



Conjugated Polyelectrolyte Materials for Promoting Progenitor Cell Growth Without Serum

Gaomai Yang, Libing Liu, Fengting Lv & Shu Wang

Beijing National Laboratory for Molecular Sciences, Key Laboratory of Organic Solids, Institute of Chemistry, Chinese Academy of Sciences, Beijing 100190, P. R. China.

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Correspondence and requests for materials should be addressed to

S.W. (wangshu@iccas.ac.cn) or L.B.L. (liulibing@iccas.ac.cn)

The discovery of new active biomaterials for promoting progenitor cell growth and differentiation in serum-free medium is still proving more challenging for the clinical treatments of degenerative diseases. In this work, a conjugated polyelectrolyte, polythiophene derivative (PMNT), was discovered to significantly drive the cell cycle progression from G₁ to S and G₂ phases and thus efficiently promote the cell growth without the need of serum. Furthermore, the fluorescent characteristic of PMNT makes it simultaneously able to trace its cellular uptake and localization by cell imaging. cDNA microarray study shows that PMNT can greatly regulate genes related to cell growth or differentiation. To the best of our knowledge, this is the first example of cell growth or differentiation promotion by polyelectrolyte material without the need of serum, thereby providing an important demonstration of degenerative biomaterial discovery through polymer design.

The study of growth and proliferation of stem cells or progenitor cells not only holds great significance in biology and medicine, but also provides new hope for the clinical treatments of degenerative diseases^{1–3}. To keep cells alive for longer periods of time and to evaluate growth and proliferation *in vitro* experiment, a basal medium must be supplemented with serum⁴. Serum is a complex containing a large number of components, such as growth factors, proteins, vitamins, trace elements, and hormones. However, due to collection from the unborn calf, seasonal and continental variations in the serum composition can produce batch-to-batch variations. Furthermore, the use of serum is restricted and controversial due to the risk of immune reaction and cross contamination of viral and other pathogens⁵. Since serum-free and component-controllable medium can overcome these limits, the stimulation using growth factors instead of human serum was performed^{6–8}. However, the removal of the components of serum is proving more challenging. Some small molecules that are able to promote cell proliferation or growth have been discovered up to date^{9–12}. However, the culture medium of these small molecules must be supplemented with serum. Furthermore, these active small molecules were obtained primarily from high-throughput screening, it is difficult to predict and obtain active compounds by molecular design. The discovery of new active compounds for promoting cell growth and differentiation in serum-free medium still remains more challenging.

Conjugated polymers (CPs) for regenerative medicine applications have aroused great attention benefiting from electrical conductivity tuning of CPs, and CP scaffolds were found to promote muscle and nerve cell growth and differentiation by electrical stimulation^{13–17}. In a recent study, cells cultured on the CP hydrogel substrates were shown to adhere and proliferate¹⁴. In this work, we discovered a conjugated polyelectrolyte, polythiophene derivative (PMNT, see its chemical structure in **Figure 1**)^{18,19} for efficiently promoting cell growth without serum. We comprehensively studied the effect of growth promotion of PMNT toward MC3T3 cells (primary mouse osteoblasts, widely used as model systems in bone biology) without serum. Flow cytometry study shows PMNT can significantly drive the cell cycle progression from G₁ to S and G₂ phases. cDNA microarray study shows that PMNT can regulate genes related to cell growth or differentiation. compared with the reported small molecules that can induce cell growth or differentiation, the fluorescent characteristic of PMNT makes it simultaneously be able to trace its cellular uptake and localization by cell imaging. To the best of our knowledge, this is the first example of cell growth or differentiation promotion by polyelectrolytes without the need of serum, thereby providing an important demonstration of future drug discovery to degenerative diseases through polymer design.

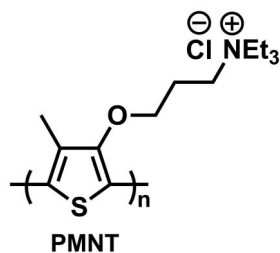


Figure 1 | Chemical structure of PMNT.

Results

The schematic illustration of cell growth promotion by PMNT is shown in **Figure 2**. Serum is the most widely used supplement for *in vitro* cell culture of eukaryotic cells, which supports cell growth and division by providing a broad spectrum of macromolecules, including bovine serum albumin (BSA), attachment factors, nutrients, hormones, and growth factors. Cells cannot undergo normal growth and proliferation in the culture medium without serum. While PMNT-treated cells can grow and proliferate in the culture medium without serum, which means that PMNT can replace the role of serum to some extent and promote the cell growth and proliferation.

To get more insights into the interaction mechanism of PMNT with cells, the location of PMNT in the MC3T3 cells investigated by fluorescence microscopy (FV1000-IX81, Olympus). The fluorescent property of the PMNT (quantum yield: 3%, maximum emission: 530 nm)¹⁸ makes it be able to monitor uptake and location in living cells, which eliminates the complicated conjugation steps with additional imaging probes^{20–22}. In this experiment, the PMNT was incubated with MC3T3 cells at 37°C for 24 h. The medium was removed and the cells were washed with phosphate buffered saline (1×PBS, pH 7.4) for three times before taking images. The cells were also stained by nucleus-specific Hoechst 33258 dye. As shown in **Figure 3**, PMNT mainly locates in the cytoplasm other than nucleus after it uptakes into the cells, which is proven by the overlap fluorescence images of PMNT and Hoechst 33258.

To test the ability of PMNT for cell growth promotion and exclude the impact of serum, MC3T3 cells were cultured in α -MEM medium containing PMNT (20 μ M) but without serum. The phase contrast images of MC3T3 cells were taken before and after incubation with PMNT for different hours. As shown in **Figure 4a**, compared with

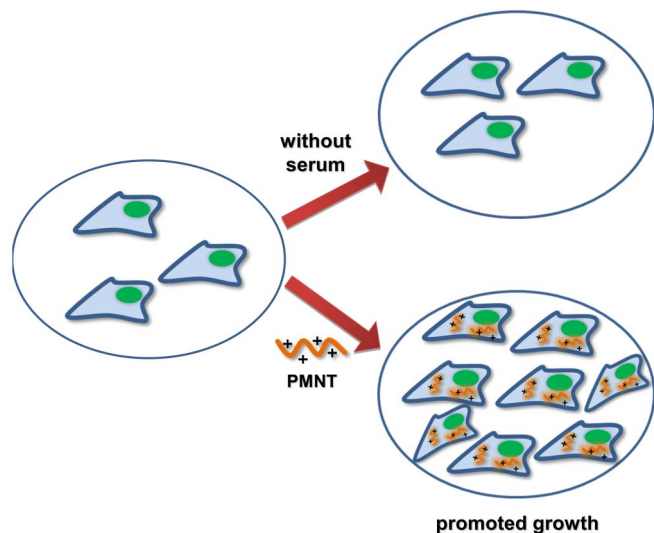


Figure 2 | Schematic representation of cell growth promotion by PMNT.

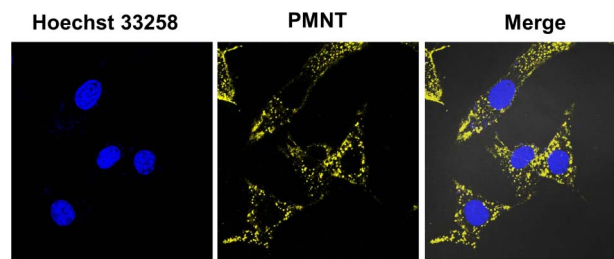


Figure 3 | Fluorescence images of MC3T3 cells stained with nuclear dye (Hoechst 33258), PMNT, and the overlapped image. The representative image of PMNT is yellow, the nuclear dye is blue.

the control, the density of MC3T3 cells treated with PMNT was increased in a time-dependent manner. The control experiment exhibits that other positively-charged polyelectrolyte, polyethylenimine (PEI) does not show cell growth promotion (**Figure 4b**). This difference of cell density was quite obvious after incubating cells with PMNT for 72 h. To further confirm that PMNT promotes the growth of MC3T3 cells, the number of cells treated with and without PMNT was counted. As shown in **Figure 4c**, no obvious increase of cell growth was observed for the control (without PMNT treatment) throughout the incubation time. However, for the cells treated with PMNT, there was an obvious increase in cell number compared to the control and 160% increase was observed after 72 h incubation, which is consistent with the results of phase contrast images. The cytotoxicity of PMNT toward MC3T3 cell line was determined by MTT assay. As shown in **Figure 4d**, PMNT shows minimal cytotoxicity, even at a high concentration (200 μ M).

The growth promotion mechanism of PMNT was further studied. Flow cytometry was used for cell cycle analysis of the cells treated with PMNT. As shown in **Table 1** and **Figure 5**, the proportion of cells in G1, S, and G2 phase for PMNT-treated cells and the control (without PMNT treatment) is identical at 4 h. With the increase of incubation time from 4 h to 16 h, it is noted that for PMNT-treated cells, the proportion of cells in the (S + G2) phase is more than that of the control in a time-dependent manner, which results in a significant increase of proliferative index for PMNT-treated cells. In the following 16–24 h, the proportion of PMNT-treated cells in (S + G2) phase is decreased as the time increases, and the value is similar to that of the control at 24 h when a complete cycle of mitosis ends. The results indicate that PMNT stimulates the growth of MC3T3 cells by driving their cell cycle progression from G1 to S and G2 phases. The PMNT-treated cells could undergo the normal cell mitosis without being cell cycle arrested in S or G2 phase.

Discussion

To examine PMNT-regulated genes involved in cell proliferation and cell cycle control, we used cDNA microarray to detect gene transcriptional expression changes associated with PMNT treatment. A typical microarray hybridization image and scatter plot of PMNT-regulated changes in gene expression were shown in **Figure 6**, where control sample label with Cy3 and PMNT-treated sample label with Cy5. As shown in **Figure 6a**, the red spots indicate that the level of gene expression in PMNT-treated cells is higher than that in the control cells, while the green spots are lower. The relative transcript abundance was expressed as Cy5/Cy3 ratios of signal intensities after background subtraction in each channel. When the ratio of fluorescence intensity of gene labeled Cy5/Cy3 is above (\geq) 2, it stands for this gene up-regulation. When the ratio of fluorescence intensity of gene labeled Cy5/Cy3 is below (\leq) 0.5, it stands for this gene down-regulation. The red color stands for the up-regulated genes with the Cy5/Cy3 ratio \geq 2.0; the green color stands for the down-regulated genes with the Cy5/Cy3 ratio \leq 0.5; the black color stands for the genes normally expressed. As shown in **Figure 6b**, PMNT treatment

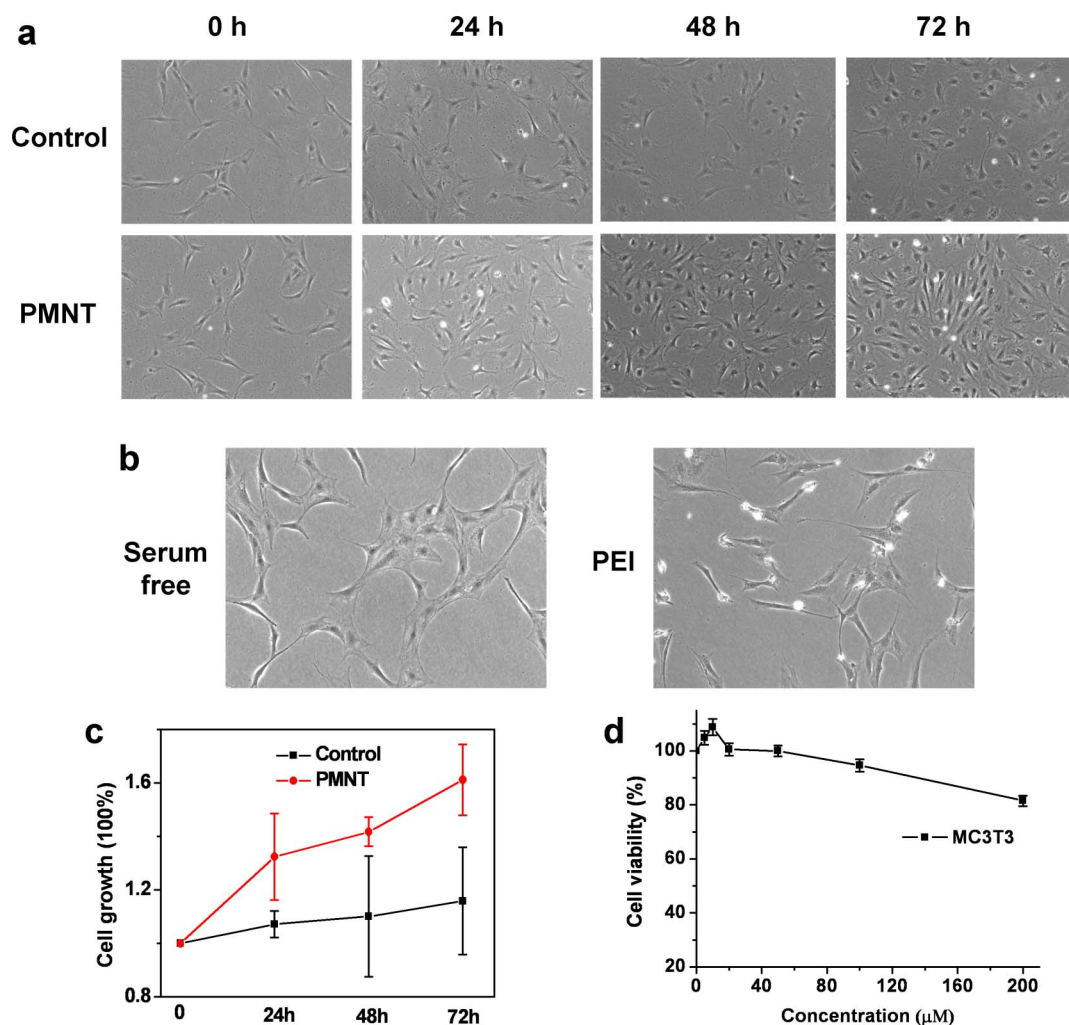


Figure 4 | (a) Phase contrast images of MC3T3 cells treated with and without PMNT. [PMNT] = 20 μM in repeat unites (RUs). (b) Phase contrast images of MC3T3 cells treated with and without PEI. [PEI] = 2 μM. (c) Cell number as a function of incubation time for MC3T3 cells treated with and without PMNT. [PMNT] = 20 μM in RUs. Error bars correspond to standard deviations from three separate measurements. (d) Cell viability of MC3T3 treated with PMNT using a typical MTT assay. Error bars correspond to standard deviations from three separate measurements.

results in the up-regulation or down-regulation of 704 genes out of the 32256 genes, which represents approximately 2.2% of the all analyzed genes. Among these 704 genes, 511 of them were up-regulated and the other 193 were down-regulated.

The genes in cellular process pathways are especially important to further understand the growth promotion mechanism of PMNT. Expression of eight representative genes mostly related to cell proliferation, apoptosis and the cell cycle are summarized in Table 2, their relative value of the gene expression level for the PMNT-treated group in comparison to that of the control group was shown in Figure 7. Inhibitors of DNA binding/differentiation (Id) family contains four members, Id1–Id4, which are identified as gene products whose function was the negative control of cell differentiation²³. The

cDNA microarray result of the PMNT sample shows that Id1–Id3 are significantly down-regulated, the Cy5/Cy3 ratios are 0.07, 0.16, and 0.04 respectively. Because the cell proliferation and differentiation are closely related, thus PMNT has both the abilities to promote cell growth and differentiation. Bone morphogenic proteins (BMPs) and Smad proteins belong to the TGF-β superfamily. BMPs can promote proliferation and migration of endothelial cells but inhibit proliferation of human aortic smooth muscle cells²⁴. Smad6 is a negative regulator of BMP signaling²⁵. The cDNA microarray result of the PMNT sample shows that both BMP-4 and Smad6 are down-regulated with the Cy5/Cy3 ratios of 0.39 and 0.16, respectively. The result is consistent with the double roles of BMP-4 and the role of negative regulator of BMP signaling for Smad6. NPM1 is an

Table 1 | Cell cycle progression of MC3T3 cells treated with PMNT for different time (%)

	4 h		8 h		12 h		14 h		16 h		24 h	
	Control	PMNT	Control	PMNT	Control	PMNT	Control	PMNT	Control	PMNT	Control	PMNT
G1	83.34	83.70	71.25	67.75	71.84	59.64	68.26	55.65	65.45	62.80	71.25	75.32
G2	0.00	0.00	2.61	1.58	6.26	5.17	10.20	19.09	15.73	19.07	12.78	10.56
S	16.66	16.30	26.14	30.66	21.90	35.18	21.54	24.26	18.82	18.13	15.96	14.12

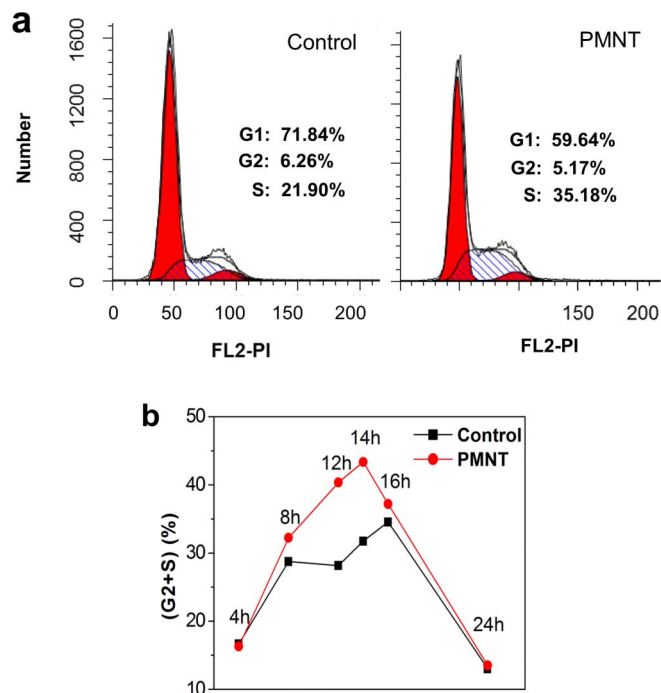


Figure 5 | (a) Typical flow cytometric analysis for cell cycle distribution of MC3T3 cells treated with or without PMNT at 12 h. (b) The proportion of cells in the (S + G2/M) phase as a function of incubation times with PMNT. [PMNT] = 20 μ M in RUs.

abundant and ubiquitously expressed phosphoprotein that plays important roles in the regulation of multiple cellular functions²⁶. Fibroblast growth factor 7 (FGF7), a member of the FGF family, is involved in a variety of biological processes and FGF7 over expression could enhance cell proliferation²⁷. In the result of the cDNA microarray, both NPM1 and FGF7 are significantly up-regulated by PMNT with the Cy5/Cy3 ratios of 3.96 and 4.28, respectively. GADD45g is a cell cycle protein that plays a key role in cell differentiation and negative control of cell growth²⁸. During the cell mitosis, GADD45g can inhibit cell proliferation at different stages, including G1-S and G2-M checkpoints²⁹. Our study shows that GADD45g is down regulated by PMNT with the Cy5/Cy3 ratio of 0.25. Therefore, the growth promotion of PMNT may be closely related to its gene regulation in signaling pathways.

Methods

Materials. All the reagents and solvents used were commercially available. Fetal bovine serum (FBS) was purchased from Sijiqing Biological Engineering Materials (Hangzhou, China). MTT was obtained from Xinjingke Biotech (Beijing, China) and dissolved in 1 \times PBS before use. Primary mouse osteoblasts cell (MC3T3) was purchased from Cell Culture Center of Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China) and cultured in α -MEM supplemented with 10% FBS. The water was purified using a Millipore filtration system. PMNT were prepared according to the procedures in the literature¹⁸.

Measurements. Phase contrast bright-field images were taken with fluorescence microscope (Olympus 1 \times 71) with a mercury lamp (100 W) as light source. The absorbance for MTT analysis was recorded on a microplate reader (BIO-TEK Synergy HT) at a wavelength of 520 nm. Confocal laser scanning microscopy (CLSM) characterization was conducted by a confocal laser scanning biological microscope (FV1000-IX81, Olympus). Flow cytometric analysis was taken by flow cytometry (FACS Calibur, BD).

Assay for cell viability. MC3T3 cells were routinely grown in α -MEM medium containing 10% FBS in a humidified atmosphere containing 5% CO₂ at 37 $^{\circ}$ C and then harvested for subculture using trypsin (0.05%, Gibco/Invitrogen). Cells were subcultured in 96-well plates the day before the experiment at a density of 4–7 \times 10⁴ cells mL⁻¹. Varying PMNT concentrations were respectively added into the cells followed by further culture for 48 h, then the culture media were discarded and MTT (1 mg mL⁻¹, 100 μ L per well) was added to the wells followed by incubation at 37 $^{\circ}$ C

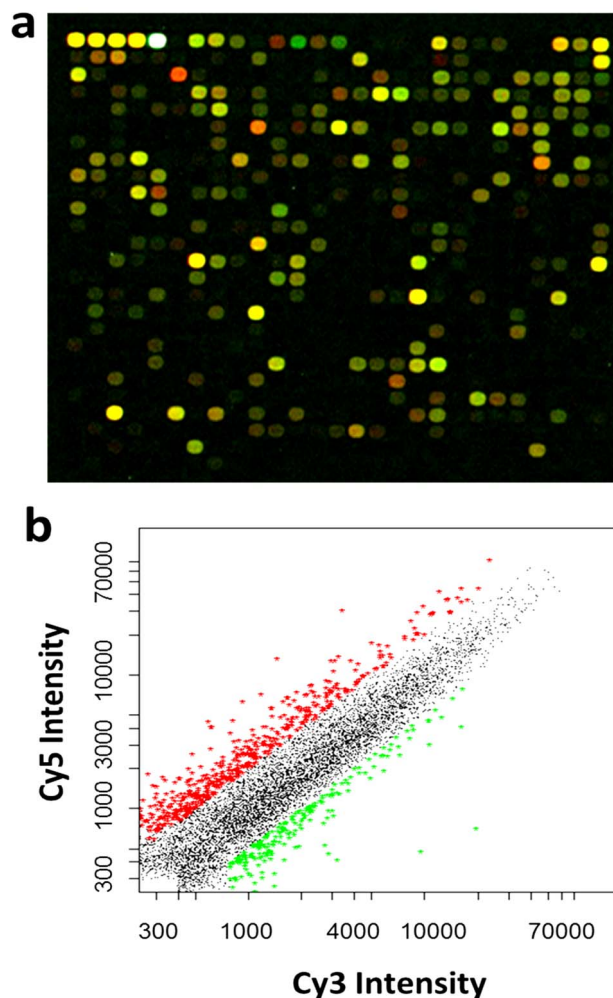


Figure 6 | (a) A typical merged microarray hybridization image of Cy3 (control sample) and Cy5 (PMNT-treated sample). (b) PMNT-regulated changes in gene expression. The Cy3 signal is shown on the x axis and the Cy5 signal is shown on the y axis. The red color stands for the up-regulated genes with the Cy5/Cy3 ratio \geq 2.0; the green color stands for the down-regulated genes with the Cy5/Cy3 ratio \leq 0.5; the color black stands for the genes normally expressed. PMNT induces a progressively larger alteration of gene expression.

for 4 h. The supernatant was abandoned and added 150 μ L DMSO per well was added to dissolve the produced formazan and the plates were shaken for an additional 10 min. The absorbance values of the wells were then read with microplate reader at a wavelength of 520 nm (Synergy HT, Bio-Tek). The cell viability rate (VR) was calculated according to the following equation, where the control group was carried out in the absence of the drugs.

$$VR(\%) = \frac{A_{\text{experimental group}}}{A_{\text{control group}}} \times 100\%$$

Confocal laser scanning microscopy (CLSM) measurements. MC3T3 cells were seeded onto 35 mm Petri dishes with glass bottoms and allowed to incubate for 24 h for attachment, and then cells were treated in α -MEM medium containing PMNT (20 μ M) but without serum for 12 h. The medium was removed and the cells were washed with phosphate buffered saline (1 \times PBS, pH 7.4) for three times. After fixation with 4% formaldehyde for 15 min, the fixation solution was abandoned and the cells were washed with 1 \times PBS for three times. Then the cells were stained by Hoechst 33258 (nuclear dye, 0.5 μ g/mL) for 20 min. After the staining solution was removed and the cells were washed with 1 \times PBS for three times, the cells were examined by confocal laser scanning microscopy (FV1000-IX81, Olympus) using a 405 nm laser for Hoechst 33258 and 488 nm laser for PMNT. The fluorescence of PMNT was highlighted in yellow and nuclear dye in blue.

Analysis of MC3T3 cells growth promotion by PMNT. The MC3T3 cells were seeded in 35 mm culture plates (Nunc) at a density of approximately 8 \times 10⁴ cells per plate for 12 h, the medium was removed and the cells were washed with 1 \times PBS



Table 2 | Differentially expressed genes related to cell growth and/or differentiation regulated by PMNT as determined by microarray analysis

Gene symbol	Gene ID	Description	Cy5/Cy3 ratio
Id1	105	Inhibitor of DNA binding 1	0.07
Id2	359	Inhibitor of DNA binding 2	0.16
Id3	25	Inhibitor of DNA binding 3	0.04
Smad6	3449	MAD homolog 6 (Drosophila)	0.17
BMP4	1447	Bone morphogenetic protein 4	0.39
NPM1	14135	Nucleophosmin 1	3.96
FGF7	7602	Fibroblast growth factor 7	4.28
GADD45g	1675	Growth arrest and DNA-damage-inducible 45 gamma	0.25

once. The cells were then cultured in 1 mL α -MEM medium containing PMNT (20 μ M) without serum. The cells cultured in 1 mL α -MEM medium without serum and PMNT as controls. Phase contrast images were taken at different hours (0–72 h) using fluorescence microscopy (IX71, Olympus). The cells for number count at different hours (0–72 h) were harvested using trypsin and collected in 1 mL culture medium.

Cell cycle analysis by flow cytometry. The MC3T3 cells were seeded in 35 mm culture plates (Nunc) for 12 h, the medium was removed and the fresh medium without serum was added for another 12 h. α -MEM medium containing PMNT (20 μ M) was added into the cells followed by further culture for different hours (4–24 h). The cells at a density of approximately 10^6 cells per plate were harvested using trypsin and collected in PBS. The PBS solution of cells was then fixed by 70% ethanol at 4°C for over 18 h. Centrifuge the ethanol-suspended cells and decant ethanol thoroughly. Suspend the cell pellet in PBS, RNase (100 μ g/mL) was added and the cells were kept in dark at 37°C for 30 min. Centrifuge the suspended cells and decant RNase. Suspend the cell pellet in 1 mL of PI (25 μ g/mL) staining solution. Keep in the dark at 4°C for 1 h. The pre-prepared cell suspensions were directly analyzed using flow cytometry (FACS Calibur, BD). Cell fragments were excluded with forward and side-scatter gating to ensure that all detected signals originated from relatively intact cells; signals PI were individually recorded in Channel FL-1. The flow cytometry diagrams presented were obtained from a population of 3×10^4 cells.

cDNA microarray analysis. 1) *Cell culture and treatment.* The MC3T3 cells were seeded in 35 mm culture plates (Nunc) for 12 h, the medium was removed and the medium without serum was added for another 12 h. α -MEM medium containing PMNT (20 μ M) was added into the cells followed by further culture for 12 h. Untreated cells were used as reference samples and referred to as controls in the analyses.

2) *RNA sample preparation.* MC3T3 cells were lysed and homogenized in Trizol reagent (Invitrogen), and total RNA was prepared according to the manufacturer's instructions.

3) *cDNA microarray hybridization.* CapitalBio 32 k Mouse Genome Array contains 32256 genes of known and unknown function (CapitalBio, Beijing, China). The reference RNA samples from MC3T3 cells were labeled with Cy3, and the test RNA samples from MC3T3 cells treated with PMNT (20 μ M) for 12 hours were labeled with Cy5. The Cy3-labeled and Cy5-labeled samples were hybridized simultaneously to the same array.

4) *Microarray analysis.* Arrays were scanned with a confocal LuxScan™ scanner and the images obtained were then analyzed using LuxScan™ 3.0 software (Both from

CapitalBio). Cy5/Cy3 ratio represents the relative abundance of a target transcript in PMNT-treated and nontreated samples respectively. A signal to control channel ratio > 2.0 was defined as upregulation by PMNT. A signal to control channel ratio < 0.4 was defined as downregulation. To determine the significant differentially expressed genes, Significance Analysis of Microarrays (SAM, version 3.02) were performed.

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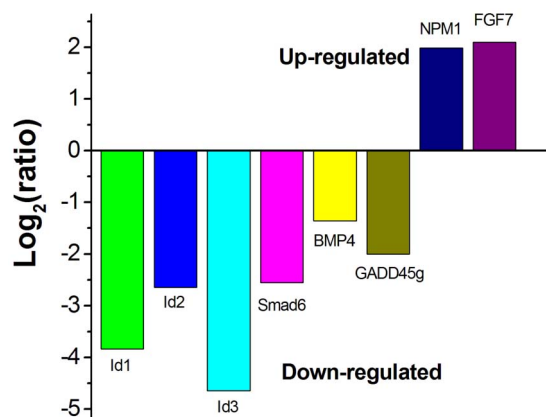


Figure 7 | The relative value of the gene expression level for the PMNT-treated group compared to that of the control group.



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Author contributions

M.G. and L.L. designed and performed experiments, analysed data and wrote the manuscript. F.L. analysed data. S.W. designed experiments, analysed data and wrote the manuscript.

Additional information

Competing financial interests: The authors declare no competing financial interests.

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