Understanding the pathogenesis of type V osteogenesis imperfecta through analysis of protein expression changes in osteoblasts during in vitro mineralization

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Abstract

The aim of the study conducted was to study the protein dynamics profile between Osteogenesis Imperfecta type V (OV-V) bone cells and normal bone cells during *in vitro* bone formation. Osteoblasts that were obtained from OI-V patients (with their consent) underwent *in vitro* mineralization. Newly synthesized proteins at three time points were labeled with SILAC and the whole cell proteins were analyzed by mass spectrometry. By extracting the 20-80 percentile from the proteome and performing global hierarchical clustering, three major clusters, Early, Late OI and Late WT, were identified. Comparing Late OI and Late WT yields proteins encoded by differentially Expressed Genes (DEGs) that are down-regulated and up-regulated in Osteogenesis Imperfecta type V (OI-V) patients. There were 21 different down-regulated proteins and 3 different up-regulated proteins that were found to be related to osteogenesis, osteoblasts, and osteocytes. The down-regulated proteins could be divided into two main process that they regulate or play a role in; cellular vesicle and cell adhesion and cytoskeleton organization.

Introduction

Osteogenesis Imperfecta (OI) is a genetic disorder that affects the bones and it characterized by fragile bones that have the tendency to break easily. OI occurs due to mutations being present in a variety of genes. In approximately 90 percent of the patients the mutations in the gene *COL1A1* and *COL1A2* cause OI. Osteogenesis Imperfecta type V has been characterized with moderate to severe fragility of the vertebral bodies and long bones. Patents of OI type V (OI-V) have a tendency to develop a hyperplastic callus upon bone fracture and healing, and calcification of the forearm interosseous membrane in long bones, forming bone bridges[1]. OI-V is dominant disorder and is not related to the mutations of the *COL1A1* and *COL1A2* genes, but mutation in the gene, interferon-induced transmembrane protein 5 (*IFITM5*)[2].

A study to analyze the proteomics data of OI-V bone-cells was conducted. Osteoblast were grown from human bone biopsies that were obtained from patients (with their consent). The study was done in an *in vitro* system rather than an *in vivo* system because the *in vitro* systems allows us to mimic the mineralization and bone formation permitting the study of changes in protein to be carried out. Stable Isotope Labeling by amino acids in cell culture (SILAC), where stable isotope-labeled amino acids are used to encode cellular proteomes for quantitative analysis, was performed. In the process the natural (light) amino acids are replaced with heavy ones and there is a characteristic mass difference which can be detected using the Mass-spectrometry analyses.

Method

In vitro mineralization of the osteoblasts obtained from patients (with their consent) occurred for 21 days. SILAC was performed on the 12th, 16th, and the 20th day. It lasted for 24 hours and the data for SILAC was collected on the 13th, 17th, and 21st day (Figure 1). After the SILAC was performed raw data was obtained and it was presented in the form of a histogram (Figure 2). The data was the further processed and a preliminary cluster analysis was performed (Figure 3). There was no clear correlation that could be identified vis the preliminary cluster analysis. This could have been due to the extreme data points that were present.

- To eliminate the extreme data points 20-80 percentile of the data was selected and a final cluster analysis (Figure 4) was performed. Form this analysis the data obtained could be separated into three different groups the early timepoint of OI and Wild-type, the late Wildtype (from Day-17 to Day-21), and the Late OI (from Day-17 to Day-21).
- Upon comparing the late wild type and the late OI, two sets of differentially expressed genes (DEGs) were found; the up-regulated one and the down-regulated one. DEGs that had the p-value larger than 0.05 were discarded.

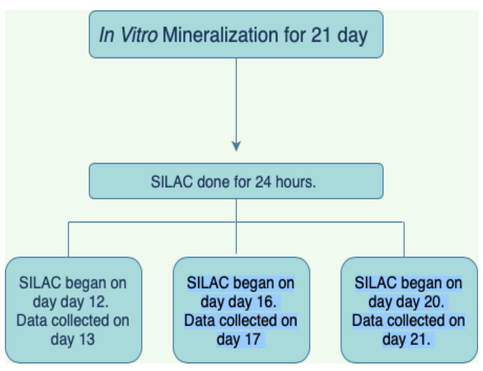

Figure 1: Flow chart showing the steps in the process of SILAC

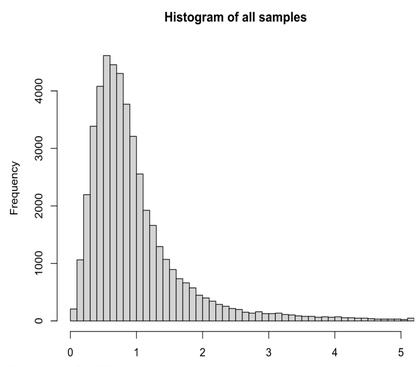

Figure 2: The histogram of the raw data obtained rom SILAC representing the raw data in terms of the ratio of light (natural) to heavy amino acids

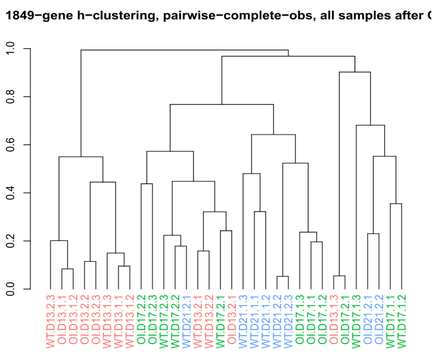

Figure 3: Preliminary Cluster Analysis

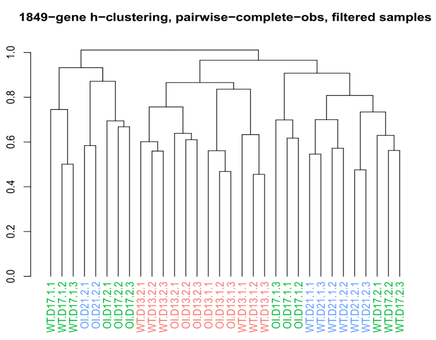

Figure 4: Final cluster analysis of the data after the selection of the data points that fall in between 20-80 percentile

Results

KEGG Mapper and GO Enrichment Analysis were performed to understand the biological meaning of DEGs. The down-regulated data-set was input into GO Enrichment which divided the DEG encoded proteins into different processes. Most of the proteins that correlated to osteogenesis, osteoblasts, or osteocytes were found to be related to processes like extracellular components, cytoplasm, and vesicles. From Go Enrichment, there were 3 categories in which the proteins could be divided into included: Biological processes, Cellular components, and Molecular Functions. From each of the categories the processes with the lowest False Detection Rate (FDR) were chose and they FDR value was log transformed (=log(FDR)) and a bar graph was made to identify the most relevant processes (figure 6-8).

The same data-set was then input onto KEGG for further analysis. A list of proteins that correlated to osteogenesis was then formed containing 20 proteins that could be grouped into 9 processes (table 1 and 2). The up-regulated data was analyzed in the same way and three DEG encoded proteins that can be grouped into three different processes were found. The up-regulated data was not analyzed further because only three proteins were identified. From the 21 DEG encoded proteins and the 9 processes, it could be established that the two most important processes were cellular vesicle and cell adhesion and cytoskeleton organization.

Process/Pathway related to osteogenesis	Specific gene from the pathway
Metabolic pathway	ACLY: ATP citrate lyase
	ADK: Adenosine Kinase
	PKM: Pyruvate Kinase M1/2
Focal Adhesion	FLNA: Filamin A
	ITGAV, integrin subunit alpha V
	THBS1, Thrombospondin 1
	TALN1, Talin 1
	TNC, tenascin C
Phagosome	CALR, Calreticulin
	LAMP2, lysosomal associated membrane protein 2
	THBS1, thrombospondin 1
Regulation of actin skeleton	CFL2, cofilin 2
	RRAS, RAS related
	VCL, vinculin
Tight Junction	ACTR2, actin related protein 2
	HSPA4, heat shock protein family A (Hsp70) member 4
Human cytomegalovirus infection	B2M, beta-2-microglobulin
Gap Junction	GNAI2, G protein subunit alpha i2
Mineral Absorption	FTL, ferritin light chain
Lysosome	CTSA, cathepsin A

Table 1: The process and proteins found from the down-regulated DEGs that are related to osteogenesis, osteoblasts, or osteocytes from KEGG

Process/Pathway related to osteogenesis	Specific gene from the pathway
Metabolic Pathways	PFKL, phosphofructokinase, liver type
Protein processing in endoplasmic reticulum	HSP90B1, heat shock protein 90 beta family member 1
Fc gamma R-mediated phagocytosis	GSN, gelsolin

Table 2: The process and proteins found from the up-regulated DEGs that are related to osteogenesis, osteoblasts, or osteocytes from KEGG

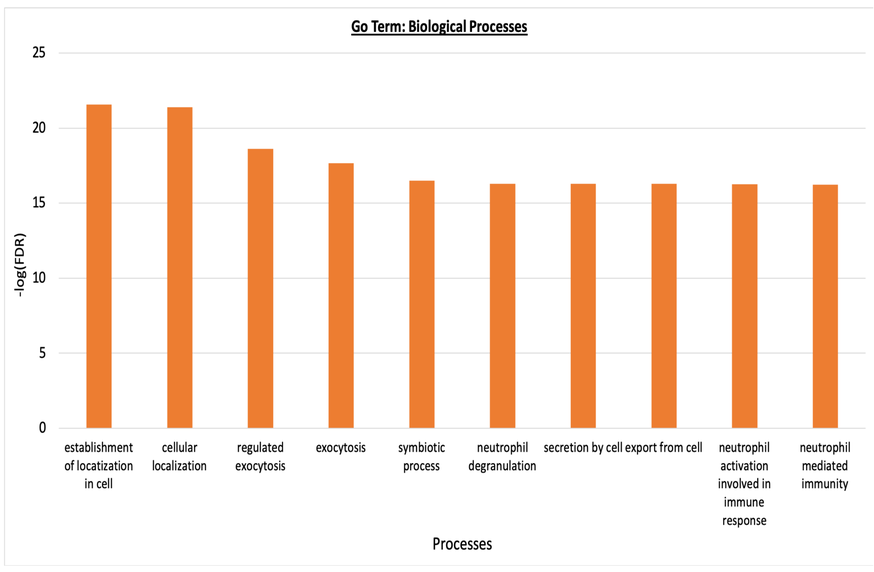

Figure 6: The processes that have the lowest FDR in the Biological Processes category obtained from Go Term

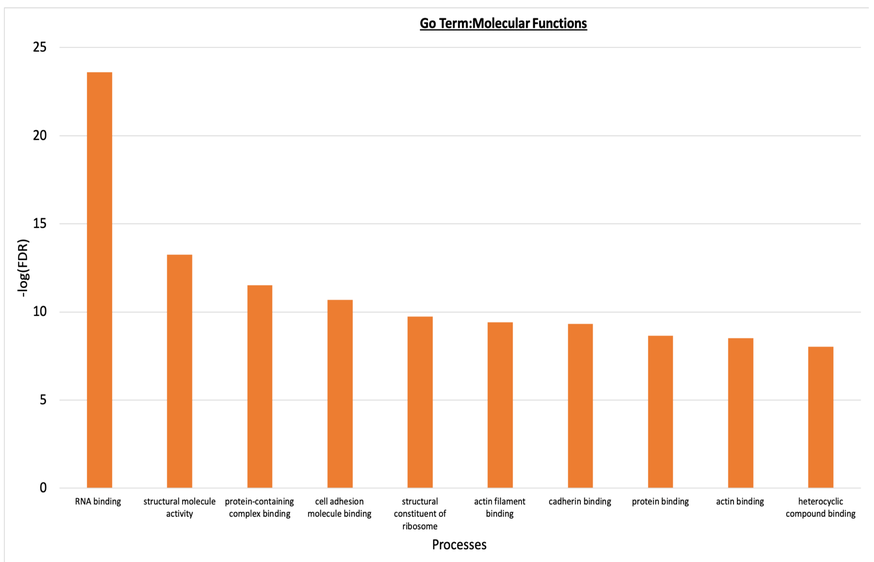

Figure 7: The processes that have the lowest FDR in the Molecular Function category obtained from Go Term

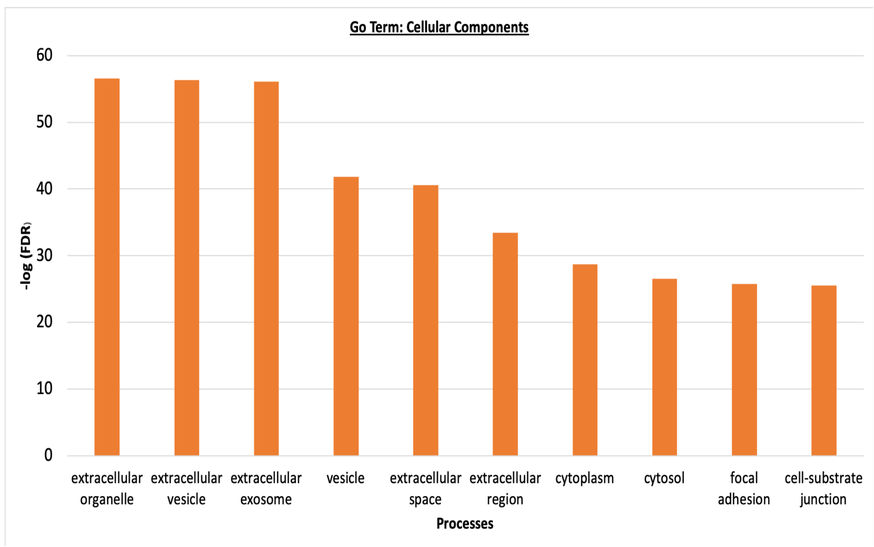

Figure 8: The processes that have the lowest FDR in the Cellular components category obtained from Go Term

Discussion

Cell adhesion and cytoskeleton organization play an important role in osteocytes. Cellular adhesion permits osteocytes to be attached to the extracellular matrix in order to perceive information and regulate the remodeling of the bone[3], and communication with other osteocytes and osteoblasts allowing them to send and receive signals affecting the bone mineralization process[4]. Integrin protein plays a crucial role in cell adhesion and allows the osteocyte to get information while remaining inside of the bone[5]. The mechanism by which osteocytes communicate and respond to the mechanical information is not fully understood. The ITGAV gene encodes for the integrin subunit alpha V, which plays a role in communication between the osteocyte and the outer atmosphere. Vinculin which is encoded by the gene VCL, plays a role in adhesion[3].

If the genes that produce the proteins that are essential for focal adhesion are down-regulated, then osteocytes will not be able to function properly, potentially altering remodeling and regulating processes[3].

Osteoblasts produce and secrete extracellular vesicles which are a group of matrix-bound nanoparticles. They have the function of acting as the center point of bone mineralization and a mode of communication. Osteoblasts play a crucial role in early mineral deposits by controlling local phosphate ion concentrations that allow osteogenesis to progress[6]. The gene THBS1 which encodes for thrombospondin-1 found to be related to phagosomes plays a role in bone remodeling[7]. Whereas lysosomal associated membrane protein 2 transactivates the RUNX2 gene expression, which plays a role is osteogenic differentiation[8]. Having these genes down-regulated in the OI-V shows that bone mineralization, bone remodeling and communication may not be taking place properly leading to fragile bones and impaired bone healing.

References

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