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ARTÍCULOS ORIGINALES / *Originals*

MLO-Y4 OSTEOCYTIC CELL CLONES EXPRESS DISTINCT GENE EXPRESSION PATTERNS CHARACTERISTIC OF DIFFERENT STAGES OF OSTEOCYTE DIFFERENTIATION

Emily G. Atkinson,[1] Alejandro Marcial,[1] Zuleima Sánchez,[1] Christian Porter,[1] Lilian I. Plotkin.[1,2*]

[1] *Department of Anatomy & Cell Biology, Indiana University School of Medicine,* [2] *Roudebush Veterans Administration Medical Center, Indianapolis, IN, USA.*

Abstract

Osteocytes are the most abundant bone cell and are formed when osteoblasts become embedded in the bone matrix. Through changes in gene expression and paracrine effects, osteocytes regulate the number of osteoblasts, bone forming cells, and osteoclasts, bone resorbing cells, which are needed to maintain bone mass. MLO-Y4 is the better characterized osteocytic cell line; however, lacks expression of sclerostin, the product of the SOST gene, which is fundamental for osteocyte function and blocks bone formation. With the objective to isolate MLO-Y4 clones with different gene expression profiles, we performed cultures at very low density of MLO-Y4 cells stably transfected with nuclear green fluorescent protein (MLO-nGFP). Cell morphology was visualized under a fluorescence microscope. Once the cells reached 80% confluency, RNA was extracted and quantitative real time PCR was performed. Clones exhibit different sizes and morphology, with some cells showing a spindle-like shape and others with abundant projections and a star-like shape. Gene expression also differed among clones. However, none of the clones examined expressed SOST. We conclude that the MLO-nGFP clones constitute a useful tool to study osteocyte differentiation and the role of osteocytes in the control of bone formation and resorption *in vitro.*

Key words: osteocytic cells, gene expression, cell morphology.

Resumen

CLONES DE LAS CÉLULAS OSTEOCÍTICAS MLO-Y4 TIENEN DIFERENTES PATRONES DE EXPRESIÓN GÉNICA CARACTERÍSTICOS DE DIFERENTES ESTADÍOS DE DIFERENCIACIÓN OSTEOCÍTICA

Los osteocitos son las células más abundantes del hueso y se forman cuando los osteoblastos se encuentran rodeados de matriz ósea. A través de cambios en la expresión génica y efectos paracrinos, los osteocitos controlan el número de osteoblastos que forman el hueso, y osteoclastos que resorben el hueso, células necesarias para mantener la masa ósea. Las células MLO-Y4 son la línea celular osteocítica más investigada; sin embargo, no expresan esclerostina, el producto del gen SOST que bloquea la formación ósea y es indispensable para la función de los osteocitos. Con el objetivo de aislar clones de las células MLO-Y4 con diferentes perfiles de

**Correspondencia: Department of Anatomy and Cell Biology, Indiana University School of Medicine, 635 Barnhill Drive, MS-5035, Indianapolis, IN 46202-5120.*
E-mail: lplotkin@iupui.edu

expresión génica, realizamos cultivos a muy baja densidad de las células transfectadas en forma estable con proteína verde fluorescente nuclear (MLO-nGFP). La morfología celular fue evaluada utilizando un microscopio de fluorescencia. Una vez que las células alcanzaron el 80% de confluencia, el ARN fue extraído y analizado por PCR cuantitativa en tiempo real. Las células de los diferentes clones tienen diferentes tamaños y morfología, algunas células son fusiformes y otras con proyecciones citoplasmáticas abundantes y en forma de estrella. La expresión de los genes también varió en los distintos clones. Sin embargo, ninguno de ellos expresó SOST. En conclusión, los clones de las células MLO-nGFP constituyen una herramienta útil para estudiar la diferenciación de los osteocitos y el rol de estas células en el control de la formación y resorción ósea in vitro.

Palabras clave: *células osteocíticas, expresión génica, morfología celular.*

Introduction

Osteoblasts, the bone forming cells, and osteoclasts, the bone resorbing cells, are needed in order to maintain bone mass.[1] If there is an increase in osteoblasts and a decrease in osteoclasts, bone mass will increase. The vice-versa is also true; if there is an increase in osteoclasts and a decrease in osteoblasts, bone mass will decrease. Coupled remodeling of osteoclasts and osteoblasts results in optimal bone turnover rate and bone mass maintenance. These processes are controlled by the actions of osteocytes, the cells buried in the bone matrix.[2] Until the late 1990s, osteocytes were poorly studied due to their localization within the bone. Further, isolation of osteocytes from bones and their culture maintaining cell morphology and gene expression profile was difficult. However, the main reason why the functional role of osteocytes remained understudied was the lack of cell lines available to study these cells *in vitro*.[3]

In 1997, Dr. Bonewald's group reported the characterization of an immortalized osteocytic cell line, MLO-Y4.[2] These cells were isolated from long bones of mice expressing a T-antigen transgene under the control of the osteocalcin promoter. Expression of the transgene renders the cells immortalized, allowing for the generation of a cell line. MLO-Y4 osteocytic cells produce high amounts of osteocalcin and low amounts of alkaline phosphatase, and have morphology consistent with osteocytes. Since their description, numerous laboratories throughout the world have utilized MLO-Y4 cells as osteocytic models to understand the mechanism of action of hormones, pharmacotherapeutic agents, and mechanical signals, as well as to investigate the cross-talk between osteocytes and other organs/tissues such as muscle and brain.[2] Because of this, MLO-Y4 cells are the better characterized osteocytic cell line.

However, further studies and the discovery of new osteocyte-specific molecules showed that MLO-Y4 cells lack expression of sclerostin, the product of the SOST gene, which is fundamental for osteocyte function and blocks bone formation.[4] In this study, we aimed to investigate whether clones within a pool of MLO-Y4 cells have different gene expression patterns and might represent a better model for osteocytes. Towards this end, we generated clones of MLO-Y4 osteocytic cells stably transfected with nuclear green fluorescent protein (MLO-nGFP), which allow us to visualize the morphology of the cells under a fluorescence microscope.[5] Although we did not find any clone expressing SOST, our studies uncovered different populations of MLO-Y4 cells that could be used to study osteoblast-osteocyte transition and the mechanism by which osteocytes regulate osteoclastogenesis.

Materials and Methods

Materials

SuperFect was obtained from Qiagen (Santa Clarita, CA). Trizol reagent was purchased

from Invitrogen (Massachusetts, MA). Primers/probes qPCR sets were purchased from Roche Applied Science (Indianapolis, IN) or Applied Biosystems (Foster City, CA). All other reagents and tissue culture material were obtained from Sigma Chemical Co (St. Louis, MO).

Establishment of MLO-Y4 cells stable transduced with green fluorescent protein

MLO-Y4 osteocytic cells were originally obtained from Dr. Lynda Bonewald[3] and maintained in our laboratory since 1998.[5] Cells were cultured on calf skin collagen type I-coated plates in αMEM media containing 2.5% fetal bovine serum and 2.5% bovine calf serum, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were plated at 1×10^4 to 2×10^4 cells/cm^2 and media was changed every 2-3 days. The generation of MLO-Y4 osteocytic cells was previously reported.[5] Briefly, a plasmid containing the retroviral nuclear GFP construct was transiently transfected into the packaging cell line Phoenix Eco using SuperFect (Qiagen, Santa Clarita, CA).[5] Supernatants containing retroviral particles were collected 24-48 h after transfection, and used to infect cell cultures. Subconfluent MLO-Y4 osteocytic cells were exposed to viral supernatants in the presence of 8 µg/ml polybrene for 8 h and then incubated in fresh culture medium for 16 h. Transduced cells were selected by culturing them in the presence of 400 µg/ml of G418 for three weeks. Cells are designated as MLO-nGFP (pool).

Cells were cultured on 10 cm collagen-coated plates[5] at very low density in order to obtain single cell cultures. From those, cell colonies growing as separated clones were picked up using a tip and transferred to 24 well plates and continue cultured. Semi-confluent cultures were then trypsinized and cells were frozen until used. Cell were thawed and plated in replicas of 6 for gene expression analysis or in 10 cm plates for imaging, as detailed below.

Microscope imaging and morphological analysis

Cells were seeded at 4.4×10^4 cells/cm^2 on collagen-coated plates, as published.[5] Twenty four hours after seeding, medium was changed to remove dead cells and debris and cells were cultured for additional 48 hours. Cells were then fixed in 10% neutral buffer formalin for 8 minutes, washed twice with PBS and stored at 4°C in PBS containing thimerosal to avoid bacterial contamination. Fluorescent images were collected on an EVOS fluorescence microscope system (Life Technologies, Carlsbad, CA),[6] and cell morphology was evaluated in 10 independent fields at 40X magnification.

RNA extraction and quantitative RT-PCR (qPCR)

Total RNA was extracted from cells using Trizol reagent (Invitrogen), as previously reported.[6,7] mRNA was converted to cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). qPCR was performed using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as house-keeping gene, and the ΔCt method. Primers and probes were designed using the Assay Design Center (Roche Applied Science) or were commercially purchased (Applied Biosystems).

Statistical Analysis

Statistical analysis was performed with SigmaPlot. One-way ANOVA on the ranks was the statistical method used and a Dunn's post-hoc test was performed. $p < 0.05$ was considered significant.

Results

MLO-nGFP clones exhibit distinct morphological features. Microscopic examination of MLO-nGFP cells revealed unique morphologic features of the clones. Indeed, whereas the pool of MLO-nGFP contained cells with various shapes and sizes, the clones exhibited more distinct morphologies, as evidenced in the images for 7 representative clones (Fig. 1).

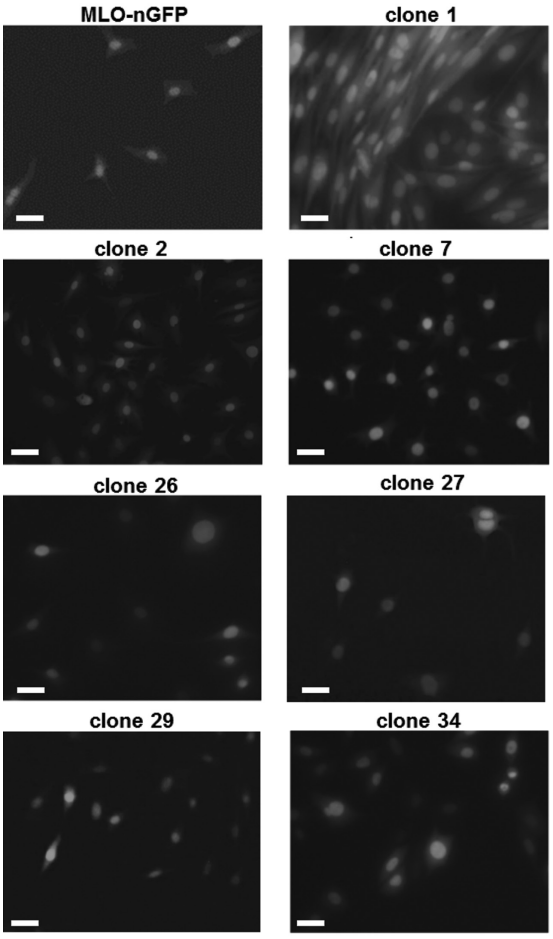

Figure 1. Clones of MLO-nGFP cells show unique morphologies. Images of MLO-nGFP osteocytic cells (pool) and representative clones under fluorescence microscopy. Scale bars correspond to 25 µm.

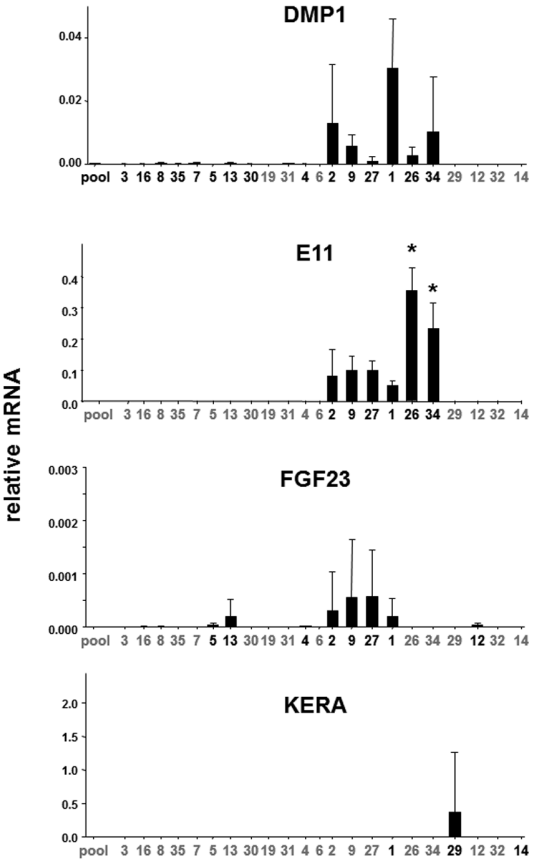

Figure 2. Distinct pattern of osteocytic/osteoblastic gene expression in MLO-nGFP clones. Expression of the indicated osteocytic and osteoblastic genes relative to GAPDH was measured by qPCR. * $p<0.05$ versus MLO-nGFP pool by one-way ANOVA. Clones that show no expression of the corresponding gene are indicated in grey.

For example, cells of clone 1 and 29 were elongated (fusiform), and cells of clones 2, 7, 26, 27, and 34 exhibited cytoplasmic projections and a star-shaped morphology. Regarding size, cells from clones 2 and 29 were overall smaller that cells in clones 24 and 34. This evidence suggests that we were able to isolate individual cell types that might exhibit a particular repertoire of genes.

Gene expression pattern varies among MLO-nGFP clones. qPCR analysis revealed the MLO-nGFP pool expressed undetectable levels of most genes investigated, with exception of RANKL and the housekeeping gene GAPDH (Fig. 2).

The reason for the lack of detection of most genes in the pool is not clear. However, we can rule out the possibility of lack of enough cDNA, since the Ct values for GAPDH were approximately 21, similar to the values for clones 26 (Ct=23) and 27 (Ct=21), for example. Nevertheless, the MLO-nGFP clones exhibit different levels of expression of osteocytic genes. Although none of the clones expressed detectable levels of SOST, we could detect mRNA expression for other osteocytic genes such as dentin matrix protein 1 (DMP1), E11 (also known as podoplanin) and fibroblast growth factor-23 (FGF23). For example, clone

34, which exhibits a star-like shape, has high expression of E11 and DMP1 (although the difference for the later did not reach significance, likely due to the high variation of the values and the multiple comparisons), markers of early osteocyte differentiation, no expression of keratocan, a gene enriched in osteoblasts; and absence of FGF23 and Sost expression, suggesting they represent early osteocytes. On the other hand, clones 1 and 29, exhibits a spindle-like shape, and, whereas clone 1 has the highest expression of DMP1 among the clones analyzed and low but detectable levels of E11, FGF23, and keratocan, clone 29 lacks E11, DMP1, and FGF23 expression and has relative high levels of keratocan, suggesting they represent late and early osteoblasts, respectively.

The expression of the genes involved in osteoclastogenesis ligand for the receptor activator of NFkB (RANKL) and osteoprotegerin (OPG) also varied among the clones (Fig. 3), with some exhibiting high (such as clone 34), and others low (such as clone 1) RANKL/OPG ratio.

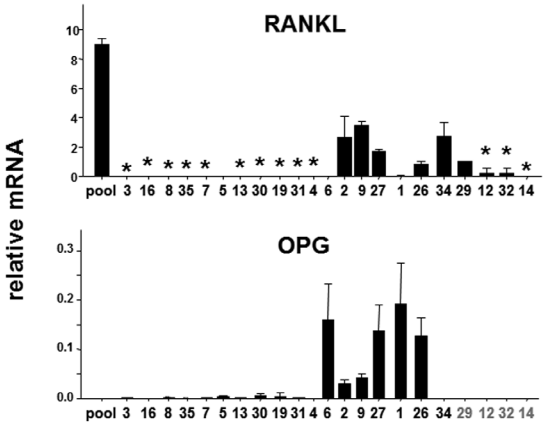

Figure 3. Osteoclastogenic cytokines are expressed at different levels in MLO-nGFP clones. Expression of the indicated osteoclastogenic cytokines relative to GAPDH was measured by qPCR. * p<0.05 versus MLO-Y4-nGFP pool by one-way ANOVA. Clones that show no expression of the corresponding gene are indicated in grey.

Discussion

Our understanding of the role of osteocytes in skeletal biology and in the interaction of bone with other tissues and organs have exhibited a substantial increase in the last 20 years.[8-10] These advances were originated in the isolation and initial characterization of the first osteocytic cell line, MLO-Y4 by Lynda Bonewald's group.[3] Since then, other osteocytic cell lines have been generated by Dr. Bonewald and other groups, including MLO-A5 and OCY454,[11-13] but MLO-Y4 cells continue to be the most utilized osteocytic cell line. However, the use of this cell line is limited by the fact that they do not express SOST/sclerostin, a gene expressed only by osteocytes in bone and that is fundamental for the role of osteocytes as regulators of bone homeostasis.

Osteocytes are derived from osteoblasts once the bone forming cells have finished their synthetic activity and become surrounded by mineralized matrix.[1] Osteocytes and osteoblasts share expression of several genes, albeit at different relative levels. The differential expression of genes in the 2 cell types has been studied by the Kalajzik's group, who performed gene array analysis to determine differentially expressed genes.[14] This later study, as well as others have shown that, in addition to SOST/sclerostin, other genes are expressed in higher levels in osteocytes compared to osteoblasts.[1] Of those, we chose to measure the levels of E11, expressed in early embedding osteocytes and responsible for dendrite formation; DMP-1, expressed in early and mature osteocytes and involved in phosphate metabolism and mineralization; and FGF-23, also expressed in early and mature osteocytes and responsible for phosphate metabolism. All these genes are also expressed in late osteoblasts, although at lower levels than in osteocytes. We also measured the levels of keratocan, a gene that is expressed at higher levels in osteoblasts compared to osteocytes and that has been used as a

marker of osteoblastic cells.[14] SOST/sclerostin, on the other hand, is expressed in late osteocytes, regulates osteoblast differentiation and bone formation, and has not been shown to be expressed in osteoblasts.[1]

In addition to the regulation of bone formation and mineralization, osteocytes control the formation and survival of osteoclasts through the production of RANKL and OPG, cytokines that promote and inhibit osteoclastogenesis, respectively.[8] Similar to the levels of osteocytic genes, the expression of RANKL and OPG varied in the different clones. RANKL is a membrane bound protein that can also act as a soluble cytokine, activating RANK in osteoclast precursors leading to osteoclast differentiation.[15] RANKL can also activate RANK in mature osteoclasts and promote osteoclast survival. OPG binds to RANKL and acts as a decoy receptor, blocking RANKL interaction with RANK and therefore, osteoclast differentiation. It is important, therefore, to estimate the ratio between RANKL and OPG, which will dictate a pro- or anti-osteoclastogenic condition. We found that some clones, such as clone 6, express relatively low levels of RANKL and high levels of OPG, which should lead to low RANKL/OPG ratio and reduced osteoclastogenic potential compared to the MLO-nGFP pool. On the other hand, clone 34 exhibits higher RANKL levels compared to OPG, which should favor osteoclastogenesis. Future co-culture studies using the MLO-nGFP clones and osteoclast precursors will determine whether these changes in mRNA levels translate into different ability to induce osteoclast differentiation *in vitro*.

Overall, cells expressing higher levels of osteocytic genes also express higher levels of RANKL and OPG. This is consistent with evidence that osteocytes are one of the main sources of cytokines associated with osteoclast differentiation.[16] Furthermore, the higher RANKL/OPG ratio could be detected in clones exhibiting higher number of projections, such as clones 2 and 34, again reinforcing the notion that osteocytes regulate osteoclastogenesis.

In summary, we found that MLO-Y4 clones exhibit different sizes and morphology, with some cells showing a spindle-like shape and others with abundant projections and a star-like shape. Clones of MLO-nGFP cells do not express SOST, and express different levels of other osteocytic genes. MLO-nGFP clones constitute a useful tool to study osteocyte differentiation and the role of osteocytes in the control of bone formation and resorption *in vitro.*

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