detected in WT and *Hyp* chondrocytes (Figure 3B). These results suggest that Pi uptake via *Pit-1* is specifically involved in the regulation of chondrogenesis including apoptosis and mineralization.

Suppression of chondrocyte differentiation by NPT inhibitor - To verify whether a decrease in Pi uptake due to reduced *Pit-1* expression is responsible for a reduction in ATP levels in *Hyp* chondrocytes, we determined the effects of phosphonoformic acid (PFA or foscarnet), which is a competitive inhibitor of Pi uptake via NPT (23), on intracellular ATP levels. PFA (10^{-5}-10^{-3} M) reduced Pi-uptake in chondrocytes in a dose-dependent manner (data not shown). PFA (10^{-5} M) profoundly reduced intracellular ATP levels (Figure 4A). Of note, PFA treatment caused disorganization of growth plate cartilage (Figure 4B, left) and significantly decreased the number of TUNEL-positive chondrocytes in the hypertrophic cartilage (Figure 4B, right and Figure 4C) in a similar manner to those seen in *Hyp* mice. Consistent with these *in vivo* results, PFA markedly inhibited mineralization of chondrocytes in a dose-dependent manner (Figure 4D, bottom and 4E), while GAG synthesis was not affected by PFA treatment (Figure 4D, top). Furthermore, PFA also inhibited caspase-9 (Figure 4F) and caspase-3 activity (Figure 4G). We determined serum Pi levels in PFA-treated mice. There was a trend of decreased serum Pi levels in PFA-treated mice but it was not significantly different (Figure 4H). The results are consistent with the notion that Pi uptake via *Pit-1* is closely associated with late chondrogenesis including apoptosis and mineralization through reducing ATP synthesis. These results also suggest an important role for intracellular Pi over extracellular Pi in the regulation of apoptosis and ATP synthesis in chondrocytes.

Suppression of chondrocyte differentiation by NPT siRNA - To further and more specifically verify the role of *Pit-1* on chondrocyte differentiation, we performed knock-down experiments using small interfering RNA (siRNA) for *Pit-1*. As control, *Npt2a* was also knocked-down. siPit-1 and siNpt2a profoundly reduced *Pit-1* and *Npt2a* mRNA levels in WT chondrocytes, respectively (Figure 5A). *Pit-1* knock-down by siPit-1 significantly decreased Pi-uptake (Figure 5B), intracellular ATP levels (Figure 5C), caspase-9 (Figure 5D) and caspase-3 activity (Figure 5E). In parallel with these, apoptosis (Figure 5F) and mineralization (Figure 5G and 5H) were also suppressed. In contrast, knock-down of *Npt2a* by siNpt2a had no effects on apoptosis and mineralization and other determinations (Figure 5B, 5C, 5D, 5E, 5F, 5G, and 5H). These data suggest that *Pit-1* specifically controls Pi uptake and following cascades of ATP-dependent caspase signaling, apoptosis and mineralization in chondrocytes.

Recovery of differentiation in Hyp chondrocytes by Pit-1 overexpression - As an alternative approach to confirm a critical role of *Pit-1* in apoptosis and mineralization in chondrocytes, we next examined the effects of *Pit-1* overexpression on *Hyp* chondrocytes. *Pit-1* overexpression significantly increased Pi uptake (Figure 6A) and intracellular ATP levels (Figure 6B) in *Hyp* chondrocytes. Furthermore, *Pit-1* overexpression also stimulated caspase-9 (Figure 6C) and caspase-3 activity (Figure 6D), apoptosis (Figure 6E) and mineralization (Figure 6F and 6G). WT chondrocytes also showed significantly increased Pi uptake (Figure 6A), intracellular ATP levels (Figure 6B), caspase-9 (Figure 6C) and caspase-3 activity (Figure 6D), apoptosis (Figure 6E) and mineralization (Figure 6F and 6G) by *Pit-1* overexpression. These results further suggest that *Pit-1* is critical in the regulation of Pi uptake and following cascades of ATP-dependent caspase signaling, apoptosis and mineralization in chondrocytes.

Suppression of chondrocyte differentiation by ATP synthesis inhibitor - To further examine the role of intracellular ATP in chondrocyte differentiation, we studied the effects of the ATP synthesis inhibitor 3-bromopyruvate (3-BrPA). 3-BrPA (10^{-6}M) significantly reduced intracellular ATP levels in WT chondrocytes in culture (data not shown). Caspase-9 (Figure 7A) and caspase-3 activity (Figure 7B) were also significantly decreased in 3-BrPA-treated chondrocytes. More importantly, 3-BrPA treatment significantly decreased the number of TUNEL-positive chondrocytes in the hypertrophic zone in mice (Figure 7C and Figure 7D). 3-BrPA inhibited chondrocyte mineralization in a dose-dependent manner (Figure 7E and 7F). However, GAG synthesis was not affected by 3-BrPA (Figure 7E). The serum Pi levels in 3-BrPA-treated mice were not significantly different from control mice (Figure 7G), suggesting an important role for intracellular Pi over extracellular Pi. These results suggest that ATP synthesis is important for chondrocytes to undergo apoptosis via caspase signaling and advance to mineralization.

DISCUSSION

In the present study, we explored the role of Pi/NPT system in chondrogenesis using *Hyp* mice compared with WT mice. We found that *Hyp* mice exhibited a widened and disorganized hypertrophic zone with reduced chondrocyte apoptosis compared with WT mice. In addition, PFA (a competitive inhibitor of *Pit-1*) or 3-BrPA (an ATP synthesis inhibitor) markedly caused elongation and disorganization of hypertrophic cartilage with reduced apoptosis in WT mice in a similar manner to *Hyp* mice. It is noted that the disorders in the hypertrophic zone were most severe in *Hyp* mice compared with PFA- or 3-BrPA-treated mice, despite that the number of TUNEL-positive cells are comparable in these mice. We postulate that the disorders in *Hyp* mice are congenital and irreversible and thus most severe, whereas the disorders seen in PFA- and 3-BrPA-treated mice are due to transient exposure of these agents and reversible and thus less severe.

Consistent with these *in vivo* results, *Hyp* chondrocytes in culture exhibited decreased activity of the apoptotic signaling including caspase-9 and caspase-3 and apoptosis and mineralization following to reduced Pi uptake and cellular ATP synthesis. Furthermore, PFA or 3-BrPA diminished caspase-9 and caspase-3 activity, apoptosis and mineralization in conjunction with a reduction in Pi uptake and ATP synthesis in WT chondrocytes. *Hyp* primary chondrocytes displayed a decrease in *Pit-1* (type III NPT) mRNA expression compared with WT chondrocytes, while there was no difference in type IIa NPT mRNA expression between WT and *Hyp* chondrocytes. WT and *Hyp* chondrocytes expressed no type I NPT mRNA. Meanwhile, GAG synthesis, which is an early event in chondrogenesis, was not reduced in *Hyp* chondrocytes and PFA and 3-BrPA or knockdown of *Pit-1* failed to decrease GAG synthesis in WT chondrocytes. *Pit-1* overexpression restored apoptosis and mineralization in *Hyp* chondrocytes. Taken together, these results suggest that Pi uptake via *Pit-1* and consequent ATP synthesis are critical in the regulation of late chondrogenesis including apoptosis and mineralization. These results also suggest that the disruption of cellular Pi homeostasis causes abnormal endochondral ossification due to a reduction of ATP synthesis in Hyp mice. In support of our study, Zalutskaya et al (32) have recently described that Pi activates mitochondrial apoptotic pathways and promotes endochondral ossification.

ATP synthesis and chondrogenesis - A notable and novel finding obtained in this study is that 3-BrPA inhibits apoptosis and mineralization in growth plate hypertrophic cartilage *in vivo* and primary chondrocytes *in vitro*. 3-BrPA is an alkylating agent that decreases cellular ATP via inhibition of hexokinase in glycolysis and is shown to promote cancer cell death through activation of mitochondrial pathway of apoptosis or necrosis (33). Of note, ATP-depleting effect of 3-BrPA is prominent only in tumor cells but not apparent in non-transformed cells (34). Hence, it has been proposed that 3-BrPA could be an anti-cancer agent for varieties of cancers. In addition to these effects on cancers, our results show that 3-BrPA inhibits the differentiation of cartilage, suggesting that ATP generation is also necessary for non-transformed chondrocytes to differentiate and that chondrogenesis is thus an energy-dependent biological event.

Decreased Pit-1 expression and Hyp skeletal phenotype - Decreased Pi-uptake in *Hyp* chondrocytes is likely primarily due to reduced *Pit-1* mRNA expression. Type IIa NPT expression was not diminished in *Hyp* chondrocytes and type I NPT was not expressed in chondrocytes. Earlier reports described that disturbed endochondral ossification was not rescued by Pi supplementation in *Hyp* mice (35-37), suggesting that intrinsic factors are involved. Miao et al (5) showed that reduced expression of PHEX and MMP-9 was associated with cartilage abnormalities in *Hyp* mice. Our results suggest that *Pit-1* is one of these intrinsic factors responsible for the abnormal chondrogenesis seen in *Hyp* mice as well.

Regulation of Pit-1 expression - The mechanism underlying down-regulation of *Pit-1* expression in *Hyp* chondrocytes is unknown. Recent studies have reported that stanniocalcin 1 (STC1) increases *Pit-1* mRNA expression in osteoblasts (38) and STC1 and STC2 have been shown to regulate Pi-uptake in chicken chondrocytes (39). STC1 stimulates renal Pi uptake and increases *Pit1* expression in osteoblasts (40), whereas STC2 inhibits the *Pit1* expression and renal Pi uptake (38). Thus, STC1 and STC2 have an opposite action in the regulation of *Pit-1* expression. Therefore, it is intriguing to examine whether STC1 or STC2 is involved in the *Pit-1* expression in chondrocytes. In preliminary experiments, we determined the expression of *Stc1* and *Stc2* mRNA in WT and *Hyp* chondrocytes using RT-PCR and real-time PCR. The *Stc2 mRNA* was expressed in both WT and *Hyp* chondrocytes at the same level (data not shown). However, the expression of *Stc1 mRNA* was decreased in *Hyp*

chondrocytes compared with WT chondrocytes (data not shown). These results suggest that STC1 but not STC2 regulates *Pit-1* expression in chondrocytes.

Involvement of fibroblast growth factor23 - Fibroblast growth factor 23 (FGF23) is a hormone that regulates serum Pi levels (41). FGF23 requires Klotho for its signaling as the co-receptors in addition to the canonical FGFR1(IIIc) (42, 43). Mice transgenic for FGF23 displayed a reduction in *Npt2a* expression in the renal proximal tubules (44), indicating that FGF23 is a negative regulator of *Npt2a* expression, raising the possibility that Klotho-dependent FGF23 signaling regulates *Pit-1* expression in chondrocytes as well. FGF23 expression was predominantly localized in osteoblasts, cementoblasts, and odontoblasts, with a sporadic expression in some chondrocytes, osteocytes and cementocytes (45). However, we were not able to demonstrate FGF23 expression in primary mouse chondrocytes by RT-PCR. Further studies are needed to elucidate the relationship between FGF23 signaling and *Pit-1* expression in cartilage.

In conclusion, we have found in the present study that chondrogenesis is modulated by cellular Pi uptake via Pit-1 and cellular ATP synthesis and thus is a biological event that depends on mitochondrial energy generation. We believe that these findings should provide us with a novel concept and alternative approaches to study the cellular differentiation that occurs in physiological conditions and also to analyze the skeletal abnormalities seen in congenital hypophosphatemic disorders such as XLH.

FOOTNOTES

We thank Dr. Kenichi Miyamoto and Dr. Hiroko Segawa (University of Tokushima Graduate School, Tokushima, Japan) for the kind gift of mouse *Pit-1* cDNA. This work was partly supported by the 21st Century COE program entitled "Origination of Frontier BioDentistry" at Osaka University Graduate School of Dentistry, supported by the Ministry of Education, Culture, Sports, Science and Technology and by Grants-in-Aid for Scientific Research from the Japanese Society for the Promotion of Science (T.Y. #A202290100).

The abbreviations used are: XLH, X-linked hypophosphatemia; Pi, phosphate; GAG, glycosaminoglycans; *Npt2a*, type IIa sodium-dependent Pi transporter; RT-PCR, Reverse transcription-polymerase chain reaction; PFA, phosphonoformic acid; ip, intraperitoneal injection; DAPI, 4',6-Diamidino-2-phenylindole; 3-BrPA, 3-Bromopyruvate; WT, wild-type; STC, Stanniocalcin; FGF23, fibroblast growth factor 23; ATPase, adenosine triphosphatase.

REFERENCES

1. Zuscik, M. J., Hilton, M. J., Zhang, X., Chen, D., and O'Keefe, R. J. (2008) *J Clin Invest* **118**(2), 429-438
2. Winters, R. W., Graham, J. B., Williams, T. F., Mc, F. V., and Burnett, C. H. (1958) *Medicine (Baltimore)* **37**(2), 97-142
3. Holm, I. A., Huang, X., and Kunkel, L. M. (1997) *Am J Hum Genet* **60**(4), 790-797
4. Eicher, E. M., Southard, J. L., Scriver, C. R., and Glorieux, F. H. (1976) *Proc Natl Acad Sci U S A* **73**(12), 4667-4671
5. Miao, D., Bai, X., Panda, D. K., Karaplis, A. C., Goltzman, D., and McKee, M. D. (2004) *Bone* **34**(4), 638-647.
6. Hayashibara, T., Hiraga, T., Sugita, A., Wang, L., Hata, K., Ooshima, T., and Yoneda, T. (2007) *J Bone Miner Res* **22**(11), 1743-1751
7. Boyde, A., and Shapiro, I. M. (1980) *Histochemistry* **69**(1), 85-94.
8. Kakuta, S., Golub, E. E., and Shapiro, I. M. (1985) *Calcif Tissue Int* **37**(3), 293-299.
9. Mwale, F., Tchetina, E., Wu, C. W., and Poole, A. R. (2002) *J Bone Miner Res* **17**(2), 275-283.
10. Shapiro, I. M., and Boyde, A. (1984) *Metab Bone Dis Relat Res* **5**(6), 317-326.
11. Virkki, L. V., Biber, J., Murer, H., and Forster, I. C. (2007) *Am J Physiol Renal Physiol* **293**(3), F643-654
12. Palmer, G., Zhao, J., Bonjour, J., Hofstetter, W., and Caverzasio, J. (1999) *Bone* **24**(1), 1-7.
13. Mansfield, K., Teixeira, C. C., Adams, C. S., and Shapiro, I. M. (2001) *Bone* **28**(1), 1-8.
14. Cecil, D. L., Rose, D. M., Terkeltaub, R., and Liu-Bryan, R. (2005) *Arthritis Rheum* **52**(1), 144-154.

15. Fujita, T., Meguro, T., Izumo, N., Yasutomi, C., Fukuyama, R., Nakamuta, H., and Koida, M. (2001) *Jpn J Pharmacol* **85**(3), 278-281.
16. Guicheux, J., Palmer, G., Shukunami, C., Hiraki, Y., Bonjour, J. P., and Caverzasio, J. (2000) *Bone* **27**(1), 69-74.
17. Montessuit, C., Caverzasio, J., and Bonjour, J. P. (1991) *J Biol Chem* **266**(27), 17791-17797.
18. Wang, D., Canaff, L., Davidson, D., Corluka, A., Liu, H., Hendy, G. N., and Henderson, J. E. (2001) *J Biol Chem* **276**(36), 33995-34005
19. Wu, L. N., Guo, Y., Genge, B. R., Ishikawa, Y., and Wuthier, R. E. (2002) *J Cell Biochem* **86**(3), 475-489
20. Magne, D., Bluteau, G., Faucheux, C., Palmer, G., Vignes-Colombeix, C., Pilet, P., Rouillon, T., Caverzasio, J., Weiss, P., Daculsi, G., and Guicheux, J. (2003) *J Bone Miner Res* **18**(8), 1430-1442.
21. Shimomura, Y., Yoneda, T., and Suzuki, F. (1975) *Calcif Tissue Res* **19**(3), 179-187
22. Rowe, P. S., Ong, A. C., Cockerill, F. J., Goulding, J. N., and Hewison, M. (1996) *Bone* **18**(2), 159-169.
23. Loghman-Adham, M. (1996) *Gen Pharmacol* **27**(2), 305-312
24. Swenson. C.L., Weisbrode, S.E., Nagode, L.A., Hayes, K.A., Steinmeyer, C.L., Mathes, L.E. (1991) *Calcif Tissue Int* **48**(5), 353-361.
25. Geschwind, J. F., Ko, Y. H., Torbenson, M. S., Magee, C., and Pedersen, P. L. (2002) *Cancer Res* **62**(14), 3909-3913.
26. Jones, A. R., Gillan, L., and Milmlow, D. (1995) *Contraception* **52**(5), 317-320.
27. Ko, Y. H., Smith, B. L., Wang, Y., Pomper, M. G., Rini, D. A., Torbenson, M. S., Hullihen, J., and Pedersen, P. L. (2004) *Biochem Biophys Res Commun* **324**(1), 269-275.
28. Gibson, G. (1998) *Microsc Res Tech* **43**(2), 191-204
29. Eguchi, Y., Srinivasan, A., Tomaselli, K. J., Shimizu, S., and Tsujimoto, Y. (1999) *Cancer Res* **59**(9), 2174-2181.
30. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. (1997) *Cell* **91**(4), 479-489.
31. Rao, N. N., Gomez-Garcia, M. R., and Kornberg, A. (2009) *Annu Rev Biochem* **78**, 605-647
32. Zalutskaya, A. A., Cox, M. K., and Demay, M. B. (2009) *J Cell Biochem* **108**(3), 668-674
33. Pelicano, H., Martin, D. S., Xu, R. H., and Huang, P. (2006) *Oncogene* **25**(34), 4633-4646
34. Xu, R. H., Pelicano, H., Zhou, Y., Carew, J. S., Feng, L., Bhalla, K. N., Keating, M. J., and Huang, P. (2005) *Cancer Res* **65**(2), 613-621
35. Ecarot, B., Glorieux, F. H., Desbarats, M., Travers, R., and Labelle, L. (1992) *J Bone Miner Res* **7**(5), 523-530.
36. Tanaka, H., Seino, Y., Shima, M., Yamaoka, K., Yabuuchi, H., Yoshikawa, H., Masuhara, K., Takaoka, K., and Ono, K. (1988) *Bone Miner* **4**(3), 237-246
37. Yoshikawa, H., Masuhara, K., Takaoka, K., Ono, K., Tanaka, H., and Seino, Y. (1985) *Bone* **6**(4), 235-239
38. Yoshiko, Y., Candeliere, G. A., Maeda, N., and Aubin, J. E. (2007) *Mol Cell Biol* **27**(12), 4465-4474. Epub 2007 Apr 4416.
39. Wu, S., Yoshiko, Y., and De Luca, F. (2006) *J Biol Chem* **281**(8), 5120-5127. Epub 2005 Dec 5123.
40. Ishibashi, K., and Imai, M. (2002) *Am J Physiol Renal Physiol* **282**(3), F367-375
41. Fukumoto, S., and Yamashita, T. (2007) *Bone* **40**(5), 1190-1195
42. Kurosu, H., Ogawa, Y., Miyoshi, M., Yamamoto, M., Nandi, A., Rosenblatt, K. P., Baum, M. G., Schiavi, S., Hu, M. C., Moe, O. W., and Kuro-o, M. (2006) *J Biol Chem* **281**(10), 6120-6123. Epub 2006 Jan 6125.
43. Urakawa, I., Yamazaki, Y., Shimada, T., Iijima, K., Hasegawa, H., Okawa, K., Fujita, T., Fukumoto, S., and Yamashita, T. (2006) *Nature* **444**(7120), 770-774
44. Shimada, T., Urakawa, I., Yamazaki, Y., Hasegawa, H., Hino, R., Yoneya, T., Takeuchi, Y., Fujita, T., Fukumoto, S., and Yamashita, T. (2004) *Biochem Biophys Res Commun* **314**(2), 409-414.
45. Yoshiko, Y., Wang, H., Minamizaki, T., Ijuin, C., Yamamoto, R., Suemune, S., Kozai, K., Tanne, K., Aubin, J. E., and Maeda, N. (2007) *Bone* **40**(6), 1565-1573

FIGURE LEGENDS

FIGURE 1. Apoptosis and related events in *Hyp* chondrocytes. *A*, Histological examination of chondrocyte apoptosis. Hematoxylin/eosin staining (left) and TUNEL staining (right) were performed using tibiae of 4-week-old WT and *Hyp* mice. Hypertrophic zone is marked with dotted line and scale bar indicates 200 μm. Representative pictures obtained out of numerous sections of four mice from each group are shown. *B*, Number of TUNEL-positive cells in tibial growth plate of WT and *Hyp* mice. *C*, Quantitative determination of chondrocyte apoptosis. Cells were cultured in the differentiation medium in 96-well plates for 7days. The determination was conducted using the Cell Death Detection ELISA PLUS after differentiation. Data are shown as apoptotic activity. *D*, Histochemical staining of WT and *Hyp* chondrocytes. Cells were cultured for 7days in the differentiation medium and stained with alcian blue for GAG synthesis (top) and with alizarin red-S for mineralization (bottom). *E*, Quantification of alcian blue staining. *F*, Quantification of alizarin red staining. Results are expressed as mean +/- SEM of four separate experiments. *Significantly different from WT chondrocytes ($p<0.05$).

FIGURE 2. Activity of apoptotic signaling pathways. *A*, Caspase-9 activity in WT and *Hyp* chondrocytes. *B*, Caspase-3 activity in WT and *Hyp* chondrocytes. Activity was measured using the Caspase-Glo 9 and Caspase-Glo 3/7 assay kit after differentiation. *C*, Intracellular ATP levels in WT and *Hyp* chondrocytes. Cells were cultured at a density of 1 x 10^4 cells/well in 96-well plates for 24 hours. ATP levels were measured using the ATP assay kit.

FIGURE 3. Characterization of *Hyp* chondrocytes. *A*, Time-course of Pi uptake in WT (open circle) and *Hyp* (solid circle) chondrocytes. Cells were cultured for 7days in the differentiation medium and Pi uptake was determined as described in Experimental Procedures. *B*, Expressions of *Npt*1, *Npt2a* and *Pit-1* mRNA in WT and *Hyp* chondrocytes. Total RNA isolated from chondrocytes cultured for 24 hours was used for RT-PCR analysis using the primer pairs. β-actin was amplified as control. *C*, Time-dependent expression of *Pit-1* mRNA by real-time PCR. *D*, Time-dependent expression of *Npt2a* mRNA by real-time PCR. The amount of *Npt2a* and *Pit-1* of WT chondrocytes at day 0 was designated as 1.0 and normalized to GAPDH. Results are expressed as mean +/- SEM of four separate experiments. *Significantly different from WT chondrocytes ($p<0.05$).

FIGURE 4. Effects of PFA on chondrocyte differentiation. *A*, Intracellular ATP levels. Cells were cultured in the presence of 10^{-5}M PFA. ATP levels were measured using the ATP assay kit. *B*, Histological examination of chondrocyte apoptosis. Hematoxylin/eosin staining (left) and TUNEL staining (right) were performed on tibial sections from 31-day-old control and PFA-treated mice. Hypertrophic zone is marked with dotted line and scale bar indicates 200 μm. Representative pictures obtained out of numerous sections of four mice from each group are shown. *C*, Number of TUNEL positive cells in tibial growth plate of control and PFA-treated mice. *D*, Histochemical staining of chondrocytes. Cells were cultured in the presence of 10^{-5} M PFA and stained with alcian blue for GAG synthesis (top) and alizarin red-S for mineralization (bottom). *E*, Quantification of alizarin red staining. *F*, Caspase-9 activity. *G*, Caspase-3 activity. Cells were cultured in the presence of 10^{-5}M PFA. Activity was measured using the Caspase-Glo 9 and Caspase-Glo 3/7 assay. *H*, Serum Pi levels. Results are expressed as mean +/- SEM of four separate determinations. *Significantly different from control ($p<0.05$).

FIGURE 5. *Npt2a* and *Pit-1* knockdown by siRNA in chondrocytes. *A*, siRNA for siNEGATIVE (Control), siNPT2a or siPIT-1 was transfected in chondrocytes and the expression of *NPT2a*, *Pit-1* or GAPDH was analyzed by RT-PCR. *B*, Pi-uptake in siNEGATIVE- (Control), siNPT2a- and siPIT-1-transfected chondrocytes was determined in the presence of 3 μCi/mL of $KH_2{}^{32}PO_4$. *C*, Intracellular ATP levels in siNEGATIVE- (Control), siNPT2a- and siPIT-1-transfected chondrocytes. *D*, Caspase-9 activity in siNEGATIVE- (Control), siNPT2a- and siPIT-1-transfected chondrocytes. *E*, Caspase-3 activity in siNEGATIVE- (Control), siNPT2a- and siPIT-1-transfected chondrocytes. *F*, Quantitative determination of chondrocyte apoptosis in siNEGATIVE- (Control), siNPT2a- and siPIT-1-transfected chondrocytes. *G*, Histochemical staining of siNEGATIVE- (Control), siNPT2a- and siPIT-1-transfected chondrocytes. Cells were cultured and stained with alcian blue for GAG synthesis (top) and alizarin red-S for mineralization (bottom). *H*, Quantification of alizarin red staining. We repeated the experiments twice using different preparation of primary chondrocytes and obtained the identical results. Results are expressed as mean +/- SEM of two separate determinations.*Significantly different from control ($p<0.05$).

FIGURE 6. Effects of *Pit-1* overexpression in chondrocytes. *A*, Empty vector (Control) or *Pit-1* was transfected in WT and *Hyp* chondrocytes. Pi-uptake was determined in the presence of 3 μCi/mL of $KH_2{}^{32}PO_4$. *B*, Intracellular ATP levels in Control or *Pit-1*-transfected WT and *Hyp* chondrocytes. *C*, Caspase-9 activity in control and *Pit-1*-transfected WT and *Hyp* chondrocytes. *D*, Caspase-3 activity in control and *Pit-1*-transfected WT and *Hyp* chondrocytes. *E*, Quantitative determination of apoptosis in control and *Pit-1*-transfected WT and *Hyp* chondrocytes. *F*, Histochemical staining of control and *Pit-1*-transfected WT and *Hyp* chondrocytes. Cells were cultured and stained with alizarin red-S for mineralization. *Pit-1* expression was confirmed by RT-PCR. *G*, Quantification of alizarin red staining. We repeated the experiments twice using different preparation of primary chondrocytes and obtained the identical results. Results are expressed as mean +/- SEM of two separate determinations. *Significantly different from WT control ($p<0.05$). $^{+}$Significantly different from *Hyp* control ($p<0.05$).

FIGURE 7. Effects of 3-BrPA on chondrocyte apoptosis and calcification. *A*, Effects of 3-BrPA on caspase-9 activity. *B*, Effects of 3-BrPA on caspase-3 activity. Cells were treated with 10^{-6} M 3-BrPA for 7 days and measured for caspase activity. *C*, Histological examination of chondrocyte apoptosis. Hematoxylin/eosin staining (left) and TUNEL staining (right) were performed on tibial sections from 31-day-old control and 3-BrPA-treated mice. Hypertrophic zone is marked with dotted line and scale bar indicates 200 μm. *D*, Number of TUNEL-positive cells in tibial growth plate of control and 3-BrPA-treated mice. *E*, Histochemical staining of chondrocytes. Cells were cultured for 7days in the presence of 10^{-6} and 10^{-5} M 3-BrPA and stained with alcian blue for GAG synthesis (top) and alizarin red-S for mineralization (bottom). *G*, Quantification of alizarin red staining. *H*, Serum Pi levels. Results are expressed as mean +/- SEM of four separate experiments. *Significantly different from control ($p<0.05$).

Figure 1

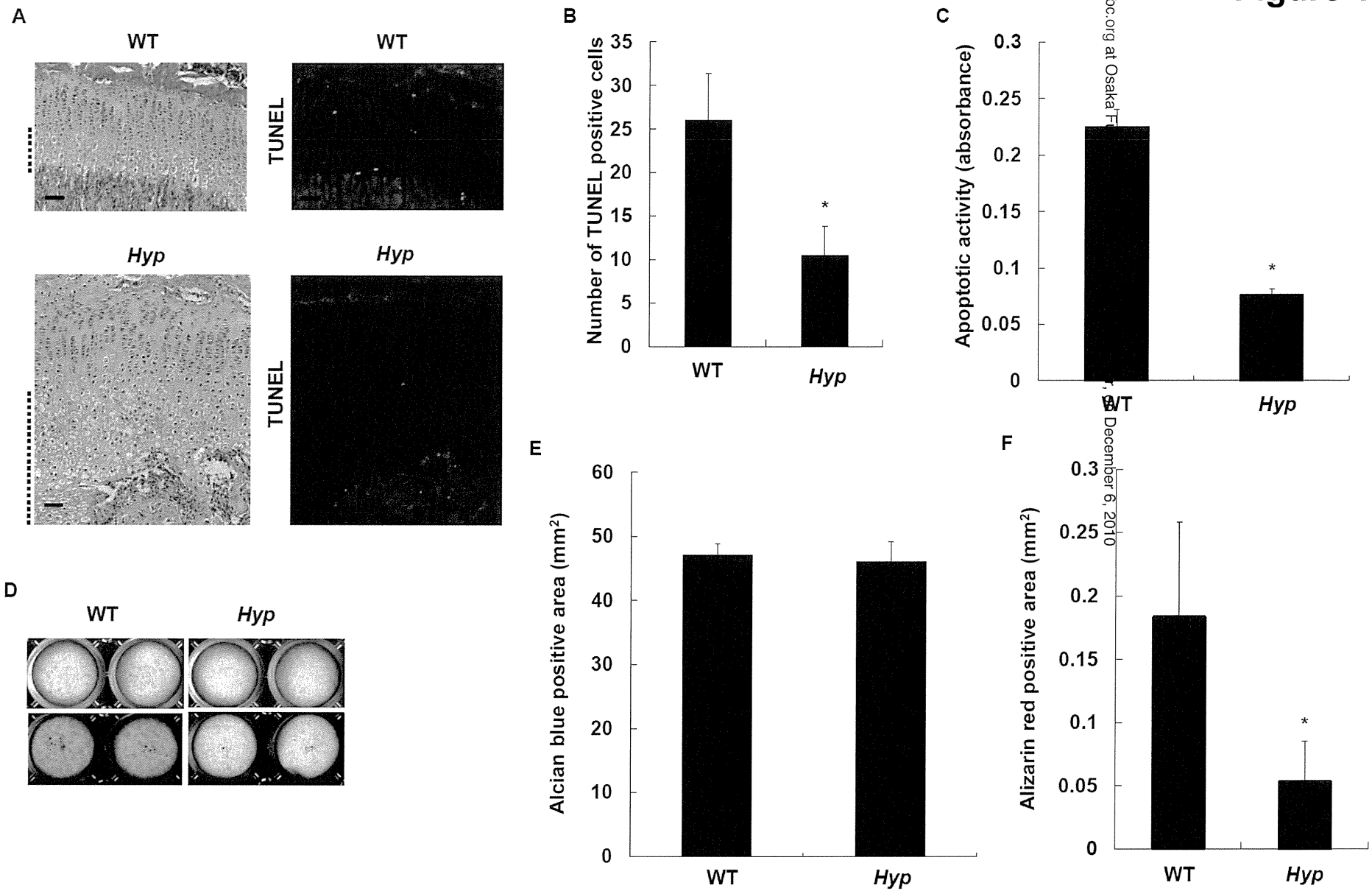


Figure 2

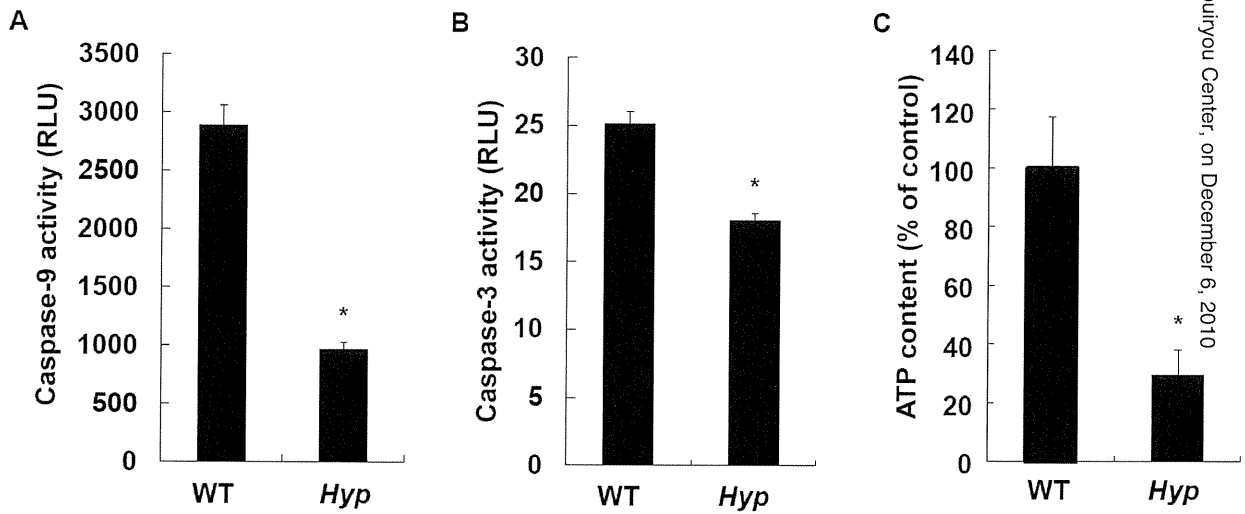


Figure 3

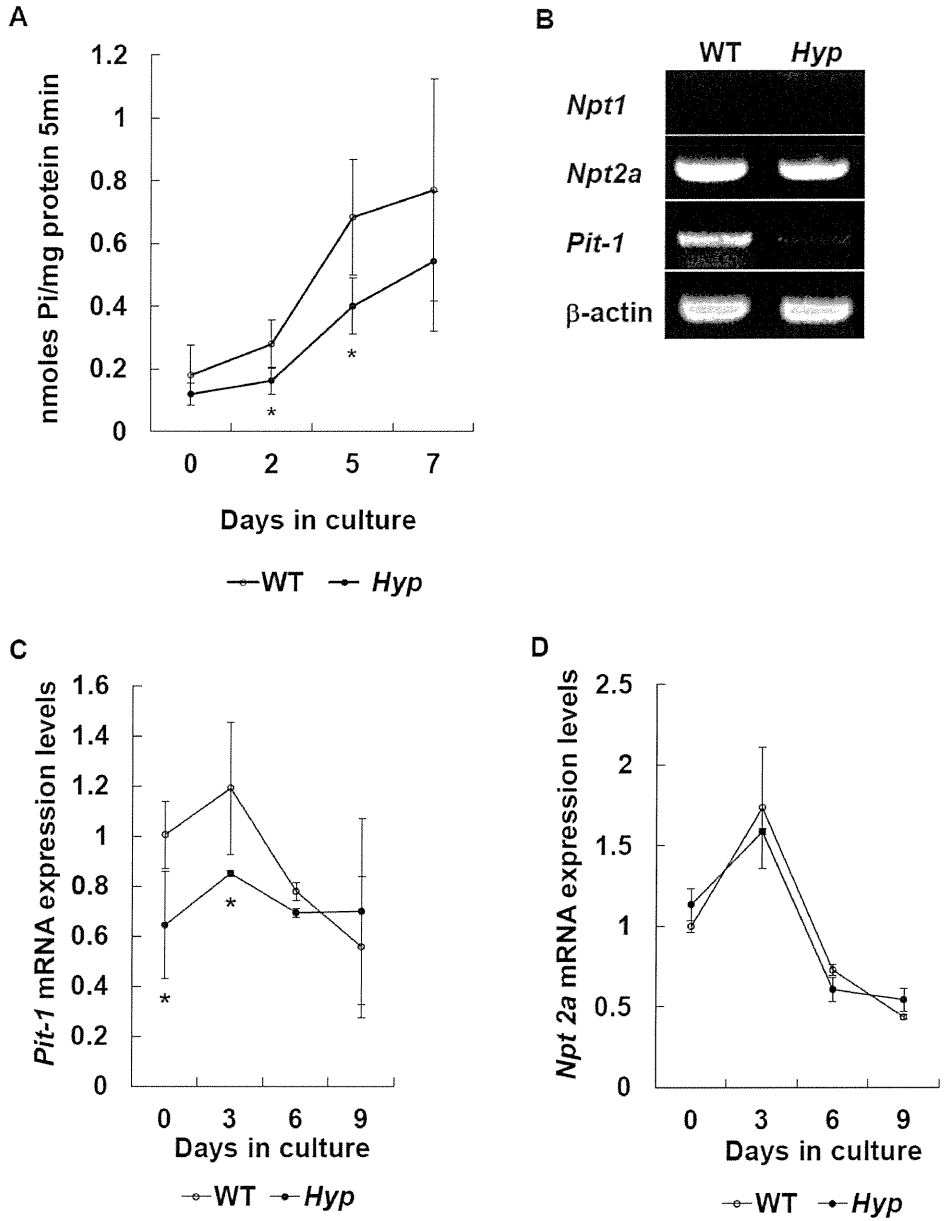


Figure 4

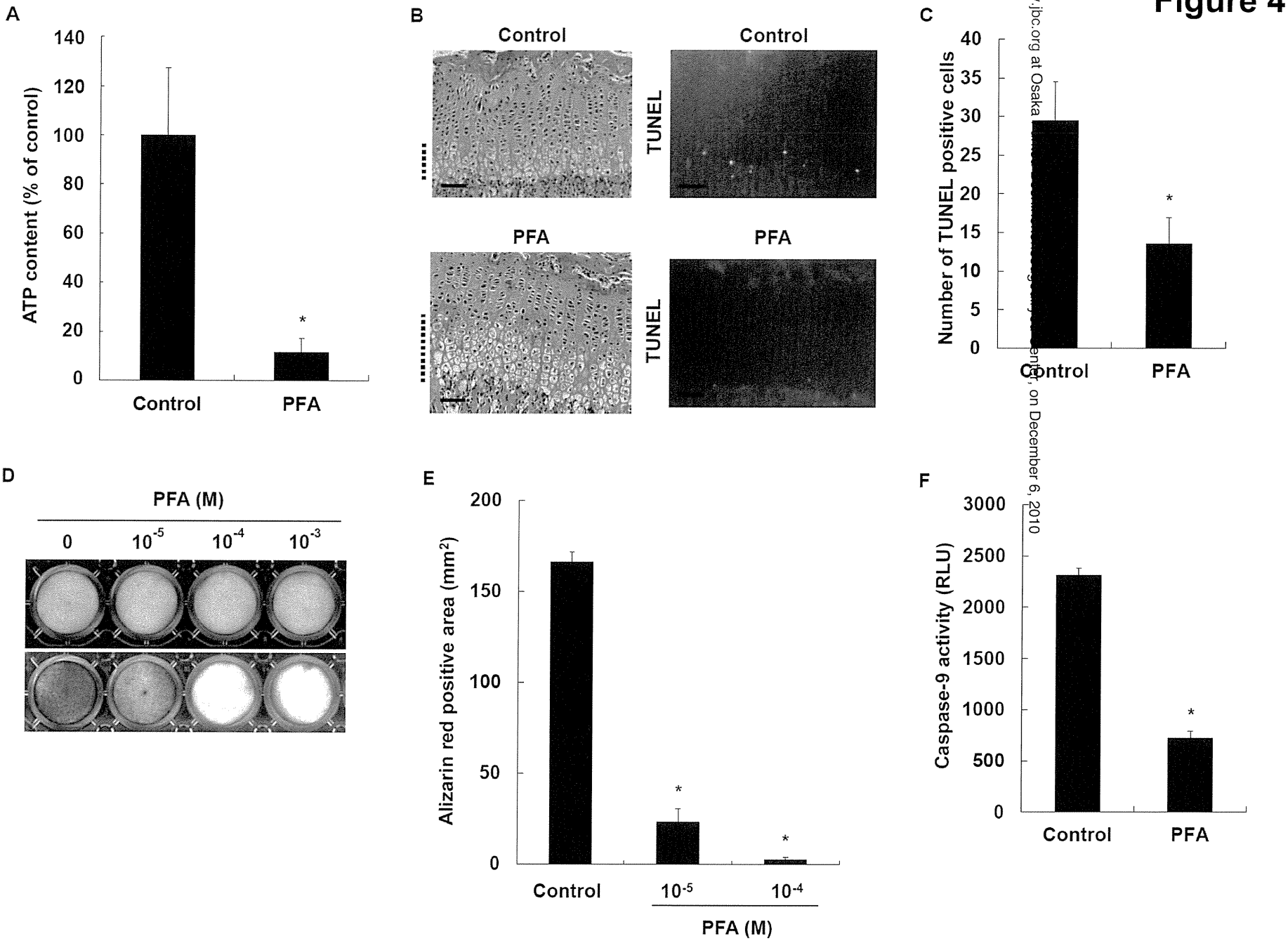


Figure 4

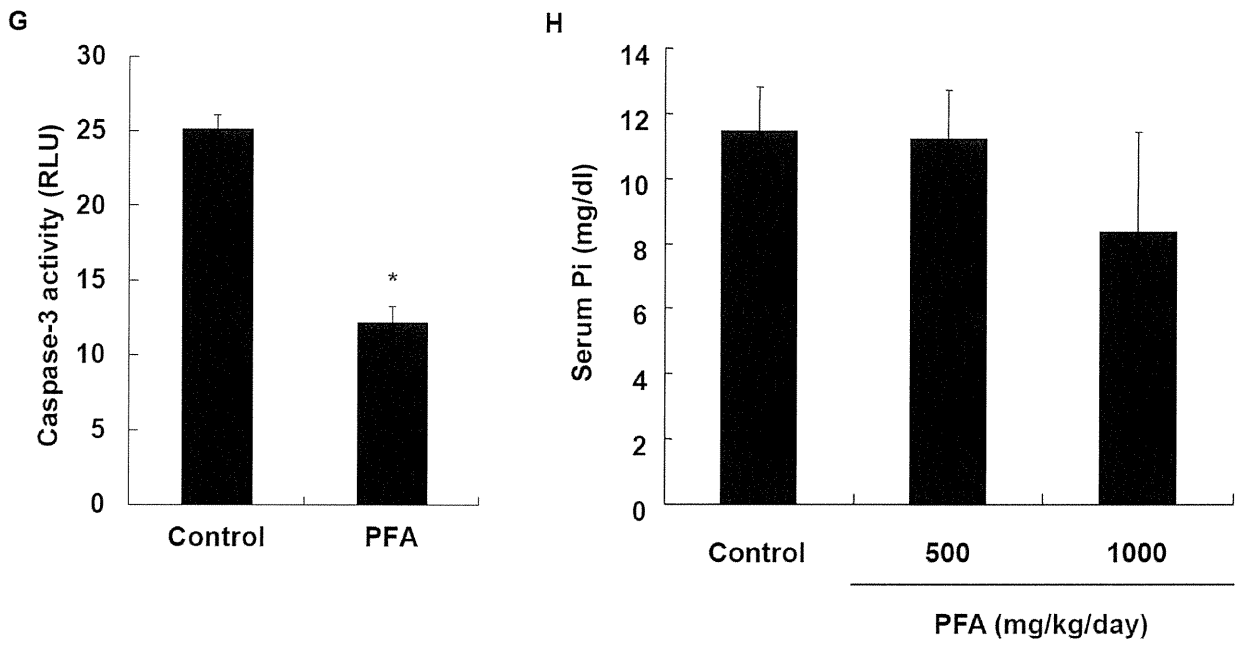


Figure 5

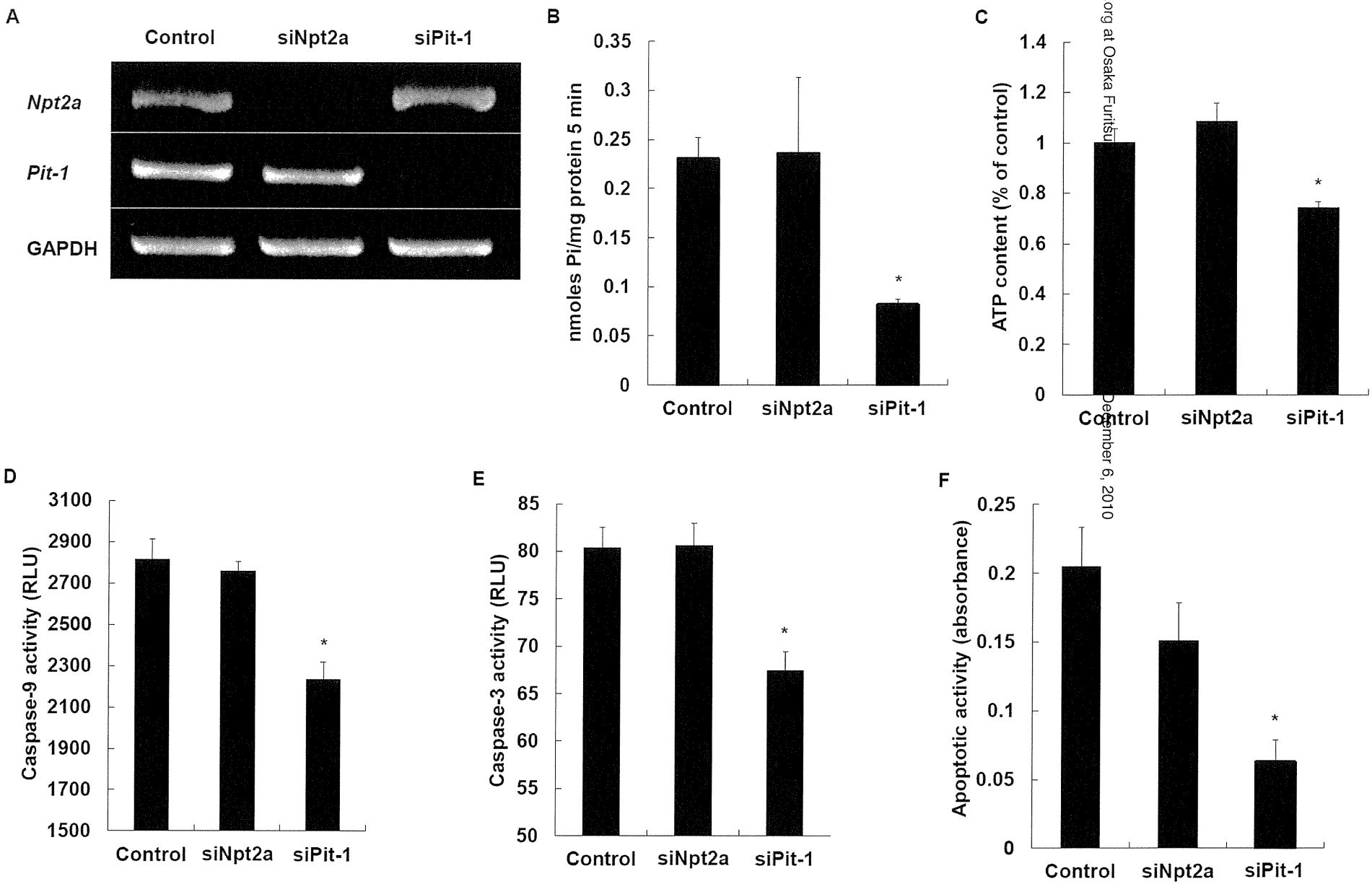


Figure 5

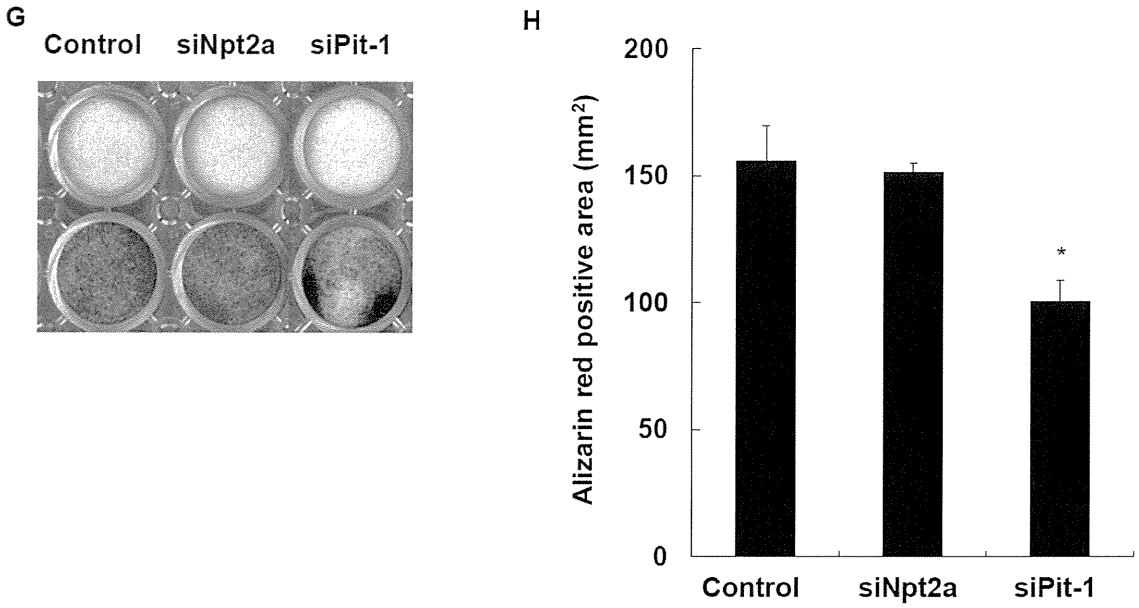


Figure 6

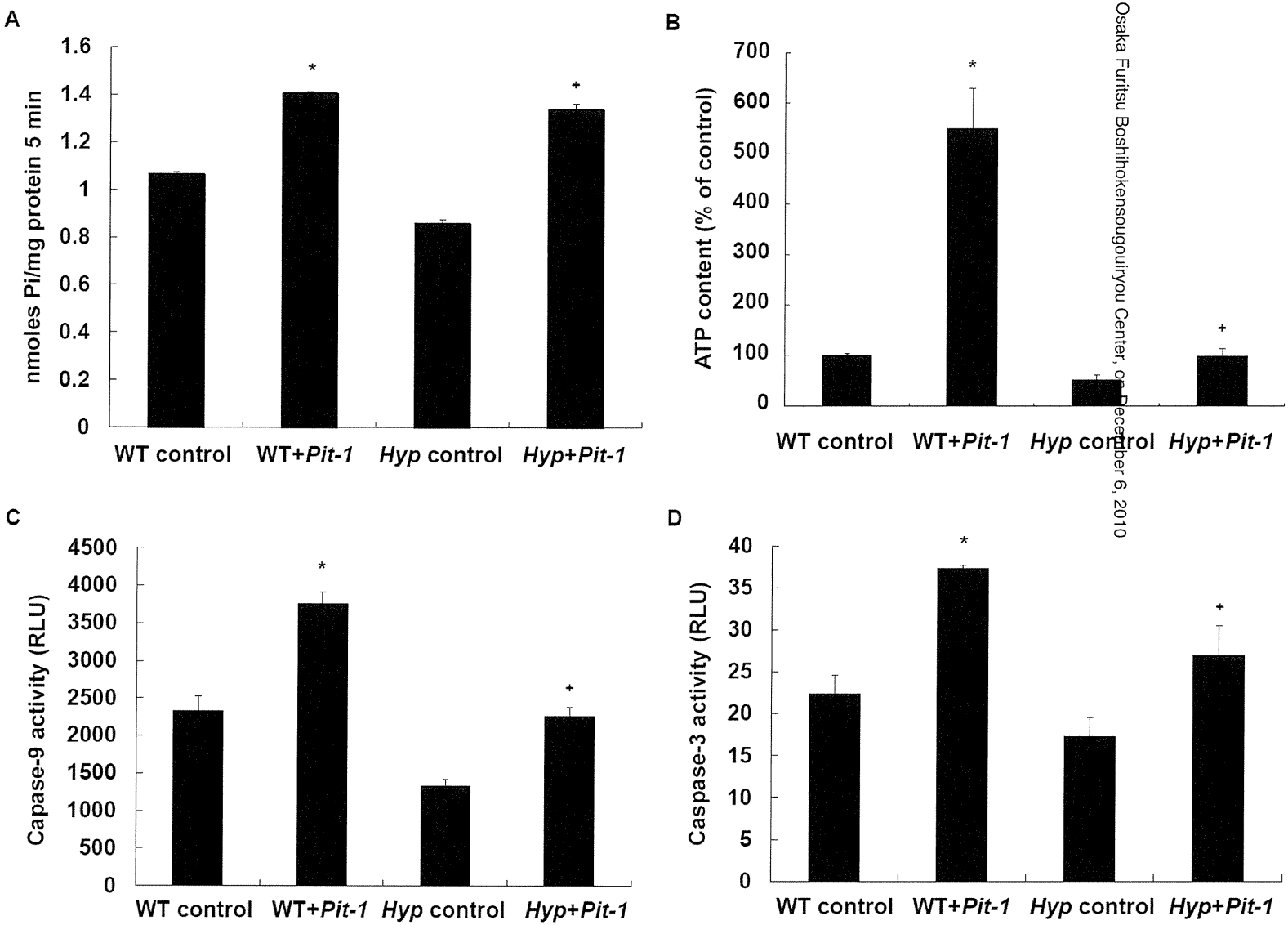
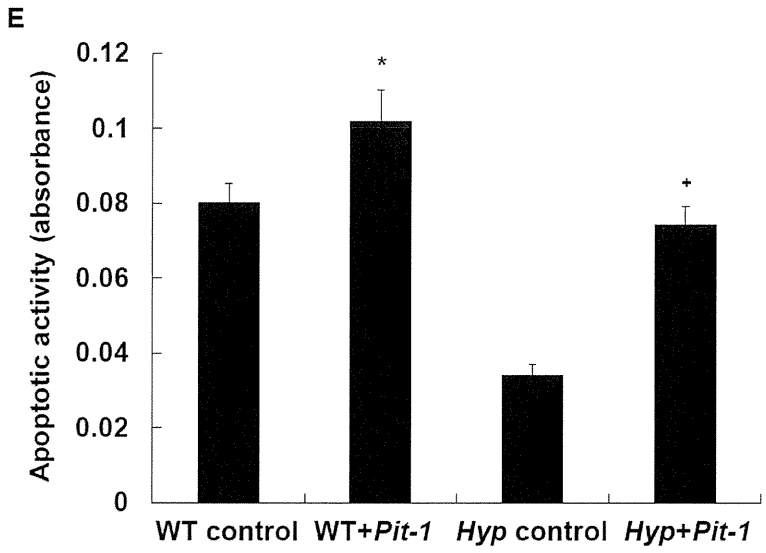
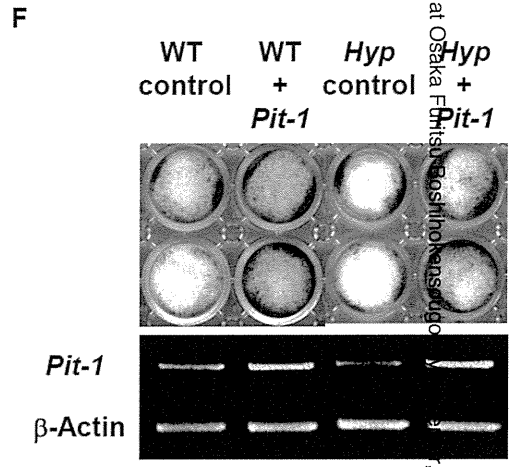


Figure 6





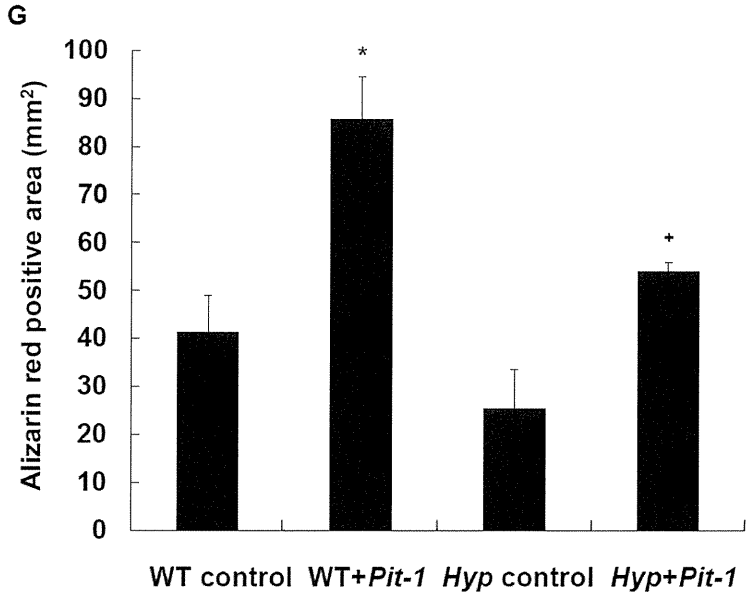
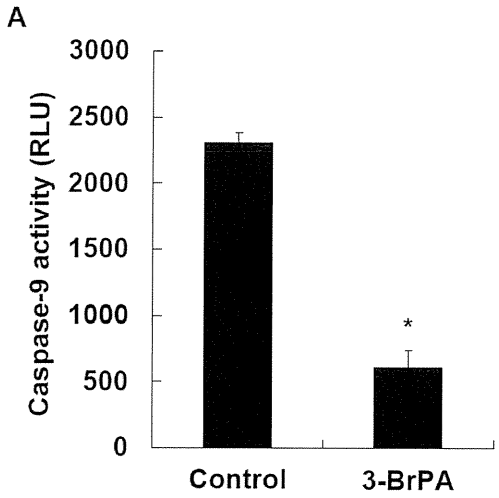
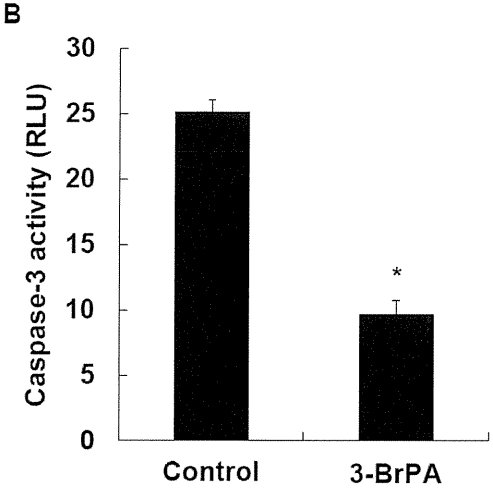
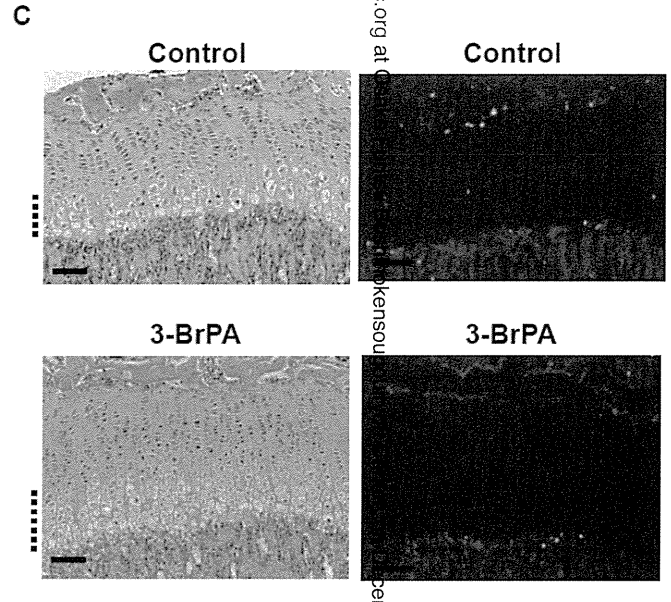
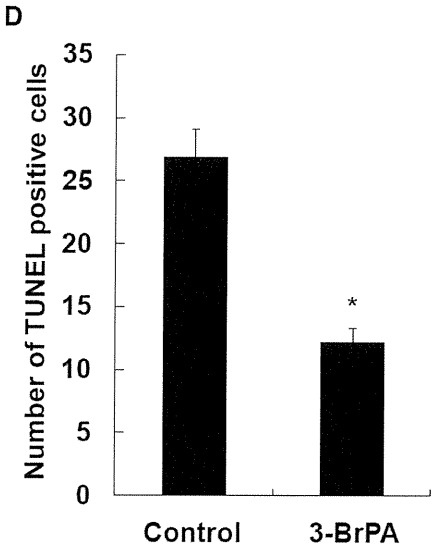


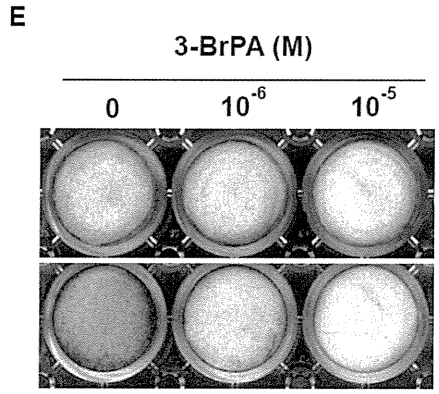
Figure 7











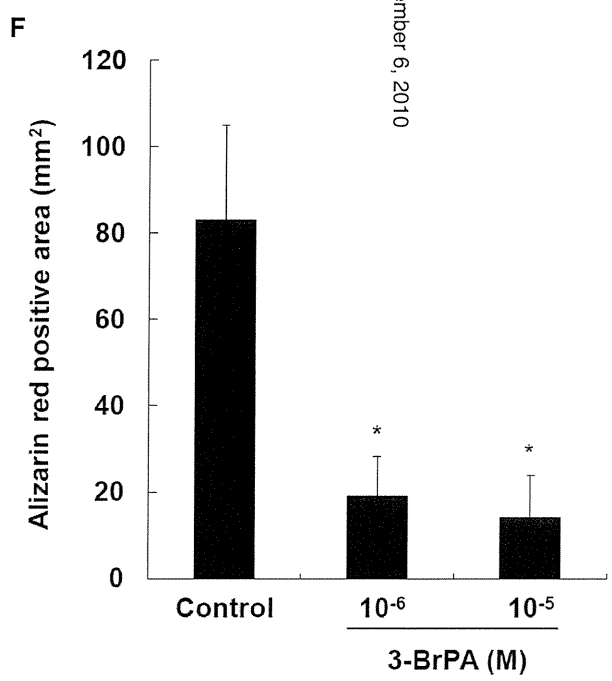
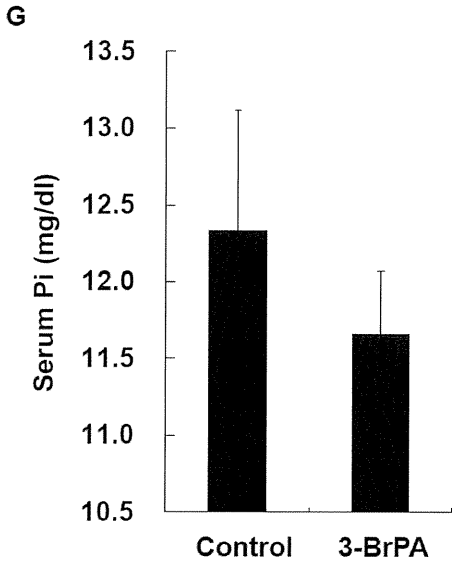


Figure 7



Successful gene therapy *in utero* for lethal murine hypophosphatasia

Hanako Sugano[1,2], Tae Matsumoto[1,2], Koichi Miyake[1], Atsushi Watanabe[1], Osamu Iijima[1], Makoto Migita[2], Sonoko Narisawa[3], José Luis Millán[3], Yoshitaka Fukunaga[2], Takashi Shimada[1].

1. Department of Biochemistry and Molecular Biology, Nippon Medical School, Tokyo, Japan

2. Department of Pediatrics, Nippon Medical School, Tokyo, Japan

3. Sanford Children's Health Research Center, Sanford-Burnham Medical Research Institute, La Jolla, CA.

Running title: Fetal gene therapy for hypophosphatasia

Corresponding author:

Takashi Shimada, M.D., Ph. D.

Department of Biochemistry and Molecular Biology, Nippon Medical School, 1-1-5, Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan

Tel: +81-3-3822-2131; Fax: +81-3-5814-8156

Email: tshimada@nms.ac.jp

Abstract

Hypophosphatasia (HPP), caused by mutations in the gene *ALPL* encoding tissue-nonspecific alkaline phosphatase (TNALP), is an inherited systemic skeletal disease characterized by mineralization defects of bones and teeth. The clinical severity of HPP varies widely from a lethal perinatal form to mild odontohypophosphatasia showing only dental manifestations. HPP model mice (*Akp2*$^{-/-}$) phenotypically mimic the severe infantile form of human HPP; they appear normal at birth but die by two weeks of age due to growth failure, hypomineralization, and epileptic seizures. In the present study, we investigated the feasibility of fetal gene therapy using the lethal HPP model mice. On day 15 of gestation, the fetuses of HPP model mice underwent transuterine intraperitoneal injection of AAV serotype 9 expressing bone-targeted TNALP. Treated and delivered mice showed normal weight gain and seizure-free survival for at least 8 weeks. Vector sequence was detected in systemic organs including bone at 14 days of age. ALP activities in plasma and bone were consistently high. Enhanced mineralization was demonstrated on X-ray images of the chest and forepaw. Our data clearly demonstrates that systemic injection of AAV9 *in utero* is an effective strategy for the treatment of lethal HPP mice. Fetal gene therapy may be an important choice after prenatal diagnosis of life-threatening HPP.

Introduction

Hypophosphatasia (HPP), caused by a deficiency in tissue-nonspecific alkaline phosphatase (TNALP), is an inherited disease characterized by mineralization defects. HPP is a clinically heterogeneous disease and is classified according to severity and age at diagnosis (Mornet, 2007; Whyte, 2010). Perinatal and infantile forms of HPP are usually severe, and life expectancy is less than one year in most cases. The major cause of death is respiratory failure associated with a narrow chest and pyridoxine-responsive seizures are also observed in some severely affected cases (Whyte, 2010; Nakamura *et al.*, 2010). The childhood and adult forms of HPP show milder phenotypes and odontohypophosphatasia causes premature loss of deciduous teeth without evidence of skeletal disease. Perinatal HPP is more common in Japan than in other countries and is the fifth most common form of fetal-diagnosed skeletal dysplasia (Satoh *et al.*, 2009).

There is no established treatment for HPP, but several experimental approaches have been attempted to treat TNALP knockout mice (*Akp2*$^{-/-}$). The phenotype of these mice mimics that of severe infantile HPP; the animals appear normal at birth, but rapidly develop growth failure, epileptic seizures, and hypomineralization, and die by two weeks of age. Recently, Millán et al. reported that *Akp2*$^{-/-}$ mice can be treated by repeated injection of bone-targeted TNALP with deca-aspartates at the C terminus (TNALP-D10) (Millán *et al.*, 2008). Based on these data, new clinical trials of enzyme replacement therapy (ERT) for patients with infantile and childhood HPP have been initiated (http://clinicaltrials.gov/). Another important possibility for treatment of HPP is gene therapy. We have recently demonstrated that a single