

**NUCLEAR LOCALIZATION OF DMP1 AND DSPP
IN VARIOUS CELL LINES**

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By
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ABSTRACT

Objective: To examine Dentine matrix protein 1(DMP1) and Dentine sialophosphoprotein (DSPP) expression and subcellular localization in various cell lines to better understand their function.

Methods: RT-PCR, immunofluorescent staining and Western-blotting analyses were used to determine the expression and subcellular localization of DMP1 and DSPP in various cell lines, including odontoblast-like (17IIA11), preosteoblast (MC3T3-E1), mesenchymal cells (C3H10T1/2), and human dental pulp stem cells (DPSC). In addition, a haemagglutinin (HA) tagged DMP1 expression construct was generated and examined for its subcellular localization in COS-7 cells.

Results: Western-blot analysis showed the presence of DMP1 and DSPP in the cytoplasmic and nuclear extracts of MC3T3-E1, 17IIA11, and C3H10T1/2 cells. DMP1 and DSPP transcripts were consistently detected in all three cell lines by RT-PCR analysis. However, immunofluorescent detection of DMP1 revealed the presence of two distinct subpopulations of cells with either nuclear or cytoplasmic staining; this phenomenon was not noticed with DSPP immunofluorescence.

Nuclear and cytoplasmic DMP1 was confirmed in MC3T3-E1 cells by immunofluorescent staining using a rabbit polyclonal antibody; and the staining was inhibited when the antibody was preincubated with the synthetic peptide used to generate the antibody, confirming the specificity of the antibody. Nuclear and cytoplasmic localization was also observed in COS-7 cells transfected with HA-tagged DMP1 expression construct when detected with an antibody against the HA tag.

Conclusion: These findings suggest that, apart from their role as a constituent of dentin/bone matrix, both DMP1 and DSPP might play a regulatory or structural role in the nucleus that is not unique to the odontoblast/osteoblast cells.

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CHAPTER 1

INTRODUCTION

Nuclear Targeting Of Secretory Proteins

Secretory proteins, like the name implies, are proteins that are manufactured in the cell and then secreted to fulfill either a signaling role by interacting with other cell surface receptors associated with signal transduction pathways, or by direct nuclear association, or play a structural role in the formation of various body tissues. Recently, some of these secretory proteins like fibroblast growth factor (FGF), epidermal growth factor (EGF) and dentine matrix protein 1 (DMP1) have been found to target the nucleus.

The localization of these proteins in the nuclei of cells suggests an extended function/s that needs to be explored. It has been found, for example, that angiogenin biological activity is conditional on its nuclear targeting (1). Others suggested roles that include transcriptional regulation, mRNA transport or translational control (2). It also proposes unconventional cellular secretory functions that can explain the mechanisms by which these proteins are translocated in the nucleus. The two major suggested pathways are either internalization of the secreted protein or the deviation of the manufactured protein from its pathway out of the cell. The internalization can be mediated through the presence of specific cell receptors on cell surfaces or certain structures in the cell membrane like caveolae that facilitate nuclear uptake(3) . A prominent example of the second mechanism is Fibroblast growth factor 3 (FGF3) that is thought to possess a nuclear localization signal (NLS). This motif

acts as a recognition site for proteins that help in the import of proteins in to the nucleus (4).

Dentine Matrix Protein (DMP1)

Dentine matrix protein was first cloned from dentine matrix (5). A few years later it was identified in osteoblasts using RT-PCR, making it not unique to dentine (6). Other studies had shown its presence in several other tissues like the kidneys and salivary glands (7, 8)

The protein is acidic in nature making it a key participant in the mineralization of bone and dentine. It is also a member of the Small integrin binding ligand N-linked glycoproteins or SIBLINGs family. These are a group of proteins that possess common characteristics like an RGD (ARG-GLY-ASP) amino acid sequence that is recognized by cellular integrins to facilitate cell-matrix attachment, and amino acid sequences that are rich in aspartic acid, glutamic acid and phosphorylated serines. Other members of the family include osteopontin (OPN), bone sialoprotein (BSP) and dentine sialophosphoprotein (DSPP) and matrix extracellular phosphoglycoprotein (MEPE). DMP1 is made of three highly conserved regions. The first region known as the GAG domain due to the presence of a serine at position 74 that links a glycosaminoglycan to the protein. The second domain is named as the cleavage domain as it contains the primary cleavage site. The third domain, that is thought to be responsible for most of the protein's function, is the C-terminal. A deletion of this region in humans is thought to cause Autosomal recessive hypophosphatemic rickets (9). Studies done by Qin et al concluded that the full form of DMP1 is a precursor to the biologically active forms identified in the extra cellular matrix of bone and

dentine (10). DMP1 is thought to be cleaved by BMP1 into an NH₂-terminal and COOH-terminal fragment (11). The N-terminal exists in two forms, the 37kDA core protein and the proteoglycan (PG) form (12). The C-terminal is a 57kDA fragment (10). Although emerging from the same parent molecule these fragments behave differently. Studies have shown that recombinant full length DMP1 and native DMP1 C-terminal fragments isolated from rat bone accelerated the nucleation process of hydroxyapatite whereas the N-terminal inhibited crystal formation and stabilized the amorphous form (13). Proteolytic cleavage of DMP1 is vital for its function. Recent studies have shown that transgenic expression of normal DMP1 driven by 3.6 kb rat type I collagen promoter was able to rescue Dmp1-null mice tooth and skeletal defects(14, 15); however, transgenic expression of the key cleavage site mutant DMP1 failed to do so, i.e. the tooth and skeletal defects persist in Dmp1-null mice expressing the mutant DMP1(16, 17). On the other hand, these mice demonstrated enlarged growth plates and condylar cartilages proposing a role for the full length DMP1 in chondrogenesis.

Biological functions of DMP1 are numerous. Its high acidic nature gives it superior calcium binding capacity and makes it an important protein in the initiation and regulation of mineralization of bone and dentine (14, 18, 19). It is hydrophilic and is rich in aspartic acid, glutamic and serine residues that are mostly phosphorylated imparting acidity to the protein (5, 20). Moreover, its RGD sequence was shown to be functional in cell- and tissue specific interactions allowing possible interactions of DMP1 with specific cells and activations of signal pathways (21).

A study by Narayanan et al, demonstrated that overexpression of DMP1 in transfected pluripotent and mesenchyme derived cells can lead to the

differentiation of these cells into functional odontoblasts that lay down a mineralizing matrix (22).

A more interesting role was suggested when DMP1 was detected in the nuclei of osteoblasts and odontoblasts (23, 24). It is thought to act as a transcription factor for osteoblast specific genes like osteocalcin and genes expressed by odontoblasts like *Dspp* (23, 25). The importance of this protein in the control of osteogenesis and dentinogenesis has been demonstrated by a series of *Dmp-1* knock –out mice experiments. Regarding bone formation, these newborn pups developed rickets, abnormalities in bone growth, short stature due to malformed epiphyses and delayed secondary ossification and osteomalacia (abnormalities in mineralization) that deteriorated with age (9, 18, 26) .

The most remarkable function of DMP1 and probably the most influential on bone development and mineralization is its effect on phosphate ion homeostasis. Loss of DMP1 in humans and mice lead to autosomal recessive hypophosphatemic rickets (ARHR). The reduced serum phosphate levels was shown to be a result of a significant increase in the levels of FGF23 expressed in osteoblasts due to DMP1 mutations (9, 26) . The low levels of Pi directly affects chondrocytes by retarding apoptosis and thereby disturbing the growth plates and delaying blood vessel invasion for secondary ossification leading to malformed epiphyses (26). Feeding *Dmp1* null mice a diet rich in Pi along with intraperitoneal injections of FGF23 neutralizing antibodies reversed the skeletal phenotype further confirming the role of DMP1, Pi and FGF23 on bone development.

DMP1 plays a pivotal role in controlling dentinogenesis by regulating differentiation and maturation of odontoblasts as well as mineralization of dentine. Mice lacking DMP1 developed teeth with defects in dentine formation

including failure of maturation of predentine into dentine, thin dentine walls, large pulp chambers, hypomineralization, dental tubular system abnormalities and a 3-fold reduction in the rate of dentine apposition (14, 27). These mice also showed a significant decrease in the levels of DSPP corroborating the results from Narayanan et al showed that DMP1 upregulates DSPP expression (25).

Its role in regeneration and stem cell stimulation is now being explored. Recent studies have shown an upregulation in the level of genes like MMP-2, alkaline phosphatase and transforming growth factor β -1 as well as increased matrix and mineralized nodule formation in DMP1 treated human periodontal ligament stem cells. This can expand the role of DMP1 to involve dental tissue engineering and regeneration (28).

DMP1 has also been associated with inflammation in dental tissues. It was detected in higher levels in the gingival crevices of orthodontically induced root resorption(29) in addition to human inflamed dental pulps. It caused an increase in the expression of IL-6 and IL-8 levels when added to pulp fibroblasts. This inflammatory effect was potentiated in the presence of LPS (30). The clinical extent of the proinflammatory influence of DMP1 remains unclear and whether this can interfere with its regenerative capacity, as previously mentioned, is still to be determined.

Apart from their functions in mineralized tissue the ubiquity of DMP1 and its SIBLINGs family members in other tissues has been proven in a series of studies. For example, it was identified in human kidneys along with its partner matrix metalloproteinases (MMP) throughout the nephrons (7). It was also identified in metabolically active ducts of salivary and sweat glands (31). Terasawa et al. reported the expression of DMP1 in the soft tissue of rats including, brain, liver, muscle, pancreas and kidney (32). The exact function of

the SIBLINGs proteins in the nephral and salivary gland tissues are yet to be determined. The hypothesis remains that they are involved (along with their partner MMP) in the normal turnover of cell surface proteins and/ or pericellular matrix proteins such as those in basement membranes (31).

The role of the SIBLINGs proteins in cancer is under investigation. BSP is the most prominent member that has been related to invasion, metastasis and angiogenesis of cancer cells. On the other hand, many studies have unveiled the involvement of DMP1 in similar processes. In vitro studies had showed the increase in the invasive ability of a colon cancer cell line in the presence of DMP1 (33). The ability of DMP1 to bind and modulate the function of MMP (specifically MMP-9) grants it a matrix degradation potential that allows for invasiveness and spread (34). Immunohistochemical and cDNA profiling studies demonstrated high levels of DMP1 in human lung and breast cancer (35-37).

On the flip side more recent studies had suggested a helpful role played by DMP1 against cancer progression and invasion. One study suggests that DMP1 can interfere with the migration of human breast cancer cells in-vitro and its expression is inversely associated with breast cancer progression (37). Another study by Pirotte et al. demonstrated the ability of DMP1 to specifically block VEGF induced, tumor associated, angiogenesis a process that is necessary for cancer growth and progression (38).

Dentine Sialophosphoprotein (DSPP)

DSPP is another protein member of the SIBLING family, discovered by cDNA cloning of mouse dentine as a single protein that is later cleaved to produce two smaller proteins (39), namely dentine phosphoprotein (DPP) and dentine sialoprotein (DSP). DSPP was formerly thought to be exclusive to dentine (40, 41), but other studies had identified it in other mineralized and non-mineralized tissues (42-45)

These two cleavage products of DSPP had been individually identified and characterized earlier on. DPP was initially reported in 1976 by Veis and Perry (46) and DSP was isolated by Butler et al in 1981 (47). They were originally considered as expressed products of different genes but the 1997 discovery by MacDougall put an end to the misconception identifying DPP and DSP as products from a single gene product, with DSP corresponding to the 5' end and DPP corresponding to the 3' end of the gene sequence (39). A study by Yamakoshi et al. characterized porcine DSPP and suggested a third cleavage product known as DGP (dentine glycoprotein) that is not present in other species(48). Although expressed by the same gene, these proteins have different, unique physical and chemical characteristics. In addition DPP shows abundance over DSP in an approximate ratio of 10:1(49) .

The protein structure of DSPP consists of a signal peptide present in the NH-2 terminus, (to help guide the protein into the endoplasmic reticulum), followed by DSP amino acid sequence the DPP sequence which contains an RGD binding domain (49). DSPP was never found in its full length. This indicates that it is rapidly broken down into its biologically active counterparts DPP and

DSP. Three isoforms of bone morphogenic protein-1 (BMP-1) has been recently reported to play a role in the proteolytic cleavage of DSPP after successful generation of its recombinant full length (50).

DPP is the most negatively charged protein with large amounts of aspartic acid and serine that is mostly phosphorylated (51). It can present in three forms depending on the degree of phosphorylation, highly phosphorylated (HP), moderately phosphorylated (MP), and low phosphorylated (LP) (52). Their physical characteristics make them ideal for the initiation and growth of apatite crystals in tissues. DPP is the most abundant NCP in dentine. It is strictly present in mineralized dentine and has been proven to be transported through the dentinal tubules to the mineralization front (53, 54). The high negative charge and the ability to bind to collagen contributes to its role in nucleation, growth and modulation of the apatite crystals. DSP is not as negatively charged as DPP, it is a glycoprotein that is scarcely phosphorylated but rich in sialic acid. The proteoglycan form of DSP (DSP-PG) is another variant of DSP that is detected in the extracellular matrix of dentine (55-57) which functions are not yet clear. DSP was initially thought to be localized in odontoblasts, predentine and dentine with a predilection around the odontoblastic process and in preameloblasts (40, 41, 58-60). However further studies identified its presence in alveolar bone, cellular cementum and periodontal ligament too (43). DSP was thought to play little or no role in mineralization, but recent studies suggest that DSP is needed for the initiation of mineralization (61). More interestingly, it was shown that overexpression of DSP in transgenic mice lead to a 20% increase in enamel hardness. The opposite effect was seen in mice over expressing DPP in their enamel organ (62).

The tooth specificity of DSPP has been challenged after studies established its presence in bone (42) sweat glands, salivary glands, cartilage, liver, kidney and brain (58) in levels higher than those of bone. DSPP was also detected in some types of cancer including, lung cancer, prostate cancer and oral squamous cell carcinoma (63-65). It is found to be more specifically associated with prostate cancer progression (65).

The importance of DSPP in dentinogenesis has been demonstrated in gene mutation studies in humans and gene knockout experiments in mice (66-68). The mutation of the *Dspp* gene in humans is associated with dentine development diseases including dentinogenesis imperfecta (DI) types II and III and dentine dysplasia types II and III (69-72). The *Dspp* null mice demonstrated phenotypic changes similar to the manifestations of DI in humans, showing hypomineralized dentine and a widened predentine layer (66).

Furthermore, DSPP is believed to play a role in signal transduction and growth factor function. More specifically DPP was shown to activate the Smad pathway leading to an upregulation of BMP-2 gene expression in adult mesenchymal stem cells (73). On the other hand DSP was shown to stimulate the differentiation and mineralization of periodontal ligament stem cells and dental papilla mesenchymal cells (74). More intriguingly, the overexpression of DSPP in adipose-derived stromal cells lead to their differentiation into odontoblast-like cells (75).

The presence of DSPP in several non-mineralized tissues had been established. It was also evident that the structure of DSPP in mineralized tissue is different from its structure in non-mineralized tissue which suggests a different role of DSPP in such tissues (76). The exact function that DSPP serves is still under investigation.

Recent cancer studies are showing DSPP to be a valuable marker in detecting the transformation of oral premalignant lesions into oral squamous cell carcinoma and predicting recurrence of oral squamous carcinoma at histologically negative margins, providing an invaluable adjunct in deciding for additional treatment means (77, 78).

Purpose Of The Study:

Some studies had commented on the subcellular localization of DMP1 but their conclusions were contradictory. The Narayanan et al. (23) study was the first to localize DMP1 in the nuclei of osteoblast like cells and identify its function as a transcription factor. In contrast a study by Farrow et al. failed to see DMP1 in the nuclei of their transfected cells (79).

DSPP has never been investigated for its subcellular localization although it shares with DMP1 certain physical, chemical and functional characteristics. Moreover it has been identified to play a role in signal transduction and cellular differentiation. The purpose of our study was to confirm the subcellular localization of DMP1 and investigate that of DSPP to help us better understand their function.

CHAPTER 2

MATERIALS AND METHODS

Cells And Constructs:

In this experiment five different types of cell lines were cultured representing different cell origins. MC3T3E1 cells (mouse pre-osteoblast), DPSC (human dental pulp stem cells), C3H10T1/2 cells (mouse embryonic undifferentiated mesenchymal cells), Cos-7 cells (monkey fibroblast like cells) and 17IIA11 (mouse pre-odontoblast cells). C3H10T1/2 mesenchymal cells, MC3T3-E1 preosteoblast cells and Cos-7 cells were obtained from American Type Culture Collection (ATCC), and 17IIA11 odontoblast-like cells were derived from E18.5 mouse molar papilla.

MC3T3E-1 and DPSC were cultured in α -MEM supplemented with 10% FBS, 1% penicillin and streptomycin and 1% glutamate. The rest of the cells were cultured in DMEM supplemented with 10%FBS, 1% penicillin/streptomycin and 1% glutamate.

All cells were grown in a humidified incubator with 5% CO₂ at a temperature of 37°C. A construct expressing HA-tagged DMP1 (referred to as "DMP1-HA") was generated by inserting a sequence, taccctacgacgtgcccgactacgcc, encoding HA tag and a HpaI recognition site (gttaac) between codon 259 and codon 260 of mouse DMP1 cDNA sequence in pCDNA3 vector (80), using a site-directed mutagenesis kit (Agilent Technologies)

Transient Transfection:

All transient transfection experiments were performed with Fugene HD transfection reagent according to the manufacturer's instruction (Roche Applied Science). For Western-blot analysis, Cos-7 cells in a 6-well plate were transiently transfected with a total of 2 μg of either an empty vector or a construct expressing DMP1-HA. Twenty-four hours after transfection, the transfection medium was replaced with serum-free DMEM, and the transfected cells were further cultured for 48 hours. The conditioned medium was then collected and analyzed by Western-blot analyses. For immunofluorescent staining, C3H10T1/2 cells in a 24-well plate were transiently transfected with 0.6 μg of DMP1-HA construct; on the next day, the transfected cells were replated into 8-well chamber slides. Twenty-four hours after replating, the transfected cells were processed for immunofluorescent staining.

Immunofluorescence

All cells were cultured in 8 well culture slides at a cell density of 2×10^4 with α -MEM or DMEM in 10% FBS. The cells were allowed to reach confluence (at least 48 hours). The cells were then washed in PBS, fixed in 4% PFA in 1% PBS for 5 min at room temperature then permeabilized with 0.1 % Triton-X100 in PBS for 5 min. 1% goat serum in PBS with 0.05% NaN₃ for was used to block the non-specific binding sites for 2-3 hours at room temperature. For detection of endogenous DMP1, cells were incubated with mouse anti-DMP1 monoclonal antibody (8G10.3; 1:800) or rabbit anti-DMP1 polyclonal antibody (857-3; 1:250),

which recognizes the C-terminal region of DMP1 (81, 82). The specificity of the polyclonal antibody was confirmed by preincubating it overnight at 4°C in the presence or absence of 4 µg/ml of the synthetic peptide used to generate the antibody. The preabsorbed primary antibody was then used to do immunofluorescent staining. For DSPP detection the cells were incubated with mouse anti-DSP monoclonal antibody (2C12.3; 1:1000).

For detection of exogenous DMP1, transfected cells were incubated with mouse anti-HA monoclonal antibody (Covance; 1:2000) or together with rabbit anti-WDR46 polyclonal antibody (Proteintech group; 1:1000) for 2 hours, followed by incubation with Alexa 555-conjugated goat anti-mouse or Alexa 488 goat anti-rabbit IgG(H+L) (Invitrogen Corporation; 1:1000) for 1 hour as secondary antibodies used in appropriation with the primary antibody applied. The nuclei were counterstained with DAPI solution. Normal mouse or rabbit IgG was used as negative control. Fluorescent-stained cells were imaged under a Nikon Eclipse TE2000-U fluorescence microscope.

Preparation Of Nuclear, Cytoplasmic And Total Cell Lysates

C3H10T1/2, Cos-7, MC3T3E-1 and 17IIA11 cells were cultured in a 100mm culture dishes at a density of 1×10^6 . Nuclear and cytoplasmic extracts were prepared using the Active Motif nuclear extraction kit following manufacturer's instructions. Total cell lysates were prepared by adding radioimmunoprecipitation assay (RIPA) buffer to the culture dishes, scraping the cells and continuously agitating for 30 min at 4°C.

The protein concentrations of the lysates were determined using the bicinchoninic acid (BCA) assay and spectrophotometry. Aliquots of the lysates were stored at -80°C.

SDS PAGE And Western Blot Analysis

20 µg of the fractioned components or cell lysates extracted from C3H10T1/2 cells, MC3T3-E1 cells and 17IIA11 cells or the conditioned medium collected from Cos-7 cells transfected with either an empty vector or a DMP1-HA construct were electrophoresed using 10% SDS-polyacrylamide gel, and separated proteins were transferred onto PVDF membranes. Membranes were then immunoblotted with rabbit anti-DMP1 polyclonal antibody (857-3; 1:2000), which recognizes the C-terminal region of DMP1 (82) or mouse anti-HA monoclonal antibody (Covance; 1:2000) or mouse anti DSP monoclonal antibody (2C12.3; 1:1000) for DSPP followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology; 1:1000) or HRP-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology; 1:1000). β -actin was immunoblotted with mouse monoclonal anti- β -actin-peroxidase antibody (Sigma; 1:20,000). The immunostained protein bands were detected with ECLTM Chemiluminescent Detection reagents (Amersham Biosciences) and imaged using a CL-XPosure film (Pierce Biotechnology Inc.). To verify the purity of the nuclear and cytoplasmic fractions the membranes were stripped then incubated with anti GADPH antibodies and detected using the technique mentioned above.

RT-PCR Analysis

Total RNA was isolated from cultured cells, C3H10T1/2, MC3T3E1 and 17IIA11 using TriZol[®] reagent (GIBCO/BRL). 1 µg of RNA was used for reverse transcription using the QuantiTect[®] reverse transcription kit according to the manufacturer's instructions. All PCR was carried out with a GeneAmp PCR System 9700 (Life Technologies Corporation). For *Dmp1* amplification AmpliTaq[®] DNA Polymerase (Life Technologies Corporation) was used, with an initial denaturation at 94°C for 1 minute, followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute. The primer sequences used were DMP1 forward primer 5'-cgagtctcaggaggaca-3', DMP1 reverse primer 5'-ctgtcctcctcactgga -3', *Gapdh* forward primer 5'- tggagccaaaagggtca -3' and *Gapdh* reverse primer 5'- cttctgggtggc. For *DSPP* PCR, Platinum[®] PCR SuperMix (Life Technologies Corporation) was used along with the primer sequences 5'- acgagtccatgcaagga -3' and reverse, 5'-tactgtcactgtcacca-3'. The amplification process of an initial denaturation step at 95°C for 5 minutes, then 45 repetitions of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 30 seconds. Finally a cycle at 72°C for 7 min before the final hold at 4°C. The final products were then run in a 10% agarose gel stained with Ethidium bromide and viewed under UV light.

CHAPTER 3

RESULTS

Expression Of *Dmp1* And *Dspp* Gene In Various Cell Lines

RT-PCR analysis detected *Dmp1* and *Dspp* transcripts in all cell lines (Fig 1). This verifies the ubiquity of the expression of these genes in cells representing different origins and that the presences of these gene products are not unique to calcifying tissue cells.

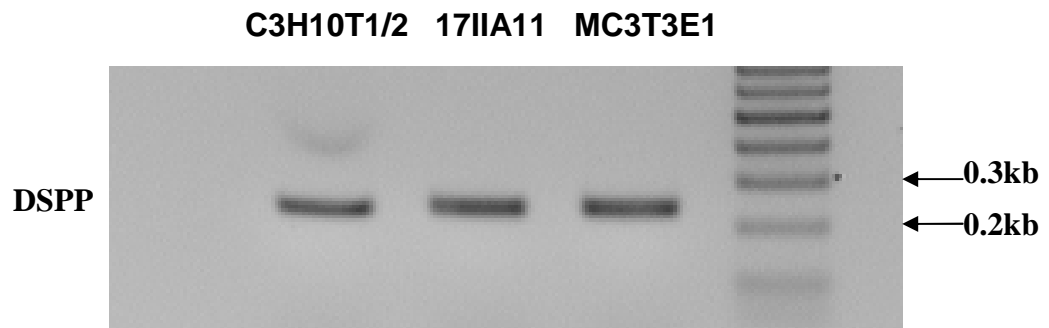


Figure 1- RT-PCR analysis of DMP1 and DSPP transcripts in various cell lines

RT-PCR analyses showing that *DSPP* transcripts were detected in mesenchymal cells (C3H10T1/2), osteoblast like cells (MC3T3-E1) and odontoblast like cells (17IIA11).

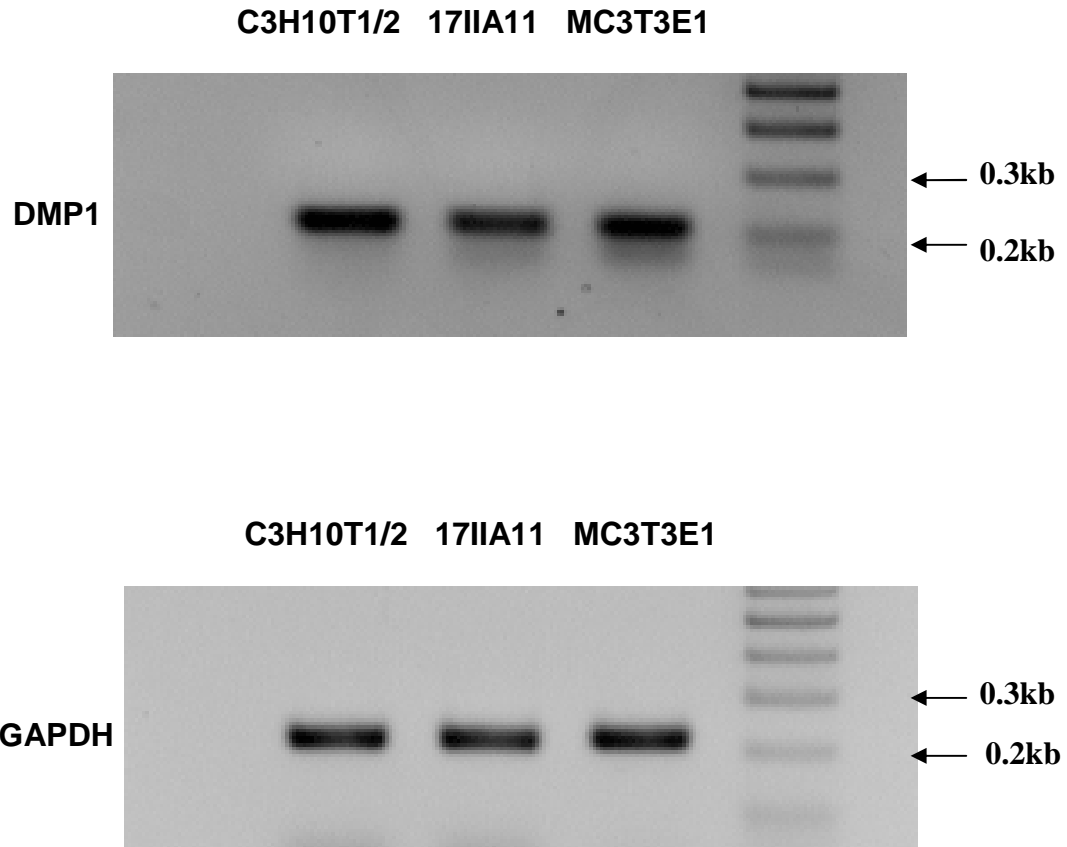
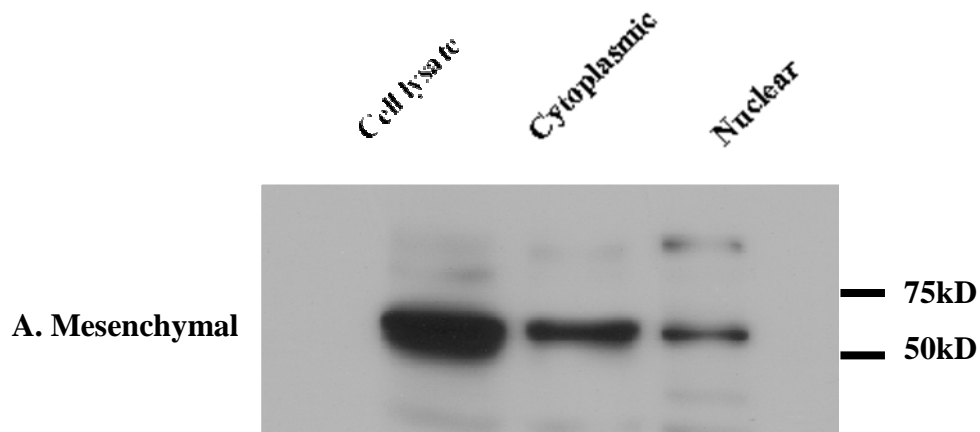


Figure 1- RT-PCR analysis of DMP1 and DSPP transcripts in various cell lines

RT-PCR analyses showing that *Dmp1* transcripts were detected in mesenchymal cells (C3H10T1/2), osteoblast like cells (MC3T3-E1) and odontoblast like cells (17IIA11). GAPDH PCR results confirm proper control.

Subcellular Distribution Of DMP1 And DSPP

DMP1 and DSPP proteins were observed in the total cell lysates of all three cell lines by western blot analyses as well as in the cytoplasmic and nuclear fractions of all cell lines. For DMP1 the protein band is at 57kDa which corresponds to the C-terminal of the cleaved full length of DMP1 (Fig.2A-C). The protein content seems to be more in the total cell lysates, followed by the cytoplasmic fraction then the nuclear portion. DSPP was detected as two bands between the 50kDa and the 75kDa and the content seems to be uniform in all three fractions. (Fig.3A-C)



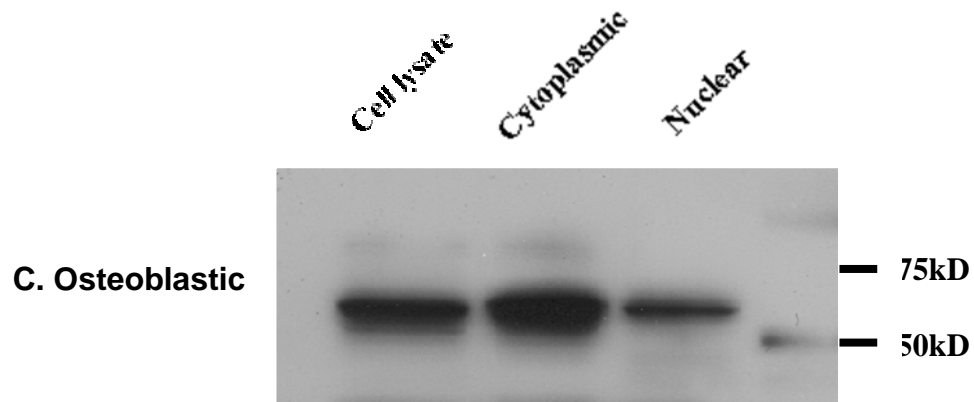
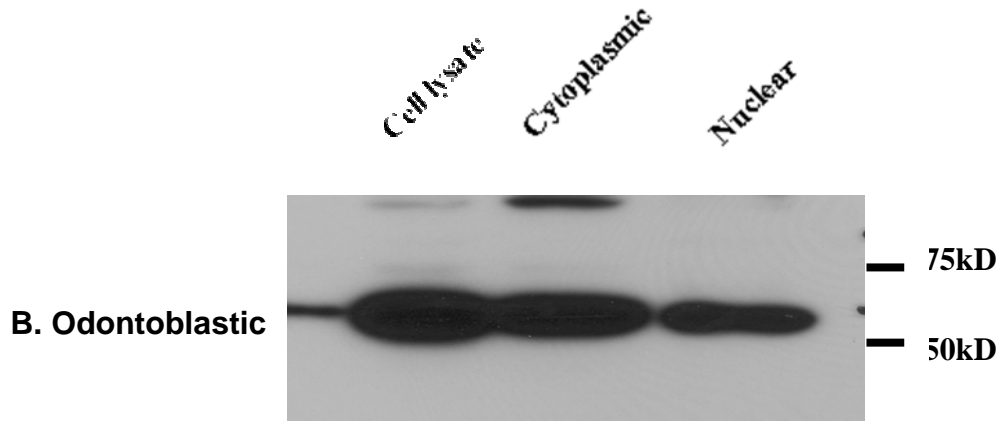


Figure 2- Western blot detection of DMP1 proteins

Western blot analyses with antibodies against the C-terminal part of DMP1 revealed a major protein band of about 57 kDa in the total cell lysates, cytoplasmic and nuclear fraction of all three cell lines (A-C).

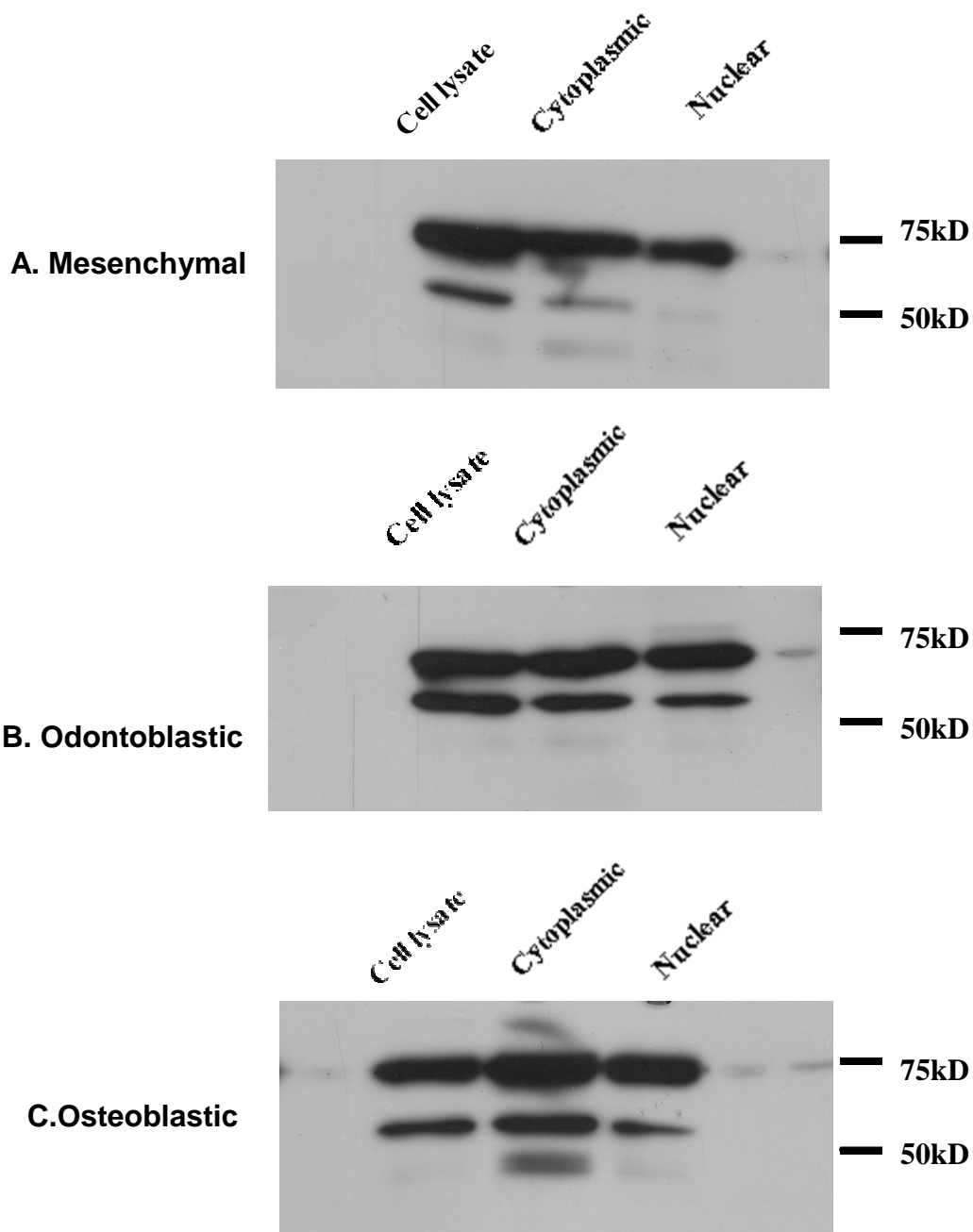


Figure 3- Western blot detection of DSPP proteins

Western blot analyses with antibodies against DSP revealed 2 major protein bands of about 50 kDa and 70kDa in the total cell lysates, cytoplasmic and nuclear fraction of all three cell lines. (A-C)

Nuclear Detection Of Endogenous DMP1 And DSPP Using Immunofluorescence

Nuclear localization of endogenous DMP1 in all three cell lines was observed by immunofluorescent staining with a monoclonal anti-DMP1 antibody against the C-terminal part of DMP1 (Fig. 4A-D). However, we found that non-synchronized cells presented two subpopulations with or without nuclear DMP1 staining (Table 1). Nuclear DMP1 was also observed in C3H10T1/2 cells (Fig. 6A) when a rabbit polyclonal antibody against the C-terminal part of DMP1 was used; and the staining was inhibited after the antibody was pre-incubated with the synthetic peptide used to generate the antibody (Fig. 6B), confirming the specificity of the antibody. Collectively, these findings suggest that endogenous DMP1 can enter the nuclei of all cell lines examined.

In addition, all the cell lines tested showed nuclear localization of DSPP (Fig. 5A-D). However the expression was uniform in all the cells of all the cell lines with hardly any cytoplasmic or nuclear distribution phenomenon.

Table 1- Nuclear expression of endogenous DMP1 in various cell lines

| Cell type | Cells with nuclear localization | Total cells counted | % cells with nuclear localization |
|------------------|--|----------------------------|--|
| C3H10T1/2 | 124 | 174 | 71.3 |
| MC3T3E1 | 202 | 249 | 81.1 |
| 17IIA11 | 92 | 139 | 66.28 |

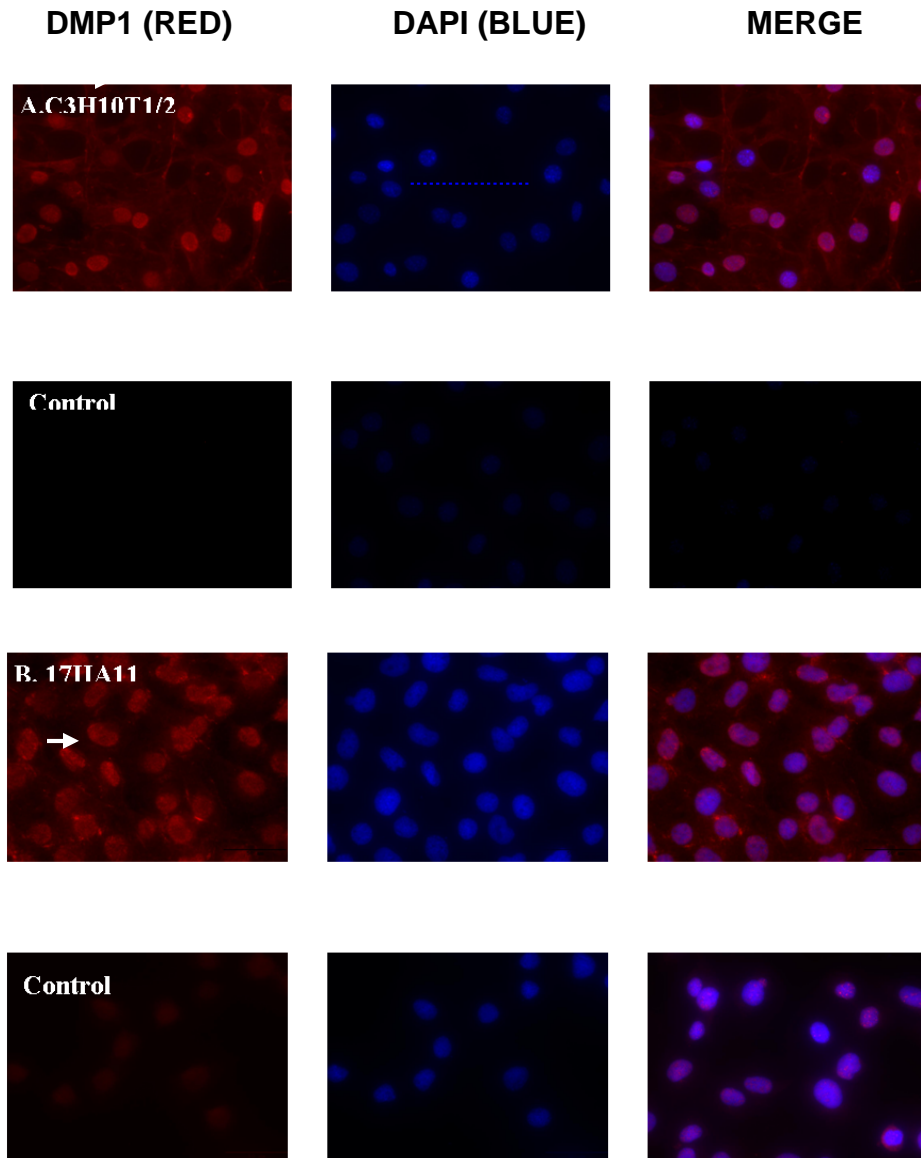


Figure 4- Immunofluorescent localization of DMP1 proteins.

Immunofluorescent staining with anti-DMP1 monoclonal antibody showed the nuclear localization of DMP1 proteins in subpopulations of mesenchymal cells (panel A), and odontoblast like cells (panel B). DMP1 signal is in red color. IgG controls show no staining signal. Nuclei were stained with DAPI (blue). Arrows indicate cells that lack nuclear DMP1 signal.

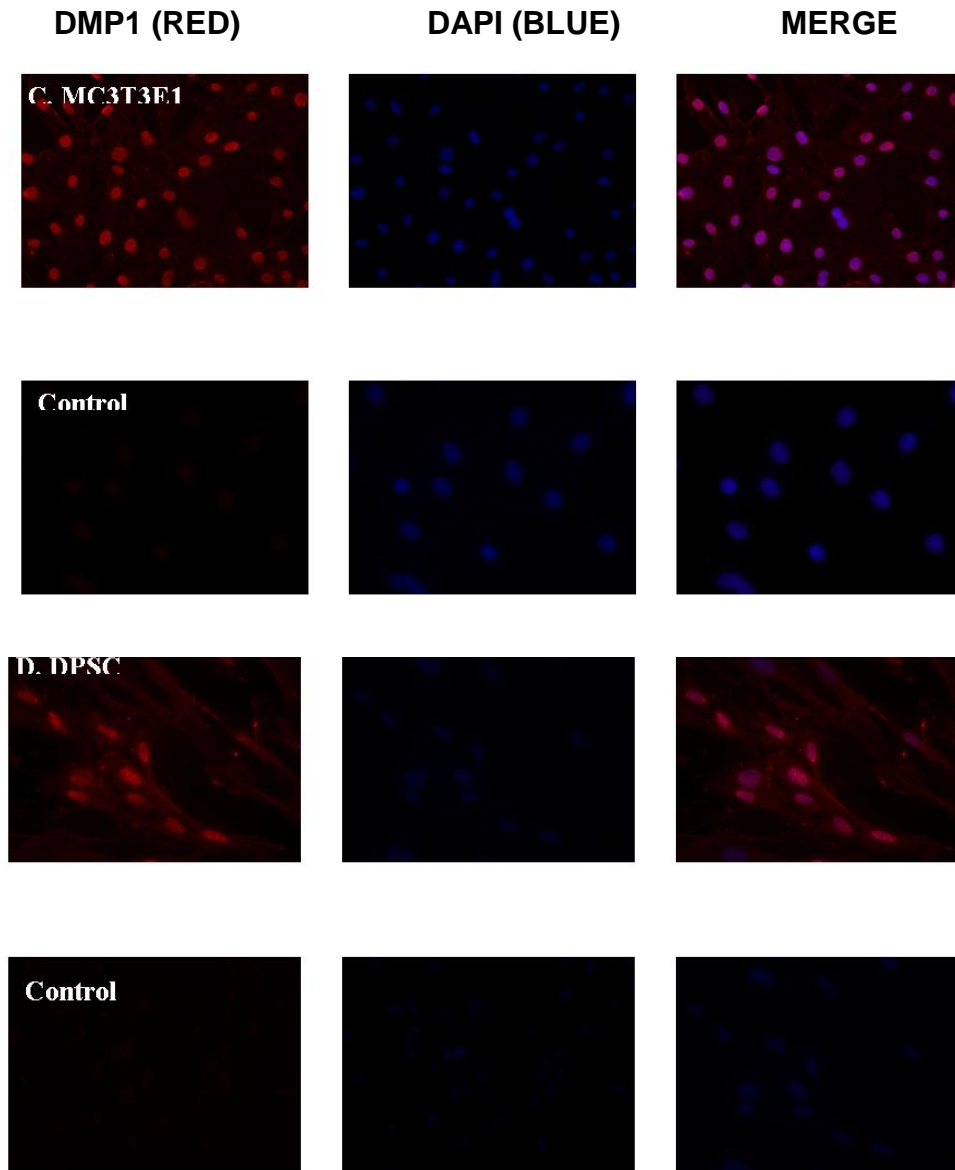


Figure 4- Immunofluorescent localization of DMP1 proteins

Immunofluorescent staining with anti-DMP1 monoclonal antibody showed the nuclear localization of DMP1 proteins in subpopulations of osteoblast like cells (panel C), and dental pulp stem cells (panel D). DMP1 signal is in red color. IgG controls show no staining signal. Nuclei were stained with DAPI (blue). Arrows indicate cells that lack nuclear DMP1 signal.

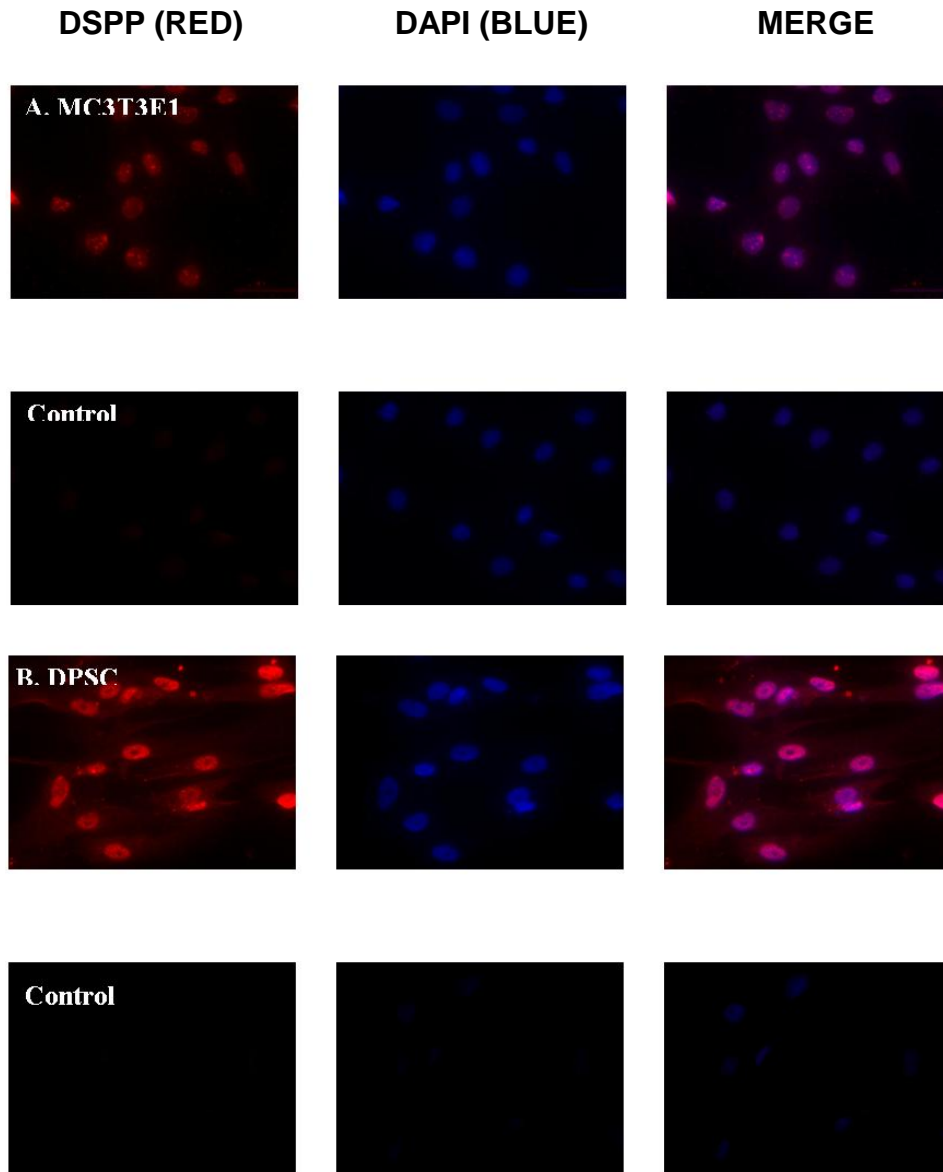


Figure 5- Immunofluorescent localization of DSPP proteins

Immunofluorescent staining with anti-DSPP monoclonal antibody showed the nuclear localization of DSPP proteins in subpopulations of osteoblast like cells (panel A), and dental pulp stem cells (panel B). DSPP signal is in red color. IgG controls show no staining signal. Nuclei were stained with DAPI (blue)

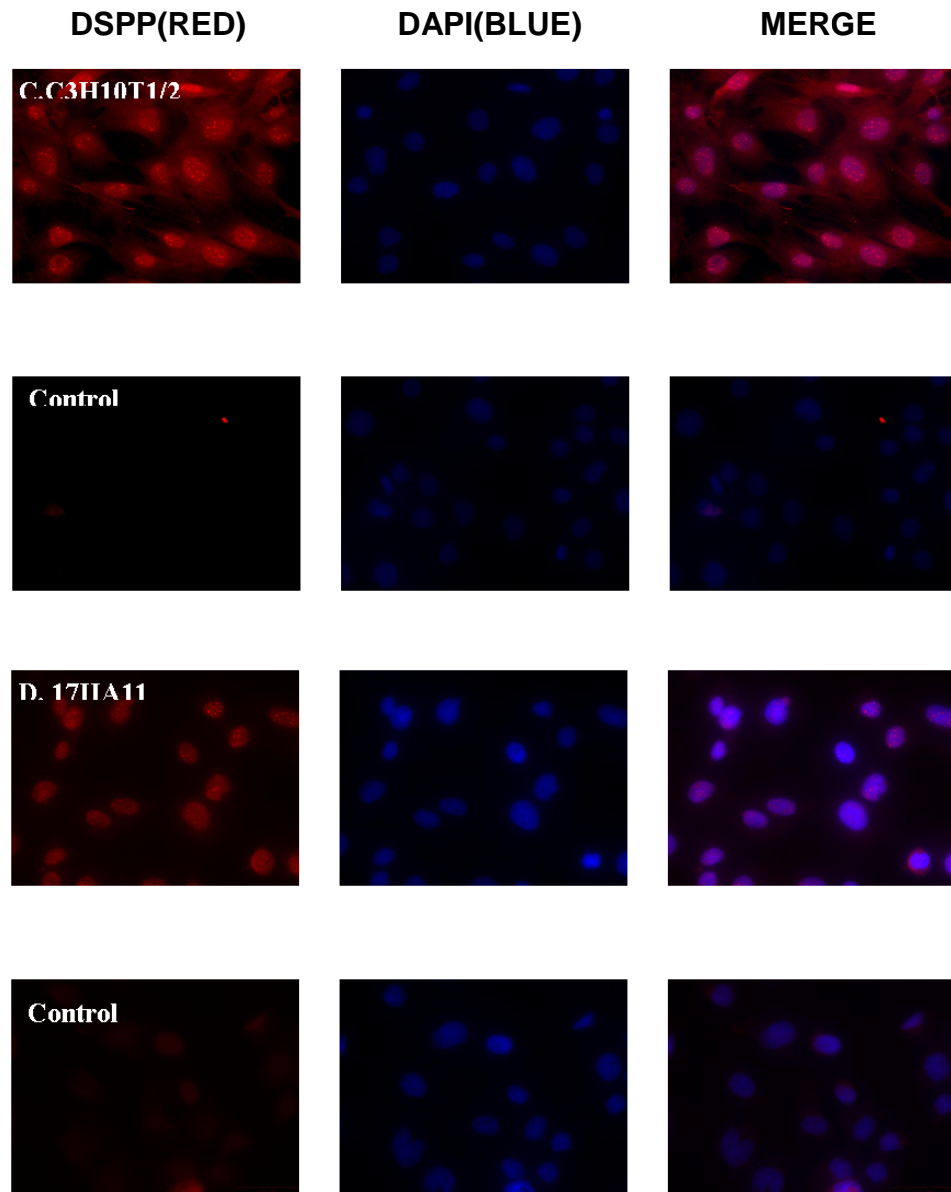


Figure 5- Immunofluorescent localization of DSPP proteins

Immunofluorescent staining with anti-DSPP monoclonal antibody showed the nuclear localization of DSPP proteins in subpopulations of mesenchymal cells (panel C), and odontoblast like cells (panel D). DSPP signal is in red color. IgG controls show no staining signal. Nuclei were stained with DAPI (blue)

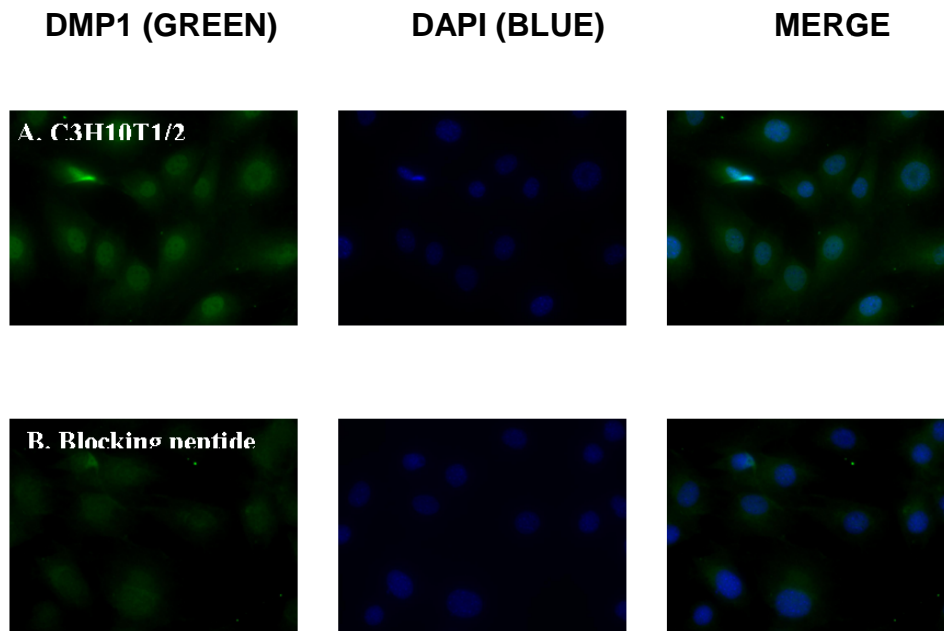


Figure 6- Immunofluorescent localization of DMP1 by polyclonal antibody

Immunofluorescent staining with anti-DMP1 polyclonal antibodies confirmed the nuclear localization of DMP1 proteins in C3H10T1/2 (panel A). The staining signal was inhibited when the polyclonal antibodies was preincubated with the peptides used to generated the antibodies (panel B). DMP1 signal is in green color. IgG controls show no staining signal (not shown). Nuclei were stained with DAPI (blue)

Exogenous DMP1

Further analysis tested whether exogenous DMP1 was able to enter the nucleus, a DMP1 expression construct was generated with a hemagglutinin (HA) tag inserted after the proteolytic cleavage sites (designated as “DMP1-HA”), so that the HA-tag would label either the full-length DMP1 (before cleavage) or the 57 kDa C-terminal fragment after cleavage (Fig. 7A). We transfected this DMP1-HA construct into the easily transfectable Cos-7 cells and analyzed the conditioned medium by Western-blot analysis with polyclonal antibodies against the C-terminal part of DMP1. We found that the HA-tag did not affect secretion, processing or posttranslational modifications of the tagged DMP1 (Fig. 7B). This construct was then transfected into the C3H10T1/2 cells, and immunofluorescent staining showed that the transfected cells presented HA staining signals in either the nuclei and/or the secretory pathway when detected with an antibody against the HA tag (Fig. 7C). These data provided strong evidence that DMP1 enters the nucleus.

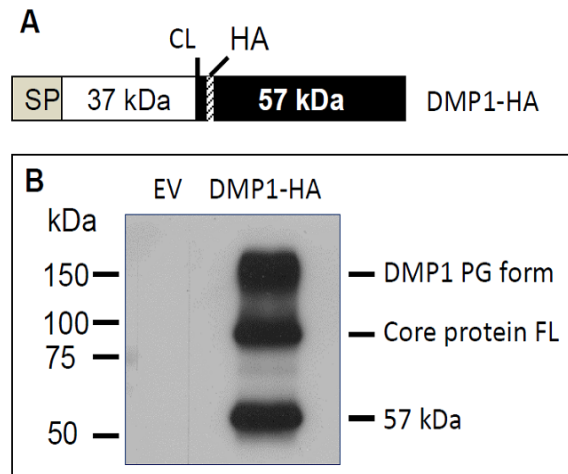


Figure 7- Nuclear localization of exogenous DMP1 proteins in C3H10T1/2 cells

A. Schematic representation of the DMP1-HA construct showing the ER entry signal peptide (SP), 37 kDa N-terminal fragment (37 kDa), key cleavage site (CL), 57 kDa C-terminal fragment (57 kDa), and the location of the hemagglutinin tag (HA). **B.** Western-blot analysis of DMP1-HA proteins. Cos-7 cells were transiently transfected with DMP1-HA expression constructs. The conditioned medium was analyzed by Western blotting using the anti-DMP1 C-terminal polyclonal antibody, showing the processed 57 kDa C-terminal fragment, the full-length (FL) core protein and the proteoglycan (PG) form. EV, empty expression vector.

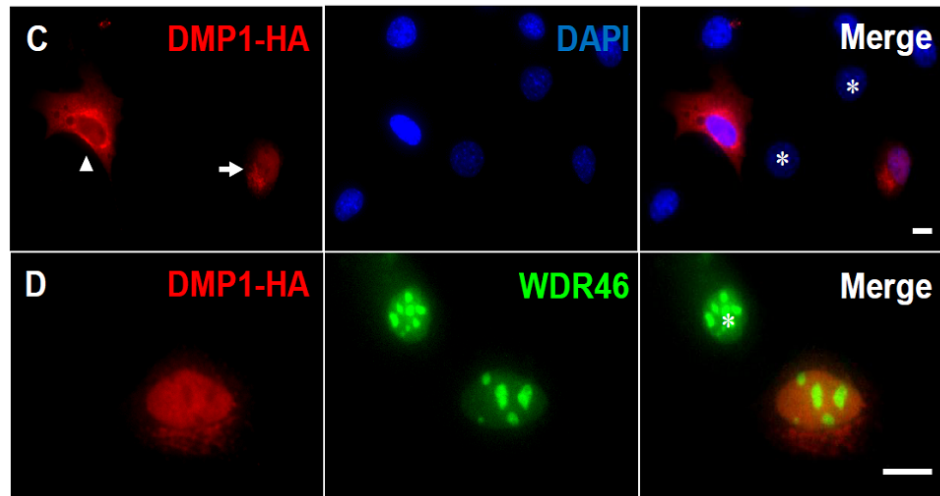


Figure 7- Nuclear localization of exogenous DMP1 proteins in C3H10T1/2 cells

Panel C. DMP1-HA expression constructs were transiently transfected into C3H10T1/2 cells, which were then immunofluorescently stained with antibodies against the HA tag (red). The nuclei were stained with DAPI (blue). The arrow indicates a cell with HA signal in the nuclear and Golgi complex; the arrowhead points to a cell without nuclear HA signal. The asterisks indicate nontransfected cells (without the constructs). **Panel D.** C3H10T1/2 cells transfected with DMP1-HA expression constructs were immunofluorescently stained with antibodies against the HA tag (red) and WDR46 (green). Scale bar = 10 μ m

Lack Of DMP1 In The Nucleolus

Though DMP1 proteins were localized in the nucleus, they appeared to be unevenly distributed throughout the nucleus (Fig. 4 and 7C). Their distribution pattern was like a negative image of the nucleolus distribution pattern (83). The nucleolus is the center for rRNA processing and ribosomal assembly. WDR46 is enriched in the nucleoli and is a good maker for nucleolus (84). Therefore, we performed co-immunofluorescent staining on cells transfected with a construct expressing HA-tagged full-length DMP1 with an antibody against HA tag and an antibody against WDR46. The staining showed that, although weak WDR46 staining was observed throughout the nucleoplasm, intense signals were localized in the nucleoli; the merged image confirmed that nuclear DMP1 was absent in the WDR46-enriched region (Fig. 7D). These observations suggest that nuclear DMP1 may not be involved in the rRNA processing or ribosomal assembly.

CHAPTER 4

DISCUSSION

DMP1 and DSPP are members of the same protein family known as SIBLING protein family. They were both first identified in the dentine, but further investigation lead to their discovery in the periodontium, bone and several other non-mineralizing tissues in addition to some types of cancer (5, 8, 19, 32, 43, 76). Research on DMP1 took a turning point after its localization in the nuclei of osteoblastic cells (23, 24) proposing an important role of DMP1 in transcription and signal transduction during the process of terminal differentiation of functional cells like osteoblasts and odontoblasts. Nonetheless its localization in the nuclei and its proposed transcription factor function has been controversial (79). DSPP shares many similarities in terms of function and structure with DMP1. This prompted us to investigate the subcellular localization of DSPP for the first time and reconfirm the endogenous and exogenous expression of DMP1 in mesenchymal cells (C3H10T1/2), pre-osteoblastic cells (MC3T3E1), odontoblast like cells (17IIA11) and DPSCs as a primary cell line to help us better understand their range of functions.

The detection of *Dmp1* and *Dspp* transcripts using RT-PCR showed the presence of the DNA coding these proteins in the cell lines representing different origins. This corroborates the finding from other studies indentifying those proteins in a variety of tissues. Having showed the ability of these cells to code for both proteins the next step was to explore their subcellular distribution. This was done using immunofluoresence and immunoblotting techniques.

The nuclear expression of DMP1 and DSPP in all the cell lines was evident with immunofluorescence. Such signal was absent in control slides. An

interesting observation was made when all cell lines presented with nuclear or cytoplasmic localization of endogenous DMP1. Such a non-synchronized expression was hardly found in anti-DSPP incubated cells. Based on the deduced protein sequence from the DMP1 and DSPP cDNA, both have a typical ER-entry signal peptide sequence that guides peptide to the secretory pathway. However, DMP1 also contains a functional NLS in the carboxyl-terminal end of DMP1 which is essential for localization of DMP1 in the nucleus (23). It has been shown that to achieve such nuclear localization, extracellular full-length DMP1 is necessary for its binding to GRP-78 receptor on the cell surface, followed by endocytosis and subsequent nuclear translocation (85). These dual signal peptides in DMP1 are consistent with its nuclear or cytoplasmic expression in the cells tested. Since cells in each cell line are genetically identical, these findings suggest that the final destination of DMP1 is highly regulated, and may be associated with the progression of the cell cycle. Furthermore, we found that cells transfected with a construct carrying the full-length DMP1 cDNA under the control of a CMV promoter presented both nuclear and cytoplasmic localization of exogenous DMP1. We also showed that nuclear DMP1 was restricted to the nucleoplasm, but was absent in the nucleolus. This subnuclear localization is consistent with its function as a transcription factor in the nucleus, regulating odontoblast differentiation (23). However, this result is opposite to the previous report showing that cells transfected with a DMP1-expressing construct did not have a nuclear localization of exogenous DMP1 (79). The underlying reason for this discrepancy is currently unknown.

To the best of our knowledge, DSPP lacks a NLS, and this suggests a different mechanism of nuclear translocation that can perhaps involve specialized receptors on the surface of the nucleus similar to the process used by insulin or

prolactin (3, 86, 87) . The exact mechanism is still elusive and requires further investigation.

The immunoblotting results confirmed the immunofluorescence results as both DMP1 and DSPP were consistently expressed in the total cell lysate, cytoplasmic and nuclear fractions. Western-blot analysis detected a major DMP1 protein band of about 57 kDa, which corresponds to the 57 kDa C-terminal fragment of DMP1 as mentioned before. This finding along with other studies showing that only the C-terminal fragment was localized in the nuclei of both osteocytes in bone and cultured cells transfected with constructs expressing full-length DMP1 when using different antibodies against either the N-terminal or C-terminal fragment of DMP1 (88, 89), suggest that the nuclear form of DMP1 may be similar, if not identical, to the 57 kDa C-terminal fragment produced by proteolytic processing of the secreted full-length DMP1. The western blot results for DSPP invariably show two bands between the 50kDa and the 75kDa that might correspond to DSP with different extents of post translational modifications. Unfortunately we were unable to successfully transfect cells with DSPP constructs to help us confirm the shape and size of the cell secreted DSPP or its cleaved products. However negative controls replacing the primary antibody with pre-immune serum did not show any signal.

In summary our results present that nuclear localization is a highly regulated event, and may be associated with the progression of the cell cycle and that nuclear DMP1 is distributed throughout the nucleoplasm but absent in the nucleolus. Another novel finding is the nuclear localization of DSPP. However more studies are required to understand the mechanism behind DSPP's localization and its exact significance. Moreover the relation to DMP1 to its nuclear/cytoplasmic expression to the cell cycle needs to be explored. Such

studies will better assist us in further understanding signal transduction and transcription mechanisms involved in tooth and bone formation and help in defining pathogenic mechanisms associated with loss of DMP1 or DSPP functions.

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