Published in final edited form as:

J Bone Miner Res. 2011 November ; 26(11): 2634–2646. doi:10.1002/jbmr.465.

Cell Line IDG-SW3 Replicates Osteoblast-to-Late-Osteocyte Differentiation *in vitro* and Accelerates Bone Formation *in vivo*

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Abstract

Osteocytes are the most abundant cells in bone yet are the most challenging to study as they are embedded in a mineralized matrix. We generated a clonal cell line called IDG-SW3 (for Immortomouse/Dmp1-GFP-SW3) from long bone chips from mice carrying a Dmp1 promoter driving GFP crossed with the Immortomouse, which expresses a thermolabile SV40 large T-antigen regulated by IFN- γ . Cells from these mice can be expanded at 33°C in the presence of IFN- γ and then allowed to resume their original phenotype at 37°C in the absence of IFN- γ . IDG-SW3 cells are Dmp1-GFP-negative and T-antigen-positive under immortalizing conditions but Dmp1-GFP-positive and T-antigen-negative under osteogenic conditions. Like osteoblasts, they express alkaline phosphatase and produce and mineralize a type I collagen matrix containing calcospherulites. Like early osteocytes, they express E11/gp38, Dmp1, MEPE, and Phex. Like late osteocytes, they develop a dendritic morphology and express SOST/sclerostin and FGF23, regulated by PTH and 1,25-dihydroxyvitamin-D3. When cultured on 3D matrices, they express Dmp1-GFP and sclerostin. When the 3D cultures are implanted in calvarial defects *in vivo*, they accelerate bone healing. This cell line should prove useful for studying osteoblast-to-osteocyte transition, mechanisms for biomineralization, osteocyte function, and regulation of SOST/sclerostin and FGF23.

Keywords

osteocyte; Immortomouse; sclerostin; SOST; FGF23

INTRODUCTION

No longer considered passive place holders in bone, osteocytes have emerged in recent years as active, versatile orchestrators of bone remodeling and mineralization. As executors of mineralization, osteocytes actively secrete factors that control mineralization of collagen

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CONFLICT OF INTEREST

All authors have no conflicts of interest.

extracellular matrix (1). As regulators of phosphate homeostasis, osteocytes express factors such as FGF23 that control local and systemic mineral metabolism and regulate phosphate homeostasis (For review see (2)). As mechanosensory cells, osteocytes respond to mechanical loading and unloading through fluid flow shear stress within the lacuno-canalicular network through PGE₂ release and the Wnt/*b*-catenin pathway (3–5). As modulators of bone formation, mature osteocytes produce sclerostin, encoded by the gene *SOST*, a negative regulator of bone formation (4,6). As regulators of bone resorption, osteocytes remodel their perilacunar matrix during lactation through activation of genes traditionally associated with osteoclasts (7). Furthermore, osteocyte apoptosis and autophagy in response to immobilization, glucocorticoid treatment, or hypoxia play important roles in maintenance of bone integrity(2). In light of this multifunctionality, better model systems are needed to study osteocytes.

Osteocytes are the most abundant bone cells in the body but are the most challenging to study because they are embedded in a lacuno-canalicular network within mineralized bone and are relatively difficult to isolate with increasing skeletal age and mineralization. Primary osteocyte isolations have very low yields, and because they are terminally differentiated, they cannot be expanded without loss of phenotype. Furthermore, primary osteocyte isolations are highly heterogeneous and may include cells of earlier or different stages of differentiation (8). Only mature osteocytes embedded within a mineralized matrix express the bone formation inhibitor and late osteocytic marker *SOST*/sclerostin (4,6,9), but mature osteocytes are most difficult to isolate.

As the importance of osteocyte viability and multifunctionality emerges, it becomes more important to have accurate tools and approaches to study this bone cell. Existing cell lines for the study of osteocytes, such as MLO-Y4 (10) and MLO-A5 (11) have limitations including transformation, constitutive expression of the large T antigen, absence of *SOST*/sclerostin or FGF23 expression, and/or absence of a mineralized matrix. Better cell model systems are needed that demonstrate the gene expression profiles and function seen in primary osteocytes. Osteocytic markers include *Dmp1*, *SOST*/sclerostin, and FGF23. *Dmp1* is an early osteocyte-selective marker that plays a role in matrix mineralization, hydroxyapatite formation, and phosphate homeostasis (5,12). *SOST*/sclerostin, secreted by mature, embedded osteocytes, is currently a major therapeutic target for bone because inhibition of *SOST*/sclerostin increases bone formation *in vivo* (4,6). FGF23, produced by osteocytes to regulate phosphate homeostasis, is a therapeutic target for defects in mineral homeostasis, such as hypophosphatemic rickets (13).

Here we report the establishment and characterization of an Immortomouse/*Dmp1*-GFP-derived bone cell line (IDG-SW3) capable of overcoming many of the limitations of existing osteocytic cell lines (12,14). Immortomouse-derived cells express a temperature-sensitive mutant of the SV40 large tumor antigen under the control of the interferon- γ -inducible *H-2K^b* promoter (*H-2K^b*-tsA58) at 33°C in the presence of γ -IFN, inducing continuous proliferation and immortalization (14). However, at 37°C in the absence of IFN- γ under differentiation conditions, the cells resume their original replicative ability and *in vivo* phenotype of a late osteoblast with the capacity to differentiate into a late osteocyte. This differentiation process faithfully replicates that of primary cells *in vivo*, especially in 3D compared to 2D culture, and therefore, will prove an extremely valuable experimental tool.

MATERIALS & METHODS

Cell Culture

Tissue culture media were purchased from GIBCO BRL, fetal bovine serum (FBS) was from BioWhittaker. Rat tail collagen type 1, 99% pure, was purchased from Becton

Dickinson Laboratories. All other reagents were purchased from Sigma Chemical Co. unless otherwise stated. Cells were expanded in permissive conditions (33°C in α MEM with 10% FBS, 100 units/ml penicillin, 50 μ g/ml streptomycin, and 50 U/ml IFN- γ) on rat tail type I collagen-coated plates or gels or bovine type I collagen sponges. To induce osteogenesis, cells were plated at 80,000 cells/cm² in osteogenic conditions (37°C with 50 μ g/ml ascorbic acid and 4 mM β -glycerophosphate in the absence of IFN- γ). Collagen-coated surfaces were used because they were found to be effective at maintaining an osteocyte-like phenotype (10).

MLO-A5 cells, used as controls, are an established model of late osteoblasts with the ability to rapidly synthesize mineralized extracellular matrix (1). MLO-A5 cells are highly responsive to mechanical loading in 3D culture (15). MLO-Y4 cells, also used as controls, are an established model of osteocytes.

Cell Isolation

Long bones were isolated from a 3-month old Immortomouse^{+/-}/Dmp1-GFP^{+/-} mouse. These mice carry an γ -IFN-inducible promoter driving expression of a thermolabile large T antigen (*H-2K^b-tsA58*), enabling conditionally immortalization of cells derived from their tissues (12). Additionally, these mice carry a mouse Dmp1 cis-regulatory system driving GFP expression, specific to long bones and calvaria, correlated with endogenous Dmp1 expression, and widely used as a marker for living early osteocytes (14). Genotype was verified by GFP expression in tail biopsies and by PCR analysis of genomic DNA as described in (16). Periosteum was scraped off. Both ends of the bones including the growth plate cartilage were removed, and the marrow was flushed. Bone pieces were cut into smaller chips and sequentially digested in 0.75 mg/ml collagenase in Hank's balanced salt solution or 10 mM EDTA pH 7.4 for 30 minutes at 37°C. Cells collected from 8 digestions were not used for cloning. Digested bone chips were cultured for 2 days at 37°C to allow cells to grow out of the bone chips.

Cells were isolated by single cell dilutions in 96-well plates and initially screened for single clones. Colonies were expanded and maintained in permissive conditions. When cells were transferred to osteogenic conditions for screening and experiments, the original cell line remained cultured in permissive conditions. Colonies not meeting screening criteria for Dmp1-GFP expression, mineralized extracellular matrix formation, and E11/gp38 expression over time, were ruled out. Over 1500 potential cell lines were screened to obtain two IDG cell lines, each derived from a single clone.

Real-time PCR

Total RNA was isolated using TRIzol Reagent (Invitrogen). DNA contaminants were removed with DNase digestion. 2–4 μ g were reverse transcribed to cDNA. Real-time PCR was conducted using TaqMan Gene Expression Mix and gene-specific TaqMan probes. Relative quantification was calculated with the $2^{-\Delta\Delta C_T}$ method.

Western Blot Analysis

Total protein lysates were isolated with RIPA buffer containing proteinase inhibitors. 7–10 μ g per sample were separated by SDS-PAGE as previously described (1) and detected with anti-SV40 T-antigen mouse monoclonal antibodies (Pab 419, sc-58665, Santa Cruz Biotechnologies), anti-E11/gp38 serum (8.1.1), anti-sclerostin antibodies (R&D Systems, #AF1589), or anti-GAPDH antibodies and the appropriate secondary HRP-conjugated goat anti-hamster, goat anti-mouse, donkey anti-rabbit, or donkey anti-goat antibodies.

Alkaline Phosphatase (ALP) Activity

Cells were lysed with 0.05% Triton-X and two freeze-thaw cycles. ALP activity was assayed with 1.5 M 2-amino-2-methyl-1-propanol (AMP) buffer (pH 10.3) and quantified against *p*-nitrophenol phosphate standard curve as a previously described (17). Absorbance at 405 nm was recorded in triplicate and normalized to protein levels.

Cell Staining

Cells were fixed in 10% formalin for 20 min and stained with 4 mM alizarin red S for 2 min to stain for calcium or 2% silver nitrate under UV light for 20 min to stain for phosphate by von Kossa. Stained area was calculated as a percent of total well area over a representative threshold level. Alizarin red S dye was extracted with sequential incubation with 10 mM HCl in 70% EtOH. Samples were quantified against a phosphate buffer standard curve on a plate reader at 405 nm.

Immunohistochemical Staining for Collagen Type I

Cells were plated on non-collagen-coated plastic tissue culture and stained as previously described (1) using rabbit serum recognizing the C-telopeptide of collagen type I, LF-68 (kindly provided by Dr. Larry W. Fisher, National Institutes of Health, Bethesda, MD), or non-immune rabbit serum and a Cy3-conjugated donkey anti-rabbit IgG.

Fluorometric Analysis

Cells were fixed in 10% formalin for 20 min and stained with 4 µg/ml 4',6-diamidino-2-phenylindole (DAPI) for 1 min to visualize nuclei. GFP-positive cells were counted per field at 20X and calculated as a percent of DAPI-stained cells. Total protein lysates were isolated with RIPA buffer containing proteinase inhibitors and quantified against a standard curve using the Bio-Rad BCA or Pierce protein assay.

Immunohistochemical Staining for Sclerostin

Samples were fixed in 10% formalin for 20 min, decalcified in 14% EDTA pH 7.1, and embedded in paraffin. Ten-micron sections were stained following the protocol described in Poole et al. 2005 (6) and counterstained with 0.5% methyl green. Non-immune goat IgG was used as a negative control.

3D Culture

Three-mm disks of bovine collagen sponges (CollaTape, Zimmer) or rat tail type I collagen gels (pH 7.4) were seeded on top with 80,000 cells/cm², and fed with osteogenic media every 2–3 days.

Scanning Electron Microscopy

Samples were fixed in 2.5% glutaraldehyde-Na cacodylate buffer, dried with HMDS, sputter-coated with gold palladium, and examined with an XL30 field emission SEM (FEI/Philips). Accelerated voltage at 15 kV for backscatter imaging. For x-ray microanalysis (energy-dispersive spectrometry), samples were carbon-coated and examined with 15 KeV accelerating voltage. X-ray spectra maps for calcium and phosphorus distribution were acquired.

Mouse Calvaria Defect Model

The animal protocol was approved by the UMKC Institutional Animal Care and Use Committee. Experiments were performed as described by Aalami (18). Fifteen 2–4-month-old wild-type C57/B6 mice were anesthetized with 0.5–4% isoflurane inhalation and

ketamine/dexdomitor (75/1mg/kg) *i.p.* injection. The calvarium was shaved, cleaned with betadine, rinsed with alcohol, and repeated 3 times. A 1-cm incision along the cranial midline and envelope flap was reflected. Bilateral, full bone thickness, critical-sized, 3-mm diameter, non-suture-associated osteotomies were centered in parietal bones with a dental bur (Brasseler) on a Dremel handpiece under copious irrigation, avoiding underlying dura mater. Defects were irrigated and randomly implanted with controls or cells on collagen sponges after 21 days differentiation. Skin was reapproximated with primary closure and sutured with 5-0 coated Vicryl (polyglactin 910). All animals were injected with Antisedan reversal agent (Atipamezole; 0.1–1.0mg/kg; *i.p.* or *s.c.*) and buprenorphine (0.01–0.05 mg/kg *s.c.*) immediately postsurgery.

MicroCT Analysis

At 2, 4, and 7 weeks after surgery, mice were anesthetized and imaged *in vivo* a Scanco VivaCT 40, following recommended guidelines from Bouxsein 2010 (19). Bone healing over time was examined. Voxel isotropic resolution was 15 μ m. X-ray energy was 55 Kvp and 72 uA. Threshold for image binarization was 220. Volumetric analysis using Scanco software included a 120 $\times$ 120 pixel diameter, 68 slice-thick VOI within the osteotomy.

Histology

Mice were sacrificed 7 weeks post-surgery by CO₂ asphyxiation, cervical dislocation, and decapitation. Calvariae were excised, fixed, and infiltrated with 15% and 30% sucrose. Undecalcified, frozen 10 μ m sections on cryotape were stored at –80°C prior to staining with alizarin red S and DAPI and visualized under fluorescent microscopy.

Statistical Analysis

A Student's t-test or one-way ANOVA with Tukey post-test was used to determine significant differences compared to controls, with $p < 0.05$. Data shown are mean $\pm$ SEM for at least 3 or more experiments.

RESULTS

Establishment of a cell line representing osteocyte differentiation

To improve upon existing cell models for studying osteocytes, initial screening criteria focused on identifying cell lines with the capacity to express Dmp1-GFP, produce a mineralized extracellular matrix, and express early osteocyte marker E11/gp38. To enrich for osteocytes, periosteum was removed, bone marrow was flushed, and bones were digested with collagenase and EDTA to remove surface cells in favor of embedded cells. Cells that migrated out of digested bone chips after 2 days culture in normal conditions were cloned. We found that cells would migrate from the bone chips only if cultured in normal tissue culture conditions at 37°C, not immortalizing conditions at 33°C in the presence of IFN- γ . Moreover, isolated cells would not divide if they were Dmp1-GFP positive.

From over 1,500 screened colonies, two clonal cell lines that met initial screening criteria were established and characterized further. This paper focuses on the establishment, characterization, and testing of one cell line, IDG-SW3, for the ability to differentiate into osteocytes.

IDG-SW3 cells express SV40 large T antigen under immortalizing conditions but not under osteogenic conditions

Overexpression of the SV40 large T-antigen is a common technique for cell immortalization and generation of cell lines (10,11). Unlike other studies, here cells were conditionally

immortalized with a thermolabile large T antigen, which induces cell division under immortalizing conditions but not under normal culture conditions. In immortalizing conditions, T-antigen is highly expressed, but T-antigen levels drop after 24 hours in normal culture conditions, and are undetectable by 3 days in osteogenic conditions by Western blot (Fig 1A). Thus, in the absence of the large T-antigen, IDG-SW3 cells more closely resemble primary bone cells in the osteogenic environment.

IDG-SW3 cells increase Dmp1-GFP expression under osteogenic conditions over time but not under immortalizing conditions

Dmp1-GFP is a marker for osteocytic differentiation (12). To measure Dmp1-GFP expression in IDG-SW3 cells, fluorescent imaging, cell counting, and fluorometric analysis were performed. When cultured in immortalizing conditions, IDG-SW3 cells proliferate and are GFP-negative (Fig 1B). In contrast, under osteogenic conditions, IDG-SW3 cells increase Dmp1-GFP expression within 3 days of culture and begin osteocytic differentiation. By 14 days GFP increased and was uniformly distributed across the culture surface (Fig 1C) without any significant change in the number of DAPI-stained cells (Fig 1D). Increasing percent GFP-positive cells (Fig 1E) correlates with increasing total GFP levels (Fig 1F), determined by fluorometric analysis of protein lysates, further demonstrating differentiation that when IDG-SW3 cells are removed from immortalizing conditions and cultured in osteogenic conditions, they differentiate toward an osteocytic phenotype.

IDG-SW3 cells express osteoblastic markers on 2D surfaces *in vitro*

To evaluate the osteoblastic activity of IDG-SW3 cells, two hallmarks of an osteoblastic phenotype were evaluated, ALP activity and type I collagen expression. ALP activity increased over time in osteogenic conditions, first elevated at Day 0, peaking at Day 14, and declining at Day 21 (Fig 2A). After 9 days of osteogenic differentiation, immunofluorescent staining of cells with a type I collagen-specific antibody revealed production of abundant type I collagen fibrils, organized in a honey-combed extracellular matrix (Fig 2B). IDG-SW3 cells appear similar to MLO-A5 cells with production of large amounts of type I collagen in conjunction with ALP expression, in contrast to MLO-Y4 cells that have nearly undetectable ALP levels, indicating that IDG-SW3 display an osteoblastic phenotype early in osteogenic differentiation.

IDG-SW3 cells increase mineralization and calcium deposition that co-localizes with Dmp1-GFP expression under osteogenic conditions on 2D surfaces *in vitro*

Von Kossa staining from 0–14 days shows focal nodular mineralization (Fig 2C), and increased percent mineralized area (Fig 2D). Alizarin red S staining demonstrates increased calcium deposition (Fig 2E), increased percent calcified area (Fig 2F), and increased quantities of total alizarin red dye extracted from stained samples (Fig 2G) from 0–14 days that co-localizes with GFP-positive cells expression on Day 14 (Fig 2H). This mineralization pattern is similar to the mineralization and calcification of MLO-A5 cells in osteogenic culture.

IDG-SW3 cells produce a dense, extracellular collagen fibril network associated with mineralized spheres on 2D surfaces *in vitro*

Upon culture in osteogenic conditions, IDG-SW3 cells produce an extensive, dense, extracellular collagen network intercalated with abundant, highly mineralized calcospherulites and nanospherulites ranging from 0.03–1 μm (Fig 2I), similar to the calcospherulites proposed to initiate collagen-mediated mineralization in MLO-A5 cells (1). Collagen fibrils project from the cell membrane (Fig 2J) in a pattern consistent with those observed in mineralizing MLO-A5 cultures, where nanospheres containing calcium and

phosphorus bud from cellular projections, associate with collagen fibrils, and enlarge, coalesce, and engulf the collagen network during mineralization (1).

IDG-SW3 cells express early osteocytic genes on 2D surfaces *in vitro*

Markers for early osteocytes include E11/gp38, Dmp1, MEPE, and Phex. E11/gp38 is a plasma membrane-bound protein that plays a direct role in formation of osteocyte dendritic processes and response to shear stress. Immunostaining with an E11 antibody shows E11 induction in IDG-SW3 cultures over time, compared to MLO-Y4 osteocytic cells, which express high E11 and MLO-A5 osteoblastic cells, which also express E11 over time in culture (Fig 3A). Similar to E11 expression in MLO-A5 cells, E11 in IDG-SW3 cells peaks near Day 7, decreases thereafter, and remains elevated over time (Fig 3B). In extended culture, IDG-SW3 cells increased Dmp1-GFP expression over time and remained elevated at Day 35 under fluorescent microscopy (Fig 3C) and by relative GFP fluorometry (Fig 3D). Real-time PCR analysis shows dramatic increases in Dmp1 mRNA expression rising in parallel with GFP levels (Fig 3E). IDG-SW3 cells also show increased MEPE (Fig 3F) and Phex (Fig 3G) expression by Day 14 to greater levels than are observed in differentiating MLO-A5 cells. This induction of early osteocytic genes in 2D culture correlates with the increase in early osteocyte functionality previously observed as early as Days 3 to 7 (Fig 2D, 2F, 2G). Together, expression of multiple early osteocytic markers shows IDG-SW3 cells differentiate into early osteocytes over time.

IDG-SW3 cells express late osteocytic markers when cultured on 2D surfaces *in vitro*

To test if IDG-SW3 cells differentiate into late osteocytes in culture, expression of SOST/sclerostin and FGF23 were measured after osteogenic culture for 0–35 days. SOST/sclerostin, a marker for late, embedded osteocytes, is a potent negative regulator of bone formation and is responsive to mechanical loading (20). IDG-SW3 cells show increased SOST mRNA expression, first detectable at day 14, peaking at Day 21, and remaining highly elevated at Day 35. C_T values at peak expression were consistently between 23–25 (Fig 4A). Western blot analysis of cell lysates demonstrates abundant sclerostin protein production first detectable at low levels at Day 14 and peaking at Day 35 (Fig 4B).

Previous studies have shown that SOST regulation may play a role in mediating PTH action in bone (21). A single injection of PTH(1–34) *in vivo* was shown to reduce SOST expression (22). Constitutive activation of the PTH receptor in osteocytes increased bone mass and reduced sclerostin expression (23). To test the effect of PTH on SOST expression in IDG-SW3 cells, cells were treated with 10nM PTH(1–34) for 24 or 48 hours at 14 or 21 days of osteogenic differentiation. Quantitative RT-PCR of extracted RNA demonstrates that a single dose of PTH dramatically inhibits SOST mRNA expression. At 14 days of differentiation, SOST expression decreased from a 23.4 ± 12.1 fold induction to undetectable levels 48 h after PTH treatment. At 21 days of differentiation, SOST expression in IDG-SW3 cells decreased from 29.2 ± 19.5 and 30.5 ± 2.4 fold induction at 24 h and 48 h after PTH(1–34) treatment, respectively, to undetectable levels (Fig 4C).

FGF23, a critical regulator of phosphate homeostasis, is secreted by osteocytes and is upregulated through 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] and through dietary phosphate (13). To measure FGF23 expression in IDG-SW3 cells, quantitative RT-PCR was performed on RNA isolated from cells cultured from 0–35 days in osteogenic conditions. IDG-SW3 cells express low FGF23 mRNA under osteogenic conditions *in vitro*, first detectable at Day 14 and increasing at Day 21 and 35. C_T values at peak expression were consistently 31–32 (Fig 4D). To test the effect of 1,25-dihydroxyvitamin D₃ on FGF23 expression in IDG-SW3 cells, cells were treated with 10nM 1,25-dihydroxyvitamin D₃ for 24 or 48 hours at 14 or 21 days of osteogenic differentiation. Quantitative RT-PCR of

extracted RNA demonstrates that a single dose of 1,25-dihydroxyvitamin D₃ significantly induced FGF23 expression (Fig 4E). In summary, expression of late osteocytic markers SOST/sclerostin, and FGF23 and regulation of these markers by PTH and 1,25-dihydroxyvitamin D₃, respectively, indicate IDG-SW3 cells display a late osteocytic phenotype *in vitro* by 21 days of osteogenic culture.

IDG-SW3 cells infiltrate and mineralize 3D collagen matrices

To determine the ability of IDG-SW3 cells to infiltrate and mineralize 3D substrates, IDG-SW3 cells were seeded at 80,000 cells/cm² on type I collagen gel (Fig 5A) or type I collagen sponge (Fig 5B) and cultured in osteogenic media for 21 days. Cells grew well and were still viable after 21 days. Transverse sections of 3D cultures show cell penetration into the gel and sponges and multilayering of cells with intervening extracellular matrix (Figs 5A, 5B). SEM and backscatter imaging of cells cultured for 21 days on collagen sponges show mineralization of fibrils and abundant mineralized spherical structures, nanospherulites, as large as 10 µm in diameter (Fig 5C). EDS analysis indicates bone-like material containing calcium and phosphorus, similar to hydroxyapatite, co-localizing in the mineralizing areas (Fig 5D).

IDG-SW3 cells express osteocytic genes and display dendritic morphology when cultured on 3D collagen matrices

To evaluate the ability of IDG-SW3 cells to undergo osteogenic differentiation on 3D substrates, cells were seeded on type I collagen sponges and cultured in osteogenic conditions for 30 days. IDG-SW3 cells express similar levels of Dmp1-GFP when cultured on 3D collagen gels and sponges for 30 days, as shown compared to 2D collagen surfaces (Fig 5E). Dmp1-GFP-positive cells with dendritic morphology are visible at multiple planes of focus within the collagen sponge, as shown under fluorescent and phase contrast microscopy (Fig 5F). IDG-SW3 cells seeded on collagen sponges produce sclerostin protein. Immunohistochemistry with an anti-sclerostin antibody compared to a non-immune IgG demonstrates increased sclerostin in the cell layers and extracellular matrix (Fig 5G). Thus, IDG-SW3 cells are viable and differentiate toward an osteocytic phenotype in both 2D and 3D collagen culture conditions.

IDG-SW3 cells accelerate bone healing *in vivo*

To evaluate the ability of IDG-SW3 cells to survive or function *in vivo*, cells were implanted into calvarial defects of congenic C57/B6 mice. Bilateral calvarial defects were left empty or implanted with collagen sponge or IDG-SW3 cells cultured on a collagen sponge. Culture for 21 days was selected for presence osteocytic markers Dmp1-GFP (Fig 5E, 5F) and sclerostin (Fig 5G). Implantation of IDG-SW3 cells enhanced bone healing at Week 7 (Fig 6A). Bone healing within the defects was minimal at 2 and 4 weeks but a significant increase in bone volume and bone density was observed at 7 weeks implantation. Control empty defects showed negligible healing at all time points (Fig 6B). Fluorescent microscopy of undecalcified frozen sections show Dmp1-GFP-positive cells regionally localized within mineralizing healing defects implanted with IDG-SW3 cells but not in control defects implanted with collagen sponge. Images of intact calvaria distant from the surgical site are shown (Fig 6C). Thus, IDG-SW3 cells accelerate bone formation *in vivo* and are still present at the site 35 days after implantation, albeit at low numbers.

IDG-SW3 cells have distinct osteocytic gene expression profiles

Markers for osteoblasts and osteocytes are expressed as they undergo the osteoblast-to-osteocyte transition. IDG-SW3 cells produce and mineralize an extracellular matrix and express Dmp1-GFP as a marker mirroring their differentiation. IDG-SW3 cells express the

entire profile from late osteoblastic to late osteocytic genes, including SOST/sclerostin and FGF23 over time in culture (Fig 7).

DISCUSSION

Osteocyte function has been difficult to study because osteocytes are embedded in a mineralized matrix. While numerous osteoblast models have been generated, only a few osteocyte models are available, and none of the available osteocyte models have completely replicated the normal *in vivo* differentiation process. We believe IDG-SW3 cultured in osteogenic conditions for 21 days represents a late osteocyte.

Osteoblastic cell lines were among the first bone cell lines established and include mouse cell line MC3T3 and rat osteosarcoma-derived cell lines ROS17/2.8 and UMR-106 (24). Later human cell lines included MG-63 and Saos-2 cells were developed (25,26). No markers for osteocytes were known at that time. In 1997 the MLO-Y4 cell line was cloned based on morphology, low alkaline phosphatase activity, and high osteocalcin expression (10). MLO-Y4 cells highly express connexin 43, a protein that comprises gap junctions (10). Later studies demonstrated expression of E11/gp38 as a marker for early osteocytes (27). MLO-Y4 cells are highly sensitive to fluid flow shear stress (28). However, MLO-Y4 cells lack an extracellular matrix and do not express late osteocyte markers such as SOST/sclerostin.

SOST/sclerostin is a mature osteocyte marker of particular interest as a potential therapeutic target. Sclerostin deficiency results in increased bone formation, as seen in the human bone diseases sclerostosis and van Buchem disease (20) and in mouse models lacking this gene (29). Sclerostin antibody treatment in osteoporotic rats and normal monkeys increases bone formation and bone quality (30). However, no previous *in vitro* cell models could reproduce the robust induction of SOST mRNA observed *in vivo* in osteocytes.

MLO-A5 cells appear to be a cell line representing early osteocytes but not late osteocytes (11). MLO-A5 cells produce a highly mineralized extracellular matrix and markers of early osteocytes such as E11/gp38. Basal SOST mRNA expression in MLO-A5 cells at Day 12 was reported (22), however, in our experience basal levels of SOST in MLO-A5 cells are very low or undetectable by real-time PCR. It was later discovered that exogenous addition of TWEAK alone or TWEAK and TNF- α co-treatment induce SOST expression *in vitro* in SaOS-2, MG-63, and MLO-Y4 cells in a dose-dependent fashion and *ex vivo* in human bone cultures (31). TNF- α treatment alone appears to induce SOST in a species-specific manner. However, induction levels were low compared to primary cells.

IDG-SW3 expresses SOST mRNA detectable within a normal range using real-time PCR and expresses sclerostin protein, easily detectable by traditional western blotting techniques. Induction of SOST/sclerostin, normally seen in deeply embedded, mature osteocytes, indicates that IDG-SW3 cells enter the latter stages of osteocytic differentiation over time *in vitro*. SOST/sclerostin is first detected at Day 14 of culture and reaches significant levels at Days 21 and 35. SOST/sclerostin expression is preceded by Dmp1 mRNA and Dmp1-GFP induction, first detected at Day 3, suggesting that IDG-SW3 cells proceed through osteogenic differentiation from less to more differentiated state in conjunction with the formation of a mineralized matrix. This is also consistent with the temporal expression of osteoblastic markers alkaline phosphatase, type I collagen- α 1, Phex, and MEPE, followed by the osteocytic gene, FGF23.

Further studies are also needed to reveal the mechanisms of regulation of SOST/sclerostin expression. Regulation of SOST/sclerostin expression is believed to play a role in mediating the opposite effects of continuous vs. intermittent PTH treatment on bone formation (32).

Previous studies showed that a single injection of PTH(1–34) *in vivo* reduces SOST expression (22). Constitutive PTH receptor activation in osteocytes increases bone mass and reduces sclerostin expression (23). PTH treatment was reported to inhibit SOST in MLO-A5 cells (22). IDG-SW3 represents a cell line with the capacity for robust SOST expression regulated by a single dose of PTH. PTH inhibition of SOST in IDG-SW3 lasted at least 48 hours and was not dependent on whether administered at 14 or 21 weeks. Therefore, IDG-SW3 cells are a model system to study PTH regulation of SOST *in vitro*.

Another osteocyte-selective marker is the hormone FGF23, highly expressed in the osteocyte. Through FGF23 release, the osteocyte directly regulates systemic mineral levels distant from the bone. In hypophosphatemia, Dmp1 and Phex, also produced by the osteocyte, downregulate FGF23 expression, thereby increasing phosphate reabsorption in the kidney and enabling the body to maintain normal phosphate levels and bone mineral content (13,33). Patients with chronic kidney disease, vitamin D deficiency, or hypophosphatemic rickets suffer from elevated FGF23 and subsequent phosphate wasting and loss of bone mineral content (13,34,35). Elucidation of the regulation of FGF23 expression in osteocytes and exact mechanisms for its control would lead to better treatment for these patients. FGF23 expression in IDG-SW3 cells is low but detectable in baseline conditions at Day 14 of culture. Consistent with *in vivo* observations, 1,25-dihydroxyvitamin D₃ treatment dramatically increases FGF23 expression in IDG-SW3 cells. Thus, IDG-SW3 cells may be a model to further understand FGF23 regulation.

IDG-SW3 may also be a model system for studying bone formation in healing defects. When IDG-SW3 cells were cultured on collagen sponges and implanted into calvarial defects in wild-type hosts, both microCT and histology show enhanced bone formation *in vivo*. GFP-positive cells are noted within the healing site in association with mineralizing bone, but in much fewer numbers than were implanted at the site. We speculate that the implanted cells may have ceased GFP expression after extended differentiation. An alternative explanation is that the IDG-SW3 cells were responsible for recruiting host cells to the defect, creating a more favorable environment for host cell attachment, and/or accelerating host cell differentiation and function. These data clearly warrant further studies to fully unravel the roles of IDG-SW3 cells and host cells in the new bone formation.

Immunorejection of the implanted cells was not observed. This is consistent with previous studies using Immortomouse-derived hepatocytes transplanted into congenic C57/B6 mice after engraftment for 2 weeks without development of a graft vs. host response (36).

The temperature-sensitive form of the large T antigen is rapidly degraded at normal mouse body temperature (37). Although the transgene is not fully inactivated *in vivo*, the Immortomouse expresses little to no T antigen in most of its tissues at body temperature, lives to adulthood, and lacks tumorigenicity. The thymus and liver showed higher expression, with the Immortomouse eventually succumbing to thymic hyperplasia, with homozygotes more affected than hemizygotes (14). Furthermore, Immortomouse-derived cells will eventually senesce at 37°C (37). This is consistent with our findings of low to undetectable T-antigen under osteogenic conditions *in vitro* and decreased numbers of Dmp1-GFP-positive mice *in vivo*, such as through the natural course of senescence.

In summary, we show the establishment and characterization of a cell line with features distinctly different from existing cell lines. First, IDG-SW3 cells were derived from normal, healthy long bones. They express a full osteogenic differentiation profile from late osteoblast to late osteocyte. During this process, they produce, mineralize, and embed in a mineralized collagen matrix. Because IDG-SW3 cells are derived from the Immortomouse, these cells possess an IFN- γ -inducible thermolabile large T-antigen and are conditionally

immortalized. Thus, the temperature-sensitive large T-antigen can be temporarily turned on for large scale production of IDG-SW3 and later turned off and rapidly degraded for experiments requiring cells with gene expression and function more closely aligned with primary cells. This represents a distinct advantage over other cell lines, such as MLO-A5 and MLO-Y4, that constitutively express the large T-antigen. Furthermore, because the cells were derived from the Dmp1-GFP transgenic mouse, IDG-SW3 cells contain a GFP reporter paralleling osteogenic differentiation, enabling live monitoring as the cells transition into osteocytes. Future experiments will explore osteocyte gene regulation and function and how IDG-SW3 interacts with host cells *in vivo*.

Acknowledgments

This work was supported by NIH NIAMS P01 AR046798. IK was supported by NIH NIAMS R03 AR053275. The American Association of Endodontists Foundation also supported this work. The authors thank Dr. Lixiang Bi and Jacquitta L. Taylor for their assistance.

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Fig. 1. Dmp1-GFP and T-antigen expression in IDG-SW3 cells in immortalizing or osteogenic conditions

(A) Total protein was isolated from cells cultured in immortalizing conditions or Day 0–14 in osteogenic conditions for Western blot analysis (left). MLO-A5 cells, which constitutively express the large T-antigen driven by the osteocalcin promoter, are a positive control. Relative densitometry of T antigen-positive bands normalized to GAPDH is shown (right). (B) Images of cells under immortalizing conditions (33°C in the presence of IFN- γ) at 10X and 20X fluorescent and phase contrast microscopy. (C) Images of cells after 14 days osteogenic culture at 37°C in the absence of IFN- γ under 4X fluorescent microscopy. (D) Time course of Dmp1-GFP-positive cells from 0–14 days. DAPI-stained cells were imaged

under fluorescent and phase contrast microscopy in osteogenic conditions. (E) Relative percent GFP-positive cells were counted in representative fields. (F) Relative GFP expression as normalized to total protein. Lysates were fluorometrically quantitated. Day 0 is the day after plating and when the cells reached 100% confluence. * $p < 0.05$ compared to Day 0

Fig 2. IDG-SW3 expression of osteoblastic markers, mineralized matrix, and calcospherulites on 2D surfaces *in vitro*

(A) Alkaline phosphatase expression over 21 days. Cell lysates were assayed for ALP activity at 405 nm, normalized to total protein. (B) Collagen expression in IDG-SW3 compared to MLO-A5. Cells cultured in osteogenic media for 9 days were incubated with rabbit serum immunized against type I collagen or non-immune rabbit serum and compared with MLO-A5 cells, previously shown to produce extracellular matrix rich in type I collagen (1). (C) Mineralization by von Kossa staining. Cells were fixed and stained at 0–14 days differentiation. (D) Percent mineralized area increased significantly over time. (E) Alizarin red stain for calcium deposition. (F) Percent calcified area correlates with extracted alizarin red dye. (G) Alizarin red dye quantitation. MLO-A5 cells were cultured for 9 days demonstrated similar mineralization, whereas MLO-Y4 osteocyte-like cells also did not mineralize. (H) Overlaid images for GFP and alizarin red staining (20X) showing GFP-positive cells closely associated with mineral. (I) Comparative secondary and backscatter images show (1,500X or 1,000X) nanospherulites and calcospherulites ranging from a few nanometers (arrows, top) to approximately 10 μ m in diameter were observed (arrows, bottom). (J) Abundant vesicle budding from the cell membrane (arrows, top) and collagen fiber production (arrows, bottom) were observed (1,000X). * $p < 0.05$ compared to Day 0.

Fig 3. IDG-SW3 expression of early osteocytic markers on 2D surfaces *in vitro*

(A) Correlation of E11 expression with Dmp1-GFP. Cells were fixed after 14 days osteogenic differentiation and incubated with an E11-specific primary antibody and a Cy3-conjugated secondary antibody. E11 staining was overlaid with GFP expression to evaluate co-localization. MLO-Y4 cells, a positive control, are highly positive for E11. (B) E11/gp38 western blot and quantitation of cell lysates, normalized to GAPDH. Also shown is relative densitometry of E11-positive bands of a representative experiment. MLO-A5 baseline cell lysates were used as a negative control. (C) Time course of Dmp1-GFP-positive cells in extended culture. Cells were fixed from Day 0–35, stained with DAPI, and representative fields were imaged under fluorescent microscopy. (D) Relative GFP expression in extended culture, as normalized to protein. Cell lysates were fluorometrically quantitated. (E) Relative fold induction of Dmp1 mRNA in extended culture. Quantitative RT-PCR analysis of RNA isolated from cells from 0–35 days, normalized to 18S, shows increased Dmp1 mRNA in parallel with GFP levels observed in culture. (F) Relative fold induction of MEPE mRNA and (G) Phex mRNA in IDG-SW3 compared to MLO-A5 by quantitative RT-PCR. * $p < 0.05$ compared to Day 0

Fig 4. IDG-SW3 expression of late osteocytic markers on 2D surfaces *in vitro*

(A) Relative fold induction of SOST mRNA in IDG-SW3 compared to MLO-A5.

Quantitative RT-PCR with a SOST-specific probe, normalized to 18S, was performed on RNA isolated after 0–35 days of osteogenic culture. MLO-A5 cells were used as controls to compare expression levels. (B) Western blot analysis of sclerostin expression. Total protein isolated at 0–35 days demonstrates abundant sclerostin protein. Relative densitometry of sclerostin-positive bands normalized to GAPDH is shown (bottom). MLO-A5 cells did not express detectable sclerostin protein. (C) PTH inhibition of SOST expression. 10 nM PTH(1–34) treatment inhibits SOST expression after 24 or 48 h. (D) FGF23 mRNA expression in IDG-SW3 cells. Quantitative RT-PCR with an FGF23-specific probe,

normalized to 18S, performed on IDG-SW3 RNA isolated after 0–35 days osteogenic culture shows low FGF23 expression over time, although levels were still higher than observed in MLO-A5 cells. (E) 1,25-dihydroxyvitamin D₃ upregulation of FGF23 expression in IDG-SW3. Treatment with 10 nM 1,25-dihydroxyvitamin D₃ upregulates FGF23 expression after 48 h. *n=3, $p<0.05$

Fig 5. Mineralization, infiltration, and expression of osteocytic markers of 3D collagen matrices *in vitro*

IDG-SW3 cells penetrating 3D matrices. H&E staining of decalcified, transverse, paraffin-embedded cells cultured on 3D collagen gel (A) or sponge (B) at 4X, 10X, and 20X magnification. Cells within the gel are indicated with arrows. (C) SEM of IDG-SW3 and underlying mineralization on collagen sponge. Secondary SEM and backscatter images are shown. Abundant calcospherulites intercalated with collagen fibers are observed in 3D culture. Magnification is 10,000X. (D) EDS analysis of highly mineralized regions detected in backscatter imaging indicates the presence of mineral containing calcium and phosphorus associated with IDG-SW3 cells in osteogenic culture. (E) Images of cells on 2D compared to 3D collagen sponge and 3D collagen gel. Cells expressed similar Dmp1-GFP levels. (F) Images of cell morphology and dendrites in 3D culture. Osteocyte-selective dendrites are observed in GFP-positive cells in 4X and 10X fluorescent and phase contrast images. (G) Sclerostin expression in 3D culture. Transverse decalcified, paraffin-embedded sections at 10X and 40X were incubated with sclerostin-specific antibodies and stained with DAB to demonstrate sclerostin protein in embedded cells and within the matrix itself after 30 days of osteogenic culture. The majority of the embedded cells showed some staining for sclerostin.

Insignificant staining was detected in deeper regions of the matrix or in samples incubated with a non-immune IgG as a negative control.

Fig 6. IDG-SW3 cells accelerate bone healing *in vivo*

(A) MicroCT reconstruction of bone healing within calvarial defects. Shown are 2D coronal slices and 3D superior and inferior views of calvaria captured from reconstructed images at Week 7. Increased bone formation is seen in defects containing IDG-SW3 cells. (B) MicroCT quantitative analysis of bone healing within defects. Significant bone healing was observed at Week 7 in IDG-SW3-implanted defects. Total volume measured within the defect appears as a single line due to superimposition of all three lines. (C) Fluorescent images of frozen sections of calvarial defects. Dmp1-GFP-positive cells are still detected at the site 7 weeks after implantation, regionally localized with healing bone.

Fig 7. Schematic diagram summarizing osteoblastic and osteocytic markers in IDG-SW3 cells over time

IDG-SW3 cells transition from late osteoblasts to late osteocytes *in vitro* on both 2D and 3D substrates.