科技部補助專題研究計畫成果報告
期末報告

微型RNA調控牙髓幹細胞分化及血管生成潛能的分子機制研究

計 畫 類 別 ： 個別型計畫
計 畫 編 號 ： MOST 104-2314-B-040-014-
執 行 期 間 ： 104年08月01日至105年07月31日
執 行 單 位 ： 中山醫學大學牙醫學系（所）

計 畫 主 持 人： 周明勇
共 同 主 持 人： 余承佳、楊惠雯

中 華 民 國 105 年 10 月 31 日

中 文 摘 要 ： 牙髓幹細胞是口腔醫學研究領域中重要的組織幹細胞之一，牙髓幹細胞具有高度增生、自我更新能力和多向分化潛能的成體幹細胞，在一定條件下可向特定的細胞類型分化。然而，調控牙髓幹細胞自我更新及分化潛能之分子機轉尚未清楚。微型RNA (microRNAs, miRNAs)是一類長度約19-24個核苷酸之非編碼RNA，可利用鹼基配對的方式與標靶mRNA的3’ UTR (untranslation region)結合而抑制mRNA的轉譯，許多證據亦顯示微型RNA在幹細胞自我更新及分化中扮演重要角色，但微型RNA於牙髓幹細胞角色仍未知，因此本計畫目的欲了解微型RNA對牙髓幹細胞幹性維持及分化潛能分子機轉，將為牙齒組織工程及牙髓組織再生提供新方向。初步結果發現miR-145於牙髓幹細胞低表達而miR-302於牙髓幹細胞高度表達。miR-145表現抑制可促牙髓細胞骨生成及脂肪生成能力。螢光素酶檢測顯示，miR-145能特異性地與Oct4及Nanog mRNA的3‘-UTR結合，抑制其螢光素酶活性。本研究成果能了解微型RNA在牙髓幹細胞調控自我更新及分化潛能分子機制，期待研究結果將有助於促進牙髓幹細胞應用於再生牙醫學。

中 文 關 鍵 詞 ： 牙髓幹細胞

英 文 摘 要 ： Dental pulp stem cells have attracted much attention because of their unique biological behaviors and potential clinical usages such as dentistry-related diseases, which remain to be major challenges for clinicians. Recently, microRNAs (miRNAs) have been proved to regulate stemness genes, stem cell lineage-specific differentiation. Therefore, the roles of miRNAs, as well as their down-stream targets, on the regulation of stemness and differentiation of DPSCs in the proposal will be studied. In the preliminary results, expression of miR-145 is down-regulated and miR-302 is up-regulated in DPSCs. Down-regulation of miR-145 enhanced the expression level of osteogenic /adipogenic induction differentiation capability. miR-145 also targets Oct4 and Nanog in DPSCs. The research will and suggests novel miRNAs-mediated mechanisms contribute stemness and differentiation properties of DPSCs. This study should open a new avenue for miRNAs research on DPSCs and lead to the rational design and development of innovative methods for regenerative dentistry and medicine. The results generated in this study may offer an insight into the miRNAs-mediated mechanisms regulating stemness and differentiation properties in DPSCs. This study should open a new avenue for miRNAs research on DPSCs and lead to facilitate the innovative design and develop regenerative medicine for various diseases.

英 文 關 鍵 詞 ： pulp stem cells

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（□期中進度報告/■期末報告）

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計畫類別：■個別型計畫　□整合型計畫
計畫編號： 104-2314-B-040-014
執行期間： 104 年 8 月 1 日至 105 年 7 月 31 日

執行機構及系所：中山醫學大學牙醫系(所)

計畫主持人：周明勇
共同主持人：余承佳
計畫參與人員：

本計畫除繳交成果報告外，另含下列出國報告，共 ___ 份：
□執行國際合作與移地研究心得報告
□出席國際學術會議心得報告

期末報告處理方式：

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中　華　民　國 105 年 10 月 31 日

中文摘要

牙髓幹細胞是口腔醫學研究領域中重要的組織幹細胞之一，牙髓幹細胞具有高度增生、自我更新能力和多向分化潛能的成體幹細胞，在一定條件下可向特定的細胞類型分化。然而，調控牙髓幹細胞自我更新及分化潛能之分子機轉尚未清楚。微型RNA (microRNAs, miRNAs)是一類長度約19-24個核苷酸之非編碼RNA，可利用鹼基配對的方式與標靶mRNA的3'UTR (untranslation region)結合而抑制mRNA的轉譯，許多證據亦顯示微型RNA在幹細胞自我更新及分化中扮演重要角色，但微型RNA於牙髓幹細胞角色仍未知，因此本計畫目的欲了解微型RNA對牙髓幹細胞幹性維持及分化潛能分子機轉，將為牙齒組織工程及牙髓組織再生提供新方向。初步結果發現miR-145於牙髓幹細胞低表達而miR-302於牙髓幹細胞高度表達。miR-145表現抑制可促牙髓細胞骨生成及脂肪生成能力。螢光素酶檢測顯示，miR-145能特異性地與Oct4及Nanog mRNA的3'-UTR結合，抑制其螢光素酶活性。本研究成果能了解微型RNA在牙髓幹細胞調控自我更新及分化潛能分子機制，期待研究結果將有助於促進牙髓幹細胞應用於再生牙醫學。

Abstract

Dental pulp stem cells have attracted much attention because of their unique biological behaviors and potential clinical usages such as dentistry-related diseases, which remain to be major challenges for clinicians. Recently, microRNAs (miRNAs) have been proved to regulate stemness genes, stem cell lineage-specific differentiation. Therefore, the roles of miRNAs, as well as their down-stream targets, on the regulation of stemness and differentiation of DPSCs in the proposal will be studied. In the preliminary results, expression of miR-145 is down-regulated and miR-302 is up-regulated in DPSCs. Down-regulation of miR-145 enhanced the expression level of osteogenic /adipogenic induction differentiation capability. miR-145 also targets Oct4 and Nanog in DPSCs. The research will and suggests novel miRNAs-mediated mechanisms contribute stemness and differentiation properties of DPSCs. This study should open a new avenue for miRNAs research on DPSCs and lead to the rational design and development of innovative methods for regenerative dentistry and medicine. The results generated in this study may offer an insight into the miRNAs-mediated mechanisms regulating stemness and differentiation properties in DPSCs. This study should open a new avenue for miRNAs research on DPSCs and lead to facilitate the innovative design and develop regenerative medicine for various diseases.

Background

Dental pulp stem cells (DPSCs), which possess multipotent-differentiation capability, are a candidate for regenerative medicine (1). DPSCs are well known to be multi-potent cells capable of differentiation along multiple lineages (1). DPSCs have the remarkable potential for multi-lineage differentiation capacity including osteoblast (2), cartilage (3), adipocyte (4), muscle (5), hepatocyte (6), and neurons (7). They described the identification of DPSCs by virtue of their clonogenic abilities, rapid proliferative rates, and capacity to form mineralized tissues both *in vitro* and *in vivo*. Compared with other stem cells such as mesenchymal stem cells (MSCs) from the bone marrow and neural stem cells (NSCs) from cadavers, DPSCs are more easily isolated (8). However, preliminary studies have identified that the stem cell or potential progenitor cell population in dental pulp comprises less than 1% of the total cells (9). In dental pulp cells, the reported markers of DPSCs include STRO-1, CD146, CD44, CD105, or CD166 (10). Therefore, an improved comprehension of the cellular and molecular mechanisms, which modulate self-renewal and differentiation properties of DPSCs, could be pursued to bring forth future progress in regenerative medicine.

In the past decade, the biological function and biogenesis of microRNAs (miRNAs) became popular topics for biomedical researches (11). miRNAs, approximately 18-25 nucleotides in length, are a group of endogenous small and noncoding RNAs (11). They are transcribed to form a primary miRNA via RNA polymerase II (11). Primary miRNA is processed into precursor miRNA (pre-miRNA) by Drosha and DRG 8 and is then exported from nucleus to cytoplasm through exportin 5 (11). The pre-miRNA is modified by Dicer and the matured miRNA forms (11). There are about 1000 different miRNAs which have been discovered and estimated in human genome (12). miRNAs can bind to target mRNAs by specific base pairing, then degrade mRNAs or inhibit protein translation, so they can participate in posttranscriptional regulation (11). Stem cells self-renewal and multiple differentiation are depending on various regulation mechanisms, and miRNAs are surely an important class of regulation factors among them. It has been reported that some kinds of miRNAs are likely important regulators for stem cells maintaining their state of self-renewal, and others may play key roles in their differentiation. miRNAs can be served as a new approach for stem cells research. In skin development, miR-205 and miR-125 promote self-renewal of hair follicle epidermal stem cells but are downregulated during differentiation (13, 14). By contrast, miR-203 is upregulated during differentiation and

targets cell cycle progression markers (SKP2, p63, MSI2 and VAV3) (15). miR-205 targets SHIP2, a negative regulator of AKT signaling, resulting in promoting AKT signaling of epidermal stem cells (13, 14). In neural development, miR-219 and miR-338 increase and target the transcription factors SOX6 and HES5 that have been correlated proliferation and oligodendrocyte differentiation (16). let-7, miR-124 and miR-9 expression is up-regulated in neuronal differentiation (17). miR-124 promotes the transition of neurogenic precursors to the neuroblast stage by directly targeting SOX9 (18). miRNAs also modulate tooth morphogenesis and ameloblast differentiation (19), suggesting their crucial roles in DPSCs.

Angiogenesis is a critical physiology event in bone formation. Vascular endothelial cells are the core of the vessels. These cells express some specific markers, such as vascular endothelial growth factor receptor2 (VEGFR2), CD31, or endothelial cell-nitric oxide synthase (ec-NOS) (20). These cells can promote the formation of tube structures in matrigel (21). Dental pulp stem cells also express endothelial cell markers and form vascular-like structure *in vitro* when cultured in low-serum culture medium added with vascular endothelial growth factor and fibroblast growth factor (22, 23). *In vivo* studies suggest that these cells can promote angiogenesis and blood perfusion of regional ischemic tissue, which further confirms that dental pulp stem cells can also be differentiated into endothelial cells (24, 25). However, the detailed molecular mechanisms involved in miRNAs in differentiation and angiogenic potentials in DPSCs are still poorly understood. Therefore, better understanding of the biological characteristics of miRNAs-mediated mechanisms in DPSCs will provide us with new clinical applications for regenerative medicine.

Results

1. Differential expression of microRNAs expression in human STRO-1+ CD146+ dental pulp stem cells.

(A) The up-regulated (*upper panel*) or down-regulated (*lower panel*) microRNAs expression in STRO-1+ CD146+ DPSCs was analyzed by microRNAs microarray analysis. MiR-145 (B) or miR302 (C) miRNA expression in STRO-1+ CD146+ and STRO-1- CD146- dental pulp cells were examined by miRNA real-time RT-PCR analysis

A

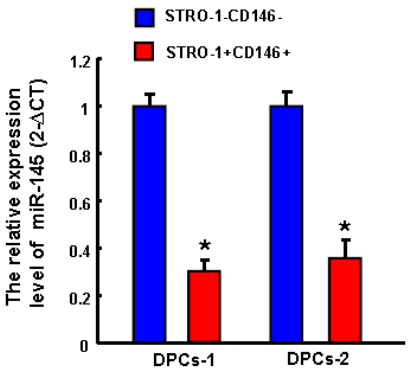
Up-regulated miRNAs in STRO-1+CD146+ DPSC

miRNAs	Fold changes	*p* value
has-miR-302	4.14	0.024
has-miR-372	3.89	0.048
has-miR-451	4.03	0.039
has-miR-203	3.79	0.031
has-miR-134	4.04	0.042

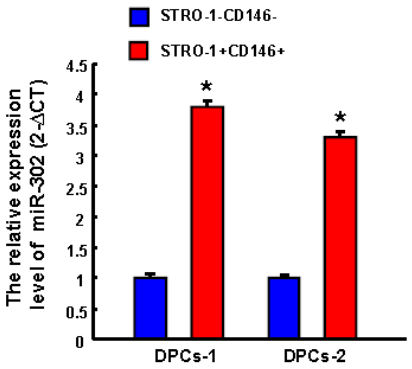
Down-regulated miRNAs in STRO-1+CD146+ DPSC

miRNAs	Fold changes	*p* value
has-miR-145	2.84	0.021
has-miR-20a	2.49	0.029
has-miR-126	2.53	0.032
has-miR-29	2.39	0.034
has-miR-4405	2.42	0.028

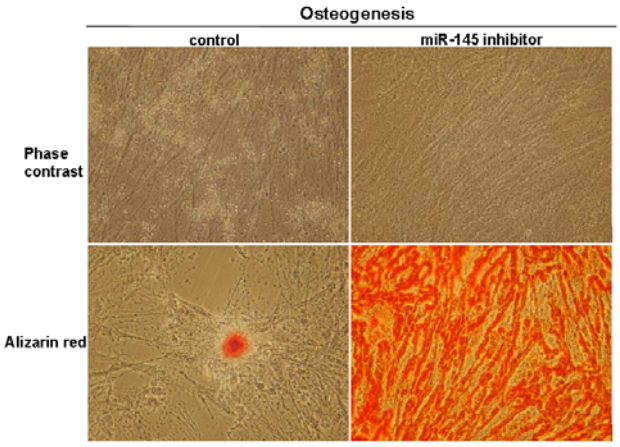
B



C

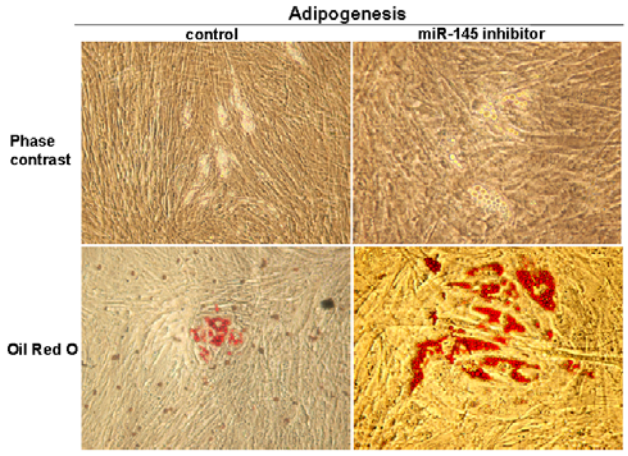


2. Depletion of miR-145 enhanced osteogenic properties in dental pulp cells. Representative image of osteogenesis capability of control or miR-145-knockdown DPC cells when cultured with osteogenic induction differentiation medium.



3. Depletion of miR-145 increased adipogenic potentials in dental pulp cells

Representative image of osteogenesis capability of control or miR-145-knockdown DPC cells when cultured with adipogenic induction differentiation medium.

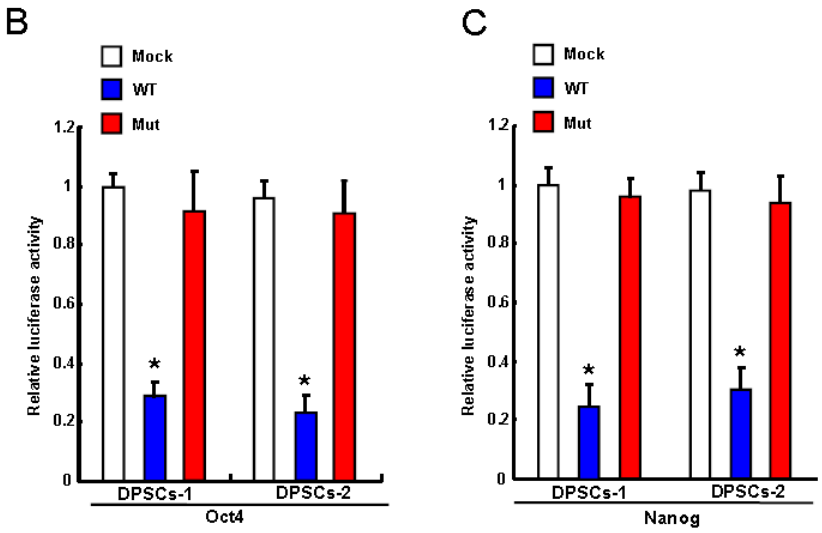


4. miR-145 directly targets Oct4 and Nanog in DPSCs. (A) miR-145 target sites were predicted within the 3'UTR regions of Oct4 and Nanog using Target Scan. We then constructed 3'UTR reporter plasmids for Oct4

(B) and Nanog (C) containing wild-type, deleted, and mutated forms of the miR-145 target sequences. The wild-type and deleted forms of the Oct4 (B) and Nanog (C) reporters were co-transfected with miR-145 or empty vector into DPSCs. The results of the luciferase assays indicated that only WT reporter activity of Oct4 (B) and Nanog (C) was inhibited by miR-145.

A

Gene	3'UTR Position	Target Site(s)	
Oct4	138-157	5′... AGGGGAGUUUGGGGCAACUGGUU...3′ 3′ UCCCUAAGGACCCUUUUGACCUG 5′ 5′... A GGAGUUU G GCAA U UU...3′	(wild type) (miR-145) (mutant)
Nanog	764-790	5′... CCUAAGGACC-CUU---UUGACCU...3′ 3′ GGAUGCCUGGUGAACCCGACUGGG 5′ 5′... UAA ACC- ---U GA U...3′	(wild type) (miR-145) (mutant)



General Materials and Methods

Tissue collection and isolation of DPSCs

Dental pulp tissues were freshly derived from a caries-free intact premolar that was extracted for orthodontic treatment purpose. All of the clinical samples in this study were collected after obtaining written informed consent and this study was reviewed and approved by the Institutional Review Board in Chung Shan Medical University Hospital. . Dental pulp tissue was immersed in phosphate buffered saline (Caisson, North Logan, UT, USA) solution and enzymatically dissociated into single cells. These cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin-G and 100 μg/mL streptomycin. (GIBCO BRL, Grand Island, NY, USA).

Quantitative Real-Time Reverse-Transcriptase (RT)-PCR

Briefly, total RNA (1 μg) of each sample was reverse-transcribed in a 20-μL reaction using 0.5 μg oligo(dT) and 200 U Superscript II RT (Invitrogen, Carlsbad, CA, USA). The amplification was carried out in a total volume of 20 μL containing 0.5 μM of each primer, 4 mM $MgCl_2$, 2 ML LightCycler™–FastStart DNA Master SYBR green I (Roche Molecular Systems, Alameda, CA, USA) and 2 μL of 1:10 diluted cDNA. PCR reactions were prepared in duplicate and heated to 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing at 55°C for 5 s, and extension at 72°C for 20 s. Standard curves (cycle threshold values *versus* template concentration) were prepared for each target gene and for the endogenous reference (GAPDH) in each sample. Quantification of unknown samples was performed using LightCycler Relative Quantification Software version 3.3 (Roche Molecular Systems). Primer sequences are listed in Table 1.

Table 1 The sequences of the primers for quantitative RT-PCR

Gene (Accession No.)	Primer Sequence (5' to 3')	Product size (bp)	Tm (°C)
ALP	F: CCACGTCTTCACATTTGGTG	99	60
(NM_000478)	R: ATGGCAGTGAAGGGCTTCTT		
DSPP	F: TCACAAGGGAGAAGGGAATG	187	60
(NM_014208)	R: CTGGATGCCATTTGCTGTGA		
OCN	F: GGCAGCGAGGTAGTGAAGAG	160	60
(NM_199173)	R: GCCGATAGGCCTCCTGAAAG		
BSP	F: AAAGTGAGAACGGGGAACCT	95	60
(NM_004967)	R: ACCATCATAGCCATCGTAGCC		
GAPDH	F: CATCATCCCTGCCTCTACTG	180	60
(NM_002046)	R: GCCTGCTTCACCACCTTC		

MicroRNA isolation and miRNA quantitative real-time reverse-transcriptase (RT)-PCR

A mirVana PARIS kit (Ambion) will be used to isolate miRNA from the total RNA according to the manufacturer's instructions. For miRNAs detection, qRT–PCR will be performed by using TaqMan miRNA assays (Applied Biosystems) with specific primer sets. All reagents and protocols will be from Applied Biosystems and detection will be performed by using a 7900HT fast real-time PCR system using RNU6B as internal controls. miRNA specific qRT-PCR will be done in triplicate and repeated three times on the ABI

Prism 7700 Sequence Detector system (Applied Biosystems).

Alkaline Phosphatase Activity (ALP)

For detecting the ALP activity of cells on original plates, cells were fixed with 80% alcohol, and then fixed cells were stained using the Vector Blue Alkaline Phosphatase Substrate Kit III (Vector Laboratories Inc., Burlingame, CA, USA) according to the manufacturer's instructions.

In Vitro Osteogenic Differentiation

Cells were cultured in DMEM-LG (Invitrogen) supplemented with 15% FBS, 50 μg/mL ascorbate-2-phosphate, 10 nmol/L dexamethasone, and 10 mmol/L β-glycerophosphate (Sigma, St. Louis, MO, USA) for 2 weeks. At the end of osteogenic induction, cells were washed twice with PBS, fixed for 10 min at room temperature with 3.7% paraformaldehyde, and stained with Alizarin red to assess osteogenic differentiation.

In Vitro Chondrogenic Differentiation

Cells were cultured in DMEM High Glucose supplemented with ITS + Premix: 6.25 μg/mL insulin, 6.25 μg/mL transferrin, 5.33 μg/mL linoleic acid, 1.25 μg/mL bovine serum albumin, 10^{-7} M dexamethasone, 50 μg/mL ascorbate-2 phosphate, 1 μM sodium pyruvate, 100 μg/mL penicillin and

10 μg/mL streptomycin) (Sigma) in the presence of TGF-β1 (10 ng/mL) (Mylteny, Bergisch, Gladbach, Germany) for 2 weeks. At the end of chondrogenic induction, cells were washed twice with PBS, fixed for 10 min at room temperature with 3.7% paraformaldehyde, and stained with Alcian Blue to assess chondrogenic differentiation.

In Vitro Adipogenic Differentiation

Cells were cultured in α-MEM supplemented with 16.6% FBS, 50 μg/mL ascorbate-2 phosphate, 10^{-7} M dexamethasone, 50 μM indomethacin, 0.45 mM 3-isobutyl-1-methyl-xanthine and 10 μg/mL insulin for 2 weeks. At the end of adipogenic induction, cells were washed twice with PBS, fixed for 10 min at room temperature with 3.7% paraformaldehyde, and stained with Oil Red O to assess adipogenic differentiation.

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科技部補助計畫衍生研發成果推廣資料表

日期:2016/10/31

科技部補助計畫	計畫名稱: 微型RNA調控牙髓幹細胞分化及血管生成潛能的分子機制研究
	計畫主持人: 周明勇
	計畫編號: 104-2314-B-040-014- 學門領域: 牙醫

無研發成果推廣資料

104年度專題研究計畫成果彙整表

計畫主持人：周明勇	**計畫編號：**104-2314-B-040-014-
計畫名稱：微型RNA調控牙髓幹細胞分化及血管生成潛能的分子機制研究	

成果項目					量化	單位	質化 （說明：各成果項目請附佐證資料或細項說明，如期刊名稱、年份、卷期、起訖頁數、證號...等）
國內	學術性論文	期刊論文			0	篇	
		研討會論文			0		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		
	技術移轉	件數			0	件	
		收入			0	千元	
國外	學術性論文	期刊論文			0	篇	
		研討會論文			0		
		專書			0	本	
		專書論文			0	章	
		技術報告			0	篇	
		其他			0	篇	
	智慧財產權及成果	專利權	發明專利	申請中	0	件	
				已獲得	0		
			新型/設計專利		0		
		商標權			0		
		營業秘密			0		
		積體電路電路布局權			0		
		著作權			0		
		品種權			0		
		其他			0		

	技術移轉	件數	0	件	
		收入	0	千元	
參與計畫人力	本國籍	大專生	0	人次	
		碩士生	0		
		博士生	0		
		博士後研究員	0		
		專任助理	0		
	非本國籍	大專生	0		
		碩士生	0		
		博士生	0		
		博士後研究員	0		
		專任助理	0		
其他成果 （無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。）			研究成果整理中，準備投稿至國際學術期刊		

科技部補助專題研究計畫成果自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現（簡要敘述成果是否具有政策應用參考價值及具影響公共利益之重大發現）或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估
   ■達成目標
   □未達成目標（請說明，以100字為限）
   　□實驗失敗
   　□因故實驗中斷
   　□其他原因
   說明：

2. 研究成果在學術期刊發表或申請專利等情形（請於其他欄註明專利及技轉之證號、合約、申請及洽談等詳細資訊）
   論文：□已發表　□未發表之文稿　■撰寫中　□無
   專利：□已獲得　□申請中　■無
   技轉：□已技轉　□洽談中　■無
   其他：（以200字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性，以500字為限）
   本研究成果能了解微型RNA在牙髓幹細胞調控自我更新及分化潛能分子機制，期待研究結果將有助於促進牙髓幹細胞應用於再生牙醫學。

4. 主要發現
   本研究具有政策應用參考價值：■否　□是，建議提供機關
   （勾選「是」者，請列舉建議可提供施政參考之業務主管機關）
   本研究具影響公共利益之重大發現：■否　□是
   說明：（以150字為限）