Liver-type of tissue non-specific alkaline phosphatase is induced during mouse bone and tooth cell differentiation

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ABSTRACT

Background and objective: Tissue non-specific alkaline phosphatase (TNSALP) contains two types—bone- and liver-type—which are produced from the same gene due to differences in splicing. These two differ in their promoter, but the amino acid sequences of the mature proteins are identical. In this study, we examined the relationship between the two types of TNSALP expression and osteoblast differentiation.

Design: Gene expression of the two types of TNSALP was observed by reverse transcription-polymerase chain reaction. MC3T3-NM4 was sub-cloned from an established mouse osteoblastic cell line in which osteoblast characters do not appear without dexamethasone. The C2C12 mouse myoblastic cell line, which can be induced to osteoblasts with bone morphogenic protein 2, and organ-cultured tooth germs were also used in this work.

Results: The gene expression of liver-type TNSALP was observed in only MC3T3-NM4 activated by dexamethasone. For C2C12, the gene expression of bone-type TNSALP was observed even in non-induced conditions where myotubes were formed, whereas the liver-type TNSALP mRNA was only expressed when C2C12 differentiated into osteoblasts by bone morphogenic protein 2. Furthermore, in the organ-cultured tooth germs, the liver-type TNSALP mRNA was expressed according to differentiation of tooth germs.

Conclusion: These results suggest that the liver-type TNSALP mRNA is induced according to differentiation of bone and tooth.

1. Introduction

Alkaline phosphatase (ALP) is an enzyme (E.C.3.1.3.1.) that degrades phosphoric acid monoester bonds in an alkaline state (pH 9–11). The measured value of this enzyme has been widely used clinically to capture abnormalities of organs (Weiss et al., 1988). There are many types of this enzyme, from *E. coli* to humans. There are three different types of genes in mammalian alkaline phosphatase: tissue non-specific ALP (TNSALP) (Matsuura, Kishi, & Kajii, 1990), in which expression is widely observed, intestinal ALP (Henthorn, Raducha, Kadesch, Weiss, & Harris, 1988), in which expression is observed in the small intestine, and placental-like ALP, similar to the human placental type. There are other placental ALP in humans (Knoll, Rothblum, & Longley, 1988). Human TNSALP is located on chromosome 1 and the human intestinal type, placental type and placental-like type are on chromosome 2, then these are all distinct genes. It has embryologically thought that TNSALP occurred first, followed by intestinal ALP, placental-like ALP, and finally placental ALP branching from primitive ALP (Sharma, Pal, & Prasad, 2014). Despite for a long time studies and numerous papers about human ALP (Fawley & Gourlay, 2016; Millán, 2006; Millán & Whyte, 2016; Poupon, 2015; Sardiwal, Magnusson, Goldsmith, & Lamb, 2013; Sharma et al., 2014), the elucidation of their functions are limited to TNSALP and intestinal types, yet other types are still unknown.

Hypophosphatasia (HPP) features rickets or osteomalacia and hypomineralization of teeth. They result from mutation of TNSALP gene. The deficiency or point mutations of TNSALP gene lead to the abnormal intracellular transport of TNSALP protein or the low activity of TNSALP enzyme (Whyte, 2017). Many of severe cases caused by the mutation in HPP are defective in intracellular trafficking. Others are ones that have low phosphatase activity or those that secreted without binding to the membrane. Currently, more than 360 mutations have been reported (http://www.sesep.uvsq.fr/database_hypo/mutation.html) in TNSALP.

TNSALP produces two mRNAs—bone- and liver-type—by splicing, but their amino acid sequences of mature proteins are the same. As shown in Fig. 1, these splices are regulated by a promoter located upstream of exon 1A concerning the bone-type and by a promoter located between exon 1A and exon 1B concerning the liver-type. Bone-type is transcribed from exon 1A to exon 2 by jumping over exon 1B, while liver-type transcription starts from exon 1B and continues to exon 2. The liver-type is further spliced to produce two mRNAs of a slightly different length. However, since exon 3, which has a translation initiation site, and the downstream are identical, translated proteins have the same amino acid sequence. TNSALP, intestinal type, placental type, and placental-like type are chemically distinguishable due to their different amino acid sequences (Harris, 1990). TNSALP is sensitive to temperature and inactivates at temperature > 65°C. Placental ALP and placental-like ALP are stable for an hour or more at 65°C. Intestinal ALP is partially stable under heating. Placental-like ALP is sensitive to L-Leucine, and L-

Levamisole is a particularly potent inhibitor of TNSALP. However, it is difficult to chemically distinguish liver- from bone-type TNSALPs having the same amino acid sequences. Clinically, liver- and bone-type TNSALPs are distinguishable based on differences in electrophoretic behavior. However, this is mainly due to post-translational modification in expressed tissues. Regardless of whether it is transcribed from either promoter, the TNSALP undergoes bone-type modification if expressed in bone and undergoes liver-type modification if expressed in the liver. Few studies have explored the amount of expression of promoter-derived bone- and liver-type TNSALPs in the same tissue. However, even though the protein and amino acid sequence are similar, different promoters will undergo different expressional regulation. Therefore, they may differ in distribution and function. To investigate how these differences in regulation occur, we generated a primer upstream from the translation start site and examined its expression. As a result, unexpectedly, liver-type TNSALP was up-regulated during osteoblast differentiation rather than bone-type TNSALP.

2. Material and methods

2.1. Harvesting of tissues

Livers, kidneys, and calvaria were obtained from newborn ddY mice. Isolated tissues were cooled and homogenized by Polytron, followed by RNA extraction.

2.2. Cell culture

MC3T3-NM4 was sub-cloned from the MC3T3-E1 cell line derived from mouse calvaria (T. T. Baba, 2000). C2C12 is an established mouse myoblast cell line. MC3T3-E1 and C2C12 were obtained from the RIKEN Cell Bank (Tsukuba, Japan). Cells were maintained under 5% CO_2 at 37°C in minimum medium α modification (Sigma Chemical Co.) supplemented with 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin (Life Technology, Inc., Grand Island, NY), and 80 μg/ml L-ascorbic acid phosphate magnesium salt (Wako Pure Chemical Industries) (Baba, Nemoto, Miyazaki, & Oida, 2008). Cells that reached confluence were cultured with 10 nM dexamethasone or 50 μg/ml bone morphogenic protein 2 (BMP2) (Maruoka et al., 1995) indicated in the text. The medium was changed every 2 or 3 days.

2.3. Organ culture

Organ cultures were created according to a previous report (Baba, Terashima, Oida, & Sasaki, 1996). A schematic figure of the method and histological views are shown in Fig. 2. In brief, ICR mouse embryos 16.5 days *in utero* were aseptically removed from pregnant females (vaginal plug = day 0). The mandibular first molar germs were dissected from the surrounding tissues under a dissecting microscope and rinsed several times with serum-free HBSS (GIBCO Laboratories, Grand Island, NY) The epithelial and mesenchymal components of the tooth germs were enzymatically dissociated by incubation in HBSS solution containing 60 units/ml collagenase (CLS II type: Worthington Biochemical Corporation, NJ) for 70 min at 37°C. The components and non-separated tooth germs were then cultured on Millipore filter (pore size, 0.45um) supported by a stainless metal grid. After the organ culture, dissociated components alone and intact tooth germs without a prior enzymic treatment were analyzed with reverse transcription-polymerase chain reaction (RT-PCR) or morphological observation.

2.4. RT-PCR

Total RNA was isolated using Isogen reagent (Nippon Gene Co., Tokyo, Japan) according to a previous report (Baba, Ohara-Nemoto, Miyazaki, & Nemoto, 2013). cDNA was prepared from 1 μg of RNA with reverse transcriptase (TAKARA BIO Inc., Otsu, Japan) in a reaction mixture (20 μl) containing 0.5 mM dNTPs and 0.2mg of oligo dT (Life Technology Inc.). The forward primers are 5'-TCCTTAGGGCTGCCGCT 3' for bone-type TNSALP and 5'- ATAGGGGACAGGGACCTGTGA -3' for liver-type TNSALP. The reverse primer is 5'- TGTACCCTGAGATTCGTCC -3', which is common in bone- and liver-type TNSALP. The primer set of amelogenins are described in a previous repot (Oida et al., 1996). RT-PCR was performed for 30 cycles according to a previous report (Baba et al., 2013). PCR products were separated on 1.8% agarose gels.

2.5. Alkaline phosphatase activity assay

ALP activity was determined according to a protocol previously described (Baba et al., 2013). In brief, MC3T3-NM4 and C2C12 cells were first plated at a density of 1.0×10^4 cells/cm^2 in 24-well plates. After reaching confluence, dexamethasone or BMP2 were added every other day. After indicated days, cells were then washed with phosphate buffered saline, lysed in solubilization buffer (80μl/well, 10 mM Tris–HCl, pH 7.4, containing 0.1% Triton X-100), sonicated for 5 min and centrifuged for 20 min at 12,000×g. Four μl of supernatant and the reaction mixture (150 μl) comprising 4mM *p*-nitrophenyl phosphate and 5 mM $MgCl_2$ in 50 mM carbonate buffer (pH 10) were incubated at room temperature for

6 min, mixed with 50 ml of 2 M NaOH to stop the reaction, after which absorbance at 405nm was determined by a microplate reader (Model 550, Bio-Rad). One unit was defined as the quantity of ALP that produced 1mmol of *p*-nitrophenol in 1 h.

2.6. Histological technique

The histological technique was performed according to the protocol described in a previous paper (Baba et al., 1996). In brief, non-separated tooth germs and separated components from tooth germs were fixed with 2.5% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4. Fixed samples were dehydrated with gradient alcohol, embedded in Technovit 7100 (Heraeus Kulzer GmbH, Wehrheim, Germany), and sectioned at 1μm thickness.

2.7. Statistical analysis

All values are presented as means ± SD. Differences between the two groups were assessed using Student's *t*-tests.

3. Results

3.1. Bone-type TNSALP mRNA is expressed in the bone, liver, and kidney, but liver-type one is mainly expressed in the liver

We investigated the gene expression of bone- and liver-type TNSALP in the kidney, liver, and calvaria of a newborn mouse. As shown in Fig. 3, bone-type TNSALP mRNA was similarly expressed in the kidney, liver, and bone. However, in the liver-type TNSALP mRNA, it was specifically expressed in the liver.

3.2. Liver-type TNSALP mRNA expression is only seen in osteoblast-like cells induced with dexamethasone

MC3T3-NM4 is a sub-cloned cell (Baba, 2000) from the mouse osteoblast MC3T3-E1 and is a mineralization quiescent cell that does not cause mineralization or any increase in ALP activity in the absence of dexamethasone. However, when 10 nM dexamethasone is added, ALP activity increases and

mineralization is also observed. Fig. 4a shows a Von Kossa stained view on day 17 after confluence and Fig. 4b shows a time-course change in ALP activity. The amount of mineralized nodules increased markedly with the addition of dexamethasone, while ALP activity was also significantly higher than that in the control group. Bone-type TNSALP mRNA was expressed on day 4 (but weak), 6, or 9 in the control group despite the absence of alkaline phosphatase activity (Fig. 4c). In the dexamethasone-added group, bone-type TNSALP mRNA expression was observed on day 4 and 6, and decreased slightly on day 9. In contrast, no expression of liver-type was observed in the control group. In a dexamethasone-added group, the expression of liver-type was observed on day 4 and a faint expression was observed on day 6. The fact that liver-type TNSALP mRNA is most strongly expressed on day 4 and increased ALP activity continues thereafter is probably attributed to the time lag between mRNA expression and the change from mRNA to active protein.

3.3. Liver-type TNSALP mRNA was detected only when BMP induces myoblasts into osteoblasts

An experiment was performed using C2C12, a mouse myoblast that differentiates into osteoblast by BMP2. When BMP2 is not added, C2C12 differentiates into myocytes and forms myotubes. However, adding 50 μg/ml BMP2 blocks myocyte differentiation and C2C12 differentiates into osteoblasts (Fig. 5a). ALP activity also increases via the addition of BMP2 (Fig. 5b). The expression of bone-type TNSALP mRNA was observed both in myocytes, which forms myotubes (cont), and in osteoblasts after differentiation by BMP2 (Fig. 5c). In contrast, for the liver-type TNSALP, gene expression is not observed at all in the control condition (cont). However, it was observed on day 5 after the addition of BMP2, as well as slight expression on day 7.

3.4. In organ-cultured tooth germs, gene expression of liver-type TNSALP accompanies the expression of amelogenin

The gene expressions of two types of TNSALPs in mouse organ-cultured tooth germs were investigated (Fig. 6). Fig. 2 shows the experimental design and histological observations. Enamel, dentin, and dental papilla in intact tooth germs organ-cultured for five days showed normal development (Fig. 2, G5), but dental epithelial and dental mesenchymal tissues organ-cultured for five days after separation were not normally developed (Fig. 2, E5 and M5). The gene expression of amelogenin, which is a specific protein of immature enamel tissue, was observed in organ-cultured tooth germs after day 6, but that of bone-type TNSALP was observed continuously throughout the culture period (Fig. 6, lanes 2 to 8). In contrast, the gene expression of liver-type TNSALP, as well as gene expression of amelogenin, occurred from day 6 (Fig. 6, lanes 6 and 8). Neither differentiation into ameloblasts nor differentiation into odontoblasts occurs

when epithelial and mesenchymal tissues are separately cultured (Baba et al., 1996). However, in this case, bone-type TNSALP mRNA expression was observed (Fig. 6, lanes E4 and M4).

4. Discussion

Human ALP is classified as TNSALP, intestinal type, placental type, and placental-like type (Harris, 1990). In mice, the ALP present in the placenta is TNSALP and classified into three types, TNSALP, intestinal type, and placental-like type. TNSALP is further classified into two ALPs—bone- and liver-types—whose promoters differ, but an identical amino acid sequence in their mature proteins (Shen, Liu, Kan, & Kam, 1988; Studer, Terao, Gianni, & Garattini, 1991; Zernik et al., 1990).

Clinically, ALP has been classified from type 1 to type 6 based on the behavior of electrophoresis of blood samples (Weiss et al., 1988). Type 1 and 2 are liver-types and type 3 is a bone-type. However, the difference here is the post-transcriptional variation based on modification in each organ. Therefore, although it is effective to know the abnormality of the organ, this does not distinguish promoter-dependent expression between bone- and liver-types. Although both the bone- and liver-type TNSALP share the same amino acid sequence in their mature protein, their promoter regions differ, which means that each TNSALP may be controlled by different regulation. However, since the mature proteins are completely identical, few studies have focused on differences in expression of the two TNSALPs.

In the promoter region of the bone-type TNSALP gene, the Sp1 binding site and TATA box are present. The former is considered to be involved in tissue-specific expression, and the latter seems to be involved in universal expression (Matsuura et al., 1990). This suggests that bone-type TNSALP may be expressed as tissue-specific and, in some cases, universally. In fact, our results show that gene expression of bone-type TNSALP increased when C2C12 was induced to osteoblasts by BMP2. This confirms that bone-type TNSALP also changes its gene expression according to cell differentiation. Conversely, neither Sp1 binding sites nor TATA boxes are found in the promoter region of liver-type TNSALP gene.
Proteins that are controlled by different promoters within the same gene are not limited to TNSALP. Amylase (Schibler, Hagenbüchle, Wellauer, & Pittet, 1983) and RUNX2 (Fujiwara et al., 1999) also share this behavior. However, due to the splicing position of these proteins, different proteins having different amino acid sequences of the amino-terminal sides are generated. In amylase, such proteins controlled by different promoters were expressed in different tissues. In the future, liver-type TNSALP may change the splicing position and produce another protein similar but not identical to bone-type TNSALP in its amino

acid sequence.

This study observed gene expression of TNSALP using the osteoblasts MC3T3-NM4, myoblast C2C12, and tooth germs. MC3T3-NM4 is a sub-cloned cell from MC3T3-E1 and is a resting osteoblast that does not cause mineralization or increases in ALP activity without dexamethasone or BMP for at least ~36 days (Baba, 2000). C2C12 is a myoblast cell line that starts to form myotubes within approximately four days after reaching confluence and differentiating into myocytes without further stimulation. Differentiated myocytes express MyoD, a specific muscle tissue protein. BMP2 can induce this cell line into osteoblasts and increase ALP activity (Katagiri et al., 1997). Tooth germs of 16.5-day mouse embryos do not produce amelogenin. Tooth germs removed from the mouse utero can develop via organ culture, where ameloblasts are differentiated from dental epithelial cells and produce amelogenin (Baba et al., 1996). Dental epithelium separated from tooth germs does not show differentiation or normal growth. Gene expression of bone-type TNSALP was observed in MC3T3-NM4 despite presence or absence of dexamethasone and in C2C12 despite presence or absence of BMP2. However, gene expression of liver-type TNSALP was only observed in MC3T3-NM4 with dexamethasone and in C2C12 with BMP2. It was surprising that gene expression of bone-type TNSALP was also observed in the myoblast cell C2C12. Similar results were obtained in experiments using tooth germs. The gene expression of bone-type TNSALP was also observed in tooth embryos with a low differentiation before gene expression of amelogenin, but the gene expression of liver-type TNSALP was accompanied by the appearance of amelogenin. Furthermore, bone-type TNSALP mRNA was also expressed in separated components of tooth germs.

It is still unknown why liver-type TNSALP mRNA, which remarkably expressed in the liver and did not express in newborn mouse calvaria, varies in its expression in osteoblasts according to its differentiation. One reason differentiation from undifferentiated cells to hepatocytes always progresses may be because the liver is a highly regenerative organ. Therefore, the gene expression of liver-type TNSALP may be high. Moreover, in bone and dental tissues, it seems that liver-type TNSALP mRNA is transiently expressed when differentiating from undifferentiated cells and convergence to bone-type TNSALP occurs when differentiation is completed.

In this study, we showed that liver-type TNSALP mRNA was expressed with osteoblast differentiation and tooth development. In addition, we also showed that bone-type TNSALP mRNA is also expressed in myotube forming C2C12. Further studies are required to elucidate the mechanism behind why gene expression of liver-type TNSALP is induced by differentiation of osteoblasts. However, liver-type TNSALP may be a better indicator of differentiation than bone-type TNSALP, as the former does not express when osteoblast differentiation is not proceeding.

Conflicts of interest

The authors report no conflicts of interest related to this study.

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Legend

Figure 1. Splicing pattern of bone- and liver-type TNSALP

The result from the splicing bone-type mRNA shows jumping of exon1B and continuation to exon2 from exon1A. The first exon of liver-type mRNA is exon1B and continues to exon2. The liver-type produces two mRNAs with minor differences in splicing at the ends of exon1B. As a result, the double band appears in RT-PCR (see Fig. 3). ATG is the location of the first transcription codon.

Figure 2. Organ culture of mouse tooth germs

Tooth germs were isolated from the 18.5-day mouse embryo jawbone and an aliquot of them were separated into epithelium and mesenchyme. Separated or non-separated tooth germs were organ-cultured on a Millipore filter. Hematoxylin-Eosin staining views of dental epithelium (E) and dental mesenchyme (M) right after separation, tooth germ (G5) for the 5-day culture, and dental epithelium (E5) and dental mesenchyme (M5) for day 5 after separation are shown.

Figure 3. Expression of TNSALPs in each tissue

Kidney, liver, and bone mRNAs were isolated from newborn mice. The bone-type TNSALP was expressed in all three tissues, whereas the liver-type was mainly expressed in the liver.

Figure 4. Expression of TNSALPs in MC3T3-NM4 induced by dexamethasone

(a) Von Kossa staining views of 17-day cultured MC3T3-NM4. (b) ALP activity in the presence (closed circle) or absence (open circle) of 10 nM dexamethasone. (c) The mRNA expression of bone- and liver-type TNSALP in MC3T3-NM4 stimulated by 10 nM dexamethasone. Liver-type TNSALP was expressed in 4-day culture induced by dexamethasone. Liver-type TNSALP was expressed only when MC3T3-NM4 was induced by dexamethasone. dex, dexamethasone; cont, control (vehicle only). *$p<0.01$ compared to control.

Figure 5. Expression of TNSALPs in C2C12 induced by BMP2

(a) Phase contrast microscope views of 6-day cultured C2C12. (b) ALP activity in the presence (closed circle) or absence (open circle) of 50 μg/ml BMP2. (c) The mRNA expression of bone- and liver-type TNSALP in C2C12 stimulated by 50 μg/ml BMP2. No liver-type TNSALP was expressed in non-stimulated myoblasts, whereas bone-type was expressed. BMP, bone morphogenic protein 2; cont, control (vehicle only). *$p<0.01$ compared to control.

Figure 6. Expression of TNSALPs during tooth germ development

The design of this experiment is shown in Fig. 2. The mRNA expressions of intact tooth germs organ-cultured for 2, 4, 6, and 8 days and isolated dental epithelium and dental mesenchyme organ-cultured for 4 days. E4; 4-day organ-culture of isolated dental epithelium; M4; 4-day organ-culture of isolated dental mesenchyme. Expression of bone-type TNSALP (BN-TNAP) was found in all cases, including separated tooth germs, whereas liver-type (LV-TNAP) coincided with expression of amelogenin. GAPDH; glyceraldehyde 3-phosphate dehydrogenase.

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Figure 1

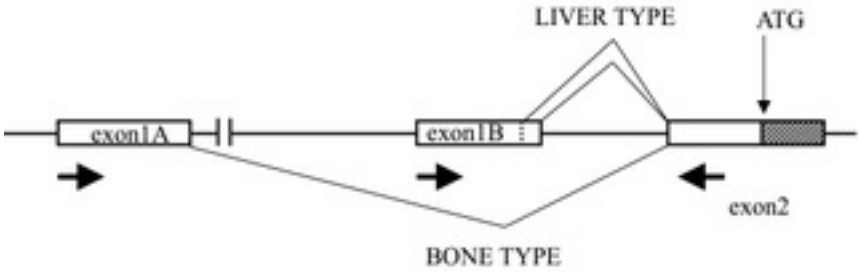
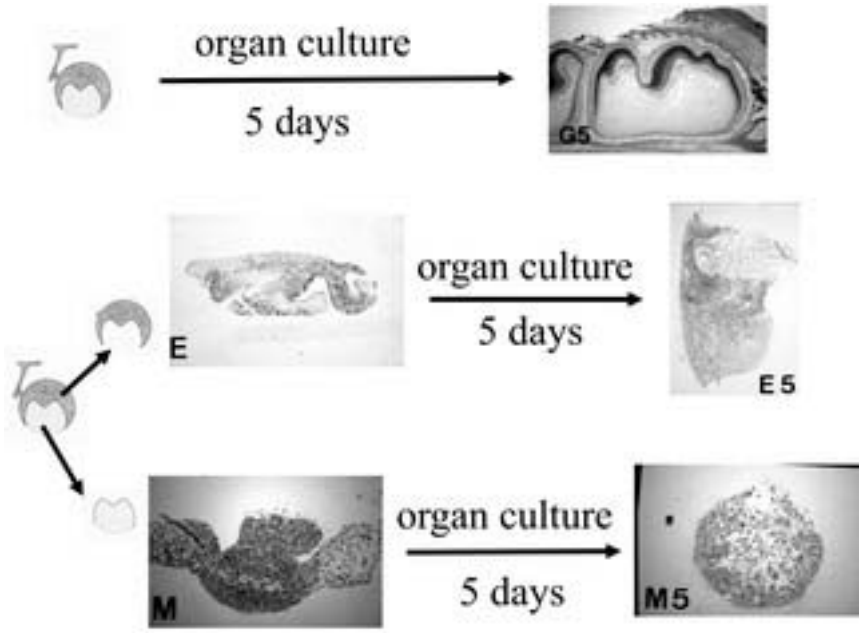


Figure 2



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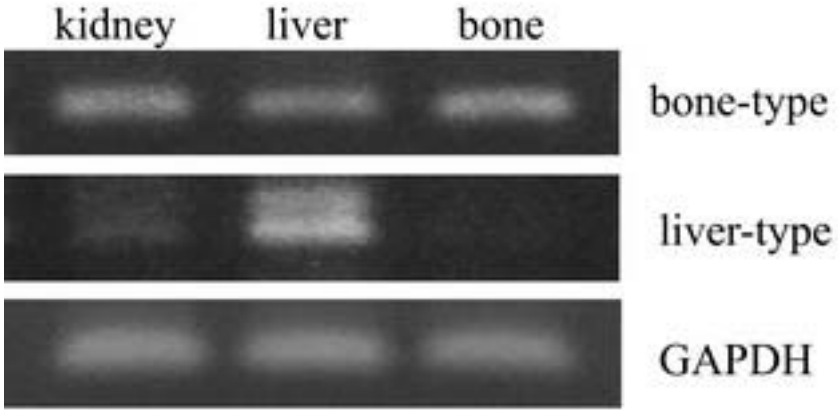

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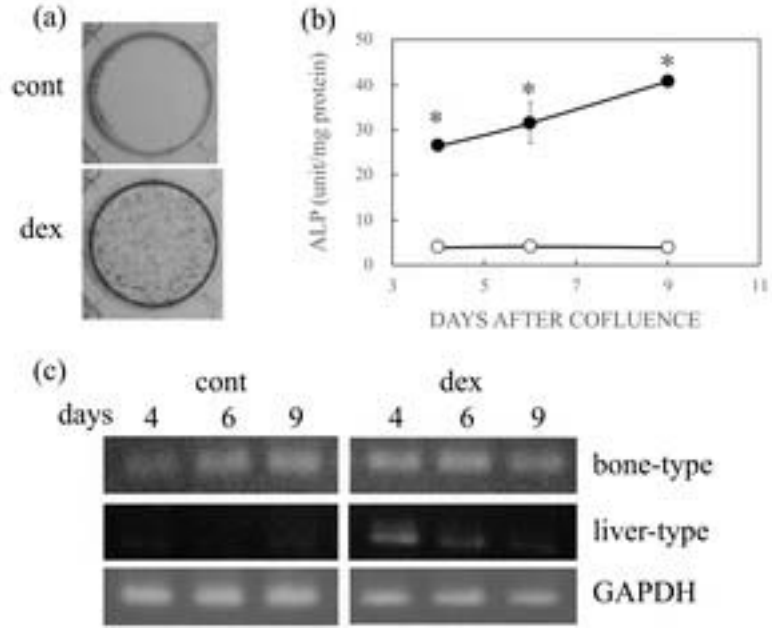

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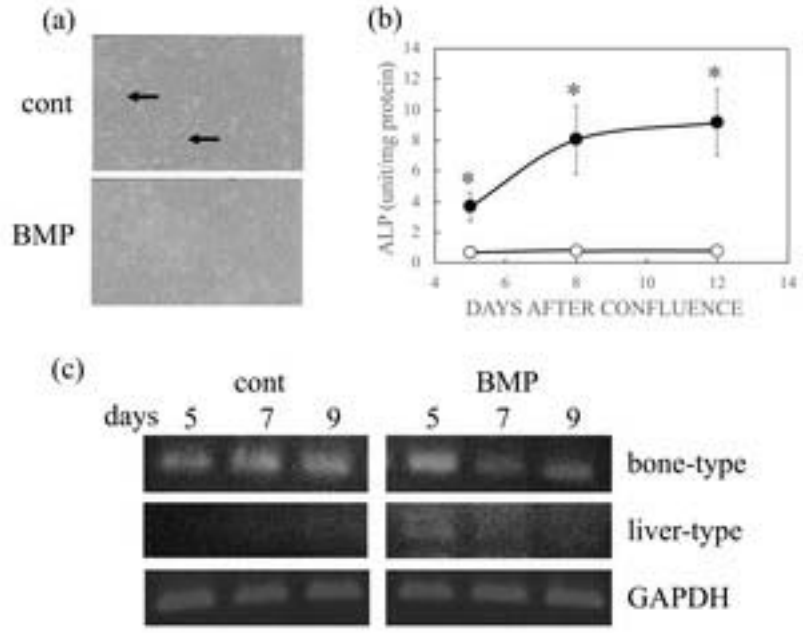
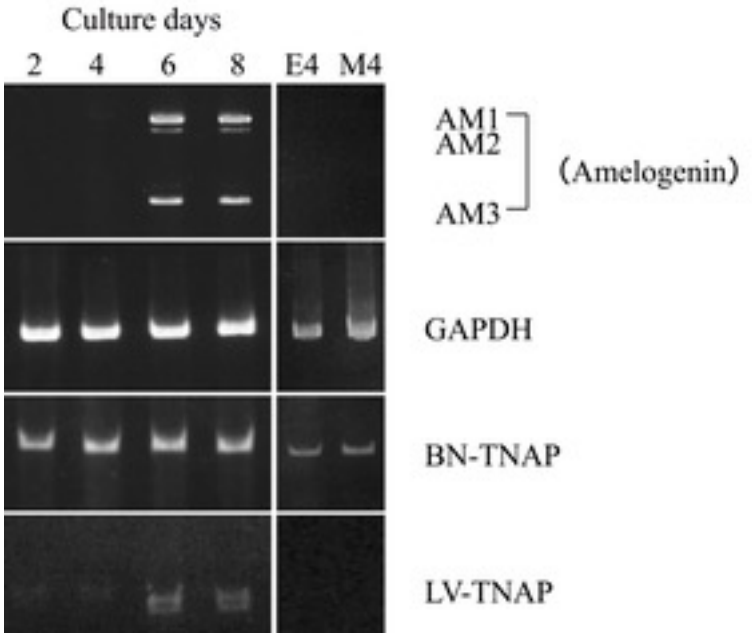


Figure 6


Culture days
2
4
6
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E4
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AM1
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(Amelogenin)
GAPDH
BN-TNAP
LV-TNAP