In vitro models of bone-forming tumours: from target to treatment

Franceschini, N.

Part I

In vitro models of bone-forming tumours

Chapter 3

Truncated FOS reduces osteogenic differentiation capacity in osteoid osteoma and osteoblastoma

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Abstract

Osteoid osteoma and osteoblastoma are non-malignant bone-forming tumours of the skeleton, characterized by the presence of irregular trabeculae of woven bone. Rearrangements in *FOS,* and less frequently *FOSB*, have recently been identified in osteoid osteoma and osteoblastoma. Identical rearrangements in *FO*S were previously detected in epithelioid hemangioma, where this leads to truncation of the FOS protein in the C-terminal domain causing increased protein stability due to impaired degradation. Since FOS plays a role in osteogenic differentiation, we investigated the effect of FOS truncation on osteogenic differentiation and proliferation in an *in vitro* model for osteoid osteoma and osteoblastoma. In this model, fetal mesenchymal stem cells have been transduced to overexpress truncated (FOSΔ) or full-length FOS as a comparison. Osteogenic differentiation - assessed by measuring mineralization, ALP expression and ALP activity - and proliferation rate were reduced in cells overexpressing truncated FOS (FOSΔ), or FOS with a deletion (FOSΔ376-377) or mutation (FOSL376N) in the C-terminal domain, compared to wild-type mesenchymal stem cells. In contrast, cells overexpressing full-length FOS showed increased proliferation and almost completely lost mineralization. These results demonstrate that the balance between differentiation and proliferation can be disrupted by changes in FOS expression, which may explain the indolent growth and formation of woven bone observed in osteoid osteoma and osteoblastoma.

Introduction

Osteoid osteoma and osteoblastoma are bone-forming tumours of the skeleton. Whereas osteoid osteomas are small in size (<2 cm) and benign, osteoblastomas are larger and can be locally aggressive (1). Despite differences in clinical presentation, the histology of these entities is identical. Both osteoid osteoma and osteoblastoma are composed of trabeculae of woven bone, which can show various degrees of mineralization of the matrix (1). These trabeculae are lined by plump osteoblast-like cells. Another benign tumour that shows morphological similarities to osteoid osteoma and osteoblastoma, but differs in clinical presentation, is cementoblastoma (2). This odontogenic tumour is characterized by the presence of immature cementum-like matrix containing activated cementoblasts, that are indistinguishable from osteoblasts.

Recently it was discovered that osteoid osteoma and osteoblastoma show overexpression of FOS or FOSB due to frequent rearrangements in *FOS* (87%) and – less frequent – *FOSB* (2%) (3). In cementoblastoma, rearrangements in *FOS* and overexpression of FOS have also been identified (2). For osteoid osteoma and osteoblastoma, the rearrangements in *FOS* involve exon 4, leading to a truncation of the FOS protein, as stop-codons are introduced near the breakpoints (**Figure 1A**). Rearrangements leading to truncation of FOS at the same exon in the gene have been previously identified in epithelioid haemangioma (4). Under normal conditions, FOS is a short-lived protein and its levels and stability are highly regulated by different mechanisms both on the RNA stability and post-translational level, including control via the 3'-UTR region, ubiquitin-dependent or ubiquitin-independent proteasomal degradation, and phosphorylation (5-11).
In epithelioid haemangioma, it was shown that truncated FOS was more resistant to proteasomal degradation, caused by the absence of a highly conserved destabilizing element (LLAL region) within the last four amino acids in the C-terminus of FOS in cells expressing truncated FOS (FOSΔ). The LLAL region acted as a signal for proteasomal degradation, causing cells carrying a mutation (FOSL376N) or deletion (FOSΔ376-377) within the LLAL region to sustain expression of FOS protein (4, 12).
Therefore the truncation of FOS found in osteoid osteoma, osteoblastoma and cementoblastoma lead to stabilization and thus sustained expression of FOS protein. Similar to epithelioid hemangioma, these tumours show strong nuclear expression of FOS in the tumour cells, and therefore overexpression of FOS can be used as a diagnostic tool (13, 14)(**Figure 1B)**.

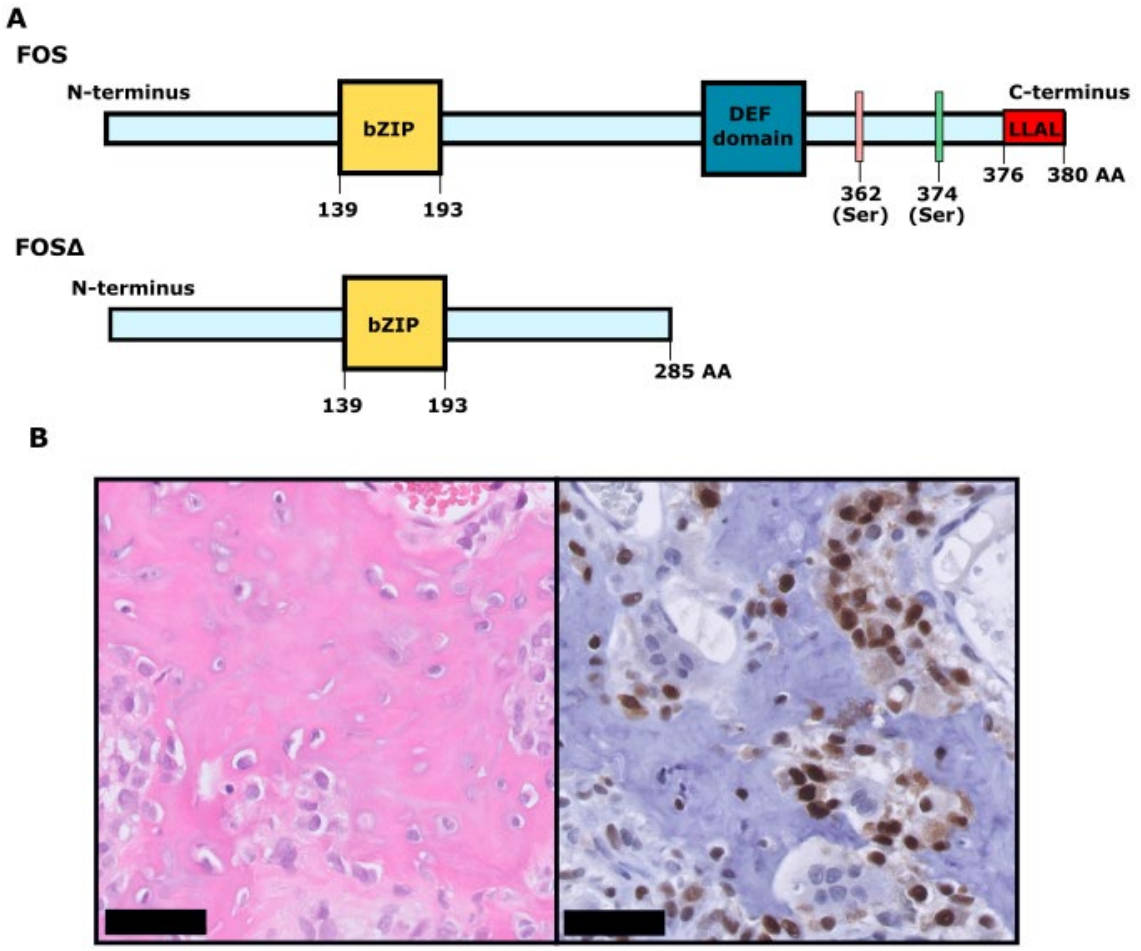

Figure 1. FOS is truncated and overexpressed in osteoid osteoma, osteoblastoma and cementoblastoma. **(A)** Schematic overview of full-length FOS protein (380 amino acids), and the truncated FOS (FOSΔ, 285 amino acids) protein found in osteoid osteoma, osteoblastoma, cementoblastoma and epithelioid haemangioma. Basic-region Leucine Zipper (bZIP) domain, DEF domain (binding site for MAPK) and the LLAL region within the C-terminus of FOS are indicated. The LLAL region is closely located to the phosphorylation sites Ser362 and Ser374. Figure adapted from (4) and (15). (**B**) Haematoxylin and eosin staining demonstrating immature woven bone deposited by osteoblasts (left panel) and FOS immunohistochemical staining (right) depicting overexpression of FOS in osteoid osteoma. Scale bar represents 50 µm.

FOS can form a heterodimer with JUN family proteins, to form the AP-1 complex, and act as a transcription factor. The AP-1 complex is involved in cell growth control and cell transformation, and a plethora of other essential cellular processes (16-18). Sustained FOS expression is sufficient for the transformation of cells (19). Interestingly, transgenic mice overexpressing FOS in bone develop osteosarcoma, although FOS overexpression itself is rarely identified in human osteosarcoma (13, 14, 20-22). The AP-1 complex and FOS also play a role in osteogenic differentiation. During osteogenic differentiation, the expression of FOS increases, which in turn further upregulates other genes involved in osteogenic differentiation (23, 24). For example, the promotors of key osteogenic genes such as alkaline phosphatase, collagen I and osteocalcin all have an AP-1 site (24). AP-1 has previously been

identified as a driver of tumorigenesis in vascular tumours epithelioid hemangioma and pseudomyogenic haemangioendothelioma, that contain similar *FOS,* or *FOSB,* rearrangements (4). It was found that HUVECs expressing truncated FOS displayed increased endothelial sprouting without changing proliferation. These results suggest AP-1 could be driving tumorigenesis both in the vascular as well as in the osteogenic lineage.

This study aims to investigate how alterations in FOS affect osteogenic differentiation and proliferation in osteoid osteoma and osteoblastoma. We hypothesized that truncation of FOS deregulates osteogenic differentiation and/or maturation and possibly proliferation leading to the characteristic presence of immature woven bone in these tumours. Since mesenchymal stem cells are the most likely progenitor cells of osteogenic tumours (25), fetal mesenchymal stem cells (fMSCs) were transduced to overexpress truncated FOS, or full-length FOS as a comparison. We carried out functional analysis of these fMSCs to assess osteogenic differentiation and proliferation. fMSCs overexpressing full-length FOS almost completely lost osteogenic differentiation capacity, whereas osteogenic differentiation was mildly impaired in fMSCs expressing truncated FOS. Furthermore, the proliferation of fMSCs expressing truncated FOS was decreased, whereas fMSCs expressing full-length FOS showed increased proliferation. These differences in osteogenic differentiation and proliferation appeared to be caused specifically by the LLAL region in the C-terminal domain of FOS, as fMSCs expressing FOS protein without the C-terminal proteasomal degradation signal showed similar impairment of proliferation and differentiation as fMSCs expressing FOS with larger truncated regions. Thus, these results demonstrate that the C-terminal domain of FOS protein plays an important role in the balance between osteogenic differentiation and proliferation and that its loss may explain the presence of woven bone and the indolent growth of osteoid osteoma and osteoblastoma.

Materials and Methods

Cell culture

Fetal mesenchymal stem cells (fMSCs) were derived from the femurs of a 22-week-old deceased fetus (abortion with unknown medical cause). fMSCs were collected based on individual written (parental) informed consent after approval by the Medical Ethics Committee of the Leiden University Medical Center (reference number: P08-087). The experiments involving human materials were performed in accordance with the principles outlined in the Declaration of Helsinki (World Medical Association). fMSCs (passage number between 5 and 13) were cultured in αMEM (Gibco) supplemented with 10% FBS, 1% non-essential amino acids (Gibco) and 1% pen/strep (Gibco) in a humidified incubator, with 5% CO_2 and at 37 °C. Cells were tested for mycoplasma.

Lentiviral transduction

FOS constructs have been generated as described previously (4). Briefly, four human FOS cDNAs were used, either containing full-length FOS (FOS FL), a FOS isoform lacking the C-terminal 95 amino acids but including the bZIP domain (FOSΔ, **Figure 1A**), a FOS isoform with a mutation (FOSL376N) or a deletion (FOSΔ376-377) within the C terminal four amino acids (LLAL) containing helical region. These cDNAs were C-terminally fused in-frame with a FLAG tag and cloned into the pLV lentiviral vector, under control of a CMV promotor. For transfection, lentivirus together with polybrene (8 µg/ml) was added to fMSCs. The next day, a medium containing lentivirus was removed and transduced cells were selected with puromycin (5 ng/ml).

Osteogenic differentiation

Cells were seeded at 5000 cells/cm^2 for osteogenic differentiation. One day after seeding, cells were treated with osteogenic compounds: β-glycerophosphate (5 mM, Sigma-Aldrich), dexamethasone (0.1 µM, Sigma-Aldrich), and ascorbate-2-phosphate (0.15 mM, Sigma-Aldrich). Medium with osteogenic compounds was refreshed twice a week. As a negative control, cells were cultured without any osteogenic compounds. Osteogenic differentiation was performed at least three times in independent experiments, by three independent researchers.

Alkaline phosphatase activity assay

After ten days of osteogenic differentiation, cells were lysed with PBS/Triton 0.1% and incubated with pNPP (P7998, Sigma-Aldrich) for 4 minutes. The reaction was stopped with NaOH. Absorption at 405 nm was measured using a microplate reader (Infinite M Plex, Tecan Group Ltd., Zürich, Switzerland). Experiments were performed in triplicate at least three times.

RT-PCR

After one, two or three weeks of osteogenic differentiation, RNA was isolated using Trizol according to the manufacturer's instructions. cDNA synthesis was performed using iScript cDNA Synthesis Kit (1708890, Bio-rad) according to the manufacturer's instructions. For RT-qPCR, iQ SYBR Green Supermix (1708880, Bio-rad) and a Thermal Cycler (Bio-rad) were used, with primers for osteogenic markers *ALPL, COL1A1, SPARC, RUNX2, SPP1* and *BGLAP*. *GAPDH* was used as a reference gene. A list of primers used can be found in **Table 1**. Relative gene expression levels to reference genes were determined with the following formula: $2^{\text{Ct value reference gene – Ct value gene of interest.}}$ Experiments were performed at least two times in duplicate.

Table 1. List of primers

Gene	Forward primer	Reverse primer
ALPL	TCACTCTCCGAGATGGTGGT	GCCTGCTTGGCTTTTCCTTC
COL1A1	AAGACGAAGACATCCCACCAAT	GTCACAGATCACGTCATCGCA
SPARC	CTGGACTACATCGGGCCTTG	CAGGACGTTCTTGAGCCAGT
RUNX2	CCCTGAACTCTGCACCAAGT	GGCTCAGGTAGGAGGGGTAA
SPP1	TTCGCAGACCTGACATCCAG	ACGGCTGTCCCAATCAGAAG
BGLAP	CCTCACACTCCTCGCCCTAT	GCTTGGACACAAAGGCTGCAC
GAPDH	TTCCAGGAGCGAGATCCCT	CACCCATGACGAACATGGG
FOS	GAGAAAAGGAGAATCCGAAGG	GTCAGAGGAAGGCTCATTGC

Alizarin Red staining

After three weeks of osteogenic differentiation, mineralization was determined by Alizarin Red staining. Cells were fixed for 1 hour in cold ethanol and incubated with Alizarin Red S staining solution (2 g Alizarin Red S (02100375, MP Biomedicals, Thermo Fisher Scientific, Waltham, MA, USA) in 60 mL water, pH 4.2) for 5 minutes. For quantification, Alizarin Red staining was rinsed with water and 10% acetic acid was added to each well for 30 minutes. Acetic acid solution containing cells was transferred to a microcentrifuge tube and vortexed. Samples were heated at 85 °C for 10 minutes and centrifuged at 12000 rpm for 15 minutes. The supernatant of each sample was transferred to a 96-well plate and 10% ammonium hydroxide was added to each well to neutralize the acid. Absorbance at 405 nm was measured using a microplate reader (Infinite M Plex, Tecan Group Ltd.). Experiments were performed in triplicate at least three times.

Proliferation assays

To determine proliferation, cells were seeded at 1500 cells per well of a 96-well plate. One, two, three, four or five days after seeding, cells were washed with PBS and incubated with Presto Blue cell viability reagent (Invitrogen, Thermo Fisher Scientific) for 60 minutes and measured using a microplate reader (Infinite M Plex, Tecan Group Ltd.). After read-out, cells were fixed with 4% formaldehyde and stained with 2 µg/mL Hoechst (Invitrogen, Thermo Fisher Scientific). Nuclei were counted using the Cellomics ArrayScan VTI HCS 700 and HCS Studio Cell Analysis Software (Thermo Fisher Scientific). Experiments were performed at least three times in triplicate.

Statistical analysis

All statistical analyses were performed using GraphPad Prism V.9. For multiple comparisons between groups, a Kruskal–Wallis test was used. Groups were compared to pLV. Comparisons were considered statistically significant using a significance level of 5%.

Results

Mesenchymal stem cells expressing truncated FOS have reduced osteogenic differentiation capacity

Fetal mesenchymal stem cells (fMSCs) transduced with constructs containing full-length FOS or truncated FOS (FOSΔ), both showed overexpression of FOS at mRNA level (**Figure 2A**). These cells were used to determine the effect of FOS truncation on osteogenic differentiation. After culture in osteogenic medium, Alizarin Red staining revealed that fMSCs expressing truncated FOS showed a reduction of mineralization ($P = 0.02$), whereas fMSCs expressing full-length FOS lost osteogenic differentiation capacity, almost completely lacking mineralization ($P \leq 0.0001$) (**Figure 2B**). The expression levels of osteogenic markers during three weeks of osteogenic differentiation were variable. Osteogenic markers *BGLAP, OPN* and *RUNX2* showed hardly any expression in fMSCs and therefore no difference between the different constructs could be determined (**Figure S1**). Gene expression of *SPARC* and *COL1A1* was induced upon osteogenic differentiation but with no marked difference between wild-type fMSCs and fMSCs overexpressing FOS (full length or truncated) (**Figure S1**). *ALPL* RNA expression and ALP activity showed a trend towards reduction in fMSCs overexpressing truncated FOS compared to wild-type fMSCs (pLV), although this was not statistically significant ($P = 0.2$ for *ALPL* expression and $P = 0.1$ for ALP activity) (**Figure 2C, 2D**). fMSCs overexpressing full length FOS further reduced *ALPL* expression (although not significant, $P = 0.2$) and ALP activity ($P \leq 0.0001$) compared to wild-type fMSCs (pLV) (**Figure 2C, 2D**).

Mesenchymal stem cells expressing truncated FOS show decreased proliferation

Since sustained expression of FOS is known to induce transformation of cells (19, 26), the proliferation rate of fMSCs expressing full length or truncated FOS was determined. Cell viability, as well as nuclei count, was assessed over a period of five days (**Figure 3A, 3B**). fMSCs expressing truncated FOS (FOSΔ) showed lower proliferation rates compared to wild-type fMSCs ($P \leq 0.05$), whereas fMSCs expressing full-length FOS showed an increase in proliferation rate ($P \leq 0.05$).

Mutations or deletions in the C-terminal region of FOS protein impair osteogenic differentiation capacity similar to truncated FOS

Proliferation rate and osteogenic differentiation capacity were determined for additional FOS constructs to explore whether disruption of the C-terminal region of FOS protein can explain the changes in proliferation and osteogenic differentiation in fMSCs expressing truncated FOS. fMSCs expressing FOSL376N or FOSΔ376-377, which are both alterations located within the LLAL region of the C-terminus of FOS, showed similarly diminished proliferation rate (**Figure 4A**) and osteogenic differentiation capacity as fMSCs expressing truncated FOS (FOSΔ), as measured by mineralization content (**Figure 4B**) and alkaline phosphatase activity (**Figure 4C**). These results indicate that disruption of the helical region within the C-terminus of FOS impairs osteogenic differentiation capacity.

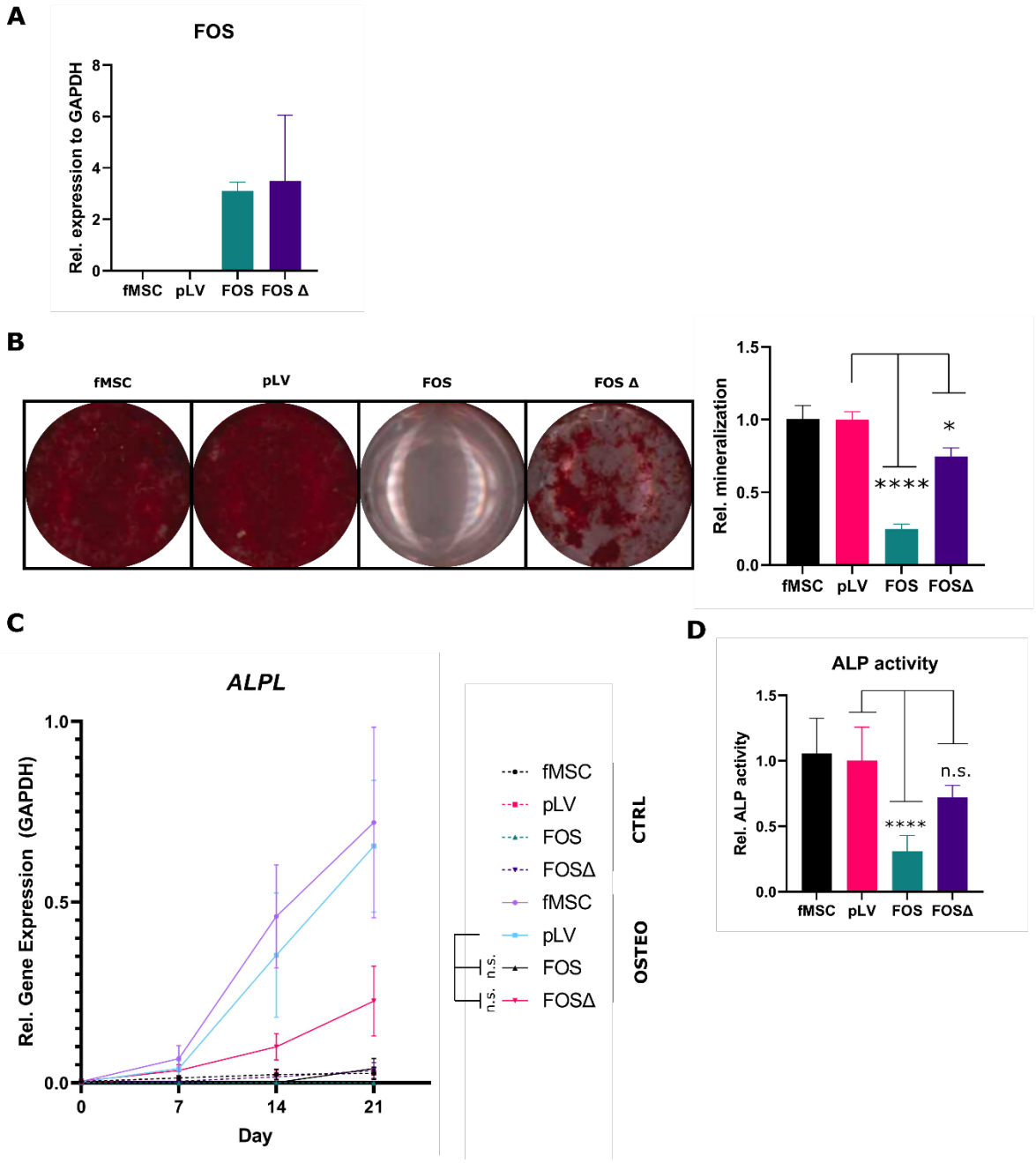

Figure 2. Osteogenic differentiation is reduced upon overexpression of truncated FOS. (**A**) FOS mRNA was overexpressed in fMSCs transduced with full-length FOS (FOS) or truncated FOS (FOSΔ). Each bar represents the mean of two experiments performed in duplicate ± standard deviation. (**B)** fMSCs expressing truncated FOS (FOSΔ) show reduced mineralization, while mineralization is almost completely absent in fMSCs expressing full-length FOS (FOS). For each condition, one representative image is shown. Mineralization was quantified relative to empty vector transduced fMSCs (pLV) and each bar represents the mean of three experiments performed in triplicate ± standard deviation. Groups were compared to pLV, using the Kruskal – Wallis test. * = P ≤ 0.05; **** = P ≤ 0.0001. (**C**) Expression of osteogenic marker *ALPL* is induced during osteogenic differentiation. Representative data from one experiment is shown. Statistical analysis was performed based on the mean of three experiments performed by two independent researchers in duplicate that all showed a similar trend as to the data shown. (**D**) Alkaline phosphatase activity was determined in fMSCs, relative to empty vector transduced fMSC (pLV). Each bar represents the mean of four experiments performed in triplicate ± standard deviation. Groups were compared to pLV, using the Kruskal – Wallis test. * = P ≤ 0.05 ; **** = P ≤ 0.0001; n.s. = not statistically significant, p > 0.05

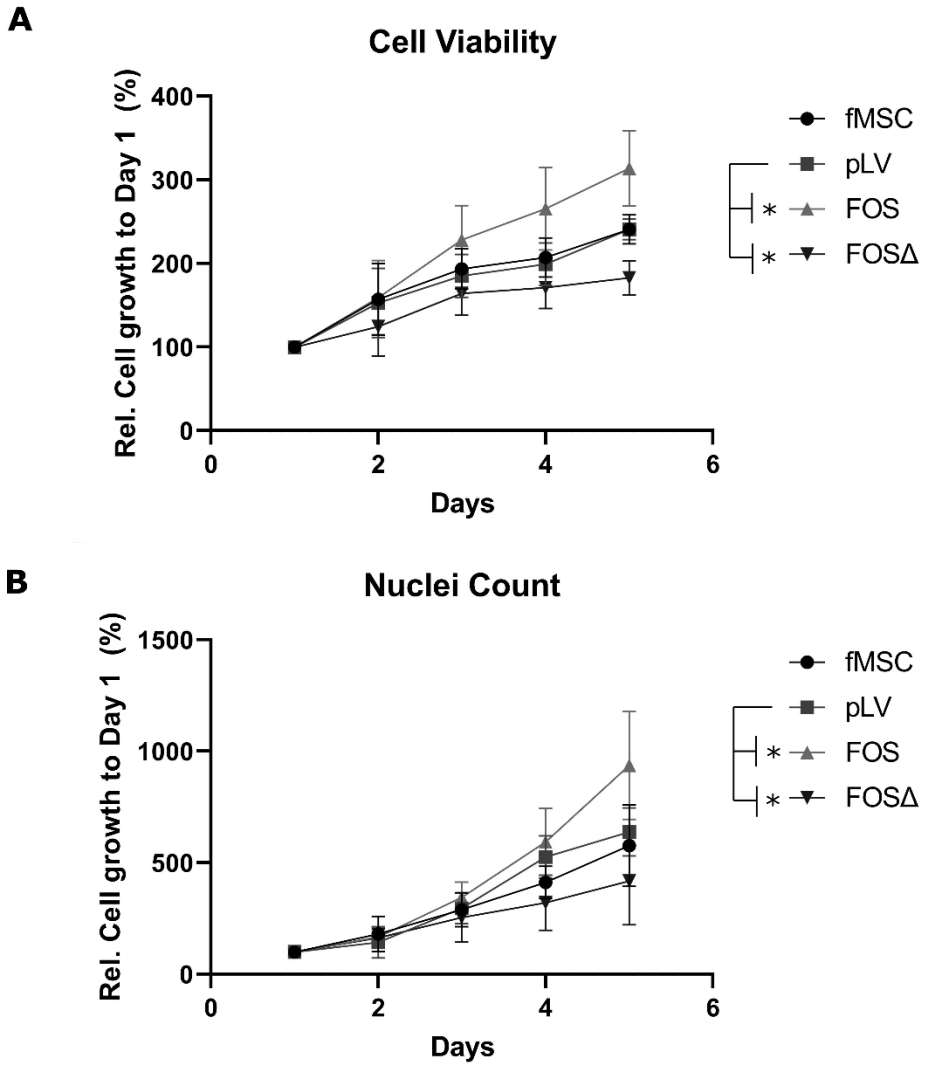

Figure 3. The proliferation rate of fMSCs with the various constructs. Both cell viability (**A**) and nuclei count (**B**) was reduced in fMSCs expressing truncated FOS (FOSΔ) compared to untransduced (fMSC) and empty vector transduced (pLV) fMSCs, whereas this was increased in cells expressing full-length FOS Each data point represents the mean of three experiments performed in triplicate ± standard deviation. Groups were compared to pLV. * = $P \leq 0.05$

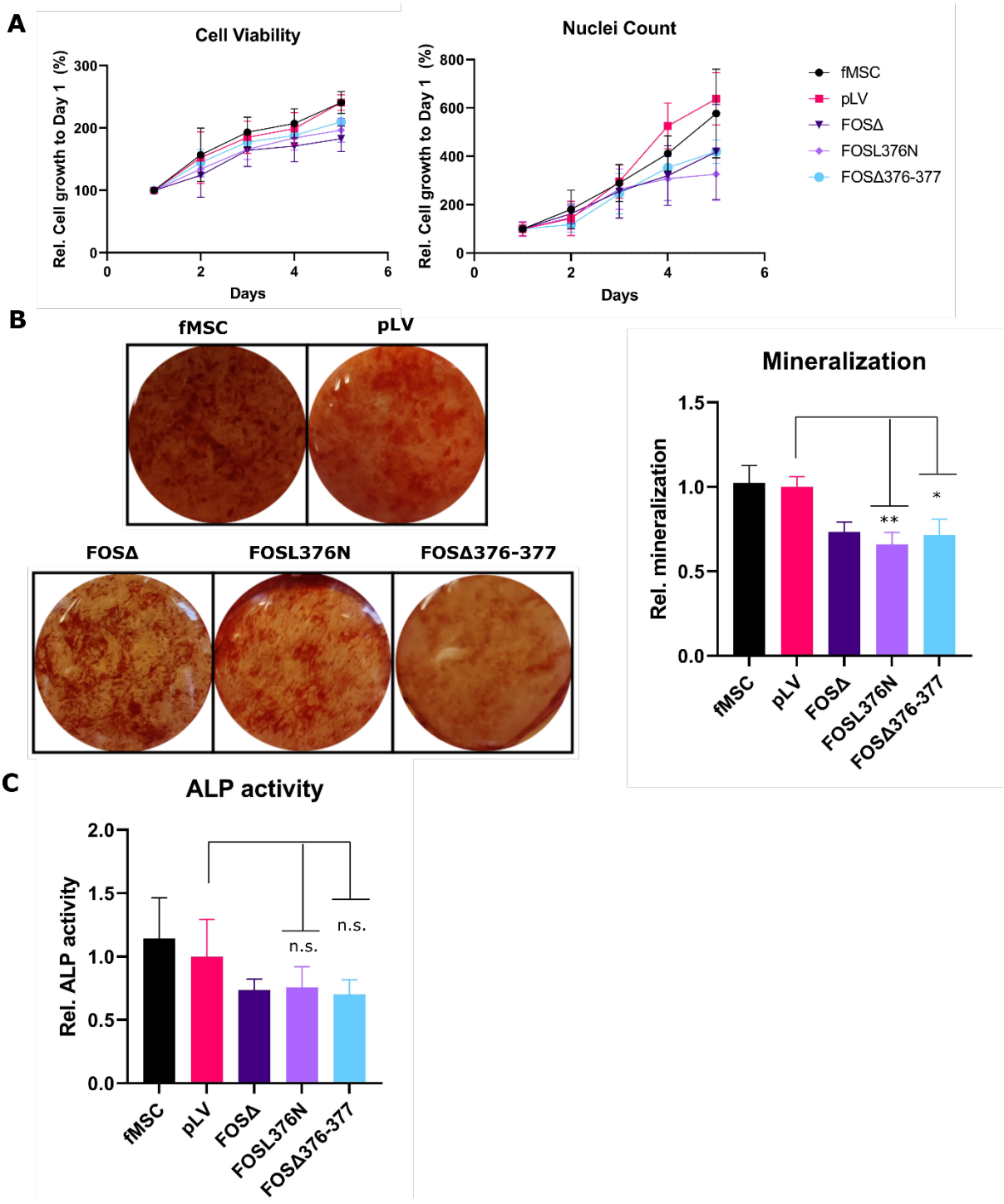

Figure 4. Osteogenic differentiation capacity and proliferation of fMSCs expressing FOS harbouring alterations in LLAL region. (**A**) Cell viability and nuclei count showed a similar proliferation rate in fMSCs expressing truncated FOS (FOSΔ), FOS with a deletion (FOSΔ376-377) or mutation (FOSL376N) in the LLAL region of the C-terminal domain. (**B**) Mineralization, as measured by Alizarin Red staining of FOSL376N or FOSΔ376-377, is similar to truncated FOS (FOSΔ). One representative image per condition is shown. Mineralization was quantified relative to empty vector transduced fMSCs (pLV) and each bar represents the mean of three experiments performed in triplicate ± standard deviation. (**C**) Alkaline phosphatase activity was determined in fMSCs, relative to empty vector transduced fMSCs (pLV), which was comparable among fMSCs expressing truncated FOS (FOSΔ), FOS with a deletion (FOSΔ376-377) or mutation (FOSL376N) in the LLAL region of the C-terminal domain. Each bar represents the mean of three experiments performed in triplicate ± standard deviation. Groups were compared to pLV using the Kruskal – Wallis test. ** = P ≤ 0.01; * = P ≤ 0.05; n.s. = not statistically significant, p > 0.05

Discussion

Osteoid osteoma, osteoblastoma and cementoblastoma show frequent rearrangements of the *FOS*- gene with various fusion partners that lead to truncation of the FOS protein. The truncation was previously identified in epithelioid hemangioma and was shown to cause resistance to protein degradation resulting in overexpression of the truncated FOS protein (4).

In this study, we investigated how truncated FOS affects osteogenic differentiation, since osteoid osteoma, osteoblastoma and cementoblastoma are characterized by the presence of immature woven bone. To investigate this, fMSCs transduced with lentiviral vectors driving expression of truncated FOS were compared to wild-type fMSCs and fMSCs overexpressing full-length FOS. In fMSCs expressing truncated FOS, mineralization was reduced, and alkaline phosphatase expression and activity showed a trend towards reduction, compared to wild-type fMSCs. Not only fMSCs expressing truncated FOS, but also fMSCs expressing FOS with a disrupted helical region of the C-terminus (FOSL376N and FOSΔ376-377) show similar results. As the helical region of FOS contains a signal for proteasomal degradation, these results suggest that the disruption of the helical region impairs osteogenic differentiation.

It was previously shown that cells expressing FOS constructs with a disrupted helical region of the C-terminus (FOSL376N and FOSΔ376-377) have impaired proteasomal degradation (4). Combined with our results, this suggests that sustained expression of FOS, either by truncation of FOS or disruption of the helical region in the C-terminus of FOS, causes the impairment of osteogenic differentiation and proliferation.
Interestingly, fMSCs that overexpress full-length FOS completely lost osteogenic differentiation capacity, which is in line with previous results that showed that human mesenchymal stem cells expressing FOS lost both osteogenic and adipogenic differentiation (27). The difference in osteogenic differentiation and proliferation as a result of overexpression of full-length FOS or truncated FOS in fMSCs is interesting. It is known that sustained expression of full-length FOS leads to the transformation of cells (19, 26). In line with this, we observed that fMSCs expressing full-length FOS increased proliferation compared to fMSCs expressing truncated FOS and wildtype cells, which is in line with a previous study using MSCs (27). As differentiation and proliferation are carefully balanced cellular processes (28), it is not surprising that the same fast-growing cells lose osteogenic differentiation capacity. The difference in proliferation and differentiation observed in fMSCs expressing truncated or full-length FOS is in line with the phenotypical difference between osteosarcoma, a malignant bone tumour, and the non-malignant bone-forming tumours osteoid osteoma and osteoblastoma (1). Osteoid osteoma and osteoblastoma have a limited growth capacity with reduced proliferation as compared to osteosarcoma and can show non-mineralized and mineralized woven bone (1). In fMSCs expressing truncated FOS, we observed slower proliferation rates compared to full-length FOS, and reduced osteogenic

differentiation capacity. Thus, fMSCs overexpressing truncated FOS can explain the more indolent growth with the presence of organized, immature woven bone observed in osteoid osteoma and osteoblastoma. These data suggest that the definitive phenotype is a careful balance between differentiation and proliferation.
A small subset of osteoid osteoma and osteoblastoma lack alterations in FOS (29). Instead, there is a subgroup that shows *FOSB* rearrangements, or loss of *NF2* (3, 29). Truncation of FOSB would likely lead to similar changes in osteogenic differentiation since both FOS and FOSB can form parts of the A.P1 complex. However, further research is needed to elucidate how FOSB and NF2 contribute to the formation of osteoid osteoma and osteoblastoma.

Another open question remains how (truncation of) FOS is able to confer these cellular changes in osteoid osteoma and osteoblastoma. FOS protein levels and protein stability are tightly regulated, and one of these regulatory processes involve phosphorylation of FOS. Phosphorylation of FOS leads to increased stability, and several phosphorylation sites at the C-terminus of FOS are binding sites for kinases of the RSK or the MAPK protein family such as ERK, JNK and p38 (30, 31). A previous study has shown that phosphorylation of FOS by RSK or MAPK usually start at Ser362 and Ser374 in the C-terminus which facilitates further phosphorylation of FOS (32). In this study we have used fetal MSCs overexpressing truncated FOS lacking the phosphorylation sites near the C-terminus. It is possible that changes in the phosphorylation status can confer changes in RSK or MAPK signalling, which alters cellular processes and ultimately leads to osteoid osteoma and osteoblastoma. Unfortunately, it was not possible to further explore this in the current study due to the lack of reliable antibodies detecting phosphorylated FOS.

In conclusion, we have demonstrated that overexpression of truncated FOS reduces osteogenic differentiation and proliferation in fMSCs. This may explain the indolent growth of osteoid osteoma and osteoblastoma and the presence of immature woven bone in these tumours.

Acknowledgements

The authors would like to thank Hans Baelde, and Pauline Wijers-Koster for technical assistance and discussion.

References

1. WHO classification of tumours of soft tissue and bone, 5th edition. Lyon, France: WHO Classification of Tumours Editorial Board; 2020.
2. Lam SW, Cleven AHG, Briaire-de Bruijn IH, Schreuder WH, Kroon HM, Savci-Heijink DC, et al. FOS Rearrangement and Expression in Cementoblastoma. The American journal of surgical pathology. 2021;45(5):690-3.
3. Fittall MW, Mifsud W, Pillay N, Ye H, Strobl AC, Verfaillie A, et al. Recurrent rearrangements of FOS and FOSB define osteoblastoma. Nat Commun. 2018;9(1):2150.
4. van IDGP, Forghany Z, Liebelt F, Vertegaal AC, Jochemsen AG, Bovee J, et al. Functional analyses of a human vascular tumor FOS variant identify a novel degradation mechanism and a link to tumorigenesis. J Biol Chem. 2017;292(52):21282-90.
5. Ferrara P, Andermarcher E, Bossis G, Acquaviva C, Brockly F, Jariel-Encontre I, et al. The structural determinants responsible for c-Fos protein proteasomal degradation differ according to the conditions of expression. Oncogene. 2003;22(10):1461-74.
6. Stancovski I, Gonen H, Orian A, Schwartz AL, Ciechanover A. Degradation of the Proto-Oncogene Product c-Fos by the Ubiquitin Proteolytic System In Vivo and In Vitro: Identification and Characterization of the Conjugating Enzymes. Molecular and Cellular Biology. 1995;15:7106-16.
7. Han XR, Zha Z, Yuan HX, Feng X, Xia YK, Lei QY, et al. KDM2B/FBXL10 targets c-Fos for ubiquitylation and degradation in response to mitogenic stimulation. Oncogene. 2016;35(32):4179-90.
8. Adler J, Reuven N, Kahana C, Shaul Y. c-Fos proteasomal degradation is activated by a default mechanism, and its regulation by NAD(P)H:quinone oxidoreductase 1 determines c-Fos serum response kinetics. Mol Cell Biol. 2010;30(15):3767-78.
9. Sasaki T, Kojima H, Kishimoto R, Ikeda A, Kunimoto H, Nakajima K. Spatiotemporal regulation of c-Fos by ERK5 and the E3 ubiquitin ligase UBR1, and its biological role. Mol Cell. 2006;24(1):63-75.
10. Bossis G, Ferrara P, Acquaviva C, Jariel-Encontre I, Piechaczyk M. c-Fos proto-oncoprotein is degraded by the proteasome independently of its own ubiquitinylation in vivo. Mol Cell Biol. 2003;23(20):7425-36.
11. Acquaviva C, Salvat C, Brockly F, Bossis G, Ferrara P, Piechaczyk M, et al. Cellular and viral Fos proteins are degraded by dierent proteolytic systems. Oncogene. 2001;20:942-50.
12. Campbell KM, Terrell AR, Laybourn PJ, Lumb KJ. Intrinsic Structural Disorder of the C-Terminal Activatin Domain from the bZIP Transcription Factor FOS. Biochemistry. 2000;39:2708-13.
13. Lam SW, Cleven AHG, Kroon HM, Briaire-de Bruijn IH, Szuhai K, Bovee J. Utility of FOS as diagnostic marker for osteoid osteoma and osteoblastoma. Virchows Arch. 2020;476(3):455-63.
14. Amary F, Markert E, Berisha F, Ye H, Gerrand C, Cool P, et al. FOS Expression in Osteoid Osteoma and Osteoblastoma: A Valuable Ancillary Diagnostic Tool. The American journal of surgical pathology. 2019;43(12):1661-7.
15. Ofir R, Dwarki VJ, Rashid D, Verma IM. Phosphorylation of the C terminus of Fos protein is required for transcriptional transrepression of the c-fos promotor. Nature. 1990;348.
16. Shaulian E, Karin M. AP-1 in cell proliferation and survival. Oncogene. 2001;20.
17. Karin M, Liu ZG, Zandi E. AP-1 function and regulation. Current Opinion in Cell Biology. 1997;9(2):240-6.
18. Ozanne BW, Spence HJ, McGarry LC, Hennigan RF. Transcription factors control invasion: AP-1 the first among equals. Oncogene. 2007;26(1):1-10.
19. Miao GG, Curran T. Cell Transformation by c-fos Requires an Extended Period of Expression and Is Independent of the Cell Cycle. Molecular and Cellular Biology. 1994;14(6):4295-310.
20. Grigoriadis AE, Schellander K, Wang Z, Wagner EF. Osteoblasts are target cells for transformation in c-fos transgenic mice. The journal of Cell Biology. 1993;122(3):685-701.

21. Wang ZQ, Lian J, Schellander K, Wagner EF, Grigoriadis AE. c-fos-induced Osteosarcoma Formation in Transgenic Mice: Cooperativity with c-jun and the role of endogenous c-fos. Cancer Research. 1995;55:6244-51.
22. Franchi A, Calzolari A, Zampi G. Immunohistochemical detection of c-fos and c-jun expression in osseous and cartilaginous tumours of the skeleton. Virchows Arch. 1998;432:515-9.
23. Kang X, Sun Y, Zhang Z. Identification of key transcription factors - gene regulatory network related with osteogenic differentiation of human mesenchymal stem cells based on transcription factor prognosis system. Exp Ther Med. 2019;17(3):2113-22.
24. McCabe LR, Banerjee C, Kundu R, Harrison RJ, DObner PR, Stein JL, et al. Developmental Expression and Activities of specific FOS and Jun proteins are functionally related to Osteoblast Maturation. Endocrinology. 1996;137(10):4398-408.
25. Mohseny AB, Szuhai K, Romeo S, Buddingh EP, Briaire-de Bruijn I, de Jong D, et al. Osteosarcoma originates from mesenchymal stem cells in consequence of aneuploidization and genomic loss of Cdkn2. J Pathol. 2009;219(3):294-305.
26. Miller AD, Curran T, Verma IM. c-fos protein van induce cellular transformation: a novel mechanism of activation of a cellular oncogene. Cell. 1984;36:51-60.
27. Abarrategi A, Gambera S, Alfranca A, Rodriguez-Milla MA, Perez-Tavarez R, Rouault-Pierre K, et al. c-Fos induces chondrogenic tumor formation in immortalized human mesenchymal progenitor cells. Sci Rep. 2018;8(1):15615.
28. Ruijtenberg S, van den Heuvel S. Coordinating cell proliferation and differentiation: Antagonism between cell cycle regulators and cell type-specific gene expression. Cell Cycle. 2016;15(2):196-212.
29. Saba KH, Cornmark L, Hofvander J, Magnusson L, Nilsson J, van den Bos H, et al. Loss of NF2 defines a genetic subgroup of non-FOS-rearranged osteoblastoma. J Pathol Clin Res. 2020;6(4):231-7.
30. Tanos T, Marinissen MJ, Leskow FC, Hochbaum D, Martinetto H, Gutkind JS, et al. Phosphorylation of c-Fos by members of the p38 MAPK family. Role in the AP-1 response to UV light. J Biol Chem. 2005;280(19):18842-52.
31. Monje P, Hernandez-Losa J, Lyons RJ, Castellone MD, Gutkind JS. Regulation of the transcriptional activity of c-Fos by ERK. A novel role for the prolyl isomerase PIN1. J Biol Chem. 2005;280(42):35081-4.
32. Chen RH, Abate C, Blenis J. Phosphorylation of the c-Fos transrepression domain by mitogen-activated protein kinase and 90-kD ribosomal S6 kinase. Proc Natl Acad Sci U S A. 1993;90:10952-6.

Supplementary Figures

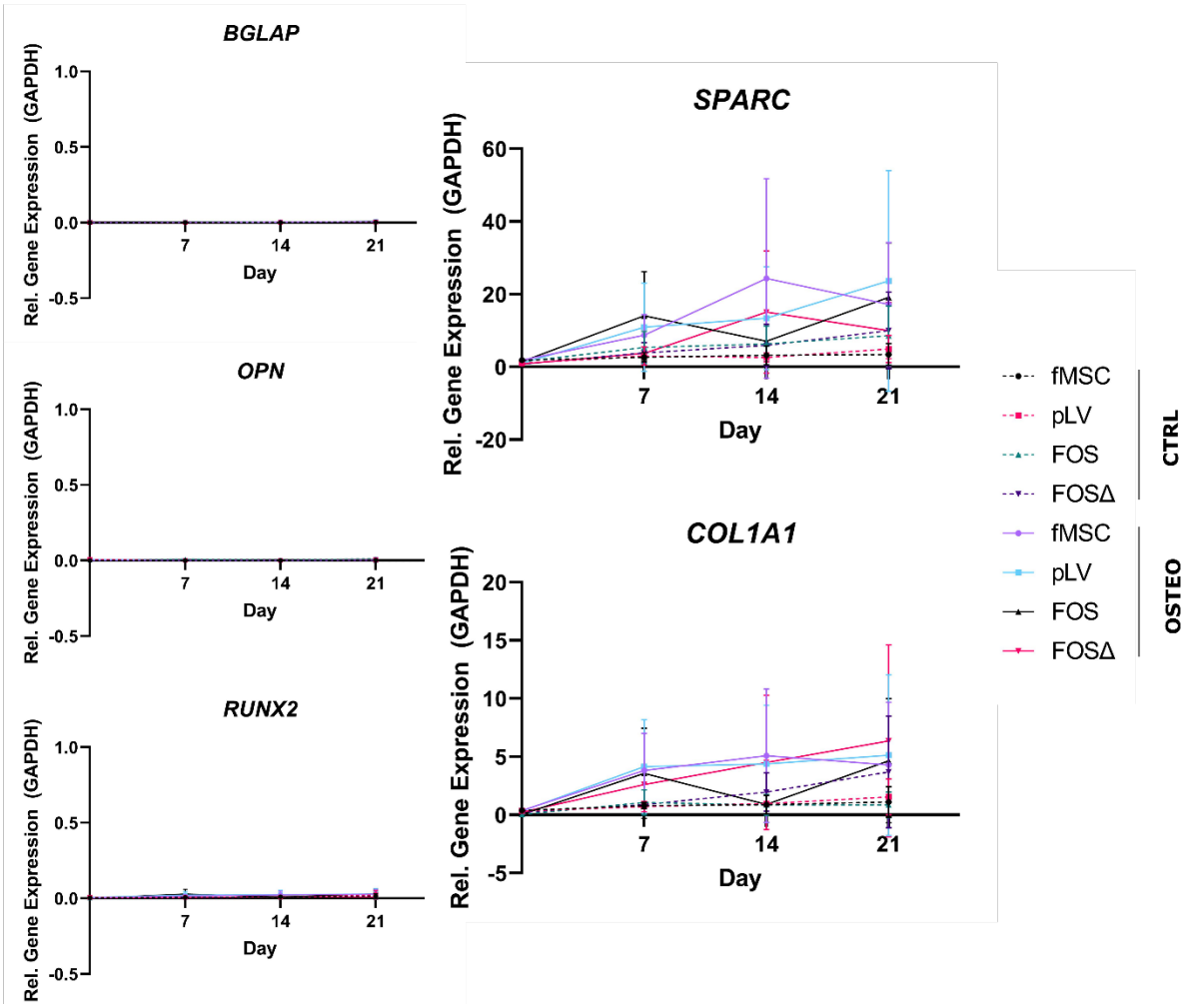

Figure S1. Gene expression of osteogenic markers *BGLAP*, *OPN* and *RUNX2* showed nearly undetectable levels in fMSCs. Gene expression of *COL1A1* and *SPARC* was highly variable.