# 学 位 論 文 の 要 旨

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学 位 論 文 名　Restoration of Cellular Function of Mesenchymal Stem Cells From a Hypophosphatasia Patient.

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## 論 文 内 容 の 要 旨

### INTRODUCTION

Hypophosphatasia is a genetic disorder characterized by impaired bone mineralization and extremely low alkaline phosphatase (ALP) activity in serum and bone. The disease is caused by mutations in the tissue non-specific alkaline phosphatase gene (*TNSALP*). The activity of ALP in serum and *TNSALP* genotype were reported to correlate with clinical forms of the disease. Depending on the age of onset and clinical symptoms, five clinical forms are categorized: perinatal, infantile, childhood, adult and odont forms. Almost all infants with severe forms of the disease (perinatal form) die in utero or shortly after birth and the disease has no established therapies. Various therapies such as cortisone, plasma and enzyme (ALP) replacement therapy have been attempted, but the results were inconsistent and did not lead to significant clinical improvement.

Mesenchymal stem cells ((MSCs), also referred to as mesenchymal stromal cells) are multipotent and can differentiate into not only mesenchymal lineages but also ectodermal and endodermal lineages. These cells have been identified in several tissues such as bone marrow, adipose tissue, synovial tissue and dental papilla tissue. We have used human MSCs from the patient's bone marrow for treating various diseases centering on bone diseases since 2001.

In hypophosphatasia, we applied MSC transplantation therapy to a female infant with the disease. The 8-month-old patient with perinatal hypophosphatasia received a BMT using fresh marrow from her father after immunosuppressive treatments. At 15 days post-BMT, MSCs and osteoblasts from her father were implanted. The patient's clinical symptoms improved after these treatments. However, the biochemical parameters of ALP and skeletal deformity did not improve. Our clinical experience of the allo-transplantation showed some degree of therapeutic effects, but

these effects were limited. Therefore, we sought to use autologous (patient's own) MSCs for the treatment of hypophosphatasia. Here, we report the restoration of the mineralization capability of normal *TNSALP* gene transduced hypophosphatasia patient MSCs in response to osteogenic differentiation.

## MATERIALS AND METHODS

### Culture of MSCs

After informed consent from parents and the permission of the local ethics committees of both AIST and the Shimane University Faculty of Medicine were obtained, about 1 ml of fresh bone marrow was harvested from a perinatal hypophosphatasia patient at the age of 8 months. The whole bone marrow was seeded in a cell culture dish with basal medium consisting of minimum essential medium alpha containing 15% fetal bovine serum and antibiotics. The culture was done in a humidified atmosphere of 95% air with 5% $CO_2$ at 37°C. After 2 weeks of culture, adherent cells were harvested and cryopreserved in liquid nitrogen before use. MSCs derived from her father and a healthy donor were prepared as described above.

### Retroviral transduction

Retroviral transduction of the *TNSALP* gene was done by using Retro-X Q Vector, pQCXIN. The plasmids with or without a *TNSALP* promoter-driven normal *TNSALP* gene were transfected into retroviral-producing cells, PT67. MSCs were incubated overnight in the virus-containing supernatants derived from the PT67 cells.

### In vitro osteogenic differentiation

MSCs were seeded at a density of $5\times10^3$ cells per $cm^2$ in 24-well culture plates in basal culture medium and cultured overnight. The medium was changed the next day to osteogenic differentiation medium, which was supplemented with 10 mM β-glycerophosphate, 0.07 mM L-ascorbic acid 2-phosphate magnesium salt n-hydrate and 100 nM dexamethasone. The medium was changed twice a week. The MSCs were also cultured in control medium, which was supplemented only with 10 mM b-glycerophosphate.

### In vivo bone formation

To examine in vivo bone formation of MSCs, we used a rat transplantation model. MSCs were suspended in basal culture medium at a density of $5\times10^6$ cells per ml. Porous hydroxyapatite (HA) disks were soaked in the suspension and incubated overnight. The MSC/HA composites were implanted into immunocompromised rats. HA disks only were similarly implanted as negative control. After 6 weeks, the implants were harvested and histologically analyzed. The animal experiment was approved by the animal care and use committee of AIST.

## RESULTS AND DISCUSSION

The cultured cells from the patient's bone marrow showed small, spindle-shaped morphology and had high proliferative activity. These cells had a mesenchymal immunophenotype: CD13+, CD14-, CD29+, CD34-, CD44+, CD45-, CD73+, CD90+, CD105+, CD166+, HLA class I+. Furthermore, the cells could differentiate into chondrocyte and adipocyte. We therefore concluded that these cells were MSCs. The ALP activity of the patient's MSCs was extremely low in spite of the expression of ALP molecules on the cell surface. The MSCs did not produce a mineralized extracellular matrix even under osteogenic culture conditions. It is considered to be the result of *TNSALP* gene mutation.

Retroviral vector was used to express normal *TNSALP* gene in patient's MSCs. *TNSALP*-transduced MSCs were similar to those of mock-transduced MSCs and non-transduced cells in the morphological characteristics. Furthermore, the panels of CD antigen expression were similar in both sets of MSCs. However, the cell surface expression of ALP molecules was strongly enhanced in the *TNSALP*-transduced MSCs.

To investigate in vitro osteogenic differentiation capability, *TNSALP*-transduced MSCs were cultured under osteogenic culture conditions for 4 weeks. The MSCs showed many ALP-stainable cells and fabricated mineralized extracellular matrices as seen in calcium stain using alizarin red S. Biochemical analysis confirmed that the *TNSALP*-transduced MSCs had higher ALP activity than did the mock-transduced MSCs. To further analyze the osteogenic differentiation, expressions of other bone-related genes were confirmed by RT-PCR analyses.

For the purpose of evaluation of bone formation in vivo, we implanted MSCs/HA composites in nude rats. Histological section of the retrieved these composites showed new bone formation. In contrast, bone formation was not detected in mock-transduced MSCs/HA composites. To confirm the origin of the newly formed bone, the bone areas of the sections were cut with laser-assisted microdissection and used for PCR analysis. The analysis showed the existence of transgene and human gene in the dissected samples.

The MSCs derived from hypophosphatasia patient's bone marrow were restored by normal *TNSALP* transduction. Cell transplantation therapy using the MSCs is thought to be an attractive candidate for clinical treatment of hypophosphatasia.

## CONCLUSION

MSCs from a hypophosphatasia patient had similar profile compared with well-reported MSCs in view points of cell morphology, immunophenotype and multipotency, except for ALP activity. The MSCs had not mineralization capability but could restore the capability by transducing the normal *TNSALP* gene. Our strategy using genetically modified autologous MSCs may be effective for treating genetic diseases.

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## 論文審査の結果の要旨

低ホスファターゼ症はアルカリホスファターゼ（ALP）の活性低下によって骨化障害をきたす常染色体劣性遺伝子疾患である。現在のところ、妊娠中または生後早期に死亡する重症型に対する根本的治療はない。これに対して骨髄移植と同種間葉系幹細胞移植とを行う新規治療の臨床研究が進められているものの、その臨床効果は十分とはいえない。申請者は患者の間葉系幹細胞（MSC）に正常ALP遺伝子を導入し、これを移植する遺伝子治療の可能性を探るために以下の実験を行った。まず、患者由来MSCを骨髄より採取し培養後、その表面抗原の発現を調べた。その結果、患者由来MSCは健常人と同様の表面抗原発現を示していた。しかし、この細胞を分化培地で培養すると軟骨・脂肪への分化能は示したが、ALP活性は低く、骨分化能（石灰化能）は失われていた。次に、患者由来MSCに正常ALP遺伝子を導入し、導入細胞の機能回復を評価した。プラスミドベクターを用いて正常ALP遺伝子を患者由来MSCに導入し、ALP遺伝子を強制発現させた。その後、骨分化培養を行ったものの、導入細胞は細胞死に至った。このため、プロモーターをALP遺伝子自身のものに変えて、患者由来MSCにALP遺伝子を導入して骨分化培養を行うと導入細胞は石灰化能を獲得した。しかし、ALP遺伝子の導入効率が低く、細胞を安定して得ることが困難であった。そこで、自己不活化型レトロウイルスベクターにALPプロモーターと正常ALP遺伝子を組み込み、患者由来MSCに導入した。その結果、この導入細胞はALP蛋白を産生し、石灰化能が回復したが、表面抗原等、他の形質は導入前後で変化しなかった。さらに、導入細胞を多孔性ハイドロキシアパタイトに注入し、ヌードラットの皮下にこれを移植したところ、*in vivo*での新生骨形成能が確認された。 以上から、本法によって正常ALP遺伝子を導入した低ホスファターゼ症患者由来MSCは、健常人由来MSCと同等の骨分化能（石灰化能）を持つことが明らかになった。本研究は、本疾患に対して拒絶されない患者自身の細胞を用いた細胞移植治療の可能性を拓くとともに、他の遺伝子疾患治療への応用にも繋がるものである。以上を総合的に評価して、本研究は学位授与に値すると判断した。