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RESEARCH ARTICLE

Efficient bone formation by gene transfer of human LIM mineralization protein-3

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LIM mineralization protein (LMP) is a novel positive regulator of the osteoblast differentiation program. In humans, three different LMP splice variants have been identified: LMP-1, LMP-2, and LMP-3. Gene transfer of human LMP-1 (hLMP-1) induces expression of genes involved in bone formation, including certain bone morphogenetic proteins (BMPs), promotes bone nodule formation in vitro, ectopic bone formation in vivo, and is therapeutic in animal models of posterior thoracic and lumbar spine fusion. To examine the osteoinductive properties of the LMP-3 in vitro and in vivo, we have generated plasmid and adenoviral vectors expressing codon-optimized hLMP-3. Here we demonstrate that gene transfer of hLMP-3 induces expression of the bone-specific genes osteocalcin, osteopontin, and bone sialoprotein and induced bone mineralization in preosteoblastic and fibroblastic cells. We also demonstrate that hLMP-3 is able to induce bone mineralization and the expression of the bone-specific genes, BMP-2, OSX, RunX2, and alkaline phosphatase in human mesenchymal stem cells in a dose-dependent manner. Finally, we demonstrate that direct gene transfer of hLMP-3 into murine skeletal muscle results in ectopic bone formation more efficiently than BMP-2. These results demonstrate that hLMP-3 gene transfer can be used to promote bone formation in cell culture and in vivo as or more efficiently than BMP-2, thus establishing feasibility and efficacy of direct gene delivery of hLMP-3 to produce bone in vivo. These results suggest that gene transfer of hLMP-3 could be developed as a bone-inductive therapeutic agent for clinical applications.

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Introduction

LIM mineralization protein (LMP) is a positive regulator of the osteoblast differentiation program,[1] containing both LIM and PDZ domains.[2] The LIM domain was named for the original three homeodomain proteins in which it was first described, Lin-11, Isl-1, and Mec-3, and appears to be involved in protein–protein interactions. LIM domains are defined by a conserved pattern of cysteine, histidine, or alternate metal-coordinating residues that define two specialized zinc fingers. The PDZ domain was also named for the three proteins in which the homologous sequences were identified, Postsynaptic density, Drosophila discs-large, and ZO-1 epithelial tight junction proteins, and acts as a modular protein–protein binding domain, able to bind specific recognition sequences.

In humans, three different LMP splice variants have been identified: LMP-1, LMP-2, and LMP-3. Human LMP-1 (hLMP-1) is a 457 aa protein containing an N-terminal PDZ domain, and three LIM domains. Whereas both the PDZ and LIM domain/motif are highly conserved with LMP from other species, the region between the PDZ domain and LIM motifs is not conserved and thus has been termed the unique region. Tissue distribution analyses have demonstrated that hLMP-1 is expressed almost ubiquitously and appears to be the predominant form expressed in lung, spleen, leukocytes, placenta, and fetal liver. Human LMP-2 (hLMP-2) has a 119 bp deletion in the unique region between bp 325 and 444, along with a 17 bp insertion at position 444. The resulting protein is 423 aa in size with intact PDZ and LIM domain/motifs. This isoform of the protein is strongly expressed in skeletal muscle, heart, and bone marrow. Finally, hLMP-3 has a 17 bp insertion at position 444, resulting in a reading frame shift that causes a stop codon to occur at bp 505–507. The resulting protein contains 153 aa, with an intact PDZ domain, but is missing approximately 30% of the unique region and has no LIM domains. This isoform is widely expressed, but is not predominant in any of the analyzed tissues.

It has been demonstrated that transfection of rat calvarian osteoblasts with a plasmid containing hLMP-1 induces bone nodule formation *in vitro*. Interestingly, a similar induction of bone formation *in vitro* was conferred by transfection of an LMP-3 expression plasmid into osteoblasts.[1,2] In addition, it has been

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demonstrated that the implantation of cells transfected with the truncated form of hLMP-1 induces ectopic bone formation *in vivo*. Taken together, these findings suggest that the LIM domains are not essential for the osteoinductive activity of hLMP-1. These results are supported by the observation that hLMP-2, which has intact LIM domains/motif, is not osteoinductive.

In addition to its ability to induce bone nodule formation *in vitro*, LMP-1 has been shown to induce ectopic bone formation *in vivo*[3] and is therapeutic in animal models of posterior thoracic and lumbar spine fusion.[4–7] In the spine models, bone marrow-derived buffy coat cells were modified to express LMP-1 by adenoviral gene transfer followed by transplantation of the genetically altered cells. However, it is unclear if LMP-3 is able to induce bone formation *in vivo* as well as induce bone-specific gene expression in culture. In addition, it is also unclear if LMP gene transfer is as effective as BMP-2 in inducing bone formation.

In this report, we have examined the ability of gene transfer of a codon-optimized, truncated LMP-3 to induce bone-specific gene expression and bone mineralization in cell culture and ectopic bone formation *in vivo* in comparison to BMP-2. Here we demonstrate that gene transfer of LMP-3 induces expression of the bone-specific genes osteocalcin (OC), osteopontin (OP), and bone sialoprotein (BSP) in both preosteoblastic and fibroblastic cells. In addition, we demonstrate that hLMP-3 is able to induce expression of the osteogenic factor BMP-2, the bone-specific transcription factor OSX, and the bone-specific marker gene alkaline phosphatase (AP) in human mesenchymal stem cells (hMSCs). We also demonstrate that hLMP-3 gene transfer is able to induce mineralization in preosteoblastic, fibroblast, and mesenchymal stem cells. Finally, we demonstrate that direct gene transfer of hLMP-3 into murine skeletal muscle results in ectopic bone formation more efficiently than BMP-2. These results suggest that gene transfer of hLMP-3 or a fragment derived from LMP-3 could be developed as an effective approach to induce bone formation *in vivo* for clinical applications.

Results

hLMP-3 gene transfer induces upregulation of bone-specific genes in NIH3T3 and MC3T3-E1 cells

To examine the bone inductive properties of LMP-3, we initially constructed a CMV expression plasmid carrying a codon-optimized LMP-3. The optimized LMP-3 was constructed using an algorithm we developed previously that designs a series of overlapping primers that are then used for PCR amplification (Figure 1a). The resulting full-length LMP-3 cDNA, optimized for efficient translation in mammalian cells, was confirmed by sequencing and inserted into the pAd.lox shuttle vector. The codon-optimized LMP-3 was 80% identical at the DNA sequence level (Figure 1b) and 100% identical at the amino-acid level (Figure 1c).

To examine the ability of LMP-3 to induce bone-specific genes, reverse transcriptase (RT)-PCR analysis was performed to determine the expression of OC, OP,

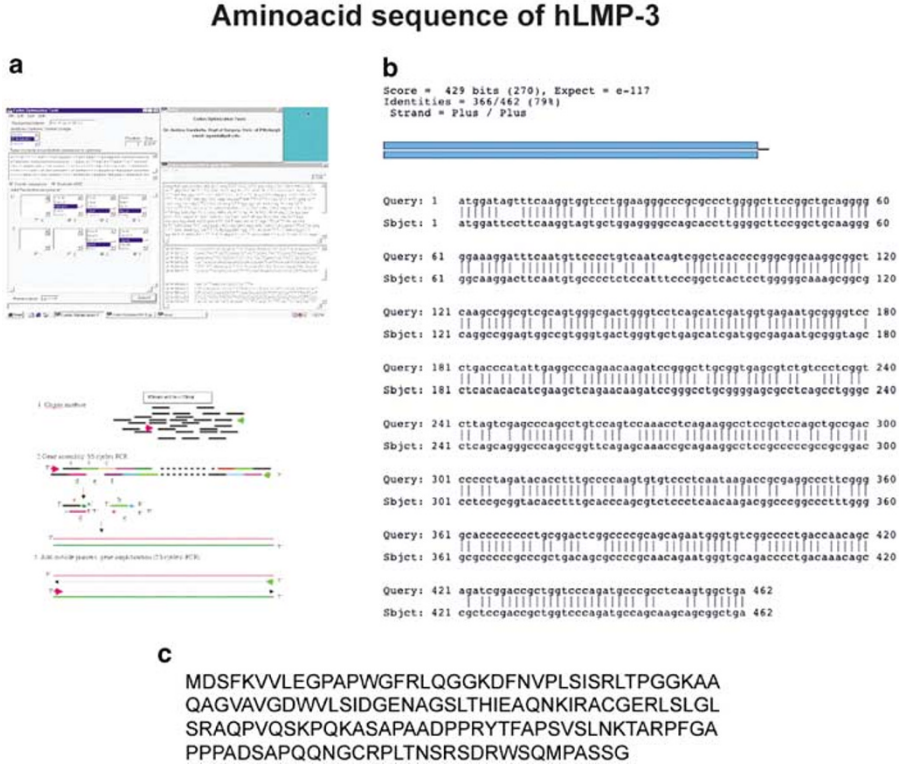

Figure 1 *Construction of a CMV expression plasmid carrying a codon-optimized LMP-3. The optimized LMP-3 was constructed using an algorithm that designs a series of overlapping primers that are then used for PCR amplification (a). The resulting full-length LMP-3 cDNA, optimized for efficient translation in mammalian cells, was confirmed by sequencing and inserted into the pAd.lox shuttle vector. The codon-optimized LMP-3 was 80% identical at the DNA sequence level (b) and 100% identical at the amino-acid level (c).*

and BSP 48 h after transfection with hLMP-3, BMP-2, and the control pAd.lox vector into MC3T3-E1 and NIH 3T3 cells. Upregulation of the expression of the three bone gene markers examined, OP, BSP, and OC, was detected in the MC3T3-E1 preosteoblastic cells treated with BMP-2 and hLMP-3, compared with empty vector-transfected cells or nontransfected control cells (Figure 2a). Semi-quantitative analyses of the RNA levels in cells transfected with BMP-2 and hLMP-3 showed comparable expression of bone marker genes. Similarly, induction of expression of OP, BSP, and OC was found after transfection of NIH3T3 cells with hLMP-3 and BMP-2 expression vectors (Figure 2b). These results suggest that transfection of LMP-3 and BMP-2 expression plasmids into either the osteoblastic cell line MC3T3-E1 or the fibroblast cell line NIH3T3 results in the induction of bone gene markers with similar efficiencies even though LMP-3 is an intracellular factor in contrast to the secreted BMP-2.

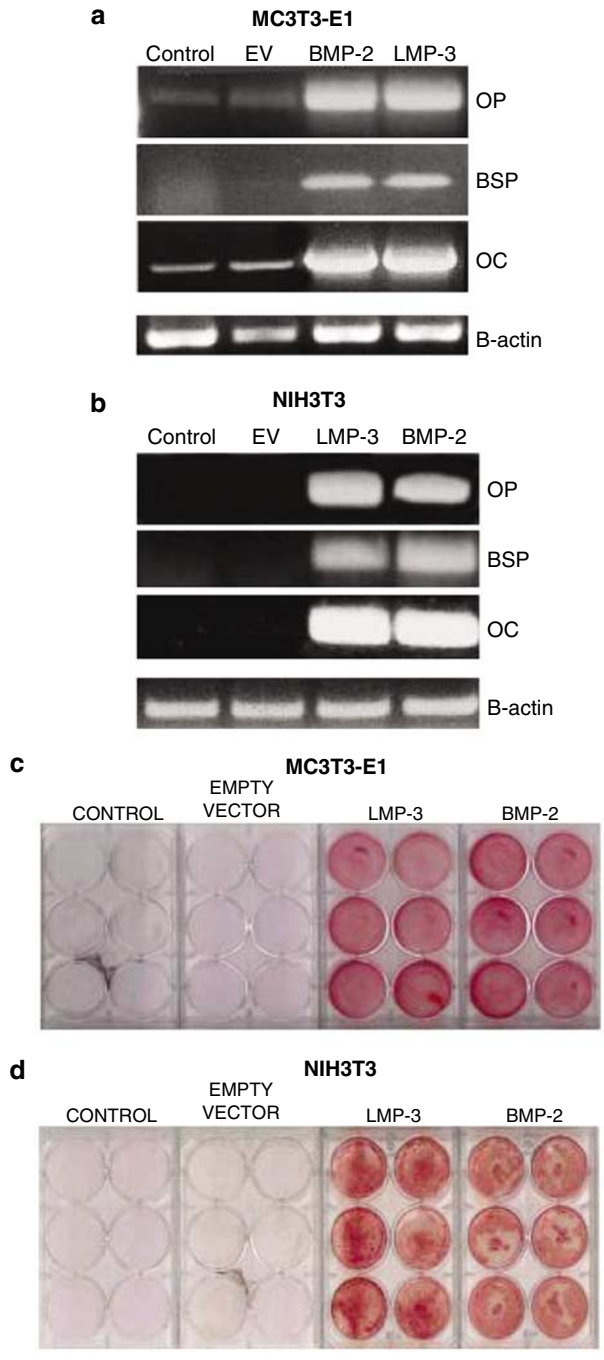

Figure 2 *Induction of specific bone genes and bone mineralization after transfection of MC3T3-E1 and NIH 3T3 cells with hLMP-3 and BMP-2. Upregulation of the three bone-specific genes examined, OP, BSP, and OC, was examined in MC3T3-E1 preosteoblastic (a) and NIH3T3 cells (b) transfected with BMP-2 and hLMP-3 expression vectors or a control empty vector (EV). In addiction, the ability of BMP-2 and LMP-3 to induce mineralization following transfection was examined in MC3T3-E1 (c) or NIH 3T3 (d) by Von Kossa staining.*

hLMP-3 transfection induces bone mineralization in NIH3T3 and MC3T3-E1 cells

The final step of osteoblast differentiation *in vitro* is characterized by the formation of mineral crystals. If treated appropriately, such as with BMP-2 or other agents,[8–11] MC3T3-E1 cells undergo a rapid and efficient mineralization process that can be detected by Von Kossa staining. Similarly, NIH 3T3 fibroblasts also do not mineralize under basal conditions, but have the ability to mineralize if grown in media supplemented with BMP-2 or $1,25(OH)_2D_3$.[12–15] Thus, in addition to examining the induction of bone gene markers, we have examined the ability of BMP-2 and LMP-3 to induce mineralization following transfection. Transfection of LMP-3 and BMP-2 into either MC3T3-E1 (Figure 2c) or NIH 3T3 (Figure 2d) cells resulted in the induction of bone mineralization compared to controls.

Ad.hLMP-3 infection induces upregulation of bone gene markers and bone mineralization in NIH3T3 and MC3T3-E1 cells

To demonstrate that adenoviral infection with Ad.LMP-3 and Ad.BMP-2, which results in a higher level of gene expression than following plasmid transfection, also can induce bone differentiation, Ad.LMP-3, Ad.BMP-2, and Ad.Ψ5 were used to infect MC3T3-E1 and NIH-3T3 cells. RT-PCR analysis demonstrated that infection of MC3T3-E1 preosteoblastic cells with the hLMP-3 gene increases the expression of OC, OP, and BSP (Figure 3a). In addition, the expression of bone gene markers was quantitatively comparable in cells infected with BMP-2 and hLMP-3. Similarly, NIH-3T3 fibroblastic cells responded to hLMP-3 and BMP-2 infection by expressing OC and OP. BSP was also weakly expressed in NIH3T3 cells treated with hLMP-3 and BMP-2, while no effect was observed after infection with the control virus Ad.Ψ5 (Figure 3b). In addition, infection of AdLMP-3 and AdBMP-2 into either MC3T3-E1 (Figure 3c) or NIH 3T3 (Figure 3d) cells resulted in the induction of bone mineralization. No mineralization was observed in cell cultures treated with Ad.Ψ5. These results demonstrate that efficient adenoviral-mediated gene delivery of LMP-3 results in the osteoblast differentiation of MC3T3-E1 and NIH-3T3 cells.

AdLMP-3 upregulates expression of BMP-2, OSX, and AP in hMSCs

In addition to examining the ability of LMP-3 to induce differentiation of MC3T3-E1 and NIH-3T3 cells, we have also examined the effect of Ad.LMP-3 on differentiation

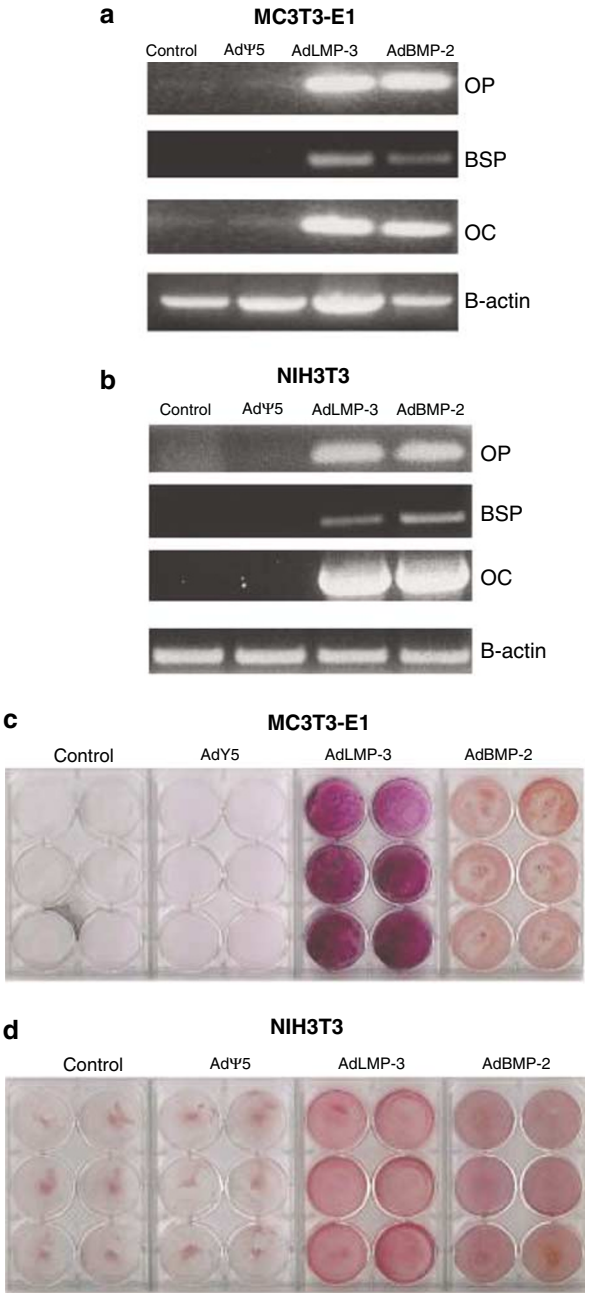

Figure 3 *Induction of specific bone genes and bone mineralization after infection of MC3T3-E1 and NIH 3T3 cells with AdLMP-3 and AdBMP-2. Upregulation of the three bone-specific genes examined, OP, BSP, and OC, was examined in MC3T3-E1 preosteoblastic (a) and NIH3T3 cells (b) infected with AdBMP-2 and AdhLMP-3 expression vectors. In addiction, the ability of BMP-2 and LMP-3 to induce mineralization following transfection was examined in MC3T3-E1 (c) or NIH 3T3 (d) by Von Kossa staining.*

of hMSCs. However, for this analysis, we used quantitative RT-PCR to determine the fold induction of gene expression at 2 and 4 days following adenoviral-mediated gene delivery. Moreover, we have also examined the ability of conditioned media (CM) from the transduced MSCs 2 days postinfection to induce gene expression in noninfected MSCs. As shown in Figure 4a, Ad.LMP-3 upregulates the expression of the bone-specific transcription factor OSX 27-fold at day 2 and seven-fold at day 4. Interestingly, the CM from Ad.LMP-3 infected cells did not induce any expression of the OSX at day 2, whereas OSX appeared to be upregulated 82-fold in cells treated for 4 days following addition of the CM. Infection of hMSCs with the control vector Ad.Ψ5 did not induce upregulation of the gene at both the time points. An upregulation of expression of endogenous BMP-2 was detected after 2 and 4 days following infection with AdLMP-3, but only after 4 days in the CM-treated cells. In particular, at day 2, cells infected with AdLMP-3 upregulated expression of BMP-2 16-fold whereas, at day 4, expression of BMP-2 increased 58-fold over the control. Similarly, the hMSCs growth in the CM showed an upregulation of 27-fold in the BMP-2 gene expression. Analysis of AP at day 4 showed a four-fold increase in expression in cells infected with hLMP-3. No expression of AP was detected in cells grown in the CM and in the control cells. Interestingly, none of the samples analyzed showed any increase in the expression of osteogenic transcription factor RunX2 at the high multiplicity of infection (MOI) of virus used in these experiments; instead LMP-3 marginally reduced expression (data not shown, but see Figure 4b).

AdLMP-3 affects bone-specific gene expression in a dose-dependent manner

The results presented above demonstrate that gene transfer of LMP-3 is able to induce bone-specific marker genes, at least at the MOI used. In order to determine if the effects of LMP are dose dependent, a dose–response analysis was performed to evaluate the effects of lower doses of Ad.LMP-3 (20 000, 2000, and 200 viral particles (vp)/cell) of LMP-3. Cells treated with medium without virus served as a negative control, and the levels of BMP-2, OSX, and RunX2 were assessed by real-time PCR (Figure 4b). The level of BMP-2 gene expression was induced at day 2 and more significantly at day 4 in a dose responsive manner, with a 16-fold induction at day 2 and increasing to 56-fold at day 4 at the highest MOI. OSX was also induced by LMP-3, but the induction at the low doses of virus was only 2- to 3-fold at 2 and 4 days. In contrast, RunX2 was actually suppressed at the high doses of Ad.LMP-3, but marginally induced at the low doses of virus (2000 and 200 vp/cell). These results demonstrate that the effects of LMP-3 are dose dependent, with expression of BMP-2 and OSX induction correlating with the dose of virus used. However, it appears that LMP-3 may regulate RunX2 differently, actually suppressing expression at high doses of Ad.LMP-3 and weakly stimulating RunX2 at lower doses. Taken together, these results demonstrate that LMP-3 regulates BMP-2, OSX, AP, and RunX2, but that the regulation may not be mediated through the same pathway for all four genes. Moreover, the fact that BMP-2 expression is higher at day 4 suggests that either LMP-3 does not directly regulate transcription of these bone-specific marker genes or that induction of these factors, such as BMP-2, results in autoinduction of bone marker gene expression.

hLMP-3 infection induces bone mineralization in vitro in hMSC cells

Similar to fibroblasts, hMSCs do not mineralize under basal condition. However, MSCs have been demon-

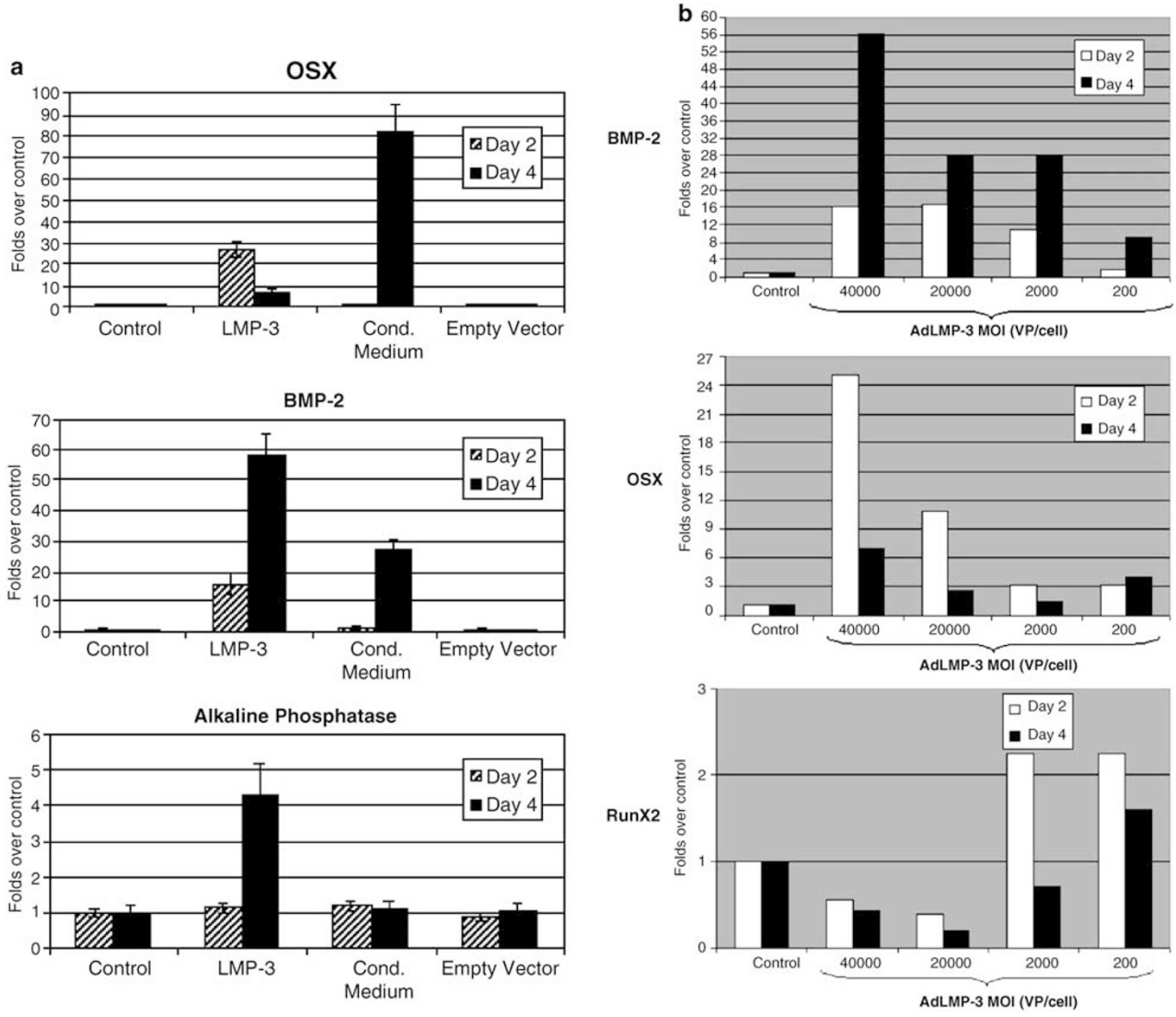

Figure 4 *Real-time RT-PCR analysis to evaluate induction of OSX, BMP-2, RunX2, and AP expression in hMSCs infected with Ad.hLMP-3. (a) MSCs were infected with Ad.LMP3 or AdΨ5 at an MOI of 4×10^4 vp/cell and gene expression analyzed at 2 and 4 days postinfection. In addition, media were removed from the infected cells at 2 days and added to noninfected cells for 2 and 4 days postaddition. The levels of expression of OSX, BMP-2, and AP were examined at the indicated time points by real-time RT-PCR. (b) MSCs were infected with Ad.LMP3 at the indicated MOIs, 40 000, 20 000, and 2000 vp/cell, and the levels of expression of OSX, BMP-2, and RunX2 was examined by real-time RT-PCR at 2 and 4 days. Cells treated with medium without virus served as a negative control for these experiments.*

strated to have the ability to mineralize if grown in media supplemented with osteogenic factors.[16–18] Similar to the results in NIH-3T3 and MC3T3-E1 cells, gene transfer of both AdBMP-2 and AdLMP-3 induced bone mineralization in hMSCs, as confirmed by the Von Kossa staining (Figure 5).

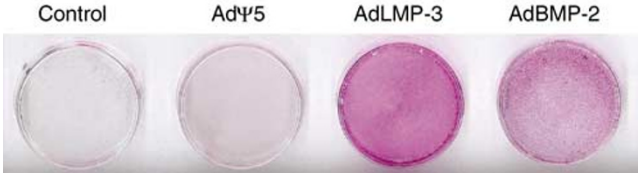

Figure 5 *Induction of bone mineralization after infection of hMSCs with AdLMP-3 and AdBMP-2. Von Kossa staining of hMSCs 10 days after infection with the indicated adenoviruses.*

Ectopic bone formation following Ad.LMP-3 intramuscular injection

To examine the ability of LMP-3 to induce bone formation *in vivo*, 1.5×10^7 PFU of AdLMP-3 virus was injected bilaterally into the exposed triceps surae musculature. As shown in Figure 6A, increases in the radiographic densities were evident at 3 and 5 weeks in mice injected with hLMP-3, although a small, weak area of radio-opacity was already detectable in the muscle after 2 weeks. Von Kossa staining of the tissue sections revealed bone formation after 5 weeks in all the mice treated with hLMP-3 (Figure 6B-c). These data were confirmed using standard hematoxylin–eosin with Von Kossa staining (Figure 6B-e). In mice treated with AdBMP-2, an increase in radiographic density and positive histology (Figure 6B-d,f) was also evident at 3

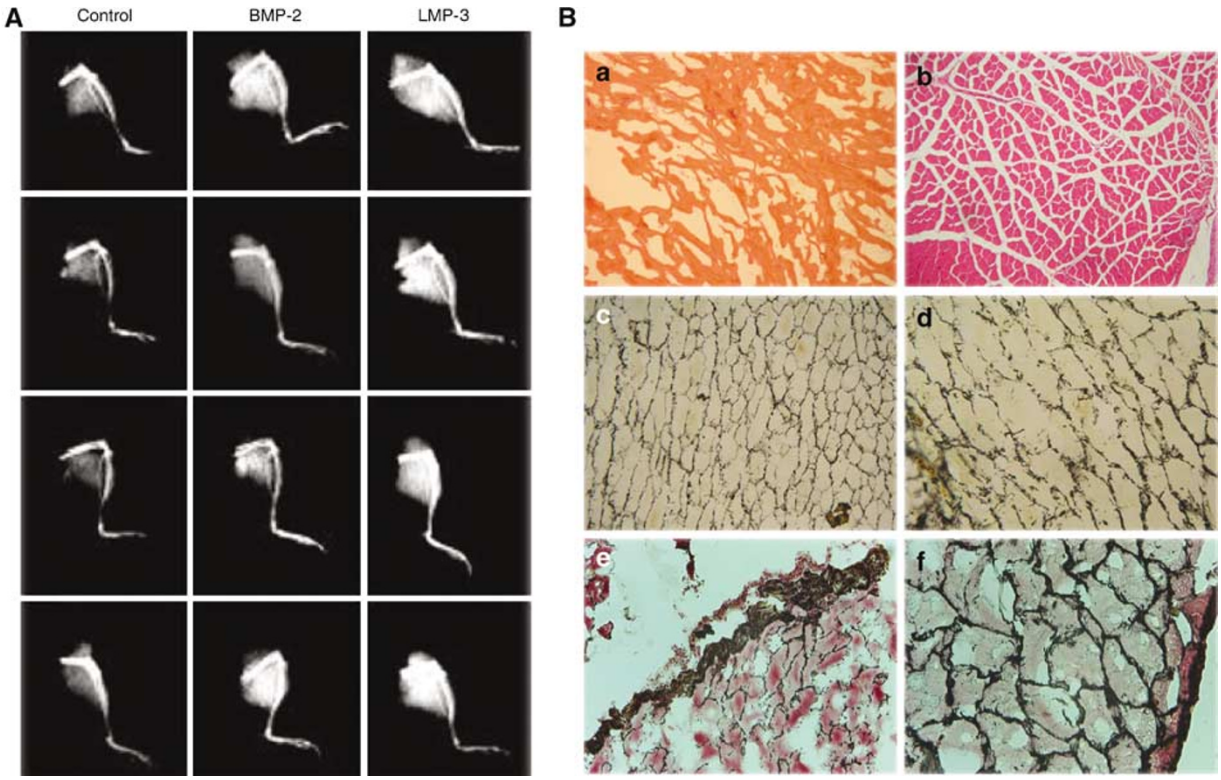

Figure 6 *Analysis of ectopic bone formation following intramuscular injection of Ad.LMP3 and Ad.BMP-2. (A) X-ray evaluation of ectopic bone formation in treated mice. (B) Hematoxylin–eosin and Von Kossa staining on muscle sections from control and treated mice at 5 weeks postinjection: (a) hematoxylin–eosin staining from a negative control; (b) hematoxylin–eosin with Von Kossa staining on negative control; (c) Von Kossa staining from an LMP-3-treated mouse; (d) Von Kossa staining from a BMP-2-treated mouse; (e) hematoxylin–eosin with Von Kossa staining on an LMP-3-treated mouse; (f) hematoxylin–eosin with Von Kossa staining on BMP-2-treated mouse.*

and 5 weeks after injection, consistent with the previous results.[19] However, the ectopic bone formation was detected after 5 weeks only in half of the animals treated with BMP-2 (4/8), a number of animals significantly smaller compared with those injected with hLMP-3 (8/8). No gross, radiographic, or histologic evidence of ectopic bone formation was present in mice injected with AdΨ5 (Figure 6B-a,b). These results demonstrate that LMP3 gene transfer to mouse muscle results in rapid and efficient ectopic bone formation, relative to the positive control BMP-2.

Discussion

In recent years, several studies have investigated which conditions and stimuli are able to influence bone gene expression, mineralization, and bone formation *in vivo*.[20–27] Gene transfer of BMP-2, BMP-4, BMP-6, and BMP-7 has been shown to induce or enhance bone formation *in vivo* in several different animal models. Interestingly, gene transfer of LMP-1, a novel intracellular regulator of bone differentiation, has also been demonstrated to induce bone formation in spine fusion models. Although the mechanism of action of LMP-1 is unknown, it has been demonstrated to induce expression, either directly or indirectly, of certain genes involved in bone formation, including several BMPs such as BMP-2, -4, and -6.[1–6] Thus it has been proposed that gene transfer of LMP may be more effective than BMPs for the induction of new bone formation *in vivo*.

Since there are at least three differentially spliced forms of LMP, termed LMP-1, -2, and -3, it is possible that each isoform has different functions *in vivo*. Recently, LMP-3, the shortest of the three isoforms, missing the LIM domain in the C-terminal portion of LMP-1, was shown to induce bone nodules. However, it was unclear if LMP-3 is able to induce bone formation *in vivo*. Thus we have examined the ability of a codon-optimized version of LMP-3 to induce bone formation *in vivo* as well as induce bone-specific gene expression in tissue culture in three different cell types, in comparison to BMP-2. We found increased expression of three bone gene markers, OC, OP, and BSP, in both preosteoblastic and fibroblast cells transfected with either hLMP-3 or BMP-2 expression plasmids. Similarly, we found that the selected bone genes were expressed *de novo*, after hLMP-3 gene transfer, in fibroblastic cells. The semiquantitative analysis demonstrated that gene transfer of hLMP-3 and BMP-2 induces similar levels of OC, BSP, and OP gene expression *in vitro*, indicating that intracellular hLMP-3 is an osteoinductive agent at least as efficient as the secreted BMP-2. Similar results were obtained when the hLMP-3 cDNA was cloned into an adenoviral shuttle plasmid and a first-generation ΔE1 E3 adenoviral vector carrying the hLMP-3 gene was used to transfect osteoblastic and nonosteoblastic cell lines.

We have also shown that hLMP-3 stimulates osteoblastic and nonosteoblastic cell lines to produce

mineralization *in vitro*. This effect may be obtained by using both the plasmid and the adenovirus to deliver the hLMP-3 cDNA. Moreover, hLMP-3 showed an ability to stimulate fibroblast cells to differentiate into osteoblastic-like cells, as determined by analysis of OC, BSP, and OP gene expression and formation of mineral deposition (data not shown). We have also shown that CM from Ad.LMP3 cells was able to induce the expression of selected bone genes and the mineralization process, suggesting that LMP3 is able to induce secreted factors such as BMP-2 and BMP-6 that are osteoinductive *in trans*. The ability of LMP-3 to induce osteogenesis *in trans* suggests that even though LMP-3 is an intracellular factor, it may be highly effective at inducing new bone formation *in vivo* following gene transfer. The osteogenic efficiency of LMP-3 may be due to the fact that the growth factors synthesized *in situ* as a result of LMP-3 gene transfer undergo authentic post-translational processing and are presented to the surrounding cells/tissues in a natural, cell-based manner.

In addition to analyzing the effects of LMP3 in murine fibroblasts and a preosteoblast cell line, we have also evaluated its effects on mesenchymal stem cells. For these experiments, we used quantitative RT-PCR to demonstrate that hLMP-3 is able to induce expression of BMP-2, OSX, and AP after infection. In particular, BMP-2 was strongly upregulated (58-fold) after 4 days, confirming the hypothesis that the LMP proteins act to stimulate expression and thus secretion of BMPs.[3] OSX, a novel zinc finger-containing transcription factor required for osteoblast differentiatio and bone formation,[28] is upregulated by hLMP-3 27-fold 2 days postinfection, but is expressed at only a seven-fold higher level at 4 days. This result suggests that OSX may be a direct target for regulation by LMP3, which in turn regulates BMP-2. Interestingly, a very high expression of OSX (82-fold) has been detected after 4 days in cells treated with the CM, suggesting that there are osteoinductive factors released from LMP-3 expressing cells that can induce expression of bone-specific genes *in trans*, including OSX. These same factors may also stimulate expression of the bone marker genes through an autocrine pathway, resulting in higher levels of expression at day 4 than at day 2. The effects of LMP-3 on OSX and BMP-2 appear to be dose dependent, with more expression at higher doses of Ad.LMP-3.

Interestingly, we observed no induction of RunX, another osteoinductive transcription factor, by LMP-3 in MSCs at the high doses of viral infection, suggesting that the ability of LMP3 to induce bone formation may be RunX independent, but possibly OSX dependent. In fact, at high doses of virus, we observed that LMP-3 actually partially suppressed basal RunX2 expression. It is important to note that the basal level of RunX2 is already extremely low, so the observed reduction may not reflect a biologically relevant effect. However, we did observe a two-fold increase in RunX2 at lower MOIs of Ad.LMP-3 in MSCs. These results suggest that the mechanism of regulation of RunX2 by LMP-3 may be different from that for BMP-2, OSX, and AP. Also, given that expression of BMP-2 and AP is higher at day 4 than at day 2, there may be an autocrine regulatory mechanism mediated by factors such as BMP-2. Thus currently we are investigating the regulation of these markers in cells expressing noggin, an inhibitor of BMP-2 activity.

In addition to the analysis in cell culture, we have also examined the ability of LMP3 gene transfer to induce ectopic bone formation *in vivo*. Time-course analysis following intramuscular injection of AdLMP3 demonstrated that ectopic bone formation becomes evident 3 weeks after gene transfer, but increases in density and size at 5 weeks after treatment. A similar time course was observed in the animals in which the BMP-2 gene was delivered, consistent with previous data in the literature.[19] However, statistical analyses, based on radiographic and histologic findings, indicate that, at 5 weeks, the number of animals that developed ectopic bone formation is significantly greater in animals treated with hLMP-3 than with BMP-2, suggesting that hLMP-3 might be more active than BMP-2 in promoting bone formation *in vivo*. However, more studies focusing on specific quantitative analyses are needed to confirm this finding. Interestingly, in preliminary experiments in a rat nonunion fracture model, AdLMP3 gene transfer results in more rapid new bone formation and healing than gene transfer of Ad.BMP-2, consistent with the results in the ectopic bone formation model.

Taken together, our results provide further evidence of an important role of LMP proteins in pathways involved in mineralization and bone formation. In particular, the ability of hLMP-3, whose structure does not include LIM domains/motif, to induce upregulation of bone-specific genes, mineralization, and ectopic bone formation supports the hypothesis that the LIM protein–protein interaction domains are not required for LMP3 function. Indeed, in preliminary studies, fusion of only 20 aa from the unique region in LMP3 to enhanced green fluorescent protein (EGFP) resulted in a protein able to induce bone-specific gene expression in culture and ectopic bone formation *in vivo*, similar to the full-length LMP3. The ability of only a 20 aa fragment from LMP3 to induce bone formation following gene transfer suggests that bone formation could be enhanced or induced by delivery of a small LMP3-derived peptide using a protein transduction domain, allowing for clinical applications of LMP3 in bone healing. Interestingly, in initial experiments using an LMP3-derived peptide fused to a protein transduction domain, induction of bone-specific gene expression was observed in cell culture experiments. Clearly, LMP3 and fragments derived from LMP3 are potent inducers of bone-specific gene expression, mineralization, and ectopic bone formation. Thus it may be possible to develop bone healing approaches using not only gene transfer of LMP-3,but also protein transduction-mediated delivery of LMP-3.

Materials and methods

Codon-optimized LMP3 gene synthesis

The codon-optimized LMP3 cDNA was generated using overlapping primers technique as previously described.[29] Briefly, oligo primers were synthesized using standard phosphoramidite chemistry. Equal volumes from each 100 pM/μl oligonucleotide solutions were combined and the mixture subsequently diluted 40-fold in 50 μl of PCR mix containing 10 mM Tris-HCl pH 9.0, 2.2 mM $MgCl_2$, 50 mM KCl, 0.2 mM each dNTP, 0.1% Triton X-100, 1 μl *Taq* polymerase/0.02 U *Pfu* polymerase. The PCR program consisted of 55 cycles at 94°C for 30 s,

52°C for 30 s, and 72°C for 30 s. The gene assembly reaction mixture was diluted 40-fold in 100 μl PCR mix containing 10 mM Tris-HCl pH 9.0, 2.2 mM $MgCl_2$, 50 mM KCl, 0.2 mM each dNTP, 0.1% Triton X-100, 5 U *Taq* polymerase/0.1 U *Pfu* polymerase and the two outside primers at a concentration of 1 μM. The outside primers were the same as the two oligonucleotides representing the 5′ ends of the plus and minus strands. The PCR program consisted of 25 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 60 s. The PCR products were separated on a 1% agarose gel. The appropriate band was purified using a Gel Extraction Kit (Qiagen), then cloned into pcDNA3.1 TOPO T/A cloning plasmid (Invitrogen), and subsequently sequenced (Figure 1).

Plasmid and recombinant adenovirus construction

E1- and E3-deleted adenoviral vector expressing human LMP3 (Ad-hLMP3) was constructed through Crelox recombination with reagents generously provided by Dr S Hardy. Briefly, a *Sal*I–*Not*I fragment containing hLMP3 from the plasmid pcDNA3.1/hLMP3 was inserted in a modified version of the shuttle vector pAdlox (GenBank U62024). E1/E3-substituted recombinant adenovirus was generated by cotransfection of *Sfi*I-digested shuttle plasmid of pAdlox/hLMP3 and Ψ5 helper virus DNA into the adenoviral packaging cell line CRE8. Adenoviruses were propagated on CRE8 cells, purified by cesium chloride density gradient centrifugation and subsequent dialyzed according to standard protocols, and stored at −70°C.

Cell culture

MC3T3-E1 cells are a murine preosteoblast cell line that undergoes osteoblast maturation when exposed to ascorbic acid *in vitro*.[30,31] These cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS with 100 U/ml penicillin and 100 μg/ml streptomycin, with a change of media every 3–4 days. The NIH3T3 fibroblast cell line was grown in DMEM medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. hMSCs were cultured in mesenchymal stem cell basal medium (MSCBM) supplemented with 10% mesenchymal stem cell growth supplement, 2% 200 mM L-glutamine, penicillin (25 U/ml), and streptomycin (25 μg/ml). All the reagents were purchased from Cambrex Bio Science Walkersville. The cells were plated at 2×10^6 cells per dish in 60 mm dishes, and grown until 80% confluent. All the cells were incubated in a humidified incubator with 5% CO_2 at 37°C.

Bone gene expression

Expression of specific bone genes (OC, OP, BSP) was evaluated in both the cell lines by RT-PCR 48 h after transfection with plasmids carrying LMP-3, BMP-2 and empty vector, and 48 h after infection with the adenoviral constructs for LMP-3, BMP-2 and empty vector (AdΨ5), respectively.

In vitro transient transfection for bone gene expression

MC3T3 and NIH3T3 cells were seeded in 60 mm^2 dishes for 72 h before transfection. Both cell lines were transfected with the pcDNA3.1 expression vector containing LMP-3. Cells transfected with the pcDNA3.1 expression vector containing BMP-2 were used as positive control, while cells transfected with the pcDNA3.1 empty expression vector were used as negative control. A pcDNA expression vector containing EGFP was used to analyze transfection efficiency. Briefly, a solution of chloroquine (100 μM) was used to prepare the cells before the transfection. Then, each plasmid was added in a mixture of 2× HBES buffer, distilled water and calcium chloride, and applied to the cells. The medium was changed 14 h post-transfection.

Adenoviral infection

Both MC3T3 and NIH3T3 cells were infected with the AdLMP-3, AdBMP-2, and AdΨ5 adenoviral vectors at an MOI of 4×10^4 vp/cell. Noninfected cells were used as negative control. The media were removed 48 h after infection to evaluate bone gene expression.

RNA isolation

For RNA isolation, growth media were removed at the indicated time points after transfection and infection, and total RNA was isolated using a Rneasy Mini Kit (Qiagen), following the manufacturer's specifications. RNA was treated with RQ1 RNase-free DNase, precipitated in a 2 mol/l LiCl solution, and then resuspended in water. RNA concentration was determined spectrophotometrically.

RT-PCR analysis

RT-PCR analysis was carried out using the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen). In all, 2 μl of the obtained cDNA was amplified in each PCR in a 50 μl reaction mixture containing 5 U/μl of *Taq* DNA polymerase, 10 μM dNTP mix, 50 μM $MgCl_2$, 5 μl 10× PCR buffer minus Mg, and 10 μM of sense and antisense primers. Amplifications were performed in a GeneAmp 9600 thermal cycler for 30 cycles (92°C for 30 s, 55°C for 30 s, 72°C for 30 s) after an initial denaturation at 92°C for 2 min. PCR products were then size separated by electrophoresis in 1% agarose gels. Primer sequences for the analyzed genes were: osteopontin (mOSPNT) Fw 5′-CATCTCAGAAGCAGAATCTC-3′, Rev 5′-GCCTATCATCTTCCTTACTC-3′; osteocalcin (mOC) Fw 5′-TGAGTCTGACAAAGCCTTC-3′, Rev 5′-CTGCTGTGACATCCATACTTG-3′; bone sialoprotein (mBSP) Fw 5′-GCATGCCTACTTTTATCCTC-3′, Rev 5′-GTTCTCGTTGTCATAGACTTC-3′.

In vitro mineralization

To evaluate the *in vitro* mineralization, both the cell lines MC3T3-E1 and NIH3T3 were transfected with LMP-3, BMP-2 and empty vector, and infected with AdLMP-3, AdBMP-2 and AdΨ5, respectively. Then, the cells were seeded in six-well plates and grown for 10 days (MC3T3) and 20 days (NIH3T3) in media supplemented with 10 μg/μl of inorganic phosphate and 10 μg/μl of ascorbic acid. Von Kossa staining was used to evaluate mineralization. In particular, cells were fixed in 10% buffered formalin for 1 h at room temperature and then washed with PBS and distilled water. After fixation, cells were treated with 5% silver nitrate and left under light for 1 h. Then, 5% sodium thiosulfate was added for 2 min and the staining was evaluated with 2.5% fuschin acid.

Infection conditions for qRT-PCR

Each cell culture was transduced with 50 µl per dish of adenoviral-construct aliquot of AdLMP-3 diluted in 4 ml of medium for a final MOI of 4×10^4 vp/cell. We used AdΨ5 as viral control. Cells that were grown within nontreated medium served as basal control. After 24 h, the medium was supplemented with 10 µg/µl of inorganic phosphate and 10 µg/µl of ascorbic acid. Two dishes were treated for 2 and 4 days reapplying the CM collected from cell culture 48 h after infection with AdLMP-3, and incubated at 50°C for 1 h to inactivate the virus.

In order to evaluate the dose-dependent effects of Ad.LMP-3 on bone gene expression, adenoviral suspension was diluted 2-, 20-, and 200-fold in 10% trealose buffer and each dilution was used for the infection of hMSCs, obtaining an MOI of 2×10^4, 2×10^3, and 2×10^2 vp/cell. Then the cells were treated as previously described for the other experiments.

RNA isolation for qRT-PCR

RNA isolation was performed at days 2 and 4 after adenoviral infection using an Rneasy Mini Kit (Qiagen) following the manufacturer's specifications. Briefly, the cells were harvested from each dish at the specified time points, lysed with 1% βME buffer and homogenized by centrifugation through QIAshredder columns (Qiagen). Then RNA was extracted by 70% ethanol precipitation and purified by three washing steps, after on-column DNase treatment performed with RNase-free DNase Set (Qiagen), and eluted in RNase-free water. RNA concentration was determined spectrophotometrically.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) analysis was performed using Taqman® One-step RT-PCR Master Mix. The reaction was carried out in a final volume of 50 µl, adding 10–30 ng of total RNA, 50–200 nM of sequence-specific primers, and 100 nM of Taqman® probes. Primers and Taqman probes used in this experiment are shown in Table 1. 18S primers and probes were designed by and purchased from Applied Biosystems. The assays were carried out in triplicate on an ABI Prism 7000 Sequence Detection System. Thermocycling conditions were as follows: the reverse transcription step at 48°C for 30 min, then the initial denaturation at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 45 s (annealing and extension). The threshold was set above the nontemplate control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted C_T).

Gene expression values were calculated based on the comparative $\Delta\Delta C_T$ method (separate tubes) detailed in Applied Biosystems Bulletin #2 (Biosystems, updated 10/2001 #57). Validation experiments demonstrated approximately the same efficiencies of target and reference gene amplification for each primer/probe set: the absolute value of the slope of log input amount *versus* $C_T < 0.1$ (data not shown). Target genes were normalized to the reference housekeeping gene, 18S. Fold differences were calculated for each treatment group using normalized C_T values for the negative control at the appropriate time point as the calibrator. If no baseline expression of

Table 1 Primers and Taqman probes

Gene	*Genebank no.*	*Forward primer*	*Reverse primer*	*Taqman probe*	*Reporter/quencher*
Human Runx2	NM 004348	AACCCACGAATGCACTATCCA	CGGACATACCGAGGGACATG	CCTTTACTTACACCCCGCCAGTCACCTC	FAM/TAMRA
Human alk. phosphatase	XM 001826	CCGTGGCAACTCTATCTTTGG	GCCATACAGGATGGCAGTGA	CATGCTGAGTGACACAGACAAGAAGCCC	FAM/TAMRA
Human OSX	AF477981	CCCCACCTCTTGCAACCA	CCTTCTAGCTGCCCCACTATTTCC	CCAGCATGTCTTGCCCCAAGATGTCTA	FAM/TAMRA
Human BMP-2	NM_001200	GGGCATCCTCTCCACAAAAG	TTACAGCTGGACTTAAGGCGTTT	AAAACGTCAAGCCAAACACAAACAGCG	FAM/TAMRA

target gene was detectable, then the total amount of RNA was calculated. The statistical analyses of the qPCR results were performed calculating the coefficient of variation (COV) from three assay replicates. For all treatment groups and target genes analyzed, the COV did not exceed 3%.

In vitro mineralization

To evaluate the *in vitro* mineralization in hMSCs, we infected the cells with AdLMP-3, AdBMP-2, and AdΨ5 at the same above-cited MOI. Noninfected cells were used as negative control. Then, the cells were seeded in six-well plates and grown for 10 days in media supplemented with 10 μg/μl of inorganic phosphate and 10 μg/μl of ascorbic acid. To evaluate mineralization, the Von Kossa staining was performed following the same above-cited protocol used for MC3T3 and NIH3T3.

In vivo ectopic bone formation

Male 8–12 weeks old C57BL/6J mice were used in the *in vivo* experiments. All the animals were anesthetized with an intramuscular injection of methoxyflurane. Both hind limbs of each animal were sterilized with 70% ethanol and a gas-tight syringe was used to inject 1.5×10^7 PFU of AdLMP-3 virus bilaterally into the exposed triceps surae musculature of 12 mice. A total of 12 mice received bilateral injections of 1.5×10^7 PFU of AdBMP-2 and were used as positive control. Finally, 1.5×10^7 PFU of AdΨ5 was injected bilaterally in 12 mice as negative control. After injections, the skin was closed with a surgical stapler and the animals were kept on a hot plate until they had recovered from anesthesia. Mice were killed at 2, 3, and 5 weeks following transfection, by injection of methoxyflurane followed by cervical dislocation. In particular, four mice, corresponding to eight injected muscles, were killed for each group at the above-cited time points. Both hind limbs of each animal were harvested and radiographs were obtained. The musculature was then dissected from the rest of the hind limbs, put in OCT Tissue-Tek compound and frozen in liquid nitrogen. In all, 8 μm thick frozen sections were cut by using a cryostat. Slides were fixed in 1% glutaraldehyde and underwent standard hematoxylin–eosin staining with Von Kossa staining.

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