Expression of Neuropeptide Receptor mRNA During Osteoblastic Differentiation of Mouse iPS cells

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ABSTRACT

Various studies have shown a relationship between nerves and bones. Recent evidence suggests that both sensory and sympathetic nerves affect bone metabolism; however, little is known about how neuropeptides are involved in the differentiation of pluripotent stem cells into osteoblastic (OB) cells. To evaluate the putative effects of neuropeptides during the differentiation of mouse induced pluripotent stem (iPS) cells into calcified tissue-forming OB cells, we investigated the expression patterns of neuropeptide receptors at each differentiation stage. Mouse iPS cells were seeded onto feeder cells and then transferred to low-attachment culture dishes to form embryoid bodies (EBs). EBs were cultured for 4 weeks in osteoblastic differentiation medium. The expression of α1-adrenergic receptor (AR), α2-AR, β2-AR, neuropeptide Y1 receptor (NPY1-R), neuropeptide Y2 receptor (NPY2-R), calcitonin gene-related protein receptor (CGRP-R), and neurokinin 1-R (NK1-R) was assessed by reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR. Among these neuropeptide receptors, CGRP-R and β2-AR were expressed at all stages of cell differentiation, including the iPS cell stage, with peak expression occurring at the early osteoblastic differentiation stage. Another sensory nervous system receptor, NK1-R, was

expressed mainly in the late osteoblastic differentiation stage. Furthermore, CGRP-R mRNA showed an additional small peak corresponding to EBs cultured for 3 days, suggesting that EBs may be affected by serum CGRP. These data suggest that the sensory nervous system receptor CGRP-R and the sympathetic nervous system receptor β2-AR may be involved in the differentiation of iPS cells into the osteoblastic lineage. It follows from these findings that CGRP and β2-AR may regulate cell differentiation in the iPS and EB stages, and that each neuropeptide has an optimal period of influence during the differentiation process.

Keywords:

induced pluripotent stem (iPS) cells

Osteoblastic (OB) cells

Sympathetic nervous system receptors

Sensory nervous system receptors

β2-adrenergic receptor (β2-AR)

Calcitonin gene-related peptide receptor (CGRP-R)

Abbreviations:

iPS cells, induced pluripotent stem cells; OB cells, osteoblastic cells; EBs, embryoid bodies; AR, adrenergic receptor; NPY1-R, neuropeptide Y1 receptor; NPY2-R, neuropeptide Y2 receptor; CGRP-R, calcitonin gene-related protein receptor; NK1-R, neurokinin 1-R.

Acknowledgments

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (23592708) to Dr. S. Kobayashi.

1. Introduction

In 2006, Yamanaka et al. reported that induced pluripotent stem (iPS) cells, with embryonic stem (ES) cell-like characteristics, could be generated from mouse skin fibroblasts via retroviral transduction of four genes: Oct3/4, Sox2, Klf4, and c-Myc [1]. The attractive feature of iPS cells is the potential for generating patient-specific cells for drug screening, cell therapy, and the study of stem cell biology in general. Further studies are required before iPS cells can be considered clinically applicable, including elucidating the factors that play a role in directing differentiation of iPS cells into various cell lineages. Among the various methods used to direct iPS cells into specific cell lineages, several have been reported for the differentiation of mouse iPS cells into osteoblast (OB) cells [2,3].

Bones are widely innervated by sympathetic and sensory nerves, which are particularly abundant in regions of high osteogenic activity, such as the growth plate. Previous reports suggest that sympathetic and/or sensory innervation is associated with regulating bone metabolism [4,5], such as in the case of sympathetic nervous system-modulated osteoblastic activity in mouse calvaria [6]. Activation of β-adrenergic receptors (ARs) on OB cells

stimulates bone resorption in intact mouse calvaria [7] and induces the expression of osteoblastic factors such as interleukin (IL)-6, IL-11, prostaglandin (PG) E2, and receptor activator of nuclear factor kappa-B ligand (RANKL) [6,8]. These findings suggest that β-AR antagonists could improve osteoporosis associated with hyperactivity of the sympathetic nervous system [9]. Various neuropeptides have been reported to be involved in bone metabolism [4]. Furthermore, axons of sensory neurons have been found to innervate bone tissue [10], and several sensory neuropeptides such as substance P (SP), calcitonin-gene-related peptides (CGRP), neurokinins, and hemokinin exhibit the potential to regulate bone metabolism [5,11]. However, it has not been clarified which neuropeptides affect the differentiation of osteoblastic stem cells into mature OB cells.

To investigate the function of neuropeptides in osteoblastic differentiation, we investigated the time-course of their receptors' expression during the differentiation of mouse iPS cells into mature OB cells. These data reveal differences between the roles of the autonomic and sensory nerves in osteoblastic differentiation, and contribute towards our understanding of how the nervous system affects bone metabolism.

2. Materials and methods

2.1 Culture of iPS cells

The mouse iPS cell line established by Dr. H. Egusa (Osaka University, Osaka, Japan), was used in the present study [12]. SNLP76.7-4 feeder cells were supplied by Dr. Allan Bradley of the Sanger Institute (London, UK). iPS cells were cultured on feeder cells in standard embryonic stem cells (ES) medium (DMEM without sodium pyruvate, Nacalai Tesque, Kyoto, Japan), supplemented with 15% fetal bovine serum (FBS; Millipore, USA), 2 mM L-glutamine (Millipore), 1×10^{-4} M non-essential amino acids (Millipore), 1×10^{-4} M 2-mercaptoethanol (Millipore), 50 units/mL penicillin, and 50 μg/mL streptomycin (Jena Bioscience, Jena, Germany). The medium was changed every day. iPS cells were harvested by trypsinization and transferred in ES medium to low-attachment culture dishes. After 3 days of floating cultivation to form embryoid bodies (EBs), EBs were treated with 1×10^{-6} M retinoic acid (RA) (Wako, Osaka, Japan) and cultured for a further 2 days. Aggregated cells were plated onto 12-well gelatin-coated tissue culture plates, and incubated in ES medium.

2.2 Osteogenic differentiation

The iPS-derived EBs differentiated by RA treatment as described above were trypsinized, placed in 12-well tissue culture plates, and cultured in osteogenic medium for up to 4 weeks. The osteogenic medium consisted of α-MEM medium (MEM Alpha with L-glutamine, ribonucleosides and deoxyribonucleosides; Life Technologies, Gaithersburg, MD, USA) supplemented with 15% FBS (Japan Bioserum Co Ltd., Hiroshima, Japan), 5 mg/mL ascorbic acid (Wako), 1 M β-glycerophosphate (Sigma-Aldrich Japan, Tokyo, Japan), 10^{-6} M dexamethasone (Sigma-Aldrich Japan), and 1% antibiotic-antimycotic (Life Technologies). The cells were maintained in culture with medium changes every 3 days. After a 4-week culture period, the medium was removed and the cells were rinsed in PBS and subsequently fixed in 10% formalin. For von Kossa staining, the plates were treated with 5% aqueous silver nitrate for 10 min, washed in distilled water, then treated with 2% sodium thiosulfate for 2 min, before examination using a scanner (EP-703A, EPSON, Tokyo, Japan).

2.3 Reverse Transcription Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was extracted using a Total RNA Extraction Miniprep System (Viogene, Sunnyvale, CA, USA), according to the manufacturer's protocol. To reduce DNA

contamination, samples were treated with RNase-free DNase I (Takara Bio, Shiga, Japan) for 3 h at 37°C. cDNA was synthesized from 2 μL total RNA in 30 μL reaction buffer containing 500 mM dNTPs, 20 U of ribonuclease inhibitor (Promega, Madison, WI, USA), and 200 U Superscript Reverse Transcriptase (Life Technologies). Cycling conditions were 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s, for 40 cycles. The primers used for amplification were *Oct3/4* GTTCTCTTTGGAAAGGTGTTC, CTCGAACCACATCCTTCTCT; *Nanog* GTACCTCAGCCTCCAGCA, CAACCACTGGTTTTTCTGC; *Brachyury* CATGTACTCTTTCTTGCTGG, GGTCTCGGGAAAGCAGTGGC; *OCN* CAAGTCCCACACAGCAGCTT, AAAGCCGAGCTGCCAGAGTT; *β-actin* TCCCTGGAGAAGAGCTACGA, ATCTGCTGGAAGGTGGACAG; *α1b-AR* CTGGTGATGTCTAGGTGTGTT, GGAATGGCCTTGTCTATAGTT; *α2b-AR* TCATCTACACCATCTTCAACC, AGGTATTCTAATCAGCCTTGG; *β2-AR* CCTCATCCCTAAGGAAGTTTA, TAGGCACAGTACCTTGACAGT; *NPY1-R* CTCGCTGGTTCTCATCGCTGTGGAACGG, GCGAATGTATATCTTGAAGTAG; *NPY2-R* TCCTGGATTCCTCATCTGAG, GGTCCAGAGCAATGACTGTC; *CALCRL* ATTGGATAGCCAGCAAATGG, CCTGGGAGTTGTTTGTGCTT; *NK1-R*

GAAGGCTATCCTCGGTTTCC, TGTGGCTGAGAATGACTTCG. PCR products were subjected to 1.5% agarose gel electrophoresis with ethidium bromide staining, and visualized under ultraviolet light illumination. The expression of β-actin was used as an internal control.

2.4 Real-time PCR

Total RNA samples were treated with DNase and reverse transcribed with random primers using a Superscript First-Strand Kit (Life Technologies). Real-time PCR was performed using the TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA, USA) and Eco Real Time PCR System (Illumina, San Diego, CA, USA). The detection of mRNA was performed using pre-developed proprietary TaqMan primers (CGRP-R [Calca: Mm00801463_g1], β2-AR [Adrb2: Mm02524224_s1], NK1-R [Tacr1: Mm00436892_m1], and β-actin [Actb: Mm00607939_s1]; Applied Biosystems). These analyses were conducted to determine the levels of mouse β-actin for data normalization. Cycling conditions were 95°C for 15 s and 60°C for 60 s, for 50 cycles. The level of target gene expression was normalized relative to that of β-actin and expressed relative to the control. The experiments were performed five times in each group.

2.5 Immunocytochemistry

Cells were fixed in 4% paraformaldehyde for 10 min at room temperature (RT). After washing in 0.1 M PBS, cells were treated with 0.1% Triton X-100 for 5 min at RT. The primary antibody was rabbit polyclonal anti-β2-AR (1:100 dilution; Santa Cruz Biotechnology, CA, USA). To assess non-specific staining, rabbit IgG (1:100 dilution; Santa Cruz Biotechnology) was used in a control condition, at the same concentration as the primary antibody. Then the cells were washed and incubated for 90 min at 37°C with the secondary antibody Alexa Fluor 488 goat anti-mouse IgG (1:500 dilution; Molecular Probes Inc., Eugene, OR, USA). Immunofluorescent labeling was observed under a fluorescent microscope and photographed. Cell nuclei were stained with DAPI (Funakoshi Co., Ltd, Tokyo, Japan).

2.6 Statistical analysis

One-way analysis of variance (ANOVA) followed by individual *post hoc* comparisons (Scheffé) was used to analyze the significant difference.

3. Results

Mouse fibroblast-derived iPS cells were seeded on SNLP76.7-4 feeder cells to generate iPS cell colonies (Fig. 1A), and subsequently transferred to low-attachment culture dishes for EB formation (Fig. 1B). EBs were treated with RA and cultured on gelatin-coated plates to generate a population enriched in mesenchymal cells. EBs were subsequently cultured in osteogenic medium, to drive differentiation into the osteogenic lineage. Differentiation into OB cells was confirmed using von Kossa staining to assess the capacity to produce mineralized tissues (Fig. 1C). Cells that were not cultured in osteogenic medium demonstrated minimal mineral deposition (data not shown). RT-PCR analysis further confirmed the differentiation from iPS cells into OB cells (Fig. 1D). These analyses clearly establish that the iPS cells generated here exhibited an ES cell-like state. The results are consistent with the established criteria for the designation of reprogrammed somatic cells as iPS cells. The pluripotency markers Oct3/4 and Nanog were expressed early and at high levels in the iPS and EB stages. The mesoderm marker Brachyury was only expressed in the EB stage, consistent with the expected effects of treatment with RA. Expression of osteocalcin (OCN), which is induced with the onset of the mineralization stage of osteoblastic differentiation, gradually increased after

EBs were cultured in osteogenic medium. These results clearly reveal that iPS cells were successfully differentiated into OB cells.

To examine the time-course of neuropeptide receptor mRNA expression during iPS cell differentiation into mature OB cells, RT-PCR analysis was performed on total RNA collected from iPS cells, EBs cultured for 3 days and 5 days, and OB cells cultured for 1 week to 4 weeks (Fig. 2A). β2-AR and CGRP-R were expressed in iPS cells, and their expression continued to the last stage of OB cell differentiation. mRNA encoding α2-AR, or NPY2-R was not detectable at any stage of cell differentiation. α1-AR mRNA was expressed weakly in late stages of OB cell differentiation. NPY1-R mRNA was rarely expressed in iPS cells. Three neuropeptides, β2-AR, CGRP-R, and NK1-R, were expressed strongly during the differentiation of iPS cells into OB cells. The expression of these receptors was subsequently examined quantitatively at each stage of cell differentiation, by real-time PCR (Fig. 2B). The expression of β2-AR and CGRP-R mRNA peaked in OB cells cultured for 1 week, after which expression gradually decreased as further differentiation took place. These findings suggest that CGRP and β2-AR may influence the early differentiation of OB cells. Furthermore, CGRP-R mRNA showed an additional small peak in EBs cultured for 3 days, which may

indicate that CGRP also plays a role in the differentiation of EBs. Unlike CGRP-R, another sensory neuropeptide, NK-1, was found to be primarily expressed in the osteogenic differentiation stage. The expression of NK1-R mRNA gradually increased over the osteogenic differentiation stage, reaching approximately nine-fold greater expression in OB cells cultured for 4 weeks relative to those cultured for 1 week.

Finally, we confirmed the distribution of β2-AR in iPS cells and OB cells cultured for 1 and 4 weeks using immunofluorescent labeling (Fig. 3). iPS cells and OB cells showed positive immunofluorescent labeling for β2-AR, whereas SNL feeder cells were scarcely positive for β2-AR. Interestingly, the immunopositive reaction for β2-AR in the cells cultured for 4 weeks was more intense than that observed in iPS cells.

4. Discussion

We found that both CGRP-R and β2-AR are expressed in iPS cells, which suggests that even undifferentiated iPS cells are likely to be regulated by both sensory and sympathetic neuropeptides. We further showed that the receptors of the sensory neuropeptides substance P and CGRP are expressed in different stages of differentiation from iPS cells into OB cells. The

expression patterns of β2-AR, CGRP-R, and NK1-R are illustrated in Figure 4.

We first tried to establish a stable method for the differentiation of mouse iPS cells into OB cells. Several studies have reported methods for the derivation of OB cells from mouse iPS cells [2,3]. Li et al. demonstrated that by culturing iPS-derived EBs in the presence of TGF-β1 and RA, it was possible to direct differentiation into OB cells via a mesenchymal stem cell intermediate [2]. Bilousova et al. generated OB cells from mouse iPS cells, and formed calcified structures in scaffolds, both *in vitro* and *in vivo* [3]. Laurence et al. induced differentiation of OB cells from mouse ES cells without generating EBs [13]. Initially, we cultured mouse iPS cells (Riken, Tsukuba, Japan) on feeder cells (SL10, Repro CELL, Japan), and tried to differentiate them into OB cells using RA or TGF-β1; however, this method failed to generate bone-forming OB cells. Subsequently, we used mouse iPS cells established by Dr. Egusa [12], and were successful in creating OB cells that produced mineralized tissue, as indicated in Figure 1. Thus, not all iPS cells have the capacity to differentiate into bone-forming OB cells using this paradigm. Protocol optimization is required to enable reproducible osteoblastic differentiation across iPS cell lines.

We evaluated the cell differentiation-dependent expression of neuropeptide receptors by

dividing the time-course of cell differentiation into four stages: the iPS stage, EB stage, and early and late osteoblastic differentiation stages. RT-PCR was performed to screen expression of sympathetic nervous system receptors (α1-AR, α2-AR, β2-AR, NPY1-R, and NRY2-R) and sensory nervous system receptors (CGRP-R and NK1-R). Here, we report for the first time that β2-AR and CGRP-R are expressed in the iPS stage. Previously, we found that substance P affects the late stages of osteoblastic differentiation [14]; however, in the present study, we found that the receptor for another sensory neuropeptide, CGRP, is expressed even in iPS cells. Because we assume that sympathetic neuropeptides affect cells in an early stage of cell differentiation, and that sensory neuropeptides affect a later stage, it was intriguing to find a sensory nervous system receptor, CGRP-R, expressed even from the pluripotent stage. As shown in previous reports, the concentration of CGRP peptide in maternal plasma is elevated both in rats and in humans during pregnancy [15,16]. These findings suggest that estrogen and progesterone elevate the circulatory concentrations of CGRP and maintain vascular adaptations during pregnancy. CGRP levels in cord blood are higher than those in the mother's plasma at term [17]. These studies suggest that CGRP may be supplied from vascular adaptations during pregnancy. It is not known whether CGRP promotes cell differentiation or maintains the

undifferentiated state.

In the real-time PCR experiments, the expression levels of β2-AR and CGRP-R were at peak levels in OB cells cultured for 1 week, and expression gradually decreased during late-stage differentiation. The expression of β2-AR was confirmed by immunofluorescent labeling of β2-AR; strong immunofluorescence was observed in the region of the colony in which OB cells are expected to localize. These findings demonstrate that both CGRP and the β2-AR ligand affect the initial differentiation of OB cells, which is consistent with the findings that β2-AR and CGRP-R are found in human OB cells [18]. Conversely, the receptor of another neuropeptide, NK1-R, is expressed primarily in the late stage of osteoblastic differentiation. Although CGRP and substance P localized to the same sensory neurons, it is possible that they may act in a different stage of osteoblastic differentiation.

Comparing the expression patterns of β2-AR and CGRP-R, we see that β2-AR shows only one peak of expression, at the initial stage of osteoblastic differentiation, whereas CGRP-R expression has an additional small peak of expression in the early EB stage. Presumably, in this stage, serum CGRP affects EBs via CGRP-R, while in the early stage of osteoblastic differentiation, CGRP from sensory neurons affects the differentiation of OBs.

We also found that receptors of both sensory and sympathetic neuropeptides were expressed even in iPS and EBs, and that their expressions peaked during the initial differentiation of OB cells. Furthermore, although CGRP and substance P are secreted from the same sensory neuron, they affect different stages of OB cell differentiation. Further study is required to elucidate the specific role of each neuropeptide in the process of OB cell differentiation.

Conclusion

To evaluate the putative effects of neuropeptides during the differentiation of mouse iPS cells into OB cells, we examined the expressions of neuropeptide receptors. We found that both the sensory neuropeptide receptor, CGRP-R, and the sympathetic neuropeptide receptor, β2-AR, were expressed starting in the iPS stage and reached maximum expression in the early stages of osteoblastic differentiation. The expression of CGRP-R showed an additional small peak in the early EB stage, which suggests that EBs may be affected by serum CGRP. Another sensory neuropeptide receptor, NK1-R, showed a totally different pattern of expression compared to CGRP-R, suggesting that substance P influences the late stages of osteoblastic differentiation. Taken together, these results suggest that sensory and sympathetic neuropeptides may take part

in the regulation of cell differentiation in the iPS and EB stages, with each neuropeptide having an optimal period of influence during osteoblastic differentiation.

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FIGURE LEGENDS

Fig. 1. The differentiation of iPS cells into OB cells. (A) Mouse iPS cells formed a colony on SNLP76.7-4 feeder cells. Scale bars = 100 μm. (B) EBs formed after iPS cells were cultured

on low-attachment culture dishes in ES medium with RA. Scale bars = 200 μm. (C) Mineralized nodules, produced by differentiated OB cells, visualized by von Kossa staining. Scale bars = 5 mm. (D) Time-course of expression of markers of pluripotency, mesoderm, and OB cells. Total RNA isolated from mouse iPS cells (lane 1), EB3d (lane 2), EB5d (lane 3), OB 1w (lane 4), OB 2w (lane 5), OB 3w (lane 6), and OB 4w (lane 7). Oct3/4 and Nanog are markers of iPS cell pluripotency. Brachyury is a marker of early mesodermal differentiation. OCN is a late osteoblastic marker. The housekeeping gene β-actin was used as an internal standard.

Fig. 2. Expression of mRNA encoding neuropeptide receptors during differentiation from iPS cells into OB cells. (A) Expression of α1-adrenergic receptor (AR), α2-AR, β2-AR, neuropeptide Y1 receptor (NPY1-R), NPY2-R, calcitonin gene-related receptor (CGRP), and neurokinin 1-R (NK1-R). Total RNA was isolated from mouse iPS cells (lane 1), EB3d (lane 2), EB5d (lane 3), OB 1w (lane 4), OB 2w (lane 5), OB 3w(lane 6), and OB 4w (lane 7). (B) Quantitative expression of CGRP-R, β2-AR, and NK1-R by real-time PCR. Data indicate expression relative to control (β-actin) (n= 5). Bars = mean ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$.

Fig. 3 Immunofluorescent localization of β2-ARs in iPS cells (A), OBs cultured for 1 week (D), and OBs cultured for 4 weeks (G). Nuclei are stained with DAPI (B, E, and H). (C, F, and I) Merged images. Scale bars = 50 μm.

Fig. 4 Schematic illustration of the expression of β2-AR, CGRP-R, and NK1-R during the differentiation of iPS cells into OB cells.

Figure 1
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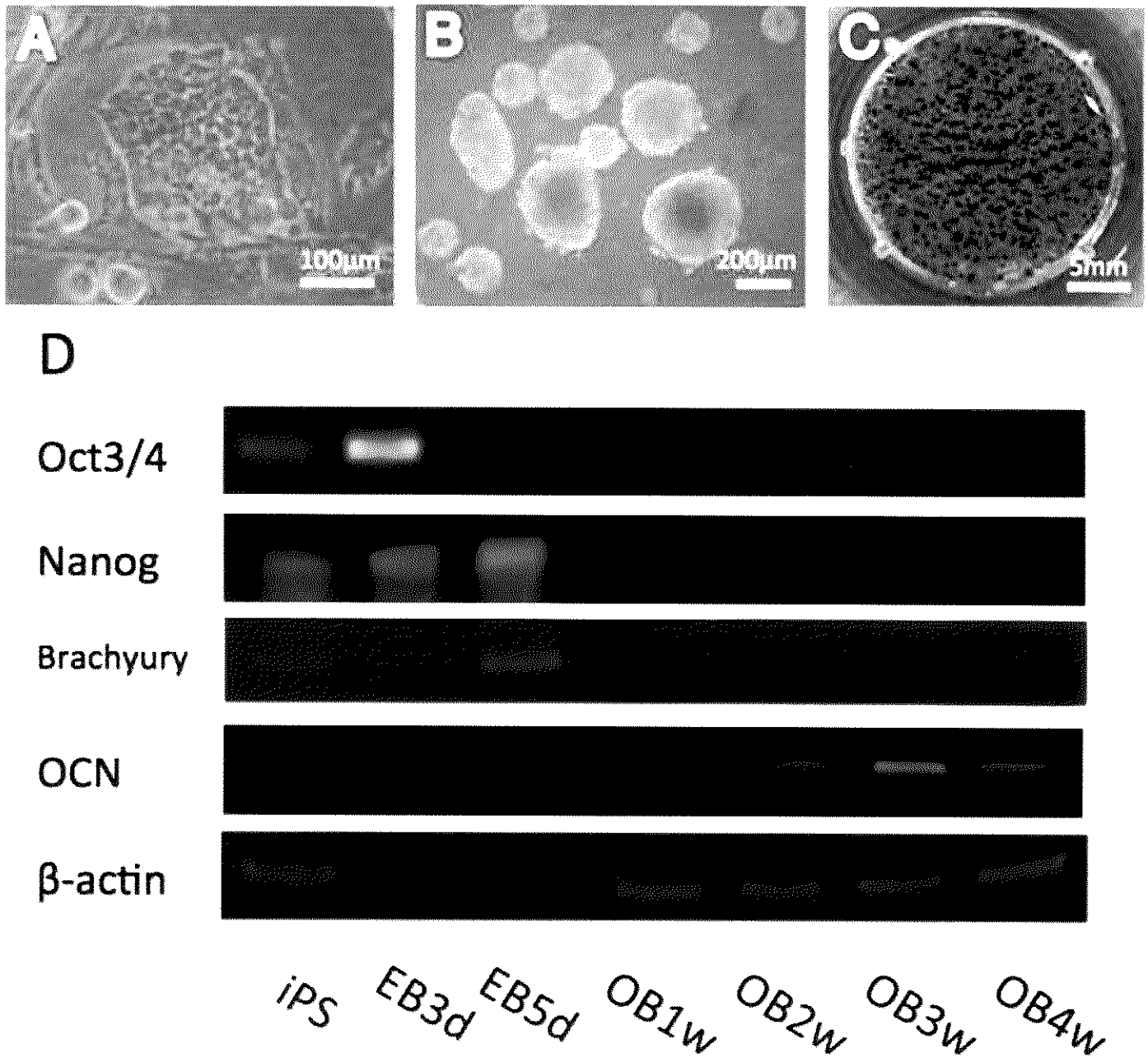

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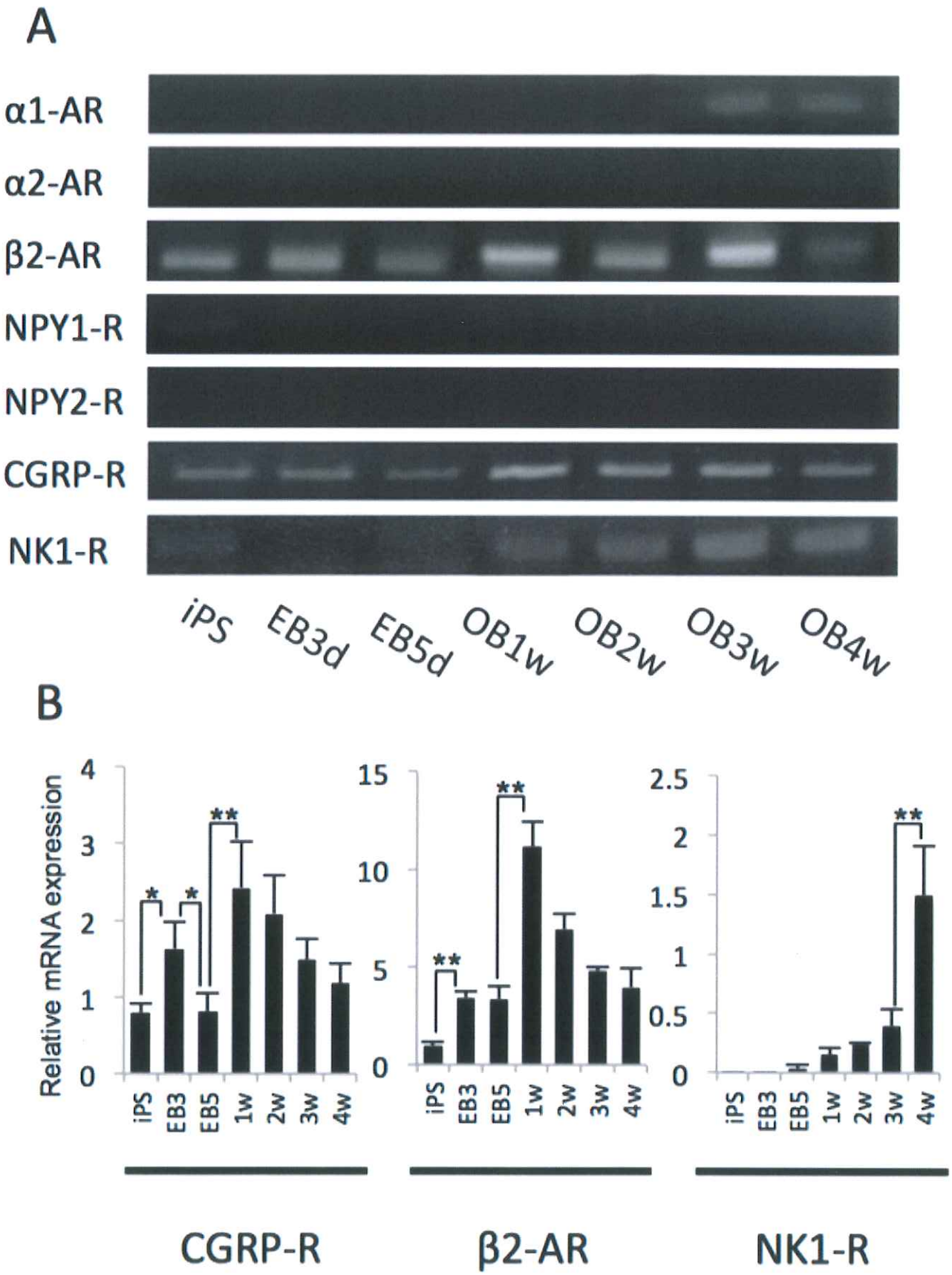

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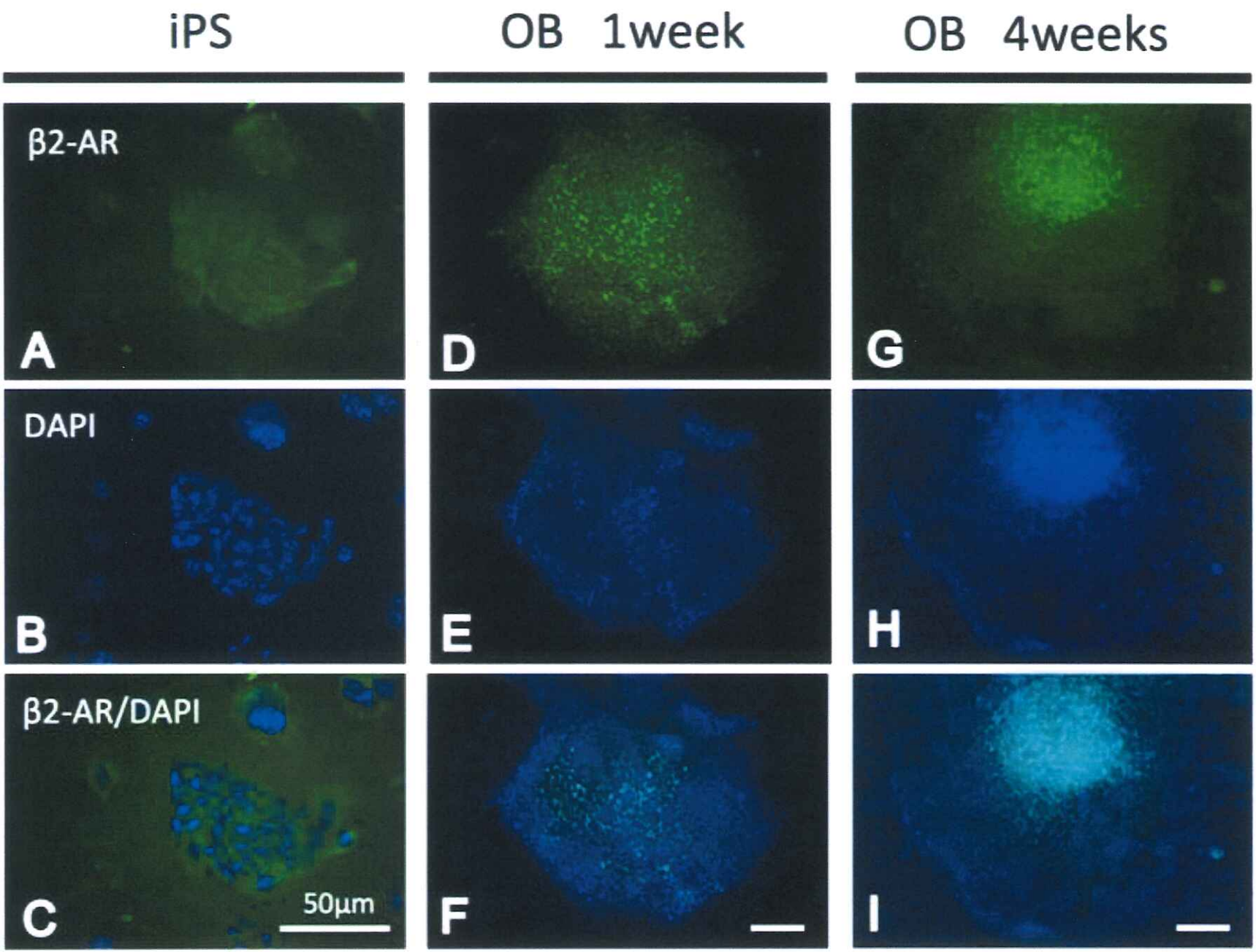

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