Short Communication

Fetal Liver Cells Transplanted *in Utero* Rescue the Osteopetrotic Phenotype in the *oc/oc* Mouse

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Autosomal recessive osteopetrosis (ARO) is a group of genetic disorders that involve defects that preclude the normal function of osteoclasts, which differentiate from hematopoietic precursors. In half of human cases, ARO is the result of mutations in the *TCIRG1* gene, which codes for a subunit of the vacuolar proton pump that plays a fundamental role in the acidification of the cell-bone interface. Functional mutations of this pump severely impair the resorption of bone mineral. Although postnatal hematopoietic stem cell transplantation can partially rescue the hematological phenotype of ARO, other stigmata of the disease, such as secondary neurological and growth defects, are not reversed. For this reason, ARO is a paradigm for genetic diseases that would benefit from effective prenatal treatment. Using the *oc/oc* mutant mouse, a murine model whose osteopetrotic phenotype closely recapitulates human *TCIRG1*-dependent ARO, we report that *in utero* transplantation of adult bone marrow hematopoietic stem cells can correct the ARO phenotype in a limited number of mice. Here we report that *in utero* injection of allogeneic fetal liver cells, which include hematopoietic stem cells, into *oc/oc* mouse fetuses at 13.5 days *post coitum* produces a high level of engraftment, and the *oc/oc* phenotype is completely rescued in a high percentage of these mice. Therefore, *oc/oc* pathology appears to be particularly sensitive to this form of early

treatment of the ARO genetic disorder. (Am J Pathol 2009, 174:727–735; DOI: 10.2353/ajpath.2009.080688)

Autosomal recessive osteopetrosis (ARO) is a severe bone disease, which, in about half of the cases, is due to mutations in the gene *TCIRG1* that codes for the $\alpha 3$ subunit of the vacuolar proton pump.¹ The clinical picture includes growth defects, osteosclerosis, pancytopenia due to absence of the marrow cavity, and subtle cranial malformations causing hydrocephalus and compression of nerves, with secondary blindness and deafness.² Unfortunately, while pancytopenia may be rescued by postnatal bone marrow transplantation, the neurological defects cannot, since they are present at birth and are not reversible.³ In addition, good engraftment of postnatal bone marrow cells requires conditioning with irradiation or chemical agents and is strictly dependent on histocompatibility. Because of the latter, treatment may be complicated by graft versus host disease, and transplant is effective only in a portion of cases.³

ARO therefore is a paradigm of genetic diseases needing prenatal treatment. In addition to preventing irreversible damage, *in utero* transplantation (IUT) of hematopoietic stem cells (HSC) does not require MHC matching, since tolerance toward grafted cells is normally acquired by exposure to donor cells during fetal life.⁴ With these considerations in mind, we performed IUT of adult bone marrow cells in the *oc/oc* mouse model, which almost perfectly recapitulates the human osteopetrotic phenotype.⁵ This strain carries a naturally occurring

Supported by grants from Eurostells (STELLAR) and FIRB/MIUR to P.V. (RBIN04CHXT) and from ISS Malattie Rare (New cell therapy approaches for infantile malignant Osteopetrosis) to P.V. and from E-rare Project. The work reported in this paper has also been funded by the Network Operativo per la Biomedicina di Eccellenza in Lombardia Program from Fondazione Cariplo to P.V. and by National Institute of Arthritis and Musculoskeletal and Skin Diseases grant AR041336.

Accepted for publication December 9, 2008.

Supplemental material for this article can be found on <http://ajp.amjpathol.org>.

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mutation that was originally selected due to the presence of osteopetrosis. It was later shown to be due to a genomic deletion in the 5' region of the gene (called *tcirg1* or *atp6i*) coding for the mouse a3 subunit of the vacuolar proton pump. This deletion causes complete absence of the corresponding protein, as occurs in human ARO patients who lack this gene product. Therefore both the mouse and the human patients bear null mutations that translate into the inability to acidify the resorbing lacuna at the osteoclast/bone interface.^{1,6,7} These genetic and biochemical identities are the basis for the similarity of their clinical picture, making the *oc/oc* strain a suitable model for *TCIRG1*-dependent human ARO.

By using this mouse model, we were able to show that injection of adult bone marrow cells at day 14.5 of pregnancy allowed complete rescue of the phenotype in two out of 14 mutant mice transplanted and partial rescue in three others.⁸ This result, establishing the principle that ARO can be completely rescued by a single *in utero* injection of unmatched HSC, prompted us to investigate whether different sources of cells can improve the degree and the percentage of cured mice. In this regard, fetal liver, which contains HSCs, was a very promising source, since it was shown in an animal model that fetal stem cells engraft much better than adult HSC cells in fetal recipients.^{9,10} Fetal liver cells have also been used in the same *oc/oc* mouse model as source of gene therapy-targeted cells for neonatal transplantation.¹¹ Use of fetal liver cells for IUT in one case of human severe immunodeficiency gave good results.¹² However, the IUT procedure has not been widely used in humans so far and, according to a recent review, only 50 cases have been reported over the past 20 years. Indeed, despite the strong rationale for IUT, there have been difficulties in achieving permanent engraftment of donor cells.¹³ On the other hand, ethical use of human fetal cells should be achievable with suitable safeguards that may be a good source for the treatment of human ARO, if proven useful in animal models. Here we show that enzymatically disaggregated fetal liver cells can cure a high proportion of *oc/oc* mice when injected *in utero*.

Materials and Methods

Mice

Two pairs of (C57BL/6JxB6C3Fe-a/aF1) *oc/+* mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) and maintained in our central animal facility in accordance with the general guidelines released by the Italian Ministry of Health. CD-1 TG(ACTB-EGFP) transgenic mice, in which the enhanced green fluorescent protein (EGFP) gene is under the control of the chicken β -actin promoter and the cytomegalovirus (CMV) enhancer, were a kind gift of Dr. Okabe.¹⁴

In Utero Transplantation

Pregnant CD-1 TG(ACTB-EGFP) females were sacrificed at 12.5 days post coitum (p.c.). The abdomen was

opened and the uterus was removed. Each GFP⁺ embryo was dissected away from the uterus and placed in a petri dish containing sterile PBS. The yolk sac was opened and the umbilicus was removed to allow exsanguination. Each embryo was then placed in fresh PBS, and the livers were isolated from the extraneous tissue under a stereomicroscopy. In the first set of trials, the livers were mechanically disaggregated with a Gilson P1000 pipettor. In the second set of trials, the livers were enzymatically dissociated as described by Rosen et al, 2002.¹⁵

Briefly, the livers were incubated in 5 ml of 0.05% trypsin for 15 minutes at 37°C. The excess solution was discarded and the disaggregated cells were resuspended by gentle pipetting. The trypsin was quenched by 5 ml of cold Dulbecco's modified Eagle's medium/F12 with 10% fetal bovine serum. The cell suspension was then passed through a 100- μ m filter and the filter was rinsed with 5 ml of medium. The cells were collected by centrifugation at $400 \times g$ for 5 minutes at 4°C, and the pellet was resuspended in 5 ml of medium. The cells were filtered again to remove material larger than 40 μ m and the cells were collected as before. The cells were rinsed with 10 ml of PBS with 2% of fetal bovine serum, pelleted, and resuspended in 1 ml of 2% fetal bovine serum/PBS. The viability was determined by trypan blue exclusion, and the cells were diluted, in 2% fetal bovine serum/PBS, to a concentration of 10^5 cells per μ l.

Pregnant recipient *oc/+* females mated with *oc/+* male mice were anesthetized with Avertin at 13.5 days p.c. Following disinfection of the abdomen with 70% ethanol, a midline incision was performed through the skin and the peritoneal wall. The uterine horns were exposed to allow the visualization of the embryos and their livers through the uterine wall. The embryos were punctured through the peritoneum in the direction of the liver with a 50- μ m diameter beveled glass capillary loaded with 2 μ l of cells suspension (2×10^5 cells). The uterine horns were reinserted in the peritoneal cavity and the incisions were sutured.

DNA Isolation and Genotyping by PCR and Southern Hybridization

Genomic DNA was obtained from tail biopsies and from liver.⁸ Heterozygotes (*oc/+*), homozygotes (*oc/oc*) and wild-type animals were identified by Multiplex PCR amplification with two pairs of primers, since *oc/oc* mice bear a 1579 bp deletion at the 5' end of *Tcirg1* gene (GenBank AF188702), spanning from intron 1 to the 5' of exon 3.⁸ The first primer pair (OCwtF: 5'-TCATGGGCTCTATGTTCCGG-3'; OCwtR: 5'-GAAGGCGCTCACGGATTCGT-3', indicated by the gray arrows in supplemental Figure S1C at <http://ajp.amjpathol.org>), detecting a sequence internal to the deleted region, identifies the wild-type allele producing a 431 bp amplification product. The second pair (OCmutF: 5'-GGCCTGGCTCTTCTGAAGCC-3'; OCmutR: 5'-CCGCTGCACTTCTCCCGCA-3', indicated by the black arrows in supplemental Figure S1C at <http://ajp.amjpathol.org>), homologous to deletion-flanking

regions, identifies the deleted allele by producing a 563 bp band in the mutant allele or a 2139 bp product in the wild-type allele, which is usually not observed due to its length. Therefore, the wild-type animals show a 431 bp band, the homozygous mutants show a 563 bp band, while the heterozygous animals have both (Supplemental Figure S1, A at <http://ajp.amjpathol.org>). PCR was performed in 25 μ l total volume with 1U Taq Polymerase (GoTaq Flexi DNA Polymerase, Promega), 1 mmol/L $MgCl_2$, 200 μ mol/L dNTP, 10pmol of each oligonucleotide and 20 ng of genomic DNA. Thermocycling consisted of a denaturation step at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 60.5°C for 30 seconds, and extension at 72°C for 1 minute.

Southern blot was performed according to the nonradioactive DIG labeling and chemiluminescent detection protocol (Roche). The probe was DIG-labeled by PCR performed on genomic DNA of a wild-type mouse (C57BL6) using the primers OCwtF and OCmutR.⁸ The probe binds a 4.7 kb fragment on the wild-type allele and a 7.0 kb on the mutated allele, because of the loss of an EcoRI restriction site in intron 1 (Supplemental Figure S1C at <http://ajp.amjpathol.org>). Five μ g of DNA from transplanted mice (tail and liver) and untreated control mice (wild-type, *oc/oc*, *oc/+* and GFP) were digested with EcoRI enzyme and blotted for the analysis.

Flow Cytometry Analysis

Cell suspensions were obtained from bone marrow by flushing of femurs and tibiae. Staining of cells for FACS analysis was performed in PBS containing 3% fetal calf serum (staining buffer) with APC-Alexa Fluor® 750-conjugated c-Kit (2B8) and APC-conjugated Sca-1 (D7) monoclonal antibodies (mAbs) obtained from eBioscience (San Diego, CA). The lineage cocktail included the following PE-conjugated mAbs purchased from BD Pharmingen: B220 (RA3-6B2), TER119 (TER-119), Mac1/CD11b (M1/70) and CD3. Percentage of donor cells within specific cell subsets was determined by detection of GFP expression. Stained cells were analyzed with a FACS Canto II flow cytometer (BD Biosciences, San Jose, CA). DIVA software (BD) was used for data acquisition and analysis.

Histology and Microcomputed Tomography

Skeletons after removal of viscera and fixation in Millonig solution were scanned by dual excitation X-ray absorptiometry (Lunar PixiMus, GE Medical Systems, Madison WI), and sections of L4–5 vertebrae, tibia, and base of the skull with mandible were cut, decalcified, and processed for paraffin embedding. Six μ m sections were then cut, deparaffinized, and stained with hematoxylin and eosin and photographed, as described.⁸ Lumbar vertebrae or maxillae of mice, fixed in 5% formaldehyde, were used for microcomputed tomography. Microcomputed tomography was performed on a Viva CT40 instrument (Scanco Medical, Bassersdorf, Switzerland) with

three-dimensional reconstruction with the instrument-specific software provided by the instrument manufacturer. The scan slice increment was 10 μ m, and three dimensional reconstructions of the spine used a density cutoff of 200 mg/cm³. For analysis of the maxilla, total mineralized tissue reconstructions used a density cutoff of 450 mg/cm³, and to determine teeth and very dense osteopetrotic bone, a density cutoff of 650 mg/cm³ was used.

Colony Assays *in Vitro*

The culture conditions were as described.⁸ Briefly, bone marrow cells were flushed from femora and tibiae in IMDM with 2% FCS and cultured at 10⁴–10⁵ per ml in Methocult medium M03434 (StemCell Technologies) in duplicate. Cultures were scored at 2, 7 and 9 days with an inverted fluorescent microscope using standard criteria.

Results

Screening and Clinical Findings in *oc/oc* Treated With *in Utero* Transplantation of Fetal Liver Cells

At 12.5 days p.c., HSCs in the fetal liver are in exponential growth and at 13.5 days p.c. they start to migrate to spleen and bone marrow, where they settle and colonize.^{16,17} Fetal liver cells were obtained from day 12.5 p.c. fetuses heterozygous for the CMV-EGFP transgene in CD-1 background.¹⁴ Cells were injected in day 13.5 p.c. fetuses originated from *oc/+* x *oc/+* mating. Mice born from IUT were initially screened by PCR on DNA extracted from tail biopsies (see supplemental Figure S1A at <http://ajp.amjpathol.org>).

Transplanted mice identified as *oc/oc* mice were analyzed by Southern blotting to confirm their genotype (see supplemental Figure S1, C–D at <http://ajp.amjpathol.org>). All of the rescued *oc/oc* mice showed only the expected mutated 7.0-kb band in their tail DNA. It is interesting to note that in the liver of mouse F4 and, to a lesser degree, of mouse F2, also a 4.7-kb band corresponding to the wild-type gene is detected. This band, derived from the transplanted GFP-positive cells, is undetectable in DNA from the tail of the same individuals and witness the high level of chimerism achieved in the liver, as determined by observation with an inverted fluorescent microscope.

Two sets of trials were performed in which alternative methods for disaggregating liver tissue (mechanical or enzymatic) were used. In the first trial, 19 injected females delivered 60 pups. Among these, only one of the

Table 1. Summary of Fetal Liver Cell *in Utero* Transplantation Experiments

Experiment	Females injected	Pups	<i>Oc/oc</i>	<i>Oc/oc</i> rescued	% Rescue
1	19	60	8	1	12.5
2	20	45	9	5	55.6
Totals	39	105	17	6	35.3

Table 2. Clinical and Pathological Parameters of IUT-Treated *oc/oc* Mice

Mouse	Body length (cm) without tail	Body weight (g)	Spleen weight (mg)	Spleen length (cm)	Age of death (weeks)	Cause of death	Mated
F1	9	22, 50	370	3, 5	30	Sacrificed	No
F2	6, 5	12, 61	320	2	16	Sacrificed	No
M3	9	25, 00	ND	ND	24	Spontaneous	Yes
F4	7, 5	17, 18	190	2	24	Sacrificed	No
F5	8	20, 57	80	1, 5	24	Sacrificed	Yes
M6	ND	ND	ND	ND	5	Spontaneous	No
FWt	8	21, 30	40	1	24	Sacrificed	ND
MWt	10	28, 0	130	1, 5	24	Sacrificed	ND

The measures of body length, body weight, spleen length, and spleen weight were taken at the age of sacrifice. ND, not determined; FWt, female wild-type; MWt, male wild-type; treated animals are numbered as in the text. Some parameters were not reported in animals which died unexpectedly.

eight *oc/oc* mutant mice survived more than 4 weeks (Table 1), which is the maximum survival age for untreated *oc/oc* mice. This female mouse (F1) was phenotypically normal, as judged by growth (see supplemental Figure S2 at <http://ajp.amjpathol.org>) and behavior. At 10 days of age, incisor teeth showed a regular eruption, making the animal able to feed (see supplemental Figure S2 at <http://ajp.amjpathol.org>); nevertheless a malocclusion of the incisors prevents them from being worn down, requiring a periodic trimming of these teeth. This aspect of tooth growth has been reported also in other papers, which obtained significant rescue of the *oc/oc* phenotype by postnatal treatment.^{11,18} The behavior of F1 appeared normal, since it explored the space around like wild-type mice. When it was sacrificed at 30 weeks of ages, it reaches the weight of 22 grams.

In the second trial, 20 females were injected and 45 pups were born (Table 1 and see supplemental Figure S2 at <http://ajp.amjpathol.org>). Five out of nine *oc/oc* mutant mice survived more than 4 weeks: three females (F2, F4, and F5) and two males (M3 and M6). F5 and M3 showed tooth eruption and performed a normal life but, like F1, developed malocclusion. F5 was sacrificed at 24 weeks when it weighed about 20 grams. M3 died of unknown causes at 24 weeks when it weighed around 25 grams. F2 and F4 didn't show tooth eruption and were fed on ground pellets and corn pudding. Despite the absence of teeth, F2 reached the weight of 12 grams and F4 17 grams. M6 died at the age of 5 weeks and it showed a partial tooth eruption. F2, M3, F4, and F5 were examined in a behavior test in comparison with an untreated *oc/oc* mutant mouse. They showed a normal capacity of exploring the surrounding environment, being able to search food, to climb toward it, and to feed. They didn't show abnormal movement or the typical turning movement of the *oc/oc* mutant mice. M3 and F5 were mated with wild-type partners. M3 produced two litters of 18 mice in total and F5 delivered four mice, all of which were *oc/+* (see supplemental Figure S1B at <http://ajp.amjpathol.org>), confirming their homozygous status and suggesting that TCIRG1-defective ARO patients have no inherent defect in germ line maturation. The difference in rescue between the two methods, one of eight *oc/oc* mice in mechanical disaggregation versus five of nine for enzymatic disaggregation, has a χ^2 3.44, which with one degree of freedom corresponds to $P = 0.06$. Relative to survival of five of 14 pups using adult marrow,⁸ the liver cells were not

significantly better, but in the rescued animals the phenotypes of surviving animals were qualitatively better (see Discussion).

Table 2 summarizes the characteristics of each animal born from IUT as compared with two wild-type mouse (C57BL/6), male and female. It is noteworthy that some of the cured animals were characterized by splenomegaly (F1, F2, and F4). The reason why a small degree of splenomegaly is maintained in mice that showed a good rescue of the phenotype is not clear. It could be that the total volume of bone marrow cavities is still below the normal values in rescued mice or, alternatively, that extramedullary spleen sites are also occupied when, during the last days of pregnancy, the transplanted cells have not yet completely remodeled the bones. Likewise, we cannot explain the relatively incomplete rescue of the tooth phenotype, although it could be hypothesized that for some reasons osteoclast function in the jaw is not properly regulated in the treated mice (see also the findings at the microCT analysis of jaws).

Skeletal Phenotype by X-Ray and Histology

In four mice the skeletal phenotype was investigated by X-ray and compared with a female wild-type mouse of the same age (Figure 1A). There was a good correlation between the skeletal appearance, the presence of marrow cavities, the mineral density and the clinical findings.

Figure 1. X-Ray analysis. **A:** X-Ray analysis of the total body of wild-type (C57BL/6) female, F4, F2, F5, and M3 mice, as indicated. **B:** Detail of the femoral head of each transplanted mouse. F2 maintains some osteopetrosis residual. The other three mice are essentially similar to the normal phenotype.

Figure 2. Histology. **A:** DEXA (Luna PixiMus) images of partially disaggregated skeletons of the same mice resolves highly localized densities in vertebral bodies and skull of some of the animals (arrows). This was in keeping with focal areas of osteosclerosis on decalcified sections. Sections of the skull were not interpretable due to problems orienting the mandible and maxilla, but the tooth eruption studies (supplementary Figure 2) were consistent with focal densities on DEXA. **B:** Sections of vertebral bodies (top row) and tibia (bottom row) for the transplanted *oc/oc* animals. Each photograph shows an 800- μ m square region. In the sections shown, arrows indicate regions of retained cartilage and woven bone consistent with osteopetrosis, but all are quite focal.

F2 female, which was the smallest of these mice, was the one showing residual signs of osteopetrosis, while F4 and F5 were essentially normal, as was the M3 mouse. In particular, normal marrow cavity was observed in the long bones from all of these three mice (Figure 1B). The animal bodies, after harvest of organs and partial disarticulation, were sent for histological analysis with the observers blinded to genotype. At this stage, dual energy X-ray absorptiometry (DEXA; Lunar PixiMus) was performed (Figure 2A), which showed only highly localized densities in some animals, consistent with treated osteopetrosis. Bone densities of F1 and F5 could not be distinguished from normal mice, and median bone densities of the animals correlated with the weight of the animals but otherwise showed no significant differences (not illustrated). Sections of the spine, tibia, and skull were also examined. This showed (Figure 2B) that most of the treated animals, including animals that were apparently completely healthy, had microscopic areas of osteopetrosis with retained mineralized cartilage and sclerotic bone, including F2; sections of F4 showed no abnormalities, but focal lesions were apparent on DEXA, particularly in the skull. The most obviously affected animals were the small male M6, with clear skull lesions on DEXA and significant osteopetrosis near the ends of long bones and vertebral growth plates. The larger male M3 had focal sclerotic lesions in the spine and long bones of focal late developing osteopetrotic lesions. The sections of spine and tibia showed straightforward changes since the shape of the bone is independent of the presence or absence of regional osteopetrosis. On blinded analysis, F1 and F5 were called normal controls. Clearly tooth growth in several animals was abnormal (see supplemental Figure S2 at <http://ajp.amjpathol.org>), but on sections

the correlation of tooth abnormalities with regional osteopetrosis (not shown) did not demonstrate clear correlation of bone density and tooth growth, although changes similar to those in the long bones were almost certainly responsible for developmental defects leading to malocclusion (cf. regional densities in mandibles or maxillae, Figure 2A).

Microcomputed Tomography

To confirm the focal recurrence of regions of osteopetrotic bone and provide quantitative information on the apparent regional osteopetrosis, microcomputed tomography was performed (Figure 3). Analysis of the lumbar spine and sections of the maxilla or mandible was performed on all animals; sections illustrating significant findings only are shown. In the M6 mouse, bone density was highest and the amount of apparent osteopetrotic bone was relatively high (Figure 3A, left), while in animals with intermediate outcomes, focal sclerotic bone was common but the skeleton was, overall, more normal (Figure 3A, middle and right panels). In animal F2, the regional variation in density was confirmed by analysis of trabecular bone density and related parameters (Table 3). As expected, the focal sclerosis increased apparent trabecular thickness and decreased trabecular number. The trabecular spacing was unchanged within the accuracy of the method (about 3%), suggesting that trabecular spacing in regions of healthy bone is unaffected by adjacent sclerotic patches. The basis of tooth eruption abnormalities was suspected to be small-scale focal sclerotic disease, and while this could not be resolved by conventional histology, microcomputed tomography was

Figure 3. Microcomputed tomography. **A:** Three-dimensional reconstructions of lower lumbar vertebrae of three of the mice show the variation in density between the surviving mice and demonstrate the focal nature of the recurrent osteopetrosis in mice with less than total phenotypic cure. The individual images show three-dimensional reconstructions of L4–L6 in M6, F2, and M3 animals, cut halfway through the dorsal aspect, with the cut surface labeled in yellow. Some regions of recurrent osteopetrotic bone are labeled (arrows); note that these regions, in animals with intermediate outcomes, occur in patches of bone frequently seen as a plate of bone within a vertebral body (arrow in F2), which are consistent with the histology (Figure 2B). **B:** Example of microscopic foci of sclerotic bone in the maxilla of an animal with apparent cure of osteopetrosis but misaligned teeth. Sections show the right maxilla of animal F1 using an intermediate density cutoff to show the overall tooth and bone structure (left) and at a high density cutoff (right) illustrating focal pathological bone even in an animal with essentially normal skeletal size and morphology by other methods.

consistent with this hypothesis (Figure 3B). When the three dimensional reconstruction was gated to eliminate normal bone, teeth, and highly focal sclerotic bone were demonstrated (Figure 3B, right panel). This suggests strongly that in animals with healthy donor and recipient osteoclast precursors, colonies of abnormal recipient cells cause small foci of osteopetrotic bone to occur in a stochastic pattern, even though the overall skeleton appears to be completely normal.

Table 3. Regional Variation in Trabecular Bone Density Reflecting the Focal Occurrence of Osteopetrotic Bone within Animals Rescued by Intrauterine Transplant

Parameter*	L5	L6
Total bone volume (mm ³)	1.23	1.10
Mineralized tissue volume (mm ³)	0.51	0.34
Fraction of volume mineralized	0.40	0.30
Trabecular number (Tb.N, 1/mm)	5.51	5.18
Mean thickness of mineral (Tb.Th, mm)	0.095	0.085
Spacing of mineral (Tb.Sp, mm)	0.199	0.196

*Trabecular bone parameters by micro-computed tomography are shown for L5 and L6 from animal F2 (Fig 3A, middle panel). Volumes analyzed exclude cortex. By this method, mineralized cartilage is not resolved from bone, and thus the parameters have slightly different meaning than in bone that is not osteoporotic. The traditional abbreviations for the parameters in normal bone are given parenthetically.

Hematological Findings and in Vitro Clonogenic Assay of Hematopoietic Progenitors

To test whether donor fetal liver cells, derived from mice constitutively expressing GFP, and transplanted *in utero*, permanently engrafted in the recipient animals, we tested hematopoietic progenitors in clonogenic assays. Mouse F1 was analyzed at 7.5 months of age to assess long-term hematopoietic reconstitution. F2 was analyzed at 4 months of age, F4 and F5 at 6 months. Cells isolated from bone marrow of *in utero* transplanted and wild-type mice were cultured in Methocult M03434 containing a cocktail of cytokines to support growth and differentiation of different types of progenitors. The results showed that the number of bone marrow cells was similar between treated mice and wild-type control, with virtually normal frequencies of erythroid and myelomonocytic progenitors (Table 4). Furthermore, in cultures from F1, F4, and F5 mice, a high percentage of the different types of colonies were fluorescent, with values similar to those detected in control cultures raised from the bone marrow of GFP mice. F2 mouse only gave rise to a smaller fraction of fluorescent colonies. M3 and M6 were not tested due to their unexpected death.

Further studies were performed only on F4 and F5 mice for technical reasons. Both mice displayed normal hemoglobin content and numbers of white cells and platelets (Table 5). Bone marrow cells were analyzed for

Table 4. Frequency of Erythroid Progenitors (CFU-E and BFU-E), Myelo-Monocytic Precursors (CFU-GM), Pluripotent (mix) Colonies (CFU-GEMM), and GFP+ Colonies, as Determined from the Clonogenic Assay

Mouse	Tot cells per leg (*10 ⁶)	Tot colonies	% Erythroid	% Myeloid	% Mix colonies	% GFP+
F1	32, 5	11	27, 3	45, 5	27, 3	27, 3
		16	50, 0	37, 5	12, 5	12, 5
F2	19, 8	216	62, 5	23, 6	13, 9	4, 6
		278	48, 6	29, 9	21, 6	5, 8
F4	3, 36*	90	35, 6	48, 9	15, 6	30, 0
F5	4, 16	100	45, 0	45, 0	10, 0	30, 0
		96	51, 0	36, 5	12, 5	33, 3
FWt	19, 59	305	60, 7	26, 9	12, 5	0
		329	59, 9	24, 6	15, 5	0
MGFP	21, 0	55	36, 4	45, 5	18, 2	56, 4
		57	28, 1	47, 4	24, 6	57, 9

*F4 BM cells were tested only once.

the presence of GFP fluorescence: in both cases about half of the cells were GFP⁺. As shown in Table 5, 0.26% (F4) and 0.08% (F5) of bone marrow cells were Lin⁻ ckit⁺ Sca-1⁺ (progenitors), which represent percentages similar to those obtained in normal mice. Among them, 58% (F4) and 49% (F5) were GFP⁺, in agreement with the data obtained with the colony formation assays, suggesting that, at least in the analyzed animals, the pancytopenia usually associated with the *oc/oc* phenotype was corrected by stem cells located in the bone marrow. However, we cannot exclude that in other mice (and in particular in F1 whose spleen was enlarged at autopsy) partial extramedullary hematopoiesis coexisted with bone marrow hematopoiesis.

Discussion

In utero treatment of human diseases is increasingly practical, with surgical procedures as well as by whole bone marrow and HSC transplantation.¹⁹ Although it is generally thought to be of limited clinical relevancy, it is likely that its applicability will increase since prenatal diagnosis performed by genetic and instrumental means will become more frequent. In fact, not only is genetic diagnosis becoming easier, more precise and less risky, but increasing sophistication in imaging techniques, such as ecography, will increase the number of diagnoses of diseases obtained prenatally. Even if most early prenatal diagnoses showing the presence of a genetic defect are followed by termination of the pregnancy, a portion of them are maintained for ethical and personal reasons; in addition, late prenatal diagnoses are not allowed to be terminated in some countries, in consideration of recent techniques that allow survival of babies born around 21 to 22 weeks.

In several genetic diseases, severe damage starts before birth and cannot be corrected by postnatal intervention. This is the case of ARO, in which, even in cases in which the hematological defects are corrected and good engraftment obtained, the skull deformities remain and compression of optic and acoustic nerve persist, with blindness and deafness. Taken together with the difficulty in obtaining matched HSCs, and with the fact that conditioning by mutagen agents is also necessary, the possibility of correcting the defect *in utero* would present several advantages. Still, the risk of graft versus host disease is not completely ruled out by the IUT approach and could be a concern for human studies, although we have not experienced this adverse effect in our mice and its occurrence in the 50 reported human trials was not high.¹³

In the present work, we compare the results obtained with fetal liver cells to those previously obtained with whole adult bone marrow. We want to emphasize that, although performed sequentially, the two set of experiments with the two different kinds of donor cells used the same strain and were evaluated in an identical way, thus making the comparison meaningful. The only modification was in the type of cells used. In this regard, we found that the results of experiments performed by preparing the fetal liver cells with enzymatic disaggregation were superior to those obtained with mechanical means. While the direct comparison has a χ^2 probability of 0.06, the expense of further experiments is not justified since the enzymatic disaggregation is clearly a very good method, even if refinement might improve outcomes with mechanical dissociation of the fetal liver cells.

In the experiments performed with whole adult bone marrow, we obtained clinically complete rescue of the phenotype in two animals and partial rescue in three in a

Table 5. Hematological Findings in IUT-Treated F4 and F5 *oc/oc* Mice

Mouse	Hemoglobin (g/dL)	WBC (10 ³ /μL)	Platelets (10 ³ /μL)	% Of GFP ⁺ bone marrow cells	% Of Lin ⁻ ckit ⁺ Sca-1 ⁺ cells on total bone marrow cells	% Of GFP ⁺ cells on Lin ⁻ ckit ⁺ Sca-1 ⁺ cells
F4	15	5, 6	875	55, 5	0.08	58, 6
F5	14, 6	3, 5	979	45, 5	0.26	49, 5
Wt*	15, 3	3, 7	750	0	0.08	0

*Reported values are the mean of seven normal mice.

total of 14 *in utero* treated *oc/oc* mice.⁸ On the two completely rescued mice, only one had detectable GFP+ cells at 5 to 6 months of age. In the present set of experiments, out of 17 treated *oc/oc* mice we obtained five animals with clinically complete and one with a partial rescue. In particular, five out of these six rescued mice were obtained with the enzymatic protocol. The difference in frequency of complete rescue with the fetal donor cells relative to adult donor cells has a χ^2 of 2.21 corresponding to $P = 0.14$. Thus, while further trials will be needed for results to be confirmed, these data suggest that enzymatically prepared fetal liver cells could be a better tool to achieve cure of ARO mice. This could be due to a better ability of fetal liver cells to engraft, since in all of the four animals tested GFP+ hematological colonies were detected, while a good engraftment was found in only one mouse in the previous work.⁸ This is not unexpected as fetal liver cells are harvested at the exact time when they are poised to migrate to bone cavities.^{16,17} Since they are ready to colonize the bone marrow, it could be that their expression pattern gives them a better ability to migrate and home to the bone than that of adult HSCs. Indeed, a recent study has pointed out the differences between fetal and adult HSCs in their ability to colonize the bone marrow, probably in relation to the cell cycle status and the cytokine expression pattern.²⁰ Apparently, fetal cells maintain a specific transcription pattern that switches abruptly to the adult type a few weeks after birth.^{21,22}

In addition to the relatively high rate of rescue using liver cells, particularly after enzymatic digestion (Trial 2), the quality of the rescue of phenotype was superior to the results of marrow transplant in cases where response was good but there was not complete cure. Despite chimerism on a large scale, focal clonal areas developed where the osteoclast precursors were derived from host osteoclast precursors, possibly due to host clonal expansion once healthy osteoclasts create sufficient marrow space. This suggests that precursor cells for a new osteoclast are mainly derived from local clones of monocyte precursors, rather than from circulating monocytes, so that once a region of bone marrow is populated by clonal expansion of recipient rather than donor cells, microscopic regions of osteopetrosis develop (Figure 2, arrows), typically near growth plates where turnover is required to remove primary spongiosa. In earlier work with fetal transplants of bone marrow,⁸ grafts were in some cases less effective after growth of some animals, possibly due to greater growth of host marrow cells. In those cases, we saw collars of osteopetrotic bone develop at growth plates in the spine and long bones.⁸ In the fetal liver transplanted animals, there are much milder changes with mainly microscopic areas of osteopetrosis. A model of this sort also supports the slow development of dental malocclusion, despite tooth eruption being in most cases initially nearly normal.

Although ARO can be taken as a paradigm of diseases needing prenatal intervention, it must be noted that it probably represents a very favorable case, as suggested also by our unexpected but very promising results. Several factors can account for this positive outcome. The

first is the already mentioned use of cells poised to migrate to bone marrow. The second is the fact that osteoclasts are multinucleated cells that arise by cell fusion. Since heterozygous patients (and mice) have essentially normal bones, this means that a ratio of one mutated to one normal nucleus present in the multinucleated osteoclasts is sufficient to give normal vacuolar proton pump activity to the syncytial cells. Finally, we speculate that given the absence of preformed cavity, endogenous stem cells have not yet occupied their niches. When a bone cavity is formed by the first exogenous osteoclasts, these niches are not already filled by endogenous cells and therefore are particularly permissive to the engraftment of donor cells. The situation therefore could be still better than in other genetic diseases in which a single hematopoietic lineage is affected, such as in RAG-deficient humans and mice.

Although these considerations are a caveat to the translation of these results to other genetic diseases, the findings reported here and in our previous paper⁸ raise the possibility that in selected situations *in utero* injection of hematopoietic fetal liver or adult stem cells might be considered as a therapeutic option in humans. Unfortunately, no osteopetrotic large animal is available to our knowledge, and this prevents testing this procedure in models more similar to humans than the rodents. In particular, the minimum number of fetal liver cells needed to achieve engraftment of therapeutic value could be tested in large animals. In the present protocol, we used a relatively high number of mouse fetal liver cells (2×10^5), which would correspond to more than 10^9 cells in humans, an amount much higher than that used so far.²³ On the other hand, at variance with primary immunodeficiency, whose symptoms do not develop before birth, osteopetrosis would greatly benefit from IUT and, even if only partial engraftment is obtained, the patients could still be treated with postnatal transplantation with minimal ablation using cells from the same donor, as suggested by other groups.¹³ An additional unexpected finding was that, even with transplants that are apparently totally successful, sclerotic bone occurs in a highly localized, random regions, including microscopic regions of sclerotic bone associated with malocclusion. This suggests that, at least in mice, osteoclast differentiation reflects mainly cells from local colonies of precursors, so that clonal variation can cause patches of sclerotic bone in animals rescued by intrauterine transplant that have grossly normal skeletons.

Acknowledgments

We thank G. David Roodman (University of Pittsburgh) for assistance with microcomputed tomography. The technical assistance of Lucia Susani and Elena Caldana is acknowledged.

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