Stem Cell Research 15 (2015) 290–298

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr

BMP4 and FGF strongly induce differentiation of mouse ES cells into oral ectoderm

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ARTICLE INFO

Article history:
Received 27 January 2015
Received in revised form 8 June 2015
Accepted 30 June 2015
Available online 2 July 2015

Keywords:
Embryonic stem cells
BMP4
FGF
Adenohypophysis
Dental primordium

ABSTRACT

During embryonic development, oral ectoderm differentiates into the adenohypophysis, dental epithelia, salivary glands, and nasal pit. Few reports exist concerning the induction of oral ectoderm from embryonic stem (ES) cells. Generally, any lot differences in fetal bovine serum (FBS) and serum replacer may affect the induction of ES cell-differentiation. Using a previously established culture strategy for differentiation, the proportion of cell aggregates containing Pitx1+ oral ectoderm varied widely between 9–36% when several different lots of FBS or serum replacer were used. We therefore tried to enhance the differentiation method. We found that bone morphogenetic protein (BMP) 4 and fibroblast growth factor (FGF) treatments improved oral ectoderm induction. Such treatment also improved the differentiation of oral ectoderm into the adenohypophysis. Furthermore, increased BMP4 treatment induced dental epithelium and mesenchyme. Such differentiation suggests that the Pitx1+ layer displays similar properties to oral ectoderm, as found in vivo. Differentiation of ES cells into oral ectoderm using different lots of FBS and serum replacer increased 78–90% after treatment with BMP4 and FGF. In summary, we have established a robust strategy for the induction of oral ectoderm differentiation from mouse ES cells.

1. Introduction

During early embryogenesis, ectoderm differentiates into surface ectoderm, neural crest, and neural tube. From the surface ectoderm, oral ectoderm and skin develop [1]. In turn, the oral ectoderm differentiates into the adenohypophysis [2,3], dental epithelia [1], salivary glands [1], and nasal pit [4]. The adenohypophysis' anlage originates as a placode in oral ectoderm and lies adjacent to the anterior neural plate [2,3]. The development of the adenohypophysis depends on tissue interaction between the oral ectoderm and ventral hypothalamus [2,3,5,6].

To our knowledge, our previous report [7] appears to be the only one to demonstrate the induction of oral ectodermal tissues from mouse ES cells: mouse ES cells were stimulated to differentiate into both oral ectoderm and hypothalamic neuroectoderm in adjacent layers within an aggregate culture. Self-organization of the adenohypophysis occurred at the interface of these two epithelia, as seen in vivo. Various endocrine cells, including corticotrophs, were subsequently produced.

Oral ectoderm differentiates into not only the adenohypophysis, but also dental epithelium. During dental regeneration, a previous study described co-culture with ameloblasts (cells derived from oral epithelium tissue which deposit tooth enamel) [8] differentiated mouse ES cells into dental, epithelial-like cells [9]. Salivary glands are also derived from oral ectoderm and, with regard to their regeneration, mouse ES cells differentiated into salivary gland cells when co-cultured with fibroblasts derived from the human salivary gland. After transplantation, the regenerative capabilities of the differentiated cells were confirmed [10]. However, a major problem with such co-culture methods is the difficulty of tissue-bed collection from human patients. This, therefore, necessitates the induction of differentiation by growth factor treatment, as opposed to co-culture systems.

ES cells are self-renewing cells, able to differentiate into the three somatic germ layers [11]. ES cells represent valuable sources of cells for applications in tissue engineering, cell therapy, and drug screening. While expanding stem cells in culture, it is critical to also maintain their self-renewing and differentiation potencies. However, the normal culture medium for mouse ES cells includes fetal bovine serum (FBS) and a serum replacer, such as KnockOut Serum Replacement (KSR®), both containing non-defined components. As a result, many studies reported that only selected lots of FBS were used [11]. Additionally, in a move more defined media formulations, most laboratories have replaced FBS with serum replacer, a serum free, proprietary formulation of KSR®, which, however,

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http://dx.doi.org/10.1016/j.scr.2015.06.011

still contains animal-derived proteins and is thus not fully defined; therefore, a lot check is still needed [12]. Although lot differences in FBS and KSR® may affect the efficiency of differentiation induction, few reports describe lot differences in detail. Here, we report a newly modified method of oral ectoderm induction, independent of lot differences in FBS and KSR®.

2. Materials and methods

2.1. ES cell culture for maintenance and differentiation

Mouse ES cells (EB5) were passaged in a maintenance medium of G-MEM supplemented with 1% FBS (Lot A: B247; Lot B: B466; Lot C: B467; Sigma, St. Louis, MO), 10% KSR®(Lot X: 1235217; Lot Y: 1241214; Lot Z: 1249477; Life Technologies, Carlsbad, CA, USA), 2 mM glutamine, 0.1 mM non-essential amino acids, 1 mM pyruvate, 0.1 mM 2-mercaptoethanol, 2000 U/mL leukemia inhibitory factor (LIF) and Blasticidin S 10 μg/mL. For SFEBq culture, mouse ES cells were dissociated to single cells in TrypLE™ Express® (1×) (Life Technologies) and quickly reaggregated in differentiation medium (10,000 cells per 100 μL per well, unless otherwise stated) using 96-well, low-cell-adhesion plates (Lipidure Coat, NOF Corp, Tokyo, Japan).

The differentiation medium was growth-factor free Chemically Defined Medium (gfCDM), which contains Iscove's modified Dulbecco's medium/Hams F12 1:1, 1× Chemically Defined Lipid Concentrate (CDLC®; Life Technologies), monothioglycerol (450 mM) and 5 mg/mL purified bovine serum albumin (BSA; > 99% purified by crystallization; Sigma). The day SFEBq culture was initiated was defined as day 0.

Growth factors were purchased from R&D Systems (Minneapolis, MN, USA), and used at the concentrations indicated in the text and legends. The concentrations and treatment times of the reagents applied to differentiation cultures were as follows: SAG (400 nM, d0-), bone morphogenetic protein (BMP) 4 (3 pM, d3-), fibroblast growth factor (FGF) 8b (250 ng/mL, d8-), and FGF10 (250 ng/mL, d8-). Apotransferrin (15 μg/mL, R&D Systems) was added to the protocol for adenohypophysis cells. KSR® (2%, d12-) was added to the protocol for ACTH-producing cells. KSR® (2%, d14-) was added to the protocol for Pit1 + cells. The oxygen concentration was raised to 40% from day 10 onwards. A half medium change was performed on days 11 and 14.

2.2. Immunohistochemistry (IHC)

Immunohistochemistry was performed as described previously [7].

For statistical analyses, all (typically 7–8) aggregates were examined in each experiment, which was repeated four times. Antibodies against the following proteins were used at the indicated dilutions: Lim3 (rabbit, 1:200; Chemicon, Billerica, MA, USA), N-cadherin (mouse monoclonal, 1:1000; BD Pharmingen, San Diego, CA, USA), E-cadherin (rat, 1:50; TaKaRa, Shiga, Japan), Rax (guinea pig, 1:2000; TaKaRa), Pit1 (mouse, 1:100; Santa Cruz, Dallas, TX, USA), ACTH (mouse, 1:200; Fitzgerald Industries, Acton, MA, USA), Brachyury (goat, 1:1000; R&D), Dlx2 (rabbit, 1:500; Abcam, Cambridge, MA, USA) and Pax9 (rat, 1:500; Abcam). Antiserum against Pitx1 was raised in guinea pigs against a synthetic peptide (DAFKGGMSLERLPEGLR1C; residues 2–18) and was not purified. Antiserum against Tbx19 was raised in rabbit against a synthetic peptide (MSELATQKAGEGTVSRLLNVVESELQAGREKGDPTEK1C; residues 1–37) and was affinity purified. DAPI was used to counterstain nuclei (Molecular Probes, Eugene, Oregon, USA). The immunostaining specificity of each antibody was confirmed by immunostaining with the appropriate embryonic tissues as a positive control under the experimental conditions used.

2.3. Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

qRT-PCR was performed on eight aggregates per sample using an Mx3000P Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA) and data were normalized to GAPDH expression. Primers used for qRT-PCR are listed in Supplementary Table S1.

2.4. Statistical analyses

Values shown on graphs represent the mean ± SE, unless otherwise described. Statistical significance was tested using JMP software (SAS, Cary, NC, USA). A two-group comparison was assessed by Student's t-test, and multiple group comparisons were assessed by Tukey's test (among each pair).

3. Results

3.1. FBS and KSR® lot differences in maintenance medium affects mES cell differentiation into oral ectoderm

Previously, we demonstrated that mouse ES cells could be induced to differentiate into oral ectoderm when they were cultured as floating aggregates (typically 10,000 cells per aggregate: large cell aggregation [LCA] culture), in a chemically defined medium lacking extrinsic growth factors (including no insulin [13,14]) and treated with the hedgehog agonist N-methyl-N9-(3-pyridinylbenzyl)-N9-(3-chlorobenzo[b]thiophene-2-carbonyl)-1,4-diaminocyclohexane (smoothened agonist; hereafter named SAG); this SAG-treated LCA-SFEBq/gfCDM culture was hereafter referred to as “the previous strategy” [7]. Because differences between lots of FBS and KSR® used in maintenance medium for passaging may affect the efficiency of differentiation induction, we assessed the effect of lot differences on the differentiation of mouse ES cells into oral ectoderm. Five kinds of maintenance media were prepared by a combination of three different lots of FBS and KSR® (Fig. 1A). Mouse ES cells were passaged for 14 days (7 times) and differentiated by the previous strategy (Fig. 1B). Markers marking a lack of differentiation, Oct3/4 and Nanog, were positive by immunostaining before the induction of differentiation (data not shown). We observed, on average, eight aggregates on day 10, and counted the proportion of aggregates that contained the Pitx1 +/E-cadherin + sequential layer as determined by IHC. E-cadherin is expressed on the surface of epithelial cells, and Pitx1 is a specific marker of oral ectoderm. IHC analysis showed that Pitx1 +/E-cadherin + oral ectoderm was induced in 9–36% of aggregates, depending on the medium used (Fig. 1C). Fig. 1D, F, G, H, and I all shows representative aggregates that failed to exhibit a Pitx1 +/E-cadherin + surface layer. These IHC results suggested that the combination of FBS and KSR® lots affected the differentiation induction efficacy. For example, more than 90% aggregates, grown in the No. 1 maintenance medium, expressed only a thin Pitx1 −/E-cadherin +/Cytokeratin + epithelial layer (Fig. 1D, J) on the surface (Cytokeratin is a specific marker of epithelium). A Rax +/Nkx2.1 +/Chx10 −/N-cadherin + ventral hypothalamic layer was observed inside each aggregate (Fig. 1E, K, L, M) (Rax is expressed in the hypothalamus and retina. Nkx2.1 is a specific marker of the ventral hypothalamus and ganglionic eminence. Chx10 is a retina marker. N-cadherin is a neuron marker.).

3.2. Mouse ES culture in BMP4 improves its differentiation into Pitx1 + oral ectoderm

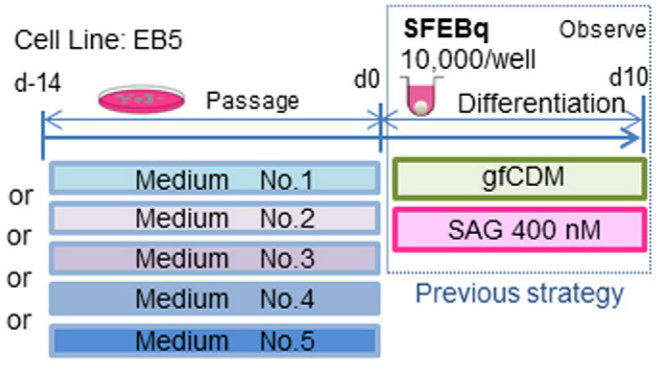
In vivo, the ventral hypothalamus expresses BMP4, FGF 8/10/18, and Wnt5a, while oral ectoderm, except in the primordium of the adenohypophysis, expresses sonic hedgehog (Shh). The opposing dorsal BMP4/FGF and ventral Shh gradient conveys proliferative and positional cues by regulating combinatorial patterns of transcription factor gene expression [2,6,15–20]. Of these growth factors, Shh and BMP4, especially, play an important role in the first step of pituitary primordium development [2,21].

The No. 1 maintenance medium was selected because it induced the lowest proportion of aggregates expressing Pitx1 (Fig. 1C). On day 7, when Pitx1 expression is initiated [7], aggregates have similar

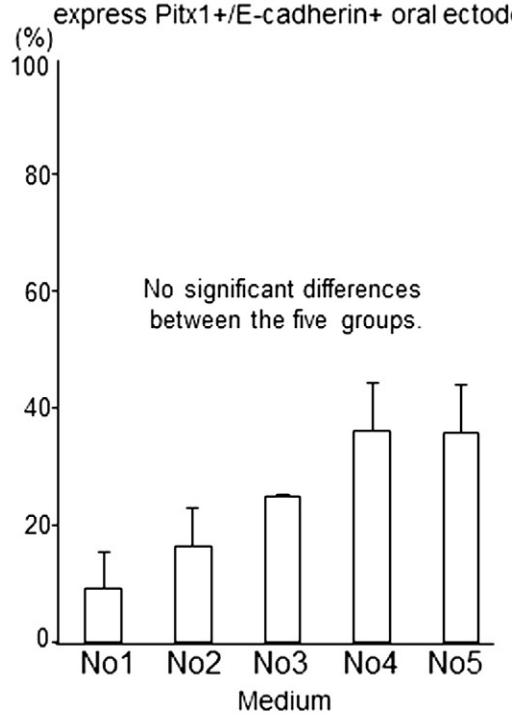
A

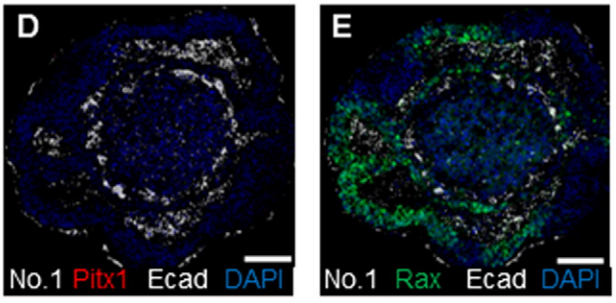
Maintenance Medium No.		KSR®		
		Lot X	Lot Y	Lot Z
FBS	Lot A	No.1		
	Lot B		No.2	No.4
	Lot C		No.3	No.5

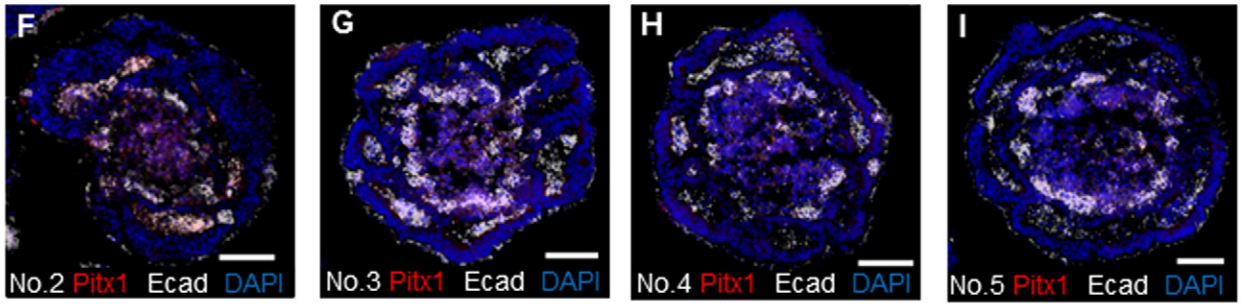
B

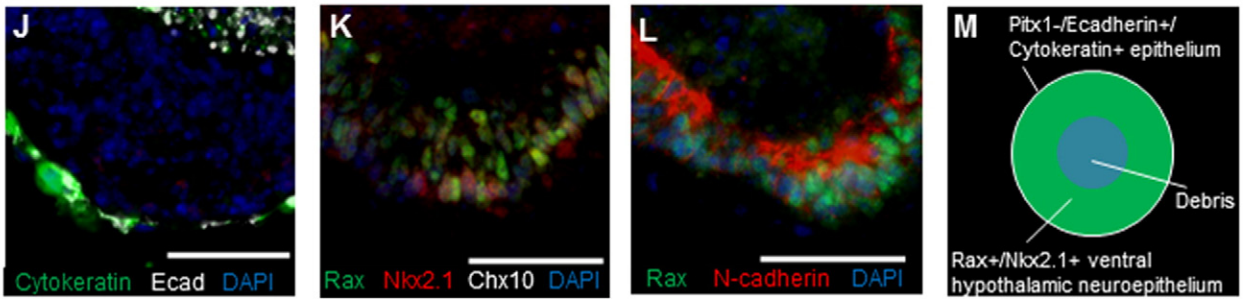


C The proportion of aggregates which express Pitx1+/E-cadherin+ oral ectoderm









characteristics to that of day 10 (Fig. 2A, B). We tested several growth factors (Shh, BMP4 and FGF8) known to affect early adenohypophysis generation (Fig. 2C). Whether cell number and nutrient components (e.g., BSA, CDLC®, and KSR®) in differentiation culture medium based on the previous strategy [7] improved differentiation efficiency were also examined. Seven or eight aggregates were observed on day 7, and the proportion of aggregates that expressed a Pitx1 +/E-cadherin + continuous layer was determined. As a result, BMP4 treatment (Fig. 2D) increased the differentiation of Pitx1 +/E-cadherin + layers characteristic of oral ectoderm (Fig. 2C).

BMP4 treatment (d2- or d3-, or more than 10 pM) promoted Pitx1 expression in oral ectoderm (Fig. 2E, G, I, K, M, arrowhead). With regard to hypothalamus induction, BMP4 treatment (d2-, or 10 pM) suppressed Rax (Fig. 2F, J, L, N). Collectively, in BMP4 (d3-, 3 pM)-treated cultures, IHC analysis showed a Pitx1 +/E-cadherin + thick layer juxtaposing a Rax + hypothalamus (Fig. 2G, H). Similarly, qPCR analysis showed that *Pitx2* (both *Pitx1* and *Pitx2* are expressed at E8.5 in the oral ectoderm [3]) expression was increased, but *Rax* expression was not inhibited (Fig. 2O, P). Thus, in BMP4 (d3-, 3 pM)-treated cultures, both Pitx1 + oral ectoderm and Rax + ventral hypothalamus formed continuous epithelia within the ES cell aggregate. This bilayer structure is similar to the adenohypophysis primordium and ventral hypothalamus in vivo.

3.3. FGF8 and FGF10 differentiate Pitx1 + oral ectoderm into adenohypophysis

In vivo, Pitx1 + oral ectoderm-derived Lim3 + (also called Lhx3) pituitary progenitors differentiate into several hormone-type-specific lineages [2,3,5,6,22,23] (Fig. 3A). In the SAG- (d0-, 400 nM) and BMP4- (d3-, 3 pM) treated LCA-SFEBq/gfCDM aggregates, both Pitx1 +/E-cadherin + oral ectoderm and Rax + ventral hypothalamus formed continuous epithelia in the aggregates. However, Lim3 + progenitors were not detected by IHC in this culture (data not shown). During mouse development, FGF8 and FGF10 are important during Lim3 + progenitor differentiation [2,5,6,16,24]. The addition of both FGF8 and FGF10 to SAG- and BMP4-treated LCA-SFEBq/gfCDM cultures (Fig. 3B) was required to efficiently increase the expression of Lim3 (Fig. 3C, E, F, K). Both a Lim3 +/Pitx1 + thick placode and a Rax + neural layer formed continuous epithelia (Fig. 3C, D). Lim3 +/Pitx1 + pouch epithelia, consisting of tall columnar cells, were located between the Rax + hypothalamus and Pitx1 +/E-cadherin + oral ectoderm (Fig. 3E, F), as also seen in vivo. Thus, in the presence of SAG, BMP4 and FGF8/10, the ES cell-derived oral ectoderm adjacent to the hypothalamic neuroepithelium differentiated into a Lim3 + Rathke's pouch. Further differentiation of the ES cell-derived oral ectoderm resulted in the expression of ACTH/Tbx19 (Fig. 3G, H, I, L) and Pit1 (Fig. 3J, M). The ACTH-producing corticotroph lineage requires the transcription factor Tbx19 [25]. Pit1 (also called Pou1f) is essential for the terminal differentiation and expansion of three lineages: somatotroph, lactotroph, and thyrotroph [26–28]. This demonstrates that the Lim3 + pouch epithelium induced by our strategy is the adenohypophysis progenitor.

3.4. Increased BMP4 allows mES cells to differentiate into both dental epithelium and dental mesenchyme

In vivo, dental epithelium also originates from the oral ectoderm [1]. Dental primordium is made from the interaction between oral ectoderm and dental mesenchyme [29–31]. Dental mesenchyme is derived from neural crest cells that arose from the ectoderm [32]. BMP4 induces differentiation of ectoderm into neural crest cells during gestation [33–36]. Similarly, in vitro, BMP4 treatment of SFEBq/gfCDM cultures induces differentiation of neural crest cells from mouse ES cells [37]. In this study, we found that an increased concentration of BMP4 (d3-, 30 pM; Fig. 3N), compared with that which induced adenohypophysis progenitors, caused the differentiation of Brachyury + neural crest cells in the outer layer of aggregates (Fig. 3O) (Brachyury is a neural crest cell marker). Furthermore, Dlx2 +/Pitx1 + dental epithelium-like cells were detected between the Pitx1 + oral ectoderm and Pax9 +/Dlx2 + dental mesenchyme (Fig. 3P) (Dlx2 is a marker of dental epithelium, dental mesenchyme and hypothalamus. Pax9 is a dental mesenchyme marker.).

3.5. BMP4 and FGF8/10 strongly induce mES cell differentiation into oral ectoderm

For SAG, BMP4 and FGF8/10-treated LCA-SFEBq/gfCDM cultures (hereafter referred to as the refined strategy), we assessed the effect of FBS and KSR® lot difference in regard to the differentiating efficacy of mouse ES aggregates into Pitx1 +/E-cadherin + oral ectoderm. As already mentioned in Fig. 1A, we prepared five kinds of maintenance media containing three different lots of FBS and KSR®. ES cells were passaged for 14 days and differentiation was assessed on day 10 (Fig. 4A). We found Pitx1 +/E-cadherin + oral ectoderm in 78–90% of aggregates cultured by the refined strategy (Fig. 4B, black columns). In all combinations of FBS and KSR® lots, the proportion of aggregates containing oral ectoderm increased in the refined strategy as compared to the previous strategy. Aggregates grown with the previous strategy expressed a thin Pitx1 −/E-cadherin + layer (Fig. 4C). In contrast, aggregates grown under the refined strategy expressed a thick, continuous Pitx1 +/E-cadherin + layer (Fig. 4D, E, F, G, H).

In conclusion, our results show that BMP4 and FGF8/10 strongly induced differentiation of mouse ES cells into oral ectoderm, and this differentiation method was not affected by lot differences between FBS and KSR®.

4. Discussion

In this study, we have demonstrated that BMP4 addition to the previous strategy [7] increased the differentiation efficacy of mouse ES cells into Pitx1 + oral ectoderm. The induction of mouse ES cells using the previous strategy resulted in only a thin, Pitx1-/E-cadherin + epithelium after using different lots of FBS and KSR®. This thin, oral epithelium resembled the oral ectoderm formation seen in the BMP4 KO mouse at E 9.5 [2]. Therefore, we speculated that BMP4 was the key molecule for the differentiation of mES cells into a thick, Pitx1 +/E-cadherin + oral ectoderm. In the meanwhile, BMP4 has been reported to suppress neural differentiation [38]. Of interest, in this study, we demonstrated that appropriate BMP4 treatment changed mES cell differentiation from a thin, Pitx1- layer to a thick, Pitx1 + layer, characteristic of oral ectoderm, while any inhibition of hypothalamic tissues was limited. A high concentration (Fig. 2F, J, N) and early dose of BMP4 (Fig. 2L) suppressed Rax.

Subsequently, both FGF8b and FGF10 treatments were found to initiate Lim3 + adenohypophysis progenitor induction. The Pitx1 + oral ectoderm and Rax + hypothalamus of aggregates in BMP4-treated culture were similar to the oral ectoderm and hypothalamus seen in the TTF-1 KO mouse [2]. In such an embryo, *Bmp4* expression was

Fig. 1. FBS and KSR® lot differences affect the efficacy of the previous strategy. (A) Five kinds of maintenance media prepared by a combination of three different lots of FBS and KSR. (B) A culture strategy to test the five kinds of maintenance media. After EB5 mES cells were passaged for 14 days, they were then cultured under the previous strategy. (C) The proportion of aggregates, which express oral ectoderm on day 10. Values shown on graphs represent the mean ± s.e.m. n = 4 individual batched experiments. No significant differences between five groups of media. (D–I) Immunostaining of day 10 aggregates cultured in each numbered medium, for Pitx1 (red), Rax (green) and E-cadherin (white). (J) Immunostaining of day 10 aggregates for Cytokeratin (green), and E-cadherin (white). (K) Immunostaining of day 10 aggregates for Rax (green), Nkx2.1 (red), and Chx10 (white). (L) Immunostaining of day 10 aggregates for Rax (green) and N-cadherin (red). (M) Schematic of immunostaining results. Scale bars, 100 μm. Ecad: E-cadherin. Nuclei are stained by DAPI (blue).

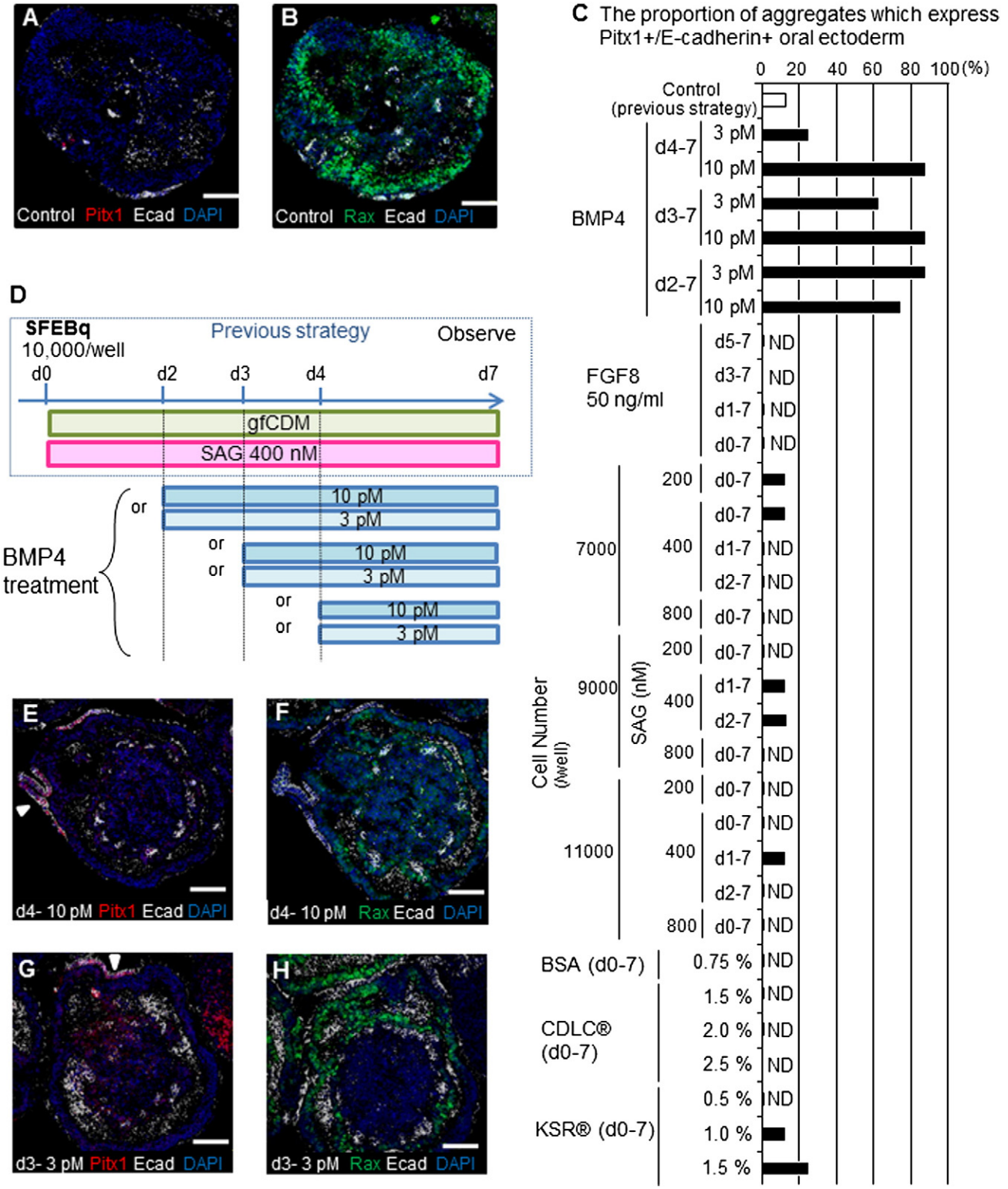


Fig. 2. BMP4 promotes differentiation into oral ectoderm. (A, B) Immunostaining of the day 7 aggregates cultured under the previous strategy, for Pitx1 (red), Rax (green) and E-cadherin (white). (C) The proportion of aggregates which displayed oral ectoderm under several different culture conditions on day 7. BMP4 increased oral ectoderm. n = 1. ND, not detectable. (D) BMP4-treated culture protocols. (E–N) Immunostaining of the day 7, BMP4 treated aggregates for Pitx1 (red), Rax (green) and E-cadherin (white). Arrowheads indicate Pitx1+/E-cadherin+ oral ectoderm. (O, P) *Pitx2* and *Rax* expression, as determined by qRT-PCR, in aggregates cultured under the previous strategy (control: white) or with BMP4 (d3-, 3 pM; black). n = 3 individual batched experiments. The values show on graphs represent the mean ± s.e.m. NS, not significant. $^{**}P < 0.01$. Scale bars, 100 μm. Ecad: E-cadherin. Nuclei are stained by DAPI (blue).

maintained, whereas *Fgf8* and *Fgf10* expressions were deleted in the hypothalamus at E 10.5. The pouch rudiment was initially formed but it was then eliminated before the formation of a definitive pouch, which expressed *Lim3*. In addition, it has been shown that FGF8 promotes *Lim3* expression in a primary culture of mouse pituitary primordium at E 10 [16]. Since the ligand specificity of FGF receptors differs for FGF8 and FGF10 [39], we therefore thought that both FGF8 and FGF10 were important in Lim3+ pituitary differentiation. Our results show

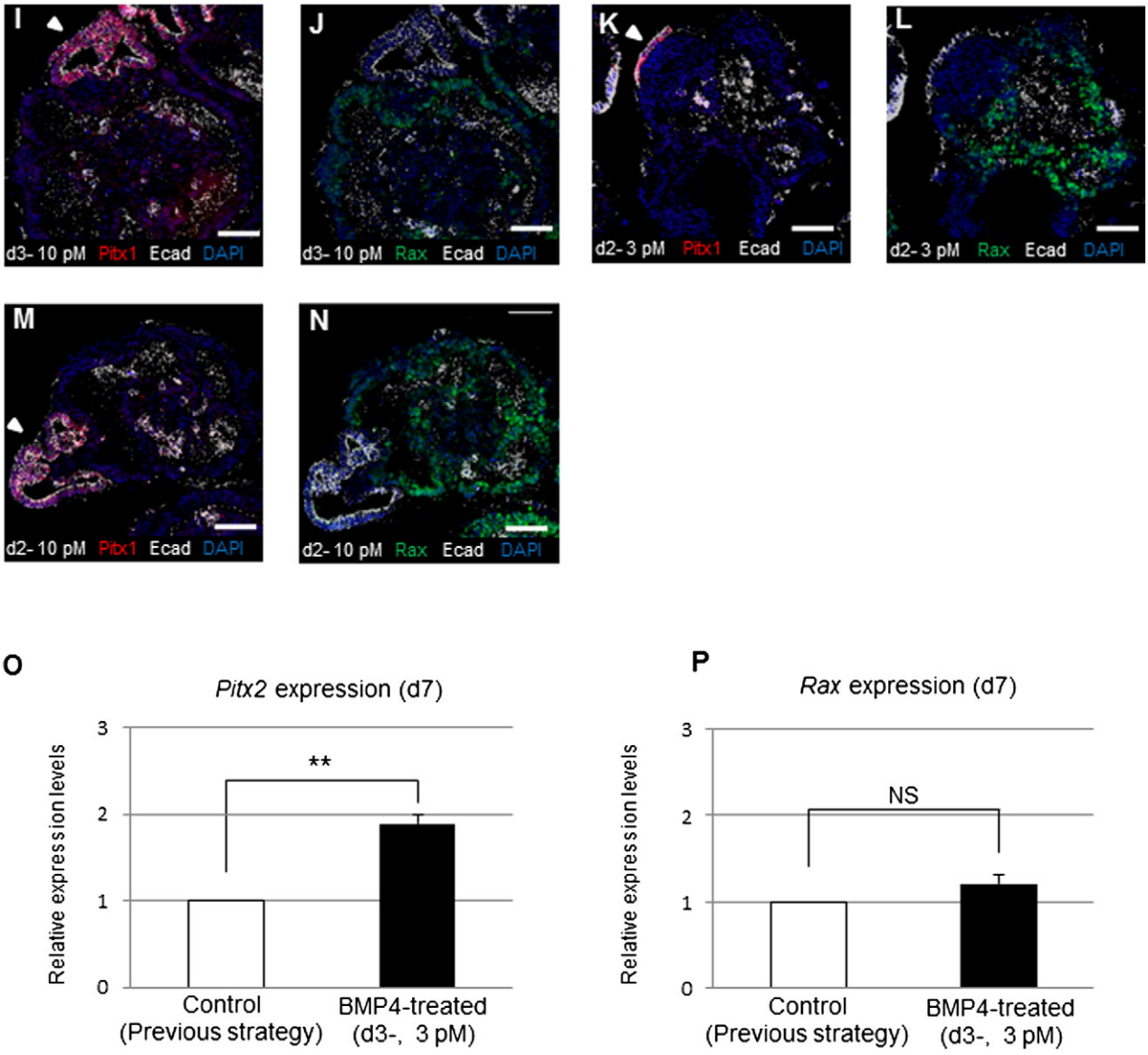


Fig. 2 (*continued*).

that both FGF8 and FGF10 induced Pitx1 + oral ectoderm to differentiate into Lim3 + pituitary progenitors, and revealed that the Pitx1 + layer of aggregates possesses similar properties to oral ectoderm in vivo.

We have established the world's first induction method to differentiate mouse embryonic stem cells into dental epithelium using a cocktail of signaling reagents. Moreover, this method induces not only dental epithelium but also dental mesenchyme, the interactions of which are important during teeth development. Such a newly described induction method may contribute to tooth regeneration.

Fig. 1 shows hardly any Pitx1 + cells in aggregates, which differs markedly from what we reported in a previous paper [7]. This dramatic difference in Pitx1 induction efficiency is because culture conditions were not strictly the same between this report and the previous paper [7]. For each passage, the medium differed biochemically. Since media contained FBS® and KSR® which are derived from animals, they would be expected to show individual differences in various growth factors and cytokines [11,12]. We postulate that FBS® and KSR® lot differences between this report and the previous paper [7] may affect induction efficiency. Because serum lots differ within the laboratory, when replicating new induction methods, it is important to confirm the effect of lot differences in assessing the reproducibility of the induction method, with the view that a modification of induction protocols may be warranted.

5. Conclusion

We confirmed that the addition of BMP4 and FGF to the previous strategy strongly induced the differentiation of mouse ES sells into oral ectoderm. The resultant, refined strategy has increased its efficacy in the induction of differentiation as compared with the previous strategy, regardless of any FBS and KSR lot difference.

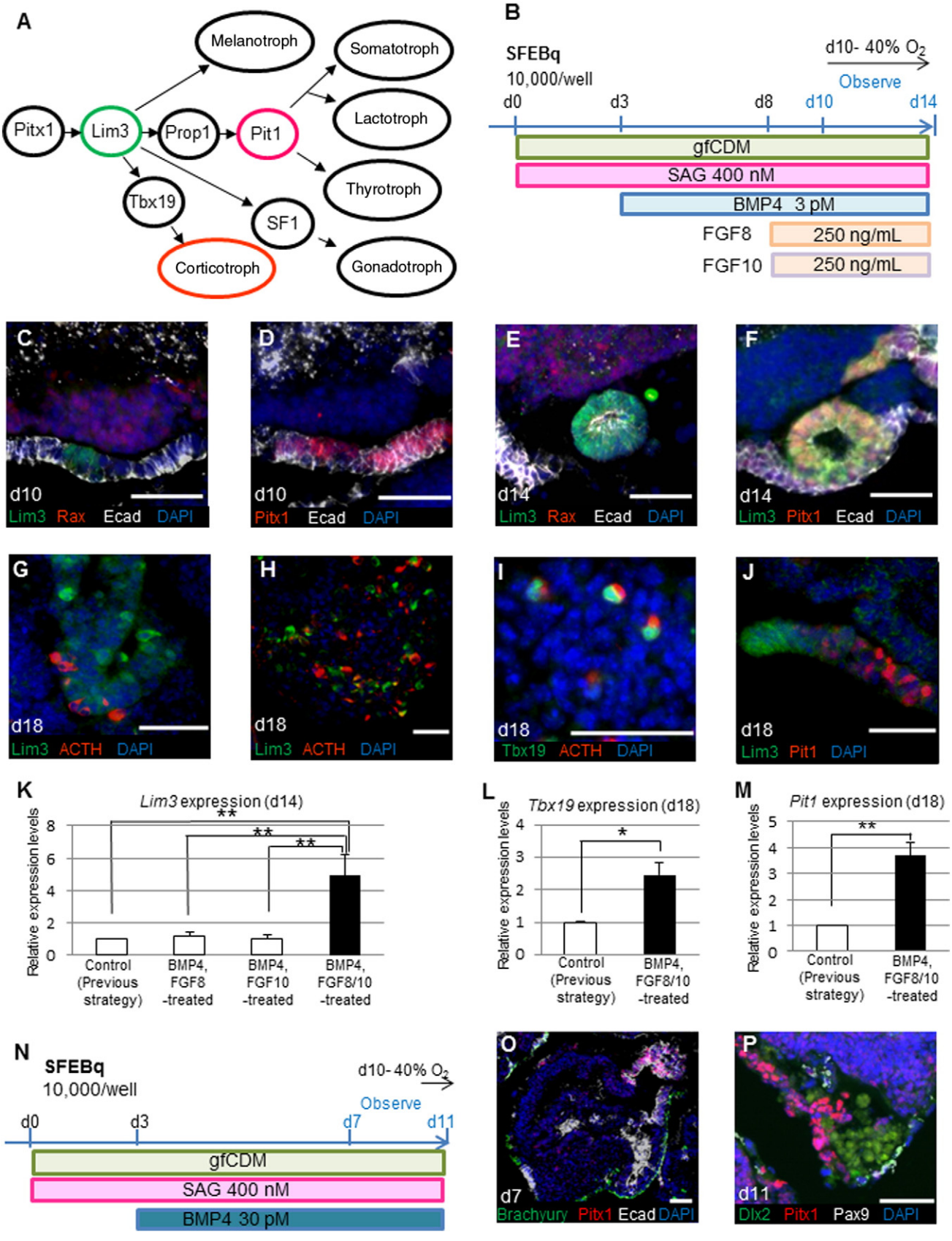
Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scr.2015.06.011.

Disclosure of potential conflicts of interest

None.

Grants

This work was supported by the following projects: Projects for Technological Development (H.S.) of the Research Center Network for Realization of Regenerative Medicine from the Japan Science and Technology Agency (JST, grant no. 15bm0404018h0003) and grants-in-aid (no. 24790955) for Scientific Research (H.S.) from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT).



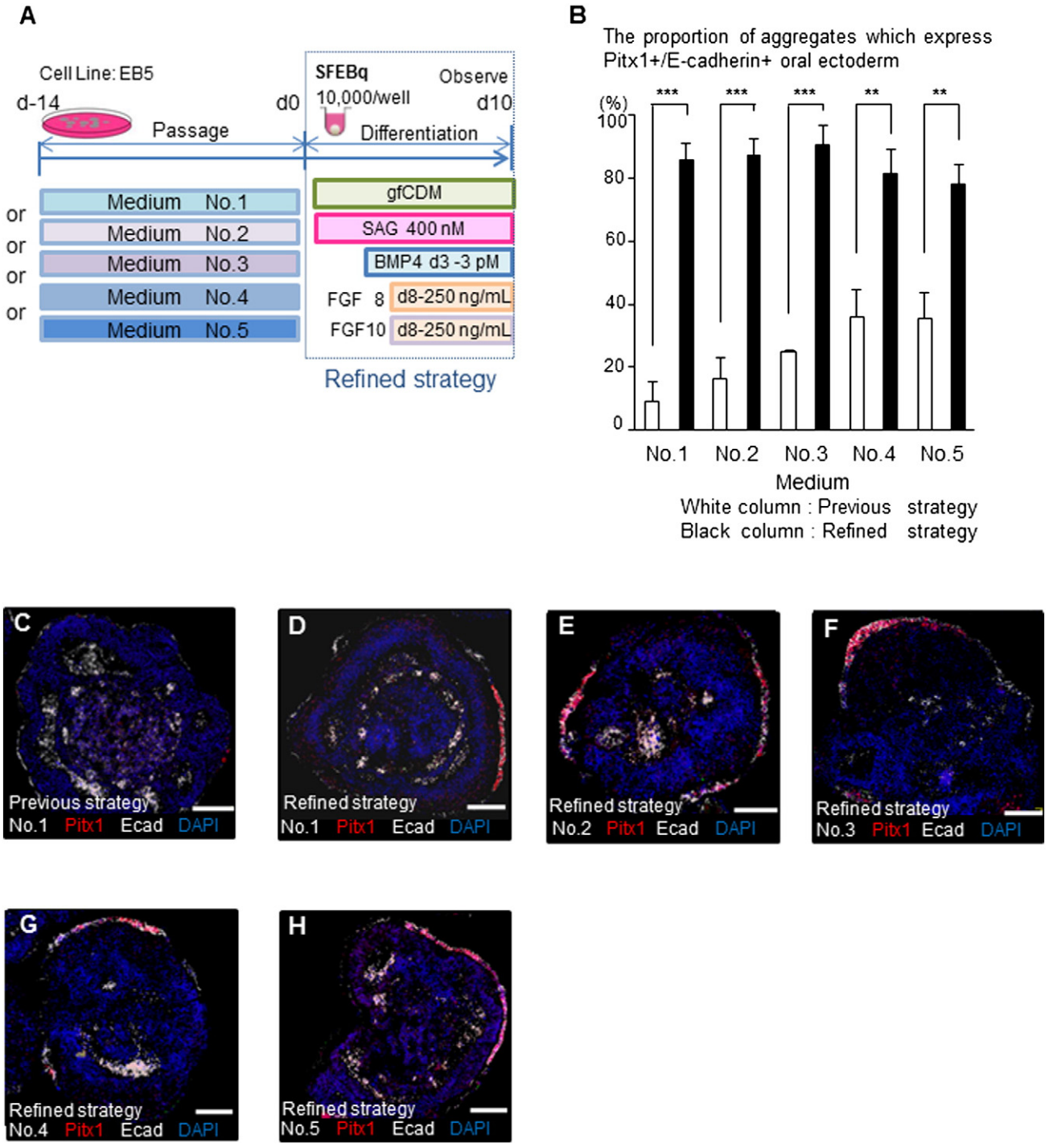


Fig. 4. BMP4 + FGF treatment of mES cultures strongly induces differentiation into oral ectoderm. (A) Culture strategies to test five kinds of maintenance media. Five kinds of maintenance media were prepared by the combination of three different lots of FBS and KSR (as mentioned for Fig. 1A). After mES cells were passaged for 14 days, they were then cultured under the refined strategy. (B) The proportion of aggregates which displayed Pitx1+/E-cadherin+ oral ectoderm on day 10. n = 4 individual batched experiments. The values shown on graphs represent the mean ± s.e.m. $^{**}P < 0.01$; $^{***}P < 0.001$. (C) Immunostaining of day 10 aggregates cultured under the previous strategy for Pitx1 (red) and E-cadherin (white). (D–H) Immunostaining of the day 10 aggregates cultured under the refined strategy for Pitx1 (red) and E-cadherin (white). Media are numbered no. 1 to no. 5. Scale bars, 100 μm. Ecad: E-cadherin. Nuclei are stained by DAPI (blue).

Fig. 3. The differentiation of ES-cell-derived pituitary progenitors into corticotropes and Pit1+ cells. (A) Schematic of pituitary endocrine cell lineages. (B) The culture strategy of SAG + BMP4 + FGF8 + FGF10-treated LCA-SFEBq/gfCDM for Lim3+ pituitary progenitors. (C, D) Immunostaining of day 10 SAG + BMP4 + FGF8 + FGF10-treated aggregates for Lim3 (green), Rax (red, C), Pitx1 (red, D), and E-cadherin (white). (E, F) Immunostaining of day 14 SAG + BMP4 + FGF8 + FGF10-treated aggregates for Lim3 (green), Rax (red, E), Pitx1 (red, F), and E-cadherin (white). (G, H) Immunostaining of day 18 aggregates for Lim3 (green) and ACTH (red). (I) Immunostaining of day 18 aggregates for Tbx19 (green) and ACTH (red). (J) Immunostaining of day 18 aggregates for Lim3 (green) and Pit1 (red). (K, L, M) *Lim3*, *Tbx19* and *Pit1* expression, as determined by qRT-PCR, in aggregates cultured under the previous strategy (control: white) or with BMP4, FGF8b and FGF10 (black). K: n = 5 individual batched experiments, L, M: n = 3 individual batched experiments. The values shown on graphs represent the mean ± s.e.m. $^{*}P < 0.05$. $^{**}P < 0.01$. (N) The culture strategy of SAG + BMP4-treated LCA-SFEBq/gfCDM for the dental primordium. (O) Immunostaining of day 7 aggregates for Brachyury (green), Pitx1 (red) and E-cadherin (white). (P) Immunostaining of day 11 aggregates for Dlx2 (green), Pitx1 (red) and Pax9 (white). Scale bars, 50 μm. Ecad: E-cadherin. Nuclei are stained by DAPI (blue).

Acknowledgments

The authors thank Ms. Michiko Yamada for her skilled technical assistance; Dr. Kazuki Fukuoka for his encouragement during the early days of the study (formerly Department of Endocrinology and Diabetes, Nagoya University Graduate School of Medicine); and Prof. Yukio Kato and Dr. Saishu Yoshida (Division of Life Science, Graduate School of Agriculture, Meiji University) for helpful discussions.

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