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SHORT COMMUNICATION

Utility of human placental alkaline phosphatase as a genetic marker for cell tracking in bone and cartilage

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Abstract It was the aim of the current study to evaluate the utility of human placental alkaline phosphatase (hPLAP) as a genetic marker for cell tracking in bone and cartilage, using transgenic Fischer 344 rats expressing hPLAP under the control of the ubiquitous R26 promoter [F344-Tg(R26-hPLAP)]. hPLAP enzyme activity was retained during paraffin and methylmethacrylate (MMA) embedding, and was best preserved using 40% ethanol as fixative. Endogenous alkaline phosphatase activity could be completely blocked by heat inactivation in paraffin and MMA sections, allowing histochemical detection of hPLAP in the complete absence of background staining. In addition, sensitive detection of hPLAP was also possible using immunohistochemistry. F344-Tg(R26-hPLAP) rats demonstrated ubiquitous expression of hPLAP in hematopoietic bone marrow cells and stromal cells such as osteoblasts, osteocytes, and chondrocytes. Osteoclasts only weakly expressed hPLAP. In conclusion, hPLAP provides superb detection quality in paraffin and plastic sections, and constitutes an excellent genetic marker for cell tracking in hard and soft tissues.

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Abbreviations

APES	3-Aminopropyltriethoxy-silane
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
EDTA	Ethylene diamino tetra acetate
F344	Fischer 344
GFP	Green fluorescent protein
hPLAP	Human placental alkaline phosphatase
MMA	Methyl methacrylate
PFA	Paraformaldehyde
R26	0.8 kb piece of the ROSA βgeo 26 promoter
Tg	Transgenic

Introduction

Cell and gene therapy are thought to revolutionize tissue repair in various organs, cancer therapy, and therapy of genetic diseases in the near future (reviewed in Prockop et al. 2003). Recent work in the field of stem cell research has suggested that cell therapy with pluripotent precursor mesenchymal cells may enhance tissue repair in various organ systems, and mesenchymal cells may also be attractive targets for ex vivo gene therapy (reviewed in Prockop 1997; Prockop et al. 2003). Regenerative therapies also hold great promise to cure or ameliorate a variety of bone and joint diseases. For example, it was reported in a goat model of osteoarthritis that injection of autologous mesenchymal stem cells harvested from bone marrow and expanded in culture stimulated regeneration of meniscal tissue and retarded progressive joint destruction (Murphy et al. 2003). To explore the therapeutic potential of these

methods, appropriate animal models are necessary that allow tracing the fate of individual donor or manipulated cells in hard tissues of the host organism.

One approach for the generation of such models is to introduce a stable genetic marker in all somatic cells. Because a stable genetic marker is expressed in whole progeny of a specific cell, this approach is especially useful for cell lineage experiments. The most widely used genetic markers are *Escherichia coli lacZ*, and green fluorescent protein (GFP) from the jellyfish *Aequorea victoria.* However, *lacZ* and GFP have several important disadvantages for histological cell tracking. *LacZ* is heat labile, and the enzyme activity is destroyed during paraffin embedding (Gossler and Zachgo 1993). A further drawback of *lacZ* is that osteoclasts have a very high β-galactosidase activity that severely hampers studies of *lacZ* gene expression in bone (Zheng et al. 1991). An important advantage of GFP is that it can easily be visualized in living cells (Cubitt et al. 1995). However, the fluorescent properties of GFP are greatly diminished during normal paraffin embedding, and sensitive detection of GFP in paraffin sections requires either sophisticated equipment (Walter et al. 2000) or immunohistochemical detection methods (Zagzag et al. 2003). Thus, the usefulness of GFP for histological studies is limited. Human placental alkaline phosphatase (hPLAP), which was initially used as a reporter enzyme in transfection assays (Henthorn et al. 1988), is a heat-stable marker enzyme that retains its enzymatic activity during routine paraffin embedding (Kisseberth et al. 1999). Enzymatic activity of hPLAP can be detected histochemically in tissue sections by post-embedding procedures. Thus, hPLAP is a genetic marker that may be highly suitable for histological studies requiring tissue sectioning and excellent tissue morphology. However, it is unknown whether the hPLAP enzyme activity is preserved during methylmethacrylate (MMA) embedding, which is required for excellent hard tissue morphology. The lack of an appropriate genetic marker suitable for the detection of labeled cells in hard tissue sections has hampered the development of regenerative treatment protocols for bone and joint diseases in the past.

Ubiquitous expression of the genetic marker is a prerequisite for a versatile animal model of genetically labeled cells. The ROSA βgeo 26 (ROSA26) gene promoter has been found to be especially useful to direct ubiquitous expression of marker genes in mice and rats (Zambrowicz et al. 1997; Kisseberth et al. 1999). Similarly, transgenic mice and rats expressing hPLAP or GFP under the control of the R26 promoter, a 0.8 kb piece of the ROSA26 promoter sequence, show ubiquitous, uniform, and stable expression of both genetic markers (Kisseberth et al. 1999). Both the R26-hPLAP and the R26-GFP constructs were shown to be developmentally neutral in transgenic rats and mice (Kisseberth et al. 1999). Using R26-hPLAP transgenic inbred Fischer 344 [F344-Tg(R26-hPLAP)] rats as an animal model, the present experiments were designed to explore the potential of hPLAP as a genetic marker for cell tracking in bone and cartilage.

Materials and methods

Animals

All experimental procedures were conducted in compliance with prevailing animal welfare regulations. Heterozygous male or female R26-hPLAP (human placental alkaline phosphatase) transgenic inbred Fischer 344 [F344-Tg(R26-hPLAP)] rats were mated with wild-type F344 rats, and the resulting wild-type and heterozygous transgenic offspring were genotyped by enzyme histochemistry using a drop of tail blood as described (Kisseberth et al. 1999). The rats were housed in pairs at 24°C and a 12 h/12 h light/dark cycle with free access to tap water and commercial rat diets (Altromin 1324 for maintenance and 1314 for breeding, Altromin, Lage, Germany).

Histology and hPLAP histochemistry

To establish the most useful method of fixation and tissue processing, liver, kidney, and bones from adult wild-type and R26-hPLAP-tg animals were harvested and were fixed in 4% paraformaldehyde (PFA) in 0.1M phosphate buffer at pH 7.4 at 4°C for 24 h, or in 40% ethanol at 4°C for 48 h. Subsequently, the tissue specimens fixed in PFA were washed overnight in 0.1M phosphate buffer, pH 7.4, containing 10% sucrose. Fixed tissues were dehydrated and embedded in paraffin or in a modified methylmethacrylate (MMA) embedding mixture that preserves enzyme activities, and that can be used for immunohistochemistry (Erben 1997). Five-µm-thick paraffin and MMA sections were cut with a HM360 microtome (Microm, Walldorf, Germany), and were mounted on slides pretreated with 3-aminopropyltriethoxy-silane (APES, Sigma-Aldrich, Deisenhofen, Germany). In order to compare hPLAP enzyme activity in decalcified, paraffin-embedded and undecalcified, MMA-embedded bone samples, bones from wild-type and R26-hPLAP-tg rats were decalcified using 0.5M ethylene diamino tetra acetate (EDTA, Sigma) in PBS, pH 8.0, containing 5% ethanol as a preservative. The decalcifying solution was changed weekly. Subsequently, the decalcified bone specimens were dehydrated and embedded in paraffin.

Paraffin sections were deparaffinated using xylene, whereas MMA sections were deplasticized using 2-methoxyethylacetate as described (Erben 1997). Deparaffinated and deplasticized sections were rehydrated and heated at 65°C for 30 min in deionized water to block endogenous

alkaline phosphatase activity. Sections were then incubated in TRIS buffer (0.1 M Tris–HCl, pH 9.5, 0.1M NaCl, 5 mM $MgCl_2$) containing 0.17 mg/ml of the substrate 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) at 37°C for 1, 2, 3, 4, 5 or 6 h, or at room temperature overnight. Subsequently, sections were counterstained with nuclear fast red (Sigma), dehydrated, and mounted using Vectamount (Vector, Burlingame, CA, USA).

Immunohistochemical detection of hPLAP expression in transgenic rats was performed on PFA- or ethanol-fixed paraffin- and MMA-embedded tissues. Sections from wild-type animals were used as controls. Sections were deparaffinized or deplasticized, and, after blocking with 20% horse serum, incubated for 2 h at room temperature with a monoclonal mouse IgG_{2a} anti-hPLAP antibody (supernatant of clone 8B6, Dako, Hamburg, Germany) diluted 1:50. Bound antibody was detected with biotinylated horse anti-mouse IgG and peroxidase-conjugated avidin biotin complex (Vector), using Vector VIP as enzyme substrate. Sections were dehydrated without counterstaining, and were mounted using Vectamount (Vector).

Cell culture

To examine the expression of hPLAP in cultured osteoblastic cells, bone marrow cells from wild type and transgenic rats were harvested from the femora and tibias, and were cultivated for osteoblastic differentiation by addition of ascorbic acid, β-glycerophosphate, and dexamethasone (Erben et al. 1997). After 1, 2, 3, 7, and 14 days of culture, cells were fixed with 40% ethanol for 10 min, washed with PBS, air-dried, and heated at 65°C for 30 min. Cells were then incubated in TRIS buffer containing 0.17 mg/ml BCIP, washed, counterstained with nuclear fast red, and air dried.

Results and discussion

Based on previous results (Kisseberth et al. 1999), expression of the human placental alkaline phosphatase (hPLAP) transgene in R26-hPLAP transgenic inbred Fischer 344 [F344-Tg(R26-hPLAP)] was expected in organs such as liver, kidney, brain, muscle, and lung. However, it was unknown whether F344-Tg(R26-hPLAP) rats express the transgene in bone and cartilage. In addition, appropriate methods for hPLAP detection in bone and cartilage were not established.

Figure 1 shows that hPLAP enzyme activity in paraffin sections of liver from F344-Tg(R26-hPLAP) rats was retained during paraffin embedding, and that the enzyme activity was much better preserved using 40% ethanol (Fig. 1a) as fixative compared with 4% paraformaldehyde (PFA) (Fig. 1b). Endogenous alkaline phosphatase activity was totally inactivated by heating the sections at 65°C for 30 min prior to staining (Fig. 1c). Therefore, tissue specimens were fixed with 40% ethanol in all subsequent experiments. In analogy to our results, previous studies have shown that other alkaline phosphatases are also better preserved using 40% ethanol as a fixative compared with PFA (Doty and Schofield 1984; Erben 1997). Methylmethacrylate (MMA) embedding did not interfere with preservation of hPLAP enzyme activity (Fig. 1e). Rather, MMA sections displayed improved morphological detail, and allowed a more precise subcellular localization of the transgene expression. For example, Fig. 1e clearly shows that the hPLAP enzyme was mainly located at the luminal membrane of renal tubular epithelium, while the staining pattern in paraffin sections was more diffuse (Fig. 1d). Similar to paraffin sections, endogenous alkaline phosphatase activity was completely inactivated in MMA sections by heat pretreatment (Fig. 1f). In addition to histochemistry, it was also possible to detect hPLAP by immunohistochemistry in PFA-fixed (Fig. 1g–h) and ethanol-fixed (Fig. 1i) paraffin- (Fig. 1g–h) and MMA-embedded (Fig. 1i) transgenic tissues, using the monoclonal antibody 8B6. Staining was absent in sections from wild-type controls (inset in Fig. 1i and data not shown). However, immunohistochemistry was not superior to histochemistry in terms of detection sensitivity of the marker gene. Therefore, we decided to use histochemistry for all further studies.

When we examined hPLAP expression in sections of MMA-embedded bones from F344-Tg(R26-hPLAP) rats, we found strong staining in all mesenchymal bone cells, i.e., in osteoblasts and osteocytes (Fig. 2a–b) as well as in growth plate and articular cartilage chondrocytes (Fig. 2c–d). Osteoblasts showed a clear membranous hPLAP-staining pattern (Fig. 2a), whereas osteoclasts demonstrated only weak membrane expression of the transgene, and no cytoplasmic hPLAP expression (Fig. 2b). In bone marrow, all cells expressed the transgene in F344-Tg(R26-hPLAP) rats, with particularly strong cytoplasmic and membrane expression in megakaryocytes (Fig. 2e). No staining was found in bone marrow, bone or cartilage cells of wild-type rats (Fig. 2f–i). Overall, the staining pattern found by histochemistry was identical to the one determined by immunohistochemical detection of hPLAP (Fig. 1i). In addition, adherent mesenchymal cells in bone marrow cultures of F344-Tg(R26-hPLAP) rats displayed strong hPLAP expression at all stages of differentiation (Fig. 3). Thus, our study confirms the usefulness of the R26 promoter to direct stable and uniform expression of marker genes to mesenchymal and hematopoietic cells (Kisseberth et al. 1999). Because of the strong expression in the mesenchymal cell lineage, F344-Tg(R26-hPLAP) rats may be very suitable donor animals for cell therapy experiments with mesenchymal precursor cells. However, a limitation of this animal

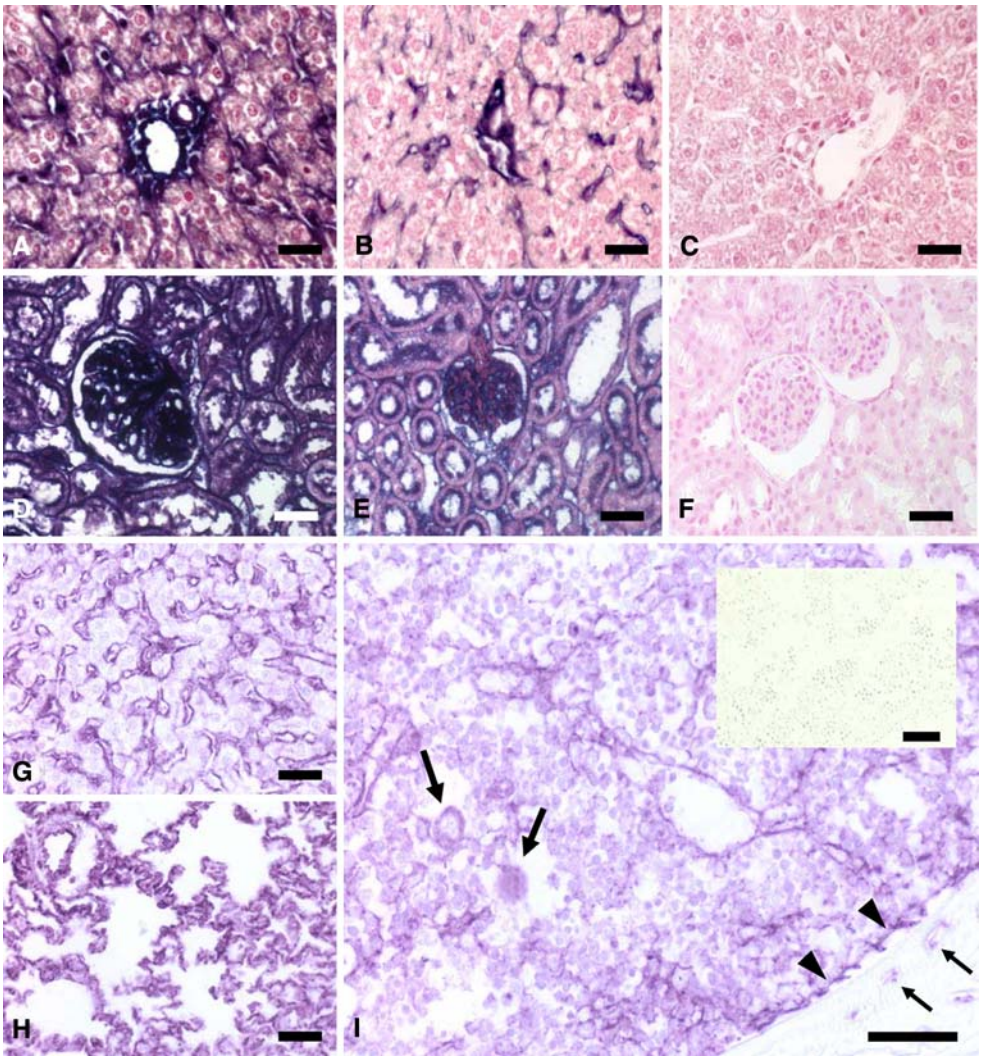

Fig. 1 Paraffin and MMA sections of liver and kidney from F344-Tg(R26-hPLAP) and wild-type rats stained for hPLAP activity after heat pretreatment. In paraffin sections of liver from transgenic rats, hPLAP staining is more intense in specimens fixed with 40% ethanol (**a**) compared with PFA fixation (**b**). No staining is observed in ethanol-fixed liver of a wild-type rat (**c**). The kidney from a transgenic rat shows comparable hPLAP-staining intensity in paraffin (**d**) and MMA-embedded specimens (**e**). However, morphological detail is improved in MMA sections, and renal tubular cells show a clear membranous hPLAP-staining pattern. Note the ubiquitous hPLAP-staining pattern in transgenic tissues. No staining is seen in ethanol-fixed, MMA-embedded kidney of a wild-type rat (**f**). Sections **a**–**f** were stained for hPLAP enzyme activity overnight at room temperature and were counterstained with nuclear fast red. Immunohistochemical detection of hPLAP (**g**–**i**) using the monoclonal antibody 8B6 revealed strong hPLAP expression in PFA-fixed and paraffin-embedded liver (**g**) and lung (**h**), as well as in the ethanol-fixed, MMA-embedded tibia from an F344-Tg(R26-hPLAP) rat (**i**). Bone sections from wild-type rats did not show any staining with 8B6 (inset in **i**). Note the intense cytoplasmic-staining pattern in megakaryocytes (*large arrows*), and the strong membranous staining of osteoblasts (*arrowheads*) and osteocytes (*small arrows*) in transgenic bone tissue (**i**). Five-μm-thick sections. Bar = 50 μm

model is that osteoclasts do not express the marker enzyme at high levels in F344-Tg(R26-hPLAP) rats. Therefore, unequivocal tracking of osteoclasts is difficult using this model.

An interesting question was whether the hPLAP enzyme activity would still be detectable after decalcification and paraffin embedding of bones. For decalcification, we used a protocol employing 0.5 M ethylene diamino tetra acetate (EDTA) as decalcifying agent, avoiding any aggressive chemicals. Because PFA fixation decreased hPLAP enzyme activity, we substituted the normally used 1% PFA by 5% ethanol in the decalcifying solution. However, when we compared hPLAP enzyme expression in undecalcified MMA-embedded bones and decalcified paraffin-embedded bones from the same transgenic animals, we found that decalcification severely diminished the enzyme activity to almost undetectable levels (data not shown).

In conclusion, our study demonstrated that hPLAP is a highly suitable marker enzyme for studies involving genetically labeled cells in bone and cartilage because it survives

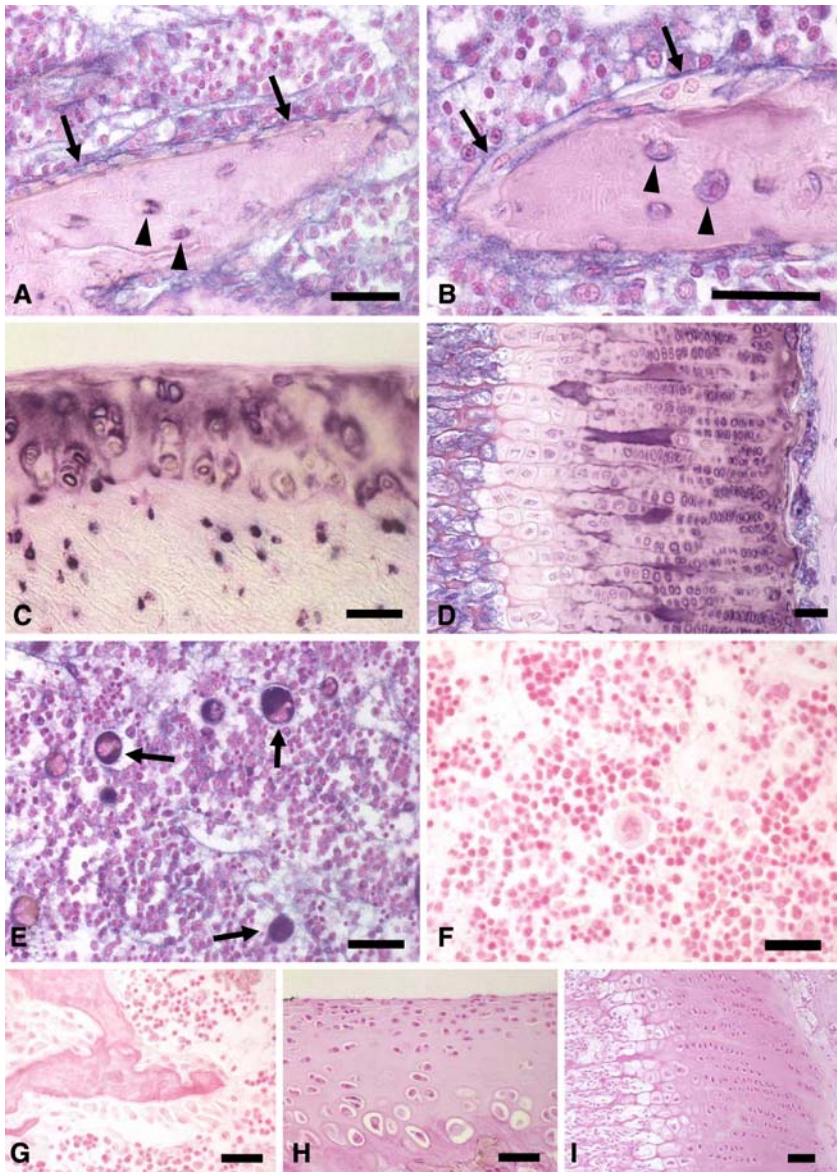

Fig. 2 Ubiquitous expression of hPLAP in bone, cartilage, and bone marrow cells of F344-Tg(R26-hPLAP) rats (**a**–**e**). Osteoblasts (*arrows*) and osteocytes (*arrowheads*, **a–b**) show intense membranous hPLAP staining in transgenic rats (**a**). Osteoclasts (*arrows*) display only weak membrane expression of hPLAP and absence of cytoplasmic staining (**b**), whereas chondrocytes in articular and growth plate cartilage are intensely stained (**c–d**). Hematopoietic bone marrow cells show ubiquitous hPLAP staining with very strong staining on the cell membrane and in the cytoplasm of megakaryocytes (*arrows*, **e**). No staining is seen in bone marrow (**f**), bone (**g**), articular cartilage (**h**), or growth plate cartilage from wild-type rats (**i**). Five-µm-thick, undecalcified, heat-pretreated MMA sections of ethanol-fixed tibias stained for hPLAP activity and counterstained with nuclear fast red. Bar = 50 µm

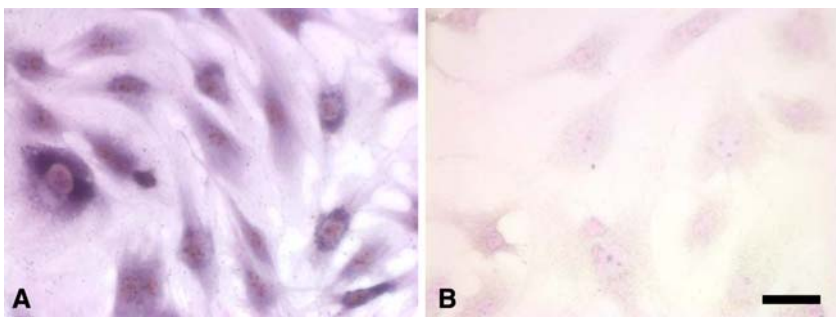

Fig. 3 hPLAP expression in cultured osteoblastic cells. Bone marrow cells from a F344-Tg(R26-hPLAP) (**a**) and a wild-type (**b**) rat were cultured for 14 days under osteoblastic differentiation conditions. Adherent osteoblastic cells from the transgenic rat show intense hPLAP staining, whereas no staining is observed in wild-type osteoblastic cells. Ethanol-fixed, air-dried, and heat-pretreated culture dishes stained for hPLAP activity and counterstained with nuclear fast red. Bar = 50 µm

MMA embedding. In addition, endogenous alkaline phosphatase activity can be totally blocked by heat inactivation. Thus, this marker enzyme provides superb detection quality of labeled cells in the total absence of background staining. The utility of hPLAP as genetic marker is further enhanced by the fact that it can readily be detected by immunohistochemistry in PFA- or ethanol-fixed specimens embedded in paraffin or MMA. In addition, hPLAP can be used for whole-mount staining of embryos (Kisseberth et al. 1999). Therefore, our results also suggest that hPLAP may be superior to other reporter genes such as *lacZ* used so far for the generation of genetically engineered animals. We believe that the excellent detection quality of this marker enzyme in paraffin and MMA sections may provide clear answers to many questions related to cell lineage and to cell and gene therapy with mesenchymal cells in bone and cartilage in the future.

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