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Lithium Chloride Exerts Differential Effects on Dentinogenesis and Osteogenesis in Primary Pulp Cultures

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Wnt/β-catenin signaling is known to play essential roles in odontoblast differentiation and reparative dentin formation. Various Wnt activators including LiCl have been increasingly studied for their effectiveness to induce repair of the dentin-pulp complex. LiCl is a simple salt thought to activate Wnt/β-catenin signaling by inhibiting GSK3β. Previous *in vitro* and *in vivo* studies showed that LiCl increased odontoblast differentiation and enhanced reparative dentin formation. However, the underlying molecular and cellular mechanisms by which LiCl regulates odontoblast and osteoblast differentiation during reparative dentinogenesis are not well-understood. Our *in vitro* studies show that exposure of early dental pulp progenitors to LiCl increased the survival and the pool of αSMA$^+$ progenitors, leading to enhanced odontoblast and osteoblast differentiation. The positive effects of LiCl in the differentiation of osteoblasts and odontoblasts from αSMA$^+$ progenitors are mediated by Wnt/β-catenin signaling. Our results also showed that continuous and late exposure of dental pulp cells to LiCl increased the expression of odontoblast markers through Wnt/β-catenin signaling, and the number of odontoblasts expressing DMP1-Cherry and DSPP-Cerulean transgenes. However, unlike the early treatment, both continuous and late treatments decreased the expression of *Bsp* and the expression of BSP-GFPtpz transgene. These observations suggest that prolonged treatment with LiCl in more mature cells of the dental pulp has an inhibitory effect on osteoblast differentiation. The inhibitory effects of LiCl on osteogenesis and *Bsp* were not mediated through Wnt/β-catenin signaling. These observations suggest that the effects of LiCl, and GSK3β antagonists on reparative dentinogenesis involve multiple pathways and are not specific to Wnt/β-catenin signaling.

Keywords: LiCl, dentinogenesis, osteogenesis, pulp culture, reporter mice

INTRODUCTION

The canonical Wnt signaling cascade (Wnt/β-catenin) begins with the binding of Wnt proteins to a heterodimeric receptor complex, consisting of frizzled (FZD) and low-density lipoprotein receptor-related protein (LRP) (1–3). Regulation of the Wnt/β-catenin signaling pathway depends on the abundance and subcellular localization of β-catenin. In the absence of Wnt proteins, β-catenin in the cytoplasm is degraded by the destruction complex consisting of adenoma polyposis coli (APC), axin, glycogen synthase kinase 3 (GSK-3), and casein kinase I (CKI) (1–3). Binding of Wnt proteins

to these receptors, results in the activation of disheveled (DVL) leading to the inactivation of the degradation complex and accumulation of β-catenin in cytoplasm. After translocation into the nucleus, β-catenin associates with T cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors and promotes the transcription of its target genes (1–3).

By controlling the expression of a broad spectrum of target genes, Wnt/β-catenin signaling regulates and coordinates a wide range of cell functions including stemness, proliferation, differentiation, inflammatory and immune responses, and the repair and regeneration of many tissues (1–4).

Several studies have implicated roles of Wnt/β-catenin signaling in reparative dentinogenesis (5–11). Wnt/β-catenin signaling is activated by injury to the dental pulp and participates in the dentin-pulp complex's stepwise healing process (5). Changes in the levels of Wnt/β-catenin signaling affect the rate and extent of reparative dentin formation (5, 8, 12–14).

Lithium chloride (LiCl) is a simple salt that is widely used in the treatment of various disorders (15–17). LiCl is thought to activate Wnt/β-catenin-mediated signaling through the inhibition of GSK3β (18) and is widely used in studies examining roles of Wnt/β-catenin signaling on multiple processes, including the repair and regeneration of the dentin-pulp complex (7, 19–22).

Studies in the pulp showed that the application of LiCl to injured and exposed pulp tissue induced reparative dentinogenesis (7, 19–22). *In vitro* studies showed that LiCl increased cell migration and mineralization of human dental pulp stem cells, and increased *Dspp* expression in human dental pulp cells (DPCs) (hDPSCs) (19).

Although the effects of LiCl on reparative dentin and odontoblast differentiation has been established, much remains to be understood about the underlying cellular events leading to increased reparative dentinogenesis. Therefore, to gain a better
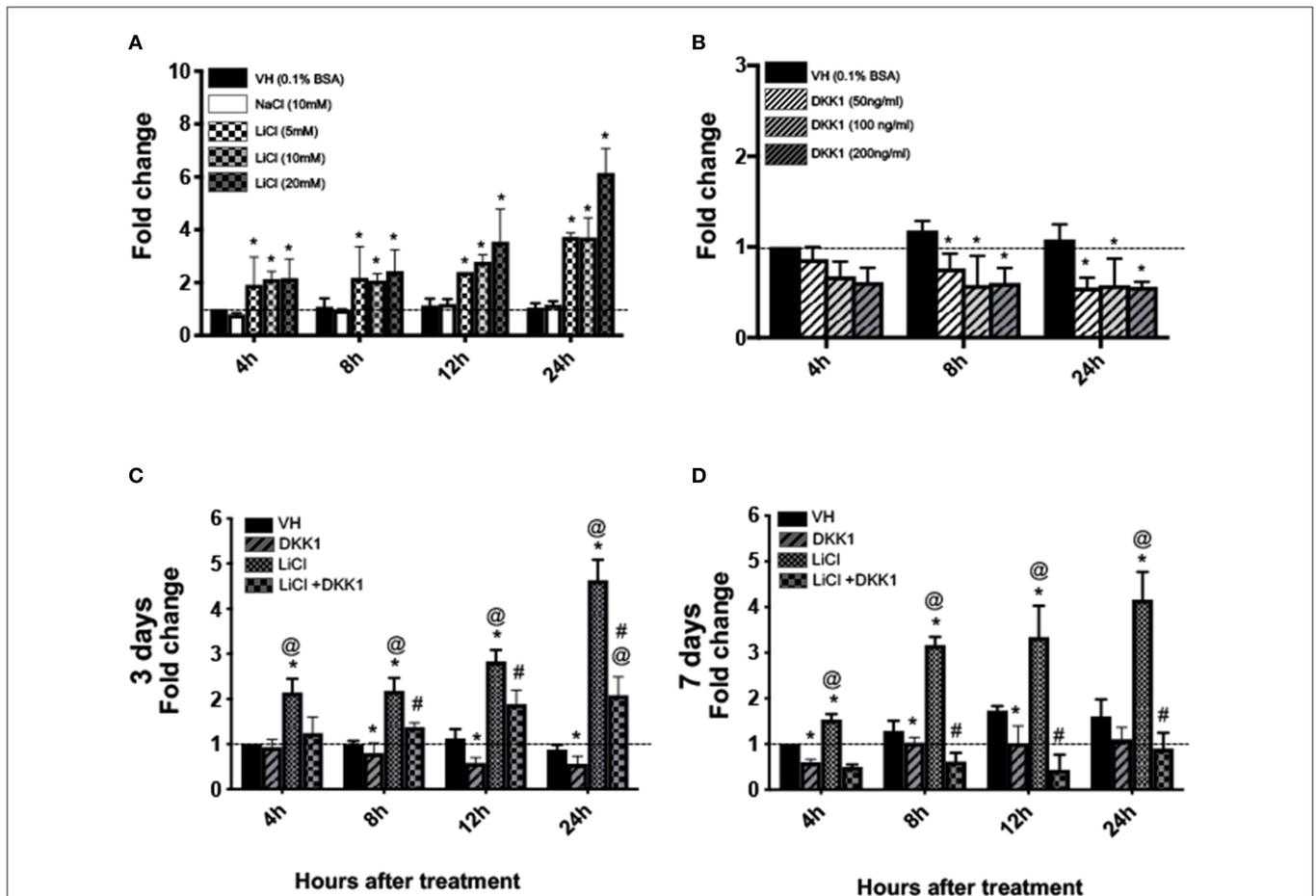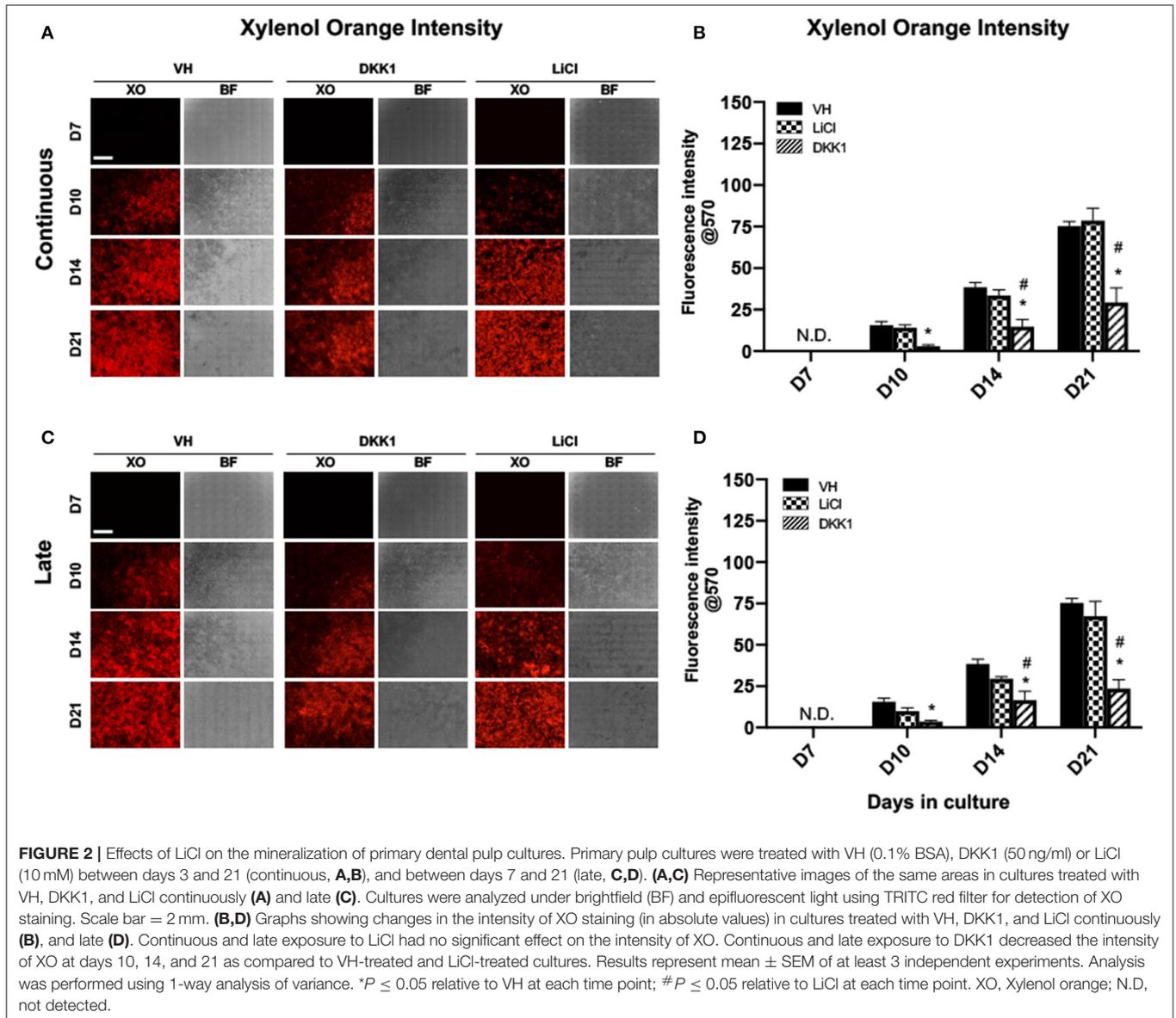

FIGURE 1 | Effects of LiCl on Axin2-expression in primary pulp cultures cultures. (A,B) Graphs showing the dose-dependent changes in the expression of *Axin2* in cultures treated with Vehicle (VH, 0.1% BSA), (A) NaCl (10 mM), LiCl (5, 10, and 20 mM), and (B) DKK1 (50, 100, and 200 ng/ml) between 4 and 24 h after treatment at various times at day 3. (C,D) Graphs showing changes in the expression of *Axin2* between 4 and 24 h in cultures with various treatments at day 3 (C) and day 7 (D). Expression of *Axin2* was normalized to 4 h of the VH-treated cultures, which is arbitrarily set to 1 and is indicated by the dashed line. Results represent mean ± SEM of at least 3 independent experiments. Analysis was performed using 1-way analysis of variance. *$P \leq 0.05$ relative to VH at each time point; @$P \leq 0.05$ relative to DKK1 at each time point; #$P \leq 0.05$ relative to LiCl at each time point.

FIGURE 2 | Effects of LiCl on the mineralization of primary dental pulp cultures. Primary pulp cultures were treated with VH (0.1% BSA), DKK1 (50 ng/ml) or LiCl (10 mM) between days 3 and 21 (continuous, **A,B**), and between days 7 and 21 (late, **C,D**). **(A,C)** Representative images of the same areas in cultures treated with VH, DKK1, and LiCl continuously **(A)** and late **(C)**. Cultures were analyzed under brightfield (BF) and epifluorescent light using TRITC red filter for detection of XO staining. Scale bar = 2 mm. **(B,D)** Graphs showing changes in the intensity of XO staining (in absolute values) in cultures treated with VH, DKK1, and LiCl continuously **(B)**, and late **(D)**. Continuous and late exposure to LiCl had no significant effect on the intensity of XO. Continuous and late exposure to DKK1 decreased the intensity of XO at days 10, 14, and 21 as compared to VH-treated and LiCl-treated cultures. Results represent mean ± SEM of at least 3 independent experiments. Analysis was performed using 1-way analysis of variance. $*P \leq 0.05$ relative to VH at each time point; $^{\#}P \leq 0.05$ relative to LiCl at each time point. XO, Xylenol orange; N.D, not detected.

understanding of the roles of LiCl on odontoblast and osteoblast differentiation, we used well-characterized dental pulp cultures from a series of previously characterized reporter mice (23–25) and examined the response of cells at various stages of differentiation to LiCl.

MATERIALS AND METHODS

Animal Models

All animal experimental protocols were approved by the Institutional Animal Care and use Committee of UConn Health Center. DSPP-Cerulean/DMP1-Cherry, BSP-GFPtpz, pOBCol2.3FP (referred to as 2.3-GFP), αSMA-GFP and non-transgenic mice have been previously described (23–25). All mice were maintained in the CD1 or C57BL/6 backgrounds. In all

experiments, pulp cells from non-transgenic littermates served as negative control.

Cell Cultures, Digital Imaging, and Epifluorescence Analysis

Primary pulp cultures were prepared from the coronal pulp of first and second molars from 5- to 7-d-old mice and grown as described in previous publications (26, 27). The cultures were grown in the presence of vehicle (VH; 0.1% bovine serum albumin), various concentrations of LiCl (Sigma), and Dickkopf1 (DKK1) (R&D Systems), with or without simultaneous LiCl between days 3 and 21 (referred to as continuous exposure), days 7 and 21 (referred to as late exposure), and days 3 and 7 (referred to as early exposure).

Mineralization in live primary cultures was examined by Xylenol orange (XO) staining as described before (24). The
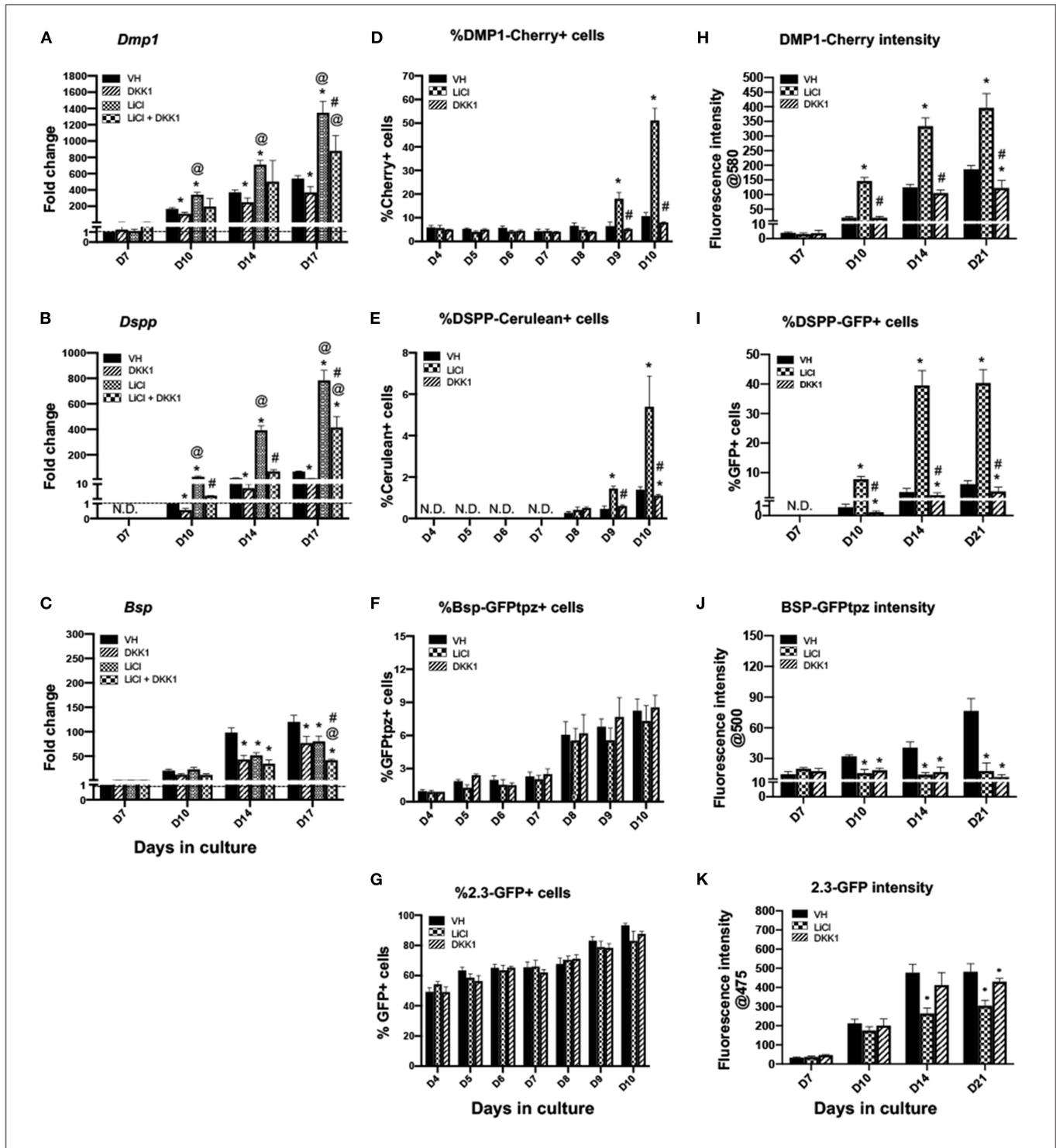
FIGURE 3 | Effects of continuous exposure to LiCl on the expression of differentiation markers and tissue- and stage-specific reporters in primary dental pulp cultures. Primary pulp cultures were treated continuously from days 3 to 21 with VH (0.1% BSA), DKK1 (50 ng/ml), LiCl (10 mM) or simultaneous LiCl (10 mM) and DKK1 (50 ng/ml). **(A–C)** Graphs showing changes in the expression of *Dmp1* **(A)**, *Dspp* **(B)** and *Bsp* **(C)** in cultures treated continuously with VH, LiCl, and DKK1 alone or with simultaneous LiCl from days 3 to 17. Continuous exposure to LiCl showed significant increases in *Dmp1* **(A)** and *Dspp* **(B)** at days 10–17, which were significantly decreased by DKK1 treatment alone. DKK1 treatment decreased the LiCl-induced increases in *Dmp1* and *Dspp*. However, continuous LiCl treatment, as well as DKK1 treatment decreased *Bsp* expression. The LiCl-induced decreases were not reversed by DKK1 **(C)**. Expression of *Dmp1* and *Bsp* was normalized to day 7 and *Dspp* was normalized to day 10 of the VH-treated cultures, which is arbitrarily set to 1 and is indicated by the dashed line. **(D–G)** Graphs showing the changes in the percentage of cells expressing DMP1-Cherry **(D)**, DSPP-Cerulean **(E)**, BSP-GFPtpz **(F)**, and 2.3-GFP **(G)** transgenes by FACS analysis between days 4 and 10

(Continued)

FIGURE 3 | (every 24 h following the start of continuous treatment). LiCl-treated cultures show significantly increased percentages in DMP1-Cherry^{+} cells and DSPP-Cerulean^{+} cells at days 9 and 10. DKK1 treated cultures show decreased percentages of DMP1-Cherry[+] and DSPP-Cerulean^{+} cells that were significant compared to VH treated controls, as well as LiCl-treated cultures (D,E). LiCl treatment decreased the percentage of BSP-GFPtpz^{+} cells and 2.3-GFP^{+} cells while DKK1 treatment showed no effects (F,G). (H–K) Graphs showing the changes in the fluorescence intensity of DMP1-Cherry (H), BSP-GFPtpz (J), and 2.3-GFP (K) transgenes by fluorometric analysis, as well as the percentage of DSPP-Cerulean^{+} cells (I), detected by immunocytochemistry following continuous treatment. Continuous exposure to LiCl showed significant increases in the fluorescence intensity of DMP1-Cherry (H) and the percentage of DSPP-GFP^{+} cells (I) at days 10–21. LiCl treatment significantly decreased the intensity of BSP-GFPtpz (J) and 2.3-GFP (K) at days 14–21. DKK1 treatment significantly decreased the intensity of DMP1-Cherry and BSP-GFPtpz, as well as the percentage of DSPP-GFP^{+} cells, at days 10–21. Results in all graphs represent mean ± SEM of at least 3 independent experiments. Analysis was performed using 1-way analysis of variance. *$P \leq 0.05$ relative to VH at each time point; @$P \leq 0.05$ relative to DKK1 at each time point; #$P \leq 0.05$ relative to LiCl at each time point. N.D, not detected.

mean fluorescence intensity of XO staining, DMP1-Cherry, and BSP-GFPtpz was measured as described before (24). Background fluorescence for XO, DMP1-Cherry and BSP-GFPtpz was measured using dental pulp cultures from non-transgenic littermates, and these values were subtracted from the respective XO and transgene measurements. Live or fixed cultures from reporter mice were imaged as described before (24) using Zeiss Axio Observer Z1 inverted microscope and appropriate filter cubes optimized for the detection of various fluorescent protein variants. The exposure times remained the same throughout the each set of experiments.

Immunocytochemistry

Pulp cultures were processed for immunocytochemistry with a 1:1,000 dilution of anti-GFP Alexa Fluor 488–conjugated antibody (Molecular Probes, Invitrogen) (25). DSPP-Cerulean^{+} cells were calculated as the ratio of cells stained with anti-GFP antibody (DSPP-Cerulean^{+} cells) to the total number of Hoechst^{+} cells (26, 28). Primary dental pulp cultures derived from the transgenic littermates without the addition of anti-GFP antibody served as controls.

RNA Extraction and Analysis

Total RNA was prepared with TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. After DNase treatment, RNA samples were processed for quantitative polymerase chain reaction (qPCR) analysis with specific primers (**Supplementary Figure 1**) purchased from Applied Biosystems (28). Gene expression was examined by qPCR analysis using 2^{-DDcT} method. For TaqMan qPCR reactions, 9 ng of cDNA was combined with 5 µl of TaqMan universal master Mix (Applied Biosystems, Nranchburg, NJ), 2.5 µl H_2O and 0.5 µl TaqMan primers (total 10 µl). All qPCR reactions were run using Biorad PCR System under the following conditions: 50°C for 2 min, 95°C for 10 min, and 40 cycles with denaturation at 95°C for 15 s and extension at 60°C for 1 min. We defined the acceptable range of CT values representing gene expression to be between 10 and 35 cycles, according to manufacturer's recommendations.

Flow Cytometric Analysis

Single-cell suspensions from pulp cultures were processed for fluorescence-activated cell sorting (FACS) analysis at different time points using gating strategies shown in **Supplementary Figure 2**. Cells growing under proliferation culture conditions were prepared by mild 0.05% trypsin/EDTA (Invitrogen, USA) digestion, while cells growing under mineralization culture conditions were prepared by incubation of cells in enzymatic solution containing 0.05% trypsin/EDTA and 2 U/ml Collagenase P for 10–20 min, followed by centrifugation. Cells were then re-suspended in 300–400 µl of staining medium (1x HBSS, 2% FBS, 10 mM HEPES, in distilled H_2O, pH 7.2) containing 1.0 µg/ml FVD eFluor780 (eBiosciences) and strained through a 70 µm strainer to obtain single-cell suspension. Approximately 20,000–100,000 live cells/sample were collected by a BD™ LSR-II FACS cytometer (BD Biosciences, San Jose, CA). Pulp cells from non-transgenic littermates served as a negative control for transgene expression in all experiments.

Proliferation and Apoptosis Assays

The proliferative cells were detected by incubation of cultures with 10 µM EdU (5-ethynyl-2′-deoxyuridine) for 4 h and Click-iT Plus Flow Cytometry Assay kit (Invitrogen) according to the manufacturer's instructions. The apoptotic cells were examined using rabbit cleaved caspase 3 antibody (1:800; Cell Signaling Technology 9661S) for 1 h and goat anti-rabbit Alexa Fluor 594 for 30 min. The number of proliferative and apoptotic cells was calculated by FACS analysis using a BD LSR-II FACS cytometer (BD Biosciences). Pulp cells processed in the absence of EdU/cleaved caspase 3 antibody and cells from non-transgenic littermates served as negative controls in all experiments.

Statistical Analysis

Analysis was performed by GraphPad Prism 8 software (GraphPad Software) using 1-way analysis of variance (ANOVA). Values in all experiments represented mean ± SEM of at least 3 independent experiments, and $P \leq 0.05$ was considered statistically significant.

RESULTS

Effects of LiCl on Wnt Responsive Cells

We first examined the effects of LiCl on the Wnt-responsive dental pulp cells by examining the levels of *Axin2* expression (29) by quantitative polymerase chain reaction (qPCR) analysis.

qPCR analysis showed marked concentration-dependent increases in *Axin2* expression between 4 and 24 h in the LiCl-treated cultures as compared to the VH-treated and
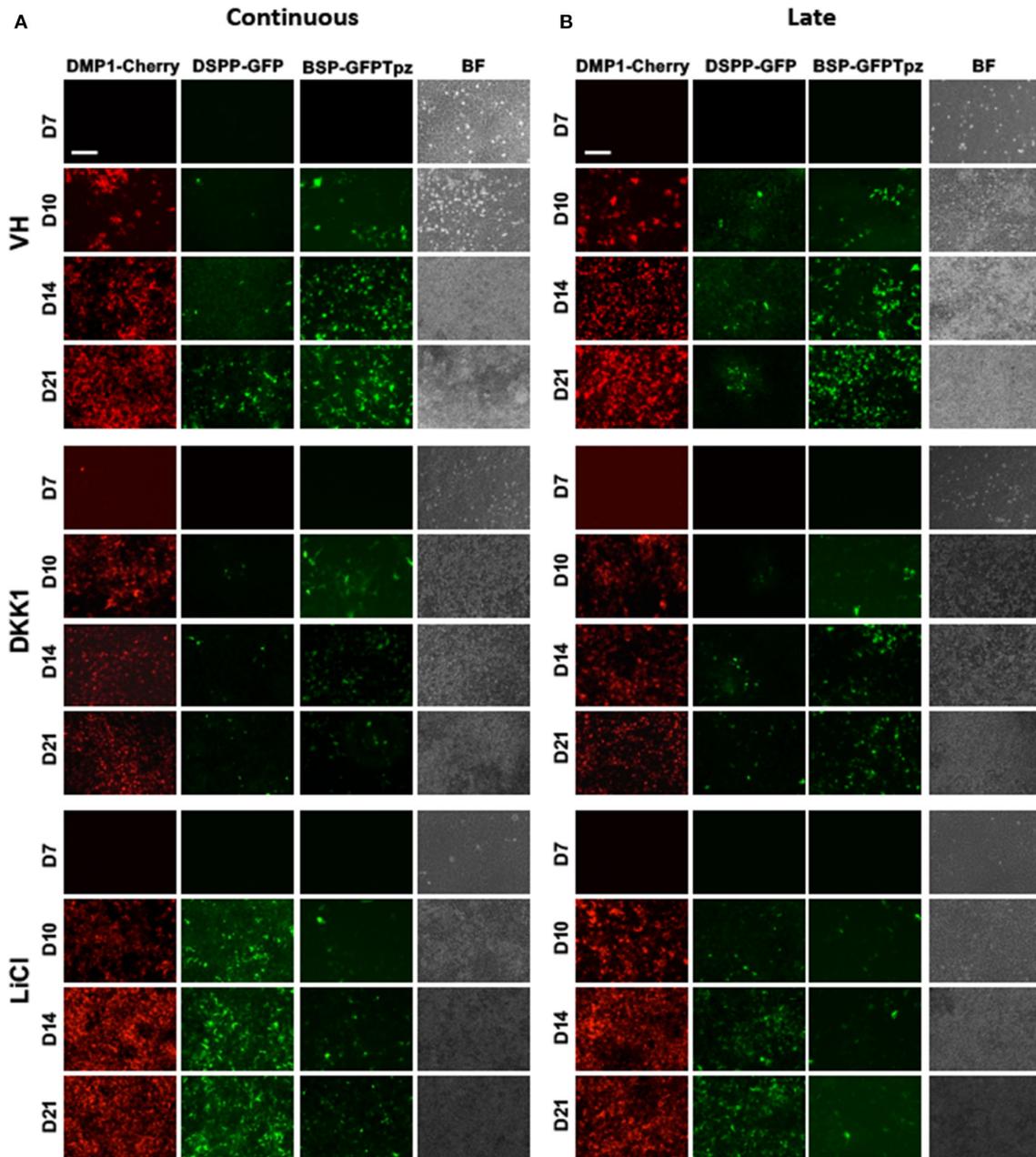
FIGURE 4 | Effects of continuous and late exposure of LiCl and DKK1 on tissue- and stage-specific reporters by epifluorescence imaging. Primary pulp cultures were treated continuously between days 3 and 21 **(A)** and late between days 7 and 21 **(B)** with VH (0.1% BSA), DKK1 (50 ng/ml) and LiCl (10 mM). **(A,B)** Representative images of the cultures from DMP1-Cherry/DSPP-Cerulean and BSP-GFPtpz transgenic mice treated with VH, DKK1, and LiCl continuously **(A)**, and late **(B)** analyzed under brightfield (BF) and epifluorescent light for the detection of DMP1-Cherry, DSPP-GFP, and BSP-GFPtpz. Continuous and late exposures to LiCl increased DMP1-Cherry and DSPP-GFP expression, which were decreased in DKK1 treated cultures. Continuous and late LiCl and DKK1 treatments decreased BSP-GFPtpz expression. Scale bar = 200 μm.

NaCl-treated controls (**Figure 1A**). Conversely, DKK1-treated cultures showed concentration-dependent decreases in *Axin2* expression as compared to controls (**Figure 1B**). The changes with the various concentrations of LiCl and DKK1 were detectable at 4 h. Based on these observations,

we selected 10 mM LiCl and 50 ng/ml DKK1 for all the following experiments.

The addition of 10 mM of LiCl to pulp cultures at days 3 and 7, induced an ∼2 to 4.5-fold increase in *Axin2* levels (**Figures 1C,D**). DKK1 decreased these increases
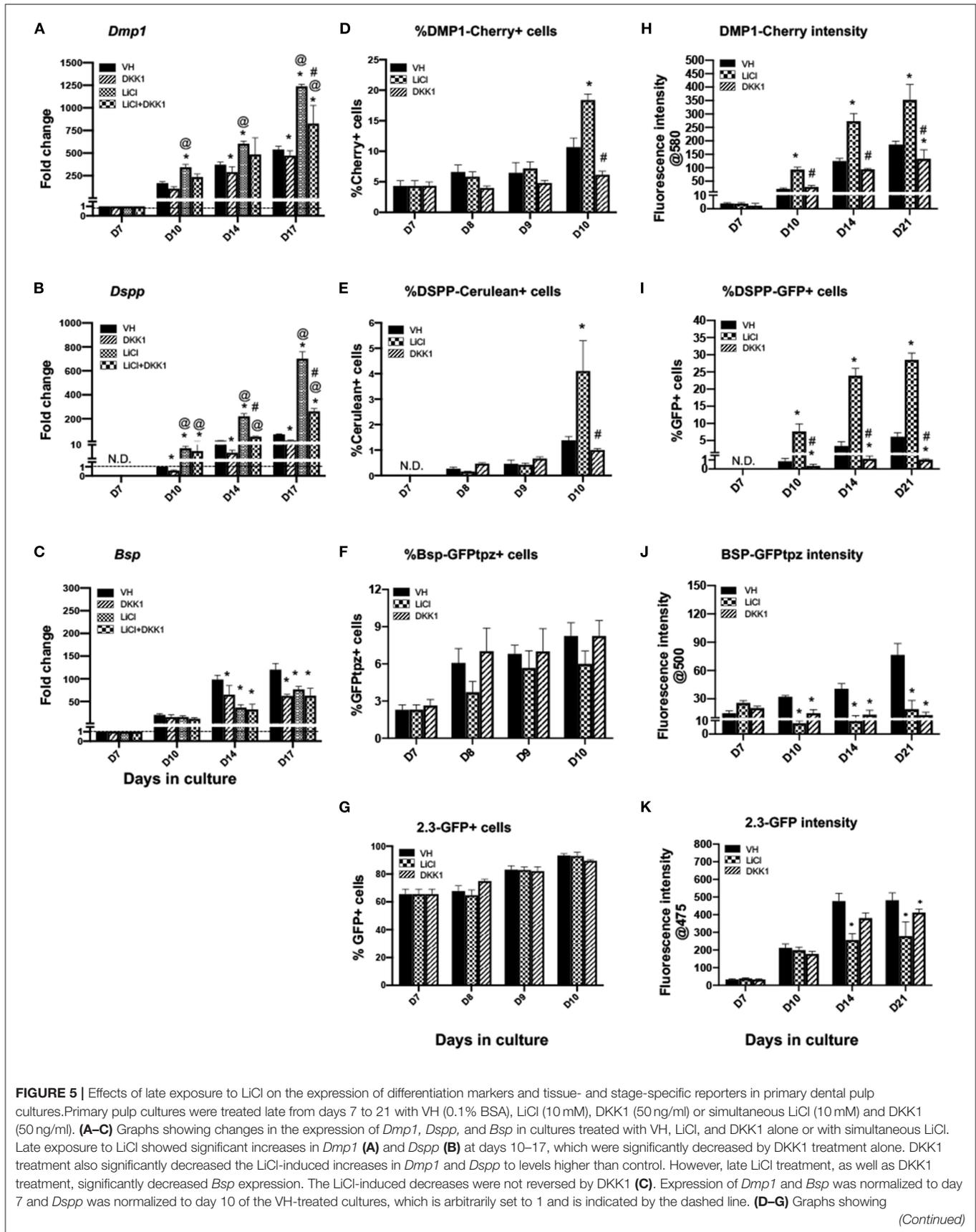
FIGURE 5 | Effects of late exposure to LiCl on the expression of differentiation markers and tissue- and stage-specific reporters in primary dental pulp cultures. Primary pulp cultures were treated late from days 7 to 21 with VH (0.1% BSA), LiCl (10 mM), DKK1 (50 ng/ml) or simultaneous LiCl (10 mM) and DKK1 (50 ng/ml). **(A–C)** Graphs showing changes in the expression of *Dmp1, Dspp,* and *Bsp* in cultures treated with VH, LiCl, and DKK1 alone or with simultaneous LiCl. Late exposure to LiCl showed significant increases in *Dmp1* **(A)** and *Dspp* **(B)** at days 10–17, which were significantly decreased by DKK1 treatment alone. DKK1 treatment also significantly decreased the LiCl-induced increases in *Dmp1* and *Dspp* to levels higher than control. However, late LiCl treatment, as well as DKK1 treatment, significantly decreased *Bsp* expression. The LiCl-induced decreases were not reversed by DKK1 **(C)**. Expression of *Dmp1* and *Bsp* was normalized to day 7 and *Dspp* was normalized to day 10 of the VH-treated cultures, which is arbitrarily set to 1 and is indicated by the dashed line. **(D–G)** Graphs showing

(Continued)

FIGURE 5 | the changes in the percentage of cells expressing DMP1-Cherry **(D)**, DSPP-Cerulean **(E)**, BSP-GFPtpz **(F)**, and 2.3-GFP **(G)** transgenes by FACS analysis between days 7 and 10 (every 24 h following the start of late treatment). LiCl-treated cultures show significantly increased the percentages of DMP1-Cherry$^+$ cells and DSPP-Cerulean$^+$ cells at day 10. DKK1 treated cultures show decreased percentages of DMP1-Cherry$^+$ and DSPP-Cerulean$^+$ cells that were significant compared to LiCl-treated cultures **(D,E)**. LiCl and DKK1 treatment showed no statistically significant effects on the percentage of BSP-GFPtpz$^+$ cells and 2.3-GFP$^+$ cells **(F,G)**. **(H–K)** Graphs showing the changes in the fluorescence intensity of DMP1-Cherry **(H)**, BSP-GFPtpz **(J)**, and 2.3-GFP **(K)** transgenes, as well as the percentage of DSPP-Cerulean$^+$ cells **(I)**, following late treatments. Late exposure to LiCl showed significant increases in the fluorescence intensity of DMP1-Cherry **(H)** and the percentage of DSPP-GFP$^+$ cells **(I)** at days 10–21. DKK1 treatment significantly decreased the intensity of DMP1-Cherry and the percentage of DSPP-GFP$^+$ cells compared to VH-treated controls, as well as LiCl-treated cultures. LiCl and DKK1 treatment significantly decreased the intensity of BSP-GFPtpz **(J)** and 2.3-GFP **(K)** at days 10–21 compared to VH-treated controls. Results represent mean ± SEM of at least 3 independent experiments. Analysis was performed using 1-way analysis of variance. $^*P \leq 0.05$ relative to VH at each time point; $^@P \leq 0.05$ relative to DKK1 at each time point; $^\#P \leq 0.05$ relative to LiCl at each time point. N.D, not detected.

(**Figures 1C,D**). Thus, primary pulp cultures contain Wnt-responsive cells and increased expression of *Axin2* by LiCl is mediated by Wnt/β-catenin signaling.

Effects of Continuous Exposure to LiCl on Primary Dental Pulp Cultures

The effects of continuous exposure to LiCl on the mineralization and differentiation of pulp cells *in vitro* were examined by addition of LiCl and DKK1 with or without LiCl between days 3 and 21 (**Figures 2A,B**).

The extent of mineralization of LiCl-treated cultures was not different from VH-treated cultures. However, continuous exposure to DKK1 markedly decreased the extent of mineralization compared to VH-treated and LiCl-treated cultures (**Figures 2A,B**).

Continuous LiCl treatment induced marked increases in the expression of *Dmp1* and *Dspp* between days 10 and 17 that were decreased by DKK1 (**Figures 3A,B**). However, continuous treatment of the cultures with both LiCl and DKK1 resulted in similarly decreased levels of *Bsp* (**Figure 3C**). These findings showed that LiCl increased the expression of *Dmp1* and *Dspp* (markers of odontoblast differentiation) but decreased the expression of *Bsp* (a marker of osteoblasts) during the mineralization phase of growth in primary pulp cultures. The decreases in the LiCl-induced changes in *Dmp1* and *Dspp* by DKK1 indicated the roles of Wnt/β-catenin signaling in LiCl-induced changes. On the other hand, the LiCl-induced decreases in *Bsp* were not regulated by Wnt/β-catenin signaling.

Primary pulp cultures from 2.3-GFP, DSPP-Cerulean/DMP1-Cherry and BSP-GFPtpz transgenic reporter mice were used to examine if the effects of LiCl on gene expression were related to changes in their levels of transcription and/or changes in the number of cells expressing these markers (**Figures 3D–K**). In these experiments, FACS analysis was used to quantify the percentages of cells expressing these transgenes 1–7 days after treatment (days 4–10 of culture) (**Figures 3D–G**) using gating strategies shown in **Supplementary Figure 2**.

No significant changes were observed in the percentage of cells expressing the various transgenes between days 4 and 7 in the LiCl- and DKK1-treated cultures compared to VH-treated cultures (**Figures 3D–G**). On the other hand, following the induction of mineralization, there were marked increases in the percentages of DMP1-Cherry$^+$ and DSPP-Cerulean$^+$ cells at days 9 and 10 in the LiCl-treated cultures. DKK1-treated cultures demonstrated significant decreases (**Figures 3D,E**). LiCl- and

DKK1-treated cultures showed no significant changes in the percentages of BSP-GFPtpz$^+$ cells (**Figure 3F**) and 2.3-GFP$^+$ cells (**Figure 3G**).

The changes in the transgene expression at later time points (days 10–21) were analyzed by fluorometric analysis and immunocytochemistry (**Figures 3H–K**, **4A**) because of the difficulties in obtaining single cells in mineralized cultures. Between days 10 and 21, LiCl-treated cultures showed continuous and marked increases in the intensity of DMP1-Cherry (**Figure 3H**), and in the percentage of DSPP-GFP$^+$ cells (**Figure 3I**) compared to VH-treated cultures. DKK1-treated cultures showed continuous and marked decreases in the intensity of DMP1-Cherry (**Figure 3H**) and in the percentage of DSPP-GFP$^+$ cells (**Figure 3I**). LiCl-and DKK1-treated cultures showed continuous decreases in the intensity of BSP-GFPtpz (**Figure 3J**) and 2.3-GFP (**Figure 3K**) compared to VH-treated cultures.

Effects of Late Exposure to LiCl on Primary Dental Pulp Cultures

Since continuous LiCl treatment demonstrated a lack of significant changes in the various differentiation markers during the first 7 days (proliferation phase), it may suggest that LiCl affects the differentiation of more mature cells. To test this possibility, we examined the effects of LiCl treatment during the mineralization of primary pulp cultures referred to as late treatment (**Figures 2**, **4**, **5**).

Late exposure of pulp cultures to LiCl (days 7–21) resulted in changes very similar to continuous treatment, albeit at a lower magnitude. LiCl did not have significant effects on the extent of mineralization (**Figures 2C,D**), increased the expression levels of *Dmp1* and *Dspp*, but decreased the levels of *Bsp* (**Figures 5A–C**). Similar to continuous treatment, late treatment increased in the percentages of DMP1-Cherry$^+$ cells (**Figure 5D**) and the intensity of DMP1-Cherry expression (**Figure 5H**) and increased the number of DSPP-Cerulean$^+$ cells (**Figures 5E,I**).

Late treatment did not have significant effects in the percentage of BSP-GFPtpz$^+$ cells and 2.3-GFP$^+$ cells (**Figures 5F,G**) between days 7 and 10 and decreased the intensity of BSP-GFPtpz and 2.3-GFP expression (**Figures 5J,K**).

Effects of LiCl on Early Progenitors' Primary Dental Pulp Cultures

Our previous observations showed that αSMA$^+$ perivascular cells in dental pulp are progenitors capable of giving rise to

TABLE 1 | Effects of early exposure to LiCl on the percentage of αSMA-GFP⁺ cells and their survival in primary dental pulp cultures.

Days in culture	VH	LiCl	DKK1
A. % aSMA-GFP⁺ cells			
4	89.53 ± 3.1	95.57 ± 1.16*	90.55 ± 3.6
5	88.68 ± 1.8	95.3 ± 0.65*	87.3 ± 2.5#
6	82.57 ± 2.65	94.2 ± 1.56*	81 ± 3.6#
7	72.67 ± 1.9	93.33 ± 1.34*	73.15 ± 4.7#
B. % aSMA-GFP⁺/EdU⁺ cells			
4	18.82 ± 1.5	26.77 ± 0.9*	14.22 ± 1.85
5	8.17 ± 0.1	17.62 ± 0.7*	8.35 ± 0.45#
6	15.9 ± 1.5	21.0 ± 1.9*	13.02 ± 0.71#
7	7.56 ± 1.7	10.23 ± 1.3*	8.9 ± 0.77
C. % aSMA-GFP⁺/cleaved caspase-3⁺ cells			
4	29.76 ± 3.2	27.43 ± 2.56	30.12 ± 2.6
5	28.72 ± 4.35	25.6 ± 1.51	34.1 ± 3.28#
6	34.62 ± 1.88	25.95 ± 4.3*	32.53 ± 7.02#
7	25.15 ± 2.78	14.42 ± 1.4*	24.63 ± 1.76#
D. % aSMA-GFP⁻/EdU⁺ cells			
4	0.8 ± 0.4	2.5 ± 0.25*	0.92 ± 0.15
5	1.47 ± 0.43	1.3 ± 0.41	0.87 ± 0.16
6	3.17 ± 0.89	1.17 ± 0.37	2.57 ± 0.24
7	8.96 ± 1.54	8.23 ± 2.94	5.35 ± 1.15*#
E. % aSMA-GFP⁻/cleaved caspase-3⁺ cells			
4	3.57 ± 1.25	2.25 ± 0.47	3.17 ± 0.58
5	3.45 ± 1.14	3.05 ± 0.33	2.7 ± 1.09
6	4.87 ± 0.99	2.52 ± 0.34	4.1 ± 0.4
7	4.02 ± 1.01	1.85 ± 0.47*	3.63 ± 0.55#

A. Cultures from αSMA-GFP transgenic mice were treated with VH, LiCl, and DKK1 from days 3 to 7 and FACS analyzed every 24 h from days 4 to 10. Note the significant increases in the percentage of αSMA-GFP⁺ progenitors in the LiCl-treated cultures. DKK1-treated cultures show no significant effects compared to VH-treated controls.
B. Cultures from αSMA-GFP transgenic mice were treated with VH, LiCl, and DKK1 from days 3 to 7, stained for EdU staining and FACS analyzed every 24 h between days 4 and 10. Note the significant increases in proliferation of αSMA-GFP⁺ populations in the LiCl-treated cultures compared to controls. DKK1 treatment had no significant effects on proliferation compared to VH-treated controls.
C. Cultures from αSMA-GFP transgenic mice were treated with VH, LiCl, and DKK1 from days 3 to 7, processed for cleaved caspase-3 staining and FACS analyzed every 24 h between days 4 and 10. Note the significant decreases in apoptosis of αSMA-GFP⁺ populations in the LiCl-treated cultures compared to controls. DKK1 treatment had no significant effects on apoptosis compared to VH-treated controls.
D and E show the effects of LiCl and DKK1 on proliferation and apoptosis of αSMA-GFP⁻ population.
*Results represent mean ± SEM of at least three independent experiments; Analysis was performed using 1-way analysis of variance. *$p \leq 0.05$ relative to the control at each time point; #$P \leq 0.05$ relative to LiCl at each time point.*

odontoblasts and osteoblasts during reparative dentinogenesis *in vivo* (30). Therefore, we examined the response αSMA⁺ perivascular cells to LiCl and DKK1 treatment.

In these experiments, the cultures from αSMA-GFP⁺ transgenic mice were treated with LiCl and DKK1 between days 3 and 7. The effects on the percentage of αSMA-GFP⁺ cells, their proliferation and apoptosis were examined by FACS analysis using gating strategies shown in **Supplementary Figure 2** (**Tables 1A–E**).

LiCl-treated cultures showed markedly increased percentage of αSMA-GFP⁺ population as compared to VH-and DKK1-treated cultures (**Table 1A**). Furthermore, LiCl increased proliferation (**Table 1B**) and decreased apoptosis (**Table 1C**) in the αSMA-GFP⁺ population. These changes were not detected in the αSMA-GFP⁻ population (**Tables 1D,E**)

Early and limited treatment with LiCl induced changes during differentiation similar to continuous and late treatment with a few important differences (**Figures 6, 7**). Unlike the continuous and late treatment, early treatment increased the extent of mineralization (**Figures 6A,B**). Early treatment also transiently increased the percentage of 2.3-GFP⁺ cells at day 8 (**Figure 7G**), unlike continuous and late treatments, which had no significant effects. The effects of early treatment with LiCl on the expression of *Dmp1* (**Figure 7A**), *Dspp* (**Figure 7B**), percentages and intensities of DMP1-Cherry⁺ and DSPP-GFP⁺ cells (**Figures 7D,E,H,I**) were similar to the other treatments, but at lower levels. However, early treatment induced increases in the expression of *Bsp* and BSP-GFPtpz (**Figures 7C,F,J**).

DISCUSSION

Using a well-characterized primary pulp culture from various reporter mice, we investigated the underlying mechanisms regulating the stimulatory effects of LiCl on odontoblast and osteoblast differentiation. Our results showed that exposure of pulp cultures to LiCl during the proliferation phase of *in vitro* growth and before induction of differentiation increased the pool of αSMA⁺ progenitors by regulating their survival. This limited and early treatment also transiently increased the number of pre-odontoblasts expressing 2.3-GFP that rapidly differentiated into odontoblasts expressing DMP1-Cherry and DSPP-Cerulean transgenes and osteoblasts expressing BSP-GFPtpz. Our results also showed that the LiCl-induced changes in the expression of *Dspp*, *Dmp1*, and *Bsp* in these early progenitors were reversed by DKK1. DKK1 is a secreted protein that, by binding to LRP5/6 and Kremen receptors, functions as a negative regulator of Wnt/β-catenin signaling (31). These observations indicated that the positive effects of LiCl in the differentiation of osteoblasts and odontoblasts from αSMA⁺ progenitors are mediated by Wnt/β-catenin signaling. The effects of LiCl on early progenitors is very similar to the effects of WNT3a (27).

Our results also showed that continuous and late exposure of dental pulp cells to LiCl increased the expression of odontoblast markers through Wnt/β-catenin signaling and the number of odontoblasts expressing DMP1-Cherry and DSPP-Cerulean transgenes. However, unlike the early treatment, both continuous and late treatments decreased the expression of *Bsp* and the expression of BSP-GFPtpz transgene. These observations suggest that prolonged treatment by LiCl in more mature cells in the dental pulp has an inhibitory effect on osteoblast differentiation. The LiCl-induced decreases in the levels of *Bsp* during the mineralization phase of *in vitro* growth were not reversed by DKK1, indicating that the inhibitory effects of LiCl on osteogenesis and *Bsp* are not mediated through Wnt/β-catenin signaling. The inhibitory effects of prolonged exposure of pulp
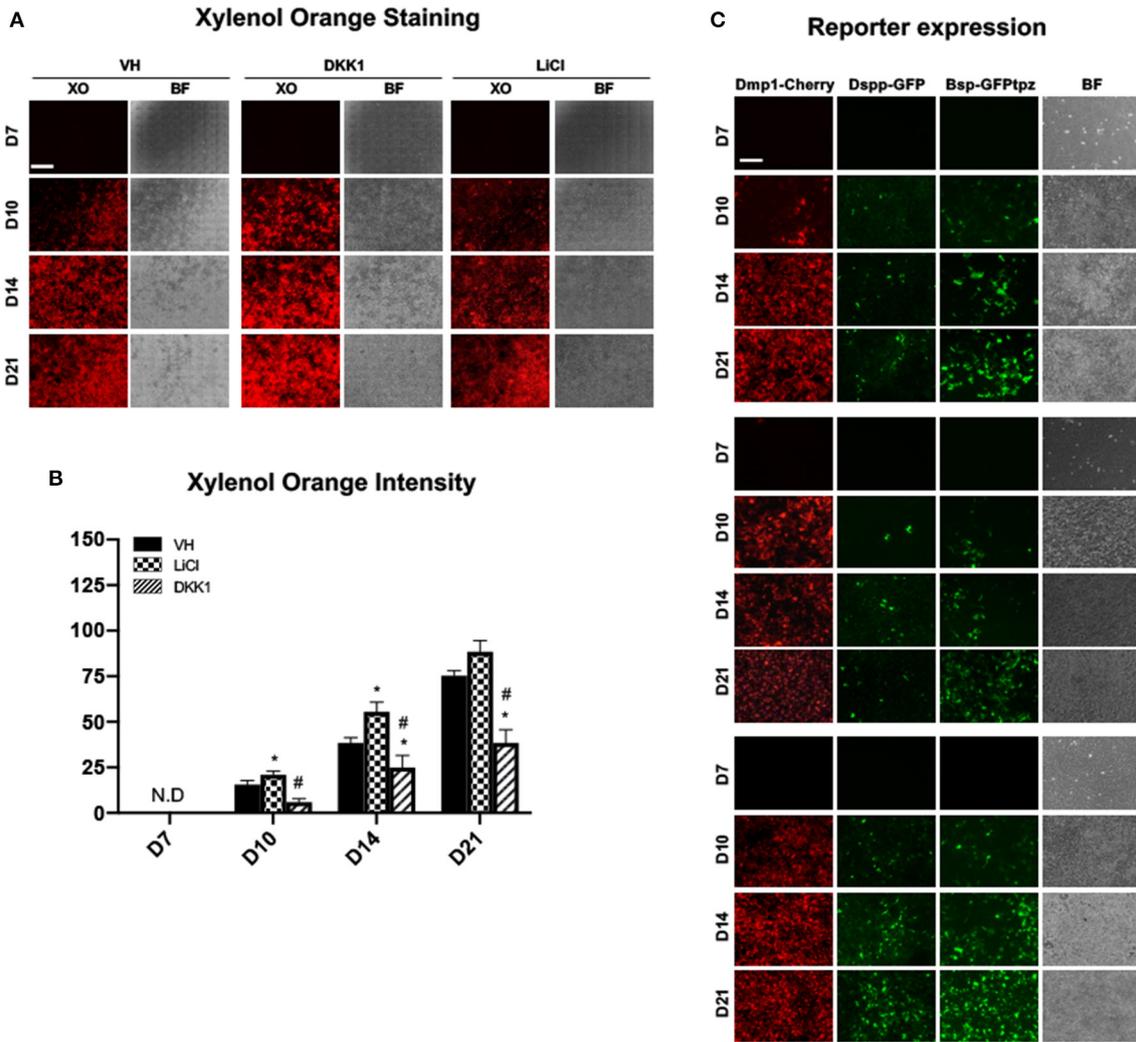
FIGURE 6 | Effects of early exposure to LiCl on mineralization and tissue- and stage-specific reporters in primary dental pulp cultures. Primary pulp cultures were treated with VH (0.1% BSA), DKK1 (50 ng/ml) or LiCl (10 mM) during the proliferation phase of *in vitro* growth between days 3 and 7. **(A)** Representative images of the same areas in cultures treated with VH, DKK1, and LiCl analyzed under brightfield (BF) and epifluorescent light using TRITC red filter for detection of XO staining. Scale bar = 2 mm. **(B)** Graphs showing changes in the intensity of XO staining (in absolute values) in cultures treated with VH, DKK1, and LiCl. Early exposure increased XO staining at days 10 and 14. The intensity of XO staining was measured at 570/610-nm wavelength (excitation/emission) and at a gain of 80. Background fluorescence for XO was measured using cultures without the addition of ascorbic acid and β-glycerophosphate, which lack mineralization potential. The background fluorescence values were subtracted from respective XO measurements. Results represents mean ± SEM of at least 3 independent experiments. Analysis was performed using 1-way analysis of variance. *$P \leq 0.05$ relative to VH at each time point; #$P \leq 0.05$ relative to LiCl at each time point. XO, Xylenol orange; N.D, not detected. **(C)** Representative images of the cultures from DMP1-Cherry/DSPP-Cerulean and BSP-GFPtpz transgenic mice treated with VH, DKK1, and LiCl and analyzed under brightfield (BF) and epifluorescent light for the detection of DMP1-Cherry, DSPP-GFP, and BSP-GFPtpz. Early exposures to LiCl increased DMP1-Cherry and DSPP-GFP expression, which were decreased in DKK1 treated cultures. Early LiCl and DKK1 treatments increased and decreased BSP-GFPtpz expression, respectively. Scale bar = 200 μm. XO, Xylenol orange; N.D, not detected; VH, vehicle; GFP, green fluorescent; GFPtpz, topaz variant of GFP.

cells to LiCl are different from the stimulatory effects of WNT3a on pulp cells (27).

Although the stimulatory effects of Wnt/β-catenin signaling on osteoblast differentiation has previously been well-established (32), the effects of LiCl on osteogenesis remains controversial. Several studies have shown the positive effects of LiCl on osteogenic differentiation. LiCl enhanced osteogenic differentiation by bone marrow-derived mesenchymal stem (33),

human periodontal ligament fibroblasts (34), and stem cells from the apical papilla (17).

In contrast, several studies reported inhibitory effects of lithium on osteogenic differentiation. LiCl inhibited the osteogenic differentiation of MC3T3-E1 pre-osteoblasts and a pluripotent mesenchymal cell line C2C12 (35, 36), bone marrow-derived mesenchymal stem cells (37), cementoblasts (38), SHED (39) and osteogenesis in the cultured embryonic palatal shelves
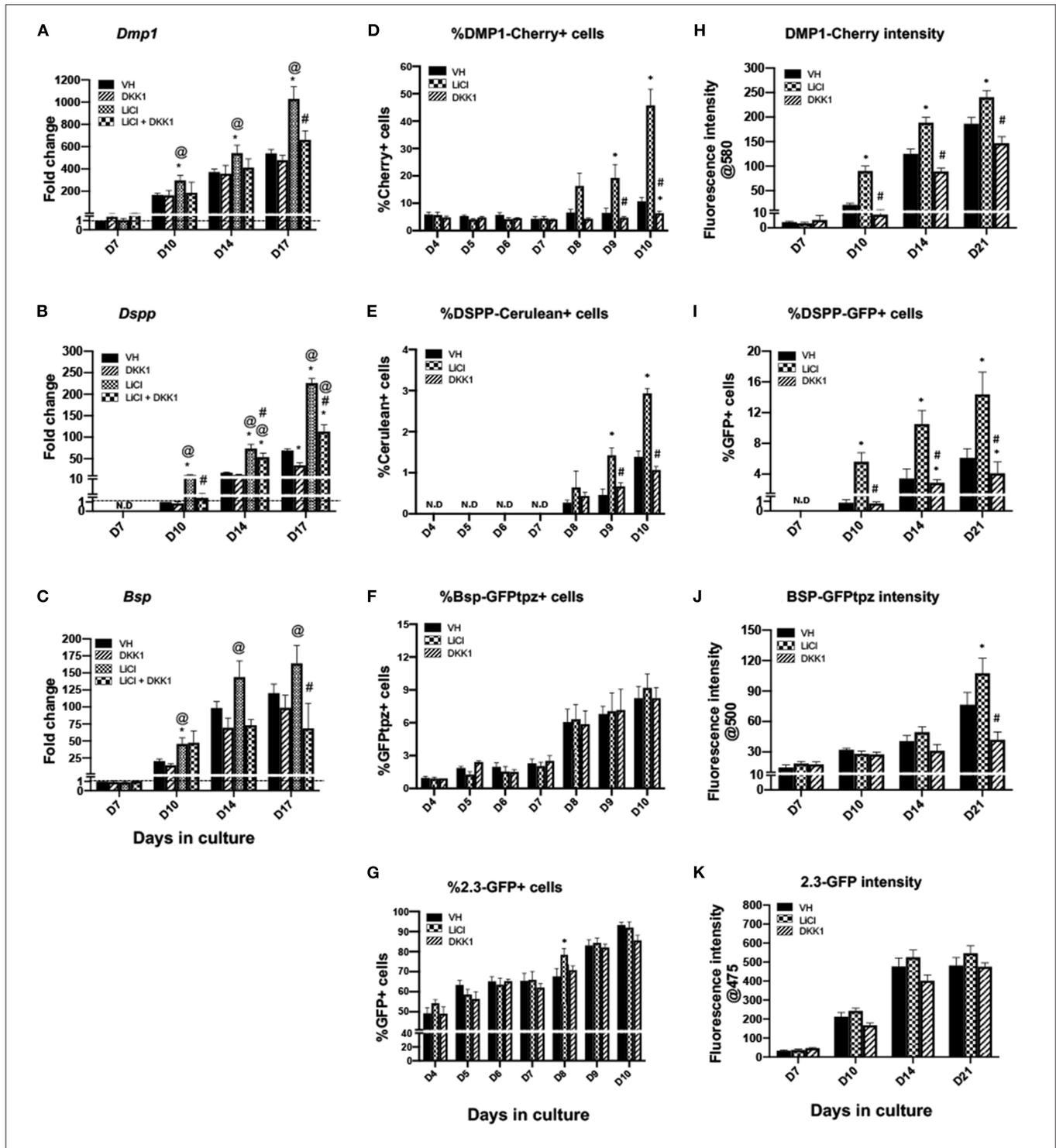
FIGURE 7 | Effects of early exposure to LiCl on the expression of differentiation markers in primary dental pulp cultures. Primary pulp cultures were treated early from days 3 to 7 with VH (0.1% BSA), LiCl (10 mM), DKK1 (50 ng/ml) or simultaneous LiCl (10 mM) and DKK1 (50 ng/ml). **(A–C)** Graphs showing changes in the expression of *Dmp1, Dspp,* and *Bsp* in cultures. Early exposure to LiCl showed significant increases in *Dmp1* **(A)**, *Dspp* **(B)**, and *Bsp* **(C)** at days 10–17, which were decreased by DKK1 treatment alone. DKK1 treatment also significantly decreased the LiCl-induced increases in *Dmp1*, *Dspp*, and *Bsp*. Expression of *Dmp1* and *Bsp* was normalized to day 7 and *Dspp* was normalized to day 10 of the VH-treated cultures, which is arbitrarily set to 1 and is indicated by the dashed line. **(D–G)** Graphs showing the changes in the percentage of cells expressing DMP1-Cherry **(D)**, DSPP-Cerulean **(E)**, BSP-GFPtpz **(F)**, and 2.3-GFP **(G)** transgenes by FACS analysis. LiCl treatment significantly increased percentages in DMP1-Cherry⁺ cells and DSPP-Cerulean⁺ cells at days 9–10, while DKK1-treated cultures show significantly

(Continued)

FIGURE 7 | decreased percentages of DMP1-Cherry$^+$ and DSPP-Cerulean$^+$ cells **(D,E)**. LiCl and DKK1 treatment showed no effects on the percentage of BSP-GFPtpz$^+$ cells and 2.3-GFP$^+$ cells **(F,G)**. **(H–K)** Graphs showing the changes in the fluorescence intensity of DMP1-Cherry **(H)**, BSP-GFPtpz **(J)**, and 2.3-GFP **(K)** transgenes, as well as the percentage of DSPP-Cerulean$^+$ cells **(I)** following early treatment with VH, LiCl, and DKK1 from days 3 to 7. Early exposure to LiCl showed significant increases in the fluorescence intensity of DMP1-Cherry **(H)**, BSP-GFPtpz **(J)**, 2.3-GFP **(K)** and the percentage of DSPP-GFP$^+$ cells **(I)** at days 10–21. DKK1 treatment significantly decreased the intensity of DMP1-Cherry, BSP-GFPtpz, and 2.3-GFP as well as the percentage of DSPP-GFP$^+$ cells, at days 10–21. Results represent mean ± SEM of at least 3 independent experiments; Analysis was performed using 1-way analysis of variance. $^*P \leq 0.05$ relative to VH at each time point; $^{@}P \leq 0.05$ relative to DKK1 at each time point; $^{\#}P \leq 0.05$ relative to LiCl at each time point. N.D, not detected.

(40). In both MC3T3-E1 and C2C12 cell lines, LiCl inhibition of osteogenesis was mediated through BMP2/SMAD signaling pathway (35). Additionally, in MC3T3-E1 cells, the negative effects of LiCl osteogenesis were mediated through GSK3b, whereas in C2C12 cells, these effects were independent of GSK3-mediated signaling (35).

Furthermore, LiCl modulates the activity of several other pathways, including inhibiting inositol monophosphates (IMPase) in the phosphatidylinositol (PI) signaling pathways, with essential positive roles in osteoblast differentiation and bone formation (41, 42). These observations suggest that the inhibitory effects of LiCl on osteogenesis is complex and may involve more than one pathway.

Considering the effects of LiCl on multiple signaling pathways, the results of the studies in which LiCl has been used to block the GSK3 activity should be interpreted carefully. GSK3β, a serine/threonine kinase, modulates many signaling pathways and transcription factors (43), and the effects of LiCl on reparative dentinogenesis cannot be considered specific to Wnt/β-catenin signaling.

A few studies have shown that reparative dentinogenesis induced by LiCl and small-molecule GSK3 inhibitor drugs following pulp exposure resulted in the formation of reparative dentin that was more similar to physiological dentin in tubularity and chemical composition (7, 8). Our observations suggest that the improved reparative dentin structure in these studies as compared to osteodentin is related to LiCl-mediated inhibition of osteogenesis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and use Committee of UConn Health Center.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fdmed.2021.649500/full#supplementary-material

REFERENCES

1. Clevers H, Nusse R. Wnt/beta-catenin signaling and disease. *Cell.* (2012) 149:1192–205. doi: 10.1016/j.cell.2012.05.012

2. Ma B, Hottiger MO. Crosstalk between wnt/beta-catenin and nf-kappab signaling pathway during inflammation. *Front Immunol.* (2016) 7:378. doi: 10.3389/fimmu.2016.00378

3. Whyte JL, Smith AA, Helms JA. Wnt signaling and injury repair. *Cold Spring Harb Perspect Biol.* (2012) 4:a008078. doi: 10.1101/cshperspect.a008078

4. Baron R, Kneissel M. Wnt signaling in bone homeostasis and disease: from human mutations to treatments. *Nat Med.* (2013) 19:179–92. doi: 10.1038/nm.3074

5. Babb R, Chandrasekaran D, Carvalho Moreno Neves V, Sharpe PT. Axin2-expressing cells differentiate into reparative odontoblasts

via autocrine wnt/beta-catenin signaling in response to tooth damage. *Sci Rep.* (2017) 7:3102. doi: 10.1038/s41598-017-03145-6

6. Hunter DJ, Bardet C, Mouraret S, Liu B, Singh G, Sadoine J, et al. Wnt acts as a prosurvival signal to enhance dentin regeneration. *J Bone Miner Res.* (2015) 30:1150–9. doi: 10.1002/jbmr.2444

7. Ishimoto K, Hayano S, Yanagita T, Kurosaka H, Kawanabe N, Itoh S, et al. Topical application of lithium chloride on the pulp induces dentin regeneration. *PLoS ONE.* (2015) 10:e0121938. doi: 10.1371/journal.pone.0121938

8. Neves VC, Babb R, Chandrasekaran D, Sharpe PT. Promotion of natural tooth repair by small molecule gsk3 antagonists. *Sci Rep.* (2017) 7:39654. doi: 10.1038/srep39654

9. Zaugg LK, Banu A, Walther AR, Chandrasekaran D, Babb RC, Salzlechner C, et al. Translation approach for dentine regeneration using gsk-3 antagonists. *J Dental Res.* (2020) 99:544–51. doi: 10.1177/0022034520908593

10. Zhao Y, Yuan X, Bellido T, Helms JA. A correlation between wnt/beta-catenin signaling and the rate of dentin secretion. *J Endod.* (2019) 45:1357–64.e1351. doi: 10.1016/j.joen.2019.07.014

11. Zhao Y, Yuan X, Liu B, Tulu US, Helms JA. Wnt-responsive odontoblasts secrete new dentin after superficial tooth injury. *J Dental Res.* (2018) 97:1047–54. doi: 10.1177/0022034518763151

12. Han N, Zheng Y, Li R, Li X, Zhou M, Niu Y, et al. Beta-catenin enhances odontoblastic differentiation of dental pulp cells through activation of runx2. *PLoS ONE.* (2014) 9:e88890. doi: 10.1371/journal.pone.0088890

13. Volponi AA, Pang Y, Sharpe PT. Stem cell-based biological tooth repair and regeneration. *Trends Cell Biol.* (2010) 20:715–22. doi: 10.1016/j.tcb.2010.09.012

14. Yokose S, Naka T. Lymphocyte enhancer-binding factor 1: an essential factor in odontoblastic differentiation of dental pulp cells enzymatically isolated from rat incisors. *J Bone Miner Metab.* (2010) 28:650–8. doi: 10.1007/s00774-010-0185-0

15. Apostu D, Lucaciu O, Mester A, Oltean-Dan D, Baciut M, Baciut G, et al. Systemic drugs with impact on osteoarthritis. *Drug Metab Rev.* (2019) 51:498–523. doi: 10.1080/03602532.2019.1687511

16. Jakobsson E, Arguello-Miranda O, Chiu SW, Fazal Z, Kruczek J, Nunez-Corrales S, et al. Towards a unified understanding of lithium action in basic biology and its significance for applied biology. *J Membr Biol.* (2017) 250:587–604. doi: 10.1007/s00232-017-9998-2

17. Wang J, Liu B, Gu S, Liang J. Effects of wnt/beta-catenin signalling on proliferation and differentiation of apical papilla stem cells. *Cell Prolif.* (2012) 45:121–31. doi: 10.1111/j.1365-2184.2012.00806.x

18. Klein PS, Melton DA. A molecular mechanism for the effect of lithium on development. *Proc Natl Acad Sci USA.* (1996) 93:8455–9. doi: 10.1073/pnas.93.16.8455

19. Ali M, Okamoto M, Komichi S, Watanabe M, Huang H, Takahashi Y, et al. Lithium-containing surface pre-reacted glass fillers enhance hdpsc functions and induce reparative dentin formation in a rat pulp capping model through activation of wnt/β-catenin signaling. *Acta Biomater.* (2019) 96:594–604. doi: 10.1016/j.actbio.2019.06.016

20. Fathke C, Wilson L, Shah K, Kim B, Hocking A, Moon R, et al. Wnt signaling induces epithelial differentiation during cutaneous wound healing. *BMC Cell Biol.* (2006) 7:4. doi: 10.1186/1471-2121-7-4

21. Li J, Yao Q, Xu Y, Zhang H, Li, L.-L., et al. Lithium chloride-releasing 3d printed scaffold for enhanced cartilage regeneration. *Med Sci Monit.* (2019) 25:4041–50. doi: 10.12659/MSM.916918

22. Zheng C, Chen J, Liu S, Jin Y. Stem cell-based bone and dental regeneration: a view of microenvironmental modulation. *Int J Oral Sci.* (2019) 11:23. doi: 10.1038/s41368-019-0060-3

23. Balic A, Aguila HL, Mina M. Identification of cells at early and late stages of polarization during odontoblast differentiation using pobcol3.6gfp and pobcol2.3gfp transgenic mice. *Bone.* (2010) 47:948–58. doi: 10.1016/j.bone.2010.08.009

24. Vijaykumar A, Dyrkacz P, Vidovic-Zdrilic I, Maye P, Mina M. Expression of bsp-gfptpz transgene during osteogenesis and reparative dentinogenesis. *J Dental Res.* (2020) 99:89–97. doi: 10.1177/0022034519885089

25. Vijaykumar A, Ghassem-Zadeh S, Vidovic-Zdrilic I, Komitas K, Adameyko I, Krivanek J, et al. Generation and characterization of dspp-cerulean/dmp1-cherry reporter mice. *Genesis.* (2019) 57:e23324. doi: 10.1002/dvg.23324

26. Sagomonyants K, Kalajzic I, Maye P, Mina M. Enhanced dentinogenesis of pulp progenitors by early exposure to fgf2. *J Dental Res.* (2015) 94:1582–90. doi: 10.1177/0022034515599768

27. Vijaykumar A, Root SH, Mina M. Wnt/beta-catenin signaling promotes the formation of preodontoblasts *in vitro*. *J Dental Res.* (2021) 100:387–96. doi: 10.1177/0022034520967353

28. Sagomonyants K, Mina M. Biphasic effects of fgf2 on odontoblast differentiation involve changes in the bmp and wnt signaling pathways. *Connect Tissue Res.* (2014) 55 (Suppl. 1):53–6. doi: 10.3109/03008207.2014.923867

29. Jho EH, Zhang T, Domon C, Joo CK, Freund JN, Costantini F. Wnt/beta-catenin/tcf signaling induces the transcription of axin2, a negative regulator of the signaling pathway. *Mol Cell Biol.* (2002) 22:1172–83. doi: 10.1128/MCB.22.4.1172-1183.2002

30. Vidovic I, Banerjee A, Fatahi R, Matthews BG, Dyment NA, Kalajzic I, et al. Alphasma-expressing perivascular cells represent dental pulp progenitors in vivo. *J Dental Res.* (2017) 96:323–30. doi: 10.1177/0022034516678208

31. Cruciat CM, Niehrs C. Secreted and transmembrane wnt inhibitors and activators. *Cold Spring Harb Perspect Biol.* (2013) 5:a015081. doi: 10.1101/cshperspect.a015081

32. Wong SK, Chin KY, Ima-Nirwana S. The skeletal-protecting action and mechanisms of action for mood-stabilizing drug lithium chloride: current evidence and future potential research areas. *Front Pharmacol.* (2020) 11:430. doi: 10.3389/fphar.2020.00430

33. Yu Z, Fan L, Li J, Ge Z, Dang X, Wang K. Lithium chloride attenuates the abnormal osteogenic/adipogenic differentiation of bone marrow-derived mesenchymal stem cells obtained from rats with steroid-related osteonecrosis by activating the beta-catenin pathway. *Int J Mol Med.* (2015) 36:1264–72. doi: 10.3892/ijmm.2015.2340

34. Heo JS, Lee SY, Lee JC. Wnt/beta-catenin signaling enhances osteoblastogenic differentiation from human periodontal ligament fibroblasts. *Mol Cells.* (2010) 30:449–54. doi: 10.1007/s10059-010-0139-3

35. Li J, Khavandgar Z, Lin SH, Murshed M. Lithium chloride attenuates bmp-2 signaling and inhibits osteogenic differentiation through a novel wnt/gsk3- independent mechanism. *Bone.* (2011) 48:321–31. doi: 10.1016/j.bone.2010.09.033

36. Shi YC, Worton L, Esteban L, Baldock P, Fong C, Eisman JA, et al. Effects of continuous activation of vitamin d and wnt response pathways on osteoblastic proliferation and differentiation. *Bone.* (2007) 41:87–96. doi: 10.1016/j.bone.2007.04.174

37. de Boer J, Siddappa R, Gaspar C, van Apeldoorn A, Fodde R, van Blitterswijk C. Wnt signaling inhibits osteogenic differentiation of human mesenchymal stem cells. *Bone.* (2004) 34:818–26. doi: 10.1016/j.bone.2004.01.016

38. Nemoto E, Koshikawa Y, Kanaya S, Tsuchiya M, Tamura M, Somerman MJ, et al. Wnt signaling inhibits cementoblast differentiation and promotes proliferation. *Bone.* (2009) 44:805–12. doi: 10.1016/j.bone.2008.12.029

39. Rattanawarawipa P, Pavasant P, Osathanon T, Sukarawan W. Effect of lithium chloride on cell proliferation and osteogenic differentiation in stem cells from human exfoliated deciduous teeth. *Tissue Cell.* (2016) 48:425–31. doi: 10.1016/j.tice.2016.08.005

40. Meng L, Wang X, Torensma R, Von den Hoff JW, Bian Z. Lithium inhibits palatal fusion and osteogenic differentiation in palatal shelves *in vitro*. *Arch Oral Biol.* (2015) 60:501–7. doi: 10.1016/j.archoralbio.2014.12.011

41. Brown KM, Tracy DK. Lithium: the pharmacodynamic actions of the amazing ion. *Ther Adv Psychopharmacol.* (2013) 3:163–76. doi: 10.1177/2045125312471963

42. Gámez B, Rodríguez-Carballo E, Graupera M, Rosa JL, Ventura F. Class i pi-3-kinase signaling is critical for bone formation through regulation of smad1 activity in osteoblasts. *J Bone Mineral Res.* (2016) 31:1617–30. doi: 10.1002/jbmr.2819

43. Beurel E, Grieco SF, Jope RS. Glycogen synthase kinase-3 (gsk3): regulation, actions, and diseases. *Pharmacol Ther.* (2015) 148:114–31. doi: 10.1016/j.pharmthera.2014.11.016