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Induction of endosomal/lysosomal pathways in differentiating osteoblasts as revealed by combined proteomic and transcriptomic analyses

Takako Taniguchi [a], Shinsuke Kido [b], Emiko Yamauchi [a], Masahiro Abe [b], Toshio Matsumoto [b], Hisaaki Taniguchi [a,*]

[a] Institute for Enzyme Research, University of Tokushima, Tokushima 770-8503, Japan
[b] Institute of Health Biosciences, University of Tokushima, Tokushima 770-8503, Japan

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ABSTRACT

We have analyzed proteome changes associated with bone-forming osteoblast differentiation by quantitative differential proteomic and transcriptomic analyses using in vitro differentiation model. Sixty nine proteins were found up-regulated (>2-fold) and 18 were down-regulated (<0.5-fold) at protein level. The mRNA levels of these proteins were then analyzed by quantitative real-time PCR combined with clustering analysis. The most prominent cluster with increased protein and mRNA levels contains endosomal and lysosomal proteins, demonstrating the drastic induction of degradative endosomal/lysosomal pathways in osteoblasts. Osteoblasts, therefore, are involved not only in the synthesis but also in the turnover of the extracellular matrix proteins such as collagens.

1. Introduction

Bone is maintained by two distinct cell types: bone-forming osteoblasts and bone-resorbing osteoclasts. Bone is constantly resorbed by the latter cells and then replaced by the former cells in a physiological process called bone remodeling. Therefore, the major physiological function of osteoblasts is to synthesize extracellular matrix proteins such as collagens, while that of osteoclasts is the degradation of the ECM proteins. The osteoblast differentiation can be divided into three stages: proliferation, maturation that is accompanied by extracellular matrix formation, and mineralization. Although several osteoblast-specific transcription factors and small number of molecular markers have been identified to be involved in the process [1], the whole molecular basis of the osteoblast differentiation has yet to be elucidated. While the DNA microarray analysis has already been applied to the in vitro models of osteoblast differentiation [2], quantitative proteomic approaches to analyze protein expression changes have not been well presented in the literature.

In the present study, we have analyzed proteomic changes of osteoblast differentiation by quantitative analysis using cleavable ICAT [3]. Proteins detected with the cICAT method were further analyzed by quantitative real-time PCR for their mRNA expression, and the expression changes both at the protein and mRNA levels were compared with the functional classification. Cluster analysis of transcriptome data were found well correlated with the proteomic changes and functional classification. One notable characteristic is the increased transcriptional and translational levels of proteins involved in the endo/exocytosis and lysosomal protein degradation. A whole series of proteins involved in the uptake and degradation of extracellular matrix proteins were found drastically induced during the osteoblast differentiation. To our knowledge, this is the first demonstration that the degradative lysosomal pathways are highly induced in the osteoblasts and that the osteoblasts are involved not only in the synthesis but also in the turnover of ECM proteins.

2. Materials and methods

2.1. Cell culture and osteogenic differentiation

Primary osteoblast cells were routinely isolated from the calvariae of newborn ICR (Institute of Cancer Research) mouse (Charles

* Corresponding author. Address: Institute for Enzyme Research, University of Tokushima, 3-18-15 Kuramoto, Tokushima 770-8503, Japan. Fax: +81 88 633 7428.
E-mail address: hisatan@ier.tokushima-u.ac.jp (H. Taniguchi).

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River Japan, Tokyo) and the osteoblast differentiation was induced with L-(+)-ascorbic acid (50 μg/ml) and 10 mM β-glycerophosphate for 0, 11 and 22 days as described previously [4,5]. The differentiation into mature osteoblasts was monitored by alkaline phosphatase and von Kossa staining (Supplementary Fig. S1). Cells were harvested and suspended in 50 mM Tris–HCl buffer (pH8.3) containing 0.1% SDS and the supernatants after centrifugation were used for the proteomic analysis.

2.2. ICAT labeling and mass spectrometric analysis

The labeling of the cellular proteins with the cleavable isotope-tagged reagent (Applied Biosystems, Foster City, CA) was performed according to the manufacturer's protocol. The control lysates labeled with the heavy ICAT reagent were combined with equal amounts of those obtained from the differentiated cells labeled with the light reagent, and subjected to the SDS–PAGE (5–18% acrylamide gradient gel, ca.250 μg protein for each lane). The gel lane was cut into 35 bands of the same size and subjected to the in-gel digestion with trypsin [6]. The extracted peptides were treated by avidin affinity cartridge to capture the ICAT-labeled peptides. After cleaving off the avidin tag with TFA, the resulting peptide mixtures were analyzed with the LC/MS/MS using an Agilent nano LC apparatus (LC1100, Agilent, Santa Clara, CA) directly connected to the nano-spray ion source of an ABI mass spectrometer (Q-StarXL, Applied Biosystems). The operating conditions for the nanoLC with column switching were as described previously [6] except that an Agilen Zorbax 300SB enrichment column (5 μm particle, 5 × 0.3 mm) and a Zorbax 300SB separation column (5 μm particle, 150 × 75 μm) were used. Data were collected in an information-dependent acquisition mode. The data were processed using a script (mascot.dll 1.6b16 for Analyst QS 1.0 SP8), and subjected to database search against the non-redundant protein database (NCBInr retrieved on 2009/10/10 containing 9868,855 sequences) using the Mascot search engine (Version 2.1, Matrix Science, London, UK). The taxonomy was restricted to *Mus musculus* (143 835 sequences). The number of missed cleavages was set to one. The first search was performed with wide tolerance (±0.60 Da for MS and MS/MS), and the data obtained with high confidence were used to recalibrate the original data [7]. The final search was conducted with reduced tolerances for MS (±35 ppm) and MS/MS (±0.10 Da) data. Typical modifications (oxidation of Met and deamidation of Asn/Gln) were considered in addition to the ICAT label as variable modifications. The cut-off score for individual MS/MS score was set to 25. The cut-off value was chosen from the false discovery rate after recalibration, which was estimated to be less than 0.37% by searching against the decoy database with reversed sequences [8]. The protein level change was calculated from the peak intensity using the ProICAT software (Applied Biosystems), and all the results were checked manually.

2.3. Quantitative real-time PCR analysis

The quantitative real-time analysis was performed with an AB 7500 real-time PCR apparatus (Applied Biosystems) using the SYBR Premix Ex Taq II kit (Takara Bio, Otsu, Japan) according to the manufacturer's protocol. Briefly, total RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA), treated with DNaseI, and reverse-transcribed with SuperScript®III First Strand Synthesis System (Invtrogen) using oligo$(dT)_{20}$ as primer. Two sets of primer pairs for each protein were used for the PCR analysis and the averages of the two data were used. When only one pair of primer set gave results, the PCR analysis was repeated with the same primer pair, and the average of data was used.

3. Results and discussion

3.1. Differential proteomic analysis

Altogether 176 proteins were detected by the cICAT analysis with confidence, of which 69 proteins showed increases (more than 2-fold), while 18 proteins showed decreased protein levels (less than 0.5) (Supplementary Table 1). The lists of proteins showing increases or decreases were separately subjected to the DAVID functional annotation tool [9]. It is evident that the major functional classes with increased protein levels include the endosomal/lysosomal (21%), signaling (18%), and extracellular matrix proteins (13%) (Fig. 1). The presence of five isoforms of 14-3-3 proteins and that of four isoforms of annexins are also noted (Supplementary Table 2). Among the ECM proteins, three proteins, i.e., fibromodulin, biglycan and dermatopontin, belonging to a family of ECM proteins with a leucine-rich repeat (LRR) motif, showed highest increases. While most of the proteins detected are cytoplasmic, 18 proteins are involved in the intracellular transport. It should be noted that the major components of the ECM such as collagen isoforms were less presented reflecting the facts that the only soluble fractions were subjected to the cICAT analysis and that the mature collagens lack Cys residue, which is used to affinity-purify labeled peptides. The identification of secreted frizzled-related protein 1 (sFRP1) as one of the highly up-regulated proteins should also be noted: the Wnt signaling plays a central role in the osteoblast differentiation [10].

While the up-regulated proteins include typical osteoblast differentiation marker proteins, the list of the down-regulated proteins includes many ribosomal proteins and several muscle-specific marker proteins such as calponin 1 and transgelin (smooth muscle protein 22α). The DAVID functional analysis predicts that the two prominent functional clusters are those of ribosomal and translational functions (Supplementary Table 2). The third annotation cluster contains three calponin-related proteins associated with muscle differentiation. Altogether, the list of decreased proteins suggests decreased overall protein synthesis compared to the proliferating progenitor cells with a clear switching to the synthesis of osteoblast-specific proteins.

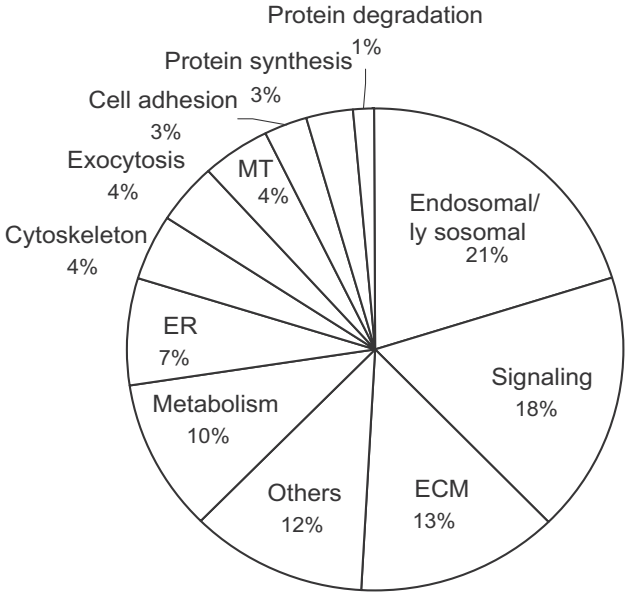

Fig. 1. Functional classification of proteins increased in differentiating osteoblasts. Functional annotation was done with the aid of DAVID annotation tool for the proteins showing increased protein levels (>2-fold).

3.2. Differential transcriptomic analysis

The expression changes of the proteins detected in the ICAT analysis were then analyzed by the real-time PCR analysis. Total RNA was extracted from the cells at 0, 3, 14 and 25 days in vitro. Out of 176 proteins, 152 proteins showed mRNA expression data with confidence. The expression level changes relative to the 3-day levels were analyzed by the hierarchical clustering analysis [11], with 89 genes showing changes larger than 1.3-fold at least one time point included in the present analysis. The high accuracy achieved by the real-time PCR analysis was demonstrated by the one-way analysis of variance (ANOVA) as shown in Supplementary Table 4. As shown in Fig. 2, the 89 genes were classified into five major clusters. The 49 genes showing increased mRNA levels can

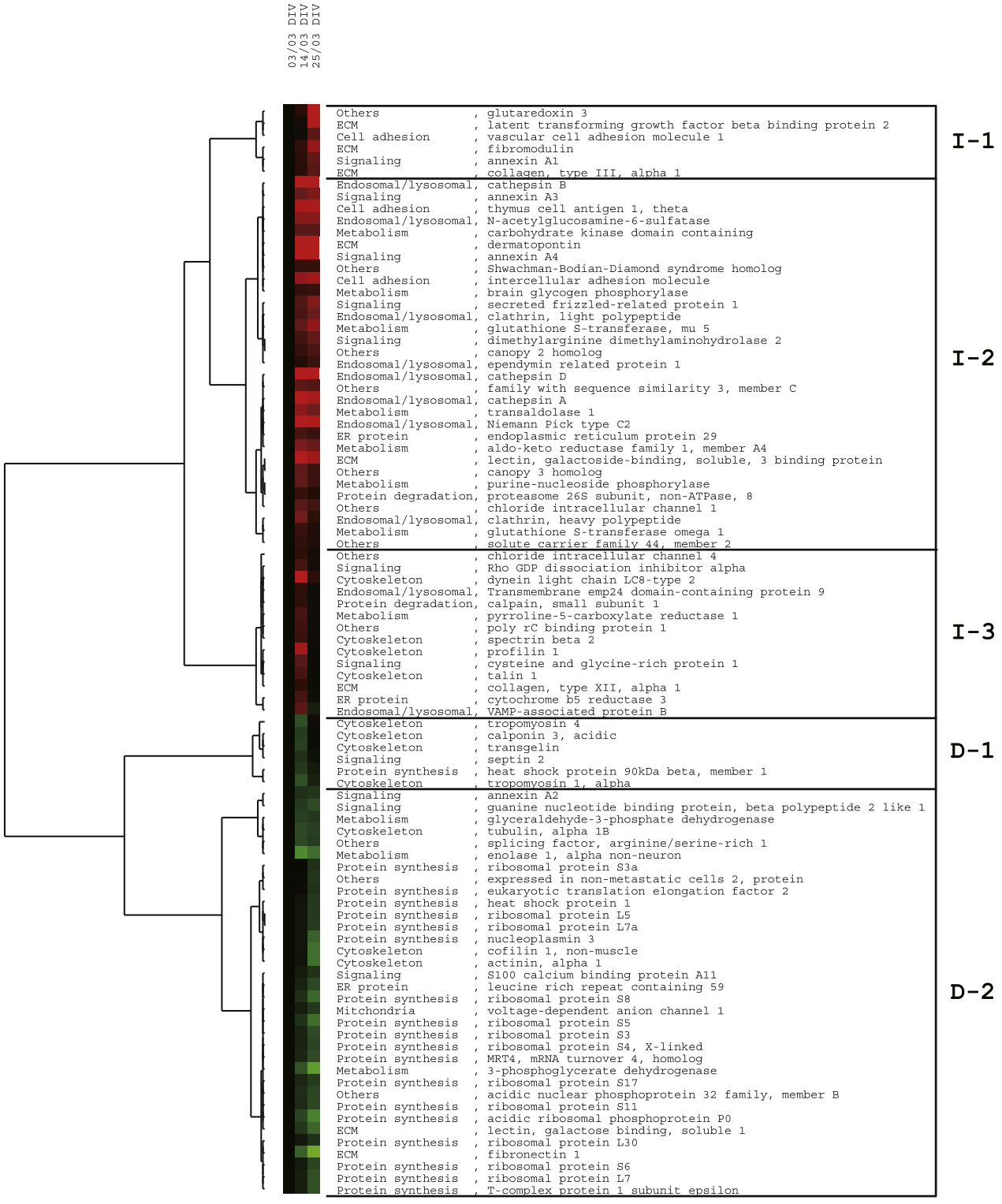

Fig. 2. Cluster analysis of mRNA expression change analyzed by real-time PCR analysis. Genes showing more than 1.3-fold changes were analyzed by hierarchal clustering. The intensity in the red and green color spectrum denoting up-regulated and down-regulated genes, respectively.

be divided into three major clusters: one with only a slight increase at 14 days and significant increase at 25 days (cluster I-1), one with a rapid increase at 14 days with saturation or a slow decrease at 25 days (I-2), and one showing a clear peak at 14 days with a decrease at 25 days (I-3). The 40 genes with decreased mRNA levels can be divided into two major clusters: one with a negative peak at 14 days (D-1) and the other with a more or less continuous decrease (D-2).

The first cluster (I-1) with slow mRNA increases is related to the extracellular glycoproteins, although the enrichment score is not high (1.09) (Supplementary Table 3). The functional annotation of the large cluster consisting of genes with rapid mRNA induction at 14 DIV (I-2) clearly associates the cluster with 'lysosome' (Supplementary Table 3). Many of the genes showing the largest mRNA increases belong to this cluster. The third cluster showing increases at 14 DIV but decreases to the initial 03 DIV levels (I-3) contains cytoskeletal proteins such as spectrin and talin. On the other hand, one small cluster with a negative peak at 14 days and recovery to the initial level (D-1) contains mostly cytoskeletal proteins, and the functional annotation associates the cluster both with 'actin-binding' and to 'muscle'. The dramatic down regulation of myoblast-related genes has been reported [2,12]. The proliferating progenitor cells, therefore, show some muscle-like characters, and the osteoblast differentiation is associated with the decrease of muscle-specific proteins. Finally, the fifth cluster containing proteins showing significant decreases at the mRNA level (D-2) is associated with 'ribosome'. The other 61 proteins, which showed only small changes (less than 1.3-fold) and not included in the clustering analysis, contain those of various functions including 11 mitochondrial proteins. All together, there is a clear tendency that the functionally-related proteins cluster together, and the largest cluster showing increased protein and mRNA levels is associated with the lysosomal functions.

3.3. Comparison between proteomic and transcriptomic analyses

The proteomic and transcriptomic data obtained for the 152 proteins were then compared (Fig. 3). There is a negative correlation between the 0 day and 03 day mRNA and protein level change (14/03 day), and the majority of the proteins analyzed showed slightly decreased mRNA levels between the 0 day and 03 days (Fig. 3A). On the contrary, a positive correlation between the protein and mRNA levels was observed with other mRNA datasets obtained for 14 and 25 DIV (Fig. 3B and C).

When the data belonging to each cluster were separately compared to the proteome data, more clear relationships between the two analyses became evident (Fig. 3D–F). The first cluster with slow increases at the mRNA level consists of seven proteins, all of which are found in the first quadrant (Fig. 3D). The 31 proteins, which belong to the major cluster with rapid increases at the mRNA level with saturation behavior, are again found only in the first quadrant (Fig. 3E). The most notable feature is the presence of five lysosomal proteins in this group (Fig. 3E, open circle). On the other hand, most of the ribosomal proteins showed slight decreases both at mRNA and protein levels (Fig. 3F).

Since a large-scale transcriptomic analysis data of a similar in vitro osteoblast differentiation model is available [2], it is of interest to compare the microarray-based results with the present

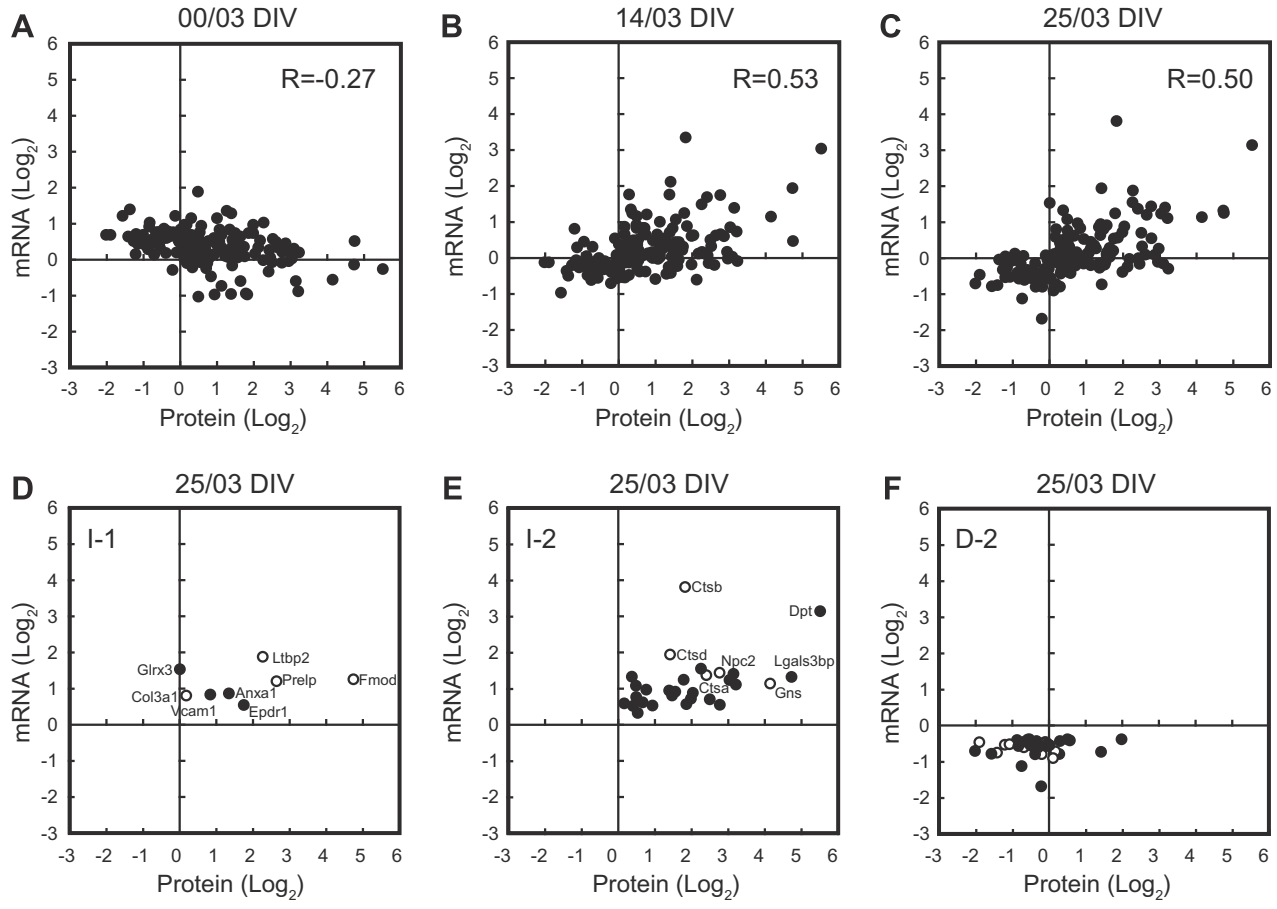

Fig. 3. Scatter plots showing the correlation between mRNA and protein expression levels. Real-time PCR data obtained at 0 (A), 14 (B), and 25 (C) DIV expressed as ratios against 03 DIV data were plotted against ICAT data (14/03 DIV). Proteins belonging to individual clusters revealed by the clustering analysis shown in Fig. 2 were plotted separately (D–F). ECM proteins (D), lysosomal proteins (E), and ribosomal proteins (F) are in open circles.

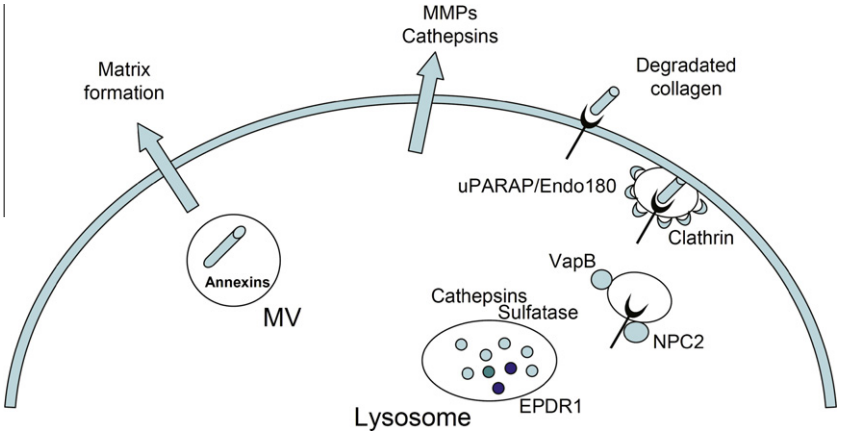

Fig. 4. Induction of degradative lysosomal pathways in the osteoblast. Not only the collagen uptake receptor but also the proteins involved in the various phases of the endosomal/lysosomal pathways are unregulated in the differentiating osteoblasts.

data. The decreases of myoblast-related genes correspond very well as discussed above. However, it is notable that except for typical ECM proteins and lysosomal proteases, which will be discussed below, there are not much in common between the two results. One reason for the discrepancy may be the way of choosing genes; in the present study, only the abundant proteins detected with the ICAT analysis were selected for further transcriptomic analysis. The absence of less abundant proteins such as various transcription factors in the present study is in good contrast to the tip-based analysis.

3.4. Proteins involved in endocytosis/lysosomes are highly induced in osteoblasts

Various proteins associated with endocytosis showed increased expression at protein and/or mRNA levels. The first one to be mentioned is the mannose receptor C type 2 (also known as Endo180/CD280/uPARAP), which is the catabolic internalization receptor of collagens [13,14]. Although the endocytic route of collagen degradation mediated by Endo180/CD280/uPARAP has been recently shown in the mesenchymal fibroblasts [15], this is the first report showing the induction of the collagen receptor in the differentiating osteoblasts. Low density lipoprotein receptor-related protein-1 (LRP-1), another catabolic endocytosis receptor for ECM proteins [16], was also found increased in the present study.

Other proteins such as Niemann Pick type C2 protein (NPC2) [17] and ependymin-related protein 1 [18], together with the three cathepsin isozymes and glucosamine (*N*-acetyl)-6-sulfatase, all involved in the degradative endosomal/lysosomal pathways, showed drastic increases both at the mRNA and protein levels (Fig. 4). It should be noted that the unexpected increases of these proteins in the differentiating osteoblasts are probably not due the contaminating osteoclasts in the present in vitro differentiation model used. One reason is the limited sensitivity of the ICAT methodology used; the inspection of the protein lists obtained, and the peak intensities observed suggest that one deals with only abundant proteins in the present study. The less abundant proteins such as osteoclast specific transcription factors detected in the highly-sensitive DNA array analysis [2] are not detected in the present study. These authors also reported increased lysosomal cathepsins, and reached the same conclusion like us [2]. Since the visual inspection and osteoblast-specific staining demonstrated that the majority of the cells show osteoblast characteristics (Fig. S1), it is unlikely that the drastic induction of degradative endosomal/lysosomal proteins observed is due to the contaminating osteoclasts.

Although the production of the ECM proteins is considered to be the major physiological function of osteoblasts, the increased uptake and degradation of collagens and other ECM proteins is clearly another feature of osteoblasts as revealed by the present combined proteomic and transcriptomic analyses. In fact, a recent cell biological study demonstrated a drastic increase in total lysosomes that is accompanied with change of cellular distribution in osteoblasts [19]. An enhanced endocytosis is also reported. Taken together, the concerted induction of endosomal and lysosomal proteins including endocytosis receptors suggests that the induced lysosomes in the differentiating osteoblasts are involved in the degradation of extracellular matrix proteins. One possibility is that the osteoblasts are involved in the degradation of aberrant or denatured collagens. Another intriguing possibility is that the osteoblasts play an important role in the remodeling of bone through the turnover of the ECM proteins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2010.07.055.

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