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Bone 41 (2007) 87–96

BONE

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Effects of continuous activation of vitamin D and Wnt response pathways on osteoblastic proliferation and differentiation

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Received 8 August 2006; revised 28 February 2007; accepted 6 April 2007
Available online 19 April 2007

Abstract

The Wnt pathway regulates cell proliferation and differentiation in development and disease, with a number of recent reports linking Wnt to control of osteoblast differentiation and bone mass. There is also accumulating evidence for interaction between the Wnt and nuclear receptor (NR)-mediated control pathways in non-osseous tissues. Calcitriol ($1,25D_3$), which is the active hormonal ligand for the vitamin D receptor (VDR), a member of the NR superfamily, induces osteoblastic cell cycle arrest and expression of genes involved in matrix mineralization *in vitro*, with over-expression of VDR in mature osteoblasts increasing bone mass in mice. To determine whether the vitamin D and Wnt control pathways interact in osteoblastic regulation, we investigated the treatment effects of $1,25D_3$ and/or lithium chloride (LiCl), which mimics canonical Wnt pathway activation, on osteoblast proliferation and differentiation. Treatments were initiated at various stages in differentiating cultures of the MC3T3-E1 osteoprogenitor cell line. Treatment of subconfluent cultures (day 1) with either agent transiently increased cell proliferation but decreased viable cell number, with additive inhibition after combined treatment. Interestingly, although early response patterns of alkaline phosphatase activity to $1,25D_3$ and LiCl were opposite, mineralized nodule formation was virtually abolished by either treatment initiated at day 1 and remained very low after initiating treatments at matrix-formation stage (day 6). By contrast, mineralized nodule formation was substantial but reduced if $1,25D_3$ and/or LiCl treatment was initiated at mineralization onset (day 13). Osteocalcin production was reduced by all treatments at all time points. Thus, vitamin D and/or canonical Wnt pathway activation markedly reduced mineralization, with additive inhibitory effects on viable cell number. The strength of the response was dependent on the stage of differentiation at treatment initiation. Importantly, the inhibitory effect of LiCl in this committed osteoblastic cell line contrasts with the stimulatory effects of genetic Wnt pathway activation in human and mouse bone tissue. This is consistent with the anabolic Wnt response occurring at a stage prior to the mature osteoprogenitor in the intact skeleton and suggests that prolonged or repeated activation of the canonical Wnt response in committed cells may have an inhibitory effect on osteoblast differentiation and function.

Keywords: $1,25D_3$; LiCl; Proliferation and differentiation; MC3T3-E1; Wnt

Introduction

Osteoporosis is an increasing health care problem in all developed countries. To date, most effective osteoporosis therapies reduce bone loss but do not restore lost bone mass and strength. Therefore, there is a pressing need for anabolic treatments to stimulate bone formation, such as intermittent parathyroid hormone administration [32]. Recently, the bone anabolic response to canonical Wnt pathway activation has become a subject of investigation. The Wnt pathway regulates cell migration, proliferation and differentiation in development of many tissues (reviewed in [20,37]). In the absence of Wnt signal, the β-catenin protein is constitutively degraded by a multicomponent destruction complex containing glycogen synthase kinase-3β (GSK-3β), which phosphorylates and targets β-catenin for proteasomal degradation [1,4,33]. Binding

Abbreviations: $1,25D_3$, 1,25-dihydroxyvitamin D3; ALP, alkaline phosphatase; OCN, osteocalcin; LiCl, lithium chloride; VDR, vitamin D receptor; TGFβ, transforming growth factor β; BMP2, bone morphogenic protein 2; GSK-3β, glycogen synthase kinase 3β; CM, conditioned medium.

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doi:10.1016/j.bone.2007.04.174

of secreted Wnt protein to the Frizzled transmembrane receptor leads to GSK-3β inhibition and thus nuclear accumulation of stabilized β-catenin, which complexes with a TCF/LEF transcription factor to increase expression of Wnt target genes. This activation of the canonical Wnt nuclear pathway can be mimicked by exposure to the potent GSK-3β inhibitor lithium chloride (LiCl) [18,48].

There is accumulating evidence that activation of the canonical Wnt/β-catenin pathway is associated with increased bone formation. A family carrying a loss of function mutation of the gene encoding Wnt co-receptor low-density lipoprotein receptor-related protein 5 (LRP5) exhibited very low bone mass [17], whereas LRP5 gain of function mutation caused a marked increase in bone mass in another [8,26]. Deletion of the secreted Frizzled related protein sFRP1, a competitive inhibitor of Wnt signalling, increased osteoblast activity in mice [6], whereas Wnt10b over-expression enhanced osteoblastogenesis in mice but Wnt10b gene deletion decreased trabecular bone mass and serum osteocalcin [5]. In addition, Wnt1, Wnt2, and Wnt3a induced alkaline phosphatase (ALP) activity in osteogenic cultures of the mouse cell lines C2C12, C3H10T1/2, and ST2 [17,39]. Taken together, these and other published data strongly support a stimulatory effect of Wnt pathway activation on bone formation. There is also, however, emerging evidence that Wnt pathway activation can have negative effects on bone formation and osteoblast function [7].

Extracellular signals from hormones and growth factors can modulate the canonical Wnt response in non-osseous tissues [38], including endocrine hormones such as estradiol, androgen, and vitamin D response pathways (reviewed in [29,53]). In skeletal development, control of osteoblast differentiation and bone formation involves interaction between Wnt and several growth factor-associated osteogenic pathways, including TGFβ, BMP2 and Notch [31,39,42,58]. It has been observed that the anabolic effect of PTH does not require Wnt/LRP5 signalling [41], although PTH may exert anti-apoptotic actions in osteoblasts by stimulating the Wnt canonical pathway [51]. In general, however, whether osteogenic hormones influence the Wnt anabolic response or, conversely, whether canonical Wnt activation affects osteoblastic hormonal responses has not been fully investigated.

The active hormonal form of vitamin D, calcitriol (1,25-dihydroxyvitamin D3, or $1,25D_3$), controls calcium homeostasis and modulates osteoblastic proliferation and differentiation (recently reviewed in [19]). Calcitriol regulates gene expression by binding to the vitamin D receptor (VDR), a ligand-modulated transcription factor. The skeletal consequences of altered vitamin D pathway activity depend on the stage of osteoblastic differentiation, as both transgenic mice with elevated VDR in mature osteoblastic cells and mice with conditional ablation of VDR from all osteoblast lineage cells exhibited increased cortical bone formation [16,56]. Together, these models suggest that vitamin D action may be inhibitory in early cells of the osteoblastic lineage but stimulatory in more mature cells. In the present study we investigated whether the vitamin D and Wnt pathways interact in the regulation of osteoblastic cell proliferation and differentiation, by initiating continuous treatments with $1,25D_3$ and/or LiCl at progressive differentiation stages in osteogenic cultures of the committed pre-osteoblastic cell line MC3T3-E1.

Materials and methods

Cell cultures

MC3T3-E1 pre-osteoblastic cell line was maintained in a humidified 5% CO_2 atmosphere at 37 °C in α-minimum essential medium (αMEM, Gibco, Invitrogen Life Technologies, Mount Waverley, Vic, Australia) supplemented with 10% Fetal bovine serum (FBS, Thermotrace, Melbourne, Australia) and 100 U/ml penicillin-100 μg/ml streptomycin (Gibco). Cell stocks were maintained in a sub-confluent state. For differentiation experiments, cells were plated at 0.7 to $1\times10^4/cm^2$ (see specific seeding conditions for individual experiments below) in alpha-MEM with 10% FBS (plating day was regarded as day 0) and supplemented from day 1 with 50 mg/L ascorbic acid and 10 mM β-glycerol-phosphate. Cells were then exposed to $1,25D_3$ in ethanol (Calbiochem, EMD Biosciences, San Diego, CA), lithium chloride in H_2O (LiCl, ICN Biomedical Ltd, Eschwege, Germany) or in combination at different phases of differentiation and for the time periods indicated. Cells were harvested at different time points up to day 27 as indicated.

L-Wnt3a and control L cell lines were purchased from ATCC. L cells were maintained in DMEM supplemented with 10% FBS. Wnt3a-producing cells (L-cells stably transfected with a pcDNA–Wnt3a construct) were grown in the same medium plus 0.4 mg/ml G418. Wnt3a-conditioned medium (Wnt3a-CM) and L-conditioned medium (L-CM) were prepared as described by the ATCC product sheet, and the activity of 5%, 10%, 20% and 30% final concentration of Wnt3a-CM was evaluated on normal L cells by assaying for increased β-catenin activity (TOPFLASH) as described previously [56]. A final concentration of 10% Wnt3a-CM was used in all our subsequent studies as it achieved a nearly maximum induction without any obvious toxicity (referred to as 10% Wnt3a-CM).

Cell proliferation

Cells were seeded on day 0 in sets of 96-well and 24-well plates (Corning Incorporated, Corning, NY) at 1×10^4 and 0.7×10^4 cells/cm2, respectively, and subjected from day 1 to continuous treatment with $1,25D_3$ and/or LiCl at various concentrations from 6 h up to 12 days. Cultures were re-fed with fresh treatment medium every other day. For proliferation studies, 96-well cultures were incubated with 100 μM bromodeoxyuridine (BrdU, final concentration: 10 μM) for 2 h prior to measurement with Cell Proliferation ELISA BrdU kit (Roche Diagnostics, Mannheim, Germany). Cells in 24-well plates were collected on the same days for cell counting using 0.4% trypan blue (Sigma) to distinguish viable and dead cells. Preliminary dose response studies indicated that LiCl at high dose (50 mM) was toxic to the cells, but lower doses were not (20, 10 and 5 mM; data not shown). Therefore intermediate concentration 10 mM LiCl was used in cell proliferation and BrdU experiments.

Alkaline phosphatase

Cells were plated into 12 well plates (0.7×10^4 cells/cm^2) and treatments were initiated at day 1, day 6 or day 13 and cultured to day 21, as above. Cell layers were washed with ice-cold 50 mM Tris-buffered saline (pH 7.4), scraped into 0.5 ml ice-cold 50 mM Tris-buffered saline containing 0.2% NP-40, and sonicated on ice (20 sec). ALP activity was measured using 2-Amino-20methy-propanol buffer (IFCC) method by Alkaline Phosphatase (ALP) Diagnostic Kit (Thermotrace, Australia). Calibration of ALP activity used standard dilutions of calf intestine ALP (Roche Diagnostics GmbH, Mannheim, Germany). DNA content from the same cell lysates was measured using the fluorescent Hoechst 33258 dye. ALP activity was normalized to DNA content and expressed as μmol *p*-NPP/min/μg DNA.

Mineralization

Von Kossa

Cells were plated ($0.7\times10^4/cm^2$) into 6-well plates (Falcon, BD Biosciences, Franklin Lakes, NJ), and subjected to continuous treatments as above. At

collection, cultures were fixed in 10% paraformaldehyde and stained with 2% silver nitrate followed by 2.5% sodium thiosulphate (both from Sigma). Stained images were captured using Olympus digital camera (Olympus, Japan) and mineralized nodule size, total area and number were quantified using the BIOQUANT Classic 95 Image Analysis System (R&M Biometrics Inc., Nashville, Tennessee, USA).

Alizarin Red S

Fixed cultures were stained with 2% Alizarin Red S (pH 4.2, Sigma) and images collected using an EPSON Perfection 4990 Photo scanner (EPSON Australia). Alizarin Red S was also quantified after elution from fixed cells with 10% (w/v) cetylpyridinium chloride (CPC, ICN Biomedicals Inc, Eschwege, Germany) and absorbance measurement at 562 nm using a DU 650 spectrophotometer (Beckman).

Osteocalcin secretion

Osteocalcin protein in culture medium was assayed using a Mouse Osteocalcin EIA Kit (Biomedical Technologies Inc, Stoughton, MA) according to manufacturer protocol.

RNA extraction and RT-PCR

Total cellular RNA was extracted from individual 6-well cultures with TRIZOL (Gibco BRL, Mount Waverley, Vic, Australia) and subjected to DNAse I treatment using DNAse Treatment and Removal Reagents (Ambion, Austin, TX). The first-strand complementary DNA (cDNA) was synthesized by random priming from 1 μg of total RNA using the Superscript II First-strand Synthesis System for RT-PCR (Invitrogen). Real-time quantitative PCR was performed using Platinum SYBR Green qPCR SuperMix-UDG kit (Invitrogen) with Rotor-gene 3000 amplification system (Corbett Research, Mortlake, NSW, Australia). Aliquots of synthesized cDNA were added to a PCR reaction mixture containing 1.5 U Platinum *Taq* DNA polymerase in 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 3 mM $MgCl_2$, 200 μM dATP, 200 μM dGTP, 200 μM dCTP, 400 μM dUTP, 1 U uracil-*N*-glycosylase (UDG), and 0.5 μM of each forward and reverse primer (Table 1) in 10 μl volume. The PCRs were initially incubated at 50 °C for 2 min followed by denaturation at 95 °C for 10 min. Annealing temperature and number of cycles for each gene are shown in Table 1. The PCR product was evaluated by melting curve analysis following the manufacturer's instructions and by checking the PCR products on 2% agarose gels. Cyclophilin A, a housekeeping gene, was used to normalize gene expression.

Protein analysis by Western blotting

Cells were harvested from 6 well cultures at subconfluent or early mineralization stage in TK lysis buffer (50 mM HEPES, 150 mM NaCl, 1% Triton-X-100, 1 mM Na_3VO_4, 30 mM NaF, 10 mM $Na_4P_2O_7$, 10 mM EDTA, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml antipain, 250 μM benzamidine and 400 μM AEBSF). Lysates were centrifuged at 5000 rpm for 5 min at 4 °C. Total protein content was quantified using the BCA protein assay (Pierce, Rockford, IL). Lysates were heated in 1x loading buffer containing 1% DTT at 100 °C for 10 min. Equal amounts of protein (50 μg) were separated and analyzed by 10% SDS-PAGE, transferred to PVDF membrane (Millipore, Billerica, MA), and blocked at room temperature in 5% skim milk/0.05% triton X-100/PBS for 1 h. All antibody incubations were performed in 2% skim milk/0.05% triton X-100/PBS. Membrane was incubated in primary antibody at 4 °C overnight. Cyclin D1 antibody (sc-450; Santa Cruz Biotechnology, Santa Cruz, CA) was used at 1:125; anti-β-tubulin antibody (T4026; Sigma-Aldrich) was used at 1:5000. Membrane was incubated at room temperature in fluorophore labelled secondary antibodies (Molecular Probes, Invitrogen, Carlsbad, CA) at 1:10,000 for 1 h. Bands were detected and quantified on the Odyssey Infrared Imager (Li-Cor, Lincoln, Nebraska).

Transient transfection and evaluation of canonical Wnt response

MC3T3-E1 cells were plated into 24-well plates at $0.75\times10^4/cm^2$. 24 h later, the TCF reporter plasmid TOPFLASH (a generous gift from Dr. Marc van de Wetering) and pRSV-βgal plasmid (250 ng/well and 10 ng/well, respectively) were transiently transfected into these cells using Lipofectamine™ (Invitrogen). 24 h after transfection, cells were treated with $1,25D_3$ and/or LiCl at various concentrations for 24 h prior to harvest. The cells were then washed with 1× PBS, and lysed in 1× lysis buffer (Promega, Madison, WI). 10 μl of cell lysates were assayed for luciferase (Promega) and β-galactosidase (Galacto-Light Assay Systems, Applied Biosystems, Foster City, CA) activities as described [14]. Luciferase values were standardized to β-galactosidase values to adjust for differences in transfection efficiency between samples.

Statistics

Assessment of the effect of treatment on nodule formation was analyzed relative to control cultures with one-way ANOVA, with $p<0.05$ considered significant. All data are expressed as mean±S.E.M.

Results

Doses of LiCl and $1,25D_3$ in continuous treatment

Treatment with LiCl doses led to comparable reductions of mineralized nodule formation in differentiating MC3T3-E1 cultures in a pilot experiment (Fig. 1A). Moreover, a test of two doses of $1,25D_3$ (1 and 10 nM) on ALP activity revealed that 10 nM $1,25D_3$ transiently stimulated and then strongly inhibited ALP production (Fig. 1B), whereas parallel stimulatory and inhibitory effects of 1 nM $1,25D_3$ were less marked. Furthermore, 27-day continuous treatment with 10 nM $1,25D_3$ with or without LiCl resulted in complete abolition of mineral deposition (Fig. 1C). Based on these pilot investigations, intermediate concentrations of LiCl (10 mM) and $1,25D_3$ (1 nM) were used for continuous treatment studies of mature osteoblastic function and gene expression responses.

Table 1
Mouse primers and conditions used in quantitative RT-PCR

Gene	GenBank accession no.	Sequence (5′–3′)	Base pairs	Annealing Tm	Cycle	Product size (bp)
c-Myc	NM 010849.3	Forward GTCTTCCCCTACCCGCTC	18	57	37	404
		Reverse CTGTCCAACTTGGCCCTC	18			
CyclinD1	NM 007631.1	Forward ACCCTGACACCAATCTCCTCAA	23	57	37	175
		Reverse ACCAGTGCCATTATGGCGTGTG	22			
Collagen type I	XR 002595.1	Forward TCTCCACTCTTCTAGTTCCT	20	52	37	250
		Reverse TTGGGTCATTTCCACATGC	19			
Cyclophilin A	NM 008907.1	Forward GCCGATGACGAGCCCTTGGGCC	22	60	37	189
		Reverse TCTTCGCACTTCTGCTCCTCAC	22			

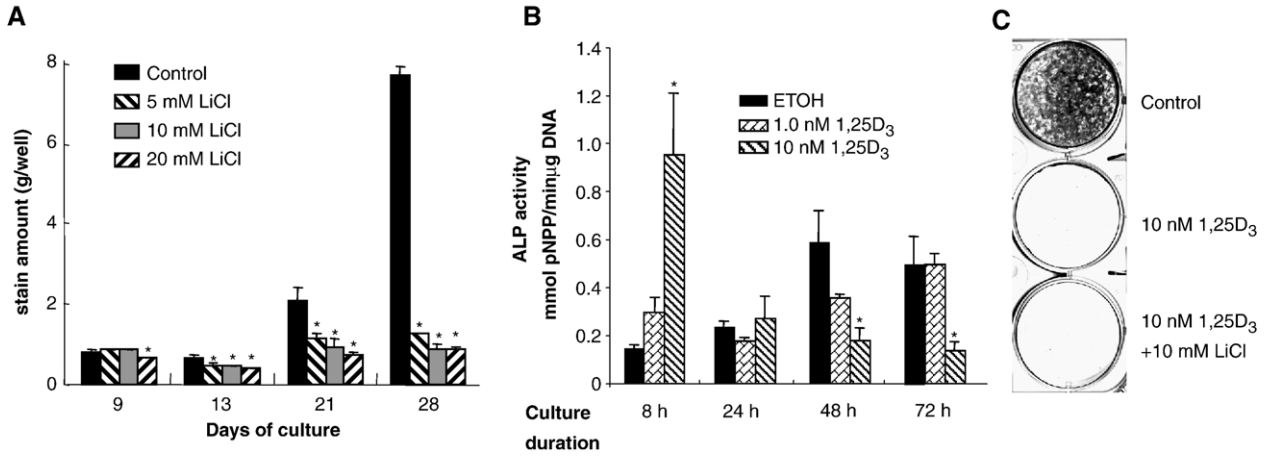

Fig. 1. Effects of increasing doses of lithium chloride or $1,25D_3$ on osteoblast differentiation. A: Effect of increasing doses of lithium chloride on formation of mineralized nodules. MC3T3-E1 cells were treated from day 1 with LiCl (5, 10 or 20 mM) continuously until the indicted time points, then stained with Alizarin Red and quantified. B: Effect of $1,25D_3$ (1 or 10 nM) on ALP activity. MC3T3-E1 cells were exposed to various concentrations of $1,25D_3$ continuously from day 1 until the indicated time points, when cell lysates were collected to conduct ALP activity. The bars represent mean±S.E.M., $*p<0.05$ versus control. C: Effect of continuous 10 nM $1,25D_3$ on mineralization, evidenced by Von Kossa stain on day 27.

Mineralization and timing of treatment

Under standard differentiation conditions, the MC3T3-E1 cells went through progressive stages of proliferation (days 1–6), multilayered bone matrix formation and maturation (days 6–13) and nodule mineralization (days 13–27) as previously reported [12]. Addition of LiCl, $1,25D_3$ or the combination during days 1–27 (initiated at day 1, treated every second day and harvested on day 27) virtually abolished mineral deposition as assessed by Von Kossa and Alizarin Red S staining, with only a very small number of tiny nodules detectable in the $1,25D_3$ and combination cultures (Fig. 2). Addition of LiCl to multilayered post-confluent cultures

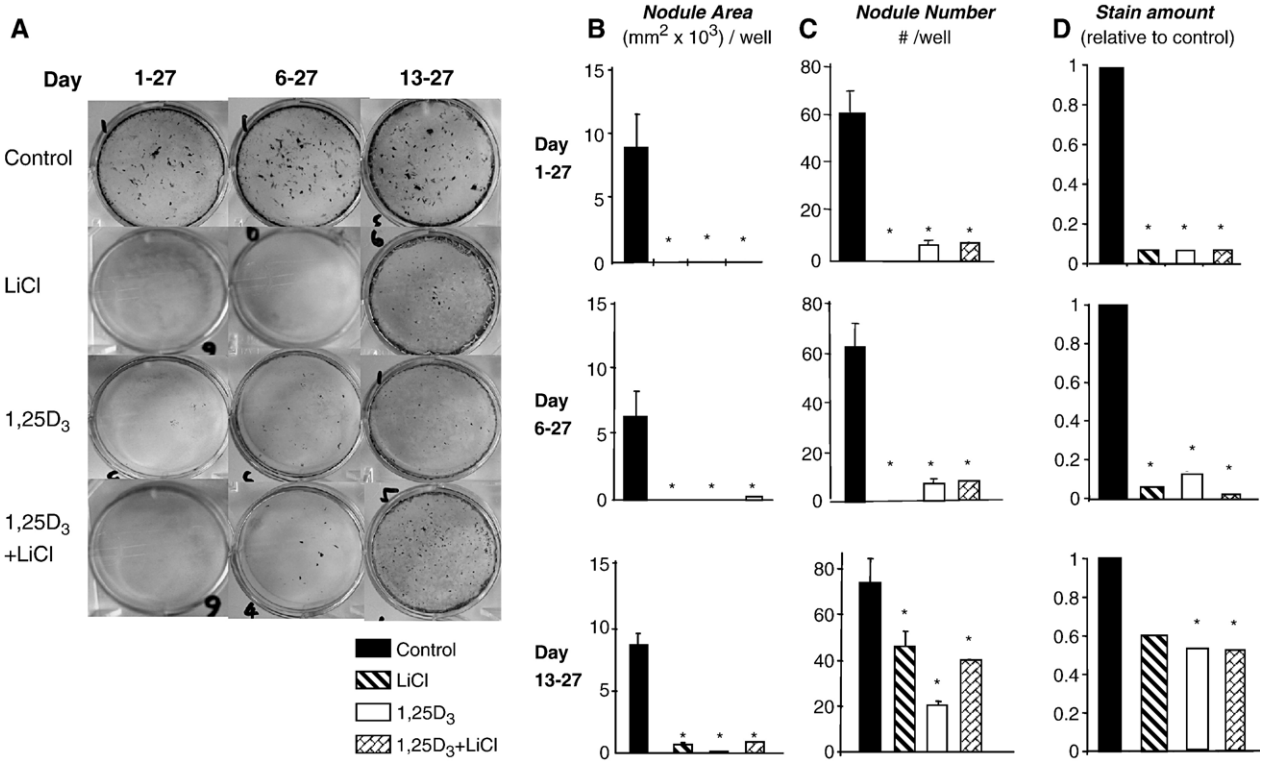

Fig. 2. Response of MC3T3-E1 cells to treatments with $1,25D_3$ (1 nM) and/or LiCl (10 mM) are dependent on the differentiation stage at treatment initiation (day 1, 6 or 13). Cultures were Von Kossa stained for mineralization at day 27. A: Nodule formation was inhibited by all treatments, although to varying degrees. Photographs of representative wells. B, C: Quantification of nodule size and number in days 1–27, days 6–27 and days 13–27 cultures. D: Parallel cultures were quantified on day 27 with alizarin red S staining, mean values normalized to control. $*p<0.05$ versus control. Results shown are representative of 3 independent experiments; mean±S.E.M., $n=3$ wells/treatment.

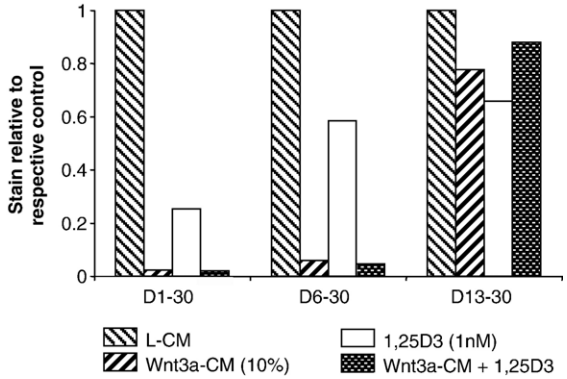

Fig. 3. Alizarin Red stain at day 30 of cultures treated with Wnt3a-conditioned medium. MC3T3-E1 cells were treated with 10% Wnt3a-CM and/or $1,25D_3$ (1 nM) continuously from day 1, 6, or 13, respectively. Alizarin Red stain was carried out at day 30, and the stain was eluted. The value was normalized to L-CM control (regarded as 1), and represents triplicate wells/treatment.

(from day 6) again blocked nodule formation, whereas $1,25D_3$ and the combined treatment significantly decreased nodule number and size. Furthermore, all treatments initiated as late as the onset of mineralization (day 13) also reduced mineral deposition. Similar inhibitory effects were observed in cultures exposed from day 1, 6 or 13 to Wnt3a-conditioned medium [55], with or without 1 nM vitamin D treatment (Fig. 3). Treatment with a lower dose of $1,25D_3$ had minimal effect alone or in combination with Wnt3a-CM treatment (data not shown). There was no obvious difference between days 1–27 and days 6–27 treatments with either LiCl or Wnt3a.

Cell proliferation

Viable cell number (Fig. 4) was reduced by treatment with $1,25D_3$ at 1 or 10 nM, or with LiCl at 10 or 20 mM (Fig. 4). Combination of $1,25D_3$ at either dose with 10 mM LiCl further

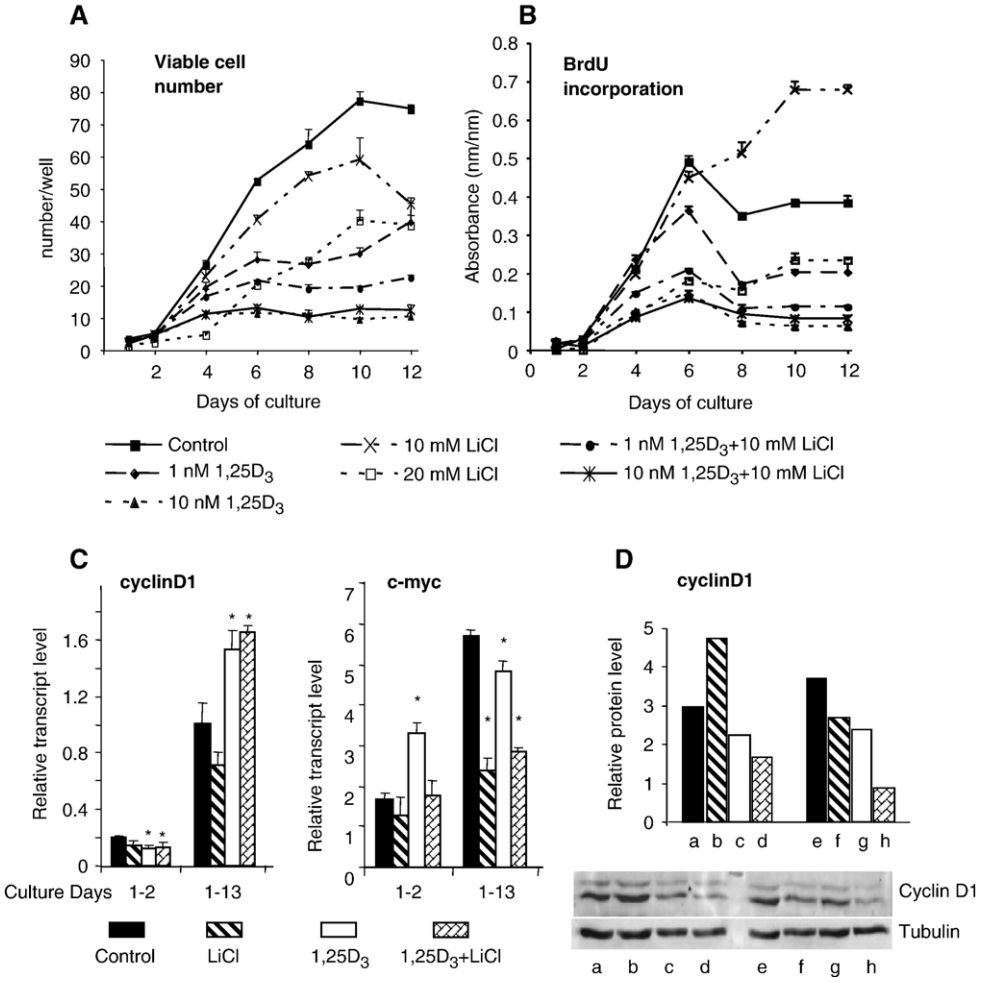

Fig. 4. Reduced osteoblast number with continuous $1,25D_3$ (1 or 10 nM) and/or LiCl (10 or 20 mM) treatment beginning on day 1. A: Viable cell number was decreased by $1,25D_3$ and LiCl, with further reduction observed in combined treatment. B: DNA synthesis, evaluated by BrdU incorporation, was inhibited by $1,25D_3$, whereas LiCl (10 mM) treatment increased cell proliferation, especially in multilayered (days 8–12) cultures. Treatment with 20 mM LiCl reduced BrdU uptake. Combined treatment further reduced BrdU uptake below that with $1,25D_3$ alone. C: Expression of the cell cycle regulators c-*myc* and cyclinD1 after acute 24 h treatment or continuous 13-day treatment with LiCl (10 mM) and/or $1,25D_3$ (1 nM). Transcription levels were normalized to cyclophilin A. $^*p<0.05$ versus control. Results are mean ± S.E.M., and representative of 3 separate experiments. D: Protein level of Cyclin D1 after acute or prolonged treatment, normalized to tubulin. Lanes a–d, acute days 1–2 treatment; e–h, continuous days 1–11 treatment. Control (a,e); 10 mM LiCl (b,f); 1 nM $1,25D_3$ (c,g); LiCl plus $1,25D_3$ (d,h). Results are representative of two separate experiments.

reduced live cell number. From BrdU incorporation it is evident that there was ongoing DNA synthesis in control cultures through day 12, the last time point assayed, with distinct responses to the different treatments. Continuous $1,25D_3$ treatment at either concentration from day 1 inhibited DNA synthesis below control levels in late stage cultures (day 6 and beyond), whereas treatment with 10 mM LiCl stimulated BrdU uptake in post-confluent cultures, becoming apparent at day 8 and persisting thereafter. Treatment with 20 mM LiCl reduced viable cell number and BrdU uptake, probably the result of Wnt-independent effects and consistent with preliminary evidence of toxicity at this concentration. Other studies were therefore conducted using only the lower LiCl concentration. The level of DNA synthesis in cultures treated with LiCl and $1,25D_3$ together did not change beyond day 4. Surprisingly, transcript levels of two direct Wnt target genes, the cell cycle regulators c-*myc* and cyclinD1, were not significantly altered 24 h after LiCl treatment on day 1 and were depressed after continuous treatment for 12 days. By contrast, initiating 24 h treatment with $1,25D_3$ at day 1 transiently increased c-*myc* but decreased cyclinD1 expression, whereas continuous treatment for 13 days led to decreased c-*myc* but elevated cyclinD1 levels. Effects of combined treatment on c-*myc* expression paralleled the LiCl response, whereas the cyclinD1 response to double treatment was more similar to the $1,25D_3$ pattern. In contrast to the reduced transcript level, cyclin D1 protein was induced by LiCl treatment at the early but not the later time point (Fig. 4D). Expression of neither the cell cycle inhibitor p21 nor the

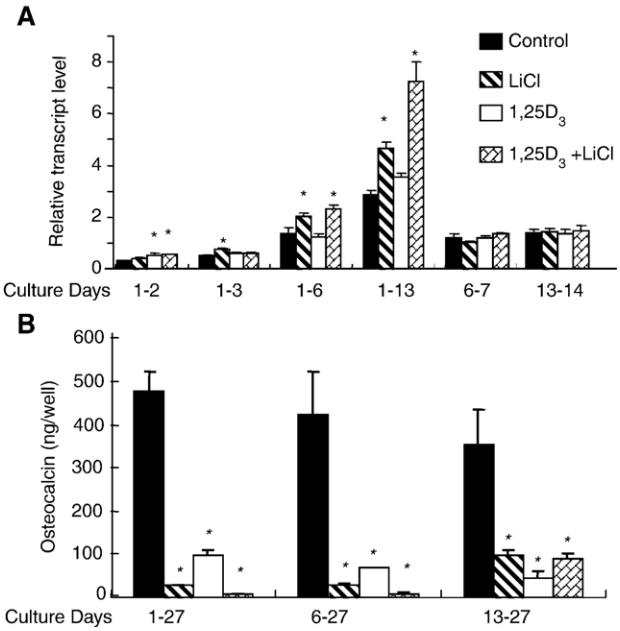

Fig. 6. Collagen expression and osteocalcin levels after initiation of treatment with $1,25D_3$ (1 nM) and/or LiCl (10 mM) at different stages of differentiation. A: LiCl initiated on day 1 increased collagen synthesis over 13 days; $1,25D_3$ modestly increased collagen expression very early but had no effect in later stage cultures, while combined treatment showed an effect similar to LiCl alone. Treatments (24 h) initiated on day 6 or day 13 had no effect on collagen I expression. mRNA values are relative to cyclophilin A. B: Osteocalcin protein levels on day 27 were markedly reduced by treatment with $1,25D_3$ and/or LiCl initiated on day 1, 6 or 13. $^*p<0.05$ versus control.

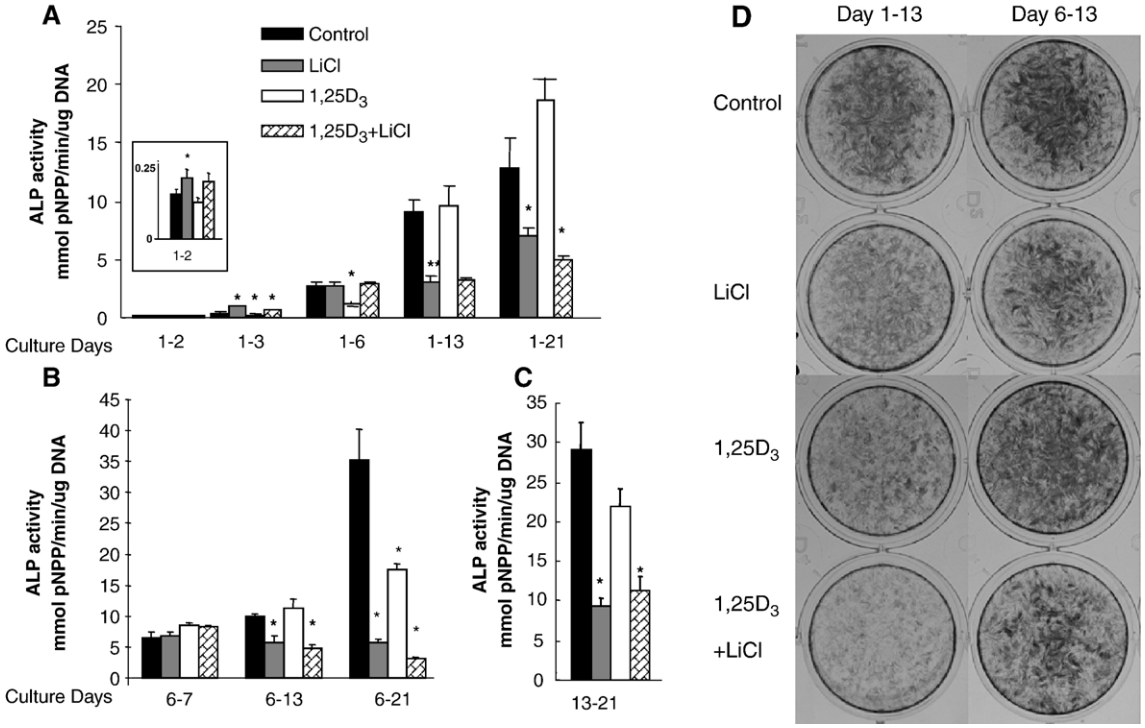

Fig. 5. Effect of continuous treatment with $1,25D_3$ (1 nM) and/or LiCl (10 mM) on the early differentiation marker, alkaline phosphatase (ALP). Cells were treated from day 1 or day 6, and stained on day 13. A, B, C: ALP activity was assayed in lysates of cultures with treatments initiated at day 1, 6 or 12 and collected at time points indicated. D: Consistent with assay results, intensity of ALP staining in the days 1–13 cultures was reduced with LiCl or $1,25D_3$ treatment and further reduced with combined treatment. Decreased staining was also observed after LiCl treatment during days 6–13, with or without concurrent $1,25D_3$ treatment. $^*p<0.05$ versus control. Results are means of triplicate values±S.E.M. and representative of 4 independent experiments.

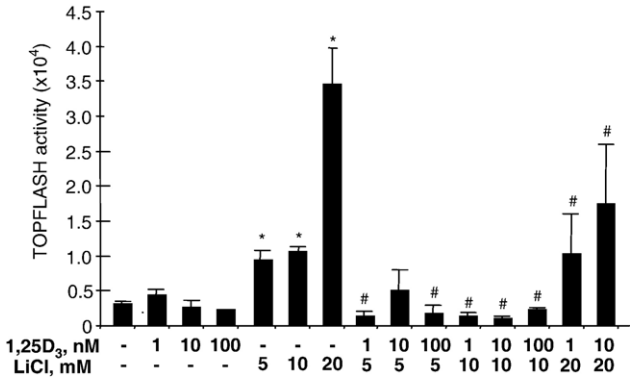

Fig. 7. 1,25D_3 reduces Wnt target promoter activity: TCF/LEF response element MC3T3-E1 cells were transiently transfected with TOPFLASH reporter construct and β-galactosidase plasmid. 24 h after transfection, cells were cultured in differentiation medium, and subjected to 1,25D_3 and/or LiCl for 24 h prior to harvest. Luciferase activity was normalized to β-galactosidase activity in the same sample. The bars represent mean ± S.E.M. *$p<0.05$ versus control, #$p<0.05$ versus respective LiCl treatment alone.

apoptosis-associated Bcl2 gene was affected by any treatment (data not shown).

Alkaline phosphatase

Alkaline phosphatase (ALP) activity was initially induced in cultures treated with LiCl from day 1 through day 3 (Fig. 5) but this increase was lost by day 6 and ALP activity was reduced in cultures continuously treated from day 1 to day 13 and beyond. Initiating LiCl treatment at later stages (day 6 or 13) produced patterns of ALP inhibition very similar to that seen for cultures treated continuously from day 1. The effect of combined treatment on ALP activity was similar to LiCl alone at all time points. By contrast, the ALP response to 1,25D_3 was a decrease with treatment from day 1 to 3 or 6 ($p<0.05$), but loss of this inhibition after 12 days of continuous treatment and varying responses thereafter, depending on stage at treatment initiation. Of particular note is the reduction in alkaline phosphatase activity in day 21 cultures after 1,25D_3 treatment that was initiated at day 6 or 13.

Collagen and osteocalcin

In cultures continuously treated from day 1 with LiCl, type I collagen transcript levels were significantly elevated at most time points, whereas with continuous 1,25D_3 treatment there was a modest increase in collagen I expression only in pre-confluent cultures (Fig. 6). Continuous combined treatment led to higher transcript levels, similar to the LiCl response. Treatment with either agent for 24 h at day 6 or day 13 did not affect collagen I expression. Osteocalcin protein in culture medium was reduced by all treatments at all time points to an extent somewhat dependent on the differentiation stage at initiation of treatment, with the LiCl effect dampened but the 1,25D_3 effect greater in later cultures.

Wnt transcriptional response element activity

Treatment of transiently transfected MC3T3-E1 cells with 1,25D_3 had minimal effect on transcriptional regulation by the Wnt-responsive TCF/LEF response element in the TOPFLASH reporter construct, whereas LiCl increased this activity in a dose-dependent manner (Fig. 7). The combined treatment response was more similar to 1,25D_3 treatment alone, except at the highest LiCl concentration tested.

Discussion

The coordinated processes of osteoblastic proliferation and differentiation involve multiple signalling pathways and transcriptional responses. In our studies of the committed osteoprogenitor cell line MC3T3-E1, repeated activation of the canonical Wnt pathway by treatment with 10 mM LiCl or 10% Wnt3a-CM throughout the culture period stimulated osteoblast proliferation in post-confluent cultures. Chronic treatment with 1,25D_3 alone reduced DNA synthesis, and in cultures treated simultaneously with both compounds, cell proliferation failed to rise to the levels achieved by either single treatment. The end result of all treatments initiated at the beginning of the culture period (day 1) was a block to osteoblastic differentiation, leading to a marked suppression of mineralized nodule formation. The responses were not identical, however, as LiCl or Wnt3a treatment from day 1 abolished nodule formation, whereas a few small mineralized nodules were seen in cultures treated from this early time point with 1,25D_3 alone or with both agents in combination. The strength of this suppression was dependent on the stage of culture at initiation, with later treatments resulting in less inhibition of matrix mineralization and osteocalcin production. Thus, LiCl/Wnt3a and/or 1,25D_3 appear to inhibit progress along the osteoblast differentiation pathway and osteoblast function at both early and late stages.

LiCl treatment had limited impact on cell proliferation prior to confluence, and mRNA levels of cyclinD1 and c-*myc*, which are both regulators of G1 to S phase cell cycle progression and direct Wnt target genes in many cell types [50,44], were not induced by LiCl treatment in the subconfluent MC3T3-E1 cultures. Consistent with this finding, cyclin D1 transcript level was similarly unaltered by Wnt β-catenin pathway activation in MC3T3-E1 cultures in another recent study [40]. In contrast to this transcriptional result, however, cyclin D1 protein was increased by LiCl treatment in the Day 1–2 samples, consistent with the subsequent rise in BrdU incorporation. This divergence between protein and transcript levels suggests post-transcriptional regulation of cyclin D1 in this experimental model. In contrast to its effect in subconfluent cultures, LiCl markedly stimulated BrdU incorporation in postconfluent cultures. In these later stage cultures, however, c-*myc* was significantly repressed and cyclin D1 showed a consistent lack of induction by LiCl treatment at transcript and protein levels. The evidence here therefore suggests that the mechanism underlying this increase in proliferation in postconfluent MC3T3-E1 osteoblastic cultures may not be the typical cyclin D1-mediated canonical Wnt pathway proliferative response.

Importantly, despite the evidence for increased DNA synthesis, viable cell number was decreased in the post-confluent LiCl treated cultures, suggesting an increase in apoptosis. Transcriptional analysis revealed no LiCl or $1,25D_3$ effects on expression of p21 (CIP1/WAF1) or Bcl2 (data not shown), but other apoptotic mechanisms have not been ruled out. Thus, the likely contribution of programmed cell death to the population dynamics in this study will be an important future area of investigation. The finding that there was no appreciable difference in mineralized nodule formation between the days 1–27 and days 6–27 cultures might be due to the treatment effects on cell proliferation and viability. It could be expected that a much longer culture period would allow mineralization to occur but this was not directly tested in the present study.

Alkaline phosphatase activity initiates at early stages of osteoblast differentiation and continues to increase until the mineralization phase, when it is decreased [3,10,49]. Here, LiCl exposure induced ALP activity in early cultures but markedly suppressed it as the cells progressed through the culture period. The stimulation in subconfluent cultures is consistent with an earlier study in C3H10t1/2 cells [2]. The reduction of ALP activity in subconfluent $1,25D_3$-treated cultures coincided with a period of increasing BrdU incorporation and thus cell proliferation, but this effect was transient, disappearing at mineralization onset and beyond. The early period of ALP reduction may be due to phenotype suppression of transcription [34]. That the early induction of ALP activity by LiCl did not lead to increased nodule formation is in agreement with a previous report that ALP expression and activity could be dissociated from the expression of late maturation genes such as osteocalcin and osteopontin [3]. Given the importance of this enzyme for hydroxyapatite deposition, however, it seems likely that the reduced ALP activity in the later stage LiCl-treated cultures could have contributed to the decreased nodule mineralization [54]. The stimulation of type I collagen expression at the later time points by LiCl treatment initiated at day 1 is not necessarily due to a direct transcriptional effect on the gene, as transcript levels were not affected by 24 h LiCl treatment in the later matrix-formation or mineralization onset stage (day 6 and day 13) cultures. An alternative explanation that would fit all osteoblastic marker data including the increase in cell proliferation and the reduced osteocalcin production is a LiCl-induced block of progression to the ALP-producing stage [10], resulting in an accumulation of immature type I collagen-producing osteoblasts. However, further investigations are necessary to test this interpretation.

Treatment with $1,25D_3$ did not alter cell proliferation in subconfluent MC3T3-E1 cultures but was inhibitory at late matrix formation and mineralization stages, consistent with its known antiproliferative effect on most osteoblastic cell lines [30,52]. There was a decrease in cyclin D1 transcripts and protein after $1,25D_3$ treatment (24 h), consistent with a recent report that after 12 h treatment of MC3T3-E1 cultures, G1 arrest was accompanied by decreased expression of cyclin D1 [13]. Cyclin D1 transcripts were increased after prolonged $1,25D_3$ treatment in the present study, paralleling the clear reduction in cell proliferation at post-confluent and mineralizing stages. Expression of c-*myc* was upregulated after 24 h $1,25D_3$ treatment, but subsequently was repressed, paralleling the decrease in cell proliferation at the multilayered stage (days 8, 10 and 12). The latter observation is compatible with a marked decrease in c-*myc* levels after prolonged $1,25D_3$ treatment (8 day) in the MCF7 breast cancer cell line [23].

Combined treatment with both LiCl and $1,25D_3$ reduced BrdU incorporation and live cell numbers in post-confluent cultures to values lower than the reduction achieved by single treatments. Moreover, the clear inconsistency with the elevation of BrdU incorporation observed after LiCl treatment suggests that $1,25D_3$ interferes with Wnt pathway stimulation of DNA synthesis. In the combined treatments, the inhibitory LiCl effect on c-*myc* was sustained, whereas the $1,25D_3$ effects on cyclin D1 persisted, suggesting that the $1,25D_3$ inhibition of cyclin D1 or other cell cycle regulators at the early stage may be the overriding change in these cultures. The inhibition of LiCl transcriptional activation of the Wnt responsive TOPFLASH reporter assay by simultaneous $1,25D_3$ treatment supports this concept. There are also other effects on osteoblastic differentiation or function that must be taken into account, however, such as Wnt inhibition of Runx2 function by direct protein interaction with activated β-catenin or vitamin D reduction of TCF reporter activity through VDR binding to and/or competition for β-catenin [35,43]. Less direct mechanisms may include vitamin D effects on expression of cadherin [35], Axin2, a negative regulator of Wnt/β-catenin signaling [21], or Lrp5, the Wnt co-receptor [15]. Roles for these and other possible mechanisms of interaction between the $1,25D_3$ and Wnt regulatory pathways in modulating osteoblast differentiation remain to be tested.

The inhibition of osteoblastic differentiation and mineralized nodule formation after $1,25D_3$ treatment of osteogenic MC3T3-E1 cultures is consistent with an earlier study in rat primary calvarial cultures [22] but not in primary and cell line models of mouse [9,11,45,46,59], or human [27] osteoblastic differentiation. These differences may be attributable to species variation or possibly to the presence of varying types of immature uncommitted multipotential cells in some primary cultures. Interestingly, the present result also differs from an increase in mineral accumulation in MC3T3-E1 cultures treated with $1,25D_3$ after mineralization onset [28,47], possibly because hormone exposure in those studies were of shorter durations. The absence of crucial paracrine or endocrine factors from the MC3T3-E1 cell line mode may also be a reason for the difference between the present results and the low bone mass phenotype of VDR knockout mice [25,57] or the high bone formation phenotype of the OSVDR transgenic mouse, in which the vitamin D response was elevated solely in mature cells of the osteoblastic lineage [16].

By the same token, the current observations differ from a number of reports of osteoblastic anabolic responses to Wnt pathway activation *in vivo* and *in vitro*, although it must be noted that the effects of GSK-3β inhibition LiCl or non-Wnt effects by this chemical may differ from direct canonical Wnt stimulation. However, in the studies using Wnt3a conditioned medium, there was a high similarity with the inhibitory effects of LiCl treatment on mineralized nodule formation. Most

studies showing positive effects of Wnt on osteogenic differentiation have employed mesenchymal progenitor cell line models rather than mature osteoblastic cell lines, with some results relating to cell fate determination rather than simple progression along the osteoblastic lineage [5,39,53]. Thus, the effects of Wnt pathway activation and downstream mechanisms of action may depend on the stage of osteoblastic maturation. Duration of treatment may also affect the outcome, as the repeated applications of LiCl and/or $1,25D_3$ to these cultures may have distinct consequences compared to the more acute activation of these pathways by shorter treatment periods. Further studies will be required to determine whether transient stimulation of either pathway can have a positive effect. Given the current state of understanding, the potential value of the Wnt and vitamin D response pathways as targets for anabolic therapies requires further investigation.

Acknowledgments

We are grateful to Ms Qing Tu for statistical assistance and Ms Alisa Poh for technical contributions, and to Drs E. Musgrove, P. Lundberg, G. Thomas and anonymous reviewers for critical comments on this manuscript. This work was supported by an Australian National Health and Medical Research Council project grant (276415) to EMG and JAE, an NHMRC International Postgraduate Research Scholarship to YCS, and Australian Postgraduate Award and ANZ Scholarship to LW.

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