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Review Article

Visual reporters for study of the osteoblast lineage☆

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ABSTRACT

Advancing our understanding of osteoblast biology and differentiation is critical to elucidate the pathological mechanisms responsible for skeletal diseases such as osteoporosis. Histology and histomorphometry, the classical methods to study osteoblast biology, identify osteoblasts based on their location and morphology and ability to mineralize matrix, but do not clearly define their stage of differentiation. Introduction of visual transgenes into the cells of osteoblast lineage has revolutionized the field and resulted in a paradigm shift that allowed for specific identification and isolation of subpopulations within the osteoblast lineage. Knowledge acquired from the studies based on GFP transgenes has allowed for more precise interpretation of studies analyzing targeted overexpression or deletion of genes in the osteoblast lineage. Here, we provide a condensed overview of the currently available promoter-fluorescent reporter transgenic mice that have been generated and evaluated to varying extents. We cover different stages of the lineage as transgenes have been utilized to identify osteoprogenitors, pre-osteoblasts, osteoblasts, or osteocytes. We show that each of these promoters present with advantages and disadvantages. The studies based on the use of these reporter mice have improved our understanding of bone biology. They constitute attractive models to target osteoblasts and help to understand their cell biology.

Contents

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1. Introduction

Bone is a highly active organ undergoing constant turnover in a coordinated process involving matrix resorption by osteoclasts followed by osteoblast-mediated bone formation. The bone forming cells, the osteoblasts represent 4 to 6% of the cellular content within the bone lineage [1]. These cuboidal-shaped cells cover active bone surfaces. Their major function is to produce new bone by synthesis and assembly of the extracellular matrix.

Osteoblasts are thought to be derived from mesenchymal stem/progenitor cells resident near the bone surface that differentiate into the osteogenic lineage, although our understanding of the exact identity, localization and heterogeneity of osteoprogenitors remains incomplete. In contrast, different stages of osteoblast differentiation have been well defined based on cellular properties and gene expression. There are two transcription factors critical for entry into, and differentiation down the osteoblast lineage, *Runx2* and osterix (*Sp7*). Mice with null alleles for both *Runx2* and osterix show total absence of bone formation with a completely cartilaginous skeleton [2,3,4]. Osterix is genetically downstream of *Runx2*, at least under some circumstances, as osterix null cells can express *Runx2*, but *Runx2* null cells never express osterix. Both factors are involved in regulation of important genes in the osteoblast lineage, including genes expressed in pre-osteoblasts such as type I collagen (*Cola1*, *Col1a2*), and alkaline phosphatase (ALP, *Alpl*), as well as markers of more mature osteoblasts bone sialoprotein (BSP, *Ibsp*), and osteocalcin (Oc, *Bglap*). During osteoblast differentiation, when *Runx2* and *Col1a1* are expressed in a pool of progenitors, a proliferation phase is engaged. During this phase, the cells start to acquire ALP activity and are considered pre-osteoblasts. The next stage of differentiation marks the transition to mature osteoblasts. Two steps are essential for the synthesis of the bone matrix: the organic matrix deposition followed by its mineralization. Osteoblasts secrete collagens (mainly collagen type I), non collagenous proteins including Oc, BSP and osteopontin (OPN), and proteoglycans such as decorin and byglycan. Osteoblasts mediate the process of mineralization by producing ALP and secreting matrix vesicles to seed hydroxyapatite crystal formation. Following completion of their matrix forming activity, mature osteoblasts can undergo apoptosis, become embedded in the matrix and differentiate into osteocytes or become quiescent bone lining cells.

The understanding of osteoblast biology is critical as numerous skeletal diseases show an impairment of their number or their function resulting in bone defects. The current knowledge of the osteoblast lineage is expanding in the area of identification of the osteoprogenitor cells, along with further defining paracrine and endocrine functions of cells of the osteoblast lineage *in vivo*. All of these studies require robust methods to identify and target cells of interest.

2. Histological methods to identify osteoblasts

The primary characteristics used to identify osteoblasts *in vivo* include their location on the bone surface as cuboidal mononuclear cells. Toluidine blue staining is often used to identify osteoblasts in paraffin sections, where surfaces with at least four adjacent labeled cuboidal cells are defined as osteoblast populated surfaces. Enzymatic staining for ALP can also be used as a more specific method of identifying osteoblasts, particularly in conjunction with mineralization labels such as calcein (green), alizarin complexone (red) or demeclocycline (yellow). ALP is fairly specific for osteoblasts, although ALP activity alone, particularly *in vitro* where it is expressed early in the osteogenic lineage as well as in embryonic stem (ES) cells, is not sufficient to demonstrate differentiation to mature fully functional osteoblasts. Immunostaining for markers including osterix, and osteocalcin has also been used in many studies to identify osteoblasts. In order to better characterize the differentiation stage of cells of the osteoblast lineage and simplify their detection, a number of transgenic visual reporter mice have been developed, and are described in more detail below.

3. Fluorescent proteins

The past decades were witnesses to the rapid development of detection and imaging tools to monitor various cellular phenomena. Fluorescent proteins (FP) have proved to be extremely useful tools both as reporters or fused to other proteins for detection and tracking of specific cells or molecules both *in vitro* and *in vivo*. Green Fluorescent Protein (GFP) was isolated from jellyfish *Aequorea victoria* in 1962 and was the first FP to be cloned in 1992. GFP presents the advantage of being a small molecule (27 kDa) that can fluoresce as a monomer without further cofactors or modifications and its detection is non invasive.

Many properties of the FPs have been improved by targeted mutations including brightness, photostability, faster folding, inducible or spontaneous photoconvertability, photoactivatability and clear cut excitation/emission properties by increasing the Stokes shift [5]. Variants of GFP covering a large spectrum from ultraviolet to far red such as YFP (yellow), CFP (cyan), BFP (blue) and RFP (red) constitute the basis of multicolor imaging [5,6]. FPs from other organisms have subsequently been cloned and modified to produce variants that can be easily distinguished from *A. victoria*-based FPs by antibodies and sequence.

FP-based reporters offer great advantages over the commonly used LacZ reporters as they can be detected by either microscopy or flow cytometry in both live and fixed cells, and, when appropriate variants are used, do not require any additional staining procedures that can produce experimental variability. In many cases, the brightness of the fluorescence signal is proportional to the level of promoter activity and can be used to subdivide cells, particularly when using flow cytometry. They also enable flow cytometric sorting of live cells for subsequent culture or transplantation on the basis of expression of intracellular proteins, expanding the range of markers that can be used with this technique. They are widely used for serial imaging of cultures, and can also be used for repeated intravital imaging assuming the tissue is interest is accessible (for example the calvaria) [7,8]. Fluorescence is retained well following fixation in formalin-based fixatives, and cryosectioning, but can be reduced by high temperature, and solvents such as methanol and acetone.

4. Generation of mice with fluorescent reporters

Fluorescent protein based reporter genes provide a powerful tool to monitor spatial or temporal patterns of gene expression *in vivo*. Multiple approaches can be utilized to generate mice with a FP under the control of a gene of interest. Historically, the most common way to generate reporter mice was a transgenic approach using specific gene promoter fragments upstream of the FP gene. This straightforward approach has a number of drawbacks, primarily related to the random insertion of genetic material which can affect the specificity and strength of reporter expression. To ensure that the reporter expression faithfully reflects endogenous gene expression, multiple transgenic lines must be screened. More recently, larger regulatory regions from bacterial artificial chromosomes (BACs) have been coupled with a transgenic approach which allows generation of reporters that more closely reflect endogenous gene expression. However, this approach also depends on the position of insertion of genetic material. The most precise method to generate fluorescent reporter mice is to insert a FP into the endogenous gene locus. Depending on where the reporter is inserted, this approach can result in haploinsufficiency of the gene of interest, which in some cases can cause a phenotype.

5. *In vivo* identification of osteoblast lineage cells

The use of selective markers labeling the osteoblast lineage at different stages of maturation is essential to allow the identification and the isolation of the cells. To date, several markers have been used as promoter driven reporters to study osteoprogenitor cells or mature osteoblasts. These are summarized in Table 1.

Table 1
Osteoprogenitor lineage directed GFP transgenic lines.

Promoter	Reporter	Labeled population	Advantages	Disadvantages	Ref.
Osteoblast precursors					
Osterix	CreERT2-GFP mCherry	Perivascular progenitors, craniofacial bone during development, osteoprogenitors, osteoblasts and osteocytes	Labeling osteoprogenitors during bone development, labels majority of fracture callus cells, and bone marrow resident progenitors for various lineages	Expressed at all stages of the osteoblast differentiation. Targets expression to tissues other than bone: adipocytes, stromal cells, kidney and intestine.	7, 8, 53
Nestin	GFP	Perisinusoidal stem cell – osteochondroprogenitors, neural progenitors, muscle satellite cells	Nestin GFP labels a population supportive of hematopoiesis with multilineage differentiation potential	Nestin targets multiple cell types. Different reporter mice (Nestin-GFP, Cre and CreERT2) identify different cell populations.	12–14
αSMA	GFP mCherry dsRed	Osteoprogenitor cells during growth and in fracture, perivascular cells, mammary stem cells, tendon progenitors	Label osteochondroprogenitor cells during fracture healing. Reporter is inactive in mature cell lineages and allows for lineage tracing. Identifies mesenchymal progenitors within periodontal tissues, and within tendon.	Labels numerous mesenchymal cell types with strong expression in smooth muscle (aorta, bladder), population of myeloid hematopoietic lineage, muscle satellite cells.	15–19, 21, 54
CTGF	GFP	Bone marrow resident mesenchymal progenitors	Label mesenchymal progenitor cells capable of multilineage differentiation *in vitro*. Expression inversely correlated with osteoblast maturation	Expression decreases with age of the animal. *In vivo* fate without culture unknown.	22
Prx1	CreERT2-GFP	Osteochondroprogenitors	Label a population of osteochondral progenitors within the periosteum	GFP expression not been well characterized, periosteal specificity has not been demonstrated.	23, 24
Pre-osteoblasts/osteoblasts					
3.6 kb Collagen1a1	GFPTopaz GFPCyan	Preosteoblasts, osteoblasts, osteocytes, odontoblasts, fibroblasts in tendon, skin	Labels pre-osteoblasts	Expressed in non-osseous tissue cells. Low expression in osteoclast lineage.	28, 29, 33
2.3 kb Collagen1a1	GFPemerald GFPcyan mRFP	Osteoblasts, osteocytes and a subset of cells in tendon, odontoblasts	Labels osteoblasts with bright GFP	Expression in a population of CD45+ fibrocytes in the bone marrow.	28, 34, 49, 50
BSP	GFPTopaz	Osteoblasts, osteocytes, cementoblasts.	Labels mature osteoblasts	Also expressed by osteocytes.	8
Oc	GFPTopaz	Osteoblasts, cementoblasts, cementocytes, odontoblasts	Different size of promoter fragments (rat 1.7 kb, human 3.8 kb) show a targeted labeling of the osteoblast lineage.	Rat 1.7 kb promoter is active in a small population of osteoblasts. 3.8 kb promoter may also label bone lining cells or another quiescent population.	31, 39
OPN	GFP	Osteoblasts, hypertrophic and articular chondrocytes	Strong GFP expression in osteoblasts	Labels chondrocytes. Specificity for osteoblasts on bone surface unproven.	42
Osteocytes					
Dmp1	GFPtopaz memGFP mCherry	Osteocytes	Generally osteocyte specific, does not label osteoblasts	Does not label all osteocytes	36, 45, 47, 48

5.1. Identification of osteoblast precursors

5.1.1. Osterix

Osterix is expressed in immature osteoblasts but not chondrocytes making it an excellent marker to study osteoblasts. The osterix promoter has been shown to drive mCherry in osteoblasts of the medaka fish allowing the study of the osteoblast function during skeletal development in this species [9]. Osterix reporters are also available to study osteoblasts in zebrafish [10,11]. The Osterix promoter has also been used to drive LacZ and GFP reporter genes in mice. Maes et al. demonstrated that Osterix-LacZ$^+$ cells are closely localized with blood vessels and display a phenotypic resemblance with pericytes [12]. OsterixGFP positive progenitor cells were abundant in the thickened periosteum 4 days post-fracture and contributed to the woven bone callus after 14 days linked with the vascular invasion of the callus. Based on these results, a perivascular osteoblast progenitor niche has been proposed [12]. Osterix-Cherry reporter mice were also generated to characterize the cells expressing osterix during skeletal development. Osterix reporter expression is first detected in craniofacial bones at E13.5 and its level of expression increased and broadened with embryo maturation. Postnatally, osterix reporter expression is strong in mature osteoblasts lining the trabecular and cortical surface and in matrix embedded osteocytes. Reporter expression is also present in cells within the bone marrow near bone surfaces and in hypertrophic chondrocytes [13]. While mainly restricted to bone, Osterix-Cherry was also transiently expressed in kidney. Osterix is a useful marker to identify or target the osteogenic lineage, but its expression throughout the lineage limits its utility as a marker of osteoprogenitors to developmental studies unless combined with another mature marker.

5.1.2. Nestin

Nestin is an intermediate filament protein discovered in neural stem cells but was found to be expressed in various cell types including pericytes, endothelial cells and neuronal lineages [14–16]. In bone marrow, Nes-GFP labels a perivascular cell population associated with catecholaminergic nerve fibers. When isolated from adult bone and expanded *in vitro*, Nestin-GFP positive cells showed multilineage differentiation potential *in vitro* and *in vivo* including the ability to support a hematopoietic niche [17]. While Nes-GFP shows significant overlap with expression of endogenous Nestin, an inducible Cre driven by the same promoter shows very inefficient targeting of reporters to Nes-GFP+ cells (4% overlap), and fails to label cells capable of CFU-F formation [18,19]. In the absence of a reliable Cre to trace the fate of Nestin expressing cells, the function and fate of this cell population *in vivo* remains controversial.

5.1.3. αSMA

Alpha Smooth Muscle Actin (αSMA) is a marker of pericytes and myofibroblastic cells. αSMA-GFP$^+$ cells are located in a perivascular niche, in the periosteum or in the cranial sutures, regions containing osteoprogenitor cells (Fig. 1A). These cells express stem cell markers such as Sca1 and CD90 and display osteogenic and adipogenic differentiation potential *in vitro* [20]. A mCherry reporter driven by the same αSMA promoter fragment labels similar populations [21]. Lineage

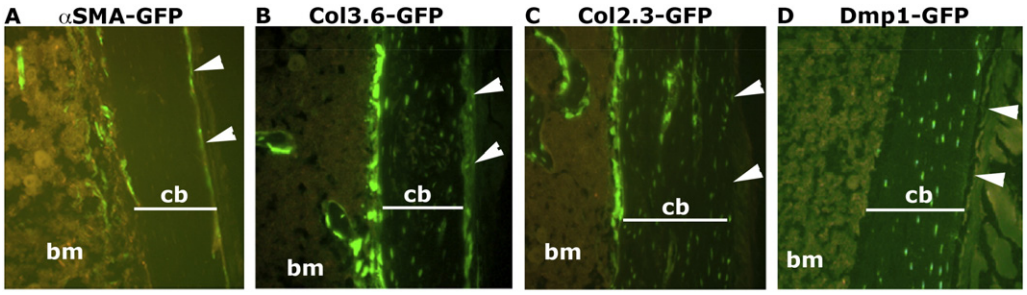

Fig. 1. GFP reporter expression in long bone GFP reporter expression in a number of transgenic reporter lines is shown. Images show mainly cortical bone (cb) from the femoral diaphysis. (A) αSMA-GFP is expressed in perivascular cells, and in the periosteum. (B) Col3.6-GFP is expressed in cells lining endocortical and periosteal surfaces, and by osteocytes. (C) Col2.3-GFP is expressed in osteoblasts lining trabecular and endocortical surfaces, and osteocytes, but is not present in periosteal fibroblasts. (D) Dmp1-GFP expression is restricted to osteocytes. Arrowheads indicate periosteum; bm, bone marrow.

tracing of αSMA+ progenitors using αSMACreER^{T2} has indicated that they represent an osteoprogenitor population *in vivo* during bone growth and osteochondroprogenitors in the periosteum during fracture healing, as well as progenitors in other tissues including periodontium and tendon [21–23]. The broad expression of the αSMA promoter makes this reporter active in number of tissues including the smooth muscle layer of blood vessels, bladder, and other myoepithelial cells such as in the mammary gland [24]. Another study using an older version of RFP αSMA-dsRed1 detected expression in a rare population of monocytes and macrophages that expressed COX-2, however this FP can induce cellular toxicity which causes noticeable morbidity in animals homozygous for the transgene [25,26]. αSMA has proved a useful reporter of progenitor cells in a number of tissues, particularly of cells contributing to healing, and is not expressed in mature osteolineage cells. However, it does not appear to label a long-term quiescent osteoprogenitor population in the bone marrow, and is not specific to ossified tissues.

5.1.4. CTGF

Connective Tissue Growth Factor (CTGF) is a multi-domain extracellular matrix protein involved in tissue fibrosis, whose function in bone development is controversial. CTGF-GFP labels a population of cells in the bone marrow residing mainly in the trabecular region. CTGF reporter expression was inversely correlated with osteoblast maturation but closely associated with the mature osteoblast layer, suggesting a progenitor function. *In vitro*, CTGF-GFP+ cells presented multipotent characteristics of mesenchymal progenitor cells by differentiating into chondrogenic, osteogenic and adipogenic lineages. When sorted, expanded *ex vivo* and transplanted in a bone defect model, the CTGF-GFP^{+} were found in the regenerated bone tissue. Over time this population did not remain GFP^{+} but its progeny contributed to osteoblasts and osteocytes [27]. Further studies are required to identify the *in vivo* function of this cell population.

5.1.5. Prx1

Paired Related Homeobox 1 (Prx1, *Prrx1*) is a transcription factor that plays a major role in limb bud formation and skeletal patterning. Cre under control of the 2.4 kb Prx1 promoter has been very widely used to knock out genes in limb bud mesenchyme, but in adult mice, expression is more restricted to the periosteum. The creation of a Prx1CreER-GFP transgenic mouse using the same Prx1 promoter allowed the identification of a GFP^{+} population of cells within the periosteum displaying chondrogenic and osteogenic differentiation potential *in vitro* and *in vivo* [28,29]. Prx1-GFP was also used to enrich for mesenchymal progenitors in neonatal calvaria, particularly in combination with staining for Sca1 [30]. Prx1-GFP expression is not exclusive to periosteum, but it is unclear from the studies whether there is expression in the bone marrow or endosteal compartments, so further characterization is necessary to prove the utility of this model [29]. Studies with Prx1-Cre show recombination in various tissues including cardiovascular, respiratory, urinary and neural system [31]. An additional concern with the inducible Cre model is the high number of tamoxifen injections required to induce Cre activity.

5.2. Identification of pre-osteoblasts/osteoblasts

5.2.1. Collagen 1

Type I collagen is the most abundant matrix protein synthesized by osteoblasts. Different domains of the *Col1a1* promoter regulate expression in different type I collagen-producing tissues. A region between −2295 and −1670 bp is required for high expression in various tissues including bone and tendon. The 3.6 kb (−3521 to +115) promoter of the rat *Col1a1* gene targets various *Col1a1* expressing tissues [32]. However the 2.3 kb promoter activity is present in calvaria but lost when calvarial cells are cultured [33]. Following these studies, we utilized Col3.6 and Col2.3 promoters to target GFP expression to different stages of the osteoblast lineage [34]. Two versions of the Col3.6 reporter (topaz and cyan variants) show expression localized to the spindle-shaped cells at early stages of osteoblast differentiation with strong expression associated with mature bone nodules, coinciding with the increase of *Col1a1* expression and ALP activity. Col3.6GFP is strongly expressed in the osteoblastic cells on endosteal and trabecular surfaces *in vivo*, and more weakly in the fibroblast-like cells in the periosteum (Fig. 1B). Expression is also present in all tissues expressing type I collagen including strong expression in tendon and weaker expression in the skin, bladder, fat, lung, and muscle. The main utility of Col3.6 is the ability to target preosteoblastic cells, while the main disadvantage of the cyan version of this reporter is its expression in osteoclasts [35].

Col2.3 generated a more osteoblast-specific reporter, with expression restricted to nodules undergoing mineralization *in vitro*, and primarily labeling mature matrix producing osteoblasts, and osteocytes in the bone *in vivo* (Fig. 1C) [34,36,37]. In addition, Col2.3GFP is also expressed by cells of other mineralized tissues such as cementoblasts and odontoblasts, and a subset of cells in the body of tendons and ligaments [22,38]. Col2.3-mRFP mice were also generated and displayed the same transgene expression pattern with fluorescent signal restricted to mature osteoblasts [39]. However, it has been recently demonstrated that a population of cells within the bone marrow can express Col2.3GFP and CD45, indicating it may identify a fibrocyte population, although in our hands, GFP intensity in CD45+ cells is much weaker than in osteoblasts (unpublished data) [40]. Despite expression in cells outside the osteogenic lineage, Col2.3 remains one of the most reliable and widely used markers to identify mature osteoblasts, and target or delete expression in these cells [41].

5.2.2. Bone sialoprotein

Bone sialoprotein (BSP) is a non-collagenous protein specifically expressed in mineralized tissue. BSP-GFPtopaz lines have been generated as a part of a larger BAC vector containing reporters for Dmp1-Cherry and Trap-CFP [42]. The Trap reporter does not function well, but lines

are available with either BSP-GFP alone, or BSP-GFP/Dmp1-Cherry dual reporters. BSP reporter expression overlaps well with endogenous gene expression, marking osteoblasts, osteocytes, and some hypertrophic chondrocytes, and is very strong in trabecular bone. Although not widely used, BSP-GFP is useful reporter for identification of osteoblasts that avoids expression in non-osseous tissues.

5.2.3. Osteocalcin

Osteocalcin is a highly expressed protein in mature osteoblasts. A construct containing a 1.7 kb rat osteocalcin promoter driving GFP was expressed later in the osteoblast lineage and in a lower number of cells than Col2.3-GFP, but appeared to label only cells with very high expression of osteocalcin and not the whole osteoblast lineage, limiting its utility [37]. Another larger construct containing a 3.8 kb fragment of the upstream sequence of the human osteocalcin promoter and 3.5 kb of the 3′-untranslated region and flanking DNA has been used to target expression of various genes to mature osteoblasts *in vivo* [43,44]. The Oc-GFPtopaz mouse generated using this promoter shows expression in osteoblasts and osteocytes, as well as cells in the periosteum not associated with mineralization label, possibly bone lining cells [45,46]. Oc-GFP has also been used to isolate cementoblasts, as it is expressed in cementoblasts, cementocytes and odontoblasts but not PDL cells [47].

5.2.4. Osteopontin

Osteopontin is a non-collagenous bone matrix glycoprotein that is secreted by pre-osteoblasts/osteoblasts during bone remodeling and by macrophages playing a role in the reversal phase of remodeling. Higashibata et al. demonstrated that a 5.5 kb fragment of the osteopontin promoter mimicked endogenous expression in a number of tissues, while shorter fragments retained bone-directed expression but also showed increased expression in chondrocytes [48]. It is unclear whether other non-osteogenic cell types are targeted, but the osteopontin promoter does not appear ideal for driving the expression of GFP specifically in osteoblasts.

5.3. Identification of osteocytes

5.3.1. Dmp1

Dentin Matrix Protein 1 (*Dmp1*) is another non-collagenous matrix protein that is regularly used as a marker of pre-osteocytes/osteocytes [49,50]. Dmp1-GFP transgenic mice were generated using the 8 kb Dmp1 promoter, and show expression in osteocytes (Fig. 1D) [51]. This promoter fragment is mechanoresponsive, with an increase in intensity and number of GFP+ cells in response to loading [50]. This transgenic model has been widely used to mark preosteocytes/osteocytes, including for the generation of an osteogenic cell line that can be used to generate GFP+ osteocyte-like cells in culture (IDG-SW3 cells) [52]. More recently a membrane bound GFP controlled by the 10 kb Dmp1 promoter has been generated to allow for detailed imaging of osteocyte dendrites [53]. These promoter fragments have also been used to drive Cre or CreERT2, however when combined with a visual reporter they show broader expression encompassing osteoblasts and osteocytes, a population of CD45+ cells in the bone marrow, and cells in the muscle [54]. Differences between the expression of Dmp1-GFP and Dmp1Cre may be due to differences in copy number, positional effects of transgene insertion, or a requirement for higher expression to visualize the GFP reporter than Cre expression to activate sensitive recombination reporter genes such as Ai9 [55]. Some studies have confirmed osteocyte specificity of Dmp1Cre by combining it with Dmp1-GFP followed by isolation of osteocytes based on GFP expression to confirm the deletion or overexpression of genes [56]. In addition, specificity of the Dmp1 directed overexpression or deletion of particular gene can be confirmed by histological detection of the targeted gene or its protein expression [57,58]. A number of BAC-based Dmp1 reporters have also been generated such as the BSP-GFP/Dmp1-Cherry mouse, and the Dmp1BAC-dsRed and SostBAC-GFP mice that effectively identify the majority of osteocytes [42,54]. Dmp1 reporters are effective tools for identifying osteocytes *in vitro* and *in vivo*, however care must be taken when interpreting results using Dmp1 promoter-driven transgenes or Cre as osteoblasts and other cell types may also be targeted.

6. Isolation of osteoblast lineage cells based on the expression of visual transgenes

Expression of GFP reporters at different stages of osteogenic lineage maturation allowed for the isolation and further characterization of these populations. Our group used primary calvarial osteoblast cultures to isolate pre-osteoblasts expressing Col3.6GFP and compare their gene expression to Col2.3GFP expressing cells (mature osteoblasts within the mineralized nodules) [59]. This study was followed by an *in vivo* isolation of calvarial osteoblasts (Col2.3CFP+ cells) and osteocytes (Dmp1-GFP+ cells). These approaches allowed for comparison of the levels of the gene expression between the cell types, and identified some of the genes that are specifically expressed in either of these populations such as Keratocan that is exclusively expressed in osteoblasts but not in osteocytes, while genes such as Reelin, Neuropeptide Y, and Calcitonin Receptor were strongly expressed in Dmp1-GFP+ osteocytic population [60,61]. These gene expression studies show the potential usefulness of the promoter-GFP transgenes in studying differential gene expression and as gene discovery tool. Osteoblast reporters also demonstrated that osteoblast differentiation is impaired in a murine model of osteogenesis imperfecta (OI), as the OI mice had greater Col3.6 expression, but less Col2.3, which could also explain at least in part the increased bone resorption observed in this OI model [62].

FP reporter mice have proved very useful for reliable identification of mature osteoblasts *in vivo*. Col2.3-GFP in particular has been widely used in lineage tracking studies to identify osteoblasts derived from various cell populations during development, adulthood, or following injury [19,21,63–65]. They are also useful to track differentiation following transplantation of cells. A number of studies have used reporters to confirm osteogenic differentiation of transplanted bone marrow stromal cells, and to evaluate contribution of donor *versus* host cells using Col2.3 with different color FPs [8,66,67]. Various reporters have also been useful for closely monitoring the fracture healing process [46,68,69]. ES cell lines have also been generated from mice harboring promoter-FP transgenes in order to monitor differentiation of ES-derived progenitor cells *in vitro* and *in vivo* [70,71]. A Col2.3GFP reporter construct has also been inserted into human ES cells to monitor osteogenic differentiation [72].

7. Conclusion

Introduction of visual transgenes into the cells of the osteoblast lineage more than 15 years ago resulted in a major paradigm shift in how we look into different stages of osteogenic lineage maturation. Use of FP reporters allowed for identification and isolation of subpopulations of cells at different stages of lineage commitment. Currently, well characterized reporters are available for pre-osteoblasts (Col3.6), mature osteoblasts (Col2.3, BSP, Oc) and osteocytes (Dmp1). A number of reporters are also available for different osteoprogenitor populations, however there is still much to be learnt about the sources and identity of osteoprogenitors *in vivo*. Studies based on the use of these reporter mice have improved our understanding of bone biology. They are particularly useful for tracking cells into the osteoblast lineage, especially in an injury or transplantation setting where bone formation is often less organized than during normal growth. We anticipate that visual osteoblast lineage reporters will continue to contribute to our knowledge of basic bone biology, and well as to evaluate regenerative potential of different cell types.

Conflict of interest

The authors have no conflicts of interest to disclose.

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