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THE *PTTG1* IS A NOVEL INHIBITOR OF OSTEOGENIC DIFFERENTIATION OF MOUSE MESENCHYMAL STEM CELLS

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Rheumatoid arthritis (RA) is a severe autoimmune inflammatory disease leading to chronic pain in the joints frequently ending in patient disability and even death. Molecular mechanisms that trigger a disease and exacerbate its progression are still poorly understood. Several signaling pathways are strongly misregulated in different cell types in joints of RA patients.

It is well known that bone morphogenetic protein (BMP) and Wnt pathway are key signaling pathways that induce and support cartilage and bone formation and maintenance. We hypothesized that pituitary tumors transforming gene 1 (PTTG1) and its partner protein — PTTG1 binding protein 1 (PTTG-BP1, also called PBF1: PTTG binding factor 1) — presents a novel key system in regulating homeostasis of joint tissues and RA pathogenesis. According to our preliminary data, overexpression of PTTG1 gene leads to a drastic inhibition of Wnt signaling in target cells. Such result suggests that PTTG1/PTTG-BP1 axis serves as a new negative regulator of bone and potentially cartilage homeostasis.

In this work, we have investigated the effect of PTTG1 gene overexpression on activation of BMP and Wnt signaling pathways. We have found that ectopic expression of PTTG1 gene inhibited hBMP2/7-induced osteogenenic differentiation of C2C12 cells and bone matrix mineralization in KS483 cells. At the same time shRNA-mediated knockdown of mRNA PTTG1 gene leads to a substantial activation of bone formation in these cells. Thus, PTTG1 is an important repressor of osteogenesis, and it may be involved in skeletal tissue destruction caused by the inflammatory processes.

Keywords: RHEUMATOID ARTHRITIS, PITUITARY TUMORS TRANSFORMING GENE 1, OSTEOGENESIS, MESENCHYMAL STEM CELLS, WNT SIGNALING PATHWAY

PTTG1 — НОВИЙ ІНГІБІТОР ОСТЕОГЕННОЇ ДИФЕРЕНЦІАЦІЇ МИШАЧИХ МЕЗЕНХІМНИХ СТОВБУРОВИХ КЛІТИН

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— ці автори зробили однаковий внесок у виконання роботи

Ревматоїдний артрит (РА) — важке автоімунне запальне захворювання, яке призводить до хронічного болю у суглобах і часто закінчується інвалідністю та навіть смертю пацієнтів. Молекулярні механізми, які викликають цю хворобу та посилюють її розвиток, залишаються маловивченими. У пацієнтів з РА виявлені порушення деяких регуляторних шляхів у різних типах клітин суглобів.

Відомо, що морфогенетичні білки кістки (МБК) і сигнальний ланцюг Wnt є ключовими сигнальними шляхами, які індукують та підтримують формування хрящової і кісткової тканин. Ми припускаємо, що ген пухлинної трансформації клітин гіпофізу 1 (PTTG1) та PTTG1 зв'язувальний білок 1 (PTTG-BP1; також має назву PBF1: PTTG binding factor 1) є новою важливою системою у регуляції гомеостазу тканин суглобів і патогенезу РА. Згідно з нашими попередніми даними, надекспресія гену PTTG1 призводить до значного гальмування регуляторних сигналів Wnt в клітинах-мішенях. Такий результат дозволяє припустити, що система PTTG1/PTTG-BP1 виконує функцію нового негативного регулятора гомеостазу кісткової та, вірогідно, хрящової тканин.

У цій роботі було досліджено вплив надекспресії гену PTTG1 на активацію сигнальних шляхів BMP і Wnt. Встановлено, що тимчасова експресія гену PTTG1 інгібує hBMP2/7-індуковану остеогенну диференціацію клітин лінії C2C12 та мінералізацію кісткового матриксу клітинами лінії KS483. У той же час shRNA-опосередкований нокдаун експресії мРНК гену PTTG1 призводить до значної активації остеогенної диференціації цих клітин. Отже, PTTG1 є важливим транскрипційним репресором остеогенезу і, можливо, задіяний у руйнуванні кісткової тканини, спричиненому запальним процесом.

Ключові слова: РЕВМАТОЇДНИЙ АРТРИТ, ГЕН ПУХЛИННОЇ ТРАНСФОРМАЦІЇ КЛІТИН ГІПОФІЗУ 1, ОСТЕОГЕНЕЗ, МЕЗЕНХІМНІ СТОВБУРОВІ КЛІТИНИ, СИГНАЛЬНИЙ ШЛЯХ WNT

PTTG1 — НОВЫЙ ИНГИБИТОР ОСТЕОГЕННОЙ ДИФФЕРЕНЦИРОВКИ МЫШИНЫХ МЕЗЕНХИМАЛЬНЫХ СТВОЛОВЫХ КЛЕТОК

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— эти авторы имет равный вклад в выполнение работы

Ревматоидный артрит (РА) — тяжелое аутоиммунное воспалительное заболевание, которое приводит к хронической боли в суставах и часто заканчивается инвалидностью и даже гибелью пациентов. Точные молекулярные механизмы, которые вызывают болезнь и усиливают ее развитие, остаются малоизученными. У пациентов с РА выявлены нарушения некоторых сигнальных путей в различных типах клеток суставов.

Известно, что морфогенетические белки кости и сигнальная цепь Wnt являются ключевыми сигнальными путями, которые индуцируют и поддерживают формирование хрящевой и костной тканей. Мы предполагаем, что ген опухолевой трансформации клеток гипофиза 1 (PTTG1) и PTTG1 связывающий белок 1 (PTTG-BP1; также имеет название PBF1: PTTG binding factor 1) является новой важной системой в регуляции гомеостаза тканей суставов и патогенеза РА. Согласно нашим предварительным данным, сверхэкспресия гена PTTG1 приводит к значительному торможению сигналов Wnt в клетках-мишенях. Такой результат позволяет предположить, что система PTTG1/PTTG-BP1 выполняет функцию нового негативного регулятора гомеостаза костной и, вероятно, хрящевой тканей.

В данной работе мы исследовали влияние сверхэкспресия гена PTTG1 на активацию сигнальных путей BMP и Wnt. Мы обнаружили, что временная сверхэкспрессия гена PTTG1 ингибирует hBMP2/7-индуцированную остеогенную дифференцировку клеток линии C2C12 и минерализацию костного мат-

рикса клетками линии KS483. В то же время shRNA-опосредованный нокдаун экспрессии мРНК гена PTTG1 приводит к значительной активации остеогенеза в этих клетках. Таким образом, PTTG1 является важным репрессором остеогенеза и, возможно, задействован в разрушении костной ткани, вызванным воспалительным процессом.

Ключевые слова: РЕВМАТОИДНЫЙ АРТРИТ, ГЕН ОПУХОЛЕВОЙ ТРАНСФОРМАЦИИ КЛЕТОК ГИПОФИЗА 1, ОСТЕОГЕНЕЗ, МЕЗЕНХИМАЛЬНЫЕ СТВОЛОВЫЕ КЛЕТКИ, СИГНАЛЬНЫЙ ПУТЬ WNT

RA is a highly morbid autoimmune disorder that debilitates the life of patients due to a progressive irreversible destruction of cartilage and bone in the joint leading to joint swelling, pain, and stiffness. During RA development, synovial fibroblast-like cells (FLS) start to overgrow and invade surrounding tissues in the joint, thus forming a pannus that leads to a severe destruction of the articular cartilage and frequently to bone erosions. Although RA has been a subject of innumerable investigations, the main cause of the disease stays unknown, and the etiology and pathogenesis of the disease remain incompletely understood [15]. Strikingly, several signaling pathways are strongly misregulated in FLS, monocytes, neutrophils, endothelial and other cells in joints of the RA patients. The bone morphogenetic protein (BMP) and Wnt pathway are known to be key signaling players that induce and support cartilage and bone formation and maintenance.

Recent studies of human rheumatic and orthopedic diseases and specific mouse models with both activating and null mutations of proteins required for a canonical Wnt signaling suggest a crucial role of this signaling pathway in the regulation of bone formation, maintenance, reparation and remodeling by regulating osteoblast and osteoclast proliferation and differentiation [4, 5, 6]. Osteoblast differentiation is predominantly supported by the BMPs — the members of the transforming growth factor β (TGFβ) superfamily and by Wnt proteins. Although BMPs are initial inducers of osteogenesis, efficient differentiation of mesenchymal precursors to the osteo- and chondrogenic lineages requires both Wnt and BMP signaling, and the canonical Wnt pathway subsequently acts as the master regulator of osteogenesis [13].

Wnt/β-catenin signaling modulates osteogenesis through multiple mechanisms. Wnts repress alternative mesenchymal differentiation pathways, such as adipocyte and chondrocyte differentiation, and promote osteoblast differentiation, proliferation, and mineralization activity, while blocking osteoblast apoptosis. By increasing a ratio of OPG/RANKL (osteoprotegerin/receptor activating NF-κB ligand), β-catenin represses osteoclastogenesis [12]. In a healthy skeleton, adjacent to joints cortical bones' formation and resorption are well balanced; however, the inflammatory arthritis leads to an imbalance between these processes. Bone formation is hampered by tumor necrosis factor α (TNFα)-mediated expression of inhibitors which suppress Wnt signals, whereas bone resorption is enhanced by the expression of RANKL [1] — a key factor for the osteoclast differentiation and activation.

In terms of commitment and differentiation of the mesenchymal stem cells (MSC), there is a cooperative crosstalk between the Wnt and BMP pathways [17]. BMP signaling is crucial for skeletogenesis and homeostasis through both development and adulthood. A crosstalk between the BMP and Wnt signaling is notoriously complex in all tissues, as it can be either synergistic or antagonistic, depending on cellular context, and bone is not an exception of this rule. In line with the complexity of their crosstalk, BMP and Wnt signaling were shown to have opposing effects in osteo-progenitors, yet they seem to function, for the most part, cooperatively in the osteoblasts.

Although a crucial role for canonical Wnt signaling has been well established, many mechanisms remain to be discovered in respect to their fine tuning and crosstalk with other pathways in bone [1].

Here we identified PTTG1/Securin and PTTG-BP1/PBF1 as important new molecules that regulate the process of bone formation and maintenance.

PTTG gene is a proto-oncogene, first identified in 1997 in rat pituitary GH4 cells [16]. Human *PTTG1* gene is located on chromosome 5 and encodes securin — a protein consisting of 202 amino acids (22 kDa) with no significant homology to other known proteins [19]. Securin is critically important in regulation of chromosome separation during a mitotic step called the anaphase [22]. *PTTG1* gene is abundantly expressed in cells of malignant tumor, as well as of some normal tissues, and potently transforms cells both *in vitro* and *in vivo*. Overexpression of *PTTG1* gene in NIH-3T3 cells increases basic fibroblast growth factor (FGF) mRNA level as well as stimulates its secretion. As a transactivator of growth factors, high *PTTG1* gene expression also induces vascular endothelial growth factor (VEGF) and other proangiogenic genes [19]. Strikingly, targeted inactivation of *PTTG1* gene leads to generation of aneuploid cells. Like any other oncogene, *PTTG* gene has important normal physiological functions that were revealed by deleting the *PTTG1* gene in mice. Mice lacking *PTTG* gene show testicular and splenic hypoplasia, thymic hyperplasia, thrombocytopenia, aberrant cell cycle progression, and premature centromere division [19, 21].

In addition, recent studies showed that *PTTG-BP1/PBF1* expression was reduced in long standing RA comparably to early RA [14]. At the same time, *PTTG-BP1/PBF1* is a target gene for osteoblast-specific transcriptional factor RUNX2 [18]. Thus, these data allow to hypothesize that PTTG1/PTTG-BP1 axis represents a novel key system in regulating homeostasis of joint tissues with involvement into pathogenesis of osteoporosis and RA progression.

According to our preliminary data, *PTTG1* overexpression leads to a drastic inhibition of Wnt signaling in target cells. Such result suggests that PTTG/PTTG-BP1 axis can serve as a new negative regulator of bone and potentially cartilage homeostasis. In this work, we investigated the effect of *PTTG1* gene overexpression on the activation of BMP and Wnt signaling pathways.

Materials and methods

Cell culture and ligands. Our studies were performed with mouse mesenchymal precursor cells of C2C12 and KS483 lines cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 10 % fetal calf serum (FCS, Sigma). Cells were grown in a 5 % CO_2-containing atmosphere at 37 °C. Upon transient transfection, cells were grown in DMEM supplemented with 4 % FCS and 16 hours later they were transferred to fresh DMEM with 10 % FCS and addition of appropriate ligands or recombinant adenoviral vectors expressing BMP2 and BMP7 to induce osteoblast differentiation. In particular, C2C12 cells in appropriate variants were treated with 10 ng/ml of recombinant TNFα purchased from R&D Systems (Minneapolis, MN, USA).

Short (small) hairpin RNA (shRNA). shRNA-expressing constructs are frequently used as a convenient substitution for siRNA specifically targeting gene expression that allows to avoid initial side effects of transfection required for siRNA delivery to the cells. The set of validated shRNA lentiviral constructs that specifically target the expression of mouse versions of *PTTG1* and *PTTG-BP1* mRNAs was purchased as a part of MISSION library from Sigma-Aldrich (St. Louis, MS, USA).

Transient transfection. C2C12 and KS483 cells were split at a density of 1.5×10^4 cells per cm^2 in 12-well plates. Next day, cells were transiently transfected with plasmid constructs expressing shRNA targeting *PTTG1* and *PTTG-BP1/PBF1* mRNA or control scrambled shRNA (0.5 μg of total DNA per well). Transfection was carried out using GeneJuice transfection reagent (Merck Millipore, USA) following manufacturer's protocol. An efficacy of shRNA-mediated knockdown was confirmed with quantitative PCR and varied from 6.5 to 8 times for most efficient variants (data not shown).

Induction of osteoblast differentiation. C2C12 and KS483 cell lines can be induced to differentiate into osteoblasts by different BMPs, including BMP2 and BMP7. 24 hours after a transient transfection, these cells lines were transduced with a combination of adenoviral

constructs encoding recombinant hBMP2 and hBMP7 at the multiplicity of infection (MOI) even to 500 for each one construct [10] to induce a production of hBMP2/hBMP7 heterodimers along with appropriate homodimers. During osteogenesis assay, C2C12 and KS483 cells were cultured in a differentiation-supporting medium supplemented with 50 μg/ml ascorbic acid for 4 and 10 days, respectively. Starting from day 10 upon induction of osteogenesis KS483 cells cultures were additionally supplemented with 5 mM β-glycerophospate for next 8 days, totaling in 18 days.

Alkaline phosphatase assay. The alkaline phosphatase activity produced by C2C12 was analyzed spectrophotometrically using a π-nitrophenylphosphate (π-NPP) as a substrate [20]. At day 7 of culturing, the supernatants were withdrawn and cells were washed twice with 0.4 ml of 1X phosphate-buffered saline (PBS) per well. The cells were lysed in 0.2 ml of alkaline phosphatase (ALP) lysis buffer (10 mM glycine, 100 μM $MgCl_2$, 10 μM $ZnCl_2$, 0.1 % Triton X-100) per well and gently agitated for 5 min. Then, 10 μl of cell lysate and 90 μl of ALP assay buffer (100 mM glycine, 1 mM $MgCl_2$, 100 μM $ZnCl_2$) supplemented with 6 mM π-NPP (Pierce-Thermo Fisher Scientific, Grand Island, NY, USA) [20], were mixed gently and incubated at room temperature until color developed. The optical density was measured at 405 nm (OD_{405}) in 96-well plate reader (BioTek, Winooski, VT, USA). Data are presented as a "Fold induction versus Control" — magnitudes of changes in times obtained after dividing of a value in each variant on a mean value in untreated control variant.

Alizarin staining. Histochemical examination of mineral deposition by KS483 cells was performed using conventional staining with Alizarin Red (Sigma-Aldrich, St. Louis, MS, USA) [20]. Cellular monolayers were washed with 1X PBS (0.4 ml/well) and fixed in 10 % (v/v) formaldehyde at room temperature for 5 min. Then, the monolayers were washed with dH_2O prior to addition of 0.4 ml of 2 % Alizarin Red S solution (pH 5.5) per well. The plate was incubated at room temperature for 2–5 min with gentle agitation. After aspiration of the unincorporated dye, the wells were washed twice with 0.4 ml of dH_2O per well and once with 3 ml of dH_2O per well while shaking for 5 and 20 min, respectively. The monolayers were then stored in 1 ml of 1X PBS and scanned. Representative wells were showed.

Statistical analysis. Statistical differences were analyzed by Student's *t*-test.

Results and discussion

We have investigated the effect of *PTTG1* gene overexpression on activation of BMP and Wnt signaling pathways. For transient overexpression of *PTTG1* and *PTTG-BP1*/*PBF1* genes, C2C12 cells were transduced with appropriate plasmids (*Fig. 1A*). These cells were also transiently transfected with shRNA plasmids that specifically target the expression of *PTTG1* and *PTTG-BP1*/*PBF1* mRNAs versus scrambled shRNA expressing plasmid that was used as a control (*Fig. 1B*).

ALP is a widely used marker of early stages in osteoblast differentiation [3, 7, 9], and we successfully used it in our preliminary studies. Treatment of C2C12 cells with TNFα completely inhibits their myoblast differentiation, as well as strongly inhibits BMP-induced osteogenesis (data not shown).

We found that the ectopic expression of *PTTG1* gene inhibits osteogenic differentiation in C2C12 cell line. At the same time, shRNA-mediated knockdown of *PTTG1* mRNA led to a substantial increase of bone formation in these cells.

However, ALP cannot be used as a marker of late stages of osteoblast differentiation for which the bone mineral deposition and nodules formation are specific. According to literature and our preliminary data (not shown), Wnt pathway is activated during late stages of osteoblast differentiation [11]. Unfortunately, C2C12 cells cannot undergo late stages of the osteoblast differentiation. Therefore, in the next experiments KS483 cells that can efficiently follow late stages of osteogenesis were used [20].

For transient overexpression of *PTTG1* and *PTTG-BP1*/*PBF1* genes, KS483 cell line were co-transduced with plasmids encoding appropriate proteins (*Fig. 2A*). It was shown that

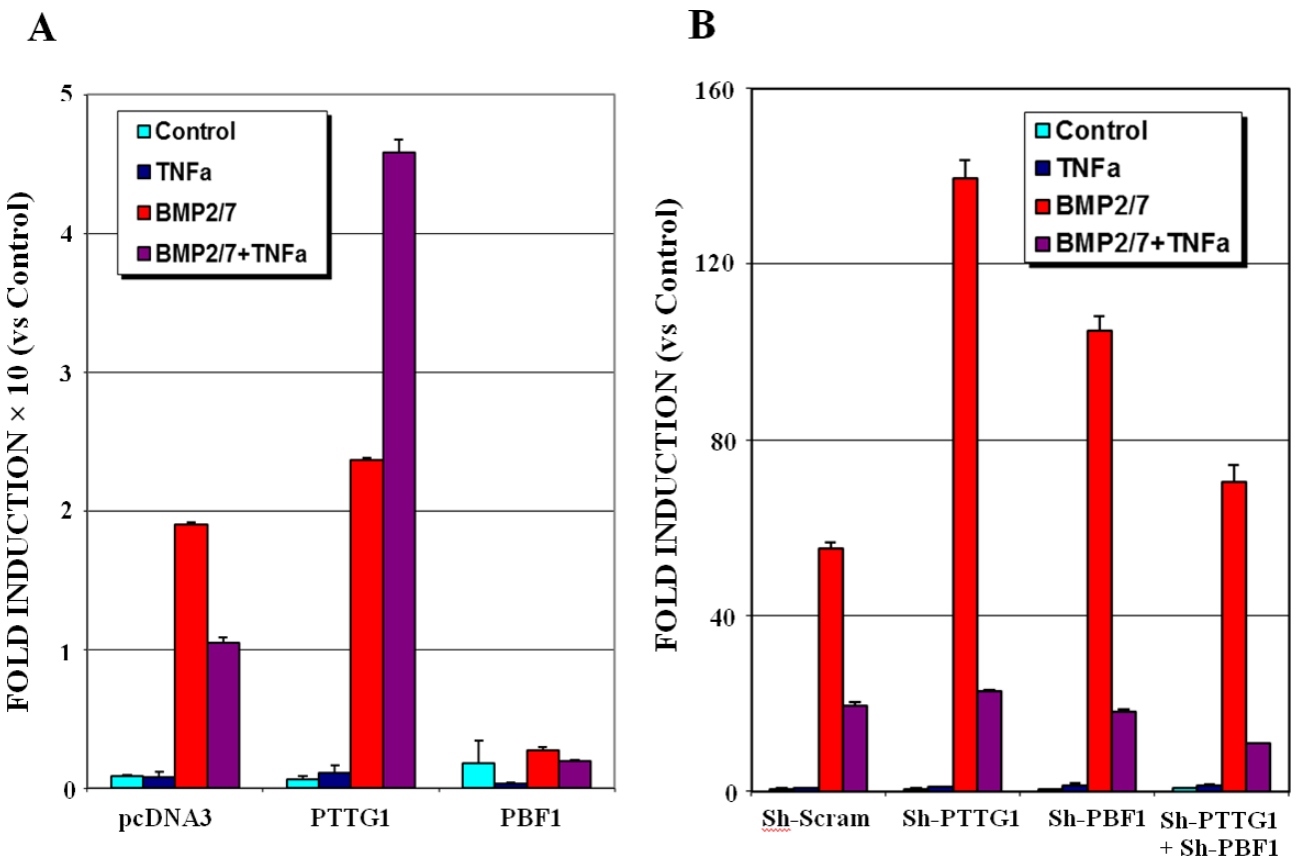

Fig. 1. Ectopic expression of *PBF1* gene (A) and shRNA-mediated knockdown of *PTTG1* and *PTTG-BP1*/*PBF1* mRNAs (B) leads to strong inhibition (A) and activation (B) of hBMP2/7-induced osteogenesis in C2C12 cells, correspondingly.

C2C12 cells were split into 12-well plates, transiently transduced with plasmids encoding *PTTG1* and *PTTG-BP1*/*PBF1* genes (A) or with plasmid constructs expressing appropriate shRNAs (B). Alkaline phosphatase activity in cell lysates was analyzed spectrophotometrically. Osteogenesis was induced with a mixture of recombinant BMP2 and BMP7 adenoviruses (1:1). Optical density at 405 nm is shown.

the ectopic expression of *PTTG1* gene inhibits activation of the Wnt signaling pathway in KS483 cells, which results in inhibition of BMP-induced bone matrix mineralization.

KS483 cells were also transiently transfected with shRNA plasmids that specifically target the expression of *PTTG1* and *PTTG-BP1*/*PBF1* mRNAs. As shown on a *Fig. 2B*, a treatment of KS483 cells with hBMP2/7 strongly intensified their late osteoblast differentiation and overexpression of shRNA constructs targeting mRNAs of *PTTG1*, *PTTG-BP1*/*PBF1* genes or their combination further potentiated osteoblast differentiation observed through nodules formation and matrix mineralization when compared with a control.

However, we were not able to combine hBMP2/hBMP7 treatment with TNFα due to massive cells death induced in KS483 cells by TNFα (data not shown). Similar effect was also observed by other investigators using different (pre)osteoblastic cell lines [2, 8].

In this study we showed that *PTTG1* gene is an important repressor of osteogenesis and can serve as an inhibitor of bone remodeling. Thus, Wnt pathway inhibition by PTTG1/Securin, for the first time may explain the phenotypic manifestations occurring due to *PTTG1* gene targeted inactivation (knockout). The mechanism described in this article could also be involved in maturation of the male reproductive system and be responsible for some forms of male infertility.

Thus, we revealed novel molecular mechanisms and functional impact of negative regulation of Wnt signaling pathway by *PTTG1* gene product. They are crucially important for proper understanding of the molecular basis of pathogenesis and progression of RA and osteoporosis. Besides that, knowing these mechanisms

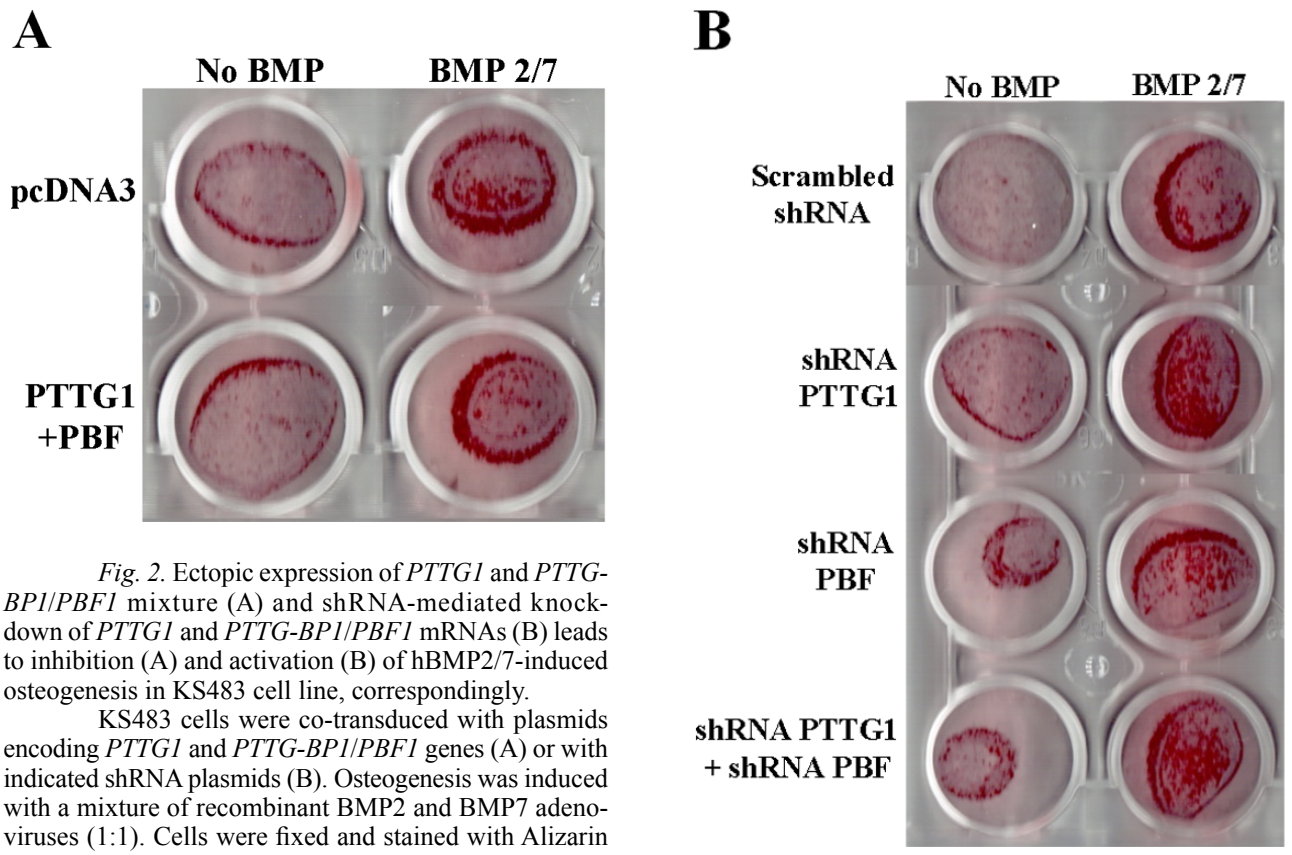

Fig. 2. Ectopic expression of *PTTG1* and *PTTG-BP1*/*PBF1* mixture (A) and shRNA-mediated knockdown of *PTTG1* and *PTTG-BP1*/*PBF1* mRNAs (B) leads to inhibition (A) and activation (B) of hBMP2/7-induced osteogenesis in KS483 cell line, correspondingly.

KS483 cells were co-transduced with plasmids encoding *PTTG1* and *PTTG-BP1*/*PBF1* genes (A) or with indicated shRNA plasmids (B). Osteogenesis was induced with a mixture of recombinant BMP2 and BMP7 adenoviruses (1:1). Cells were fixed and stained with Alizarin Red. Representative fields (1x) are shown.

can become a basis for development of new strategies in diagnostics and treatment of these and other related autoimmune disorders.

Conclusions

1. Ectopic expression of *PTTG1* gene inhibits hBMP2/7-induced osteogenic differentiation in mouse mesenchymal precursor cells of C2C12 line and shRNA-mediated knockdown of *PTTG1* mRNA leads to a substantial increase of bone formation in these cells.

2. Ectopic expression of *PTTG1* gene inhibits activation of the Wnt signaling pathway. Such inhibition results in reduction of BMP-induced bone matrix mineralization and overexpression of shRNA constructs targeting mRNAs of *PTTG1*, *PTTG-BP1*/*PBF1* genes or their combination further potentiated of hBMP2/7-induced osteogenesis in KS483 cells.

3. *PTTG1* gene is an important repressor of osteogenesis, and it may be involved in skeletal tissue destruction caused by inflammatory processes.

Perspectives for further research. We will validate the biological significance of our results using experimental models of PTTG knockout mice.

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