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研究課題名（和文）Dentin regeneration by dental pulp stem cells using nephronectin

研究課題名（英文）Dentin regeneration by dental pulp stem cells using nephronectin

研究代表者
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研究成果の概要（和文）：本研究では、象芽細胞様細胞およびヒト歯髄幹細胞（hDPSC）に対するネフロネクチンの添加効果を調べ、以下のような結果を得た。即ち、ネフロネクチン0.1～10μg／mLで表面をコーティングしたポリスチレン製培養容器でMDPC-23細胞を培養したところ、何もコーティングしていない培養容器で培養したものと比べて、以下の結果が得られた。1）濃度依存的（特に10μg／mL）に細胞の増殖を促進した。2）細胞形態には変化は無かった。3）10μg／mLのコーティングで、細胞の増殖だけではなく細胞の分化も促進した（象牙質関連の諸遺伝子の発現、および石灰化の指標であるアルカリホスファターゼの発現が上昇した）。

研究成果の学術的意義や社会的意義
本研究の目的は、歯髄保存療法において用いることができる、水酸化カルシウムに比べて安全かつ強力に象牙質再形成を誘導する生体親和性の高い新たな材料を提供することである。今回、ネフロネクチンが象牙芽細胞とヒト歯髄幹細胞の増殖と分化を促進することを見出し、この知見に基づいて本研究を完成させた。結果によれば、水酸化カルシウムに比べて安全かつ強力に象牙質再形成を誘導する生体親和性の高い新たな材料を提供することができ、この材料は、う蝕治療剤、特に歯髄保存療法におけるう蝕治療剤として有効である。

研究成果の概要（英文）：Nephronectin (Npnt) is a novel RGD-containing extracellular matrix protein originally discovered in mouse embryonic kidney that is expressed in a variety of embryonic and adult tissues including tooth germ. The effects of Npnt in odontoblasts or human dental pulp stem cells (hDPSCs) remain elusive. Our study showed Npnt in its coated form promoted the proliferation, differentiation and mineralization of MDPC-23 cells and DPSCs, providing new insight into the functional roles of Npnt in dentin regeneration.

研究分野：保存歯科

キーワード：nephronectin　dentin regeneration

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１．研究開始当初の背景

Traditional therapy of exposed teeth by direct pulp capping often involves the direct delivery of calcium hydroxide to the exposed site to promote wound healing. However, methods based on this approach suffer from undesirable side effects, including high alkalinity, porous dentin formation, and poor adhesion to dentin, which could lead to unsatisfactory clinical prognosis over time. In order to improve the success rate of pulp capping treatment and the life quality for patients, efforts have been ongoing attempting to identify novel biocompatible pulp capping reagents. Materials capable of inducing odontoblast proliferation and differentiation are particularly promising. An an RGD-containing cell adhesion protein, Npnt is capable of mediating adhesion and affecting the proliferation of a variety of cell types, such as mouse vascular endothelial cells, human fibrosarcoma cells, and human neuroglioma cells. Moreover, it was reported Npnt is expressed in the outer enamel epithelium, stellate reticulum, and the inner enamel epithelium of mouse tooth germ. Tooth development is a reciprocal process involving a mutual interaction between the epithelium and mesenchyme. Given the expression of Npnt in the inner enamel epithelium, under which odontoblasts are located, it is thus of interest to investigate how Npnt interacts with odontoblasts or stem cells derived from dental pulp. Elucidation on the roles of Npnt in tooth could be useful for direct pulp capping.

２．研究の目的

In this project, we investigated the effects of Npnt on the proliferation, differentiation and mineralization of odontoblast-like cells and human dental pulp stem cells.

３．研究の方法

Cell culture

MDPC-23 cells (Passage 20-30) and human dental pulp stem cells (hDPSCs) were used. The cells were cultured in dulbecco modified eagle medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37°C, 5% CO2 in a humidified atmosphere. The media were changed every other day.Recombinant Npnt was purchased from R&D systems, reconstituted in phosphate buffered saline (PBS) at a stock concentration of 100μg/mL and used freshly. The control group is defined as cells that were treated only with vehicle (PBS).

Npnt coating on Non-tissue culture polystyrene

Non-tissue culture polystyrene plates (96-well plate,12-well plate) coated with Npnt were prepared by soaking the plates in 10μg/mL Npnt. After 2hrs, the solution was aspirated and the wells were washed twice with PBS. The cells were rinsed with PBS, trypsinized, and

seeded into each plate.Mineralization reagents (10mmol/L β-glycerophosphate and 50 μg/mL ascorbic acid; Wako) was added from day 5 on.

CCK-8 assay

After incubation of cells for 24 hours, CCK-8 reagent (10μL/well) was added to each well at prescribed time points and incubated for 1 hour 45 minutes, then, the adsorbance was read at 450 nm.

ALP activity

Cells were removed from plates using Triton-X 100 (0.1%, w/w, in distilled water) (Sigma-Aldrich) and sonicated (Bioruptor Diagenode) for 10 minutes on ice. The lysates were centrifuged for 15 minutes at 10,483*g* at 4°C (Hitachi Koki). The resulting supernatant was diluted and assayed for ALP activity (Wako) and BCA protein quantification (Thermo Scientific) according to the manufacturer's instructions. Absorbance was read at 405 nm and 570 nm for ALP assay and protein assay, respectively.

Alizarin red staining

Cells were fixed using 10% neutral buffer formalin (Wako) for 20 minutes, then, the cell monolayer was stained with alizarin red S solution (1%, pH 4.1, Wako). The staining intensity was quantified using cetylpyridinium chloride (CPC, 10%, Sigma-Aldrich).

Real time RT-PCR

Total RNA was isolated using Trizol reagent (Invitrogen). Briefly, the cell monolayer was wahsed twice with PBS and homogenized by the addition of Trizol into culture plates at a proper volume.RNA was separated from protein and DNA using chloroform (08402-55, Nacalai Tesque), precipitated with 2-propanol (or isopropanol) (29113-95, Nacalai Tesque), and washed with ethanol (75% in water) (051-00476, Wako). The purified RNA was resuspended in RNase- and DNase-free water (10977-015, Invitrogen) and incubated at 55°C for 15 minutes to allow complete dissolution. The purified RNA was used for complementary DNA (cDNA) synthesis or stored at -80°C. The messenger RNA (mRNA) levels of ALP, DMP-1, BSP, Runx-2, OCN, OPN etc., were measured using a quantitative reverse-transcription polymerase chain reaction (RT-PCR) machine (LightCycler, Nano).

４．研究成果

1) Npnt in its coated form promoted the proliferation, differentiation and mineralization of MDPC-23 cells.

2) Npnt facilitates hDPSCs adhesion and spreading to hydrophobic nontissue culture plates. The adhesion of hDPSCs to Npnt was partially mediated by RGD. Moreover, Npnt acts as a bioactive signal to initiated the upregulation of ITGA1, ITGA4, ITGA7, and ITGB1 as early as day five. Further, mRNA expression of DSPP was slightly downregulated by Npnt,

while BSP was significantly enhanced. Finally, late stage mineralization was significantly enhanced in cells cultured on Npnt.

５．主な発表論文等

〔雑誌論文〕　計6件（うち査読付論文　6件／うち国際共著　0件／うちオープンアクセス　4件）

１．著者名	４．巻
Tang J, Saito T.	44
２．論文標題	５．発行年
iMatrix-511 stimulates the proliferation and differentiation of MDPC-23 cells into odontoblast-like phenotype	2018年
３．雑誌名	６．最初と最後の頁
Journal of Endodontics	1367-1375
掲載論文のDOI（デジタルオブジェクト識別子）	査読の有無
https://doi.org/10.1016/j.joen.2018.05.018	有
オープンアクセス	国際共著
オープンアクセスではない、又はオープンアクセスが困難	－

１．著者名	４．巻
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Archives of Oral Biology	54～61
掲載論文のDOI（デジタルオブジェクト識別子）	査読の有無
https://doi.org/10.1016/j.archoralbio.2018.06.017	有
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https://doi.org/10.1155/2017/2546261	有
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３．雑誌名	６．最初と最後の頁
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掲載論文のDOI（デジタルオブジェクト識別子）	査読の有無
https://doi.org/10.1155/2018/2370438	有
オープンアクセス	国際共著
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1．著者名 Jia Tang, Takashi Saito	4．巻 Advance Publication
2．論文標題 Laminin-1 acts as an adhesive for odontoblast-like cells and promotes their differentiation toward a hard tissue-forming phenotype	5．発行年 2018年
3．雑誌名 Journal of Oral Science	6．最初と最後の頁 1-9
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オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 －

1．著者名 Jia Tang, Takashi Saito	4．巻 2018
2．論文標題 A novel fragment derived from laminin-411 facilitates proliferation and differentiation of odontoblast-like cells	5．発行年 2018年
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3．学会等名 International Collaborative Symposium on Development of Human Resources in Practical Oral Health and Treatment, Jakarta, Indonesia（国際学会）
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1．発表者名 Jia Tang, Takashi Saito
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1．発表者名 Jia Tang, Takashi Saito
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〔図書〕　計0件

〔出願〕　計1件

産業財産権の名称	発明者	権利者
ラミニン断片を含有する歯の象牙質及び/又は歯髄の疾患、障害又は症状を治療又は予防するための医薬	唐佳、斎藤隆史	同左
産業財産権の種類、番号	**出願年**	**国内・外国の別**
特許、特願2018-67591	2018年	国内

〔取得〕　計0件

〔その他〕

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6．研究組織

	氏名 （ローマ字氏名） （研究者番号）	所属研究機関・部局・職 （機関番号）	備考