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Wnt10b Activates the Wnt, Notch, and NFκB Pathways in U2OS Osteosarcoma Cells

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ABSTRACT

Although osteosarcoma represents the most common bone malignancy, the molecular and cellular mechanisms influencing its pathogenesis have remained elusive. Recent evidence has suggested that the Wnt signaling pathway may play a crucial role in osteosarcoma. This study employed a microarray approach to discover novel genes and pathways involved in Wnt signaling in osteosarcoma. We developed a Wnt10b-expressing cell line using the human U2OS osteosarcoma model (U2OS-Wnt10b) and performed microarray and pathway analyses using parental U2OS cells as control. Differential expression of 1,003 genes encompassing 28 pathways was noted. The Wnt, NFκB, and Notch pathways were chosen for further study based on their known importance in bone biology. Known Wnt-responsive genes Axin-2 (4.9-fold), CD44 (2.1-fold), endothelin-1 (4.2-fold) and sclerostin domain containing-1 (43-fold) were regulated by Wnt10b. The proinflammatory cytokines interleukin-1α and tumor necrosis factor-α, known inducers of NFκB, were upregulated both at the transcript and protein level, and NFκB reporter activity was stimulated 3.8-fold, confirming NFκB activation. Interestingly, genes involved in Notch signaling [Notch-1 (2.4-fold) and Jagged-1 (3.1-fold)] were upregulated, whereas the Notch inhibitor, lunatic fringe, was downregulated (8.2-fold). This resulted in the activation of the classic Notch-responsive genes, hairy and enhancer of split-1 (Hes-1; 2.2-fold) and hairy/enhancer-of-split related with YRPW motif-1 (Hey-1; 2.5-fold). A Hey-1 reporter construct was regulated 9.1-fold in U2OS-Wnt10b cells, confirming Notch activation. Interestingly, Wnt3a failed to induce the Notch and NFκB pathways, demonstrating Wnt-specificity. In conclusion, our data demonstrate that Wnt10b, but not Wnt3a, stimulates the NFκB and Notch pathways in U2OS osteosarcoma cells. J. Cell. Biochem. 112: 1392–1402, 2011.

KEY WORDS: Wnt10b; OSTEOSARCOMA; BONE; MICROARRAY; NOTCH

Osteosarcoma represents the most common malignant bone tumor and accounts for ∼5% of all pediatric cancers in the United States. The current preferred treatment remains preoperative adjuvant chemotherapy followed by surgery where the overall survival rate is ∼60–75% in localized disease [Link et al., 2002]. However, ∼20% of all patients presenting with osteosarcoma exhibit metastatic disease with pulmonary lesions representing the major site of metastasis [Link et al., 2002]. Although adjuvant chemotherapy has drastically decreased mortality in patients with metastatic disease, 30–50% of patients still die of pulmonary metastasis [Glasser et al., 1992] and the overall 5-year survival rate is only ∼20% [Goorin et al., 1984; Glasser et al., 1992]. The molecular mechanisms of the pathogenesis of osteosarcoma have largely remained elusive. Identification of specific genes and cellular pathways that are associated with, or potentiate, osteosarcoma would greatly benefit the future management of both localized and metastatic disease.

The Wnt/β-catenin signaling pathway plays an important role in bone cell differentiation, proliferation, and apoptosis [Baron and Rawadi, 2007; Johnson and Kamel, 2007], and recently has been identified as potential target in both human and mouse osteosarcoma models [Kansara et al., 2009; Rubin et al., 2010]. The canonical Wnt pathway relies on stabilization of β-catenin. In the absence of a Wnt signal, a multiprotein complex containing the kinase glycogen synthase kinase (GSK)-3β, Axin-1/-2, adenomatous polyposis coli and disheveled, mediate the degradation of excess β-catenin through phosphorylation [Westendorf et al., 2004]. Wnt binding to a coreceptor complex containing low-density lipoprotein receptor-related protein-5/6 and a member of the frizzled family results in the inhibition of GSK-3β activity and leads to the nuclear translocation of hypophosphorylated β-catenin, where it associates with members of the Tcf/Lef family of transcription factors to regulate gene expression [Westendorf et al., 2004]. Deregulation of Wnt signaling has been implicated in the development and pathogenesis of a wide

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array of cancers [Reya and Clevers, 2005], including osteosarcoma. Recent evidence has demonstrated that suppression of secreted frizzled related protein 3 (Sfrp3) [Mandal et al., 2007] or Wnt inhibitory factor 1 (Wif1) [Kansara et al., 2009; Rubin et al., 2010], both negative regulators of canonical Wnt signaling, plays a role in osteosarcoma progression by leaving the effects of β-catenin unchecked. Indeed, osteosarcomas often exhibit increased levels of intracellular β-catenin [Haydon et al., 2002] which are associated with metastatic potential [Iwao et al., 1999; Iwaya et al., 2003]. These data collectively suggest that osteosarcomas are more sensitive to the effects of Wnts; however, the genes and pathways regulated by specific Wnt ligands in osteosarcoma are unknown.

Recent interest in the interactions between bone and Wnt signaling has focused on mouse studies involving Wnt10b, a canonical Wnt signaling molecule. Wnt10b deficiency is associated with a decrease in bone mineral density in mice, whereas transgenic overexpression of Wnt10b causes increases in bone formation [Bennett et al., 2005; Bennett et al., 2007]. Wnt10b is also important for maintaining adult bone mass through maintenance of mesenchymal progenitor cells, as Wnt10b deficiency results in age-dependent bone loss and a reduction in these progenitor cells [Stevens et al., 2010]. Collectively, these lines of evidence demonstrate that Wnt10b is a positive regulator of bone formation through the maintenance of a progenitor pool of preosteoblastic cells in the bone marrow. However, relatively little is known concerning the molecular targets of Wnt10b in osteoblast or osteosarcoma cell models.

In this study, we employed microarray analysis to identify the genetic targets regulated by Wnt10b in the human U2OS osteosarcoma cell model in an effort to better understand the cellular effects of increased Wnt signaling in osteosarcoma. Quantitative PCR (QPCR) was used to validate a subset of Wnt10b-regulated genes. To understand the how these regulated genes affect cellular processes, pathway analysis was used to identify changes in groups of interacting genes or pathways responding to Wnt10b. Using this approach we provide evidence that the Wnt, NFκB, and Notch pathways are specific Wnt10b targets in U2OS osteosarcoma cells. Understanding the pathways regulated by Wnts may illuminate therapeutic targets for clinical interventions in the treatment of osteosarcoma.

MATERIALS AND METHODS

CELL CULTURE AND REAGENTS

U2OS osteosarcoma cells were cultured in phenol red-free Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 containing 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic (Invitrogen). The U2OS-Wnt10b cell line was cultured in selective media [same media supplemented with 5 mg/L blasticidin S (Roche Diagnostics) and 500 mg/L zeocin (Invitrogen)] to maintain selection of the Tet-repressor and Wnt10b-pcDNA4/TO plasmids, respectively. The Wnt10b transgene was induced using 100 ng/ml doxycycline (Sigma) for 24 h prior to and during experimentation. Parental U2OS cells used as a control were treated in the same manner. Recombinant mouse Wnt3a was purchased from R&D Systems.

U2OS-Wnt10b STABLE CELL LINE PRODUCTION

Wnt10b expression plasmid (Addgene plasmid 1831) [Lane and Leder, 1997] was subcloned into the *Eco*RI site of pcDNA4/TO (Invitrogen). Following *Pvu*I linearization, the construct was transiently transfected into U2OS-Tet^{R} cells [Monroe et al., 2003] at a density of 50% using FuGENE6 according to the manufacturer's protocol (Roche Diagnostics). Forty-eight hours later the cells were split into selective media. Resistant cell clones were isolated and Western blot analyses performed to confirm expression of Wnt10b. The resultant cell line used in this study is designated as U2OS-Wnt10b.

ILLUMINA MICROARRAY AND PATHWAY ANALYSIS

U2OS and U2OS-Wnt10b cells (n = 6 each) were plated in 10-cm culture dishes at 50% confluence and treated with 100 ng/ml Dox for 24 h. Total RNA was isolated using Trizol reagent (Invitrogen) and treated with RNase-free DNase (Qiagen) using standard procedures. Isolated RNA (1 μg) was submitted for microarray analysis using the Illumina Whole Genome Human Sentrix 6 BeadArray Chips that profile 48,701 human transcripts annotated from the National Center for Biotechnology Information, Reference Sequence, and the UniGene databases. The preparation of the samples and microarray hybridizations was performed by the Advanced Genomic Technology Center Microarray Shared Resource at the Mayo Clinic. Analysis of variance (ANOVA) statistical modeling was used to categorize differentially expressed genes between U2OS and U2OS-Wnt10b cell lines. In general, all genes regulated at $P < 0.05$ were considered statistically significant and were categorized by >1, 2, or 3 standard deviations (SD) from the mean. To increase the power of our analysis, we only considered genes regulated at >2 SD from the mean. MetaCore pathway analysis was performed on this geneset using these criteria, and pathways regulated as a whole ($P < 0.05$) were considered significant.

QUANTITATIVE RT-PCR (QPCR)

U2OS and U2OS-Wnt10b cells (n = 6 each) were treated and total RNA was isolated as described above. Isolated RNA (1 μg) was used in a reverse transcriptase (RT) reaction using the iScript cDNA Synthesis Kit (Biorad). RT reactions were diluted fivefold and 2 μl used for QPCR using FastStart Universal SYBR Green Master (Roche Diagnostics) on a 7900HT Fast Real-Time PCR System (Applied Biosystems). Individual genes for each sample were run in triplicate reactions in 10 μl. Specific QPCR primers were designed using Primer Express software (Applied Biosystems) and the sequences are available on request. Amplification conditions were 95°C for 15 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Median values of the triplicates were normalized to the TBP reference gene and the formula $2^{-\Delta Ct}$ was used to obtain relative changes in gene expression among samples. Negative RT controls were included to check for genomic DNA contamination (data not shown).

WESTERN BLOTTING

Whole cell extracts of U2OS and U2OS-Wnt10b cells were prepared by lysing the cells for 30 min on ice in lysis buffer [20 mM Tris (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet-P40] supplemented with Complete Mini EDTA-free Protease Inhibitor tablets and PhosStop Phosphatase Inhibitor tablets (Roche Diagnostics). Fractionated cytoplasmic and nuclear extracts were prepared as previously described [Cicek et al., 2005]. Protein concentrations were determined using a BCA Protein Assay Kit (Pierce). Equal amounts of protein were subjected to Western blot analysis as previously described [Monroe et al., 2003]. Blots were probed with a 1:1,000 dilution of either Wnt10b (MAB2110; R&D Systems), β-actin (5C5; Santa Cruz) or hypophosphorylated-β-catenin (05-665; Millipore) antibodies. Species specific anti-IgG secondary antibodies linked to horseradish peroxidase (Sigma) were used at a 1:5,000 dilution. Washed blots were visualized using enhanced chemiluminescence (Amersham Pharmacia).

PRODUCTION OF TOP-FLASH AND FOP-FLASH REPORTER CONSTRUCTS

The Wnt-responsive TOP-Flash reporter was produced by inserting three copies of the Tcf/Lef binding site GATCAAAGG (each site separated by GGGTAA) upstream of the firefly luciferase gene in the pLuc-MCS reporter vector (Agilent Technologies) using the Quik

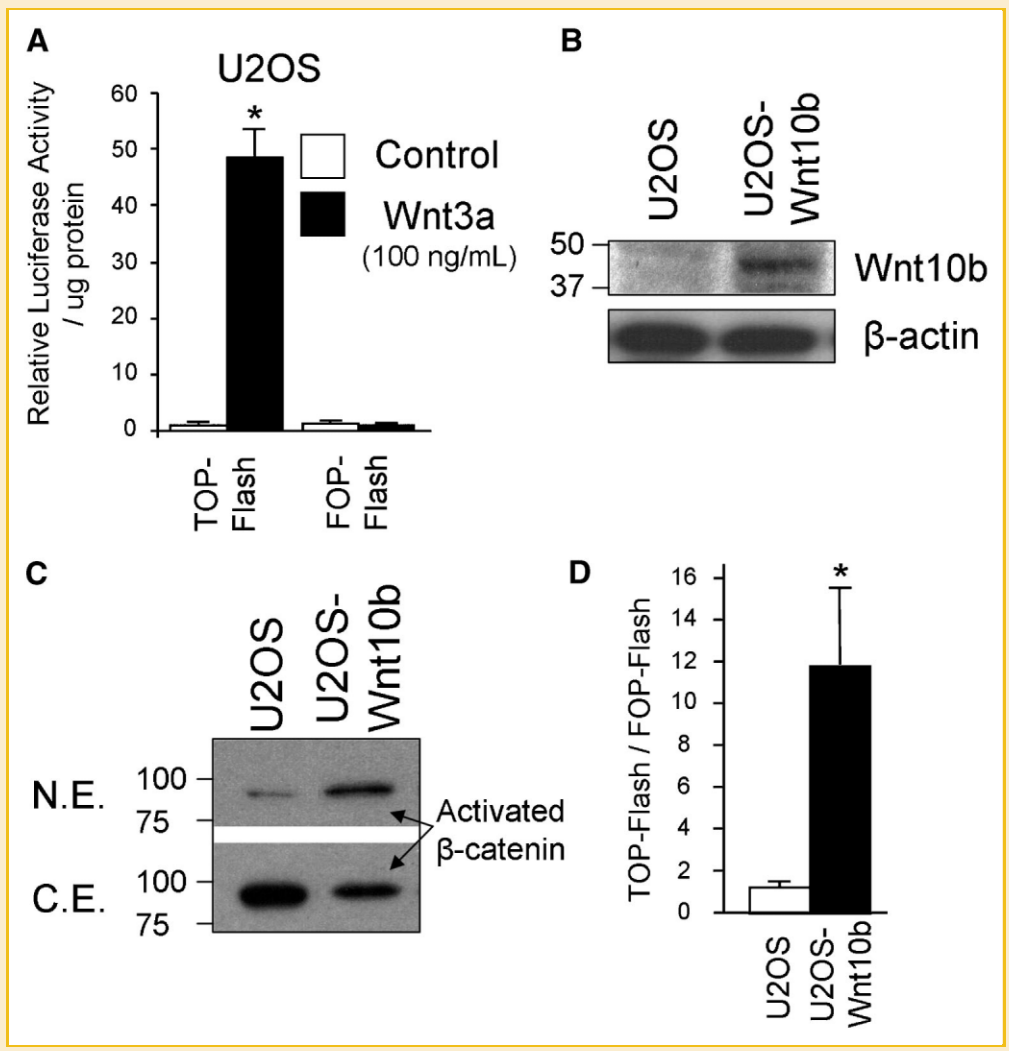

Fig. 1. Production and characterization of U2OS cells expressing Wnt10b. A: U2OS cells were transiently transfected with either TOP-Flash or FOP-Flash luciferase constructs, treated with control media or 100 ng/ml purified Wnt3a and luciferase assays performed. The bars represent mean ± SD and statistically significant differences of $P < 0.05$ (Student's *t*-test) are indicated with an asterisk. B: Twenty micrograms of total protein lysate from control U2OS and U2OS-Wnt10b cells were immunoblotted with a specific antibody directed against Wnt10b or β-actin. The molecular weight markers are indicated in kDa. C: Five micrograms of nuclear (N.E.) and 15 μg of cytoplasmic (C.E.) protein extracts from control U2OS and U2OS-Wnt10b cells were immunoblotted with a specific antibody directed against the activated, hypophosphorylated form of β-catenin. D: U2OS and U2OS-Wnt10b cells were transiently transfected as in (A) and luciferase assays performed. The TOP-Flash to FOP-Flash ratio was normalized to the U2OS cells.

Change II Site-Directed Mutagenesis Kit (Stratagene). The Wnt non-responsive FOP-Flash reporter was made in a similar manner using the mutated Tcf/Lef sequence GGCCAAAGG (the mutation is underlined).

TRANSIENT TRANSFECTION AND CONSTRUCTS

U2OS or U2OS-Wnt10b osteosarcoma cells plus 100 ng/ml doxycycline were plated at a density of 50% in 12-well plates the day before transfection. Two-hundred and fifty (250) ng TOP-Flash, FOP-Flash or pHey-1-Luc (−2,839 to +87) reporter constructs were transiently transfected (n = 3–6) using FuGENE 6 transfection reagent (Roche Diagnostics). Following incubation at 37°C for 48 h, cells were harvested in 1× Passive Lysis Buffer and equal quantities of protein extracts were assayed using Luciferase Assay Reagent on a GloMax® 96 Microplate Luminometer (Promega). Protein concentrations were determined using a BCA Protein Assay Kit (Pierce).

CONDITIONED MEDIA PRODUCTION AND ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

U2OS and U2OS-Wnt10b cells were plated at a density of 50% in T-150 flasks in serum-free DMEM/F12 and treated with 100 ng/ml Dox for 3 days. Conditioned media was collected and ELISA assays performed for TNFα and IL1α (Quantikine Human Assays, R&D Systems, interassay CV for both <9%) according to manufacturer protocol.

STATISTICAL ANALYSES

Statistical analysis was performed using a Student's *t*-test. A *P*-value of <0.05 was considered significant.

RESULTS

DEVELOPMENT OF A Wnt10b-EXPRESSING U2OS HUMAN OSTEOSARCOMA CELL MODEL

As an initial step in identifying the transcriptional targets of Wnt10b signaling in osteosarcoma, we first determined whether the U2OS cell model was capable of responding to a canonical Wnt signal. U2OS cells were transiently transfected with the synthetic β-catenin-dependent reporter plasmid TOP-Flash, or the mutated control FOP-Flash. Treatment with Wnt3a elicited a 48-fold increase in reporter activity while no increase was observed in cells transfected with FOP-Flash (Fig. 1A), demonstrating that the U2OS cell model responds to canonical Wnt signaling in a β-catenin-dependent fashion.

Since purified Wnt10b protein is not currently commercially available, we stably introduced the Wnt10b gene into U2OS cells generating the U2OS-Wnt10b cell clone. Western blot analysis confirmed expression of the Wnt10b protein (Fig. 1B). Activation of canonical Wnt signaling was confirmed through the observation that Wnt10b-dependent signaling results in the nuclear accumulation of hypophosphorylated, and therefore activated, β-catenin protein (Fig. 1C) and since U2OS-Wnt10b cells exhibited an 11.8-fold increase in TOP-Flash/FOP-Flash activity (Fig. 1D).

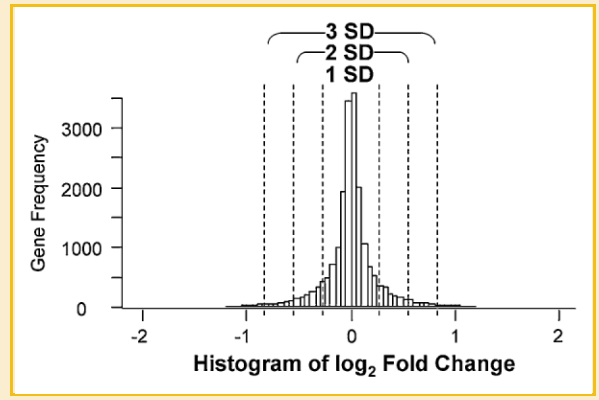

Fig. 2. Statistical categorization of differentially regulated genes in the U2OS-Wnt10b cell line. All 3,387 differentially expressed genes regulated $P < 0.05$ were categorized and displayed as a histogram of fold change in $\log_2$ scale with cutoffs at 1, 2, or 3 SD from the mean ($\log_2 = 0$).

These data demonstrate the production of a Wnt10b-expressing cell system that accurately models Wnt-dependent cellular processes, and therefore is a suitable system for the investigation of Wnt10b signaling.

MICROARRAY ANALYSIS DEMONSTRATES Wnt10b-SPECIFIC REGULATION OF GENES IN THE Wnt, Notch, AND NFκB SIGNALING PATHWAYS

To determine the transcriptional effects of Wnt10b signaling in osteosarcoma, gene expression profiling between U2OS and U2OS-Wnt10b cells was performed using Illumina microarray technology. The analysis revealed 3,387 differentially expressed genes ($P < 0.05$) of which 1,648 were upregulated and 1,739 were downregulated. The gene list was further categorized as 1 SD, 2 SD, or 3 SD from the U2OS control mean (Fig. 2 and Table I). Interestingly, the number of genes upregulated or downregulated were similar in all statistical categories, suggesting that Wnt10b signaling does not have an inherent bias for activation or repression of transcription and/or downstream effector pathways. Due to the large number of genes and to increase the strength of our analysis, we chose to focus our subsequent investigation on the 1,003 genes present in the 2 SD and 3 SD statistical categories. Fifty randomly chosen genes from this dataset were analyzed by QPCR in independent samples to validate the microarray data and regulation of 49/50 (98%) genes were confirmed (Table II), demonstrating that the microarray analysis was robust and accurate. Table III lists the most highly differentially expressed genes regulated >3.0-fold in the microarray analysis.

TABLE I. Microarray Gene Expression Changes ($P < 0.05$) in the U2OS-Wnt10b Cell Line

	1 SD from mean	2 SD from mean	3 SD from mean
Upregulated	1,139 (47.8%)	324 (51.2%)	185 (50%)
Downregulated	1,245 (52.2%)	309 (48.8%)	185 (50%)
Totals	2,384	633	370

TABLE II. QPCR Confirmation of Selected, Microarray-Identified Genes in the U2OS-Wnt10b Cell Line

Gene symbols	Gene name	Microarray fold-change[a]	QPCR fold-change[a]
ANXA10	Annexin A10	23.56	320
IL8	Interleukin 8	8.60	47.9
IGFBP7	Insulin-like growth factor binding protein 7	5.91	15.8
TAGLN	Transgelin	4.24	8.74
TGM2	Transglutaminase 2	3.79	7.48
MMP3	Matrix metallopeptidase 3	3.76	8.91
RAC2	CG8556 gene product from transcript CG8556-RA	3.13	6.28
ITGA2	Integrin, alpha 2	3.09	8.52
TLR4	Toll-like receptor 4	3.07	9.67
SEMA3A	Semaphorin 3A	2.96	28.2
EDN1	Endothelin 1	2.94	4.15
CTGF	Connective tissue growth factor	2.88	6.85
AXIN2	Axin 2	2.81	4.91
TNFRSF21	Tumor necrosis factor receptor superfamily member 21	2.80	5.37
BCL2L1	Bcl2-like 1	2.50	4.26
MSX1	Msh homeobox 1	2.46	4.53
CYP24A1	Cytochrome P450, family 24 subfamily A polypeptide 1	2.46	15.9
PPAP2B	Phosphatidic acid phosphatase type 2B	2.27	4.89
EMP1	Epithelial membrane protein 1	2.25	3.03
IL7R	Interleukin 7 receptor	2.20	3.27
CCNE1	Cyclin E1	2.17	1.37
SFRP1	Secreted frizzled-related protein 1	2.09	2.63
NKD2	Naked cuticle homolog 2	2.01	10.9
IL1α	Interleukin 1, alpha	1.95	8.72
SOX4	SRY-box containing gene 4	1.90	1.58
CDH5	Cadherin 5	1.85	3.28
NKD1	Naked cuticle homolog 1	1.87	22.2
TNFRSF14	Tumor necrosis factor receptor superfamily member 14	1.87	5.10
SOSTDC1	Sclerostin domain containing 1	1.86	44.4
COL4A2	Collagen, type IV, alpha 2	1.86	2.94
CDH11	Cadherin 11	1.84	2.66
NOTCH1	Notch 1	1.81	2.41
CD44	CD44 antigen	1.79	2.14
NOV	Nephroblastoma overexpressed gene	−1.82	−2.12
DDR2	Discoidin domain receptor family, member 2	−1.95	−3.83
ATP5O	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit	−1.97	−2.20
VWF	Von Willebrand's factor	−2.02	−8.47
THBS1	Thrombospondin 1	−2.02	−2.13
EGR1	Early growth response 1	−2.03	−2.60
OLFML3	Olfactomedin-like 3	−2.07	−2.32
MMP9	Matrix metallopeptidase 9	−2.07	−7.05
NUPR1	Nuclear protein 1	−2.15	−4.23
TGFBI	Transforming growth factor, beta-induced, 68 kDa	−2.29	1.56[b]
LY96	Lymphocyte antigen 96	−2.32	−11.6
COL5A2	Collagen, type V, alpha 2	−2.39	−3.86
CSPG4	Chondroitin sulfate proteoglycan 4	−2.40	−5.43
BCL2L13	BCL2-like 13 (apoptosis facilitator)	−2.44	−2.51
PDGFRB	Platelet derived growth factor receptor, beta polypeptide	−2.74	−3.39
LFNG	Lunatic fringe homolog	−6.54	−8.16
CXCL14	Chemokine (C-X-C motif) ligand 14	−8.27	−17.4

[a]Ratio of expression in U2OS-Wnt10b and U2OS cell lines, minus sign indicates downregulation.
[b]Microarray-identified gene which failed QPCR confirmation.

In order to place the microarray results in the proper cellular context and to further understand the cellular ramifications of Wnt10b expression in the U2OS osteosarcoma cell line, we performed pathway analysis of the 1,003 genes in the 2 SD and 3 SD statistical categories. Figure 3 displays significantly regulated ($P < 0.05$) pathways ranked in ascending P-value order as determined by the pathway analysis. Pathway analysis of the differentially expressed genes in the U2OS-Wnt10b osteosarcoma cell model highlighted pathways of diverse cellular processes such as extracellular matrix remodeling, cell adhesion, ubiquitination, and cell cycle regulation. However, identification of the Wnt, NFκB, and Notch pathways were of particular interest since they are all implicated in both normal and pathological bone metabolism [Tezuka et al., 2002; Reya and Clevers, 2005; Hilton et al., 2008; Zanotti and Canalis, 2010].

GENES FROM THE Wnt, NFκB, AND Notch PATHWAYS ARE DIFFERENTIALLY REGULATED IN THE U2OS-Wnt10b OSTEOSARCOMA CELL MODEL

Wnt10b is a known bone anabolic agent that signals through the canonical Wnt signaling cascade to activate genes involved in osteogenesis. It is perhaps no surprise that the Wnt pathway is differentially regulated in the U2OS-Wnt10b cell model; however, we wanted to confirm the regulation of endogenous Wnt gene targets to further validate our model system. A significant, 4.9-fold, upregulation of Axin-2, a classical Wnt responsive gene involved in negative regulation of canonical Wnt signaling through degradation of β-catenin [Leung et al., 2002], was observed in the U2OS-Wnt10b cell model (Fig. 4). Other differentially regulated genes implicated in Wnt signaling include CD44 (2.1-fold) [Wielenga et al., 1999], endothelin-1 (4.2-fold) [Kim et al., 2005], and Sost-dc1 (43-fold) [Itasaki and Hoppler, 2010].

TABLE III. Genes At Least 3.0-Fold Upregulated or Downregulated in U2OS-Wnt10b Cells

Gene symbol	Gene name	Microarray fold-change[a]	*P*-Value[b]
ANXA10	Annexin A10	23.56	3.97E−12
IL8	Interleukin 8	8.60	1.88E−12
TM4SF18	Transmembrane 4 L six family member 18	8.38	5.73E−12
ESM1	Endothelial cell-specific molecule 1	8.32	9.04E−12
IL1RAPL1	Interleukin 1 receptor accessory protein-like 1	7.20	1.32E−12
IGFBP7	Insulin-like growth factor binding protein 7	5.91	1.80E−11
PI3	Peptidase inhibitor 3, skin-derived	5.66	1.62E−12
KISS1	KiSS-1 metastasis-suppressor	5.14	6.72E−09
TRIML2	Tripartite motif family-like 2	4.99	1.86E−12
KRT8	Keratin 8	4.63	1.49E−10
ARHGDIB	Rho GDP dissociation inhibitor (GDI) beta	4.62	1.41E−11
TAGLN	Transgelin	4.24	4.57E−11
GNG11	Guanine nucleotide binding protein (G protein), γ11	4.09	9.15E−12
LEPROTL1	Leptin receptor overlapping transcript-like 1	4.03	8.15E−12
LOC644743	Keratin 8 pseudogene	4.00	1.06E−08
PHACTR3	Phosphatase and actin regulator 3	3.90	2.03E−10
ANGPT2	Angiopoietin 2	3.85	1.04E−10
TGM2	Transglutaminase 2	3.79	4.39E−13
MMP3	Matrix metallopeptidase 3	3.76	1.12E−09
LPXN	Leupaxin	3.74	1.10E−10
GTPBP6	GTP binding protein 6	3.61	5.08E−10
FBXL13	F-box and leucine-rich repeat protein 13	3.59	1.65E−10
DCLK1	Doublecortin-like kinase 1	3.54	5.98E−11
DCTN6	Dynactin 6	3.45	7.74E−11
C7ORF10	Chromosome 7 open reading frame 10	3.36	3.95E−12
STS-1	Ubiquitin associated and SH3 domain containing B	3.32	6.84E−10
HIST1H1C	Histone cluster 1, H1c	3.28	3.13E−09
COL13A1	Collagen, type XIII, alpha 1	3.24	7.25E−11
SLC2A3	Solute carrier family 2, member 3	3.22	1.53E−12
TMEM154	Transmembrane protein 154	3.14	6.51E−10
RAC2	CG8556 gene product from transcript CG8556-RA	3.13	1.48E−11
ITGA2	Integrin, alpha 2	3.09	6.64E−09
WDR72	WD repeat domain 72	3.07	1.63E−10
TLR4	Toll-like receptor 4	3.07	1.38E−10
EXTL3	Exostoses (multiple)-like 3	3.03	3.06E−09
LMCD1	LIM and cysteine-rich domains 1	−3.04	2.13E−09
MIAT	Myocardial infarction associated transcript	−3.06	8.59E−10
HSF2BP	Heat shock transcription factor 2 binding protein	−3.13	6.07E−12
ALDOC	Aldolase C, fructose-bisphosphate	−3.15	4.62E−11
CCDC62	Coiled-coil domain containing 62	−3.17	7.22E−11
SYT11	Synaptotagmin XI	−3.19	1.66E−11
HSD17B8	Hydroxysteroid (17-beta) dehydrogenase 8	−3.21	1.90E−09
ALOX5	Arachidonate 5-lipoxygenase	−3.26	4.07E−12
KIF1A	Kinesin family member 1A	−3.36	1.54E−10
LOXL4	Lysyl oxidase-like 4	−3.43	3.74E−13
BCYRN1	Brain cytoplasmic RNA 1	−3.47	7.21E−05
GPR132	G protein-coupled receptor 132	−3.49	1.31E−11
HAPLN1	Hyaluronan and proteoglycan link protein 1	−3.71	2.44E−12
LRRC17	Leucine rich repeat containing 17	−4.17	8.08E−11
SERPINB7	Serpin peptidase inhibitor, clade B, member 7	−4.22	2.05E−11
CT45-4	Cancer/testis antigen family 45, member A4	−4.23	1.17E−11
C7ORF41	Chromosome 7 open reading frame 41	−4.42	3.41E−13
COL6A3	Collagen, type VI, alpha 3	−5.01	1.12E−10
GALNTL1	UDP-N-acetyl-alpha-D-galactosamine/polypeptide N-acetylgalactosaminyltransferase-like 1	−5.05	1.72E−13
DHRS2	Dehydrogenase/reductase (SDR family) member 2	−5.12	1.73E−10
LMTK3	Lemur tyrosine kinase 3	−5.49	1.11E−12
C6ORF15	Chromosome 6 open reading frame 15	−5.85	2.63E−13
CXCR7	Chemokine (C-X-C motif) receptor 7	−6.05	9.61E−13
LFNG	Lunatic fringe homolog	−6.54	7.06E−11
CXCL14	Chemokine (C-X-C motif) ligand 14	−8.27	6.13E−10

[a]Ratio of expression in U2OS-Wnt10b and U2OS cell lines, minus sign indicates downregulation.
[b]Student's *t*-test.

Another significant Wnt10b-regulated pathway was the "Notch1-Mediated Pathway for NFκB Activity Modulation" (pathway 5 in Fig. 3, $P = 0.003$), suggesting both the Notch and NFκB pathways are coordinately activated by Wnt10b in U2OS osteosarcoma cells. Precedence for the interaction of the Notch and NFκB pathways exists since activated NFκB dimers are known to activate Jagged-1, a ligand for the Notch receptors, in B- and T-cells [Bash et al., 1999]. Therefore, we first focused on activation of the NFκB pathway. Viral transduction of an NFκB-luciferase reporter resulted in 3.8-fold increased activity in U2OS-Wnt10b cells (Fig. 5A), confirming the microarray and pathway analyses indicating increased NFκB activity. Similar experiments in U2OS cells using Wnt3a failed to show activation of the NFκB pathway (Fig. 5A), indicating Wnt-specificity even among other canonical Wnts. Interleukin (IL)-1α and tumor necrosis factor (TNF)-α, both classic NFκB activators [Polzer et al., 2010], were upregulated in U2OS-

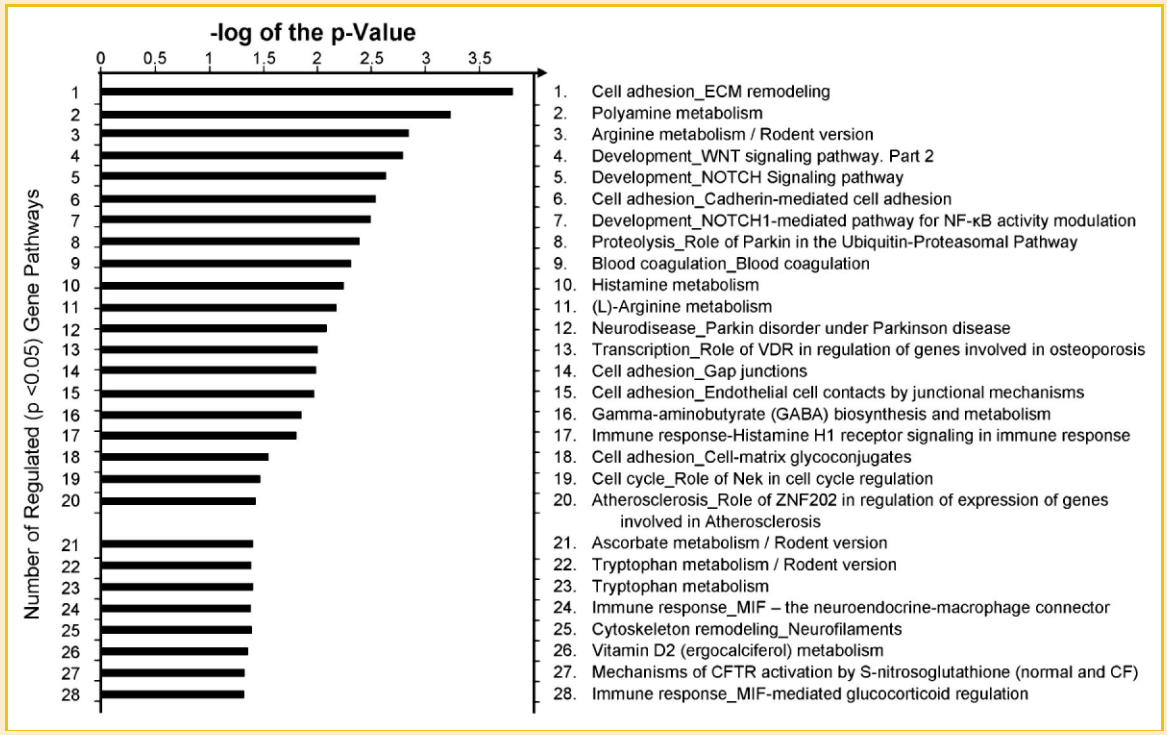

Fig. 3. Pathway analysis of differentially regulated genes in the U2OS-Wnt10b cell line. The 1,003 genes identified as differentially regulated from the 2 SD and 3 SD statistical categories were subjected to pathway analysis. The 28 regulated pathways at the $P < 0.05$ level are presented as increasing P values (−log) and MetaCore descriptions of each pathway are indicated.

Wnt10b cells 8.7- and 7.6-fold, respectively (Fig. 5B). To determine whether increased IL1α and TNFα transcripts correspond to increases in secretion and therefore possible autocrine or paracrine signaling, ELISAs of conditioned media demonstrated a statistically significant increase in both IL1α and TNFα secretion by the U2OS-Wnt10b cells (Fig. 5C). Taken together, these data indicate that Wnt10b activates the NFκB pathway possibly through the activation and secretion of TNFα and IL1α.

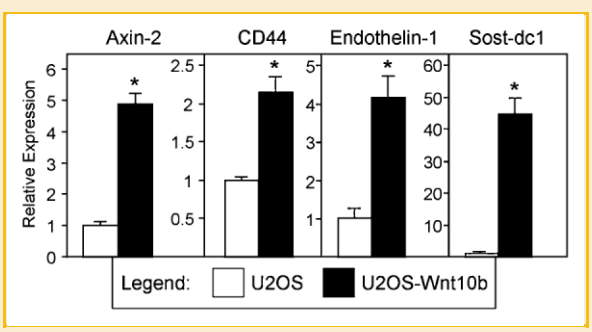

Fig. 4. The Wnt pathway is activated in U2OS-Wnt10b cells. QPCR analysis of genes in the Wnt pathway was performed in U2OS and U2OS-Wnt10b cells using primers specific for the indicated genes. The bars represent the fold change between U2OS and U2OS-Wnt10b cells ± SD and statistically significant differences of $P < 0.05$ (Student's t-test) are indicated with an asterisk.

We next focused on the Notch pathway, since pathway analysis identified two distinct gene sets involving Notch signaling (pathways 5 and 7 in Fig. 3, $P = 0.002$ and 0.003 respectively). As seen in Figure 6A, both Hes-1 and Hey-1, classic downstream targets of Notch signaling in bone and other systems [Maier and Gessler, 2000; Tezuka et al., 2002; Zanotti and Canalis, 2010], were upregulated 2.2- and 2.5-fold, respectively. Jagged-1 ligand and Notch-1 receptor were also significantly upregulated in U2OS-Wnt10b cells by 3.1- and 2.4-fold, respectively. Notch pathway activation is further supported by an 8.2-fold downregulation of lunatic fringe (Lfng), which has been previously shown to suppress Jagged-1/Notch-1 signaling [Hicks et al., 2000; Moloney et al., 2000; Chen et al., 2001]. A luciferase construct containing the mouse Hey-1 promoter region (−2,839 to +87, relative to the transcriptional start site) was transiently transfected in U2OS and U2OS-Wnt10b cells. As is observed in Figure 6B, a statistically significant, 9.1-fold activation of the reporter was observed in U2OS-Wnt10b cells, clearly confirming regulation of the Notch pathway by Wnt10b in this osteosarcoma cell model, possibly through the NFκB-dependent activation of Jagged-1, as has been previously described [Bash et al., 1999].

The inability of Wnt3a to activate the NFκB-luciferase reporter observed in Figure 5A suggested that the activation of the NFκB pathway by Wnt10b may be specific, and not a common feature of canonical Wnt signaling. Therefore, U2OS cells were treated with Wnt3a, a canonical Wnt, and QPCR performed for the Wnt10b-responsive genes described in Figures 4–6. Interestingly, only Axin-

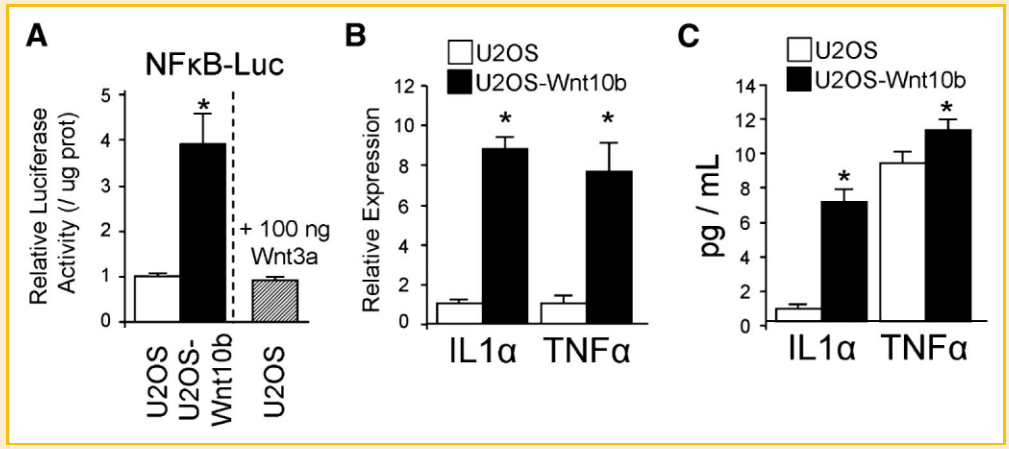

Fig. 5. The NFκB pathway is activated in U2OS-Wnt10b cells. A: U2OS and U2OS-Wnt10b cells were transduced with an NFκB-dependent luciferase reporter adenovirus. In a subset of U2OS cells, Wnt3a (100 ng/ml) was added (hatched bars). Forty-eight hours later, the cells were assayed for luciferase activity. B: QPCR analysis of U2OS and U2OS-Wnt10b cells was performed using primers specific for IL1α and TNFα. C: IL1α and TNFα in conditioned media of U2OS and U2OS-Wnt10b cells were determined using specific ELISA assays. For all panels, the bars represent mean ± SD and statistically significant differences of $P < 0.05$ (Student's *t*-test) are indicated with an asterisk.

2 was significantly regulated by Wnt3a (Fig. 7A), whereas the other Wnt-, Notch-, and NFκB-dependent genes did not respond to the Wnt3a ligand (Fig. 7A–C). This suggests that in U2OS osteosarcoma cells, the activation of the Notch and NFκB pathways may be Wnt10b-specific.

DISCUSSION

Osteosarcoma remains a significant public health concern, especially in children, and the molecular mechanisms of its pathogenesis and progression are not well understood. Recent studies have demonstrated suppression of the secreted Wnt inhibitors Srfp3 [Mandal et al., 2007] or Wif1 [Kansara et al., 2009; Rubin et al., 2010] in both human and mouse osteosarcoma models, suggesting that increased Wnt pathway activity or sensitivity may be involved in the disease process. Consistent with these observations, osteosarcomas often exhibit increased levels of intracellular β-catenin [Haydon et al., 2002], suggesting that Wnt activity is important in the etiology of osteosarcoma. Since primary osteosarcomas originate from the bone, we postulated that Wnt signaling molecules known to be expressed and secreted in the bone microenvironment are likely candidates to affect the osteosarcoma disease process. Therefore in this study, we investigated the effects of Wnt10b, a canonical Wnt signaling molecule known to have important effects in bone, on the gene expression profile of the human U2OS osteosarcoma cell model in an effort to understand how Wnts may affect osteosarcomas at the molecular level. In this

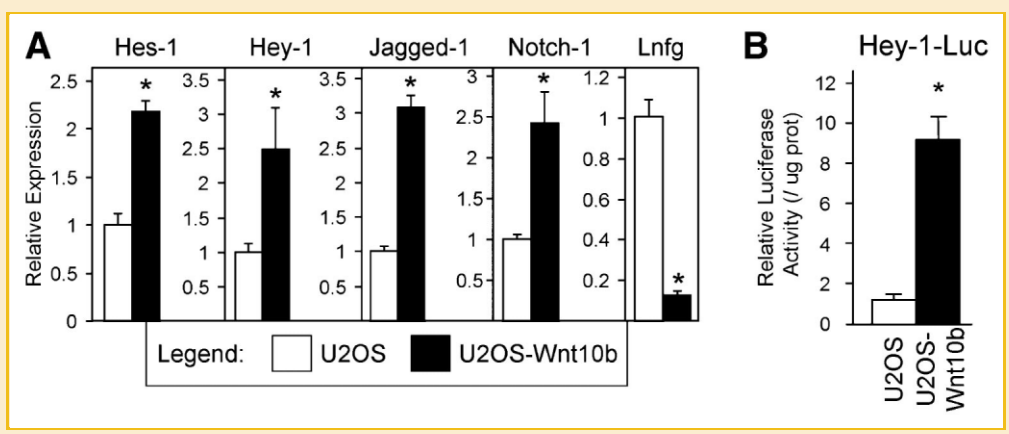

Fig. 6. The Notch pathway is activated in U2OS-Wnt10b cells. A: QPCR analysis of genes in the Notch pathway was performed in U2OS and U2OS-Wnt10b cells using primers specific for the indicated genes. B: U2OS and U2OS-Wnt10b cells were transiently transfected as in Figure 1A with a luciferase construct containing the mouse Hey-1 promoter region (Hey-1-Luc; −2,839 to +87, relative to the transcriptional start site) for 48 h and luciferase assays performed. The bars in both panels represent the fold change between U2OS and U2OS-Wnt10b cells ± SD and statistically significant differences of $P < 0.05$ (Student's *t*-test) are indicated with an asterisk.

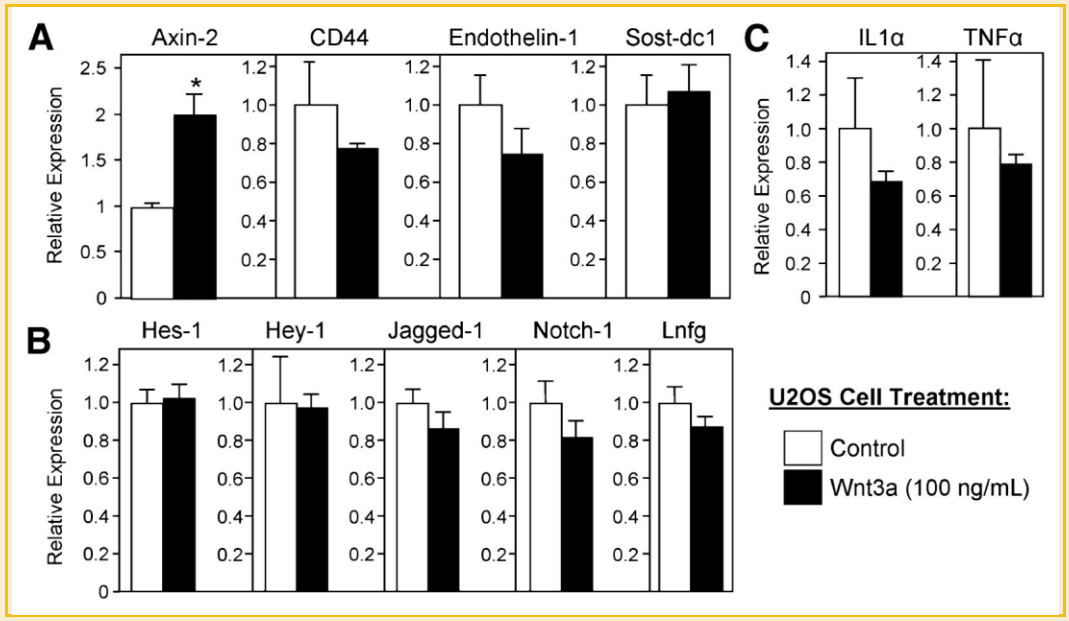

Fig. 7. Lack of activation of the Notch and NFκB pathways in U2OS cells by Wnt3a. U2OS cells were treated with Wnt3a (100 ng/ml) for 48 h and QPCR was performed using primers specific for the indicated genes for (A) Wnt targets, (B) Notch targets, and (C) NFκB targets as described in Figures 4–6. The bars represent relative expression levels ± SD and statistically significant differences of $P < 0.05$ (Student's *t*-test) are indicated with an asterisk.

report, we identified the Wnt, NFκB, and Notch pathways as significantly regulated by Wnt10b in the U2OS osteosarcoma cell model.

Recent studies have examined the gene expression profiles using microarray technology comparing osteosarcoma patients exhibiting differential responses to chemotherapy [Ochi et al., 2004; Man et al., 2005; Mintz et al., 2005; Walters et al., 2008] and human osteosarcoma xenografts treated with various chemotherapeutic agents [Bruheim et al., 2009]. Very little overlap was observed in the gene lists generated from these studies suggesting that primary osteosarcomas are most likely heterogenous in nature. However, similar functional categories of gene expression are observed, such as extracellular matrix remodeling and cell cycle regulation. Indeed, in a canine model of osteosarcoma, Selvarajah et al. [2009] found deregulation of the Wnt and inflammatory pathways mediated by cytokine signaling. Additionally, O'Donoghue et al. [2010] also demonstrated differential regulation of Wnt and NFκB targets in an independent model of canine osteosarcoma. This is in concordance with our data demonstrating Wnt10b regulation of the Wnt and NFκB pathways and the data collectively suggest that alterations in these pathways may be a common factor in the etiology and progression of osteosarcoma. A recent study found that Wnt10b is associated with increased metastatic potential resulting in poor patient prognosis [Chen et al., 2008], further supporting the role of the Wnt pathway, specifically Wnt10b, in osteosarcoma progression. The data presented herein expand the list of Wnt10b deregulated pathways to include the Notch pathway.

There is considerable data demonstrating that activation of Wnt signaling may be critical for expanding the pool of osteoprogenitor cells [Boland et al., 2004; Baksh et al., 2007]. Indeed, Stevens and colleagues recently demonstrated that Wnt10b$^{-/-}$ mice have a reduction in mesenchymal progenitor cells during aging [Stevens et al., 2010], suggesting a loss of osteoblastic precursors may be responsible for bone loss in this model. Our data suggest that Wnt10b may accomplish this function through the activation of the Notch pathway, which plays a similar role in the maintenance of mesenchymal progenitor cells through suppression of osteoblastic differentiation [Hilton et al., 2008]. Furthermore, Hilton et al. [2008] demonstrated that Notch signaling may function in this capacity by inhibiting Runx2 activity via direct interaction with the Notch-dependent factors, Hes-1 and Hey-1. This is reminiscent of our data where we observe a 2.2- and 2.5-fold induction of Hes-1 and Hey-1, respectively.

Activation of the NFκB pathway may seem counterintuitive in terms of the bone anabolic activity of Wnt10b, since NFκB classically promotes bone resorption [Boyce et al., 2005; Kaneki et al., 2006]. However, several examples exist of NFκB activation linked to bone anabolism, depending on cellular context [Iqbal et al., 2006]. Additionally, Bash et al. [1999] demonstrated that activated NFκB dimers induce the expression of Jagged-1 which subsequently activates the Notch pathway. Our data are consistent with this observation since Jagged-1 is induced 3.1-fold in our U2OS-Wnt10b model. It is tempting to speculate that Wnt10b maintains mesenchymal progenitor cells or transformed cells (e.g., osteosarcoma) in an undifferentiated state through activation of the Notch pathway via NFκB-dependent activation of Jagged-1, although further studies are clearly needed to directly test this hypothesis.

While our findings are the first to link Wnt and Notch signaling in osteoblastic cells, such a connection has previously been noted in colorectal cancer. In this disease model, tumorigenesis is dependent on β-catenin-mediated upregulation of Jagged-1 inducing Notch activation [Rodilla et al., 2009]. Although the investigators do not

mention the possible involvement of NFκB as an intermediary factor, their data does not preclude the possibility that the essential link between Wnt and Notch signaling is NFκB-mediated induction of Jagged-1, rather than a direct induction via β-catenin. Interestingly, Popivanova et al. [2008] demonstrated that blocking TNFα activity resulted in fewer intestinal tumors, demonstrating that TNFα is a crucial mediator of the initiation and progression colon carcinogenesis. Thus, it is plausible that a similar mechanism could operate in osteosarcoma, although determination of the importance of this novel pathway in osteosarcoma, as well as the requirement for Jagged-1 induction, requires further investigation.

It is of interest that Wnt3a failed to activate the NFκB and Notch pathways in our experiments, since both Wnt10b and Wnt3a are canonical Wnts, defined by dependence on nuclear translocation of β-catenin. Since signaling by these molecules elicits unique transcriptional responses, this suggests that the activation of β-catenin occurs through different cellular routes each with unique facets. One possibility is that preferential activation of the Frizzled receptors, of which 10 are identified, leads to altered cellular responses. To our knowledge, this is the first direct demonstration that two canonical Wnt molecules elicit vastly different transcriptional responses; however, the molecular mechanisms of this phenomenon remain unknown.

In summary, we determined the gene expression patterns and cellular pathways regulated by Wnt10b in the human U2OS osteosarcoma cell line using gene expression microarrays and pathway analysis. In addition to regulation of the Wnt pathway, we identified the NFκB and Notch pathways as novel downstream targets of Wnt10b signaling in the U2OS osteosarcoma model. Since both the Wnt and Notch pathways are involved in maintaining the pool of mesenchymal progenitor cells, we propose that Wnt10b functions through the canonical Wnt pathway to activate the Notch pathway as a mechanism to inhibit differentiation in osteosarcoma, perhaps leading to a more aggressive tumor. Collectively, our findings point to the need for further studies aimed at better defining the role of Wnt-induced activation of Notch signaling in the pathogenesis of osteosarcoma. Moreover, given the recently identified critical role of Notch signaling in normal bone biology [Hilton et al., 2008], the contribution of Wnt-induced Notch activation in maintaining the pool of mesenchymal progenitor cells during growth and aging warrants further investigation.

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