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Technical Article

Transgenic mice ubiquitously expressing *Human Placental Alkaline Phosphatase (PLAP)*: an additional reporter gene for use in tandem with *β-galactosidase (lacZ)*

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ABSTRACT A fundamental keystone of developmental biology has been the growing use of reporter genes in model transgenic systems. Their use has greatly facilitated investigations of cell lineage and cell fate in addition to aiding experiments aimed at determining patterns of gene expression, gene interaction and gene regulation. Through construction of transgenic mice, ubiquitously expressing *human placental alkaline phosphatase (PLAP)*, we demonstrate the suitability of *PLAP* as a reporter gene for use in conjunction with, or as an alternative to, *β-galactosidase (lacZ)*. Our findings demonstrate that over-expression of *PLAP* has no adverse effects on mouse development or viability, despite a widespread pattern of expression. This technology provides a simple yet effective mechanism based on eukaryotic reporter gene technology to facilitate the identification of transgenic cells within complex *in vivo* systems

KEY WORDS: *placental alkaline phosphatase, reporter, lacZ*

Introduction

In recent years, the use of reporter genes in transgenic mice has underpinned much of the analysis of gene expression. The primary requirements of reporter genes are that their gene products are readily detectable, with low background activities from endogenous gene products, and that high ectopic levels of reporter gene expression have no adverse effects on either the physiology or development of the individual. The most widely used reporter in mice to date has been the E. *coli* gene *β-galactosidase (lacZ)*. *LacZ* fulfils the criteria described above as it may be expressed ubiquitously without measurable detrimental effects on physiology and gives sensitive detection with chromogenic substrates in whole-mount embryos and frozen tissue section preparations (Beddington *et al.*, 1989). Other reporter genes that have been used previously include: *luciferase* (De Wet *et al.,* 1987), *chloramphenicol acetyl transferase* (Gorman *et al.*, 1982), *horseradish peroxidase* (Connolly *et al.*, 1994) and *green fluorescent protein* (Peters *et al.,* 1995); however, these gene products are less amenable to *in situ* analysis. We have been interested in using a second reporter gene that can be used as an alternative to, or in combination with, *lacZ*. For this purpose we have tested the efficacy of the *human placental alkaline phosphatase gene (PLAP)* as a reporter gene in transgenic mice.

In humans PLAP, a heat stable metalloenzyme (EC 3.1.3.1), is expressed in the placenta on the surface of the syncytiotrophoblast between the 12th week of pregnancy and term (Fishman *et al.*, 1976). In addition, ectopic re-expression of PLAP has frequently been reported in human carcinomas, in the lung, ovaries, cervix and breast (Fishman *et al.*, 1968; reviewed in Millan and Fishman, 1995). Indeed, such ectopic expression has been a focus for the development of diagnostic tests for tumour antigenicity and immunotherapeutics using ricin-A coupled anti-PLAP monoclonal antibodies (Tsukazaki *et al.*, 1985).

In mice, alkaline phosphatases are encoded by a multi-gene family with 3 active loci, designated the *embryonic, intestinal* and *tissue-non-specific alkaline phosphatases* (Terao and Mintz, 1987; Manes *et al.*, 1990; Narisawa *et al.*, 1994). Significantly for our experiments, mice do not possess a placental isoform similar to

Abbreviations used in this paper: PLAP, placental alkaline phosphatase; RSV, roux sarcoma virus; x-gal, 5-bromo-4-chloro-3-indoyl-β-D-galactoside; NBT, nitroblue-tetrazolium salt; X-phos, 5-bromo-4-chloro-3-indoyl phosphate toluidinium salt; PBS, phosphate buffered saline; ES cells, embryonic stem cells; EDL, extensor digitorum longum; TA, tibialis anterior.

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that found in humans and great apes. Therefore, the introduction of *PLAP* constructs into mice affords no possibility of cross-interaction from an endogenous murine homologue. In addition, PLAP activity is readily distinguished from that of other phosphatases by the inclusion of a simple heat-inactivation step in the staining protocol. Whereas most alkaline phosphatases are heat labile, PLAP is an extremely heat stable enzyme surviving heating to 80°C for prolonged periods and organic solvent processing during sample preparation for wax histology. PLAP enzyme activity can be detected using different chromogenic substrates that result in the production of either purple, black or red insoluble precipitates. In addition, fluorogenic substrates and antibodies to PLAP are widely available commercially (Sigma, UK).

PLAP has been used previously in a number of applications. These include, as an effective reporter gene in transfection studies (Henthorn *et al.*, 1988) in retroviral vectors for use in lineage analysis (Fekete and Cepko, 1993) and in transgenic studies (DePrimo *et al.,* 1996, Sharpe *et al.*, 1998). Also, truncated forms of the PLAP protein, which lack part of the carboxy terminus, have been used either as purification tags, to facilitate the secretion and subsequent purification of fusion proteins, or as a means of rapid quantitation of protein expression (Doronin *et al.*, 1993).

To test the suitability of *PLAP* as a reporter gene for a wide range of applications, we have generated transgenic mice that express high levels of *PLAP* from the roux sarcoma virus promoter (RSV). These mice develop and breed normally, despite ectopic ubiquitous *PLAP* expression. In addition, we have tested the potential for the use of *PLAP* in combination with *lacZ*, by generating chimeras between transgenic strains that exhibit widespread *lacZ* and *PLAP* expression. Finally, we describe one use of material from *PLAP* transgenics, namely as a source of labelled cell populations for cell mediated gene transfer experiments. Specifically, in following the fate of trans-differentiating dermal fibroblasts as they enter the muscle lineage following transfer to a muscle environment (Watt *et al.*, 1994; Gibson *et al.*, 1995).

Experimental Protocols

Plasmid construction

A 4995bp HindIII-SalI genomic fragment containing the entire *PLAP* coding sequence (Henthorn *et al.*, 1988) was subcloned, using standard techniques (Sambrook *et al.*, 1989), into pB-RSV (Kamlin *et al.*, 1991), a mammalian expression vector containing the Roux Sarcoma Virus (RSV) promoter, designated pRSV-PLAP. For microinjection, the construct was excised from the vector (pRSV-PLAP), separated by agarose gel electrophoresis and gel purified using glassmilk (Qiagen, Germany). DNA was further purified using an Amicon 30 micopure/microcon microconcentrator (Millipore, UK). DNA concentration was estimated by reference to DNA concentration standards following agarose gel electrophoresis and diluted to a final injection concentration of 2.5 ng/μl in 10 mM Tris-HCl (pH 7.4), 0.1 mM EDTA.

Transgenic mouse production

Transgenic mice were produced by standard pronuclear injection techniques (Hogan *et al.,* 1994). Briefly, fertilized mouse eggs from super-ovulated F1 mice (C57/BL6xCBA/Ca) mated to F1 males were visualised using differential interference contrast optics on an inverted Nikon microscope. DNA was introduced into the male pronucleus using Leitz manual micro-manipulators and glass capillary micro-pipettes. Eggs were cultured overnight to the two cell stage and subsequently transferred to the oviducts of pseudopregnant F1 recipient female mice (Hogan *et al.,* 1994).

Identification of transgenic mice

Transgenic mice were identified by Southern blot analysis of genomic DNA isolated from tail biopsies. 0.5 cm of tail was removed from mice after weaning under general anesthesia and digested overnight at 55°C in lysis buffer [100 mM Tris-HCl (pH 8.5), 5 mM EDTA (pH8.0), 0.2% SDS, 200 mM NaCl and 100 μg/ml Proteinase K (Sigma, UK)]. Undigested material was removed by centrifugation and the supernatant extracted with an equal volume of phenol/chloroform. Genomic DNA was precipitated by the addition of an equal volume of isopropanol and spooled into 200 μl of TE [10 mM Tris-HCl (pH 8.0), 1 mM EDTA].

Thirty μl of DNA was digested overnight with EcoRI and fragments resolved on horizontal 0.8% agarose gels. After transfer to Hybond N+ nylon membrane (Amersham, UK) by alkali capillary blotting, membranes were hybridised in Church Buffer [0.5 M Phosphate Buffer (pH 7.5), 7% SDS, 1 mM EDTA] overnight with a random primed [α^{32}P]dCTP (Amersham, UK) labeled 5.0 kb HindIII/SalI fragment spanning the entire *PLAP* gene, isolated from pRSV-PLAP. Hybridisation was performed at 65°C in a rotary oven (Hybaid, UK). Post hybridisation, the filters were washed in Church Wash (40 mM Phosphate buffer, 1% SDS) three times for 10 min each, wrapped in saran wrap and exposed to x-ray film [X-OMAT (Kodak-Eastman)] using intensifying screens at –70°C.

Tissue culture

D3 Embryonic stem cells (ES) were maintained using standard protocols as previously described (Robertson, 1987; Allen *et al.*, 1994).

Stable ES cell lines expressing *PLAP* (plapES) were made by calcium phosphate mediated co-transfection of RSV-PLAP with pMCNeopA (Stratagene) using a DNA ratio of 10 μg RSV-PLAP: 0.5 μg pMCNeopA. Briefly, linearised DNAs were dissolved in 1 ml of transfection buffer [137 mM NaCl, 21 mM HEPES, 0.62 mM Na_2HPO_4 (pH 6.95 at 37°C)]; 2.5 M $CaCl_2$ was added drop by drop (approximately 60 μl total) until a fine precipitate was formed. This was added dropwise to a semi-confluent lawn of freshly plated ES cells. After 5 h the medium was changed and the cells placed under G418 selection [200 μg/ml (Gibco-BRL)] for 10 days. Resulting colonies were clonally selected and tested for their expression of PLAP by histological staining with NBT/x-phos as described below.

Embryoid body cell aggregates were made by plating approximately 10^6 ES cells from plapES and Rosa26 [an ES cell line expressing lacZ (Allen *et al.*, 1994; Zambrowicz *et al.*, 1997)] lines together for 48 h on 6 cm bacteriological grade tissue culture dishes (Sterilin). Resulting embryoid bodies were subsequently plated on gelatin coated tissue culture grade dishes (Nunc) and allowed to grow out for a period of up to one week prior to staining.

Embryoid body outgrowths for β-galactosidase or PLAP staining were washed in PBS and subsequently fixed for 30 sec with methanol: acetone (1:1) and air dried prior to staining (see below).

Skin culture, to prepare dermal fibroblasts for implantation into mdx muscles, was performed as previously described (Watt *et al.*, 1994; Gibson *et al.*, 1995).

Production of PLAP<->lacZ chimeras

Chimeras between *PLAP* and *lacZ* expressing cells were made by aggregating 8-cell embryos derived from the transgenic lines PLAP26 and ROSA26 (Hogan *et al.*, 1994). Briefly, the zona pellucidae of 8-cell embryos were removed in acid tyrodes solution (Sigma) and subsequently aggregated by contact in microwells in KSOM culture medium at a ratio of 1 PLAP26: 1 ROSA26. Chimeric blastocysts were then transferred to day 3 recipient foster mothers.

Histology

Tissues for cryo-sectioning were embedded in OCT cryo-mountant (BDH), snap frozen in isopentane, cooled in liquid nitrogen, and stored at -80°C. 8-10 μm frozen sections were cut, collected on gelatin coated glass slides, fixed in 3% formaldehyde in phosphate buffered saline (PBS) (pH 7.4) for 15 min and washed in two changes of PBS (2x5 min each wash) prior to staining. In dual-staining experiments, staining for β-galactosidase activity was always performed first, due to the heat lability of the β-galactosidase enzyme, followed by staining for alkaline phosphatase activity.

To detect β-galactosidase activity, fixed sections were incubated at 37°C overnight in 2 mM MgCl2, 4 mM $K_3Fe(CN)_6$, 4 mM $K_4Fe(CN)_6$, 1 mg/ml X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside) in PBS.

To detect alkaline phosphatase activity, sections were washed twice in PBS, heat-inactivated in PBS at 80°C for 2 h and equilibrated in 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$ for 5 min. Subsequently, sections were incubated in alkaline phosphatase stain: 100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_2$, 0.34 mg/ml nitroblue tetrazolium salt (NBT) (Sigma), 0.17 mg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt (x-phos) (Sigma, UK) for between 10 min and overnight.

Embryos for wax embedding were fixed overnight in 4% paraformaldehyde in PBS at 4°C, washed twice in PBS, before dehydrating in a graded series of alcohols and transfer to toluene. After clearing embryos were incubated in a mixture of 50% toluene: 50% paraffin wax at 65°C for 1 h, mounted and 12 μm sections cut onto gelatin coated glass slides. Slides were then stained for alkaline phosphatase activity, as described above, with the exception that no heat-inactivation step was required.

Muscle implantations

Muscle implantations were performed as described in Watt *et al.* (1994). A mdx nude mouse (Bulfield *et al.*, 1984) was irradiated at 4 weeks of age and 12 days later the extensor digitorum longum muscle (EDL) removed. A donor EDL from another irradiated mdx nude irradiated at 4 weeks of age was grafted into the EDL bed. This EDL had been implanted 10 days earlier with $3x10^5$ *PLAP* dermal fibroblasts at passage 3 (i.e., grown in culture to passage 3 from explant cultures of skin from PLAP26 mice). Following whole muscle grafting of the implanted donor EDL by suturing of its distal tendon round the tendons of neighbouring peroneal muscles of the mdx nude host, Spongostan, a gelatin mesh, was inserted between the implanted EDL containing the *PLAP* dermal fibroblasts and the host tibialis anterior muscle (TA). The host TA muscle was injured by scalpel scoring and the muscle left *in situ* for 24 days. Muscles were then removed en-bloc with the Spongostan still intervening between the TA and the EDL muscle and cryostat sections stained for PLAP activity.

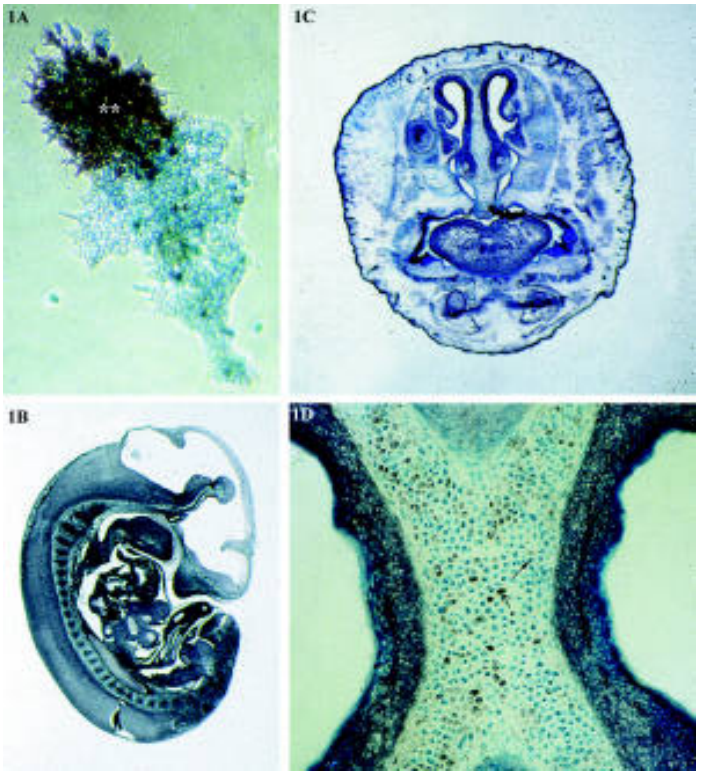

Fig. 1. Combined use of *PLAP* and *lacZ* reporter genes *in vitro* and *in vivo*. (A). *Embryoid body outgrowths, from PLAP expressing (plap-ES) and lacZ expressing (ROSA26) ES cells, stained with NBT/x-phos and x-gal. PLAP positive cells are stained brown/purple (white asterisk) whilst lac-Z positive cells are stained blue (black crosses). This illustrates that the staining protocols for the two reporters may be used in combination and that both reporters are compatible for dual use.* **(B)** *Sagittal section through wax embedded embryonic day 12 (E12) embryo from PLAP26 line of transgenic mice, in which expression of the PLAP reporter is driven by the RSV promoter, stained with NBT/x-phos. Note, staining of in all tissues of the embryo, suggesting ubiquitous expression of the transgene.* **(C)** *Coronal cryostat section through the anterior head region (including the nasal septum, mouth, and jaw) of a newborn (postnatal day 1) PLAP26<->ROSA26 chimera, stained for PLAP activity with NBT/X-phos (purple/brown) and lacZ activity with x-gal (blue) (low magnification x1.5).* **(D)** *High power (magnification x40) image of coronal section through the nasal septum of PLAP26<->ROSA26 chimera, stained for PLAP activity with NBT/x-phos (purple/brown) and lacZ activity with x-gal (blue). Note the high resolution between lac-z stained cells (→) and PLAP stained cells (➔).*

Results and Discussion

Expression of PLAP in ES cells

To determine potential toxic effects of high levels of ectopic PLAP expression and the efficacy of using PLAP as a reporter molecule in murine cells, pRSV-PLAP was first stably transfected into embryonic stem (ES) cells (white asterisk, Fig. 1A). D3 ES

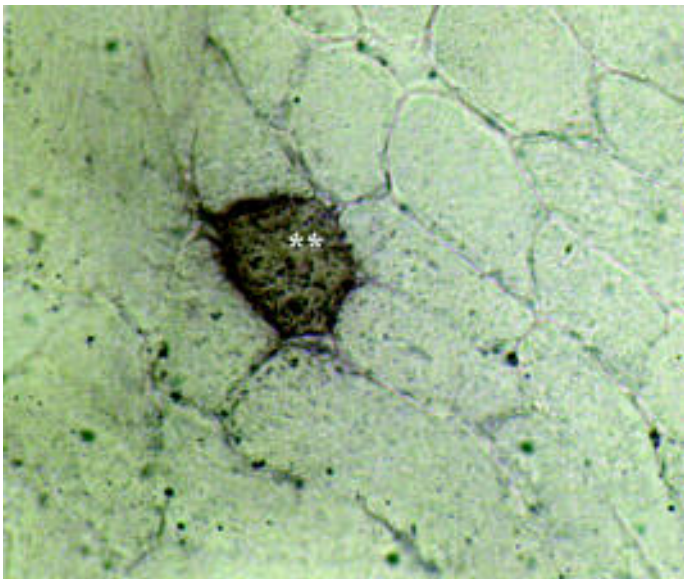

Fig. 2. Use of PLAP as a cellular marker: PLAP positive muscle fibres are found in TA muscles following implantation of dermal fibroblasts into adjacent EDL muscles. *Eight μm cryostat section through the TA muscle of an mdx nude mouse stained for PLAP activity using NBT/X-phos. The TA muscle was taken 24 days after the transplantation of the EDL muscle, previously implanted with PLAP labeled dermal fibroblasts derived from skin outgrowths from PLAP26 mice, to a second mdx nude mouse. Note, PLAP activity is detectable in one muscle fibre in this preparation, stained brown (white asterisk), whilst surrounding fibres are unstained. PLAP positive dermal fibroblasts have therefore migrated from the EDL graft, across a Spongostan barrier and into the TA muscle of the mdx host, where they have "trans-differentiated" to give rise to chimeric PLAP positive muscle fibres.*

cells were co-transfected with RSV-PLAP and pMCNeopA (Stratagene). Following selection, high levels of PLAP activity were detected in G418 resistant colonies using NBT/x-phos staining, with no detectable background in control ES cell cultures. Expression was also confirmed by RT-PCR analysis of *PLAP* positive clones using primers spanning intron/exon boundaries of the *PLAP* transgene (data not shown). In addition, the efficacy of using the two reporter systems, *PLAP* and *lacZ* together in combination was clearly shown by staining embryoid body outgrowths derived from *PLAP* transfected (white asterisk, Fig. 1A) and Rosa26 (black cross, Fig. 1A) ES cells.

Production of transgenic mice expressing PLAP

To further assess the *in vivo* effects of *PLAP* expression, transgenic mice were generated by pronuclear microinjection of the pRSV-PLAP construct. Seven founder transgenic mice were identified by *PLAP* expression in tail biopsy tissue, and confirmed by Southern analysis of tail biopsy DNA.

All founder mice were bred successfully to F1 mice (C57/BL6xCBA/Ca) with transgenic founders producing litters of normal size with Mendelian segregation of the transgene.

All lines were analysed for expression of the transgene and PLAP26, which exhibited the highest level of reporter gene activity, selected for further studies. PLAP26 expressed PLAP activity in all tissues examined and in embryos at all stages examined (E9-14), suggesting ubiquitous expression of the transgene (Fig. 1B). Importantly this line, in addition to one other high expressing founder line PLAP34, has been bred extensively for several months with no deleterious effects that could be associated with high levels of ubiquitous *PLAP* expression.

Combined use of PLAP and lacZ in chimeras

Chimeras were made by aggregating PLAP26 embryos with ROSA26 embryos (which express *lacZ* ubiquitously) to demonstrate the potential for the use of two reporter genes, namely *lacZ* and *PLAP*, in combination *in vivo*. Dual labelling was assessed *in vitro* in PLAP26/ROSA26 ES cell co-cultures (Fig. 1A) and then in frozen sections taken through the heads of new born PLAP26<->ROSA26 chimeras (Fig. 1C and D). A complimentary staining pattern was obtained in such chimeras between lacZ (arrow → Fig. 1D) and PLAP (arrow ➔ Fig. 1D) stained cells. The high cellular resolution between the reaction products from the two reporters is illustrated in a section through the developing nasal septum (Fig. 1D). Within this section lacZ stained cells appear blue and PLAP stained cells appear purple/brown.

Implantation of dermal fibroblasts into skeletal muscle

Following outgrowth of dermal fibroblasts from sections of neonatal PLAP26 skin, PLAP positive cells were harvested and grafted into EDL muscles of mdx nude mice. Twenty four days after transplantation of implanted EDL muscles to muscle beds of new recipient mdx mice, adjoining TA muscles were removed and stained for the presence of *PLAP* positive muscle fibres. The presence of reporter activity within TA muscle (white asterisk, Fig. 2) indicates that *PLAP* positive dermal fibroblasts have migrated from the grafted EDL muscle, across the intervening Spongostan, and into the adjoining TA muscle, where they have fused with host muscle fibres.

Our evaluation of *PLAP* as a reporter gene in transgenic mice shows that high levels of ubiquitous expression can be achieved and tolerated with no adverse effects on development, physiology or germline transmission. This is in keeping with data previously reported using the β-actin promoter to over express PLAP *in vivo* (DePrimo *et al.,* 1996) and *PLAP's* use in combination with lacZ to investigate *Hoxb* gene expression through transgenesis (Sharpe *et al.,* 1998). In particular, ectopic PLAP expression is not tumourigenic, as has been found previously for over-expression of the germ-cell alkaline phosphatase (Narisawa *et al.*, 1993). Therefore, *PLAP* could be widely used in transgenics derived by either pronuclear injection or by ES cell manipulation. Furthermore, we have assessed PLAP expression in a wide variety of tissues and no background staining problems have been observed following simple heat inactivation of endogenous mouse alkaline phosphatases.

For certain applications *PLAP* may have significant advantages over the use of *lacZ* as a reporter gene. For example, PLAP retains enzyme activity following wax embedding, whereas lacZ enzyme activity is lost, thus facilitating histological studies. However, of greater importance, *lacZ* has been found to interact with and down-regulate the activity of certain promoters in transgene constructs, producing misleading profiles of reporter gene activity and distribution (*Paldi et al.*, 1993; Cui *et al.,* 1994). It is proposed that this repressive activity may be a consequence of the prokaryotic origin of the *lacZ* sequence (a 3 kb CpG rich,

intron-less gene) (Clark *et al.,* 1997). In contrast, the genomic *PLAP* construct we have used is a "typical" eukaryotic gene comprising 11 exons with intervening intronic sequence and attendant splice sites. This eukaryotic sequence may circumvent disadvantages inherent with use of bacterially derived *lac-Z*. Indeed, in support of such a hypothesis, β-actin *PLAP* transgenes have been shown to express more consistently than corresponding *lacZ* based constructs *in vivo* (Nilsson and Lendahl, 1993; Cui *et al.,* 1994; DePrimo *et al., 1996).* However, probably the most important use of *PLAP* demonstrated here is its use in combination with other reporter transgenes for use in lineage studies. The high cellular resolution achievable when *lacZ* and *PLAP* transgenes are used in combination *in vivo* affords the possibility of following the distribution of either two separately labelled cell populations or two separate transgene-tagged reporter-constructs *in vivo*. To this end we demonstrate the feasibility of using *PLAP* labeled cells derived from *PLAP* transgenic mice in a cell mediated gene transfer experiment. Specifically, that *PLAP* labelled dermal fibroblasts can migrate from sites of implantation within skeletal muscles to adjacent muscles and in so doing trans-differentiate to enter the skeletal muscle lineage and fuse with host muscle fibres. These experiments support and confirm previous experiments using alternative reporter genes e.g., *lacZ* (D. Watt, personal communication) and provide the experimental framework to enable the fate of multiple populations of separately transgene tagged mixed cell populations to be determined *in vivo* following transplantation.

The ability to discriminate particular populations of cells, by means of reporter genes, is of critical importance to developmental biology; permitting questions concerning cell lineage, cell fate and cell migration, gene expression and gene regulation to be addressed. We have demonstrated efficient use of *PLAP* with *lacZ* in a dual-labelling study in chimeras. The combined use of two reporter gene systems may be of particular value in future studies of cell-cell interactions and lineage analysis in chimeras.

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