F7503, Sigma) for 30 min. We then incubated the cells with Alizarin S Red for 45 min before washing the cells four times with PBS to remove any background staining. The Alizarin Red absorbance at 450 nm was quantified using a Synergy HT Multi-Mode Microplate Reader (Biotek, Winooski, Vermont, USA). Alkaline Phosphatase (ALP) staining was also used to assess osteoblast differentiation, as this enzymatic activity is present in mature bone matrix-secreting cells. We dissolved one BCIP/NBT tablet (B5655-5ATB, Sigma) in 10 ml miliQ water and added this solution to DPSCs fixed for 1 min with 10 % formalin. We then washed the cells with PBS containing 0.05 % Tween 20 (STBB3609, Sigma). ALP activity was quantified by absorbance at 405 nm with a Synergy HT Multi-Mode Microplate Reader.

Adipogenic differentiation of DPSCs

To induce adipogenic differentiation, we treated DPSC cultures with 0.5 mM IBMX (I5879, Sigma), 1 µg/ml insulin (91077C, SAFC Biosciences, St. Louis, Massachusetts, USA) and 1 µM dexamethasone (D4902, Sigma) for four weeks after a preconditioning treatment with DMSO, DAPT, MBIO or BIO for 48 h. The DPSCs were exposed to this differentiation cocktail and subsequently fixed with 10 % formalin for 10 min and then washed with PBS containing 60 % Isopropanol. Lipid droplets in mature adipocytes were detected using Oil Red staining solution, which contained 5.14 µM Oil Red Stock (O-0625, Sigma) in miliQ water. The cells were stained for 10 min, and Oil Red absorbance was measured at 490 nm using a Synergy HT Multi-Mode Microplate Reader (Biotek).

RNA extraction, conventional RT-PCR and quantitative Real-Time PCR (qPCR)

Cell pellets were frozen and stored at -80°C. Total RNA was extracted from the cells using the RNeasy Kit (74104, Qiagen, Hilden, Germany) and checked for purity by calculating the 260/280 ratio via the Nanodrop Synergy HT (Biotek). cDNA (50 ng/µl) was obtained by reverse transcription of total extracted RNA using the iScript cDNA Kit (1708890, BioRad, Hercules, CA, USA) with the following reagents: iScript reverse Transcriptase (1µl), 5x iScript Reaction Mix (4µl) and Nuclease Free water (variable) to a final volume of 20 µl. We analyzed gene expression using 1µlof cDNA(5 ng/µl) diluted in4 µl of My TaqTM Red Mix (BIO-25043, Bioline, St. Petersburg, Russia), 1 µl of primers (0.625 µM) and Nuclease Free Water for a total volume reaction of 10 µl, for conventional RT-PCR. Amplification products were separated by electrophoresis in a 2 % agarose gel. Quantitative Real-Time PCR experiments were conducted in an iCyclerMyiQ^{TM} Single-Color Real-Time PCR Detection System (BioRad, USA), using4.5 µl of Power SYBR® Green PCR Master Mix 2x (4367659, Applied Biosystems ^{TM,} Applied Biosystems, Carlsbad, CA, USA), 0.5 µl of primers (0.3125 µM), 0.3 µl of cDNA (1.5 ng/µl), and Nuclease Free water for a total volume reaction of 10 µl. All primers were obtained from public databases and checked for optimal efficiency (> 90 %) in the qPCR reaction under our experimental conditions. The relative expression of each gene was calculated using the standard $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001) normalized with respect to the average between*β-Actin* and *Gapdh* as internal controls. All reactions were performed in triplicate. qPCR was run on ABI PRISM® 7000 (Thermo Fisher Scientific, Thermo Fisher Scientific, Boston, MA, USA). Data were processed by CFX Manager™ Software (BioRad, USA). We assessed that all qPCR reactions yielded only one amplification product by the melting curve method. We used the following primer pairs for different genes obtained via the Primer-Blast method (Primer Bank):

Primers		**Sequence 5´ - 3´**	**Annealing (ºC)**	**Amplicon (bp)**
β-Actin	Upstream	GTTGTCGACGACGAGCG	58.5	93
	Downstream	GCACAGAGCCTCGCCTT	59.7	
GAPDH	Upstream	CTTTTGCGTCGCCAG	60.3	131
	Downstream	TTGATGGCAACAATATCCAC	60.8	
DNMT1	Upstream	CGTAAAGAAGAATTATCCGAGG	60.5	123
	Downstream	GTTTTCTAGACGTCCATTCAC	57.7	
DNMT3A	Upstream	GAAGAGAAGAATCCCTACAAAG	57.6	136
	Downstream	CAATAATCTCCTTGACCTTGG	60	
DNMT3B	Upstream	CTTACCTTACCATCGACCTC	57.7	167
	Downstream	ATCCTGATACTCTGAACTGTC	54.7	
NNMT	Upstream	CTGACTACTCAGACCAGAAC	53.6	113
	Downstream	TCTGTTCCCTTCAAGATCAC	59.3	
KAT8/HAC	Upstream	GAAATATGAGAAGAGCTACCG	57.2	123
	Downstream	ATCTTATGGTCTTTGCCATC	58	
SIRT1/HDAC	Upstream	AAGGAAAACTACTTCGCAAC	57.6	89
	Downstream	GGAACCATGACACTGAATTATC	59.7	
Hes1	Upstream	GGTCCTAGGGAATTGCAGC	57.9	144
	Downstream	CTCAAGATGTCCCTCAGCCT	58.8	
Lef1	Upstream	AGATCTCAATTACTGTGGGAC	57.1	88
	Downstream	GCAGGAACACTGATATTTGTC	58.7	
Lin28	Upstream	CTGGTGGAGTATTCTGTATTG	56.2	81
	Downstream	ACCTGTCTCCTTTTGATCTG	58.3	
Rex1	Upstream	TATCTCAACCTGTTCATCGAG	59.3	130
	Downstream	CCACATTCAGGTAGATGTTC	56.9	
Ssea1/Fut4	Upstream	ACAAAATCATCTGTTGGGAC	58.9	85
	Downstream	AGCAGATAAGCACTTTCAAC	56.2	

Protein extraction

The cells were washed with hand-warm PBS several times, and the proteins were lysed on ice with 200 µl Lysis Buffer (50 mM Tris-HCl pH 7.5, 1mM EDTA, 150 mM NaCl, 0.5 % sodium deoxycholate, 0.1 %SDS, 1 % IGEPAL® CA-630 in dH_2O), and Proteinase Inhibition (1:100, 539134, Calbiochem) and phosphatase inhibitor cocktail (1:100, 539134, Calbiochem). After an incubation of 5 minutes lysates were scrapped thoroughly and transferred to a prechilled 1.5 mL tube and submitted to homogenization in a Bandelin Plus sonicator using a 1, 5 MS probe. After three sonication bursts on ice of 20 seconds with 1 min of repose between each one of them at a 90 % amplitude, lysates were cleared by centrifugation at 20000 rcf 10 min at 4ºC. Supernatants were quantified using $CuSO_4$-BCA in 50:1 ratio (B9643, Sigma). BSA (A7906, Sigma) was used for performing a linear relationship with concentration and absorbance at 490nm. Samples were read in Nanodrop Synergy HT (Biotek).

Western blot (WB).

The samples with 30 µg of protein concentration were diluted in loading buffer (62,5 mM Tris-HCl pH 6, 8, 2,5 % SDS; 10 % glycerol; 5 % β-mercaptoethanol and 0,002 % bromophenol blue). After electrophoretic separation (electrophoresis bufer formulation: 25 mM Tris, pH 8, 3; 193 mM glycine, 0, 1% SDS) under constant 120 V, proteins were blotted during 3 hours (250 V max, 600 W max, constant 400 mAmps. Transfer buffer formulation: 25 mM Tris pH 8,3; 192 mM glycine; 20% methanol; 0, 1% SDS) onto 0,2 µm-pore nitrocellulose membranes using the Mini-PROTEAN tetra system and Mini Trans-Blot cell respectively fed by a PowerPac HV™ High-Current Power Supply. Once correct protein transfer was confirmed by Ponceau S protein staining, membranes were washed with TBST (10 mM Tris-HCl pH 8; 150 mM NaCl; 0,05 % Tween20) until all dye was gone and submitted to blockage using 1 % BSA diluted in TBST during 1 hour at room temperature under constant agitation. For Western Blot analyses, we used anti α-TUBULIN (1:3000, 4967, Cell Signaling), anti-H3K9me3 (1:2000, 9542S, Cell Signaling, Massachusetts, USA), anti-H3K4me3 (1:1000, ab12209, Abcam, Massachusetts, USA), anti-H3K27me3 (1:1000, ab6002, Abcam) and anti-H3AC (1:2000, 06–599, Millipore). The secondary antibodies used were: mouse IgGκ light chain binding protein HRP conjugated 1:5000 (Santa Cruz sc-516102), anti-rabbit-HRP 1:5000 (Santa Cruz sc-2357), and anti-rat-HRP 1:4000 (Santa Cruz sc-2006). The blots

were developed using the Luminata Crescendo Western HRP Substrate (Millipore WBLUR0500). Western blot images were taken in a Syngene G: BOX CHEMI XR^5 system (Syngene, Cambridge, UK). The membranes were stripped using Red Blot (M2504, Inmmobilon® EMD Millipore).

DNA extraction

Cell lyssates were done with DNA lyssis Buffer (100mM Tris-HCl, 50mM EDTA, 200mM NaCl and 0, 2% SDS) and Proteinase K (AM2546, Thermo Fisher Scientific) which was used at 100 mg/ml and samples were incubated overnight at shaking. The samples were treated with 5 µl RNAse at 10mg/ml (800-325-3010, Roche, Basilea, Switzerland), during1h at 37ºC.DNA extraction was performed using phenol-chloroform methodology with phenol (P1037, Sigma) and chloroform (288306, Sigma). After all, DNA concentration and purity was checked purity by calculating the 260/280 ratio via the Nanodrop Synergy HT (Biotek).

Quantification of DNA methylation by Mass Spectroscopy

The, DNA was lead to hydrolysis and DNA hydrolysis samples (10 µl typically containing 50 ng of digested DNA) were run in reverse phase UPLC column (Eclipse C18 2.1 × 50 mm, 1.8 µ particle size, Agilent) equilibrated and eluted (100 µl/min) with water/methanol/formic acid (95/5/0.1, all by volume). The effluent from the column was added to an electrospray ion source (Agilent Jet Stream) connected to a triple quadrupole Mass Spectrometer (Agilent 6460 QQQ). The machine had operated in the positive ion multiple reaction monitoring mode using previously optimized conditions, and the intensity of specific MH+→fragment ion transitions were measured and recorded (5mC m/z 242.1→126.1, 5hC 258.1→142.1 and dC m/z 228.1→112.1). The measured percentage of 5mC in each experimental sample was calculated from the MRM peak area divided by the combined peak areas for 5mC plus 5hmC plus C (total cytosine pool).

Cell Cycle phase determination

Cells were trypsined and dilute in ethanol 100%. Determination of cell cycle was assessed using Propidium Iodide0.5mg/ml (P4170, Sigma) and Ribonuclease RNAse 10 µg/ml (R4642, Sigma). Samples were read using CytoFLEX Flow Cytometer (Beckman

Coulter, California, USA) and analyzed with Kaluza G for Gallios Acquisition Software (Beckman Coulter).

Statistical analyses

Statistical analyses were performed with Excel, IBM SPSS Statistics v.9 (SPSS, Chicago, IL, USA) and Graph Pad v.6 software (Graph Pad Inc., USA). All data sets were subjected to a Kolmogorov-Smirnov normality test prior to analysis. For small sample sizes non-parametric tests were chosen by default. Comparisons between only two groups were made using U-Mann Whitney test.

RESULTS

Wnt activity is required for the expression of essential core pluripotency factors in DPSCs

We culture DPSCs in DMSO, DAPT, MBIO, BIO and WNT-3A treatment conditions for 48 hours. Indeed, we exposed DPSCs to different well-established osteogenic and adipogenic differentiation media protocols. After these treatments, we did not detect differentiation either in shape or in morphology. As positive control of differentiation, established *in vitro* protocols can be used to induce terminal differentiation of DPSCs to mature osteoblasts and adipocytes (Gronthos et al., 2002; Langenbach and Handschel, 2013). After cells exposition to osteoblastic differentiation medium during 3 weeks, we verified formation of calcium deposit by Alizarin S Red staining (Figure 1A). After 4 weeks of adipogenic differentiation, we performed Oil Red staining to ensure lipid droplet formation (Fig.ure 1A). Real Time RT-PCR analysis revealed that As for DAPT-treated cells suffered a significant decrease in the gene expression of pluripotent core factors *c-MYC, SOX2, OCT4* and *NANOG*. Furthermore, in some cases such as *SOX2, OCT4* and *NANOG* gene expression, it was decreased by more than 50 % with respect to the control conditions (DMSO, MBIO) (Figure 1C). After the induction with differentiation protocols mentioned before, we confirmed strong decrease in the expression of pluripotent core factors. Interestingly, in cells treated with WNT-3A and BIO, the relative gene expression of core factors *c-MYC, SOX2, OCT4* and *NANOG* was upregulated (Figure 1C).

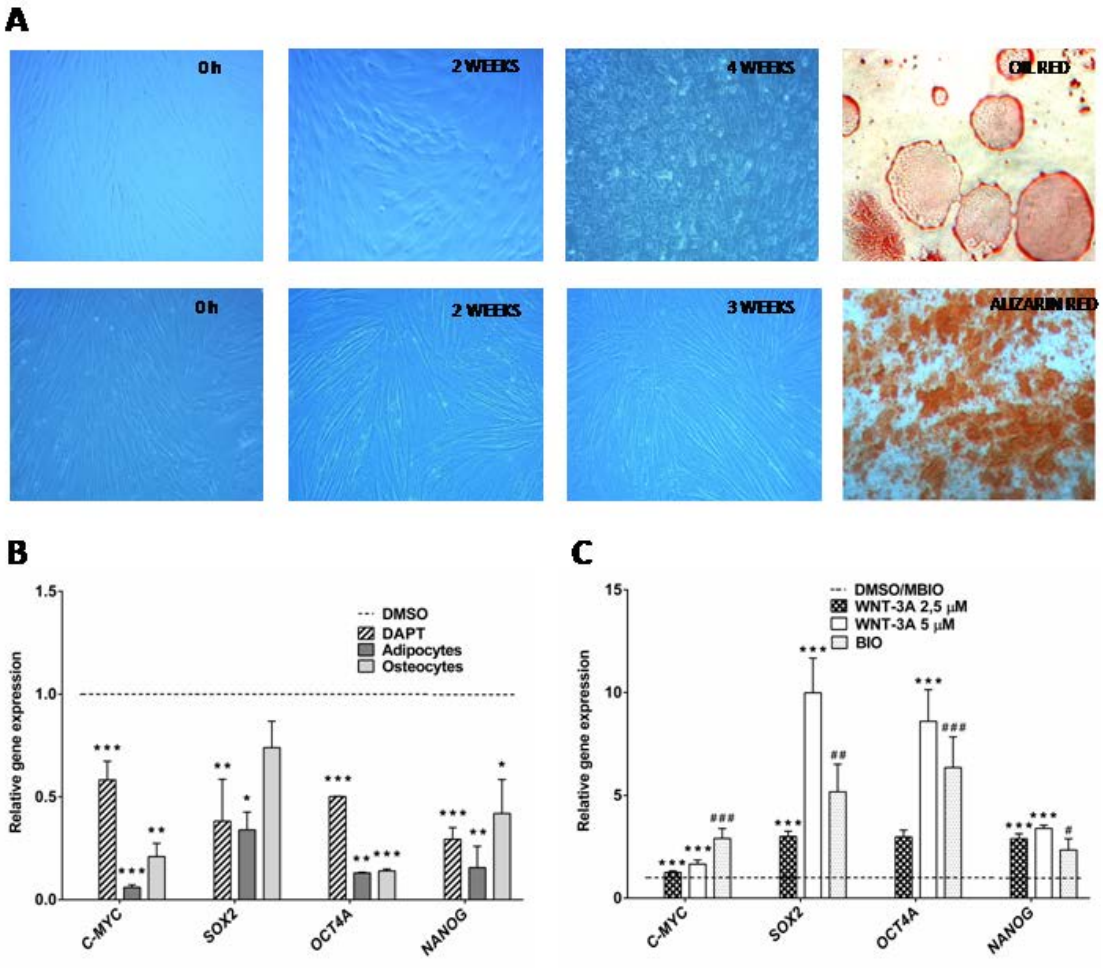


Figure 1. Enhancement of stemness and differentiation of DPSC by DAPT, BIO, WNT-3A, osteinduction and adipoinduction media. (A): Phase Contrast Microscopy and Alizarin S Red and Oil Red staining showed clear differences in morphology during the treatments with osteoinduction and adipoinduction media. Scale bar=100µm **(C):** Q-PCR analysis confirmed differences in the expression of core pluripotent factors *c-Myc*, *Sox2*, *Oct4a* and *Nanog* transcripts between the control (DMSO, MBIO) and DAPT, BIO, WNT-3A and differentiation media conditions. Data are normalized to reference *β-ACTIN* and *GAPDH* levels and presented as the mean +SEM (n=3). The dashed line represents normalized gene expression to control conditions. *: p<0.05;**: p<0.01;***: *p*<0.001. U-Mann Whitney test.

Notch and Wnt/β-Catenin signaling affected cell cycle and its regulation in DPSC

Notch and Wnt signaling both activation and inactivation, showed to alter the ratio of DPSCs proliferation (Uribe-Etxebarria et al., 2017). To confirm this data, we studied G0/G1, S and G2/M cell cycle phases after Notch and Wnt treatments. Data compiled from DPSCs in passage 5, showed the % of number cells in G0/G1, S and G2/M where each graph made a comparison between treatment and its respective control (Figure 1A-C). Bar graph showing DPSC control cells (DMSO, MBIO) compare to WNT-3A and BIO - treated cells at low passages (P3-P5) (Figure 1D). Surprisingly, data obtained by Flow Cytometry did not show significant changes after DAPT treatment. Interestingly,

BIO and WNT-3A-treated cells showed 7% and 4% lower of cells in G1/G0 phase respectively (Figure 1D). To obtained more data about cell cycle, we studied the gene expression of *CYCLIN D*, a key regulator of G0-G1, G1-S and S-G2/M phases, after DAPT, BIO and WNT-3A exposure. *CYCLIN D* transcription decreased significantly in DAPT-treated cells, whereas cells treated with WNT-3A and BIO expressed higher levels of *CYCLIN D* respect to DMSO and MBIO (Figure 1E-F). Consistently, DPSC treated with the two differentiation culture media showed lower gene expression of *CYCLIN D* by 80-90 % of their respective control values (Figure 1E).

Wnt signaling activation by WNT-3A altered methylases and demehtylases gene expression and leads to DNA hyper-methylation of DPSCs.

To investigate whether Notch and Wnt signaling would also control epigenetic profile of DPSC, we studied DNA methylation pattern by high resolution Mass Spectroscopy after these treatments. Then, we observed higher significant % 5mC in osteoinduction (2. 713 ± 0.025: $p<0.01$) and adipoinduction (2.812 ± 0.0148: $p<0.01$) media respect to control DMSO (2.412 ± 0.058) (Figure 3. A). Interestingly, cells treated with WNT-3A cells diminished % 5mC (2.168 ± 0.098: $p<0.05$) whereas we did not show significant changes in BIO (2.228 ± 0.143) respect to control DMSO (2,633 ± 0.062) and MBIO (2.215 ±0.108) (Figure 3. B). Real-Time RT-PCR determined that DAPT-treated cells increased transcript levels of methylases DNMT1, DNMT3A AND DNMT3B. Consistently, similar results were obtained when cells were exposed to osteoinduction and adipoinduction media (Figure 3C). Regarding to Wnt activators WNT-3A and BIO, the gene expression of DNMT1 was lower respect to controls (DMSO, MBIO) (Figure 3D). Surprisingly, DNMT3A and DNMT3B were higher to controls DMSO and MBIO (Figure 3B).

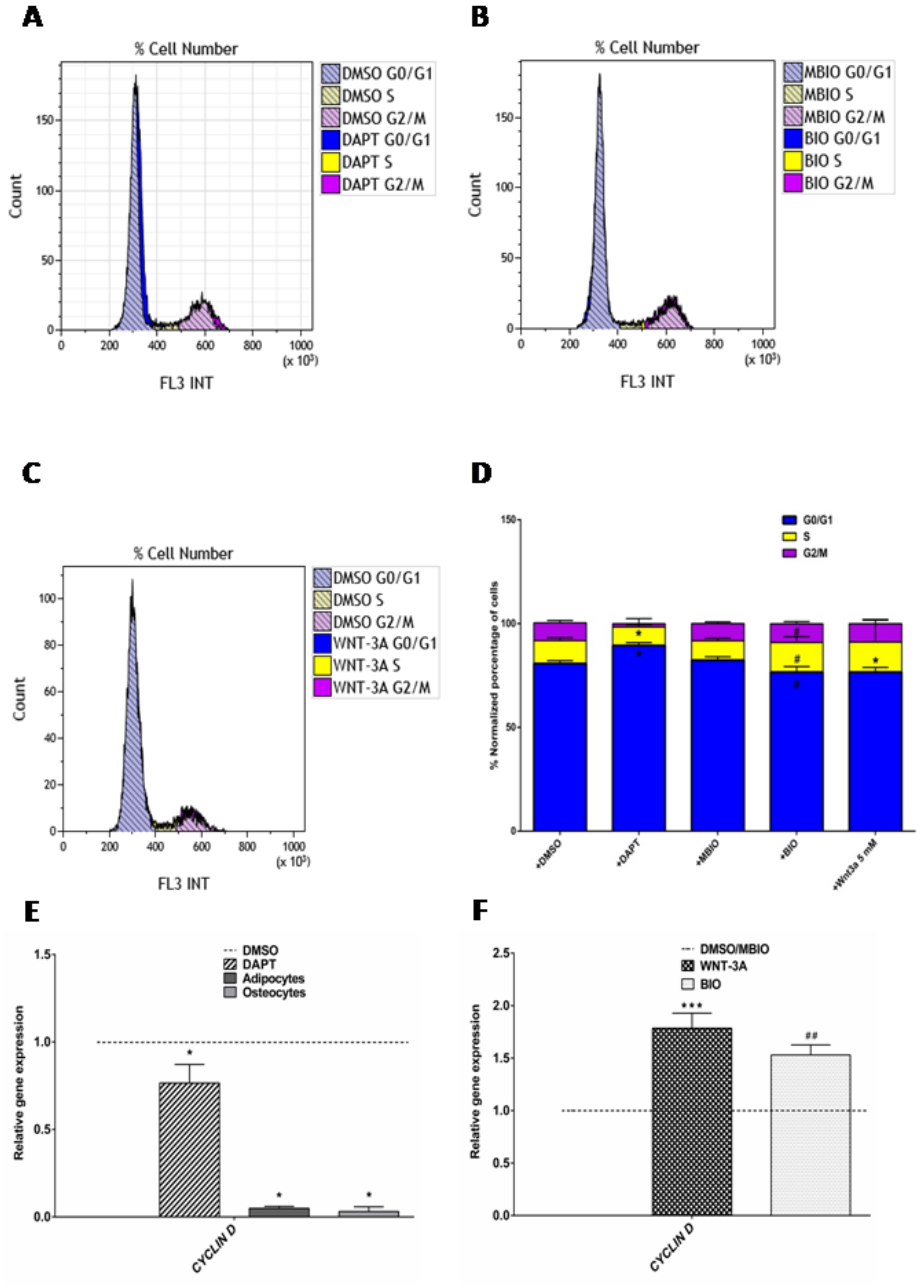


Figure 2. Notch/Wnt treatment effect in cell cycle progression in DPSCs. (A, B, C): Flow Cytometry analysis of cell cycle revealed some differences between the different treatments (DMSO, DAPT, MBIO, BIO, WNT-3A). **(D):** Representation of all cell cycle phase G1/G0, S, G2/M revealed small but significant differences between the different conditions. Data are presented as the mean +SEM (n=8). **(e)** Q-PCR analysis with cells treated with DAPT and induction media conditions confirmed a decrease in *Cyclin D* respect to the control (DMSO). **(f)** Q-PCR analysis showed higher expression of *Cyclin D* in BIO and WNT-3A-treated cells when compared to the control (MBIO). Data are normalized to reference *β-Actin* and *GAPDH* levels and presented as the mean +SEM (n=3). The dashed line represents normalized gene expression in control conditions. *: p<0.05;**: p<0.01;***: *p*<0.001. U-Mann Whitney test.

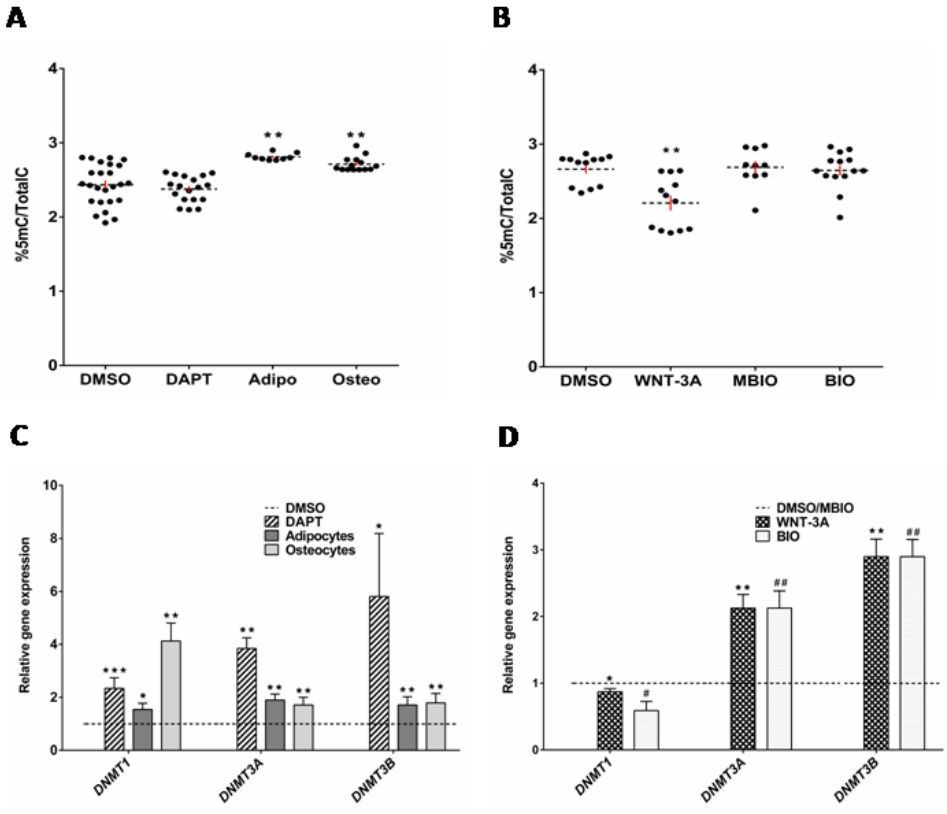


Figure 3. Notch and Wnt signaling pathways control DNA methylation and demethylation of DPSCs. (A, B): Q-PCR showing relative differences in *Dnmt1, Dnmt3a and Dnmt3b* expression between control (DMSO, MBIO) and DAPT, BIO, WNT-3A, Adipoinduction and osteoinduction media. Data are normalized to reference *β-Actin* and *GAPDH* levels and represented as the mean +SEM (n=3). **(C, D):** DNA hypermethylation patterns showing % 5mC in control (DMSO, MBIO) respect to DAPT, BIO, WNT-3A and Osteoinduction media. Data are presented as the mean +SEM (n=8). *: $p<0.05$;**: $p<0.01$;***: $p<0.001$. U-Mann Whitney test.

Wnt activation modifies acetylation pattern.

Then we wondered to know whether histone acetylation profile would also be affected in these conditions. We assessed protein expression by WB and Acetyl-Histone H3 was higher WNT-3A and BIO-treated cells compare to controls (DMSO, MBIO) (Figure 4 C). To confirm these data we assessed Real-Time PCR and DAPT-treated DPSCs had lower gene expression of acetylase *HAT/KAT8* and higher de-acetylase *HDAC/SIRT1* compare to control (DMSO) (Figure 4. A). Consistently, differentiation media increased gene expression of *HDAC/SIRT1*; approximately, two times, whereas *HAT/KAT8* was clearly downregulated (Figure 4A). Contrarily, when cells were exposed to WNT-3A and BIO, *HAT/KAT8* highly increased their expression (Figure 4B), and the *HDAC/SIRT* gene expression was not significant affected (Figure 4C). We have studied also Nicotinamide N-methyltransferase or NNMT, which clearly is downregulated in

DAPT, and upregulated in two fold change BIO and WNT-3A almost 10 fold change respect to control (DMSO, BIO) (Figure 4 D, E).

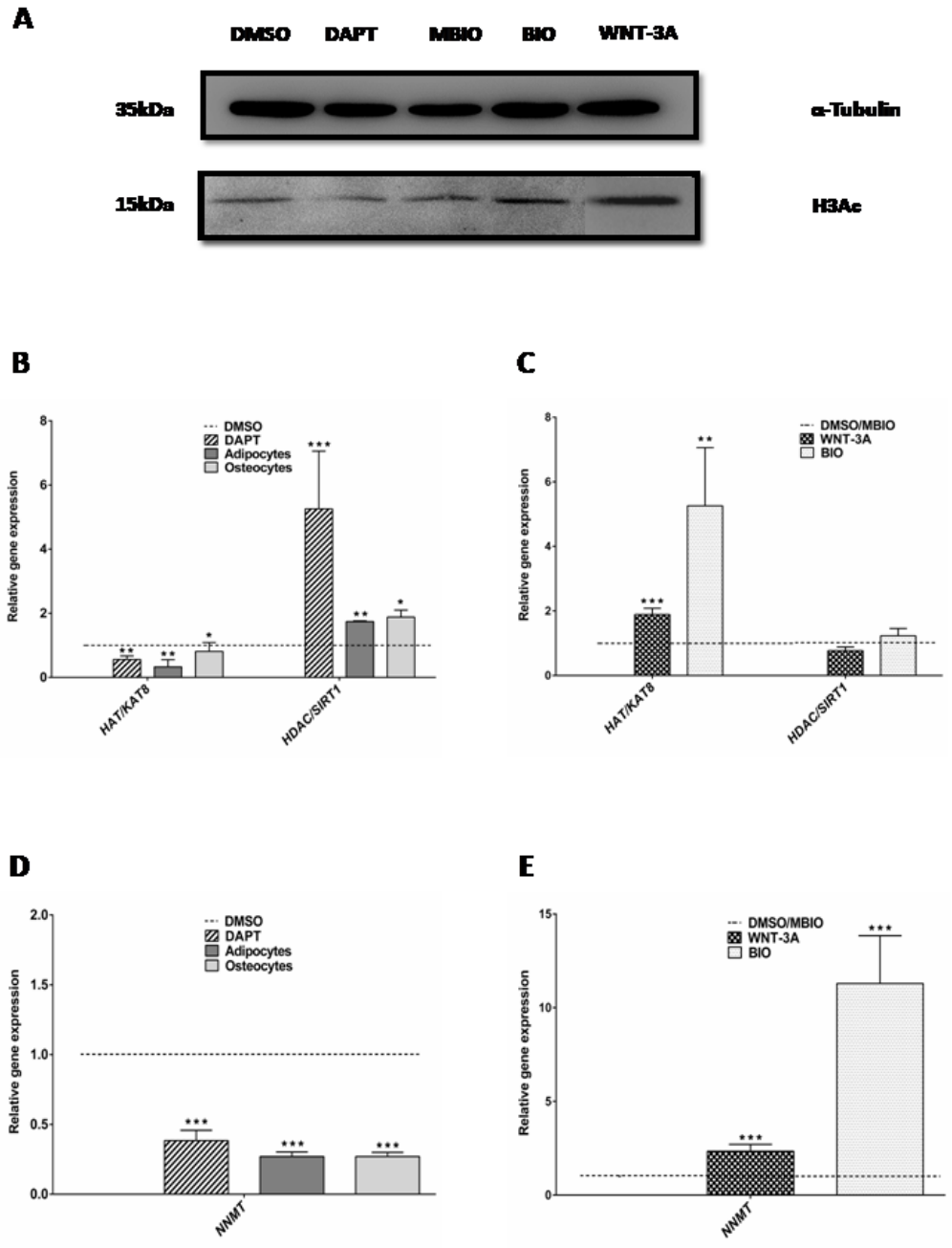


Figure 4. Notch and Wnt signaling affects DNA acetylation. (A, B): Q-PCR showing relative differences in *Hat/Kat8, Hdac/Sirt1 and Hes1* expression between control (DMSO, MBIO) and DAPT, BIO, WNT-3A, Adipoinduction and osteoinduction media. Data are normalized to reference *β-Actin* and *GAPDH* levels and represented as the mean + SEM (n=3). **(C)** Representative WB showing an increase in H3AC. WNT-3A-treated DPSCs respect to control (DMSO, MBIO), while no changes were observed in DAPT. α-TUBULIN was used as a protein loading control.*: $p<0.05$;**: $p<0.01$;***: $p<0.001$. U-Mann Whitney test.

Wnt activation modifies Histone H3 methylation pattern.

Immunoblotting analysis was assessed to determine if Histones H3 changed epigenetic tri-methylation. Firstly, we studied the most common Histones H3 such as H3K4me3, H3K27me3 and H3K9me3, protein expression profiles showed an increase in methylation in H3K4me3, H3K27me3 and H3K9me3 in BIO and WNT-3A (Figure. 5A). Regarding DAPT, no changes were detected in cells trated with γ-secretase inhibitor. Then, we analyzed, the gene expression of their catalytic enzymes subunits MLL (H3K4me3), EZH2 (H3K27me3) and EHMT2 (H3K9me3). No changes were observed in DAPT (Figure 5B). Regarding to WNT-3A, gene expression of MLL, EZH2, and EHMT2 was higher compare to control DMSO (Figure 5C).

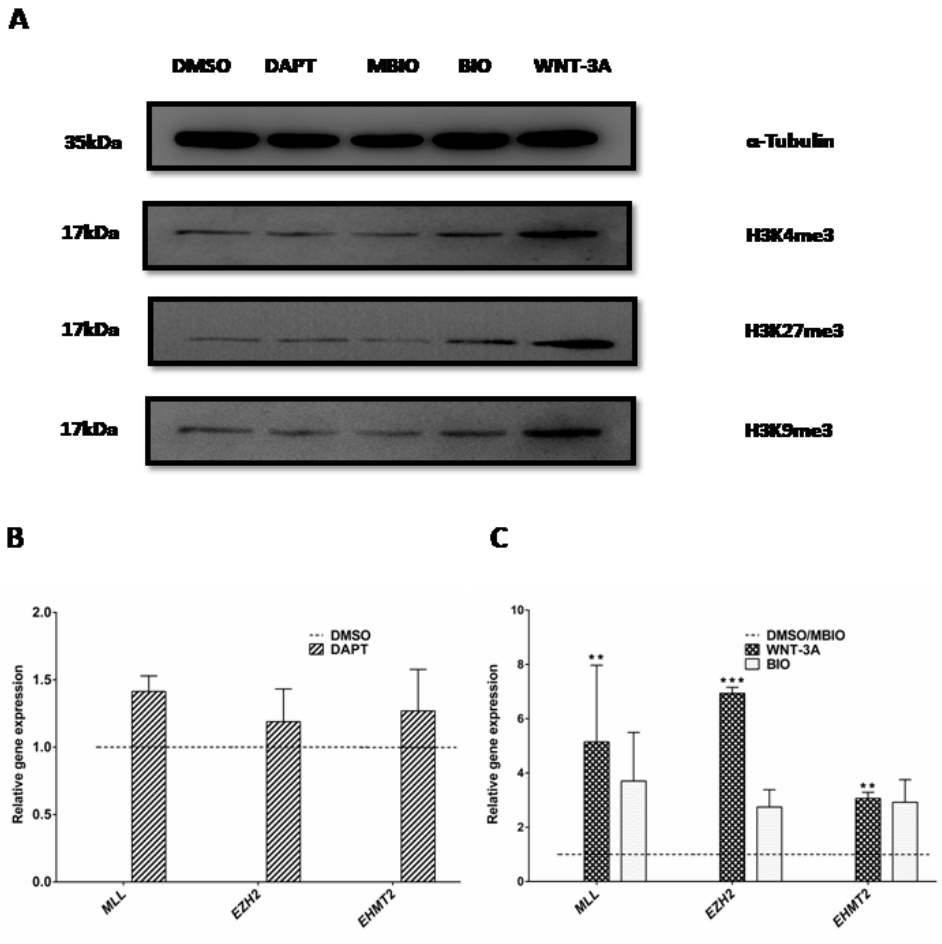


Figure 5. Notch/Wnt alteration affects histone H3 methylation in DPSCs. (**A, B, C):** Q-PCR showing relative differences in expression of catalytic enzymes of H3K4me3 (Mll), H3K27me3 (Ezh2), and H3K9me3 (Ehmt2) in DMSO, BIO and WNT-3A treatment for 48 h. Data are normalized to reference *β-ACTIN* and *GAPDH* levels and represented as the mean +SEM (n=3). **(D):** WB showing methylated H3K4me3, H3K27me3, and H3K9me3. α-TUBULIN was used as a protein loading control. *: p<0.05;**: p<0.01;***: p<0.001. U-Mann Whitney test.

DISCUSSION

Several studies have described canonical Notch and Wnt signaling pathways as pivotal regulators of stemness and pluripotency (Cartwright *et al.*, 2005; Clevers et al., 2014; Dravid *et al.*, 2005; Fox *et al.*, 2008; Li and Chen, 2012; Lowell *et al.*, 2006; Lluis *et al.*, 2008; Park *et al.*, 2008; Simandi *et al.*, 2016; Yan *et al.*, 2010), and their pharmacological manipulation has already been tested as a strategy to enhance either cell differentiation (Kitajima *et al.*, 2016) or cell reprogramming (Ichida *et al.*, 2014; Uribe-Etxebarria et al., 2017).

Notch inhibition by γ-secretase blockers has been described to enhance the efficiency of keratinocyte reprogramming (Ichida et al., 2014). In our DPSC model, however, Notch inhibition by DAPT decreased the expression of pluripotency core factors and did not altered significantly self-renewal although γ-secretase inhibitor is still used as an antitumour treatment (Dai *et al.*, 2018). Moreover, Notch effects have long been known to be extremely context-dependent; the same Notch signal can have very different outcomes depending on the cell type, physiological state, and extracellular environment. Regarding Wnt signaling pharmacological activation, mouse iPSCs have been associated with increased cell differentiation (Kitajima et al., 2016) Nevertheless, in our model system, BIO and WNT-3A application significantly increased the expression of core factors, increased self-renewal and altered epigenetics marks which could enhance reprogramming efficiency. Other authors have tested Wnt reprogramming properties in PLDSCs and DPSCs altering DNA methylation (Liu et al., 2016; Zhang et al., 2015). Again, we attribute these differences to the various cell culture systems that were tested.

The pluripotent network consists of a core set of transcription factors, including Oct4 (Pou5f1), Sox2, and Nanog, which serve to establish the undifferentiated state and the self-renewing capacity of pluripotent stem cells (Boyer et al., 2005). This network interacts with the cell cycle machinery. For example, cMyc acts as the regulator of many cell cycle genes (Kim *et al.*, 2008; Kim *et al.*, 2010) and as a target of Oct4 and Nanog is crucial to the maintenance of pluripotency network (Cartwright et al., 2005; Hishida *et al.*, 2011; Loh *et al.*, 2006). It is widely described cell cycle arrest when cells are treated with DAPT (Ferrari-Toninelli *et al.*, 2010). However, DPSCs did not suffer significant changes when expose to DAPT, giving a clue about a quiescent state. In PSCs cell cycle is defined by a rapid progression through the cell cycle and a minimal

time spent in G1, similar to ESCs (Coronado *et al.*, 2013; Ghule *et al.*, 2011; Li and Kirschner, 2014). Several studies have shown that enriching for proliferating cells enhances reprogramming (Guo *et al.*, 2014; Roccio *et al.*, 2013; Ruiz *et al.*, 2011).In DPSCs treated with BIO and WNT3-A, we did not show more than 7% of change in G1/G0 phase cell cycle compare to controls DMSO and MBIO. But interestingly, this tendency of less % of cells G1/G0 phase and more into cell cycle progression was maintaining after numerous experiments and explains results obtained in studies before regarding cell proliferation (Uribe-Etxebarria et al., 2017). One explanation of this could be non-tumorigencing DPSCs properties that could let them the chance to maintain cell cycle less affected (Wilson et al., 2015). Several studies place Cyclins as fundamental to proper reprogramming (Abe et al., 2012; Edel et al., 2010; McLenachan et al., 2012). On the other hand, CyclinD, one of the most studied cyclins because of its importance in G1 phase length regulation (Dong et al., 2018), is clearly upregulated in BIO and WNT-3A-treated DPSCs although cell cycle is not highly affected. More studies about this would explain why DPSCs are able to control cell cycle machinery despite pluripotency network upregulation.

Cell reprogramming and cell differentiation is accompanied by drastic changes in the epigenetic patterns. The epigenetic changes that occur during somatic cell reprogramming are also distinct from the epigenetic changes required to maintain pluripotency. Whereas somatic cell reprogramming involves global DNA demethylation and a complex remodeling of histone modifications, pluripotency maintenance requires an enrichment of histone acetylation to maintain chromatin plasticity, relative to differentiated cells (Mattout and Meshorer, 2010).

Several studies associate epigenetic marks with odontogenic differentiation (Gopinathan et al., 2013; Huynh *et al.*, 2017; Lesot *et al.*, 2001; Zhou *et al.*, 2018). In oral tissues such as DPLCs and DPSCs the inhibition of methylases leaded to reprogramming of cells and also was observed a high Wnt-βCatenin activity (Liu et al., 2016; Zhang et al., 2015). This is consistent with our results in which we observed a DNA demethylation in WNT-3A treated cells, Analyzing gene expression of three different methylases we observed that DNMT1 is down-regulated in BIO and WNT-3A treated cells. *DNMT1* binds to hemi-methylated DNA (DNA with only one stand methylated), at CpG sites and is expressed abundantly in adult cells (Gnyszka *et al.*, 2013; Robertson *et al.*, 1999). Although, the gene expression of DNMT3A and DNMT3B are up-regulated after BIO

and WNT-3A treatment, it is known that both enzymes are required for the genome-wide de novo methylation of DNA, less abundant in adult cells than *DNMT1* (Okano *et al.*, 1998), therefore less important for somatic cell reprogramming.

As for acetylation pattern, the deacetylase SIRT1 have been involved through Wnt/β-Catenin in odontogenic differentiation (Feng *et al.*, 2016). Similarly, according to other authors, increment of histone deacetylases (HDACs) enhances odontodifferentiation and their use could be a strategy with promising potential for regenerative endodontic therapy (Jin et al., 2013; Klinz et al., 2012; Paino *et al.*, 2014; Zhang et al., 2015). In our system in BIO and WNT3-A treated cells acetylation in H3 is higher respect to control. This was corresponded to gene expression results with lower levels of SIRT1 and higher levels of the acetylase HAT/KAT8. No changes were observed in acetylation of H3 in cells treated with DAPT, although SIRT1 and HAT/KAT8 are altered. In our system in DAPT-treated cells, SIRT1 is upregulated whereas not significant changes were observed in BIO and WNT-3A. Interestingly, SIRT1 gene expression is positive regulated by Nicotinamide N-methyltransferase or NNMT (You *et al.*, 2018) an enzyme that is crucial in One Carbon Metabolism transforming SAM into SAH (Xu *et al.*, 2016). In cells treated with DAPT, we observed less gene expression of NNMT whereas BIO and WNT-3A would overexpress NNMT in DPSCs. Although SIRT1 is not affected in BIO and WNT-3A, levels of NNMT are upregulated, indicating that metabolism could play an essential role in epigenetics. Moreover, this step is crucial in metabolism because SIRT1 depends on NAD pool provided by metabolic process involved in energy production such as lipid anabolism, gluconeogenesis, and mitochondrial respiration (Canto and Auwerx, 2012). More studies would be necessary to understand this complex network between metabolism and epigenetics.

Some studies have shown that during these process of remodelling cells in PSCs usually had high levels of methylated H3K4, combined with H3K27 trimethylation. H3K27 methylation is functionally important for preventing genes expression in ES cells whereas H3K4 methylation acts as activator mark for gene transcription (Azuara *et al.*, 2006; Boyer *et al.*, 2006). Histone marks in DPSCs revealed that H3K4me3, H3K9me3 and H3K27me3 become more important during the different stages of differentiation (Gopinathan et al., 2013). In our treated-DPSCs methylation of these histones became more important when cells were exposed to BIO and WNT-3A. H3K4, H3K27 and H3K9 tri-methylation is higher although the strongest effect is produced in cells treated

with WNT-3A. Involving catalytic subunits of key enzymatic regulators of these histones methylation (*MLL*, *EZH2* and *EHMT2*), in BIO and WNT-3A seemed to be upregulated. One explanation to this could be that this type of methylation represents the global state of histones H3 and depend on the genes that are silenced by H3K9me3 and H3K27me3 and the genes that remain active through H3K4me3. Moreover, H3K4me3 and H3K27me3 epigenetic markers could be bound to bivalent domain that could control gene activation and gene repression of specific genes (Bernstein et al., 2006). Nicotinamide N-methyl transferase (NNMT), through EZH2 catalytic enzyme, regulates H3K27me3 repressive marks (Sperber et al., 2015). In our system, H3K27me3 marks increase in Wnt overactivation.

In this article, we hypothesized that epigenetic remodelling during Wnt activation with the recombinant protein WNT-3A, did not only involve histone modifications and DNA methylation, this overview gives more clues about how reprogramming, pluripotency and epigenetics cooperates. Our results demonstrate that Wnt activation induces DPSCs to increase their stemness, enhances proliferation and remodels epigenetics. This could have important implications to optimize the clinical use of these cells. The fact that treatment with WNT-3A change epigenetic barrier gives a clue on how pluripotent network cooperates with cell cycle, global methylation of DNA and Histone methylation and acetylation. This is a very important finding because it proves that epigenetic barrier is essential to DPSCs reprogramming and it allows developing reprogramming strategies more efficient and safe to cell therapy.

CONCLUSION

Our data show that Notch and Wnt signaling are required for maintenance of core pluripotency factor expression in DPSCs. Wnt signaling activation by WNT-3A for 48h significantly enhanced the cell cycle progression and remodels epigenetic barrier by leading to less hyper-methylated DNA pattern, more acetylation and histone modifications such as H3K4me3, H3K27me3 and H3K9me3 which seem to allow all together DPSCs reprogramming. Conversely, Notch inhibition decreased stemness factors of DPSCs but did not alter cell cycle or epigenetic machinery in a functional way. These changes that involve less levels expression of core did not correlated with an enhanced differentiation potential of DPSC. Epigenetic study could be used alone or in conjunction with other methods to ensure cell reprogramming efficiency and safety allowing cell use for cell therapy.

REFERENCES

Abe S, Hamada K, Miura M, Yamaguchi S (2012) Neural crest stem cell property of apical pulp cells derived from human developing tooth. Cell biology international, 36:927-936.

Androutsellis-Theotokis A, Leker RR, Soldner F, et al. (2006) Notch signalling regulates stem cell numbers in vitro and in vivo. Nature, 442:823-826.

Atari M, Barajas M, Hernandez-Alfaro F, et al. (2011) Isolation of pluripotent stem cells from human third molar dental pulp. Histology and histopathology, 26:1057-1070.

Atari M, Gil-Recio C, Fabregat M, et al. (2012) Dental pulp of the third molar: a new source of pluripotent-like stem cells. Journal of cell science, 125:3343-3356.

Aurrekoetxea M, Garcia-Gallastegui P, Irastorza I, et al. (2015) Dental pulp stem cells as a multifaceted tool for bioengineering and the regeneration of craniomaxillofacial tissues. Frontiers in physiology, 6:289.

Azuara V, Perry P, Sauer S, et al. (2006) Chromatin signatures of pluripotent cell lines. Nat Cell Biol, 8:532-538.

Bernstein BE, Mikkelsen TS, Xie X, et al. (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell, 125:315-326.

Borghese L, Dolezalova D, Opitz T, et al. (2010) Inhibition of notch signaling in human embryonic stem cell-derived neural stem cells delays G1/S phase transition and accelerates neuronal differentiation in vitro and in vivo. Stem cells, 28:955-964.

Boyer LA, Lee TI, Cole MF, et al. (2005) Core transcriptional regulatory circuitry in human embryonic stem cells. Cell, 122:947-956.

Boyer LA, Plath K, Zeitlinger J, et al. (2006) Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature, 441:349-353.

Canto C, Auwerx J (2012) Targeting sirtuin 1 to improve metabolism: all you need is NAD(+)? Pharmacological reviews, 64:166-187.

Cartwright P, McLean C, Sheppard A, Rivett D, Jones K, Dalton S (2005) LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. Development, 132:885-896.

Clevers H, Loh KM, Nusse R (2014) Stem cell signaling. An integral program for tissue renewal and regeneration: Wnt signaling and stem cell control. Science (New York, N.Y.), 346:1248012.

Coronado D, Godet M, Bourillot PY, et al. (2013) A short G1 phase is an intrinsic determinant of naive embryonic stem cell pluripotency. Stem Cell Res, 10:118-131.

Chambers I, Tomlinson SR (2009) The transcriptional foundation of pluripotency. Development (Cambridge, England), 136:2311-2322.

Dai G, Deng S, Guo W, et al. (2018) Notch Pathway Inhibition Using DAPT, a gamma-secretase inhibitor (GSI), Enhances the Antitumor Effect of Cisplatin in Resistant Osteosarcoma. Molecular carcinogenesis.

David L, Samavarchi-Tehrani P, Golipour A, Wrana JL (2011) Looking into the black box: insights into the mechanisms of somatic cell reprogramming. Genes, 2:81-106.

Dong P, Zhang C, Parker BT, You L, Mathey-Prevot B (2018) Cyclin D/CDK4/6 activity controls G1 length in mammalian cells. PLoS One, 13:e0185637.

Dravid G, Ye Z, Hammond H, et al. (2005) Defining the role of Wnt/beta-catenin signaling in the survival, proliferation, and self-renewal of human embryonic stem cells. Stem cells, 23:1489-1501.

Edel MJ, Menchon C, Menendez S, Consiglio A, Raya A, Izpisua Belmonte JC (2010) Rem2 GTPase maintains survival of human embryonic stem cells as well as enhancing reprogramming by regulating p53 and cyclin D1. Genes Dev, 24:561-573.

Feng G, Zheng K, Song D, et al. (2016) SIRT1 was involved in TNF-alpha-promoted osteogenic differentiation of human DPSCs through Wnt/beta-catenin signal. In vitro cellular & developmental biology. Animal, 52:1001-1011.

Ferrari-Toninelli G, Bonini SA, Uberti D, et al. (2010) Targeting Notch pathway induces growth inhibition and differentiation of neuroblastoma cells. Neuro-oncology, 12:1231-1243.

Ferro F, Spelat R, D'Aurizio F, et al. (2012) Dental pulp stem cells differentiation reveals new insights in Oct4A dynamics. PloS one, 7:e41774.

Fox V, Gokhale PJ, Walsh JR, Matin M, Jones M, Andrews PW (2008) Cell-cell signaling through NOTCH regulates human embryonic stem cell proliferation. Stem cells, 26:715-723.

Ghule PN, Medina R, Lengner CJ, et al. (2011) Reprogramming the pluripotent cell cycle: restoration of an abbreviated G1 phase in human induced pluripotent stem (iPS) cells. J Cell Physiol, 226:1149-1156.

Gnyszka A, Jastrzebski Z, Flis S (2013) DNA methyltransferase inhibitors and their emerging role in epigenetic therapy of cancer. Anticancer research, 33:2989-2996.

Gopinathan G, Kolokythas A, Luan X, Diekwisch TG (2013) Epigenetic marks define the lineage and differentiation potential of two distinct neural crest-derived intermediate odontogenic progenitor populations. Stem Cells Dev, 22:1763-1778.

Gronthos S, Brahim J, Li W, et al. (2002) Stem cell properties of human dental pulp stem cells. J Dent Res, 81:531-535.

Gronthos S, Mankani M, Brahim J, Robey PG, Shi S (2000) Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proceedings of the National Academy of Sciences of the United States of America, 97:13625-13630.

Guo S, Zi X, Schulz VP, et al. (2014) Nonstochastic reprogramming from a privileged somatic cell state. Cell, 156:649-662.

Hackett JA, Surani MA (2014) Regulatory principles of pluripotency: from the ground state up. Cell Stem Cell, 15:416-430.

Hishida T, Nozaki Y, Nakachi Y, et al. (2011) Indefinite self-renewal of ESCs through Myc/Max transcriptional complex-independent mechanisms. Cell Stem Cell, 9:37-49.

Hoang M, Kim JJ, Kim Y, et al. (2016) Alcohol-induced suppression of KDM6B dysregulates the mineralization potential in dental pulp stem cells. Stem Cell Res, 17:111-121.

Huang GT, Gronthos S, Shi S (2009) Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. Journal of dental research, 88:792-806.

Huynh NC, Everts V, Ampornaramveth RS (2017) Histone deacetylases and their roles in mineralized tissue regeneration. Bone reports, 7:33-40.

Ibarretxe G, Crende O, Aurrekoetxea M, Garcia-Murga V, Etxaniz J, Unda F (2012) Neural crest stem cells from dental tissues: a new hope for dental and neural regeneration. Stem cells international, 2012:103503.

Ichida JK, Tcw J, Williams LA, et al. (2014) Notch inhibition allows oncogene-independent generation of iPS cells. Nature chemical biology, 10:632-639.

Janebodin K, Horst OV, Ieronimakis N, et al. (2011) Isolation and characterization of neural crest-derived stem cells from dental pulp of neonatal mice. PloS one, 6:e27526.

Jin H, Park JY, Choi H, Choung PH (2013) HDAC inhibitor trichostatin A promotes proliferation and odontoblast differentiation of human dental pulp stem cells. Tissue engineering. Part A, 19:613-624.

Kaukua N, Shahidi MK, Konstantinidou C, et al. (2014) Glial origin of mesenchymal stem cells in a tooth model system. Nature, 513:551-554.

Kellner M, Steindorff MM, Strempel JF, Winkel A, Kuhnel MP, Stiesch M (2014) Differences of isolated dental stem cells dependent on donor age and consequences for autologous tooth replacement. Archives of Oral Biology, 59:559-567.

Kerkis I, Kerkis A, Dozortsev D, et al. (2006) Isolation and characterization of a population of immature dental pulp stem cells expressing OCT-4 and other embryonic stem cell markers. Cells, tissues, organs, 184:105-116.

Kim J, Chu J, Shen X, Wang J, Orkin SH (2008) An extended transcriptional network for pluripotency of embryonic stem cells. Cell, 132:1049-1061.

Kim J, Woo AJ, Chu J, et al. (2010) A Myc network accounts for similarities between embryonic stem and cancer cell transcription programs. Cell, 143:313-324.

Kitajima K, Nakajima M, Kanokoda M, et al. (2016) GSK3beta inhibition activates the CDX/HOX pathway and promotes hemogenic endothelial progenitor differentiation from human pluripotent stem cells. Experimental hematology, 44:68-74.e61-10.

Klinz FJ, Korkmaz Y, Bloch W, Raab WH, Addicks K (2012) Histone deacetylases 2 and 9 are coexpressed and nuclear localized in human molar odontoblasts in vivo. Histochemistry and cell biology, 137:697-702.

Langenbach F, Handschel J (2013) Effects of dexamethasone, ascorbic acid and beta-glycerophosphate on the osteogenic differentiation of stem cells in vitro. Stem Cell Res Ther, 4:117.

Lesot H, Lisi S, Peterkova R, Peterka M, Mitolo V, Ruch JV (2001) Epigenetic signals during odontoblast differentiation. Advances in dental research, 15:8-13.

Li J, Chen B (2012) Oct4 was a novel target of Wnt signaling pathway. Molecular and cellular biochemistry, 362:233-240.

Li VC, Kirschner MW (2014) Molecular ties between the cell cycle and differentiation in embryonic stem cells. Proc Natl Acad Sci U S A, 111:9503-9508.

Liu J, Yu F, Sun Y, et al. (2015) Concise reviews: Characteristics and potential applications of human dental tissue-derived mesenchymal stem cells. Stem cells (Dayton, Ohio), 33:627-638.

Liu Z, Chen T, Sun W, et al. (2016) DNA Demethylation Rescues the Impaired Osteogenic Differentiation Ability of Human Periodontal Ligament Stem Cells in High Glucose. Scientific reports, 6:27447.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif.), 25:402-408.

Loh YH, Wu Q, Chew JL, et al. (2006) The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nature genetics, 38:431-440.

Lowell S, Benchoua A, Heavey B, Smith AG (2006) Notch promotes neural lineage entry by pluripotent embryonic stem cells. PLoS biology, 4:e121.

Lluis F, Pedone E, Pepe S, Cosma MP (2008) Periodic activation of Wnt/beta-catenin signaling enhances somatic cell reprogramming mediated by cell fusion. Cell stem cell, 3:493-507.

Marmorstein R, Zhou MM (2014) Writers and readers of histone acetylation: structure, mechanism, and inhibition. Cold Spring Harbor perspectives in biology, 6:a018762.

McLenachan S, Menchon C, Raya A, Consiglio A, Edel MJ (2012) Cyclin A1 is essential for setting the pluripotent state and reducing tumorigenicity of induced pluripotent stem cells. Stem Cells Dev, 21:2891-2899.

Mizutani K, Yoon K, Dang L, Tokunaga A, Gaiano N (2007) Differential Notch signalling distinguishes neural stem cells from intermediate progenitors. Nature, 449:351-355.

Nashun B, Hill PW, Hajkova P (2015) Reprogramming of cell fate: epigenetic memory and the erasure of memories past. EMBO J, 34:1296-1308.

Okano M, Xie S, Li E (1998) Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nature genetics, 19:219-220.

Paino F, La Noce M, Tirino V, et al. (2014) Histone deacetylase inhibition with valproic acid downregulates osteocalcin gene expression in human dental pulp stem cells and osteoblasts: evidence for HDAC2 involvement. Stem cells, 32:279-289.

Paino F, Ricci G, De Rosa A, et al. (2010) Ecto-mesenchymal stem cells from dental pulp are committed to differentiate into active melanocytes. Eur Cell Mater, 20:295-305.

Park IH, Zhao R, West JA, et al. (2008) Reprogramming of human somatic cells to pluripotency with defined factors. Nature, 451:141-146.

Perdigoto CN, Bardin AJ (2013) Sending the right signal: Notch and stem cells. Biochimica et biophysica acta, 1830:2307-2322.

Pierdomenico L, Bonsi L, Calvitti M, et al. (2005) Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp. Transplantation, 80:836-842.

Polo JM, Anderssen E, Walsh RM, et al. (2012) A molecular roadmap of reprogramming somatic cells into iPS cells. Cell, 151:1617-1632.

Reya T, Clevers H (2005) Wnt signalling in stem cells and cancer. Nature, 434:843-850.

Robertson KD, Uzvolgyi E, Liang G, et al. (1999) The human DNA methyltransferases (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissues and overexpression in tumors. Nucleic acids research, 27:2291-2298.

Roccio M, Schmitter D, Knobloch M, Okawa Y, Sage D, Lutolf MP (2013) Predicting stem cell fate changes by differential cell cycle progression patterns. Development, 140:459-470.

Rosa V, Dubey N, Islam I, Min KS, Nor JE (2016) Pluripotency of Stem Cells from Human Exfoliated Deciduous Teeth for Tissue Engineering. Stem cells international, 2016:5957806.

Ruiz S, Panopoulos AD, Herrerias A, et al. (2011) A high proliferation rate is required for cell reprogramming and maintenance of human embryonic stem cell identity. Current biology : CB, 21:45-52.

Scheller EL, Chang J, Wang CY (2008) Wnt/beta-catenin inhibits dental pulp stem cell differentiation. Journal of dental research, 87:126-130.

Simandi Z, Horvath A, Wright LC, et al. (2016) OCT4 Acts as an Integrator of Pluripotency and Signal-Induced Differentiation. Molecular cell, 63:647-661.

Sperber H, Mathieu J, Wang Y, et al. (2015) The metabolome regulates the epigenetic landscape during naive-to-primed human embryonic stem cell transition. Nat Cell Biol, 17:1523-1535.

Takahashi K, Okita K, Nakagawa M, Yamanaka S (2007) Induction of pluripotent stem cells from fibroblast cultures. Nature protocols, 2:3081-3089.

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell, 126:663-676.

Tessarz P, Kouzarides T (2014) Histone core modifications regulating nucleosome structure and dynamics. Nature reviews. Molecular cell biology, 15:703-708.

Thekkeparambil Chandrabose S, Sriram S, Subramanian S, et al. (2018) Amenable epigenetic traits of dental pulp stem cells underlie high capability of xeno-free episomal reprogramming. Stem Cell Res Ther, 9:68.

Uribe-Etxebarria V, Luzuriaga J, Garcia-Gallastegui P, Agliano A, Unda F, Ibarretxe G (2017) Notch/Wnt cross-signalling regulates stemness of dental pulp stem cells

through expression of neural crest and core pluripotency factors. Eur Cell Mater, 34:249-270.

Vasanthan P, Govindasamy V, Gnanasegaran N, Kunasekaran W, Musa S, Abu Kasim NH (2015) Differential expression of basal microRNAs' patterns in human dental pulp stem cells. Journal of cellular and molecular medicine, 19:566-580.

Wang A, Du Y, He Q, Zhou C (2013) A quantitative system for discriminating induced pluripotent stem cells, embryonic stem cells and somatic cells. PLoS One, 8:e56095.

Wang T, Liu H, Ning Y, Xu Q (2014) The histone acetyltransferase p300 regulates the expression of pluripotency factors and odontogenic differentiation of human dental pulp cells. PLoS One, 9:e102117.

Wilson R, Urraca N, Skobowiat C, et al. (2015) Assessment of the Tumorigenic Potential of Spontaneously Immortalized and hTERT-Immortalized Cultured Dental Pulp Stem Cells. Stem cells translational medicine, 4:905-912.

Wu W, Zhou J, Xu CT, Zhang J, Jin YJ, Sun GL (2015) Derivation and growth characteristics of dental pulp stem cells from patients of different ages. Molecular medicine reports, 12:5127-5134.

Xu Y, Liu P, Zheng DH, et al. (2016) Expression profile and prognostic value of NNMT in patients with pancreatic cancer. Oncotarget, 7:19975-19981.

Yan X, Qin H, Qu C, Tuan RS, Shi S, Huang GT (2010) iPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin. Stem cells and development, 19:469-480.

Yiew NK, Chatterjee TK, Tang YL, et al. (2017) Novel role for Wnt inhibitor APCDD1 in adipocyte differentiation: implications for diet-induced obesity. The Journal of biological chemistry.

You Z, Liu Y, Liu X (2018) Nicotinamide N-methyltransferase enhances the progression of prostate cancer by stabilizing sirtuin 1. Oncology letters, 15:9195-9201.

Young RA (2011) Control of the embryonic stem cell state. Cell, 144:940-954.

Yu J, Vodyanik MA, Smuga-Otto K, et al. (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science (New York, N.Y.), 318:1917-1920.

Zhang D, Li Q, Rao L, Yi B, Xu Q (2015) Effect of 5-Aza-2'-deoxycytidine on odontogenic differentiation of human dental pulp cells. J Endod, 41:640-645.

Zhou VW, Goren A, Bernstein BE (2011) Charting histone modifications and the functional organization of mammalian genomes. Nature reviews. Genetics, 12:7-18.

Zhou Y, Zheng L, Li F, et al. (2018) Bivalent Histone Codes on WNT5A during Odontogenic Differentiation. J Dent Res, 97:99-107.

4. DISCUSSION

As we mentioned before, DPSCs as a population that comes from the neural crest (NC) express markers from neural crest progenitors (Kiraly et al., 2009; Schiraldi et al., 2012) and pluripotency factors expression (Abe et al., 2012; Janebodin et al., 2011; Miletich and Sharpe, 2004; Simoes-Costa and Bronner, 2015, Atari et al., 2012; Ferro et al., 2012; Janebodin et al., 2011; Kerkis et al., 2006; Rosa et al., 2016; Uribe-Etxebarria et al., 2017).One explanation to this is that evolution of the NC-specification program would have enabled cells at the neural plate border to acquire multipotency and migratory ability (Green et al., 2015). These pluripotency factors are crucial to maintaining stem cell pluripotency in broad type of cells (Chambers and Tomlinson, 2009; Hishida et al., 2015; Takahashi et al., 2007). Some authors, remarks and highlight the use of DPSCs as they would present some superior features with respect to other multipotent stem cell populations of the adult human body due to the broad set of pluripotency factors expressed at basal conditions (Atari et al., 2011; Atari et al., 2012; Rosa et al., 2016). These pluripotency stem cells factors (especially Oct4, Sox2, c-Myc, Klf4) have described to be necessary for not only maintain stemness and self-renewal (Hackett and Surani, 2014; Young, 2011)., but also for inducing reprogramming of somatic cells into iPSCs(Takahashi and Yamanaka, 2006a). These crucial transcription factors form an intrinsic core-regulatory circuit in precise levels that maintains ESCs in a pluripotent state (Boyer et al., 2005; Ivanova et al., 2006; Loh et al., 2006; Rao and Orkin, 2006).Importantly, enhancing the expression of these pluripotent factors by different strategies have demonstrated to improve different differentiation media protocols (Han et al., 2014; Liu et al., 2015a).Other methodologies have worked as a pre-treatment strategy before the exposure to the differentiation media(Uribe-Etxebarria et al., 2017). One interesting approach is to modulate signalling pathways as a new strategy to modulate reprogramming under control conditions, although different crosstalks have been described (Uribe-Etxebarria et al., 2017).Other authors have described also the dependency Wnt, FGF, E2Fs and PI3K signaling, which also promotes pluripotency (Hishida et al., 2015; O'Connor et al., 2011; Sun et al., 1999; Yeo et al., 2011). The Wnt signaling pathway has been shown to elevate the level of cMyc(Cartwright et al., 2005). WNT-3A and BIO treatments have demonstrated to increase pluripotent markers expression and to enhance adipocytes and osteocytes differentiation media protocols (Uribe-Etxebarria et al., 2017).This complex network of crucial factors also interacts with cell cycle machinery, including cMyc(Huskey et al., 2015; Kim et al., 2008; Kim et al., 2010; Lavagnolli et al., 2015; Rahl et al., 2010), Oct4 (Huskey et al., 2015; Kim et al.,

2010; Zhang et al., 2009) and Nanog(Zhang et al., 2009).Yamanaka and colleagues observed a similar G1 phase between Induced Pluripotent Stem Cells (iPSCs) and ESC cells (Takahashi et al., 2007), with minimal G1 phase. Other authors detected a phenomena called “mitotic advantage”, in which mitotic nuclei are reprogrammed at higher significantly rates than interphase nuclei (Guo et al., 2014; Roccio et al., 2013, Halley-Stott et al., 2014)due to the de-ubiquitination of histones during mitosis resulting in gene derepression (Halley-Stott et al., 2014). Modulating the proper levels of Cyclins, especially CyclinD is fundamental to proper reprogramming in cancer stem cells (Abe et al., 2012; Edel et al., 2010; McLenachan et al., 2012, (Oh et al., 2018). Studies in core factors, cell cycle phase and cyclins could give more information about how reprogramming works through pluripotency and cell cycle. During reprogramming, metabolism suffers a significant remodelling including high glucose and glutamate/glutamine metabolism, mitochondria remodelling without ROS reactive species production and turn-over of NADH/NAD^+ and lipid droplets formation (Figure 5).

Pluripotent cell property	Human DPSCs FBS + DMSO	Human DPSCs FBS + BIO	Human DPSCs FBS + WNT-3A
Pluripotency markers (NANOG, KLFs OCT4, CMYC, LIN28, SOX2)	Low	High	High
Hystone acetylation	Low	High	High
Global DNA hypomethylation	No	No	Yes
Hystone methylation H3K4me3, H3K37me3, H3K9me3.	Low	High	High
Dependence on DNMT1, DNMT3A, DNMT3B	Yes/Yes/Yes	Yes/No/No	Yes/No/No
Metabolism	Glycolytic	OxPhos/Glycolytic	OxPhos/Glycolytic
Production of NADH, FADH2	Low	High	High
ATPase activity	Yes	Yes	Yes
Mitochondrial membrane activity and depolarization	Low	High	High
Perinuclear mitochondria	No	No	Yes
Lipid droplets	Low	High	High
Oxidation of fatty acids	Low	High	High

Figure 5. Genetic, metabolic and epigenetics features of Dental Pulp Stem Cells. DPSCs suffer a metabolic and epigenetic remodelling during Wnt overactivation with BIO and WNT-3A. We studied some features such as Pluripotency markers (NANOG, OCT4, CMYC, LIN28, SOX2), Hystone acetylation, Global DNA hypomethylation, Histone methylation (H3K4me3, H3K37me3, H3K9me3, Dependence on DNMT1, DNMT3A and DNMT3B, Metabolism, Production of NADH, FADH2, ATPase activity,

Mitochondrial membrane activity and depolarization, Perinuclear mitochondria, Lipid droplets and Oxidation of fatty acids.

This metabolic profile did not suit at all with Warburg effect described in cancer cells (Warburg, 1956). This model is based on rapid glycolysis followed by lactate synthesis even under aerobic conditions. In addition, the pyruvate-lactate step is necessary to recycle the rate-limiting NAD^+ coenzyme and keep the redox reactions in glycolysis running rapidly (Koppenol et al., 2011; Vander Heiden et al., 2009; Warburg, 1956). However, in our system the production of lactate did not increased and OXPHOS worked functionally. One explanation to this point besides in that pluripotency is not rate-limiting, is not needed the pyruvate-lactate step, but pyruvate-acetyl-CoA step is crucial for the production of intermediaries (Moussaieff et al., 2015b). Indeed, it has been described that mitochondria remodelling occurs during the induction of reprogramming in a phenomena called “mitochondria rejuvenation” (Folmes et al., 2012; Folmes et al., 2011; Gu et al., 2016; Ma et al., 2015; Prigione et al., 2010; Suhr et al., 2010; Varum et al., 2011; Zhou et al., 2012). These findings could explain the temporal elevation of mitochondrial proteins in cells undergoing reprogramming (Hansson et al., 2012). This transient “hyper-energetic” state seems to be required for reprogramming, which is accompanied with an increment in reactive oxygen species (ROS) production and enhancement of the glycolytic rate. However, Warburg effect builds up anabolic intermediates for proliferation while minimizing ROS-induced damage (Koppenol et al., 2011; Vander Heiden et al., 2009; Warburg, 1956). Although some authors have highlighted the relevance of ROS generation for efficient reprogramming in DPSCs, the rate of ROS remains constant even metabolic remodelling (Zhou et al., 2016). The upregulation of glycolysis occurs in early steps during iPSC reprogramming, before a small fraction of cells acquires pluripotency (Folmes et al., 2011; Shyh-Chang et al., 2013). Moreover, acetyl-coA metabolite suffers a rapid decrease during differentiation of ESC suggesting the relevant role of this metabolite in the balance between pluripotency and differentiation (Mathieu et al., 2014). It is also to remark the existence of two different types of populations when cells undergo reprogramming; primed and naive stem cells, which follows distinct metabolism. We have represented a schematic model showing principal differences between DPSCs after Wnt treatments comparing to this two type of populations described in Weinberger model (Weinberger et al., 2016) (Figure 6). Primed stem cells mainly depend on glycolysis and have less OXPHOS whereas naive stem cells

retain high levels of OXPHOS during high glycolysis (Mathieu et al., 2014; Sperber et al., 2015; Wanet et al., 2015; Zhou et al., 2012).Several studies have linked naive stage of reprogramming to an increased in glycolysis accompanied by a transient burst of OXPHOS activity (Burk and Schade, 1956; Hawkins et al., 2016; Kida et al., 2015). According to our data, we hypothesize that DPSCs treated with Wnt activators, remains some primed and naive stem cells characteristics. Indeed, we added other features that can help to understand more about metabolism and epigenetic and how it works with reprogramming.

During the years several recipes to enhance reprogramming has been described; Oct4 and Sox2 together with Klf4 and cMyc (OSKM) or Nanog and Lin28 (OSNA), among others (Burk and Schade, 1956; Park et al., 2008; Takahashi et al., 2007; Takahashi and Yamanaka, 2006b; Yu et al., 2007). Regarding genes and their relationship with this complex network, Lin 28 gene has been reported to play a relevant role in PSC metabolism maintaining the low mitochondrial function associated with primed pluripotency and in regulating one-carbon metabolism, nucleotide metabolism, and histone methylation (Zhang et al., 2016). Interestingly, other authors have described that the stem cells transcription factors Oct4 and Lin28 regulate stem cell metabolism and conversion to pluripotency through several molecular mechanisms interacting with other core factors such as Sox2 and Nanog(Matoba et al., 2006; Zhang et al., 2016).

Pluripotent cell property	Naive pluripotent cell	Primed pluripotent cell	Human DPSCs FBS + BIO	Human DPSCs FBS + WNT-3A
MERK-ERK dependence	No	Yes	Unknown	Unknown
Long-term dependence on FGF signalling	No	Yes	Unknown	Unknown
Long-term dependence on TGB or Activin A signalling	No	Yes	Unknown	Unknown
Dominant OCT4 enhancer	Distal	Proximal	Unknown	Unknown
H3K27me3 on developmental regulators	Low	High	Unknown	Unknown
Global DNA hypomethylation	Yes	No	No	Yes
X chromosome inactivation	No	Yes	Don´t know	Don´t know
Dependence on DNMT1, DICER, METTL3, MBD3	No	Yes	Yes	Yes
Priming markers (OTX2, ZIC2)	Low	High	Unknown	Unknown
Pluripotency markers (NANOG, KLFs ESRRB)	High	Low	High	High
TFE nuclear localization	High	Low	Unknown	Unknown
CD24/MHC class 1	Low/low	High/low	Unknown	Unknown
Expressed adhesion molecules	E-cadherin	N-cadherin	Unknown	Unknown
Promotion of pluripotency maintenace by NANOG or PRDM14	Yes	No	Yes	Yes
Metabolism	OxPhos/Glycolytic	Glycolytic	OxPhos/Glycolytic	OxPhos/Glycolytic
Mitochondrial membrane activity and depolarization	High	Low	High	High
Hypomethylation of promoter and enhancer regions	Yes	No	Unknown	Unknown
Competence as initial starting cells for PGCLC induction	High	Low	Unknown	Unknown
Capacity for colonization of host pre-implantation ICM and contribution to advanced embryonic chimeras	High	Low	Unknown	Unknown
KIT	Yes	No	Unknown	Unknown
Tolerance for abscence of exogenous L-glutamine	Yes	No	Unknown	Unknown
Competence as initial starting cell for TSC induction	High	Low	Unknown	Unknown

Figure 6. Characteristics of naïve and primed stem cells according to gene expression, metabolism and epigenetics. Pluripotent Stem cells could be divided according to some features showed in the following table in naïve and primed stem cells. Weinberger model explained the differences between these two populations of pluripotent stem cells studying gene patter expression, behaviour in metabolism and epigenetic(Weinberger et al., 2016). We observed some of these features in our treated DSPCs, but some of them observed in Weinberger model are still unknown in our system model.

These pluripotency stem cells factors are also related to epigenetic landscape, for example, cMyc is described to be associated with histone acetyltransferase (HAT) complex and induces global histone acetylation(Takahashi and Yamanaka, 2006a). Indeed, Oct4 acts in inactivation of X chromosome to induce reprogramming(Navarro et al., 2008), interacts with several polycomb group proteins (Wang et al., 2006) and regulates H3K9 methylation status of the pluripotency factors Tcl1 and Nanog, respectively, to maintain stem cell identity (Loh et al., 2007). Another relevant core factor, Nanog regulates pluripotency Gata4 and Gata6 genes important for differentiation and linked to epigenetics(Chambers et al., 2003; Mitsui et al., 2003). Klf4 might contribute to activation of Nanog and other ES cells-specific genes through p53 repression (Takahashi and Yamanaka, 2006a). These all factors induce somehow somatic cell de-differentiation thanks to the chromatin remodelling.

Epigenetic alterations suffered in Wnt overactivation conditions could be crucial for efficient reprogramming as epigenetic barrier work together to ensure stability in gene expression. Despite some progress in this field, relatively little is known about the epigenetic pattern and consequent pleiotropic effects that drive DPSCs into both stemness and specialized lineages. To search for the elusive keys for DPSC cells reprogramming, it is crucial to understand how behaves epigenetic in PSCs during reprogramming. It is well known that features of chromatin in PS cells are different from the somatic cells, including nuclear architecture, chromatin structure, chromatin dynamics, and histone modifications. As an example, PSCs chromatin displays open conformation of chromatin besides high levels of acetylated H3K9 and methylated H3K4 enablingpluripotent nuclei the early replication of lineage-specific genes during differentiation(Boyer et al., 2006; Meshorer and Misteli, 2006).Indeed, H3K27 methylation is functionally important for preventing differentiation genes expression in ES cells (Azuara et al., 2006; Bernstein et al., 2006).DNA methylation at CpG islands remains as another gene silence mechanism of epigenetics relevant for gene expression. Silencing of certain genes by DNA methylation is required for induction of differentiation of PSCs whereas DNA hypomethylation is required for stemness condition maintenance (Jackson et al., 2004). Interestingly, hypomethylation in ES cells allows cells to maintain high level of Oct4 expression, thus keeping them in pluripotent state (Taranger et al., 2005). In DPSCs levels of methylation are lower in Wnt treatments giving further evidence about the plasticity of chromatin when needs remodelling.

Finally, there is also a link between metabolism and epigenetic. For start, several studies places glycolysis apart from generating ATP, as a crucial producer of glycolytic intermediates necessary for the production of new biomass and metabolites required for epigenetic changes in transcription (Wellen et al., 2009). Wellen have attributed glycolysis-derived acetyl-CoA, through ATP citrate lyase (ACLY), the enhancer of the histone acetylation during adipogenesis(Wellen et al., 2009). The acetyl-CoA generated in glycolysis, apart from its contribution to the initiation of the TCA cycle and the de novo synthesis of lipids, would have an important role in acetylation of specific amino-acid residues (predominantly lysine, K) on both histone and non-histone proteins trough HATs(Koopman et al., 2014; Lunt and Vander Heiden, 2011). Indeed, mitochondrial acetyl-CoA from fatty acid oxidation needed glucose to serve as a substrate for histone acetylation (Wellen et al., 2009). Moreover, acetate, an acetyl-CoA precursor, prevented histone deacetylation and blocked the differentiation of ESCs (Moussaieff et al., 2015b). These links between glycolysis and histone acetylation described before, have been observed in different cells such as ESCs (Moussaieff et al., 2015b), skeletal muscle stem cells (Ryall et al., 2015), tumour cells (Liu et al., 2015b) and yeast (Friis et al., 2009). The activity of the Histone Deacetylase Sirtuin 1 or SIRT1 (HDAC) has also been shown to be crucial for reprogramming and regulates PSCs pluripotency (Tang and Rando, 2014). Interestingly, there are several metabolites that contribute in regulation of histone modifications and pluripotency apart from acetyl-CoA.In PSCs differentiation pyruvate becomes fully oxidized in mitochondria, leading to acetyl-coA depravation, loss of histone acetylation and subsequent loss of pluripotency markers expression (Moussaieff et al., 2015a; Moussaieff et al., 2015b). These findings suggest that cytosolic acetyl-CoA does not always correlate with stemness and in this case, switch might exist to modulate histone acetylation and chromatin plasticity during cellular differentiation. Another example is α-ketoglutarate, derived from glutamine, which promote histone and DNA demethylation in order to maintain relaxed chromatin state (Sperber et al., 2015). SAM acts as the substrate for methyl transferases and as a result is a key regulator for maintaining the undifferentiated state of PSCs(Shyh-Chang et al., 2013). SAM levels are controlled by nicotinamide N-methyl transferase (NNMT), converting them into SAH, which through EZH2 catalytic enzyme, regulates H3K27me3 repressive marks (Sperber et al., 2015). We incorporate metabolism signalling and its network with epigenetics in the following figure as recent research and our finding in our DPSCs system (Figure 6). Here we hypothesized NNMT role to control SAM levels and methylation in DPSCs

(Figure 6). In our system, H3K27me3 marks increase in Wnt overactivation. One explanation to this could be that the function of H3K27me3 marks remains elusive. One Perhaps in the absence of other repressive mechanisms such as DNA methylation or H3K9me3, H3K27me3 can compensate their missing activities. For example, H3K27me3 becomes enriched at repetitive elements such as ERVs in PGCs where DNA methylation is globally depleted (Lindroth et al., 2008; Ng et al., 2013).

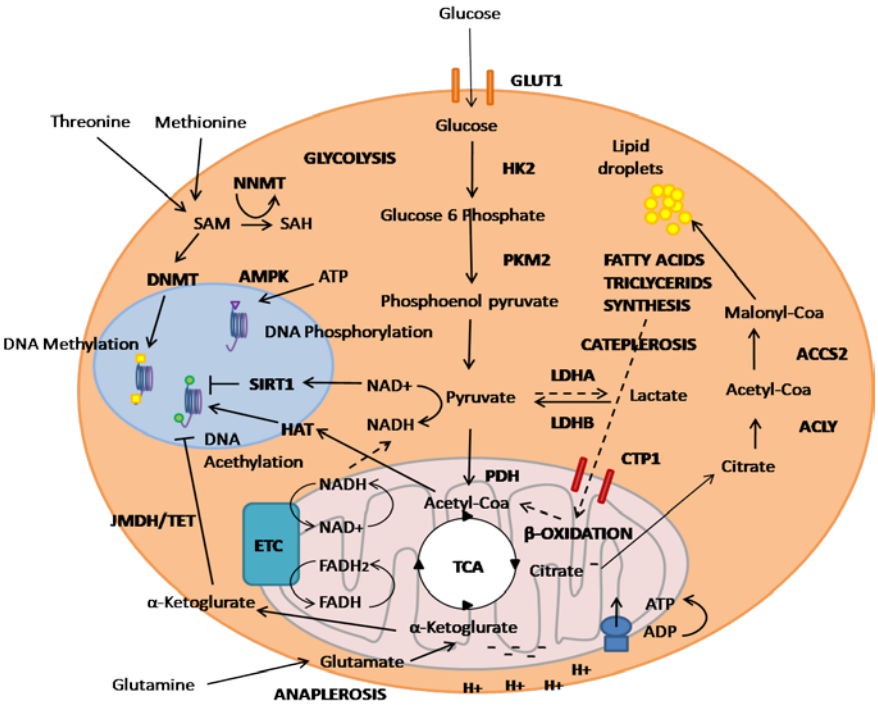


Figure6. Representation of epigenetic and metabolic changes during reprogramming in DPSCs. Wnt signaling activation by either BIO or WNT-3A increases glucose consumption by overexpression of glycolytic enzymes HK2 and/or PKM2. LDHA and LDHB contribute to lactate-pyruvate conversion. LDHB is overexpressed while LHDA is downregulated in Wnt activated DPSCs. Pyruvate dehydrogenase complex subunits are also upregulated in BIO/WNT-3A treated DPSCs and this leads to fuel the mitochondrial TCA cycle. These “hyper-energized” DPSCs show a net accumulation of lipids and a mitochondrial hyperpolarization. Overexpression of cytosolic ACLY and ACSS2 enzymes suggests cataplerosis leading to cytosolic accumulation of acetyl-coA, which can be then used for lipid biosynthesis. This excess of acetyl-coA is used for histone acetylation. The total amount of NAD^+ is used by SIRT 1 to quit acetylation marks. Contrarily, SAM from the One Carbon Metabolism acts as contributor for DNA methylation and NNMT convert SAM to SAH. This NNMT indirectly contributes to DNA de-methylation whereas then level of acetylation is higher in these conditions thanks to HAT and SIRT1 activities fueled by NAD+/NADH. Interestingly, ATP AN CONTRIBUTE TO DNA phosphorylation, but high levels can contribute to block AMPK, blocking DNA phosphorylation. Meanwhile, mitochondria consume amino acids such as glutamine and glutamate to replenish TCA metabolites in a coordinated cycle of cataplerosis

and anaplerosis. Cytosolic fatty acids also appear to participate in this process, as suggested by the overexpression of CPT1 and β-oxidation enzymes at mRNA level. DPSCs reprogrammed with BIO or WNT-3A thus show a boost in glycolysis without the characteristic lactate accumulation observed in the classic Warburg effect.

In support with this hypothesis, in DPSCs treated with Wnt overactivators, there are some key points in common with PSCs in metabolism and epigenetic behaviour during reprogramming as well as different remarks. Curiously, during reprogramming of DPSCs, these pluripotency core factors remain higher when glucose is continuously fully oxidized and OXPHOS work functionally and mitochondria are at their highest membrane potential. The epigenetic changes that occur during somatic cell reprogramming are also distinct from the epigenetic changes required to maintain pluripotency. Whereas somatic cell reprogramming involves complex global remodelling of histone modifications and DNA methylation, pluripotency maintenance requires an enrichment of histone acetylation to maintain chromatin plasticity, relative to differentiated cells (Mattout et al., 2015). In our system, we observe histone methylation and acetylation patterns modification with DA methylation remodelling.

More studies will be necessary to understand gene expression, metabolism and epigenetic network to design more safety and efficient methods of reprogramming. Notch and Wnt/β-Catenin work as fundamental signalling pathways in DPSCs maintenance of stemness and self-renewal. Modulation of Wnt signaling by recombinant protein WNT-3A would be a good alternative to control reprogramming in different times and concentrations. This strategy could be used for creating new more efficient and faster differentiation media protocols to achieve different type of cells for cell therapy from Dental Pulp Stem Cells.

5. CONCLUSIONS

After doing this thesis, we have reached the following conclusions:

1- The signalling pathways Notch and Wnt/β-Catenin regulate the stemness of Dental Pulp Stem Cells, controlling their expression of pluripotency core factors and self-renewal.

2-A Wnt signalling overactivation pre-treatment improves the capacity of DPSCs to subsequently differentiate to mature cell types such as osteocytes and adipocytes.. This strategy allows the creation of faster and more efficient differentiation media protocols.

3- Notch inhibition by DAPT slows down glycolytic metabolism.

4- Wnt overactivation by BIO or WNT-3A accelerates glycolytic metabolism, bringing Dental Pulp Stem Cells to a hyper-energetic state characterized by increases in mitochondrial tricarboxylic acid cycle (TCA) activity, mitochondrial membrane potential, lipid biosynthesis, and in the generation of reducing nucleotide species (NADH).

5- The overactivation of Wnt pathway decreases DNA methylation and increases histone methylation and acetylation in Dental Pulp Stem Cells.

6. The overactivation of Wnt pathway leads to a partial reprogramming of Dental Pulp Stem Cell cultures, as part of a signalling network controlling the expression of pluripotency core factors, and metabolic and epigenetic plasticity.

6. REFERENCES

Abe S, Hamada K, Miura M, Yamaguchi S. 2012. Neural crest stem cell property of apical pulp cells derived from human developing tooth. Cell biology international 36(10):927-936.

Adler PN, Taylor J. 2001. Asymmetric cell division: plane but not simple. Current biology : CB 11(6):R233-236.

Ai T, Zhang J, Wang X, Zheng X, Qin X, Zhang Q, Li W, Hu W, Lin J, Chen F. 2018. DNA methylation profile is associated with the osteogenic potential of three distinct human odontogenic stem cells. Signal transduction and targeted therapy 3:1.

Andersen P, Uosaki H, Shenje LT, Kwon C. 2012. Non-canonical Notch signaling: emerging role and mechanism. Trends in cell biology 22(5):257-265.

Androutsellis-Theotokis A, Leker RR, Soldner F, Hoeppner DJ, Ravin R, Poser SW, Rueger MA, Bae SK, Kittappa R, McKay RD. 2006. Notch signalling regulates stem cell numbers in vitro and in vivo. Nature 442(7104):823-826.

Armstrong L, Tilgner K, Saretzki G, Atkinson SP, Stojkovic M, Moreno R, Przyborski S, Lako M. 2010. Human induced pluripotent stem cell lines show stress defense mechanisms and mitochondrial regulation similar to those of human embryonic stem cells. Stem cells 28(4):661-673.

Arthur A, Rychkov G, Shi S, Koblar SA, Gronthos S. 2008. Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues. Stem cells 26(7):1787-1795.

Atari M, Gil-Recio C, Fabregat M, Garcia-Fernandez D, Barajas M, Carrasco MA, Jung HS, Alfaro FH, Casals N, Prosper F, Ferres-Padro E, Giner L. 2012. Dental pulp of the third molar: a new source of pluripotent-like stem cells. Journal of cell science 125(Pt 14):3343-3356.

Aurrekoetxea M, Garcia-Gallastegui P, Irastorza I, Luzuriaga J, Uribe-Etxebarria V, Unda F, Ibarretxe G. 2015. Dental pulp stem cells as a multifaceted tool for bioengineering and the regeneration of craniomaxillofacial tissues. Frontiers in physiology 6:289.

Azuara V, Perry P, Sauer S, Spivakov M, Jorgensen HF, John RM, Gouti M, Casanova M, Warnes G, Merkenschlager M, Fisher AG. 2006. Chromatin signatures of pluripotent cell lines. Nat Cell Biol 8(5):532-538.

Bayarsaihan D. 2016. Deciphering the Epigenetic Code in Embryonic and Dental Pulp Stem Cells. The Yale journal of biology and medicine 89(4):539-563.

Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, Cuff J, Fry B, Meissner A, Wernig M, Plath K, Jaenisch R, Wagschal A, Feil R, Schreiber SL, Lander ES. 2006. A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 125(2):315-326.

Borggrefe T, Lauth M, Zwijsen A, Huylebroeck D, Oswald F, Giaimo BD. 2016. The Notch intracellular domain integrates signals from Wnt, Hedgehog, TGFbeta/BMP and hypoxia pathways. Biochimica et biophysica acta 1863(2):303-313.

Boutros M, Paricio N, Strutt DI, Mlodzik M. 1998. Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. Cell 94(1):109-118.

Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG, Gifford DK, Melton DA, Jaenisch R, Young RA. 2005. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 122(6):947-956.

Boyer LA, Plath K, Zeitlinger J, Brambrink T, Medeiros LA, Lee TI, Levine SS, Wernig M, Tajonar A, Ray MK, Bell GW, Otte AP, Vidal M, Gifford DK, Young RA, Jaenisch R. 2006. Polycomb complexes repress developmental regulators in murine embryonic stem cells. Nature 441(7091):349-353.

Buck MD, O'Sullivan D, Klein Geltink RI, Curtis JD, Chang CH, Sanin DE, Qiu J, Kretz O, Braas D, van der Windt GJ, Chen Q, Huang SC, O'Neill CM, Edelson BT, Pearce EJ, Sesaki H, Huber TB, Rambold AS, Pearce EL. 2016. Mitochondrial Dynamics Controls T Cell Fate through Metabolic Programming. Cell 166(1):63-76.

Burk D, Schade AL. 1956. On respiratory impairment in cancer cells. Science 124(3215):270-272.

Cacchiarelli D, Trapnell C, Ziller MJ, Soumillon M, Cesana M, Karnik R, Donaghey J, Smith ZD, Ratanasirintrawoot S, Zhang X, Ho Sui SJ, Wu Z, Akopian V, Gifford CA, Doench J, Rinn JL, Daley GQ, Meissner A, Lander ES, Mikkelsen TS. 2015. Integrative Analyses of Human Reprogramming Reveal Dynamic Nature of Induced Pluripotency. Cell 162(2):412-424.

Carey BW, Finley LW, Cross JR, Allis CD, Thompson CB. 2015. Intracellular alpha-ketoglutarate maintains the pluripotency of embryonic stem cells. Nature 518(7539):413-416.

Cartwright P, McLean C, Sheppard A, Rivett D, Jones K, Dalton S. 2005. LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. Development 132(5):885-896.

Clevers H. 2006. Wnt/beta-catenin signaling in development and disease. Cell 127(3):469-480.

Clevers H, Loh KM, Nusse R. 2014. Stem cell signaling. An integral program for tissue renewal and regeneration: Wnt signaling and stem cell control. Science (New York, NY) 346(6205):1248012.

Coronado D, Godet M, Bourillot PY, Tapponnier Y, Bernat A, Petit M, Afanassieff M, Markossian S, Malashicheva A, Iacone R, Anastassiadis K, Savatier P. 2013. A short G1 phase is an intrinsic determinant of naive embryonic stem cell pluripotency. Stem Cell Res 10(1):118-131.

Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A. 2003. Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 113(5):643-655.

Chandel NS, Jasper H, Ho TT, Passegue E. 2016. Metabolic regulation of stem cell function in tissue homeostasis and organismal ageing. Nat Cell Biol 18(8):823-832.

d'Aquino R, De Rosa A, Laino G, Caruso F, Guida L, Rullo R, Checchi V, Laino L, Tirino V, Papaccio G. 2009. Human dental pulp stem cells: from biology to clinical applications. Journal of experimental zoology Part B, Molecular and developmental evolution 312B(5):408-415.

D'Souza B, Meloty-Kapella L, Weinmaster G. 2010. Canonical and non-canonical Notch ligands. Current topics in developmental biology 92:73-129.

Endoh M, Endo TA, Endoh T, Fujimura Y, Ohara O, Toyoda T, Otte AP, Okano M, Brockdorff N, Vidal M, Koseki H. 2008. Polycomb group proteins Ring1A/B are functionally linked to the core transcriptional regulatory circuitry to maintain ES cell identity. Development 135(8):1513-1524.

Ferro F, Spelat R, D'Aurizio F, Puppato E, Pandolfi M, Beltrami AP, Cesselli D, Falini G, Beltrami CA, Curcio F. 2012. Dental pulp stem cells differentiation reveals new insights in Oct4A dynamics. PloS one 7(7):e41774.

Folmes CD, Dzeja PP, Nelson TJ, Terzic A. 2012. Mitochondria in control of cell fate. Circulation research 110(4):526-529.

Folmes CD, Martinez-Fernandez A, Faustino RS, Yamada S, Perez-Terzic C, Nelson TJ, Terzic A. 2013. Nuclear reprogramming with c-Myc potentiates glycolytic capacity of derived induced pluripotent stem cells. Journal of cardiovascular translational research 6(1):10-21.

Folmes CD, Nelson TJ, Martinez-Fernandez A, Arrell DK, Lindor JZ, Dzeja PP, Ikeda Y, Perez-Terzic C, Terzic A. 2011. Somatic oxidative bioenergetics transitions into pluripotency-dependent glycolysis to facilitate nuclear reprogramming. Cell metabolism 14(2):264-271.

Friis RM, Wu BP, Reinke SN, Hockman DJ, Sykes BD, Schultz MC. 2009. A glycolytic burst drives glucose induction of global histone acetylation by picNuA4 and SAGA. Nucleic acids research 37(12):3969-3980.

Fukunaga-Kalabis M, Hristova DM, Wang JX, Li L, Heppt MV, Wei Z, Gyurdieva A, Webster MR, Oka M, Weeraratna AT, Herlyn M. 2015. UV-Induced Wnt7a in the Human Skin Microenvironment Specifies the Fate of Neural Crest-Like Cells via Suppression of Notch. The Journal of investigative dermatology 135(6):1521-1532.

Gao P, Tchernyshyov I, Chang TC, Lee YS, Kita K, Ochi T, Zeller KI, De Marzo AM, Van Eyk JE, Mendell JT, Dang CV. 2009. c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. Nature 458(7239):762-765.

Garcia-Castro MI, Marcelle C, Bronner-Fraser M. 2002. Ectodermal Wnt function as a neural crest inducer. Science (New York, NY) 297(5582):848-851.

Gascon S, Murenu E, Masserdotti G, Ortega F, Russo GL, Petrik D, Deshpande A, Heinrich C, Karow M, Robertson SP, Schroeder T, Beckers J, Irmler M, Berndt C, Angeli JP, Conrad M, Berninger B, Gotz M. 2016. Identification and Successful Negotiation of a Metabolic Checkpoint in Direct Neuronal Reprogramming. Cell Stem Cell 18(3):396-409.

Gattazzo F, Urciuolo A, Bonaldo P. 2014. Extracellular matrix: a dynamic microenvironment for stem cell niche. Biochimica et biophysica acta 1840(8):2506-2519.

Ghule PN, Medina R, Lengner CJ, Mandeville M, Qiao M, Dominski Z, Lian JB, Stein JL, van Wijnen AJ, Stein GS. 2011. Reprogramming the pluripotent cell cycle: restoration of an abbreviated G1 phase in human induced pluripotent stem (iPS) cells. J Cell Physiol 226(5):1149-1156.

Gronthos S, Brahim J, Li W, Fisher LW, Cherman N, Boyde A, DenBesten P, Robey PG, Shi S. 2002. Stem cell properties of human dental pulp stem cells. J Dent Res 81(8):531-535.

Grottkau BE, Purudappa PP, Lin YF. 2010. Multilineage differentiation of dental pulp stem cells from green fluorescent protein transgenic mice. Int J Oral Sci 2(1):21-27.

Gu S, Liang J, Wang J, Liu B. 2013. Histone acetylation regulates osteodifferentiation of human dental pulp stem cells via DSPP. Frontiers in bioscience 18:1072-1079.

Gu W, Gaeta X, Sahakyan A, Chan AB, Hong CS, Kim R, Braas D, Plath K, Lowry WE, Christofk HR. 2016. Glycolytic Metabolism Plays a Functional Role in Regulating Human Pluripotent Stem Cell State. Cell Stem Cell 19(4):476-490.

Hackett JA, Surani MA. 2014. Regulatory principles of pluripotency: from the ground state up. Cell Stem Cell 15(4):416-430.

Han SM, Han SH, Coh YR, Jang G, Chan Ra J, Kang SK, Lee HW, Youn HY. 2014. Enhanced proliferation and differentiation of Oct4- and Sox2-overexpressing human adipose tissue mesenchymal stem cells. Experimental & molecular medicine 46:e101.

Hansson J, Rafiee MR, Reiland S, Polo JM, Gehring J, Okawa S, Huber W, Hochedlinger K, Krijgsveld J. 2012. Highly coordinated proteome dynamics during reprogramming of somatic cells to pluripotency. Cell reports 2(6):1579-1592.

Hari L, Miescher I, Shakhova O, Suter U, Chin L, Taketo M, Richardson WD, Kessaris N, Sommer L. 2012. Temporal control of neural crest lineage generation by Wnt/beta-catenin signaling. Development (Cambridge, England) 139(12):2107-2117.

Harvey AJ, Rathjen J, Gardner DK. 2016. Metaboloepigenetic Regulation of Pluripotent Stem Cells. Stem Cells Int 2016:1816525.

Hawkins KE, Joy S, Delhove JM, Kotiadis VN, Fernandez E, Fitzpatrick LM, Whiteford JR, King PJ, Bolanos JP, Duchen MR, Waddington SN, McKay TR. 2016. NRF2 Orchestrates the Metabolic Shift during Induced Pluripotent Stem Cell Reprogramming. Cell reports 14(8):1883-1891.

Hishida T, Nakachi Y, Mizuno Y, Katano M, Okazaki Y, Ema M, Takahashi S, Hirasaki M, Suzuki A, Ueda A, Nishimoto M, Hishida-Nozaki Y, Vazquez-Ferrer E, Sancho-Martinez I, Izpisua Belmonte JC, Okuda A. 2015. Functional compensation between Myc and PI3K signaling supports self-renewal of embryonic stem cells. Stem cells 33(3):713-725.

Hishida T, Nozaki Y, Nakachi Y, Mizuno Y, Okazaki Y, Ema M, Takahashi S, Nishimoto M, Okuda A. 2011. Indefinite self-renewal of ESCs through Myc/Max transcriptional complex-independent mechanisms. Cell Stem Cell 9(1):37-49.

Hori K, Sen A, Artavanis-Tsakonas S. 2013. Notch signaling at a glance. J Cell Sci 126(Pt 10):2135-2140.

Huang GT, Gronthos S, Shi S. 2009. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. Journal of dental research 88(9):792-806.

Hui T, A P, Zhao Y, Wang C, Gao B, Zhang P, Wang J, Zhou X, Ye L. 2014. EZH2, a potential regulator of dental pulp inflammation and regeneration. J Endod 40(8):1132-1138.

Huskey NE, Guo T, Evason KJ, Momcilovic O, Pardo D, Creasman KJ, Judson RL, Blelloch R, Oakes SA, Hebrok M, Goga A. 2015. CDK1 inhibition targets the p53-NOXA-MCL1 axis, selectively kills embryonic stem cells, and prevents teratoma formation. Stem Cell Reports 4(3):374-389.

Ibarretxe G, Crende O, Aurrekoetxea M, Garcia-Murga V, Etxaniz J, Unda F. 2012. Neural crest stem cells from dental tissues: a new hope for dental and neural regeneration. Stem cells international 2012:103503.

Ichida JK, Tcw J, Williams LA, Carter AC, Shi Y, Moura MT, Ziller M, Singh S, Amabile G, Bock C, Umezawa A, Rubin LL, Bradner JE, Akutsu H, Meissner A, Eggan K. 2014. Notch inhibition allows oncogene-independent generation of iPS cells. Nature chemical biology 10(8):632-639.

Ivanova N, Dobrin R, Lu R, Kotenko I, Levorse J, DeCoste C, Schafer X, Lun Y, Lemischka IR. 2006. Dissecting self-renewal in stem cells with RNA interference. Nature 442(7102):533-538.

Jackson M, Krassowska A, Gilbert N, Chevassut T, Forrester L, Ansell J, Ramsahoye B. 2004. Severe global DNA hypomethylation blocks differentiation and induces histone hyperacetylation in embryonic stem cells. Molecular and cellular biology 24(20):8862-8871.

Janebodin K, Horst OV, Ieronimakis N, Balasundaram G, Reesukumal K, Pratumvinit B, Reyes M. 2011. Isolation and characterization of neural crest-derived stem cells from dental pulp of neonatal mice. PloS one 6(11):e27526.

Jang H, Kim TW, Yoon S, Choi SY, Kang TW, Kim SY, Kwon YW, Cho EJ, Youn HD. 2012. O-GlcNAc regulates pluripotency and reprogramming by directly acting on core components of the pluripotency network. Cell Stem Cell 11(1):62-74.

Jin H, Park JY, Choi H, Choung PH. 2013. HDAC inhibitor trichostatin A promotes proliferation and odontoblast differentiation of human dental pulp stem cells. Tissue engineering Part A 19(5-6):613-624.

Kallas A, Pook M, Trei A, Maimets T. 2014. Assessment of the Potential of CDK2 Inhibitor NU6140 to Influence the Expression of Pluripotency Markers NANOG, OCT4, and SOX2 in 2102Ep and H9 Cells. International journal of cell biology 2014:280638.

Kerkis I, Kerkis A, Dozortsev D, Stukart-Parsons GC, Gomes Massironi SM, Pereira LV, Caplan AI, Cerruti HF. 2006. Isolation and characterization of a population of immature dental pulp stem cells expressing OCT-4 and other embryonic stem cell markers. Cells, tissues, organs 184(3-4):105-116.

Kida YS, Kawamura T, Wei Z, Sogo T, Jacinto S, Shigeno A, Kushige H, Yoshihara E, Liddle C, Ecker JR, Yu RT, Atkins AR, Downes M, Evans RM. 2015. ERRs Mediate a Metabolic Switch Required for Somatic Cell Reprogramming to Pluripotency. Cell Stem Cell 16(5):547-555.

Kim H, Jang H, Kim TW, Kang BH, Lee SE, Jeon YK, Chung DH, Choi J, Shin J, Cho EJ, Youn HD. 2015. Core Pluripotency Factors Directly Regulate Metabolism in Embryonic Stem Cell to Maintain Pluripotency. Stem cells 33(9):2699-2711.

Kim J, Chu J, Shen X, Wang J, Orkin SH. 2008. An extended transcriptional network for pluripotency of embryonic stem cells. Cell 132(6):1049-1061.

Kim J, Woo AJ, Chu J, Snow JW, Fujiwara Y, Kim CG, Cantor AB, Orkin SH. 2010. A Myc network accounts for similarities between embryonic stem and cancer cell transcription programs. Cell 143(2):313-324.

Kim JW, Zeller KI, Wang Y, Jegga AG, Aronow BJ, O'Donnell KA, Dang CV. 2004. Evaluation of myc E-box phylogenetic footprints in glycolytic genes by chromatin immunoprecipitation assays. Molecular and cellular biology 24(13):5923-5936.

Kiraly M, Porcsalmy B, Pataki A, Kadar K, Jelitai M, Molnar B, Hermann P, Gera I, Grimm WD, Ganss B, Zsembery A, Varga G. 2009. Simultaneous PKC and cAMP activation induces differentiation of human dental pulp stem cells into functionally active neurons. Neurochem Int 55(5):323-332.

Kitajima K, Nakajima M, Kanokoda M, Kyba M, Dandapat A, Tolar J, Saito MK, Toyoda M, Umezawa A, Hara T. 2016. GSK3beta inhibition activates the CDX/HOX pathway and promotes hemogenic endothelial progenitor differentiation from human pluripotent stem cells. Experimental hematology 44(1):68-74.e61-10.

Koopman R, Ly CH, Ryall JG. 2014. A metabolic link to skeletal muscle wasting and regeneration. Front Physiol 5:32.

Koppenol WH, Bounds PL, Dang CV. 2011. Otto Warburg's contributions to current concepts of cancer metabolism. Nature reviews Cancer 11(5):325-337.

Kuhl M, Sheldahl LC, Malbon CC, Moon RT. 2000. Ca(2+)/calmodulin-dependent protein kinase II is stimulated by Wnt and Frizzled homologs and promotes ventral cell fates in Xenopus. J Biol Chem 275(17):12701-12711.

Kwon C, Cheng P, King IN, Andersen P, Shenje L, Nigam V, Srivastava D. 2011. Notch post-translationally regulates beta-catenin protein in stem and progenitor cells. Nature cell biology 13(10):1244-1251.

La Noce M, Mele L, Tirino V, Paino F, De Rosa A, Naddeo P, Papagerakis P, Papaccio G, Desiderio V. 2014. Neural crest stem cell population in craniomaxillofacial development and tissue repair. Eur Cell Mater 28:348-357.

Lavagnolli T, Gupta P, Hormanseder E, Mira-Bontenbal H, Dharmalingam G, Carroll T, Gurdon JB, Fisher AG, Merkenschlager M. 2015. Initiation and maintenance

of pluripotency gene expression in the absence of cohesin. Genes Dev 29(1):23-38.

Ledesma-Martinez E, Mendoza-Nunez VM, Santiago-Osorio E. 2016. Mesenchymal Stem Cells Derived from Dental Pulp: A Review. Stem Cells Int 2016:4709572.

Leung AW, Murdoch B, Salem AF, Prasad MS, Gomez GA, Garcia-Castro MI. 2016. WNT/beta-catenin signaling mediates human neural crest induction via a pre-neural border intermediate. Development (Cambridge, England) 143(3):398-410.

Li F, Wang Y, Zeller KI, Potter JJ, Wonsey DR, O'Donnell KA, Kim JW, Yustein JT, Lee LA, Dang CV. 2005. Myc stimulates nuclearly encoded mitochondrial genes and mitochondrial biogenesis. Molecular and cellular biology 25(14):6225-6234.

Li VC, Kirschner MW. 2014. Molecular ties between the cell cycle and differentiation in embryonic stem cells. Proc Natl Acad Sci U S A 111(26):9503-9508.

Lima RL, Holanda-Afonso RC, Moura-Neto V, Bolognese AM, DosSantos MF, Souza MM. 2017. Human dental follicle cells express embryonic, mesenchymal and neural stem cells markers. Arch Oral Biol 73:121-128.

Lindroth AM, Park YJ, McLean CM, Dokshin GA, Persson JM, Herman H, Pasini D, Miro X, Donohoe ME, Lee JT, Helin K, Soloway PD. 2008. Antagonism between DNA and H3K27 methylation at the imprinted Rasgrf1 locus. PLoS genetics 4(8):e1000145.

Liu J, Yu F, Sun Y, Jiang B, Zhang W, Yang J, Xu GT, Liang A, Liu S. 2015a. Concise reviews: Characteristics and potential applications of human dental tissue-derived mesenchymal stem cells. Stem cells (Dayton, Ohio) 33(3):627-638.

Liu L, Wu L, Wei X, Ling J. 2015b. Induced overexpression of Oct4A in human dental pulp cells enhances pluripotency and multilineage differentiation capability. Stem cells and development 24(8):962-972.

Liu W, Le A, Hancock C, Lane AN, Dang CV, Fan TW, Phang JM. 2012. Reprogramming of proline and glutamine metabolism contributes to the proliferative and metabolic responses regulated by oncogenic transcription factor c-MYC. Proc Natl Acad Sci U S A 109(23):8983-8988.

Liu XS, Little JB, Yuan ZM. 2015c. Glycolytic metabolism influences global chromatin structure. Oncotarget 6(6):4214-4225.

Loh YH, Wu Q, Chew JL, Vega VB, Zhang W, Chen X, Bourque G, George J, Leong B, Liu J, Wong KY, Sung KW, Lee CW, Zhao XD, Chiu KP, Lipovich L, Kuznetsov VA, Robson P, Stanton LW, Wei CL, Ruan Y, Lim B, Ng HH. 2006. The Oct4 and Nanog transcription network regulates pluripotency in mouse embryonic stem cells. Nature genetics 38(4):431-440.

Loh YH, Zhang W, Chen X, George J, Ng HH. 2007. Jmjd1a and Jmjd2c histone H3 Lys 9 demethylases regulate self-renewal in embryonic stem cells. Genes Dev 21(20):2545-2557.

Lunt SY, Vander Heiden MG. 2011. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. Annu Rev Cell Dev Biol 27:441-464.

Ma T, Li J, Xu Y, Yu C, Xu T, Wang H, Liu K, Cao N, Nie BM, Zhu SY, Xu S, Li K, Wei WG, Wu Y, Guan KL, Ding S. 2015. Atg5-independent autophagy regulates mitochondrial clearance and is essential for iPSC reprogramming. Nat Cell Biol 17(11):1379-1387.

Mathieu J, Zhou W, Xing Y, Sperber H, Ferreccio A, Agoston Z, Kuppusamy KT, Moon RT, Ruohola-Baker H. 2014. Hypoxia-inducible factors have distinct and stage-specific roles during reprogramming of human cells to pluripotency. Cell Stem Cell 14(5):592-605.

Matoba R, Niwa H, Masui S, Ohtsuka S, Carter MG, Sharov AA, Ko MS. 2006. Dissecting Oct3/4-regulated gene networks in embryonic stem cells by expression profiling. PLoS One 1:e26.

Mattout A, Aaronson Y, Sailaja BS, Raghu Ram EV, Harikumar A, Mallm JP, Sim KH, Nissim-Rafinia M, Supper E, Singh PB, Sze SK, Gasser SM, Rippe K, Meshorer E. 2015. Heterochromatin Protein 1beta (HP1beta) has distinct functions and distinct nuclear distribution in pluripotent versus differentiated cells. Genome biology 16:213.

Meshorer E, Misteli T. 2006. Chromatin in pluripotent embryonic stem cells and differentiation. Nature reviews Molecular cell biology 7(7):540-546.

Miletich I, Sharpe PT. 2004. Neural crest contribution to mammalian tooth formation. Birth defects research Part C, Embryo today : reviews 72(2):200-212.

Mitsui K, Tokuzawa Y, Itoh H, Segawa K, Murakami M, Takahashi K, Maruyama M, Maeda M, Yamanaka S. 2003. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113(5):631-642.

Mizutani K, Yoon K, Dang L, Tokunaga A, Gaiano N. 2007. Differential Notch signalling distinguishes neural stem cells from intermediate progenitors. Nature 449(7160):351-355.

Moussaieff A, Kogan NM, Aberdam D. 2015a. Concise Review: Energy Metabolites: Key Mediators of the Epigenetic State of Pluripotency. Stem cells 33(8):2374-2380.

Moussaieff A, Rouleau M, Kitsberg D, Cohen M, Levy G, Barasch D, Nemirovski A, Shen-Orr S, Laevsky I, Amit M, Bomze D, Elena-Herrmann B, Scherf T, Nissim-Rafinia M, Kempa S, Itskovitz-Eldor J, Meshorer E, Aberdam D, Nahmias Y. 2015b. Glycolysis-mediated changes in acetyl-CoA and histone acetylation control the early differentiation of embryonic stem cells. Cell metabolism 21(3):392-402.

Navarro P, Chambers I, Karwacki-Neisius V, Chureau C, Morey C, Rougeulle C, Avner P. 2008. Molecular coupling of Xist regulation and pluripotency. Science 321(5896):1693-1695.

Neganova I, Tilgner K, Buskin A, Paraskevopoulou I, Atkinson SP, Peberdy D, Passos JF, Lako M. 2014. CDK1 plays an important role in the maintenance of pluripotency and genomic stability in human pluripotent stem cells. Cell Death Dis 5:e1508.

Ng JH, Kumar V, Muratani M, Kraus P, Yeo JC, Yaw LP, Xue K, Lufkin T, Prabhakar S, Ng HH. 2013. In vivo epigenomic profiling of germ cells reveals germ cell molecular signatures. Developmental cell 24(3):324-333.

O'Connor MD, Wederell E, Robertson G, Delaney A, Morozova O, Poon SS, Yap D, Fee J, Zhao Y, McDonald H, Zeng T, Hirst M, Marra MA, Aparicio SA, Eaves CJ. 2011. Retinoblastoma-binding proteins 4 and 9 are important for human pluripotent stem cell maintenance. Exp Hematol 39(8):866-879 e861.

Oh SJ, Cho H, Kim S, Noh KH, Song KH, Lee HJ, Woo SR, Kim S, Choi CH, Chung JY, Hewitt SM, Kim JH, Baek S, Lee KM, Yee C, Park HC, Kim TW. 2018. Targeting Cyclin D-CDK4/6 Sensitizes Immune-Refractory Cancer by Blocking the SCP3-NANOG Axis. Cancer Res 78(10):2638-2653.

Osthus RC, Shim H, Kim S, Li Q, Reddy R, Mukherjee M, Xu Y, Wonsey D, Lee LA, Dang CV. 2000. Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. J Biol Chem 275(29):21797-21800.

Panopoulos AD, Yanes O, Ruiz S, Kida YS, Diep D, Tautenhahn R, Herrerias A, Batchelder EM, Plongthongkum N, Lutz M, Berggren WT, Zhang K, Evans RM, Siuzdak G, Izpisua Belmonte JC. 2012. The metabolome of induced pluripotent stem cells reveals metabolic changes occurring in somatic cell reprogramming. Cell Res 22(1):168-177.

Park IH, Zhao R, West JA, Yabuuchi A, Huo H, Ince TA, Lerou PH, Lensch MW, Daley GQ. 2008. Reprogramming of human somatic cells to pluripotency with defined factors. Nature 451(7175):141-146.

Perdigoto CN, Bardin AJ. 2013. Sending the right signal: Notch and stem cells. Biochimica et biophysica acta 1830(2):2307-2322.

Petrovic V, Stefanovic V. 2009. Dental tissue--new source for stem cells. TheScientificWorldJournal 9:1167-1177.

Pierdomenico L, Bonsi L, Calvitti M, Rondelli D, Arpinati M, Chirumbolo G, Becchetti E, Marchionni C, Alviano F, Fossati V, Staffolani N, Franchina M, Grossi A, Bagnara GP. 2005. Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp. Transplantation 80(6):836-842.

Prigione A, Fauler B, Lurz R, Lehrach H, Adjaye J. 2010. The senescence-related mitochondrial/oxidative stress pathway is repressed in human induced pluripotent stem cells. Stem cells 28(4):721-733.

Rahl PB, Lin CY, Seila AC, Flynn RA, McCuine S, Burge CB, Sharp PA, Young RA. 2010. c-Myc regulates transcriptional pause release. Cell 141(3):432-445.

Rao S, Orkin SH. 2006. Unraveling the transcriptional network controlling ES cell pluripotency. Genome biology 7(8):230.

Reya T, Clevers H. 2005. Wnt signalling in stem cells and cancer. Nature 434(7035):843-850.

Rodas-Junco BA, Canul-Chan M, Rojas-Herrera RA, De-la-Pena C, Nic-Can GI. 2017. Stem Cells from Dental Pulp: What Epigenetics Can Do with Your Tooth. Front Physiol 8:999.

Rogers CD, Jayasena CS, Nie S, Bronner ME. 2012. Neural crest specification: tissues, signals, and transcription factors. Wiley interdisciplinary reviewsDevelopmental biology 1(1):52-68.

Rosa V, Dubey N, Islam I, Min KS, Nor JE. 2016. Pluripotency of Stem Cells from Human Exfoliated Deciduous Teeth for Tissue Engineering. Stem cells international 2016:5957806.

Ryall JG, Cliff T, Dalton S, Sartorelli V. 2015a. Metabolic Reprogramming of Stem Cell Epigenetics. Cell Stem Cell 17(6):651-662.

Ryall JG, Dell'Orso S, Derfoul A, Juan A, Zare H, Feng X, Clermont D, Koulnis M, Gutierrez-Cruz G, Fulco M, Sartorelli V. 2015b. The NAD(+)-dependent SIRT1 deacetylase translates a metabolic switch into regulatory epigenetics in skeletal muscle stem cells. Cell Stem Cell 16(2):171-183.

Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. 2004. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. Nature medicine 10(1):55-63.

Scheller EL, Chang J, Wang CY. 2008. Wnt/beta-catenin inhibits dental pulp stem cell differentiation. Journal of dental research 87(2):126-130.

Schiraldi C, Stellavato A, D'Agostino A, Tirino V, d'Aquino R, Woloszyk A, De Rosa A, Laino L, Papaccio G, Mitsiadis TA. 2012. Fighting for territories: time-lapse analysis of dental pulp and dental follicle stem cells in co-culture reveals specific migratory capabilities. Eur Cell Mater 24:426-440.

Sheldahl LC, Park M, Malbon CC, Moon RT. 1999. Protein kinase C is differentially stimulated by Wnt and Frizzled homologs in a G-protein-dependent manner. Current biology : CB 9(13):695-698.

Shim H, Dolde C, Lewis BC, Wu CS, Dang G, Jungmann RA, Dalla-Favera R, Dang CV. 1997. c-Myc transactivation of LDH-A: implications for tumor metabolism and growth. Proc Natl Acad Sci U S A 94(13):6658-6663.

Shiraki N, Shiraki Y, Tsuyama T, Obata F, Miura M, Nagae G, Aburatani H, Kume K, Endo F, Kume S. 2014. Methionine metabolism regulates maintenance and differentiation of human pluripotent stem cells. Cell metabolism 19(5):780-794.

Shyh-Chang N, Locasale JW, Lyssiotis CA, Zheng Y, Teo RY, Ratanasirintrawoot S, Zhang J, Onder T, Unternaehrer JJ, Zhu H, Asara JM, Daley GQ, Cantley LC. 2013. Influence of threonine metabolism on S-adenosylmethionine and histone methylation. Science 339(6116):222-226.

Simoes-Costa M, Bronner ME. 2015. Establishing neural crest identity: a gene regulatory recipe. Development 142(2):242-257.

Sperber H, Mathieu J, Wang Y, Ferreccio A, Hesson J, Xu Z, Fischer KA, Devi A, Detraux D, Gu H, Battle SL, Showalter M, Valensisi C, Bielas JH, Ericson NG, Margaretha L, Robitaille AM, Margineantu D, Fiehn O, Hockenbery D, Blau CA, Raftery D, Margolin AA, Hawkins RD, Moon RT, Ware CB, Ruohola-Baker H. 2015. The metabolome regulates the epigenetic landscape during naive-to-primed human embryonic stem cell transition. Nat Cell Biol 17(12):1523-1535.

Stevens A, Zuliani T, Olejnik C, LeRoy H, Obriot H, Kerr-Conte J, Formstecher P, Bailliez Y, Polakowska RR. 2008. Human dental pulp stem cells differentiate into neural crest-derived melanocytes and have label-retaining and sphere-forming abilities. Stem Cells Dev 17(6):1175-1184.

Stuhlmiller TJ, Garcia-Castro MI. 2012. Current perspectives of the signaling pathways directing neural crest induction. Cellular and molecular life sciences : CMLS 69(22):3715-3737.

Suhr ST, Chang EA, Tjong J, Alcasid N, Perkins GA, Goissis MD, Ellisman MH, Perez GI, Cibelli JB. 2010. Mitochondrial rejuvenation after induced pluripotency. PLoS One 5(11):e14095.

Sun H, Lesche R, Li DM, Liliental J, Zhang H, Gao J, Gavrilova N, Mueller B, Liu X, Wu H. 1999. PTEN modulates cell cycle progression and cell survival by regulating phosphatidylinositol 3,4,5,-trisphosphate and Akt/protein kinase B signaling pathway. Proc Natl Acad Sci U S A 96(11):6199-6204.

Takahashi K, Okita K, Nakagawa M, Yamanaka S. 2007. Induction of pluripotent stem cells from fibroblast cultures. Nature protocols 2(12):3081-3089.

Takahashi K, Yamanaka S. 2006a. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663-676.

Takahashi K, Yamanaka S. 2006b. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126(4):663-676.

Tang AH, Rando TA. 2014. Induction of autophagy supports the bioenergetic demands of quiescent muscle stem cell activation. EMBO J 33(23):2782-2797.

Taranger CK, Noer A, Sorensen AL, Hakelien AM, Boquest AC, Collas P. 2005. Induction of dedifferentiation, genomewide transcriptional programming, and epigenetic reprogramming by extracts of carcinoma and embryonic stem cells. Molecular biology of the cell 16(12):5719-5735.

Uribe-Etxebarria V, Luzuriaga J, Garcia-Gallastegui P, Agliano A, Unda F, Ibarretxe G. 2017. Notch/Wnt cross-signalling regulates stemness of dental pulp stem cells through expression of neural crest and core pluripotency factors. Eur Cell Mater 34:249-270.

Vander Heiden MG, Cantley LC, Thompson CB. 2009. Understanding the Warburg effect: the metabolic requirements of cell proliferation. Science 324(5930):1029-1033.

Varum S, Rodrigues AS, Moura MB, Momcilovic O, Easley CAt, Ramalho-Santos J, Van Houten B, Schatten G. 2011. Energy metabolism in human pluripotent stem cells and their differentiated counterparts. PLoS One 6(6):e20914.

Vasanthan P, Govindasamy V, Gnanasegaran N, Kunasekaran W, Musa S, Abu Kasim NH. 2015. Differential expression of basal microRNAs' patterns in human dental pulp stem cells. Journal of cellular and molecular medicine 19(3):566-580.

Wagers AJ, Christensen JL, Weissman IL. 2002. Cell fate determination from stem cells. Gene therapy 9(10):606-612.

Wanet A, Arnould T, Najimi M, Renard P. 2015. Connecting Mitochondria, Metabolism, and Stem Cell Fate. Stem Cells Dev 24(17):1957-1971.

Wang J, Rao S, Chu J, Shen X, Levasseur DN, Theunissen TW, Orkin SH. 2006. A protein interaction network for pluripotency of embryonic stem cells. Nature 444(7117):364-368.

Warburg O. 1956. On the origin of cancer cells. Science 123(3191):309-314.

Weinberger L, Ayyash M, Novershtern N, Hanna JH. 2016. Dynamic stem cell states: naive to primed pluripotency in rodents and humans. Nature reviews Molecular cell biology 17(3):155-169.

Wellen KE, Hatzivassiliou G, Sachdeva UM, Bui TV, Cross JR, Thompson CB. 2009. ATP-citrate lyase links cellular metabolism to histone acetylation. Science 324(5930):1076-1080.

Wilson R, Urraca N, Skobowiat C, Hope KA, Miravalle L, Chamberlin R, Donaldson M, Seagroves TN, Reiter LT. 2015. Assessment of the Tumorigenic Potential of Spontaneously Immortalized and hTERT-Immortalized Cultured Dental Pulp Stem Cells. Stem cells translational medicine 4(8):905-912.

Yeo HC, Beh TT, Quek JJ, Koh G, Chan KK, Lee DY. 2011. Integrated transcriptome and binding sites analysis implicates E2F in the regulation of self-renewal in human pluripotent stem cells. PLoS One 6(11):e27231.

Yiew NK, Chatterjee TK, Tang YL, Pellenberg R, Stansfield BK, Bagi Z, Fulton DJ, Stepp DW, Chen W, Patel V, Kamath VM, Litwin SE, Hui DY, Rudich SM, Kim HW, Weintraub NL. 2017. Novel role for Wnt inhibitor APCDD1 in adipocyte differentiation: implications for diet-induced obesity. The Journal of biological chemistry.

Young RA. 2011. Control of the embryonic stem cell state. Cell 144(6):940-954.

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA. 2007. Induced pluripotent stem cell lines derived from human somatic cells. Science (New York, NY) 318(5858):1917-1920.

Zhang H, Gao P, Fukuda R, Kumar G, Krishnamachary B, Zeller KI, Dang CV, Semenza GL. 2007. HIF-1 inhibits mitochondrial biogenesis and cellular respiration in VHL-deficient renal cell carcinoma by repression of C-MYC activity. Cancer cell 11(5):407-420.

Zhang H, Ryu D, Wu Y, Gariani K, Wang X, Luan P, D'Amico D, Ropelle ER, Lutolf MP, Aebersold R, Schoonjans K, Menzies KJ, Auwerx J. 2016a. NAD(+) repletion improves mitochondrial and stem cell function and enhances life span in mice. Science 352(6292):1436-1443.

Zhang J, Ratanasirintrawoot S, Chandrasekaran S, Wu Z, Ficarro SB, Yu C, Ross CA, Cacchiarelli D, Xia Q, Seligson M, Shinoda G, Xie W, Cahan P, Wang L, Ng SC, Tintara S, Trapnell C, Onder T, Loh YH, Mikkelsen T, Sliz P, Teitell MA, Asara JM, Marto JA, Li H, Collins JJ, Daley GQ. 2016b. LIN28 Regulates Stem Cell Metabolism and Conversion to Primed Pluripotency. Cell Stem Cell 19(1):66-80.

Zhang X, Neganova I, Przyborski S, Yang C, Cooke M, Atkinson SP, Anyfantis G, Fenyk S, Keith WN, Hoare SF, Hughes O, Strachan T, Stojkovic M, Hinds PW, Armstrong L, Lako M. 2009. A role for NANOG in G1 to S transition in human

embryonic stem cells through direct binding of CDK6 and CDC25A. J Cell Biol 184(1):67-82.

Zhao R, Deibler RW, Lerou PH, Ballabeni A, Heffner GC, Cahan P, Unternaehrer JJ, Kirschner MW, Daley GQ. 2014. A nontranscriptional role for Oct4 in the regulation of mitotic entry. Proc Natl Acad Sci U S A 111(44):15768-15773.

Zheng X, Boyer L, Jin M, Mertens J, Kim Y, Ma L, Ma L, Hamm M, Gage FH, Hunter T. 2016. Metabolic reprogramming during neuronal differentiation from aerobic glycolysis to neuronal oxidative phosphorylation. eLife 5.

Zhou G, Meng S, Li Y, Ghebre YT, Cooke JP. 2016. Optimal ROS Signaling Is Critical for Nuclear Reprogramming. Cell reports 15(5):919-925.

Zhou W, Choi M, Margineantu D, Margaretha L, Hesson J, Cavanaugh C, Blau CA, Horwitz MS, Hockenbery D, Ware C, Ruohola-Baker H. 2012. HIF1alpha induced switch from bivalent to exclusively glycolytic metabolism during ESC-to-EpiSC/hESC transition. EMBO J 31(9):2103-2116.

Zhu S, Li W, Zhou H, Wei W, Ambasudhan R, Lin T, Kim J, Zhang K, Ding S. 2010. Reprogramming of human primary somatic cells by OCT4 and chemical compounds. Cell Stem Cell 7(6):651-655.