Musculoskeletal Pathology

Osteoclasts from Patients with Autosomal Dominant Osteopetrosis Type I Caused by a T253I Mutation in Low-Density Lipoprotein Receptor-Related Protein 5 Are Normal *in Vitro*, but Have Decreased Resorption Capacity *in Vivo*

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Autosomal dominant osteopetrosis type I (ADOI) is presumably caused by gain-of-function mutations in the *LRP5* gene. Patients with a T253I mutation in *LRP5* have a high bone mass phenotype, characterized by increased mineralizing surface index but abnormally low numbers of small osteoclasts. To investigate the effect of the T253I mutation in *LRP5* on osteoclasts, we isolated CD14⁺ monocytes from ADOI patients and assessed their ability to generate osteoclasts when treated with RANKL and M-CSF compared to that of age- and sex-matched control osteoclasts. We found normal osteoclastogenesis, expression of osteoclast markers, morphology, and localization of proteins involved in bone resorption, such as *Clc-7* and cathepsin K. The ability to resorb bone was also normal. *In vivo*, we compared the bone resorption and bone formation response to T₃ in ADOI patients and age- and sex-matched controls. We found attenuated resorptive response to T₃ stimulation, despite a normal bone formation response, in alignment with the reduced number of osteoclasts *in vivo*. These data demonstrate that ADOI osteoclasts are normal with respect to all aspects investigated *in vitro*. We speculate that the mutations causing ADOI alter the osteo-

blastic phenotype toward a smaller potential for supporting osteoclastogenesis. (Am J Pathol 2005; 167:1341–1348)

Bone remodeling is performed mainly by two cell types, the osteoclasts that resorb bone and the osteoblasts that form bone. Autosomal dominant osteopetrosis type I (ADOI) is a fully penetrant disease, with a benign pathological appearance,¹ characterized by bone and back pain, as well as complications such as cranial nerve compression.¹ The bone phenotype is characterized by a generalized osteosclerosis, which is most clearly seen in the cranial vault. Histological and metabolic studies of the ADOI patients, demonstrated increased trabecular and cortical thickness and a decrease in both osteoclast number and size.^{1–7}

The mutation causing ADOI was localized to chromosome 11q12-13⁸ and later shown to be caused by gain-of-function mutations in the low-density lipoprotein receptor-related protein 5 (*LRP5*).⁹ Several other mutations in *LRP5* were shown to lead to various osteopetrosis-like phenotypes, such as the high bone mass phenotype in G171V patients^{10,11} and endosteal hyperostosis in A242T mutations.⁹ Accordingly, loss of function mutations in *LRP5* lead to osteoporosis-pseudoglioma syndrome,¹² which is a disease characterized by a very low bone mass, but with normal bone cell number, in contrast to the high bone mass observed in the ADOI patients. Furthermore, polymorphisms in *LRP5* gene were shown to influence the bone mineral density in both mice and man, and influence the fracture rates,^{13–16} implicating *LRP5* as an important regulator of bone turnover and strength.

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Studies using mouse genetics have shown that mutations in *Lrp5* mimic the bone phenotype in humans close to 100%, so that loss of *Lrp5* function in mice leads to osteoporosis-pseudoglioma syndrome,^{17,18} and overexpression of a gain-of-function mutation in *Lrp5* leads to a phenotype mimicking the high bone mass phenotype in the patients with a G171V mutation.^{10,19} These mouse studies have provided detailed histological analyses of the osteoblast phenotype, and they showed that *Lrp5* tightly regulates osteoblast proliferation and life span through the binding of Wnt.¹⁹

Measurements of bone turnover markers further indicate that the high bone mass phenotype in G171V patients is caused by altered osteoblast activity, as Boyden and colleagues¹⁰ found increased osteocalcin levels, but normal resorption levels, in patients with a G171V mutation in *LRP5*. However, other studies in patients with a T253I mutation in *LRP5* failed to find increased osteocalcin levels.^{3,4} Circulating levels of the osteoclast markers tartrate-resistant acid phosphatase (TRAcP) and creatine kinase BB were found to be normal in patients with both mutations.^{10,20,21} However, the patients with a T253I mutation have lower numbers of abnormally looking osteoclasts *in vivo*,² and whether this osteoclast phenotype in the T253I patients with ADOl is due to endogenous or exogenous factors is not yet known. To investigate the differentiation and function of osteoclasts from the T253I patients, we have previously characterized clinically and genetically,^{1-7,22,23} we isolated CD14+ monocytes and cultured them under conditions promoting osteoclast development. We found no differences between ADOl and control osteoclasts *in vitro*, strongly suggesting that the ADOl phenotype in patients with a T253I mutation is caused by reduced ability of osteoblasts to support osteoclast development.

Materials and Methods

Patient Material for *in Vitro* Studies

Patients were ascertained from a Danish family with ADOl due to a T253I mutation in the *LRP5* gene.⁹ Eight mutation-positive members (three women and five men) aged 28 to 60 years were included as well as eight age- and sex-matched controls for the *in vitro* experiments. These patients have previously been characterized both genetically and clinically.^{1-7,22,23} The study was approved by the Danish regional Ethical Committee (registration number 2473-03).

Cell Culture

Isolation of CD14+ Human Monocytes

The CD14+ isolation was performed as previously described.²⁴ Briefly, the monocytes were isolated from peripheral blood by centrifugation on a Ficoll-Paque gradient (Amersham Pharmacia, Hillerød, Denmark), and magnetically sorted using a CD14+ magnetic bead isolation kit (Dynabeads M-450; Dynal Biotech, Oslo, Nor-

way). The cells were then seeded in 75-cm² flasks, and cultured in α -minimal essential medium containing 10% serum, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 25 ng/ml of M-CSF (R&D Systems, Minneapolis, MN) for 3 days, then they were lifted, reseeded, and cultured until day 10 in the presence of 25 ng/ml of M-CSF and 25 ng/ml of RANKL (R&D Systems) unless otherwise stated.

Cell Fusion

Cell fusion was determined by seeding equal numbers of CD14+ monocytes isolated from either the ADOl patients or healthy controls, and culturing them for 3, 6, 9, or 12 days in the presence of RANKL and M-CSF. The cells were fixed using 3.7% formaldehyde in phosphate-buffered saline (PBS) for 15 minutes. The nuclei of the cells were then visualized using Ehrlich's hematoxylin (BDH Laboratory Supplies, Poole, UK) for 2 minutes, followed by washing in tap water. Fusion scorings were performed using an Olympus IX-70 light microscope, and a video camera linked to a computer using CAST-Grid software (Olympus, Glostrup, Denmark). Osteoclasts fusion was detected by the formation of cells with three or more nuclei.

TRAcP Assay

The TRAcP activity assay was performed as follows: 20 μ l of cell culture medium was added to a 96-well plate and 80 μ l of freshly prepared reaction buffer (0.33 mol/L acetic acid, 0.167% Triton X-100, 0.33 mol/L NaCl, 3.33 mol/L ethylenediamine tetraacetic acid at pH 5.5, 1.5 mg/ml of ascorbic acid, 7.66 mg/ml of Na₂Tartrate, 3 mg/ml of 4-nitrophenylphosphate) was added. The reaction was left 1 hour at 37°C in the dark, and then stopped with 100 μ l of 0.3 mol/L NaOH. The colorimetric changes were measured at 405 nm with 650 nm as the reference using an enzyme-linked immunosorbent assay (ELISA) reader.

Immunoblotting

Total cell lysates were prepared by lysing the cells in RIPA+++ buffer as previously described²⁵ for 5 minutes. The lysates were centrifuged at 15,000 $\times g$ for 30 minutes to remove any cell debris left. Protein concentrations were measured using the Bio-Rad DC protein measurement assay (Bio-Rad, Hercules, CA). Thirty μ g of total protein in sample buffer containing 80 mmol/L dithiothreitol was loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, and then electroblotted onto nitrocellulose membranes. The quality of the protein loading was always checked by Ponceau Red (Sigma-Aldrich, Copenhagen, Denmark) staining. The membranes were then blocked in TBS-T (50 mmol/L Tris-base pH 7.5, 100 mmol/L NaCl, 0.1% Tween-20) containing 5% skim milk powder for 1 hour at ambient temperature. This was followed by overnight incubation at 4°C with the correct dilution of the primary antibodies against either TRAcP (Zymed, South San Francisco, CA), cathep-

sin K (Chemicon Int., Temecula, CA), CIC-7 (Nordic Bioscience A/S, Herlev, Denmark), and p38 MAPK (Cell Signaling Technology, Beverly, MA), which was used as a constant control.²⁶ This was followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibody for 1 hour at ambient temperature. Finally, the results were visualized using the ECL kit (Amersham Pharmacia Biotech).

Immunocytochemistry

Mature CD14⁺ isolated osteoclasts were seeded on cortical bovine bone slices and cultured for 2 days, fixed in 3.7% paraformaldehyde in PBS for 20 minutes, and then washed thoroughly in PBS. The specimens were blocked in Tris-buffered saline containing 2.5% casein and 0.1% Triton X-100 for 30 minutes at ambient temperature. This was followed by incubation with the primary antibodies CIC-7, cathepsin K, MMP-9, TRAcP, or the corresponding control IgG or preimmune serum overnight at 4°C in a moist atmosphere, after washing in TBS the bone slices were incubated in Rabbit EnVision (DakoCytomation, Glostrup, Denmark) for 30 minutes at ambient temperature. Finally, the peroxidase activity was visualized using DAB⁺, and the nuclei were counterstained using Ehrlich's hematoxylin. Finally, the bone slices were then dehydrated through a gradient of alcohol (70 to 99%) and toluene, and mounted in DPX. The pictures were taken using an Olympus BX-60 light microscope equipped with an Olympus C-2000 Zoom digital camera.

For visualization of the actin rings, we did as previously described.²⁷ Briefly, we seeded the cells on glass coverslips, and otherwise followed the procedure described above, using tetramethyl-rhodamine isothiocyanate-phalloidin (Sigma Chemicals) as the primary antibody, omitting the secondary antibody and performing the counterstaining of the nuclei by 4,6-diamidino-2-phenylindole (Sigma Chemicals) for 3 minutes. Finally, the cells were mounted and the results were visualized using an Olympus BX-60 microscope equipped with a $\times 20$ objective and filters suitable for tetramethyl-rhodamine isothiocyanate and 4,6-diamidino-2-phenylindole. The pictures were taken using an Olympus C-5050 Zoom camera, and mounted in Corel Draw.

Resorption Assays

The CD14⁺ monocytes were seeded at a cell density of 350,000 cells/cm² and the cells were cultured in the presence of M-CSF and RANKL until day 12. The culture supernatants were kept for measurements of CTX-I levels and TRAcP activity. The resorption pits were visualized by brushing of the osteoclasts using a cotton swab, and then the bone slices were washed in distilled water, and the pits visualized by staining with Mayer's hematoxylin (Bie & Berntsen A/S, Rødovre, Denmark), followed by washing in water. The pictures were taken using an Olympus C5050 Zoom digital camera, and are shown in inverted colors. The release of the C-terminal type I collagen fragments (CTX-I) from mineralized bone slices was

determined using the CrossLaps for Culture kit (Nordic Bioscience Diagnostics), which was used according to the manufacturer's instructions.

Patients and Experimental Design

The patient samples used in this study have previously been used.⁴ Briefly, eight ADOI patients (three women and five men, 20 to 49 years of age) and 10 normal controls (five women and five men; mean age, 35.4 years) participated. All were clinically and biochemically euthyroid before entering the study. All participants were treated with 100 μ g of triiodothyronine (T₃) for 7 days as described previously.³ The study was approved by the local ethical committee and conducted according to the declaration of Helsinki II as described before.^{3,4}

Biochemical Markers of Bone Turnover

Serum CTX-I was measured as a marker of bone resorption using the Serum Crosslaps ELISA (Nordic Bioscience Diagnostics). Serum osteocalcin was measured as a marker of bone formation using the N-MID osteocalcin ELISA (Nordic Bioscience Diagnostics) according to the manufacturer's instructions.

Statistics

The *in vitro* data are presented as averages of pentaplicates $\pm$ SEM and the statistical analyses were performed using an unpaired Student's *t*-test, with normal distribution and equal variance. The statistical analyses on the *in vivo* material were performed using the Mann-Whitney test. Statistical significance is shown by asterisks, $P < 0.05$.

Results

The Osteoclastogenesis of ADOI Osteoclasts Is Normal

Patients with a T253I mutation in LRP5 have low levels of abnormally small osteoclasts *in vivo*.² We investigated whether fusion of ADOI osteoclasts from CD14⁺ monocytes was normal, and we found that the generation of multinuclear osteoclasts investigated by counting of cell fusion was comparable to that of healthy sex- and age-matched controls (Figure 1).

The Expression of Osteoclast Markers in ADOI Cells Is Normal

To investigate the expression of the osteoclastic markers, cathepsin K, TRAcP, and MMP-9 we cultured monocytes from controls and ADOI patients in the presence of RANKL and M-CSF and prepared cell lysates at different days of differentiation, followed by protein level analysis by immunoblotting. The expression of cathepsin K,

Figure 1. Osteoclastogenesis is normal *in vitro* in ADOI patients. Peripheral blood was obtained from ADOI patients and age-matched control individuals, and the monocytes were isolated by Ficoll-Paque gradient centrifugation and CD14 magnetic bead isolation. The monocytes were cultured for 3 days in the presence of 25 ng/ml of M-CSF and then lifted and reseeded at a cell density of 100,000 cells/cm² and cultured for 3, 6, 9, or 12 days with M-CSF and 25 ng/ml of RANKL. The cells were then fixed in 3.7% formaldehyde and the number of fused cells was scored. The results are representative of three individual experiments performed in quadruplicates.

TRAcP, and MMP-9 increases during osteoclastogenesis at comparable rates in both controls and ADOI cells (Figure 2), as expected from other studies.^{24,28} As internal control for correct loading of proteins we used p38, which has previously been shown not to change during osteoclastogenesis.^{24,26} These data further indicate that ADOI osteoclasts are normal *in vitro*.

Figure 2. The expression of osteoclast markers during differentiation is normal. Peripheral blood was obtained from ADOI patients and age-matched control individuals, and the monocytes were isolated by Ficoll-Paque gradient centrifugation and CD14 magnetic bead isolation. The monocytes were cultured for 3 days in the presence of 25 ng/ml of M-CSF and then lifted and reseeded at a cell density of 100,000 cells/cm² and cultured for 3, 5, 7, or 10 days with M-CSF and 25 ng/ml of RANKL. This was followed by lysis of the cells in RIPA+++ buffer at the indicated time points. Twenty μ g of total cell lysate was subjected to sodium dodecyl sulfate gel electrophoresis and immunoblotting with antibodies against TRAcP, cathepsin K, MMP-9, and the p38 MAPK as described in the Materials and Methods section. The immunoblots are representative of three different individuals in each group.

Figure 3. The formation of resorption lacunae is normal. Healthy volunteer and ADOI monocytes were isolated as previously described. The monocytes were cultured in the presence of RANKL and M-CSF until mature osteoclasts were obtained. These were lifted and reseeded on glass coverslips and cultured for 2 days. The cells on glass were fixed and immunostained by phalloidin to visualize the actin cytoskeleton.

The Formation of Actin Rings Is Normal in ADOI Osteoclasts

In vivo the ADOI osteoclasts do not form resorption lacunae,² thus we investigated the ability of the ADOI osteoclasts to form actin rings, as an indication of the formation of a resorption lacuna *in vitro*.²⁹ Visualization of the actin rings by phalloidin staining, showed that the ADOI osteoclasts are normal morphologically (Figure 3), showing normal formation of the resorption lacuna.

The Localization of the Components of the Resorption Machinery Is Normal

To further characterize the ADOI osteoclasts, we investigated whether the localization of CIC-7, cathepsin K, TRAcP, and MMP-9 on bone slices was altered. We found that the complete array of proteins localized normally in the ADOI osteoclasts compared to the healthy osteoclasts. In alignment we found that CIC-7, cathepsin K, and MMP-9 all localized in a gradient toward the resorption lacunae (Figure 4; a to c and e to g).^{24,30–35} TRAcP localized in intracellular vesicles corresponding to transcytotic and endocytotic vesicles as

Figure 4. The localization of the resorption machinery is normal. Peripheral blood was obtained from ADOI patients and healthy volunteers, and the monocytes were isolated by Ficoll-Paque gradient centrifugation and CD14 isolation and cultured until mature osteoclasts were present as described in the Materials and Methods section. The osteoclasts were lifted by trypsin digestion and seeded on bone slices. They were allowed to grow for 3 days, and then fixed and immunostained against CIC-7 (a, e), cathepsin K (b, f), MMP-9 (c, g), and TRAcP (d, h) according to the procedures described in the Materials and Methods section.

Figure 5. Resorptive activity is normal in ADOI osteoclasts. Peripheral blood was obtained from ADOI patients and age-matched control individuals, and the monocytes were isolated by Ficoll-Paque gradient centrifugation and CD14 magnetic bead isolation. The monocytes were cultured for 3 days in the presence of 25 ng/ml of M-CSF and then lifted and reseeded at a cell density of 300,000 cells/cm² on cortical bovine bone slices. **a:** Resorption of bone measured as CTX-I fragments released into the culture supernatant by the CrossLaps ELISA during the experiment. The data are shown as accumulated resorption in percentage of endpoint values in the controls. **b:** Pictures showing the morphology of the resorption pits by control osteoclasts (**left**) and ADOI osteoclasts (**right**). **c:** TRAcP activity measured in the culture supernatant during culture. The results are pooled from the measurements of three ADOI patients and three control individuals, with each condition performed in pentaplicates. The **asterisks** show significant differences in TRAcP levels between control and ADOI osteoclasts.

previously published (Figure 4, d and h) in both ADOI and normal osteoclasts.³⁶ Thus, these data further support the view that osteoclasts from ADOI patients are normal.

The Resorption by ADOI Osteoclasts *in Vitro* Is Normal

We investigated the ability of ADOI osteoclasts from three different individuals to resorb bone by measurement of the C-terminal crosslinked C-telopeptide of collagen type I (CTX-I) and compared it to matched controls. Osteoclasts from ADOI patients and controls resorbed bone to the same extent (Figure 5a). Furthermore, osteoclasts from the ADOI patients formed normal looking pits (Figure 5b), at the same rate as the control osteoclasts, confirming that the osteoclasts are normal with respect to resorptive function. In addition, we measured the TRAcP activity in the conditioned medium, because ADOI osteoclasts are less TRAcP-positive *in vivo*,² and although we observed a slightly lowered TRAcP activity in the ADOI osteoclasts (Figure 5c), we attribute this to the person-to-person variation.

ADOI Osteoclasts Fail to Respond to Induction of Bone Turnover *in Vivo*

To characterize the ADOI osteoclasts ability to degrade bone *in vivo*, we examined serum samples from a previously published study, in which the CTX-I levels were measured in urine samples,⁴ using the Serum Crosslaps ELISA (CTX-I) and compared to the serum levels of osteocalcin measured using the N-MID osteocalcin ELISA. In the study, the thyroid hormone T₃ was used to induce bone turnover in ADOI patients and their corresponding normal controls. We measured samples at baseline and after 1 week for CTX-I and osteocalcin. We found no significant differences in the baseline levels of CTX-I and

osteocalcin in the T253I ADOI patients (data not shown), in accordance with previous publications.^{3,4}

We found that T₃ enhanced bone resorption significantly in the controls when compared to the increase in the ADOI patients after 1 week of stimulation (Figure 6a). This finding corresponds to a lower number of small and dysfunctional osteoclasts in the ADOI patients as previously reported.² Finally, we found that the T₃ treatment increased the bone formation, measured by osteocalcin, to the same extent in both groups (Figure 6b), showing that osteoblastic responses to bone turnover induction are normal as expected from the previous study.^{3,4}

Discussion

This is the first *in vitro* study of osteoclasts from patients with a presumed gain-of-function mutation in the *LRP5*

Figure 6. Activation of resorption in ADOI patients is attenuated *in vivo*. ADOI patients and corresponding controls were treated with T₃ to induce bone turnover as described in the Materials and Methods section, and blood samples were collected at the beginning of the experiment and after 1 week, and the biochemical markers were measured at both time points. **a:** CTX-I levels measured in serum. **b:** The osteocalcin levels in serum. The data are presented as changes in release from day 0 to day 7 measured in six patients and nine controls. The **asterisk** indicates a significant difference between control CTX-I at baseline and after 1 week of T₃ stimulation.

gene. We isolated CD14+ monocytes from patients with a T253I mutation in LRP5. The ADOI phenotype is manifested by decreased numbers of osteoclasts, which have fewer nuclei and lower TRAcP activity than their corresponding controls.² We have investigated human osteoclast differentiation and function using cells from ADOI patients in depth, to clarify whether the LRP5 mutations directly affect osteoclast function.

We characterized the fusion of the osteoclasts using a group of previously published approaches,^{24,26,27} and we found that all of the following parameters were normal in the ADOI osteoclasts: fusion indices, expression of osteoclast markers, and the formation of actin rings. These data demonstrate that ADOI osteoclasts undergo fusion at normal rates *in vitro*, when cultured in the presence of the pro-osteoclastic cytokines RANKL and M-CSF. We further found that the TRAcP activity and the bone resorption levels, measured by CTX-I release and pit scoring, were normal. Small differences from person to person were observed, and as seen in Figure 5c the TRAcP levels in the ADOI group are lower than the control levels. However, this is likely due to the small interperson variations observed in osteoclast differentiation experiments due to the difficulty in controlling the cell density 100%,²⁶ rather than to an actual lowering of the actual TRAcP activity in the ADOI osteoclasts. Taken together, the ADOI osteoclasts assessed in this study are normal, both with respect to differentiation, morphology, marker expression, and resorption *in vitro*.

In vivo, we present resorption data indicating that activation of ADOI osteoclastic response *in vivo* is attenuated, whereas the bone formation response is normal, in alignment with the study showing fewer osteoclasts *in vivo*.² This is not in complete alignment with the resorption and formation data previously published from the T₃ activation study in ADOI patients.^{3,4} However, the analyses presented in this study were performed using serum samples instead of urine samples potentially explaining the small difference. Our finding that the baseline osteocalcin levels are unchanged in the T253I mutation ADOI patients is not in alignment with the findings of Boyden and colleagues,¹⁰ who found increased osteocalcin levels in the G171V ADOI patients. In mice with a G171V mutation in Lrp5 the bone formation is increased due to increased osteoblast activity, and the osteoclast number is normal.¹⁹ This correlates well with the G171V patients, indicating that the different mutations lead to different phenotypes, however, this remains to be studied in detail. Another difference between the T253I and G171V patients has also been found, namely the G171V patients have normal OPG levels,¹⁰ whereas the T253I patients have a 40%, albeit nonsignificant, increase in OPG levels.³⁷

A recent publication³⁸ describes the osteoblast phenotype of mice with a stabilized expression of β -catenin specifically in mature osteoblasts. These mice have a constitutively active Wnt/Lrp5 pathway, which causes osteopetrosis due to an increase in the osteoblastic production of OPG, which impairs osteoclast formation as previously seen in OPG transgenic mice,³⁹ and therefore osteopetrosis. Furthermore, osteoblast number and func-

tion were normal, correlating well with our findings in T253I ADOI patients, namely normal osteocalcin (this study) and a tendency toward increased OPG levels.³⁷ Furthermore, another publication used ectopic expression of Wnt14 to show that constitutive activation of Wnt/Lrp5 signaling, at early developmental stages, leads to increased osteoblast activity.⁴⁰ This is in alignment with the G171V mutant mice as well as humans with the high bone mass phenotype.^{10,19} Taken together, it appears that the G171V and T253I mutations affect different stages of osteoblast function, most likely corresponding to increased β -catenin activity in either developing osteoblasts⁴⁰ or mature osteoblasts,³⁸ respectively, and thereby different outcomes in the osteoblasts.^{38,40} The difference between the G171V and the T253I mutation, and their effects of β -catenin activity, could be explained by binding of different ligands to these two regions in LRP5. The G171V mutation affects binding of Mesd,⁴¹ and the inhibitory effect of DKK1,¹⁰ whereas the effect of the T253I mutation on ligand binding is yet unknown. The T253I mutation is located in another YWTD domain than the G171V,⁹ thus possibly affecting ligands yet to be identified. Finally, mutations in the different YWTD domains of the first YWTD/EGF domain lead to different phenotypes, such as the high bone mass in G171V,¹⁰ endosteal hyperostosis in A242T, and ADOI in T253I.⁹ This further supports the view that different mutations in LRP5 lead to different phenotypes in the osteoblasts, with some affecting the osteoblasts maturation and survival (eg, G171V), and at least one affecting the ability of the osteoblasts to support osteoclastogenesis, via the up-regulation of OPG secretion (T253I).

An interesting finding in relation to the osteoclast phenotype in the ADOI patients is the increased level of transforming growth factor- β ,³⁷ which has been shown to inhibit osteoclast maturation and thereby function,²⁶ and induce apoptosis.⁴² This finding correlates well with the lower number of small osteoclasts found in the T253I ADOI patients.²

Thus, we speculate that the reason for the reduced number and function of osteoclasts in T253I ADOI patients *in vivo* is a switch in the osteoblast phenotype causing the increased level of transforming growth factor- β and OPG,² which both are known to inhibit osteoclast maturation and function and induce apoptosis.^{26,39,42-44} In conclusion, we have shown that the *in vivo* osteoclastic phenotype of the ADOI patients with a T253I mutation is indirect and likely mediated via the osteoblasts, as we present data confirming that monocytes differentiate into osteoclasts normally and the osteoclasts are normal both morphologically and with respect to resorptive activity.

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