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Expression of the invertebrate sea urchin P16 protein into mammalian MC3T3 osteoblasts transforms and reprograms them into “osteocyte-like” cells

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Abstract

P16 is an acidic phosphoprotein important in both sea urchin embryonic spicule development and transient mineralization during embryogenesis, and syncytium formation and mineralization in mature urchin tooth. Anti-P16 has been used to localize P16 to the syncytial membranes and the calcite mineral. Specific amino acid sequence motifs in P16 are similar to sequences in DSPP a protein common to all vertebrate teeth, and crucial for their mineralization. Here we examine the effect of P16 on vertebrate fibroblastic NIH3T3 cells and osteoblastic MC3T3 cells. Transfection of NIH3T3 cells with P16 cDNA resulted in profound changes in the morphology of the cells. In culture the transfected cells sent out long processes that contacted processes from neighboring cells forming networks or syncytia. There was a similar change in morphology in cultured osteoblastic MC3T3 cells. In addition, the MC3T3 developed numerous dendrites as found in osteocytes. Importantly, there was also a change in the expression of the osteoblast and osteocyte specific genes. MC3T3 cells transfected with P16 showed an 18 fold increase in expression of the osteocyte specific Dentin matrix protein (DMP1) gene, accompanied by decreased expression of osteoblast specific genes: Bone sialoprotein (BSP), osteocalcin (OCN) and β -catenin decreased by 70%, 64% and 68 %, respectively. Thus, invertebrate urchin P16 with no previously known analog in vertebrates was able to induce changes in both cell morphology and gene expression, converting vertebrate-derived osteoblast-like precursor cells to an “osteocyte-like” phenotype, an important process in bone biology. The mechanisms involved are presently under study.

INTRODUCTION

In the formation of echinoderm calcitic mineralized structures; the spicules, Aristotle's' Lantern, and especially in the teeth, the mineralization takes place in relation to a highly developed cellular syncytium. As shown in the extensive work on spicule formation and mineralization by Etensohn and colleagues (Illies et al, 2002, Cheers and Etensohn, 2005),

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a protein, P16, plays some prominent roles in spiculogenesis. In our own studies of urchin tooth mineralization, we have shown that P16 also is prominent in the earliest stages of mature tooth development. We have isolated protein P16 from the mineral occluded phase of the *Lytechinus variegatus* (*L.v.*) (phylum Echinodermata, class Echinoidea), tooth (Alvares et al, 2009). The *L.v.* is a camarodont, a member of the class of keeled tooth urchin, which, among other features have a unique mineralized Aristotle's lantern structure housing the five teeth of the urchin. The teeth grow continuously in a vectorial fashion changing from an initial highly cellular aboral structure called the plumula that elongates in the adoral direction into a highly organized and complex mineralized mature T-shaped and mechanically rigid functional tooth. Each tooth displays all stages of development along its length. The plumula forms within the coelomic fluid containing a mixed monocytic population of coelomocytes. Individual monocytes condense at the outer surface of the plumula (Kniprath, '74), just under the epithelial layer, where they fuse and form sheet-like syncytial layers (Alvares et al, 2007) in which they become multinucleated cells. Calcitic mineralization of the sea urchin tooth begins on the syncytial membranes. The mineral then grows into the syncytial spaces (Alvares et al 2009, Alvares 2014).

We cloned and sequenced *Lv* P16 and found it to be a very complex multi-domain protein (Alvares et al, 2009), similar, but not identical in sequence to the P16 of *Strongylocentrotus purpuratus* (*Sp*) (Illies et al, 2002). *Lv* P16 has a signal peptide, followed by a highly acidic phosphorylated extra-cellular domain flanked by more hydrophobic sequences, a membrane spanning region and a short intra-syncytial cytosolic domain. The role of P16 in skeletogenesis was clearly shown by Cheers and Etensohn (2005) who microinjected morpholino antisense oligonucleotides (MOs) for P16 into fertilized eggs of *S. purpuratus* and *L. variegatus*, blocking the synthesis of the P16 mRNA. Under these conditions there was an inhibition in skeletal rod mineralization and elongation. Using a GFP-tagged form of P16, they also showed that the protein was enriched in the plasma membrane and in the perinuclear region that may be the Golgi apparatus, suggesting that it might function to receive signals required for skeletogenesis or play a more direct role in the deposition of mineral. The presence of a membrane spanning region and the localization of the GFP-tagged protein at the plasma membrane, suggested that P16 is a membrane anchored protein. Using antibodies raised against the recombinant protein we have previously shown that immuno-staining of the plumula, at the region where the syncytial membranes are beginning to form P16 is present on the syncytial membranes, associated with calcite mineral deposition (Alvares, 2014). In addition, P16 is also seen along the mineralized primary plates, and the developing secondary plates where mineralization is occurring (Alvares et al, 2009).

One of the questions driving our studies (Veis, 2003) on the mechanisms of biomineralization in general was the nature of the mineral-related proteins, since in both vertebrates and invertebrates the proteins were apparently very similar in properties although giving rise to calcium phosphates in the vertebrates, calcium carbonates in the invertebrates (Veis DJ et al. 1986, George and Veis, 2008, Alvares et al, 2009), and silicates in silicified organisms such as Demosponges (Perry 2003). Early on, just after developing an antibody specific to the vertebrate tooth dentin phosphophoryn (DPP), and having also extracted the

total mineral related proteins of the *Lv* tooth, we showed (Veis DJ et al. 1986) that anti-DPP could label both mineralized urchin tooth sections, and the mineral-related *Lv* tooth proteins. Now that we have an antibody specific to *Lv* P16 which labels the syncytial membranes and newly formed calcite plates we are finally in position to explore more fully the relationships between the proteins inducing calcium carbonate (calcite) mineralization and those involved in calcium phosphate (apatite) induction. Put most simply and directly: Could a calcite biomineral-inducing protein isolated from an invertebrate induce mineralization in a vertebrate cell system? As discussed here we chose the urchin *Lv* P16, which has no known related vertebrate counterpart, to transfect into vertebrate fibroblasts and osteoblasts, and see if the P16 could induce either the morphogenic and/or biochemical conversion of the cells into a syncytium, a cell communication network or a mineralization system.

A few comments on bone mineralization are necessary at this point to explain the experimental approach. In vertebrate bone formation each bone surface-lining-osteoblast (OB) secretes a mineralizing matrix which ultimately surrounds the OB. The matrix enclosed cell then undergoes both biochemical and morphological transformations and becomes an osteocyte (OC). The gene expression pattern changes and the OC develop numerous thin dendritic filopodia-like processes that penetrate the mineralized matrix and join the filopodia on adjacent OC forming a network of cell-cell communication (Franz-Odenaal et al, 2006, Dallas and Bonewald, 2010). The factors involved in the OB to OC transformation and the mechanism of process development are yet to be determined (Franz-Odenaal et al, 2006, Dallas and Bonewald, 2010). The OC network is the system which carries out the metabolic work of the bone, keeping the mineralized bone matrix alive and responsive to the bone environment. It is this complex interconnected network of cells that is responsible for sensing mechanical loading or lack of loading (Dallas et al, 2013). Thus, the criteria for evaluating the possible utilization of the urchin protein for activity in a vertebrate cell system were the alteration of the gene expression from that of the OB to that of the OC, the development of the morphology of the OC type of cell-cell communication system, and the possible accumulation of mineral in the cell matrix. As shown below, even at this preliminary level of the investigation, all three of these criteria are met upon the transfection of vertebrate osteoblastic MC3T3 cells with the *Lv* P16 in culture.

METHODS

Transfection of NIH3T3 cells

The P16 cDNA was introduced into the BamH1/Apa1 site of the mammalian expression vector pcDNATM3.1/V5-His. The P16-pcDNATM3.1/V5-His construct was transfected into NIH3T3 cell lines (ATCC[®] CRL-1658TM) using a Lipofectamine[®] 3000 transfection kit (Life Technologies) according to the manufacturer's instructions. Following transfection, cells were grown in 800 µg/ml G418 for selection of neomycin resistance clones. Sterile glass rings were used to select surviving colonies. The isolated colonies were trypsinized and transferred to a new flask where the cells were allowed to expand. Over 6 weeks, we isolated 8 colonies of NIH3T3, stably transfected with the *P16* gene. Control NIH3T3 cells and the stable transfected cell lines were then grown on rat tail type 1 collagen coated coverslips for 48 hours. At this time the cells were fixed in ice cold methanol for 30 min and

then subjected to immunofluorescence analysis using the P16 antibody characterized previously (Alvares et al, 2009).

Transient transfection of MC3T3 osteoblast cells

MC3T3 cells (ATCC® CRL-2593™) were cultured in a 12-well plate with α -modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen). The P16-pcDNA™3.1/V5-His construct was transfected into MC3T3 cell lines using a Lipofectamine® 3000 Transfection kit (Life Technologies) according to the manufacturer's instructions. Medium was changed 48 hours after transfection to selection medium containing 800 μ g/ml G418 and cells grown for a further 72 hours. At this time the cells were fixed in ice cold methanol for 30 min and then subjected to immunofluorescence analysis using the P16 antibody characterized previously (Alvares et al, 2009).

Immunostaining and fluorescence microscopy

For immunostaining, NIH3T3 cells or cells transformed with P16 were grown on rat tail type 1 collagen (BD Biosciences) coated coverslips for 48 hours. Adherent cells were rinsed with PBS, fixed, and permeabilized with methanol at -20°C for 30 min. The cells were then blocked by treatment for 4 hours with PBS containing 3% bovine serum albumin and 1% normal donkey serum. Normal donkey serum was used, as the secondary antibody was raised in donkeys. After 4 hr. the anti-P16 antibody at a concentration of 1:200 in blocking buffer was added to the experimental slides and reacted at 4°C overnight. In control slides, the incubation was carried on in blocking buffer without the primary antibody. The next day sections were washed four times (15 min per wash) with PBS and reacted for one hour at room temperature with Alexa Fluor 568 donkey anti rabbit IgG (Invitrogen, Carlsbad, CA) in blocking buffer. Following the secondary antibody incubation, sections were once again washed 4 times (15 min per wash) with PBS. Secondary antibody incubation and subsequent washings were performed in the dark to minimize loss of fluorescent signal due to photo bleaching. The sections were incubated with DAPI at a concentration of 5 μ g/ml for 30 min. and washed with PBS three times. After the final washing slides were mounted with ProLong Gold Antifade (Invitrogen, Carlsbad, CA) and immunoreactive areas were visualized and photographed using an Olympus X-Cite series 120Q microscope.

Alizarin Red Staining of NIH3T3-P16 Cells

To determine whether there was an increase in the amount of calcium accumulated in the cultures, control NIH3T3 cells or cells transfected with P16 were seeded at the same density (1×10^5 cells) in 6 well plates. Control and cells transfected with P16 were then cultured as described above for 14 days. Cell layers were fixed in ice-cold 70% ethanol for 1 h and rinsed with deionized H_2O . Cells were stained with 40mM alizarin red-S, pH4.2, for 10 min with gentle agitation. Cell layers were rinsed five times with deionized H_2O and then rinsed for 15 min with PBS and gentle agitation and then photographed

Quantitative Real-time PCR

Total RNA was extracted from the normal and transfected MC3T3 cells using RNeasy® Mini Kit (Qiagen) according to the manufacturer's protocol and then reverse-transcribed

into cDNA, followed by real-time PCR analysis. The genes analyzed included dentin matrix protein 1 (*Dmp1*), Bone sialoprotein (*Bsp*), osteocalcin (*Ocn*) and β -catenin, with GAPDH as an internal control. The details of the primer sequences were as follows:

<i>Dmp1</i> primer,	5'-AGT GAG TCA TCA GAA GAA AGT CAA GC-3' (forward) 5'-CTA TAC TGG CCT CTG TCG TAG CC-3' (reverse);
<i>Bsp</i> primer,	5'-GAG ACG GCG ATA GTT CC-3' (forward) 5'-AGT GCC GCT AAC TCA A-3' (reverse);
<i>Ocn</i> primer,	5'-CTC TGT CTC TCT GAC CTC ACA G-3' (forward) 5'-GGA GCT GCT GTG ACA TCC ATA C-3' (reverse);
β -catenin primer,	5'-TGC CAC CAC CAC AGC TCC TT-3' (forward) 5'-GGA ACA TGG CAG CTC GGA CCC-3' (reverse);
GAPDH primer,	5'-GGT GTG AAC CAC GAG AAA-3' (forward) 5'-TGA AGT CGC AGG AGA CAA-3' (reverse).

Statistical Analysis

Real-time PCR were repeated 4 times for each gene. Data analysis was performed using student T-tests between the two groups (P16 transfected MC3T3 cells and non-transfected cells). The quantified results are presented as means $\pm$ S.E. (standard error of the mean). A $p < 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Expression of P16 in NIH3T3 fibroblasts

We were interested to see the effect on mineralization when we introduced a novel invertebrate “biomineralization” protein into a totally unrelated mammalian cell. To that end, a construct was made to drive the expression of P16 under control of the CMV promoter when introduced into NIH3T3 mouse embryonic fibroblast cell line. The transfected NIH3T3 fibroblasts were then treated with G418 to generate permanent cell lines. Over the course of 6 weeks, 8 clones were expanded, three of which are shown here. The cells were grown on collagen coated slides and analyzed by immunofluorescence to check for the expression of P16. Figure 2 shows the expression of P16 in three of the permanent clones. In control NIH3T3 cells there was absolutely no reaction with the P16 antibody (Fig 2a). To our surprise expression of P16 dramatically changed the morphology of the cells. The transfected cells expressing P16 sent out long processes (white arrows Fig. 2b and d) that connected with processes emanating from neighboring cells forming a cell network. In some instances the cells also appeared to be forming true syncytia, in which many cells are connected by the processes (green arrow Fig 2c). Similar changes in cell morphology were not observed when the alpha 1 C-propeptide of collagen type 1 chain, (an unrelated protein) was transfected into NIH3T3 cells (data not shown). P16 expressing NIH3T3 cells changed in morphology to resemble the primary mesenchymal cells (PMC) of the sea urchin embryo which express copious amounts of P16 (Cheers and Ettensohn, 2005). When cultured on glass slides the urchin PMC's have many long and branched filopodia. In the embryo these filopodia undergo fusion during gastrulation, forming a cable-like structure within which the spicules are subsequently secreted (Hodor and Ettensohn, 1998). It has recently been demonstrated that specialized filopodia (cytonemes) play an important role in cell-cell communication (Roy et al, 2014). Moreover, staining with Alizarin Red (Fig. 3) of

cells grown for 14 days, showed much more intense staining for calcium in the transfected cells expressing P16 as compared to the untransfected NIH3T3 cells. The slight red staining in the control could be background nonspecific staining. Unfortunately at the current time we have been unable to determine the nature of the mineral formed. It could be amorphous calcium, calcite or apatite. The change in the NIH3T3 cell morphology by the introduction of only one protein is remarkable. This change in morphology is reminiscent of the change, noted earlier, that occurs when an osteoblast differentiates into an osteocyte. This leads us to speculate that the change from an osteoblast cell to an osteocyte (with many dendritic projections) could be the result of the expression of a single protein.

In the *in-vivo* situation osteoblasts (OB) secrete a mineralizing matrix which completely surrounds and isolates each OB. The trapped OB, now osteocytes (OC), then undergo a transformation, in situ, by which they sprout narrow processes that permeate the matrix and connect to each other, forming a functional (syncytial?) network (canaliculi) through which the OC can communicate. Thus although mainly isolated from the circulation, the mineralized bone matrix is accessible to the bone surface and body fluids, responsive to metabolic changes and stimuli. This osteocyte network carries out the majority of the normal remodeling of mineralized bone. Although the osteocyte network has been recognized and studied for many years, the mechanism of conversion of OB to OC-network remains to be understood. Recently, however, a number of studies using proteomics and gene array technologies have shown that there is a difference in gene expression between the osteoblasts and osteocytes (van Bezooijen et al, 2005, Paic et al. 2009, Woo et al, 2011, Bonewald and Wacker, 2013 and Dallas et al, 2013). Obviously, the matrix entrapment of the OB leads to a reprogramming of the OC regulatory network. Could the protein responsible for the osteoblast to osteocyte transition be similar to P16? A protein that has a membrane spanning region and a highly acidic domain exposed to the extracellular matrix.

Effect of P16 expression in MC3T3 osteoblasts

We turned next to the MC3T3 osteoblastic precursor cell line. MC3T3 is an osteoblast precursor derived from mouse calvaria, and a good model for studying osteoblast differentiation and behave similar to primary calvarial osteoblasts.

When urchin P16 was transfected into MC3T3 cells using the same conditions as described above for the NIH3T3 cells, we observed a similar transformation of the osteoblastic cells. Transfection was performed when the cells reached 70% confluence levels. 48 hours after transfection G418 selective medium was added and cells were cultured for another 72 hours. The osteoblast cells went from spindle-like cells (Fig 4a) to flattened cells having long processes that joined neighboring cells (red arrows in 4 B) forming a network of cells. In some areas (Fig 4C, yellow arrows) the cells show dendritic like processes emerging from the cells. Once again the cells were connected to one another by these long processes that are clearly evident in figure 2b. The control MC3T3 cells and cells transfected with P16 were subjected to immunofluorescence analysis to determine the expression of P16. As seen in Figure 4D the control cells had absolutely no P16 present. However as seen in Figures 4E and F, the long processes that connect to neighboring cells are filled with copious amounts of P16. The cells appeared to be definitely multinucleated, as one would expect of cells

forming a syncytium. Although osteocytes do not form a true syncytium, each cell is connected to its neighbor by gap junctions to form a functional syncytium. It remains to be determined whether transformation with P16 directs transformation to a true syncytium or whether the cells interact with each other through gap junctions. Whatever the case, transformation of MC3T3 cells with P16 changed the morphology from that of an osteoblast to that resembling an osteocyte cell type. P16 is a transmembrane protein. However when expressed in NIH3T3 and MC3T3 cells we do see appreciable amounts in the cytosol. In the sea urchin, P16 is expressed in the primary mesenchymal cells (PMC). Confocal microscopy shows it to be predominantly localized to the cell membrane although there is diffuse staining throughout the cytoplasm. Moreover when the PMC's are isolated and cultured on glass slides, the cytoplasmic staining increases and the filopodia become filled with P16 (Cheers and Etensohn 2005) similar to what we see in the NIH3T3 or MC3T3 cells transfected with P16. We believe that appreciable amounts in the cytosol on transfection are due to the system being overwhelmed by the fact that we have overexpressed P16 in these cell lines. In our experiments introduction of P16 into mammalian cells resulted in the cells producing long filopodia that connected neighboring cells. Using morpholino anti sense oligonucleotides to knockdown the levels of P16, Cheers and Etensohn, 2005 have shown that P16 is responsible for spicule rod elongation but not required for PMC specification, ingression, migration, or fusion. However, morpholino oligonucleotides reduce the amounts but do not completely knockout the specific RNA. As the authors themselves pointed out they did not measure levels of P16 and it remains possible that some P16 protein was expressed and complete elimination of all P16 expression might completely suppress biomineral deposition. It is also possible that complete elimination of P16 might also prevent cell fusion and syncytium formation.

The next question we asked was whether there was just a change in the morphology of the cells or whether the cells were being converted to an osteocytic lineage? To do this we looked at osteoblast and osteocyte specific genes by quantitative PCR. Total RNA was extracted from either MC3T3 cells (control) or MC3T3 cells transfected with P16 as described above and then reverse transcribed into cDNA followed by quantitative real-time PCR analysis. The genes analyzed were the osteocyte specific Dentin Matrix Protein 1 (DMP1), and osteoblast specific Bone Sialoprotein (BSP), osteocalcin (OCN) and β -catenin (B-Cat). GAPDH was run as an internal control. The results shown in Figure 5 are an average of 4 separate experiments. Blue is control untransfected MC3T3 cells while red is cells transfected with P16 cDNA. There was an 18 fold increase in expression of the osteocyte specific DMP1 gene. This was accompanied by a decrease in expression of the osteoblast specific genes. The osteoblast specific genes BSP, OCN and β -catenin decreased by 70%, 64% and 68 %, respectively. Clearly transfection of osteoblasts with P16 does more than just change the morphology of the cells. There is a tremendous increase in the expression of the osteocyte specific DMP1 gene, accompanied by a decrease in expression of the osteoblast specific BMP, OCN and β -catenin genes, indicating that P16 transforms the cells from osteoblasts to an "osteocyte-like" cell. It should be noted that the change in morphology and gene expression occurred at a time point much earlier than it would take for the culture to become mineralized which normally occurs around 14 days. Moreover the transfected and untransfected cells were grown for the same amount of time before being

analyzed for gene expression changes. Thus the change in gene expression could be only due to the introduction of P16 into the cells. Although P16 is an invertebrate protein, without an apparent mammalian counterpart, it brings about a specific change in the morphology and gene expression in the mammalian fibroblastic and osteoblastic cells. The osteoblast, after transfection with P16, now resembles an osteocyte.

Based on our observation that transfection of osteoblasts with P16 results in a shift from an osteoblastic to an osteocytic lineage, we propose that a protein similar to P16 is what triggers the osteoblast to osteocyte transition. One of the likely candidates would be the E11 (podoplanin) protein. Although the amino acid sequences between P16 and E11 are entirely different, structurally they have similar domains. Like P16 E11 has a signal peptide, an acidic extracellular domain followed by a transmembrane region and a small cytosolic domain. MLO-Y4 cells subjected to fluid flow shear stress show an increase in mRNA for E11, an increase in dendricity and elongation of dendrites that is blocked by small interfering RNA specific to E11 (Zang et al, 2006). Moreover overexpression of E11 drives morphological changes in osteoblast ROS 17/2.8 cells, leading to the generation of extended cytoplasmic processes reminiscent of the long cytoplasmic processes observed in osteocytes (Sprague et al, 1996). In addition E11 was detected at plasma membranes of osteocytes and their processes, but not at those of osteoblasts (Schulze et al 1999). E11 is present in various tissues where it is known by different names. In all cases, it is associated with the plasma membrane projections such as filopodia, lamellipodia and ruffles (Scholl et al. 1999), Scholl et al. 2000). Although E11 seems a likely candidate, it has yet to be determined whether transfection with P16 leads to expression or overexpression of E11 results in changes in gene expression. It would be interesting to see whether transfection of osteoblasts with E11 would result in changes in gene expression. If this were not to occur then another protein similar to P16 should be involved. Identification of this putative protein would be of prime importance in determining the trigger that is responsible in the osteoblast to osteocyte transition. The expression of several other osteoblast and osteocyte proteins, such as sclerostin, also need to be determined but were beyond the scope of the present work. Detailed examination of the created filopodia, and of the type of junctions between them (at the EM level) is another future direction for examination. These questions relate to the mechanism of cell communication and the difference between a true syncytium as seen in the urchin teeth and the cell-cell communication networks suggested in figures 2 and 4.

The data presented here, although preliminary, convincingly show that the transmembrane protein, P16, with cytosolic and extracytosolic domains, and providing highly acidic, phosphorylated domains in the extracytosolic spaces, as depicted in Fig. 1, is sufficient to bring about a morphologic change and generate the filopodia and perhaps mineralization sites, and to epigenetically alter the host cell gene expression. The change in gene expression could be occurring via two mechanisms. It is known that the shape of a cell is affected by interactions with either the extracellular matrix or neighboring cells, which leads to restructuring of the cytoplasmic cytoskeleton. Altering cytoskeletal structure may in turn change the availability of regulatory or catalytic sites of key signal transducing molecules leading to alterations in gene expression. Some of the classical examples illustrating the effect of cell shape changes on gene expression include a switch from type I to type II collagen expression by chondrocytes upon a shift from growth on fibronectin to growth in

suspension (Benya and Shaffe '82), as well as the repression of liver-specific gene expression in dispersed hepatocytes and its resumption upon cell aggregation (Clayton et al '85). Alternatively the acidic extracellular domain of P16 may be interacting with the extracellular matrix. This interaction may then signal through the cytosolic domain to bring about the change in gene expression. Experiments are underway to determine which of these two mechanisms are involved. This should shed light on the mechanism involved in the transformation from osteoblasts to osteocytes.

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MKTIIALFAFVAVAAAVPGTGTGGFQGTD
GTQFNFGNGGGGGEIGVNDAYGGGGAMG
TSDYGTSGSADAMGGTSYGGSDTSSDT
GSDDDSIDDDGSSDDSSSEDNSGGAGR
NGLSNLGSMTAQKSGMAFGIIFAVGAVVA
AAGVG YFVYRK RQNGAQMLSNA

Figure 1. Amino acid composition of P16

Amino acid composition of sea urchin P16 protein. Signal peptide is underlined. The region to which the antibody was raised is depicted in green. Blue is extra-cellular acidic domain. The transmembrane region is shown in red.

Figure 2. Immunofluorescence localization of P16 in control (a) and P16 transfected (b–d) NIH3T3 cells

Immunofluorescence analysis of NIH3T3 cells expressing sea urchin P16. (a) is control NIH3T3 cells. b, c and d are three permanent cell lines expressing P16. The permanent cell lines were established by treatment with G418 over a period of six weeks. Blue is staining for DAPI, while red is staining with Anti P16 followed by staining with anti-rabbit Alexa flour 568. The transfected cells sent out long processes (white arrows Figure 2b and d) that connected with processes with neighboring cells. In some instances the cells also appeared to be forming true syncytia, where many cells are connected by the processes (green arrow Figure 2c).

Figure 3. Alizarin red staining of control (a) and Transfected (b) NIH3T3 cells
NIH3T3 cells (a) and cells expressing P16 (b) were grown for 14 days as described, and then stained with Alizarin red to determine accumulation of calcium.

Figure 4. Transfection of MC3T3 cells with P16. A and D are control while B, C, E and F are transfected cells

Immunofluorescence analysis of MC3T3 cells transiently transfected with sea urchin P16. (A and D) are control MC3T3 cells. B, C, E and F are 4 separate transfections. Panels A, B and C are light microscopic images to show morphology. Red arrow in B shows the long processes that joined neighboring cells forming a network of cells. In some areas (Fig 4C yellow arrows) the cells show dendritic like processes emerging from the cells. Figures D, E and F are immunofluorescence images. In D, E and F blue is staining for DAPI, while red is staining with Anti P16 followed by staining with anti-rabbit Alexa fluor 568. The osteoblast cells now have numerous processes that joined neighboring cells (white arrows in 4E and 4F).

Figure 5. Quantitative PCR

Quantitative real time PCR was carried out as described in the methods. Blue bars are control MC3T3 cells while red bars are MC3T3 cells transfected with P16. A is dentin matrix protein 1 (DMP1), B is Bone sialoprotein (BSP), C is osteocalcin (OCN), while D is β -catenin (B-Cat).