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Cell therapy of a patient with type III *Osteogenesis imperfecta* caused by mutation in *COL1A2* gene and unstable collagen type I*

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ABSTRACT

The allogenic bone marrow derived mesenchymal stem cells transplantation was given to the newborn girl diagnosed with *osteogenesis imperfecta* type III, with multiple bone fractures, extreme shortness and limbs deformities. The treatment was performed at the age of 4 and 6 weeks. The clinical diagnosis was supported by biochemical analysis of collagen type I recovered from culture medium of cultivated patient's skin fibroblast, which revealed its triple helix instability at temperature about 2˚C lower than normal. Sequencing of both genes encoding procollagen type I revealed heterozygous substitution G23569A in *COL1A2* gene causing change of glycine at position 517 to aspartate. The donor of mesenchymal stem cells was the girl's father. She received two intravenous infusions of suspended cultured mesenchymal cells in 16 days apart without any side effects. An analysis of procollagen type I secreted to the culture medium by bone marrow-derived mesenchymal stem cells obtained from the patient, 3 months following transplantation revealed its normal triple helix stability. During the subsequent two years of follow up two new bone fractures were noted. Currently a two-year-old girl's presents extreme growth and weight deficiency. The motoric development is also retarded, but the patient constantly improves and makes progresses.

Keywords: Bone Mineralisation; Cell Therapy; Collagen Type I; *Osteogenesis imperfecta*; Triple Helix Stability

*No conflict of interest exists for each author.
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1. INTRODUCTION

Osteogenesis imperfecta (*OI*) is a genetic disease determined by autosomal dominant negative mutation of type I collagen, which is commonly know as "brittle bone disease". The overall birth prevalence of *OI* is estimated to range between 1 in 20,000 and 1 in 30,000. Clinically *OI* is heterogenous disorder with wide range of manifestations, from very mild, through moderate, to fatally severe, including lethal cases [1]. It is a systemic disorder affecting primarily bones but also teeth and eyes as well as leading to progressive hearing impairment [2]. Bones in this condition are fragile such that affected individuals sustain fractures after very mild trauma and frequent fractures, from several to tenths are observed throughout the patients live [3]. Multiple skeletal features and poor healing is responsible for short stature, relative macrocephaly, scoliosis and bowing of the long bones. Two types of the *OI* are represented by most severe clinical outcome [4]. The type II *OI*, which is the most severe form of *osteogenesis imperfecta* and is almost uniformly lethal. This form of the disorder is characterised by extreme bone fragility, leading to fetal demise or death in perinatal period [5,6]. Second one in severity of clinical symptoms is type III of *OI*, which is leading to serious skeletal deformities and makes the patient to be wheelchaired or bed ridden. The frequency of type II and III *osteogenesis imperfecta* is estimated to be 1 in 10000.

The gold standard for postnatal diagnosis of *OI* is based on clinical and radiological features, confirmed subsequently by molecular diagnosis of collagen triple helix stability and detection of mutation [7]. In the majority of cases the cause of the *OI* is the mutation in

one of the two genes encoding procollagen type I polypeptide chains, namely *COL*1*A*1 and *COL*1*A*2. In recent years, however, new evidence surfaced shading light on other genes, which when mutated might lead to autosomal recessive forms of *OI* although both procollagen genes do not show the mutation [8-11]. The incorrect genes both encoding procollagen type I and potential disturbances in its posttranslational processing are the major causes of poor quality of extracellular matrix, regardless lower collagen content or its poor stability.

Conventional management for patients with *OI* consisted of supplementation with vitamin D and minerals, or administration of bisphosphonates [8,12-15]. However, these methods did not ameliorate the symptoms of *OI* [12]. Moreover, in young teenage patients the bisphosphonates cannot be used continuously, because they slow down bone turnover and as consequence child's growth and healing of eventual bone fractures [16]. Another option of supportive treatment of *OI* patients is surgical and orthopedic intervention or protection with the use of braces, steel rods and other orthopedic equipment [reviewed in 11]. Either, supportive treatment of *OI* did not provide sufficient clinical improvement, especially in the most severe forms. Neither the medications or the surgical treatment can lead to permanent amelioration of the *OI* symptoms. The obvious reason is the genetic nature of this disorder.

For patients with *OI* type II and III a cell or gene therapy could be offered as the only chance of life saving and prevention of severe disability. Already more than 10 years ago first clinical trials were conducted for treating the severe type of *OI* with cell therapy [17]. The trials were then further expanded on larger group of *OI* patients [18-20].

Here, we report a case of a girl with *OI* type III treated at the age of 4 and 6 weeks by transplantation of allogeneic bone marrow derived mesenchymal stem cells (MSC). The diagnosis of *OI* was established on the base of clinical and radiological features, and confirmed subsequently by molecular diagnosis which revealed procollagen thermal instability and detected a missense mutation in *COL*1*A*2 gene.

2. MATERIAL AND METHODS

2.1. Patient

A girl patient was born to a G2, P1 healthy mother at 39/40 weeks of gestation. A child was delivered by caesarean section, because of abnormal fetal heart rate pattern, pelvic position, and severe deformities of upper and lower extremities detected prior to the child's birth during regular monitoring of pregnancy by ultrasound examinations. Both parents are healthy and unrelated individuals. The first pregnancy of this couple was a miscarriage. The analysis of family pedigree did not show any cases of *OI* or any other genetic diseases. The birth parameters of the patient were as follow: birth weight 2490 g, length 44 cm, head circumference 34 cm, Apgar score 3, 4, 5, 7 points. In delivery room the child was successfully resuscitated. The patient required support of ventilation (nasal CPAP) shortly after birth and during the first three days of life. Neither mechanical ventilation nor oxygen therapy were needed later on.

At the age of three days, the child was admitted to the Neonatal Intensive Care Unit of the Department of Pediatrics, Chair of Pediatrics, Jagiellonian University and hospitalized for subsequent three months. Clinical evaluation of the patient on admission revealed short stature, severe lower limb deformities, relative macrocephaly, triangular face and blue sclera. X-ray examination (baby-gram) showed 27 bone fractures, multiple deformities and reduced ossification of the skull calvarion. Based on the clinical and radiological findings of multiple bone fractures and severe limb deformities, the preliminary diagnosis of type II *OI* was made according to the classification by Sillence [21]. The anthropometric measurements were conducted and the dermatoglyphic patterns were analyzed. Genomic DNA was isolated from cultured fibroblasts for the molecular analysis of the *COL*1*A*1 and *COL*1*A*2 genes. The skin biopsy was performed to assess the stability of produced by fibroblasts collagen triple helix. The levels of serum bone turn-over markers (osteocalcin (OC), procollagen type I N-propeptide (P1NP), collagen type I crosslinked C-telopeptide (Ctx)) were assessed. Then, on the basis of the natural cause of the disease, the result of molecular analysis revealing heterozygous mutation in exon 26 of *COL*1*A*2 gene and the diminished thermal stability procollagen triple helix, the diagnosis was changed to type III *osteogenesis imperfecta*. The patient was qualified to a clinical trial entitled "Cell therapy in treatment of the patients with type II and type III *osteogenesis imperfecta*". The entry criteria for the study were as follow: 1) diagnosis of type II or III *osteogenesis imperfecta*, 2) parents agreement for participation in the clinical study, 3) approval of the Bioethics Committee of the Jagiellonian University (KBET/108/B/2007).

2.2. Patient's Samples

The anthropometric measurements were conducted and the dermatoglyphic patterns were analyzed before mesenchymal cells transplantation was performed. A blood samples were taken to assess the level of serum bone markers (OC, P1NP, and Ctx). The skin biopsy was performed to assess the stability of collagen triple helix and genomic DNA purification for molecular analyses of *COL*1*A*1 and *COL*1*A*2 genes.

2.3. Survey of Collagen Genes for Mismatches

DNA fragments of the collagen *COL*1*A*1 and *COL*1*A*2 gene were amplified using primers described previously [22] and listed in **Table 1**. The primers were designed and verified using PrimerSelect in DNAStar programme package version 5.0. The DNA was isolated from the proband's cultured fibroblasts, using BloodMini DNA purification kit (A & A Biotechnologies, Gdansk, Poland). The DNA was recovered from the kit column with 1 mM EDTA buffered with 10 mM Tris-HCl pH 8.5 at final volume 150 µl and its concentration was determined spectrophotometrically at the wave length 260 nm and 280 nm. Quality of the DNA was verified following its electrophoresis in 0.8% agarose gel. Until further analyses the DNA samples were stored at −20˚C. Concentra-tions of primers were calculated based on the sample absorbance at the wave length 260 nm and adjusted to the primer compositions. PCRs were performed according to the conditions described elsewhere [22]. Briefly, the reaction mixture included 300 ng double stranded patient genomic DNA, 1 µl 10 mM dNTPs (PCR nucleotide Mix, Roche, Germany), 1 µl (5 pmol) of each primer, 2.5 µl 10x Taq polymerase buffer, 1U Taq polymerase and nuclease free water (all from FastStart Taq DNA Polymerase, Roche, Germany) to the total volume of 25 µl. The mixture was denatured for 5 minutes at 95˚C, and incubated for 34 cycles of: 30 seconds at 95˚C, 30 seconds at 60˚C or 65˚C, depending of the Tm of the specific pair of primers (**Table 1**), 30 seconds to 2 minutes at 72˚C depending on the length of the amplified DNA fragment. When the last cycle was completed the samples were incubated for 7 minutes at 72˚C then cooled down and left at 4˚C. The PCR products (3 µl) were separated on 1% agarose gel (Agarose, LE, Analytical Grade, Promega, USA) in TAE buffer.

2.4. PCR Conditions for DNA Sequencing

The PCR for sequencing was conducted according to recommendations provided by the DNA Analyser 3130 × l ABI Prism, manufacturer (Applied Biosystems, USA). Briefly 10 to 20 ng of the PCR product was purified using Shrimp Alkaline Phospatase (Fermentas, USA) and Exonuclease I, *E. coli* (Fermentas, USA). Clean product was then combined in semi-skirted 96-well PCR plate with 5 pM of primer, 8 µl of the ready to use mixture-provided by the manufacturer BigDye Terminator v3.1

Table 1. Sequences of primers used for fragment amplification and for sequencing of the *COL*1*A*1 human gene.

Fragment	Exon No. and position	Primers for fragment amplification (Amplified fragment length in bp)	Primers for sequencing
1	1 420 - 522 2 1986 -2180 3 2322 - 2356 4 2468 - 2494	M1f 305-F GACGGGAGTTTCTCCTCGGGGTC N3R 2927-R GCGCAAAAGAGCCTGATGTTA [2622]	M1f M2fk GGGAGGGGCGGGAAGTGAAAAAT N3R GCGCAAAAGAGCCTGATGTTA
2	5 2585 - 2686 6 3409 - 3480 7 3708 - 3752 8 3911 - 3964 9 4128 - 4181	N5F 2831-F GTTTGTGACATGTGCTTTATTAGT 2RA1 4049-R GCTGGGATTGAAGGGAAGAGGTAA [1218]	N6F AGTGCCGTCTTCTGCCTTTCA 2RA1
3	10 4680 - 4733 11 4850 - 4903 12 5234 - 5287 13 5376 - 5420 14 5537 - 5590	N7F 3983-F GATTCATACTCCTTCTACAAAC 3RA1 5647-R CTGGCCAGTCCCTAGAGTTCCT [1664]	N7F N7R GGCACCATCCAAACCACTGAAA 3RA1 4FA1 GAGCCTGGCAGCCCTGGTGAAAAT
4	15 5705 - 5749 16 5928 - 5981 17 6239 - 6337	4FA1 5379-F GAGCCTGGCAGCCCTGGTGAAAAT 5RA1 6436-R ACCAGGCTGTCCATCAGCAC [1057]	4FA1 5RA1

Continued

5	18 6426 - 6470 19 6574 - 6672 20 6804 - 6857 21 7076 - 7183 22 7278 - 7331	5FA1 6134-F CTGATCATTGCTCTCCTGTCCCTGT 4RA1 7332-R CCCGGCCGCAAGGAGAGGTTAC [1198]	5FA1 W9FA1 ACACCCTCAGCCCCTCGTCTC 4RA1
6	23 7457 - 7554 24 7721 - 7774 25 7863 - 7961 26 8858 - 8911 27 9055 - 9108	6FA1 7166-F GGACCCCCTGGCGAGCGTGTAA 6RA1 9166-R GGGGAGGCCGAGGAGACGAG [2000]	6FA1 8FA1 AGGCCCTGGCTTCTCACTTCA
7	28 9212 - 9265 29 9377 - 9430 30 9882 - 9926 31 10020 - 10118 32 10416 - 10523	W9FA1 9153-F ACACCCTCAGCCCCTCGTCTC W9RA2 11221-R AGCCTCCCCTCCTTCTGGT [2068]	W9FA1 M30F GGGTTCCTCTCTAATCACGGCCAGACC W8FA1 GCGGGGCTTAGGGCTGTGACC W9RA2
8	33/34 10982 - 11089 35 11310 - 11363 36 11527 - 11580 37 11799 - 11906 38 11995 - 12048 39 12175 - 12228 40 12369 - 12530 41 12632 - 12739 42 12897 - 13004	9FA1 10501-F GCTCCCATCTCTGCCTGCTTTGA W10RA3 13247-R TGGCGGGGAGAGCAGGGGAATA [2746]	W9RA3 ACGCCTTTGTCCTCATTC W9RA4 CCCACCCAGCACCCCCAACCTA W10FA2 TGTTCTCCCTCTGACTGTTCCTAT W10RA3
9	43 13112 - 13165 44 13270 - 13377 45 13758 - 13811 46 13924 - 14031 47 14370 - 14423 48 14784 - 14891	W10FA3 12800-F GCGGAGCCAAGGAGAACAGAT W11RA3 15311-R GCATCATCAGCCCGGTAGTAGC [2511]	W10FA3 M46r GGGGAAAGAATGACTATCCAG W11FA2 CCCCTGGCCCTCCTGTAAGTATGC W11RA3
10	49 14983 - 15265 50 15398 - 15588 51 15877 - 16119 52 16249 - 16395	N9F 14922-F GCCCTCCAGCCCCCATAGCACT N10R 16599-R GGTTTTTGGTCATTGTTCGGTTGGT [1677]	P48 CATGTCCCTTCTGAGCACTGGGCTA N10F AGGTCCCTGCTAGTGGTTCA M51F GGACCCTGGACAGGAGGCCAGCAGG

Cycle Sequencing Kit (Applied Biosystems, USA) containing polymerase, dideoxy-NTP in the buffer and the final volume was obtained by adjusting it with water up to 20 µl. The mixture was incubated in the thermocycler by Eppendorf for 35 cycles with the following steps in the single cycle: denaturation for 30 seconds at 96˚C, annealing 15 seconds at 50˚C, elongation for 3 minutes at 60˚C. After completing the last cycle the sample was cooled down to 4˚C. The sequencing PCR product were purified by BigDye XTerminator Purification Kit (Applied Biosystems, USA) according to manufacturers manual and put in the instrument for the DNA sequencing. The sequences were compared with reference sequence (NG_007400.1 COL1A1 and NG_007405 COL1A2) using MegAlign in DNAStar programme package version 5.0. The differences were verified for final confirmation by analysis of the raw chromatogram sequencing data using Chromas Lite version 2.01 freeware.

2.5. Procollagen Type I Production and Purification

Procollagen type I was produced using skin fibroblasts prior and following the bone marrow mesenchymal stem cells transplantation. The skin biopsies of the size of 5 mm^2 were obtained using surgical blade. The sample was minced into small pieces and placed on a 100 mm in diameter Petri dish containing 10 ml of Dulbeco's Modified Eagle's Medium supplemented with 4.5 g/L glucose, (high glucose DMEM) 10% foetal calf serum (FCS), 10,000 units penicillin, 10 mg units streptomycin, 25 µg/ml amphotericin B (all reagents from PAA The Cell Culture Company, Pasching, Austria). The medium was changed every other day. On day 7 of the culture the pieces of skin were removed and the fibroblasts were cultured until confluence. At 100% density the cells were transferred into T150 culture flask and maintained in the same medium until reaching 100% confluence when half of the culture was frozen, whereas, the other half was further cultured until 6 flasks of about 70% confluent were obtained. The procedure was followed for each sample. The fibroblast cultures were rinsed twice with sterile phosphate buffered saline (PBS) and filled with 15 ml of serum-free high glucose DMEM supplemented with ascorbic acid 50 µg/ml and all the other supplements as described above. The medium was collected every day and replaced with the fresh one for three consecutive days. Then for 24 hours the culture was fed on high glucose DMEM with serum. The procedure of washing with PBS, harvesting the serum-free medium and feeding with total complete medium were repeated 4 times.

Harvested medium was processed as previously described [23] with subsequent modifications described elsewhere [22,24]. The volume of sample containing procollagen I was reduced to the appropriate collagen concentration using Amicon cell under nitrogen pressure fitted with the membrane with the size cut off 100,000 kDa, following double dialysis against 200 volumes of so called storage buffer containing 0.4 M NaCl, 0.1 M Trizma Base (pH 7.4) supplemented with 0.025 M EDTA and 0.04% NaN_3.

2.6. Preparation of Collagen Type I and Its Triple Helix Thermal Stability Assay

The procollagen type I sample was treated with acetic acid to adjust pH to about 2.0. Pepsin was added to the acidified procollagen to a final concentration 200 µg/ml. The digestion with pepsin was carried at 10˚C for 18 hours, than terminated by raising pH to 7.5. The concentration of collagen was determined using kit by Biocolor (Ireland). Stability of collagen triple helix was assessed using J-815 Spectropolarimeter (Jasco) at 220 nm, using a 0.5 mm path-length thermostated cell. The temperature was controlled by a circulating water-bath. The temperature changes were controlled by a programmable controller (Jasco Peltier) which increasing temperature of collagen from 20˚C to 45˚C.

Stability of collagen triple helix was calculated by using Spectra Manager Program and Statistica 8.

2.7. Bone Mineralization Assay

Osteoblasts were detected by staining for the presence of hydroxyapatite in the culture using Alizarin Red-S (AR-S), following fixation in cold 70% ethanol at −20˚C. At indicated osteoblast's differentiation time points, the medium was removed and the culture was washed with PBS followed by fixation in ice-cold 70% ethanol for at least 1 hour. Subsequently, the ethanol was removed, and fixed cells were rinsed with de-ionized water, then stained with 40 mM AR-S (pH 4.2) for 10 minutes at room temperature. After staining the cells were further processed by rinsing them five times with water followed by 15 minutes wash with PBS to remove nonspecifically bound AR-S. The specimens were photographed and immediately processed for minerals content. Therefore, bound AR-S was solubilized with10% cetylpyridinium chloride (CPC) in 10 mM sodium phosphate, pH 7.0, for 15 minutes at 25˚C. Recovered AR-S was diluted 10-fold in 10% CPC solution, and the AR-S concentration was determined by measuring the absorbance at 562 nm wave length using an AR-S standard curve in the same solution [25,26]. The values were normalized per 1000 cells. The measurements were conducted in quadruplicates.

2.8. Isolation of BM-MSCs

Bone marrow derived mesenchymal stem cells (BM-MSCs) were obtained from healthy patient's father after written consent according to the protocol approved by the Jagiellonian University Bioethical Committee (KBET/108/B/2007). Bone marrow (150 ml) was har-

vested under anesthesia from the posterior superior iliac crest by multiple aspirations. Aspirates were collected in a Bone Marrow Collection Kit with Pre-Filter and Inline Filters (Baxter, USA) and further processed. Light-density mononuclear cells (MNC) were separated on a Lymphocyte Separation Medium (PAA Laboratories GmbH, Goetzis, Austria) by density gradient centrifugation at 400 g for 30 minutes at room temperature. MNC were washed twice with Dulbecco's phosphate buffered saline (PAA Laboratories) and suspended in culture medium.

Culture of BM-MSCs

Bone marrow mononuclear cells were plated into vented 75 cm^2 tissue culture flask (Sarstedt, Newton USA) with Dulbecco's-Modified Eagles Medium (DMEM, Sigma-Aldrich Germany D5523) supplemented with 10% FBS (Stem Cell Technologies, Vancouver Canada 06472) and antibiotics (PAA Laboratories GmbH, Goetzis, Austria). Flasks were incubated at 37˚C in a humidified atmosphere containing 5% CO_2 and after 7 days medium was replaced with a fresh one. Than the cells were cultured with a medium change every week until confluence. On reaching confluence, the adherent cells were detached using 0.25% trypsin and re-seeded at 75 cm^2 flask (first passage). At the end of second passage when the cells reached confluence, they were trypsinized and checked for heamatopoietic contamination [27].

2.9. Flow Cytometry

MSC phenotype was analyzed with mouse antibodies specific for human CD45 and CD3 antigens (Becton Dickinson). Briefly, to 1×10^5 cells suspended in 100 µl of staining buffer (PBS, 2% FBS) 20 µl of mouse antibodies was added. Next, the cells were incubated in the dark for 30 minutes at 4˚C. Stained cells were washed, collected and analyzed using FACS Calibur cytometer (Becton Dickinson, USA).

2.10. Infusion of the MSC

Cells were harvested after the second passage in two parts in two weeks time. Each time cells were suspended in 10 ml of normal saline and passed through a filter before infusion. The patient received two 15- to 30-minutes intravenous infusions of MSCs 16 days apart. Target doses for the first and second infusions were 4.0×10^6 cells/kg of body weight and 3.9×10^6 cells/kg, respectively. Directly before infusion the patient received hydrocortisone. The girl was evaluated for toxicity (physical examination, complete blood count, clinical chemistry panel-BUN, glucose, AST, ALT, ALKP, urine analysis, before, and 24 hours after, each infusion. She was monitored at the bedside during, and for 6 to 8 hours after, each infusion. Nine days after first infusion she received a course of the Vancomycine because of positive blood culture for Staph. Epidermidis. During the bacteremia period she was stable and in a good general status. The period of two weeks after the second infusion was uneventful. Then she developed $G^{(-)}$ sepsis with *Enterobacter cloacae* treated with Meropenem and III generation cephalosporin antibiotics. She recovered from sepsis after two weeks and her general status was quite good.

Three months after MSC transplantation a bone marrow biopsy (for assessment the chimerism and cellular differentiation) and a skin biopsy (for evaluation the stability of collagen triple helix) were performed. Another blood sample was taken to molecular analysis of *COL*1*A*1 and *COL*1*A*2 genes.

The patient was finally discharged from hospital at the age of four months. She has had regular appointments in a genetic out-patient clinic (at the beginning every month, now every two months). Each visit consists of: history taking, physical examination, anthropometric measurements and collecting blood sample to assess the level of serum bone turn over markers.

3. RESULTS

3.1. Cells Expansion

Expansion of the cells resulted in harvesting 4×10^6 cells and 3.9×10^6 cells. Two infusions with collectively of 7.9×10^6 MSC were performed. Expanded cells contained 7.3% of hematopoietic contamination, but did not contain lymphocyte contamination (**Figure 1**).

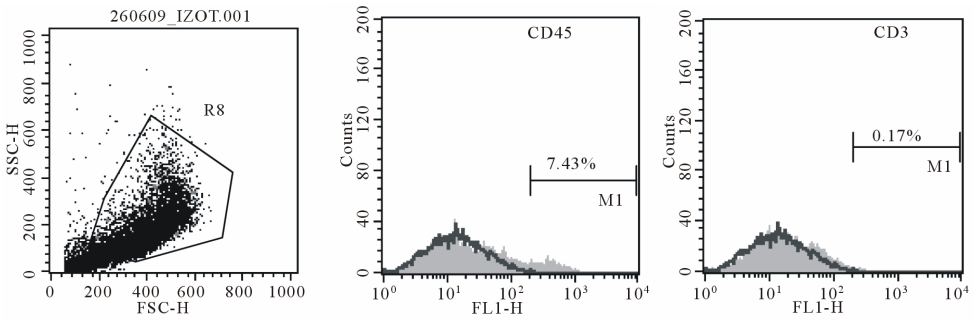

Figure 1. Majka M. *et al.* Cell therapy of a patient with type III *osteogenesis imperfecta*...

3.2. Patient Follow-Up

Patients follow up was performed monthly. Physical examination of the patient revealed short stature, severe lower limb deformities, relative macrocephaly, triangular face, blue sclera and *dentinogenesis imperfecta*. The antropometric measurements show extremely retarded growth (**Table 2**). The child's psychomotoric development was not synchronic—motoric development is retarded, while the speech development and cognitive functions are adequate to the chronological age. The patient is under the constant care of physiotherapist, orthopedic surgeon, psychologist and dentist. An objective audiology (ABR, AOE) examination was not possible to perform so far. Nevertheless, there is no signs of hearing loss in our patient.

The patient has had six bone fractures since her birth of which five were identified during the performance of diagnostic bone marrow biopsy after the MSC infusion. During the afterwards follow up over the period of two years two new fractures were diagnosed. The results of serum bone markers levels did not revealed any clinically significant tendencies.

Stability of collagen triple helix assessed in skin fibroblasts taken prior to bone marrow MSC transplantation showed temperature of 2˚C lower than normal. Stability of collagen triple helix assessed in skin fibroblasts taken after the MSC transplantation returned to the normal temperature range (**Figure 2**).

The molecular analysis of the gene *COL*1*A*1 performed prior to and following the MSC transplantation did not reveal any mutation. A missense mutation G23569A changing glycine at position 517 to aspartate (p.Gly517Asp) was detected in the exon 26 of *COL*1*A*2 gene before and after the MSC transplantation. This particular mutation was previously identified in one patient with type III *osteogenesis imperfecta* reported to *Osteogenesis Imperfecta Variant Database.*

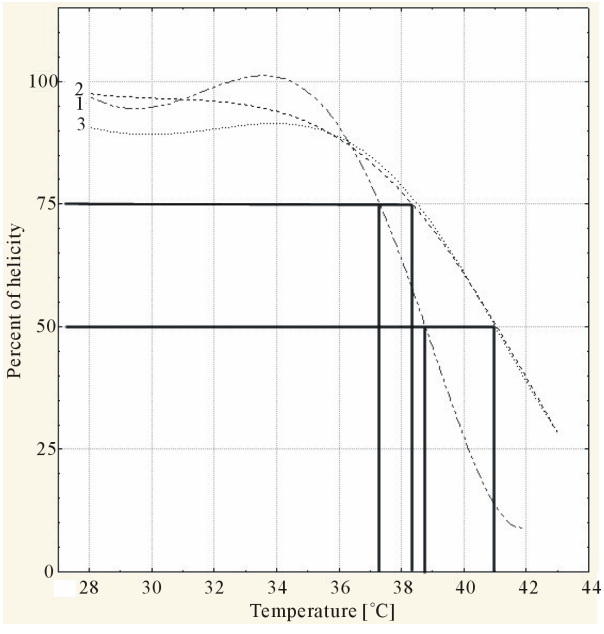

Figure 2. Majka M. *et al.* Cell therapy of a patient with type III *osteogenesis imperfecta*...

3.3. Bone Mineralization

Data on differentiation of bone marrow cells to osteoblasts measured indirectly as mineral deposition are inconclusive. Although the bone marrow MSC collected following transplantation could be successfully expanded during process of differentiation their number decreased with time. The experiments were repeated three times in quadruplicates and none of them was successful. The control sample in this experiment was established culture of stem cells purified from human normal placenta. The cells grew accordingly during differentiation and revealed increasing mineral deposition expressed as amount of calcium ion bound to AS-R (**Figure 3**).

3.4. Chimerism

Genetic profile of transplanted patients were studied using AmpFlSTR SGM which recognized 11 STR loci. Only DNA of patient was detected indicating low engraftment of donor MSC.

4. DISCUSSION

Type III *OI* is known as progressive disease leading to serious skeletal deformations and severe handicap. Its presentation at birth is phenotypically similar to the milder end of the spectrum of type II disease. Although this form of the disorder is usually not life threatening, some children with type III *OI* die in infancy because of respiratory distress, whereas others die in childhood due to pneumonia, pulmonale or trauma. For those, who survive, there is progressive deformity of long bones and spine, which in combination with compression of verte brae, contributes to marked short stature. Most of the patients with type III *OI* are severely physically handicapped and have traditionally required multiple orthopedic procedures and wheelchairs for mobility.

In fact, there is no effective treatment for the patients with *OI* so far [27-30]. That is why, it seems to be of crucial importance to continue research studies investigating the role of new methods of therapy in patients with this disease. A development of new treatment could improve the prognosis and reduce unnecessary exposure to potentially painful therapies.

We conducted a study examining the role of cell therapy in patients with type II and III *OI*. Till now, we enrolled one trial subject. MSC were administered

Table 2. Sequences of the primers used for fragment amplification and for sequencing of the *COL*1*A*2 human gene.

Fragment	Exon No. and position	Primers for fragment amplification (Amplified fragment length in bp)	Primers for sequencing
1	1 5742 - 5541	2FA2 5364-F AGTTGGAGGTACTGGCCACGACTG 2RA2 7289-R TTAGGTGGCCAAGAAGTGCGTTAT [1925]	2FA2
2	2 8188 - 8198 3 8822 - 8836 4 9489 - 9524 5 10636 - 10728	3FA2 6944-F TTGAGAGTGGGAGGAAAGAGTCG N2RA2 11159-R GTCAAGGAGAATAAAAAGCGTAGG [4216]	4FA2 TGCTGATCCCTGCCATACTTTTGAC JK3F GTGAAGGTATATTTGTATACTACAC JK3R TGTTATCTTAAACATCAAAGCTAC JK4F CATTGTAGTTACATCAGTCTTACC 7FA2 N2RA2
3	6 12007 - 12060 7 16996 - 15040	7FA2 10526-F TCCACCCTACTTGCACATAGAAAGG 7RA2 13393-R TTAGGGAATTAGAAGTCGTCAA [2867]	JK6F TCGGCCAAGTTTTTGACGTACAGCT JK6R TGGCGTGGTAAAATGTGACATAAAA
4	8 15133 - 15186 9 15279 - 15332 10 15639 - 15692 11 16113 - 16166 12 16690 - 16743 13 18287 - 18331	9FA2 14753-F TAAGGAACCTATCTGCCCCGTCTA 10RA2 18544-R TTCCAACCTGTACAAGAATACACT [3791]	9FA2 JK10/11F ACCAAGATTCCCCCATTTGTGCTGA 9RA2 GAAGTCCAGTGTTCCAGGTCCCAGC JK12R TGGAGGTCATGGGGAATTTCAATCA 11FA2
5	14 18623 - 18676 15 18776 - 18820 16 19210 - 19263 17 19762 - 19860 18 20004 - 20048 19 20163 - 20261	11FA2 18118-F GAACCTGGATATGTGGTACTATCTG N5RA2 21572-R CAGGGCCACTTGCACCACGACTA [3455]	JK14F CTTGTACAGGTTGGAAACTGAAC JK14R CCACGGGCACCCTAAGAAGA JK15F CCGTGGGCTTCCTGGTGAGAG JK16F CACCCTGGATACCATGAATGTC 11RA2 TTCTTCTTCTTCCCCATACTCCAC 12FA2 JK18FB CGTTGGACCTCCTGTAAGTAG JK19F TAATGTGTGCTGCCTCTACAGC
6	20 20682 - 20735 21 20860 - 20967 22 21329 - 21382 23 21496 - 21594	12FA2 19891-F AGTAAAAGAAAACAAAGGTGGAGT 12RA2 22245-R TGTATATGGGCAAAATGTATCACG [2354]	JK20F CTTGAGCTTCTCTTTACCTTGAC JK21F CGTAAGTAGCTCTATCATCAC JK22/23F GGGTTGGGTGAAGTGTTTTGGCTTG JK23RB GCAACTGTCAGCAAGACTAC
7	24 22508 - 22561 25 23024 - 23122	13FA2 22124-F CCACAAACAAGTAATAGGGACACC 13RA2 23663-R	13FA2 JK24F AAAAAGTCGGGGGAAAAGGTGCCTT JK25/26F

Continued

	26 23523 - 23576	CAAAATGATATCAGATGGTGTAAA [1539]	TTTCATCCGTGGCAGCATCATAAGC JK25R CTGAGACTGGACTGATTCGCAG JK26R TATCAGATGGTGTAAAAAAAAAAGTGTGGTTCT- TAGATG JK26FB TGGAGCTGCATGGTGATGGATC
8	27 24130 - 24183 28 24334 - 24387	14FA2 23556-F CTGGTCTTGCTGGTGCTCGGGTAG 14RA2 25707-R TGGACCAGCAGGACCAGGGAGACC [2151]	14FA2 JK27/29F GCTTTCGTGGGAACCCACAATGAGT JK27RB TAGCAACGTATGTCACCACTG JK28RB TGCTTCAGTCCTGAAATCATGT
9	29 24662 - 24705 30 25666 - 25110 31 26845 - 26943	15FA2 24540-F GCTTAATAACATACAATCGTGCTC 15RA2 27582-R ACATCCTTCCATACACTAAAATAG [3042]	15FA2 JK30F TGCACTCATGTAGATACTGCCAGGT JK31F AAACCAGGGCTCGGAAGCTACACAA
10	32 28164 - 28271 33 28939 - 28992 34 29938 - 29991	16FA2 27185-F GGTTGTCACTCTTTTCTCCTCACG 16RA2 30032-R TTTTAGATATTGCTGTTGGCTTAG [2847]	JK32F TCTCCCTCCTTTCAATAGCCCAGCC JK33F GAATGGTAAGGAATCGAGACATTGC JK34F GGAGTACCCTCCTTCTGAGAGTGGC
11	35 30673 - 30726 36 30831 - 30884 37 30981 - 31088 38 31449 - 31502	JK35F 30535-F ACTCTGTGAGATGTGCGTCAG JK40RB 33651-R CATCAACAAATAGATGCCACTTG [3116]	JK35F JK38F TGCGGGAATGATCCACTTGAAGAA JK36/37F GAAGCCCTGTAAGTAAGAACCTG JK37R CTTCCGTTATTTTCCATCTTCTATC JK38R TCGGAATTGCTCTGAATAGAATGAA
12	39 32339 - 32392 40 33397 - 33558 41 34776 - 34883	17FA2 31958-F GGTCATTTGTTATTCTTTTGTCCA N13RA2 35305-R GATGGTTTCTAGTTGTTGTCACG [3348]	17FA2 JK39RB GAAAAGCTGACTTCAGACCAGGAG JK39FB GAAATTCCCATCTTACCCAAATTCTTG JK40RB JK40FB CATAAAGGAAGACAGGAGTTGC 17RA2 CTCATTTATTGTCTTCCCGATTTG JK41F CTCACAATCTTCAAGCCAACCTGTG JK41R TCTGTCACATTTGAAGTGGCAGCTT N13RA2
13	42 35557 - 35664 43 36050 - 36103 44 36190 - 36297 45 36438 - 36491	18FA2 35302-F TTTGGGATACTGAATGACACG 18RA2 36985-R CTACAGTTTACTTACAGCGATACC [1683]	18FA2 JK43F AGGGTTCGTTACTGAGCACTG JK44F ATGGTCAACCCGGACACAAG JK45FB GTGGGCTTCACTTCTGACTTC
14	46 36863 - 36970 47 37448 - 37501 48 37628 - 37735	N15FA2 25691-F GTGAAGCTGGTCGTGATGTGAGTC 19RA2 38998-R TATTTAGCAGAAGGAGAGGTGTTT [3308]	JK46F GCAGATTACCAGCAGAGGTGAGAGC JK47R GACACCAGGTACATGTGAGCTG JK47/48R TGGGGCTAACTTTAATGGGTTGTC

Continued

15	49 38067 - 38325 50 38733 - 38917 51 39628 - 39870 52 40687 - 40833	20FA2 38643-F CTCTTCCTCTTAAATATGGGGTAG N16RA2 41458-R AAAAATACACCACACGATACAAC [2815]	JK49FB GAACATGCTTCCGTGTGAAGCTC JK48F CAAGAGAAGACAGTTCATCTCTG JK50FB TGTTACTTATGAGAGTCAGTATCTTTC JK51F CCCTTTTCCTAAGCTTGGATCTGAG JK51R TTAACCCCCCTTTAGACCCCCCTTG JK52FC GGACAGACATCTTCAGAATGAC

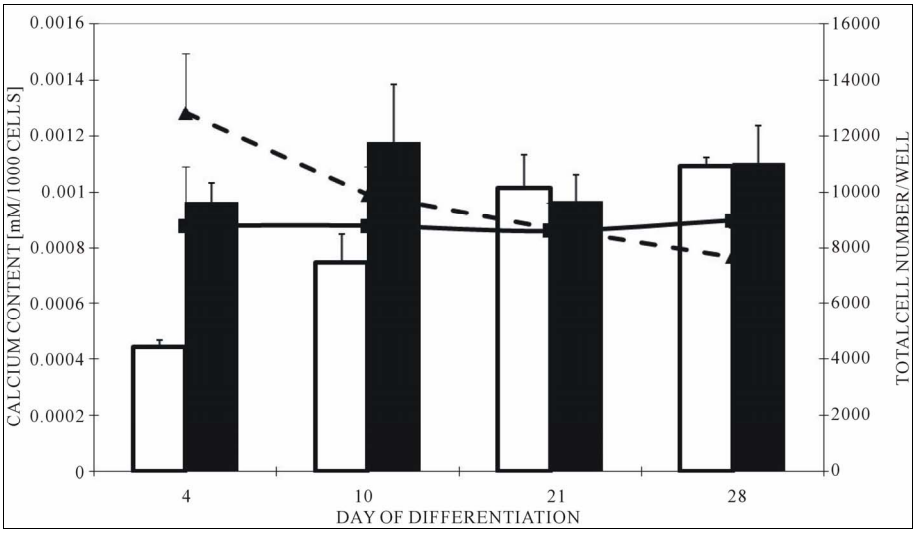

Figure 3. Majka M. *et al.* Cell therapy of a patient with type III *osteogenesis imperfecta*...

Intravenously. The most significant observation in the present study is the association between the MSC transplantation and the improvement of stability of collagen triple helix, and a decrease in a number of fractures observed in our patient. Stability of collagen triple helix after the MSC transplantation was normal, and there was only two bone fracture noted two years after the bone marrow stem cells transplantation. To validate this finding we measured serum bone markers levels, but they did not revealed any observable tendency. Nevertheless, during the first 2 years of life, the child had significant growth deficiency. Anyway, we can speculate that the cell therapy in patient with type III *osteogenesis imperfecta* looks promising. However, further evaluation is necessary.

Failure in detecting a chimerism in the patient could be a result that the threshold of the detection method used is 5%, thus, presence of donor cells could not be excluded if the cell engraftment fell under 5% and the donor DNA was not detected.

In summary, the stability of patient's collagen triple helix assessed in skin fibroblasts taken after the mesenchymal cells transplantation was within a normal temperature range. The girl has had only two bone fractures two years after the transplantation. No cases of rejection reaction when the father's MSCs are injected into the girl's body were previously reported from other trials with the use of purified cells [17,18]. Therefore, in this study the graft versus host disease symptoms also were not observed. In conclusion, although, the assay of *in vitro osteogenesis* by minerals deposition was inconclusive, altogether the results indicate that they might be considered as an objective indicators of partial success of the cell therapy. It is very important for further clinical trials of cell therapy in the *OI* because no reports are available on remission of this disease, especially of its most severe type III.

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