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Differential Regulation of the Mouse and Human *Wnt5a* Alternative Promoters A and B

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Wnt5a is an extracellular glycoprotein that activates Wnt signaling pathways, important in development and tissue homeostasis. *Wnt5a* expression is often misregulated during cancer progression. In this study, we analyzed the transcriptional regulation of two of the *Wnt5a* alternative promoters, termed A and B. Transient transfection of promoter A and B luciferase reporter constructs in to NIH3T3 and Caco-2 cells indicated that the separated promoters are both functional and that 300–450 base pair (bp) of upstream sequence is sufficient for activity. Promoter B constructs displayed distinct patterns of expression in the two cell types. The endogenous levels of promoter A-derived transcripts were found to be greater than the promoter B transcripts by four- to sixfold in fibroblast cells. Treatment of NIH3T3 cells with tumor necrosis factor (TNF)-alpha leads to an increase in both promoter A and B activities, but promoter B was more responsive. Using inhibitors of TNF-alpha effector proteins, we provide evidence that the transcription factor nuclear factor-kappaB and the MEK1/2 and p38 kinases have distinct roles in determining the activity levels of promoters, A and B. These results support the conclusion that *Wnt5a* promoters, A and B, are differentially regulated and provide a model for complex transcriptional regulation of *Wnt5a*.

Introduction

Wnt5a is a lipid-modified glycoprotein belonging to the Wnt family of secreted protein ligands. Wnt5a binds to distinct membrane receptors of the Fz family, along with the Ror2 coreceptor, and activates the noncanonical Wnt signaling pathways, PCP/CE and Ca^{2+} (reviewed in Kikuchi *et al.*, 2011). In some cells, depending on the receptor context, Wnt5a has also been shown to block or activate the canonical β-catenin pathway (Mikels and Nusse, 2006; McDonald and Silver, 2009). Wnt5a has important functions during development, particularly in events that require cell movement and rearrangements (Yamaguchi *et al.*, 1999; Hardy *et al.*, 2008; Blakely *et al.*, 2011). Wnt5a also functions in postnatal cellular differentiation and tissue homeostasis. In particular, Wnt5a has a role in hematopoiesis (Liang *et al.*, 2003) and mesenchymal stem cell differentiation (Yang *et al.*, 2003; Bilkovski *et al.*, 2010). Mechanistically, Wnt5a-initiated cell signaling leads to altered cell function through rearrangements of the cytoskeleton and/or changes in gene expression (reviewed in Kikuchi *et al.*, 2011).

Significantly, *WNT5A* expression is altered in numerous cancers, including lung (Huang *et al.*, 2005), breast (Iozzo *et al.*, 1995; Pukrop *et al.*, 2006), thyroid (McCall *et al.*, 2007), osteosarcoma (Nakano *et al.*, 2003; Enomoto *et al.*, 2009), gastric (Saitoh *et al.*, 2002), colorectal (Ying *et al.*, 2008; Hibi *et al.*, 2009), leukemias (Martin *et al.*, 2010; Deng *et al.*, 2011), skin (Taki *et al.*, 2003; Weeraratna *et al.*, 2010), pancreatic (Ripka *et al.*, 2007), melanoma (Iozzo *et al.*, 1995; Weeraratna *et al.*, 2002), esophageal (Li *et al.*, 2010), and prostate (Wang *et al.*, 2007). *WNT5A* has been found to be both overexpressed and downregulated. *WNT5A* overexpression has been associated with metastatic behavior in various cancers (Weeraratna *et al.*, 2002; Kurayoshi *et al.*, 2006; Ripka *et al.*, 2007; Enomoto *et al.*, 2009).

And, upregulation of *WNT5A* expression has been found to induce an epithelial–mesenchymal transition in melanoma cells (Dissanayake *et al.*, 2007). Clearly, understanding of the mechanisms regulating *WNT5A* expression is of particular importance.

Current published data indicate that changes in *WNT5A* expression during cancer progression do not involve genetic (DNA) changes, such as gene mutation and rearrangements, but rather nongenetic changes. Hypo- and hypermethylation of the *WNT5A* gene has been detected in tissue derived from diverse tumor types. Hypermethylation of Wnt5a is more common and has been detected in the early stages of colorectal cancer (Ying *et al.*, 2008; Hibi *et al.*, 2009), myeloid and acute lymphoblastic leukaemia (Roman-Gomez *et al.*, 2007; Martin *et al.*, 2009) and esophageal squamous cell carcinoma

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(Li *et al.*, 2010). In contrast, Wnt5a was found to be hypomethylated in prostate cancer tissue (Wang *et al.*, 2007).

Alternatively, there is evidence that *WNT5A* upregulation in certain cancers involves changes in specific signaling pathways and transcription factors. In two studies, the drug phenylmethimazole, which inhibits the Toll-like receptor 3, also coordinately decreased the level of *WNT5A* mRNA in papillary thyroid carcinoma (McCall *et al.*, 2007) and pancreatic cancer and melanoma (Schwartz *et al.*, 2009), leading to decreased cell proliferation and migration. Similarly, the CUTL1 transcription factor, which is a target of transforming growth factor β and when overexpressed enhances cancer cell motility and invasiveness (Michl *et al.*, 2005), increases *WNT5A* transcription (Ripka *et al.*, 2007). The nuclear factor (NF)-kappaB has also been implicated in the regulation of *WNT5A* transcription (Saitoh and Katoh, 2002; Ge *et al.*, 2011; Rauner *et al.*, 2011). NF-kappaB is known to mediate the expression of many genes that influence growth and inflammation and is often upregulated in cancer (Basseres and Baldwin, 2006).

The human and mouse *Wnt5a* gene region generates transcript variants derived from distinct transcription start sites and alternative splicing (see Table 1 and Katoh and Katoh, 2009). Little is known regarding the differential regulation of the Wnt5a alternative promoters. It is likely, however, that distinct patterns of *Wnt5a* expression can be achieved through the activity of gene regulatory proteins that affect the transcription from one, but not the other promoter and that altered *Wnt5a* transcript levels, particularly in cancer cells, can be achieved via multiple pathways involving these distinct promoters. Indeed, the alternative promoters of various mammalian genes have been found to display distinct activities at particular developmental stages, in specific tissue types, and in cancer cells (Liang *et al.*, 2005; Oh *et al.*, 2005; Banday *et al.*, 2011; Bee *et al.*, 2011; Demura *et al.*, 2011). In this study, we focused on two of the *Wnt5a* alternative promoters that are common in mouse and human to address the question of Wnt5a differential promoter regulation. The genomic upstream regions of these promoters were separately cloned for individual analysis and promoter-specific transcript levels were quantified by quantitative real-time (qRT)-PCR. We focused on the general activity of the separated promoters and on the response of each promoter to tumor necrosis factor (TNF)-alpha-induced cell signaling and the role of NF-kappaB and other effector proteins in the response to TNF-alpha.

Materials and Methods

Cell lines and culture

NIH3T3 mouse fibroblasts were cultured in the Dulbecco's Modified Eagle's Medium (DMEM) and supplemented with 10% calf serum (Atlanta Biologicals, Lawrenceville, GA) and 1% penicillin/streptomycin (5000 I.U./mL and 5000 μg/mL). Caco-2, a human colorectal adenocarcinoma-derived cell line, was grown in the RPMI 1640 medium containing 10% fetal bovine serum (Gibco, Carlsbad, CA) and 1% penicillin/streptomycin. GM03349 cells are normal human dermal fibroblast cells obtained from the National Institute of General Medical Sciences (NIGMS) Human Genetic Mutant Cell Repository (Corriell, Camden, NJ). They were grown in the Minimum Essential Medium (MEM; Mediatech, Inc., Manassas, VA), supplemented with 10% fetal bovine serum (Gibco), 2 mM L-glutamine, and 1% penicillin/streptomycin. All cell types were cultured in a 37°C and 5% carbon dioxide humidified incubator.

Table 1. Comparison of Mouse and Human *Wnt5a* Genes

	Mouse	*Human*
Location	Chromosome 14 29,317,936–29,340,633 Forward	Chromosome 3 55,499,743–55,523,973 Reverse
Number of transcripts[a]	6	8
Transcript size (name and ID)[b]	7009 bp (Wnt5a-001;ID-063465) 3650 bp (Wnt5a-002;ID-112272)	6042 bp (WNT5A-201;ID474267) 1299 bp (WNT5A-005;ID497027)
Proteins produced[a]	2	6
Protein Length (AA) and ID[c]	380 residues (ID-064878) 360 residues (ID-107891)	380 residues (ID-417310) 365 residues (ID-420104)
N-terminus of protein[b,d]	*MKKPIGILSPGVALGTAGGA (20)* MSSKFFLMALATFFSFAQVV…	*MKKSIGILSPGVALG (15)* MAGSAMSSKFFLVALAIFFS…
Exons and introns (bp)[b,e]	*Wnt5a*-001 Exons: 1365, 134, 251, 293, 4966 Introns: 5706, 1184, 4894, 3903 *Wnt5a*-002 Exons: #1–19, #5–2953 Intron: #1–399	*WNT5A*-201 Exons: 324, 134, 251, 293, 4835 Introns: 6061, 1220, 4684, 3786 *WNT5A*-005 Exons: #1b-63, #5–558 Introns: #1–412

Source: Ensembl Mouse *Wnt5a* ENSMUSG00000021994 and Human *WNT5A* ENSG00000114251.

[a]Total number of transcripts or proteins generated from the *Wnt5a* genomic region.

[b]For the two mouse and human transcripts analyzed in this study, Transcript ID is preceded by ENSMUST000000 for mouse or ENST00000 for human.

[c]Derived from the two transcripts analyzed. ID is preceded by ENSMUSO00000 for mouse or ENSP00000 for human.

[d]Italicized AA sequence and the number in parenthesis indicate the additional AA's and N-terminus on the longer transcript ([b]). The AA sequence of the longer transcript includes all the AA's shown and is continuous. Bottom sequence includes the N-terminus and first 20 AA's of the shorter transcript ([b]).

[e]Only the unique exons and introns for the shorter transcript are included; all others are identical to the longer transcript.

WNT5A *promoter A and B luciferase reporter constructs*

Sequences upstream of the human *WNT5A* alternative transcription start sites referred to as *h*promoter A and *h*promoter B (see Fig. 2) were PCR amplified from purchased human genomic DNA (Promega, Madison WI). *Hin*dIII restriction sites were included on the primers for the *h*promoter A sequences and *Bgl*II sites for the *h*promoter B sequences and used for cloning of the PCR fragments into the vector, pGL4.17 [luc2/Neo] (Promega). Five constructs were generated for the *h*promoter A containing 2178, 1707, 1358, 773, 420 bp upstream of the end of the cDNA as defined in NM_003392 (Fig. 2). Four constructs were generated for the *h*promoter B containing 1981, 1257, 817, 356 bp upstream of the cDNA defined in AK290869 (Fig. 2). The primers used to generate the *h*promoter A and B constructs are shown in Table 2. The clones were verified by DNA sequencing.

Putative transcription factor binding site identification

Genomic sequences included in the luciferase reporter construct, p2178 and p1981 (see Fig. 2), were analyzed for a select group of transcription factors (NF-kappaB, CutL1, Pax2, AP-1, STAT3, c-Fos, Sp1, and c-Myb) with the online analysis program PROMO, which uses the TRANSFAC database, and MacVector (MacVector, Inc., Cary, NC) nucleic acid subsequence analysis. Only those sites that were at least 95% similar to the consensus sequence were mapped on to the sequence. Transcription factors with shorter consensus sequence binding sites generated a greater number of potential sites than those with longer sequences.

Transient transfections and dual luciferase assays

NIH3T3 cells (6×10^4 cells/well) and Caco-2 cells (4×10^4 cells/well) were plated in 24-well plates and incubated for 24 h. The *h*promoter A and B constructs (0.5 μg) and the parental plasmid pGL4.17 (0.5 μg) were transiently transfected into NIH3T3 cells using TransIT-LT (Mirus, Madison, WI) and Caco-2 using Nanojuice (Novagen, San Diego, CA), along with 10 ng of a *Renilla* control vector (phRL-SV40; Promega, Madison, WI) according to instructions. Each construct was transfected into four separate wells of cells. After 48 h, the media was removed and the cells were washed with 1× phosphate-buffered saline (PBS). 1× Passive Lysis Buffer (Promega) was added and the samples incubated at 37°C for 15 min and stored at −80°C. Samples were assayed for firefly and *Renilla* luciferase activity on a Synergy 2 multimode microplate reader (BioTek, Winooski, Vermont) using the Dual-Luciferase Assay System (Promega). Promoter activity was expressed as the average value of the ratio

Table 2. Primer and Probe Sequences for PCR and qRT-PCR

Primers for Wnt5a *reporter constructs*	*Primer sequences*
*h*promoter A	
p2178	F-GCTAAGCTT/GCGTGTGTGTGTCTTCACCTTG
p1707	F-GCTAAGCTT/CAGTGGGAGCGGTCTGGGATACGC
p1358	F-GCTAAGCTT/GAGGGAGAGAGTAAGGCAGTTGG
p773	F-GCTAAGCTT/CTCACCGCACAGCAATAAGTTCCGG
p420	F-GCTAAGCTT/CGCCAAGAGTTTGCAGAATCTGTGG
	R-GCTAAGCTT/GAACCGGAGCTGAAGCGGGCACTGGCG
*h*promoter B	
p1981	F-GCTAGATCT/TTGCACTGGGATTGAAGAGGGAAGA
p1257	F-GCTAGATCT/TCTTTGAGAAGCCCCGAAGCGTCCA
p817	F-GCTAGATCT/GGAATCGATGCGCCCAGCTGCGGCTCG
p356	F-GCTAGATCT/CCAGTTTCCTTGGTTGGGAGACCCGA
	R-GCTAGATCT/TTGATTGACTGCGCTTCTCCTCCGT
Primers and probes for qRT-PCR	*Primer sequences*
*h*promoter A	
Forward	TCGGGTGGCGACTTCCT
Reverse	CAACTCCTGGGCTTAATATTCCAAT
Probe	CGCCCCCTCCCCCTCGCCATGAAG
*h*promoter B	
Forward	CCTCTCGCCCATGGAATT
Reverse	GGGCTTAATATTCCAATGGACTTC
Probe	CTGGCTCCACTTGTTGCTCGGCC
*m*promoter A	
Forward	GTGGCGACTTCCTCTCCGT
Reverse	CGGTCCCCAAAGCCACT
Probe	CCCCTCGCCATGAAGAAGCCCA
*m*promoter B	
Forward	ACTTGTTGCTCCGGCCC
Reverse	CGGTCCCCAAAGCCACT
Probe	AGAAGCCCATTGGAATATTAAGCCCGG

All primers are shown 5′ to 3′. The diagonal lines demarcate the added extra nucleotides with restriction sites from actual primer sequence. Reverse primers (R) were paired with each of the associated forward (F) primers.

of firefly luciferase to *Renilla* luciferase activity for the four replicas. The transfection was repeated three independent times. The fold-change relative to the longest construct was determined for each independent trial. These values were averaged and the standard error and statistical significance determined using the Student *t*-test.

Stable WNT5A h*promoter A and B cells and compound treatments*

NIH3T3 cells were cotransfected with *WNT5A h*promoters A p2178 or *h*promoter B p1981 reporter constructs and the selection vector pMC1neo Poly A (Agilent Technologies, Santa Clara, CA) at a mass ratio of 4:1 using the GenPORTER transfection reagent (Genlantis, San Diego, CA). Three days after transfection 500 μg of geneticin bisulfate (G418) was added. Following initial cell death, the resistant cells were replated in a medium with G418 and after 3 or 4 weeks, cells were collected as a group and expanded once more before being stored in liquid nitrogen.

For treatment experiments, p2178 and p1981 NIH3T3 cell lines were plated in 48-well plates at 2×10^4 cells per well and allowed to grow 1 day. The cells were treated with 5 ng/mL TNF-alpha, with and without a NF-kappaB inhibitor (JSH-23, 30 μM), MAP (mitogen-activated protein) kinase/ERK kinase (MEK) 1/2 inhibitor (U0126, 10 μM), mitogen-activated protein kinase p38 inhibitor (SB203850, 10 μM), and Jun N-terminal kinase (JNK) inhibitor (SP600125, 20 μM) for 6 and 12 h. Cells were also treated with the same concentration of inhibitors, but without TNF-alpha. Control cells were treated with dimethyl sulfoxide (DMSO) only. All wells contained the same concentration of DMSO. The cells were collected by removing the media and washing with 1× PBS. 100 μL of 1× Cell Lysis Buffer (Promega) for 15 min at 37°C. 20 μL of lysate from each sample was added to a black-sided 96-well plate (Corning, Inc., Corning, NY) to be assayed for luciferase activity on a Synergy 2 multimode microplate reader using the Luciferase Assay System (Promega). Each study was independently replicated three to four times. Standard error values were determined and significance using the Student *t*-test ($p<0.05$) for replicas.

Verification and amplification efficiencies of custom TaqMan primer–probe sets

Amplification (PCR) products from TaqMan qRT-PCR using the mouse and human promoter A and B custom primer–probe sets were analyzed on a 1% DNA agarose gel to determine the product size. The PCR products were then gel purified and quantified by reading the absorbance at 260 nm. An initial qRT-PCR amplification was conducted to adjust the cycle numbers. A 5-log dilution series of the purified PCR products, including five points, was prepared based on the test amplification such that the resulting cycle numbers were between 10 and 32. Each dilution was amplified in triplicate using the appropriate primer–probe set. The replica values for each concentration were averaged. A graph of concentration versus the average cycle number was generated and the slope of the line determined. The efficiency value (E) was calculated using the equation $E=10^{(-1/\text{slope})}-1$. Efficiency values between 90% and 100% with an established standard deviation value of ±10% are considered acceptable (Applied Biosystems, 2006).

RNA isolation and quantitative real-time PCR

For comparison of promoter A and B transcript levels in mouse and human fibroblast cells, NIH3T3 cells and GM03349 cells were grown to near 80% confluency in 100-mm plates. RNA was isolated from the cells as described below. For analysis of transcript levels in TNF-alpha-treated cells, NIH3T3 cells were plated in 100-mm plates and either untreated or treated with TNF-alpha and various inhibitors as previously described. The total RNA was isolated from the cells using the SV Total RNA Isolation Kit (Promega). 1–3 μg of RNA was converted to cDNA using the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA) or the Maxima Reverse Transcriptase (Fermentas, Glen Burnie, Maryland). qRT-PCR was conducted using the Applied Biosystem TaqMan Assay (Applied Biosystems, Foster City, CA). For NIH3T3 cells, primers used for amplification included mouse cyclin D1 (Mm00432358) and custom-made primer–probes that amplified mouse *Wnt5a* transcripts derived specifically from *m*promoter A and *m*promoter B (Table 1). For the human GM03349 cells, human cyclin D1 (Hs00765553) and custom-human Wnt5a primer–probes that amplified transcripts derived from *h*promoter A and *h*promoter B were used (Table 1). Each amplification set included the internal control primer for GAPDH (mouse-Mm03302249_g1; human- Hs99999905). qRT-PCR was conducted on the Applied Biosystems StepOne Real-Time PCR System thermal cycling block. Amplification conditions were 95°C for 15 s and 60°C for 1 min for 40 cycles. Amplifications were in triplicate for each sample and primer set. The C_T ($\Delta\Delta C_T$) value was determined for each cDNA sample and primer set, in comparison to the internal control, and relative change determined.

Results

Comparison of mouse and human Wnt5a *genes*

We compared the mouse and human *Wnt5a* gene regions using data provided by Ensembl (Flicek *et al.*, 2012) (Table 2 and Fig. 1). Multiple transcripts are generated from the mouse and human *Wnt5a* genes. Of the 6 mouse transcripts, only two generate proteins and these transcripts were selected for analysis (Fig. 1). In contrast, 6 of the 8 human

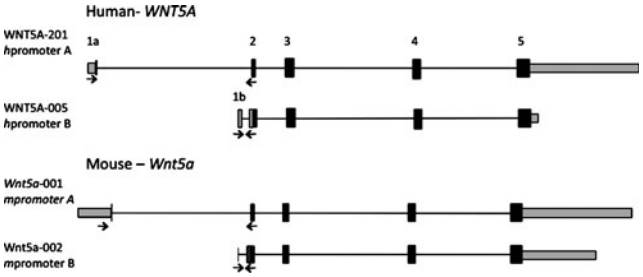


FIG. 1. Genomic structure of selected human and mouse *Wnt5a* transcripts. These structures were derived from Ensembl: Mouse *Wnt5a* ENSMUSG00000021994 and Human *WNT5A* ENSG00000114251. Coding exon regions are shown as black boxes. Gray boxes are 5′ or 3′ untranslated regions. Lines are intron sequences. The sizes are proportional to sequence length. The exons are numbered for the human transcripts. The arrows indicate the relative position of custom primers for quantitative real time (qRT)-PCR used to detect the specific transcripts.

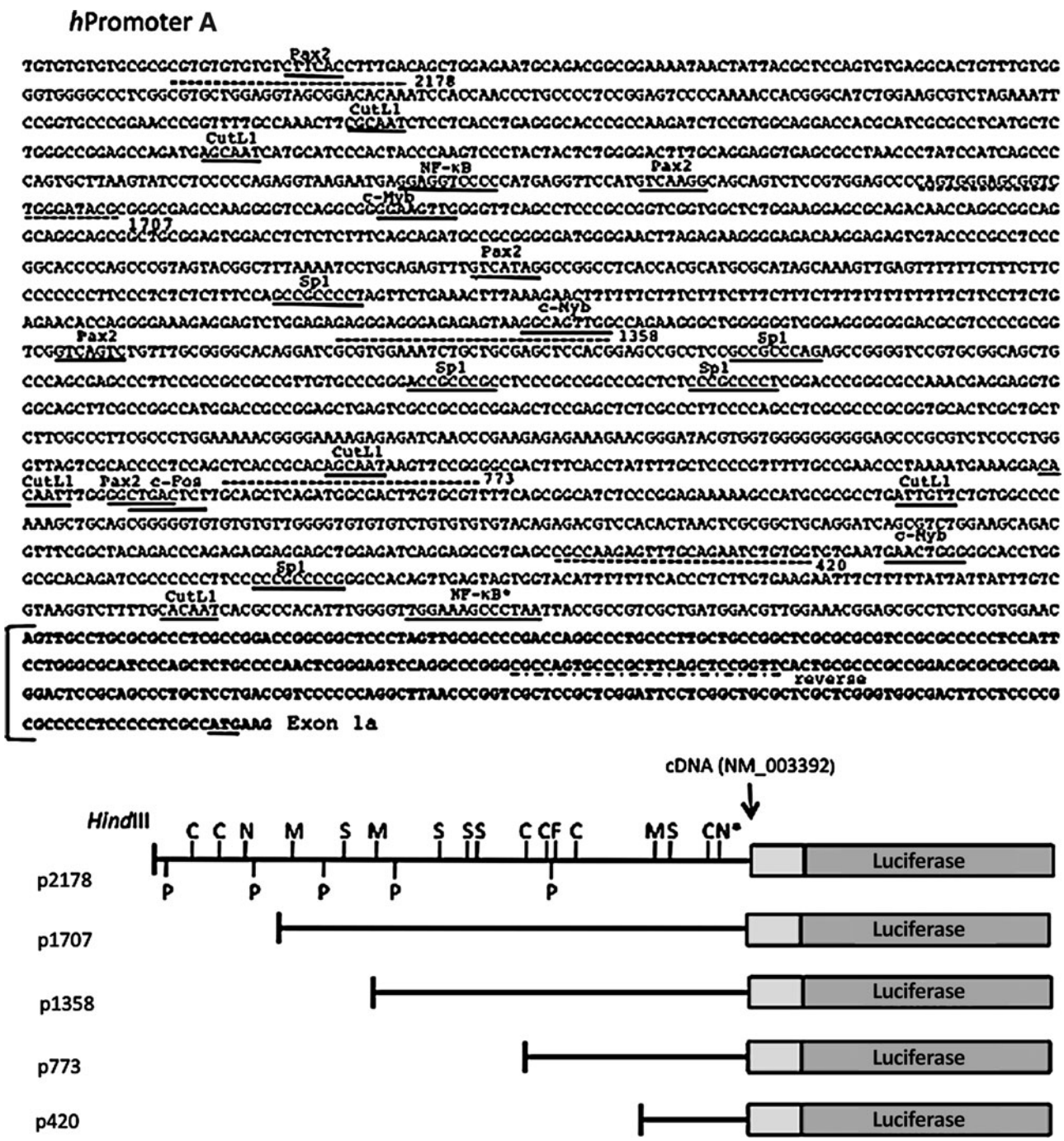


FIG. 2. Human *WNT5A* *h*promoter A and *h*promoter B luciferase reporter constructs. Genomic sequences: The included genomic sequences for the p2178 and p1981 constructs are shown. Putative transcription factor binding sites with names are underlined. Some sites are on the opposite strand. Dotted lines indicate the positions of the PCR primers used to generate the luciferase reporter constructs. Exon sequences are shown in bold and the ATG start codons are underlined. Luciferase reporter constructs: The *WNT5A* upstream sequences are represented by the black line. The numbers are base-pair (bp) upstream from the first nucleotide of the cDNA, indicated with the NCBI accession number. The gray boxes are sequences downstream of the first nucleotide. The indicated *Hin*dIII and *Bgl*II sites were used for cloning. The *h*promoter B constructs include a 412-bp intron, unique to the *h*promoter B-derived transcript. The vector sequences are not to scale. Positions of the selected transcription factor binding sites are indicated. A, AP-1; C, CutL1; F, c-Fos; M, c-Myb; N, nuclear factor (NF)-kappaB; P, Pax2; S, Sp1. NF-κB* is the site identified in Ge *et al.*, (2011).

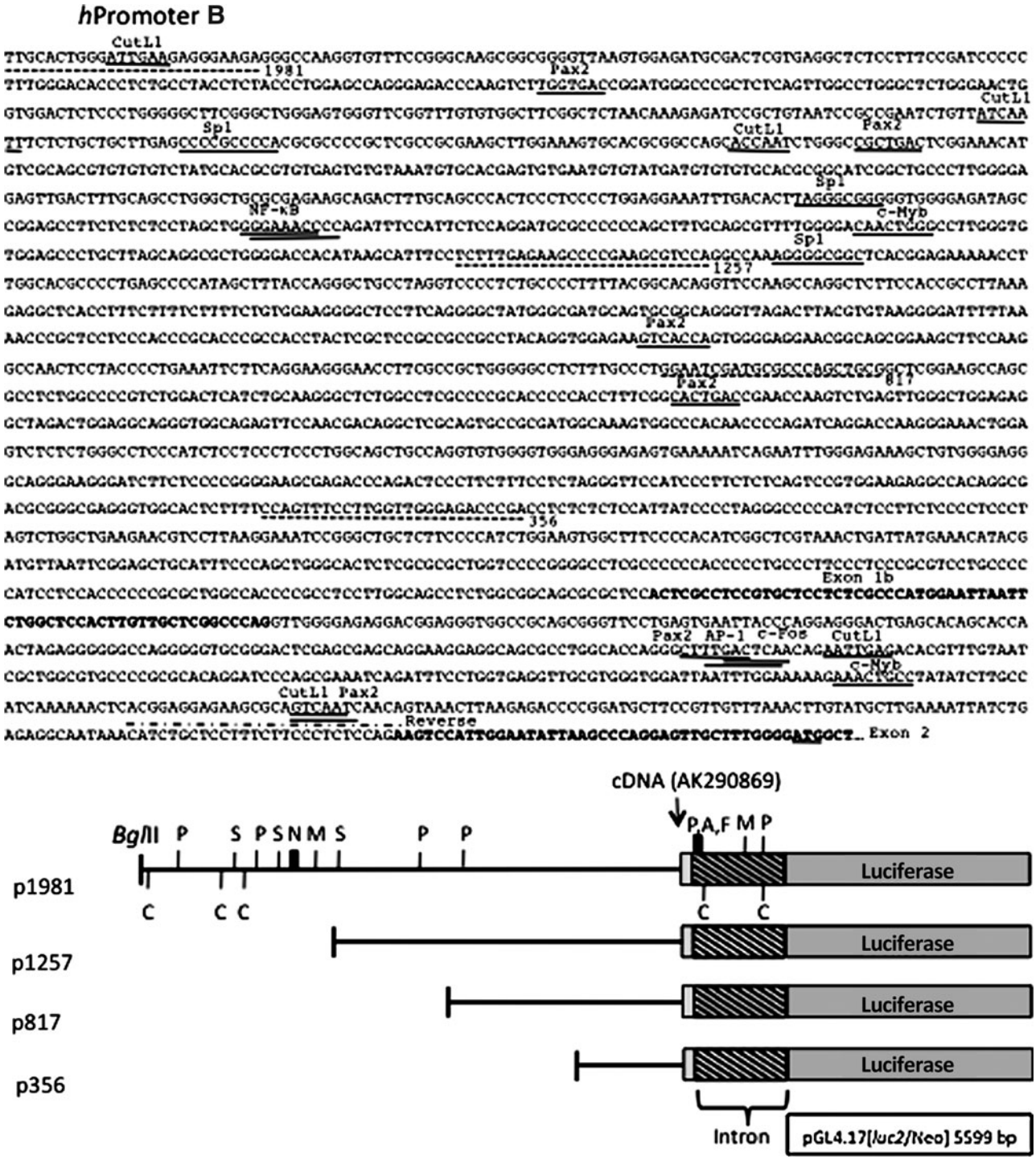


FIG. 2. (Continued).

WNT5A transcripts produce proteins. These 6 transcription units have unique transcription start sites (Ensembl ENSG000001142510).

We selected for analysis the two human transcripts that share similarity to the mouse transcripts (Fig. 1). The different transcripts include distinct first exons; the longer human transcript (*WNT5A*-201) includes exon 1a, whereas the shorter transcript (*WNT5A*-005) includes exon 1b. These transcripts share exons 2, 3, 4, and a portion of 5. *WNT5A*-201 has a longer 3′ untranslated regions. The *WNT5A*-201 and -005 transcripts have different starts of translation. The alternative transcription start site for the shorter mouse transcript (002) is in a similar relative genomic location, in comparison to the human transcript (005). One distinction is that the mouse transcript (001) has a larger exon 1 in comparison to the human exon 1a. The proteins generated from the longer transcripts include an extra 20 AA's on the N-terminus for the mouse and 15 AA's for the human (Table 2). It is important to

note that the longer human transcript *WNT5A*-201 is associated with the cDNA RefSeq NM_003392, which is the transcript most often studied.

In this study, we refer to gene regulatory sequences associated with the long and short Wnt5a transcripts as promoter A (long) and promoter B (short) and a qualifying *h* for the human gene and *m* for the mouse. The promoter A and B designation was adopted by Katoh and Katoh (2009) in their comparison of the Wnt5a genomic regions of human, chimpanzee, mouse, and rat.

Transient transfection of WNT5A h*promoter A and B constructs*

To examine the activity and regulation of the separated human *WNT5A* alternative *h*promoters A and B, different amounts of upstream regions for each promoter were cloned into a luciferase reporter vector (Fig. 2). The genomic sequences included in the longest constructs (p2178 and p1981) are shown. Putative binding sites for a select group of transcription factors are positioned on the sequence and mapped on the constructs. These may or may not represent functional sites. The *h*promoter B construct p1981 includes 2337 bp of the *h*promoter A 6061 bp intron 1.

The constructs in Figure 2 were transiently transfected into NIH3T3 and Caco-2 cells, along with a *Renilla* luciferase control vector. After 48 h, the cells were assayed for both firefly and *Renilla* luciferase activity. The fold-change relative to the longest construct (p2178 for *h*promoter A and p1981 for *h*promoter B) was graphed (Fig. 3). *h*Promoter A and B constructs are active in both cell types at relatively the same level, although the *h*promoter B appears more active in Caco-2 cells. A low level of activity was not detected for any of the *h*promoter A and B constructs, indicating that 425 bp (*h*promoter A) and 356 bp (*h*promoter B) of sequence are sufficient for transcriptional initiation and a relatively high level of activity.

The *h*promoter A constructs displayed a similar pattern of activity in both NIH3T3 and Caco-2 cells (Fig. 3). A significant increase in activity was measured comparing the p2178 and p1707 constructs. This indicates that a negative acting sequence is contained in the removed sequences. A putative NF-kappaB site is missing from the p1707 construct (Fig. 2). Activity dropped for the p1358 and p773 constructs, both in NIH3T3 and Caco-2 cells, suggesting removal of positive acting sequences. In NIH3T3 cells, an increase in activity was observed for the p425 construct, suggesting removal of a negative acting sequence.

The *h*promoter B constructs displayed distinctive patterns of activity in NIH3T3 and Caco-2 cells (Fig. 3). In NIH3T3 cells, the expression levels from the four constructs were not significantly different. In the Caco-2 cells, there was increasing activity as more of the *h*promoter B upstream sequences were removed with maximal activity (about a 3.5-fold increase) measured with the shortest construct, p356. These data indicate the presence of negative acting sequences that are functional in Caco-2 cells, but not NIH3T3.

Endogenous promoters A and B are active in human and mouse fibroblastic cells

The transient transfection data show that both *h*promoter A and *h*promoter B are active when assayed separately. We

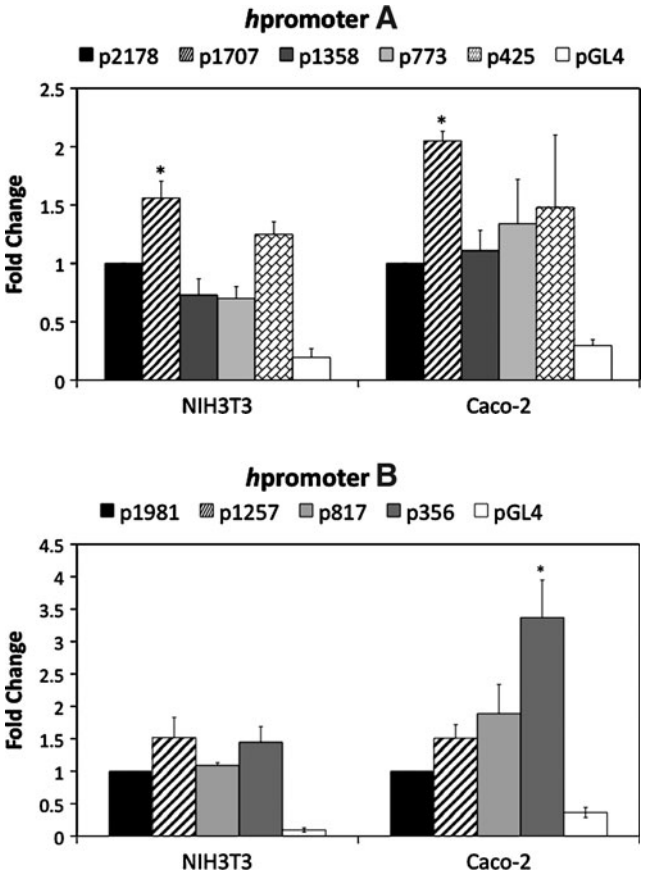
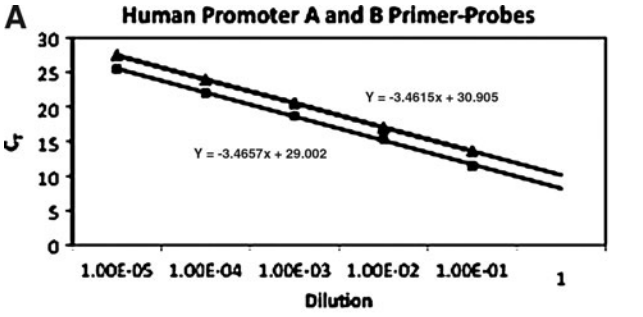


FIG. 3. Transient transfection of *h*promoter A and *h*promoter B constructs into NIH3T3 and Caco-2 cells. The constructs shown in Figure 2 and the empty vector pGL4-17 were transiently transfected into the cells, along with a *Renilla* control vector. The ratio of firefly to *Renilla* luciferase was determined for four replicas and fold-change determined relative to the longest constructs, p2178 and p1981. The mean fold-change for three or four independent transfections is shown. The bars are ± S.E.M. The asterisks indicate significance ($p < 0.05$) in comparison to the longest construct.

designed TaqMan primer–probe sets specific to the human and mouse promoter A- and B-derived transcripts to determine the level of endogenous promoter-specific transcripts (Table 1). The amplified product flanks the exon 1 and exon 2 splice junction. The TaqMan forward primer and probes for the *h*promoter A and *h*promoter B transcripts are homologous to sequences in the unique exon 1a and 1b, respectively, and the reverse primer in exon 2 (Fig. 1). The mouse primer–probe sets are similar with the exception that for the *m*promoter B transcript, the probe is located entirely in exon 2. Amplification with these primer–probe sets generated products of the expected length (Fig. 4C). The amplification efficiencies of the primer–probe sets were determined to be between 90% and 100% (Fig. 4A, B), as recommended by Applied Biosystems (2006).

The level of endogenous promoter A- and B-specific transcripts were determined by qRT-PCR using our verified primer–probe sets in mouse (NIH3T3) and human (GM03349) fibroblast cells. The threshold cycle numbers for the promoter A- and promoter B-specific amplifications were compared.



B Primer-probe pair amplification efficiency values

	Human	Mouse
Promoter A	94.5 ±10 %	98.50 ±10 %
Promoter B	94.3 ±10 %	92.0 ±10 %

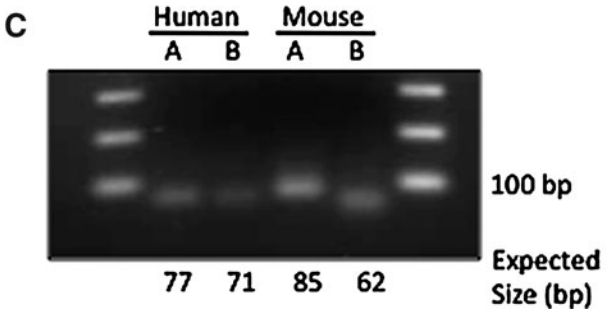


FIG. 4. Verification and amplification efficiencies of *Wnt5a* promoter A and B TaqMan primer–probe sets. **(A)** Purified human and mouse promoter A- and B-specific PCR products were diluted as indicated and used for TaqMan amplification with each primer–probe set. C_T (cycle number) values were plotted. Squares are promoter A primer–probes; triangles are promoter B primer–probes. The slopes of the lines were determined and used to calculate the amplification efficiencies shown in **(B)**. **(C)** DNA agarose gel of mouse and human promoter A and promoter B PCR products, showing the expected product sizes.

Cycle numbers for NIH3T3 were 26.34, 28.31, and 29.27 for total Wnt5a, *m*promoter A, and *m*promoter B transcripts, respectively. Cycle numbers for GM03349 cells were 28.59, 28.90, and 30.90 for the same target transcripts. The cycle numbers for human and mouse promoter B were less by 2 and 2.7, respectively, than those for promoter A, indicating 4 and 6.5 times the number of transcripts derived from promoter A. These results indicate that both mouse and human promoter A and B are active in fibroblast cells, but there are a greater number of promoter A-specific transcripts than B.

Involvement of TNF signaling pathway in the regulated activity of the Wnt5a *alternative promoters A and B*

There is evidence indicating that the TNF-alpha signaling pathway affects Wnt5a transcription. However, these studies are limited and it is not clear which of the TNF-alpha effector proteins (NFkappa-B, p38, ERK, or JNK) are involved and if Wnt5a alternative promoters A and B are differentially

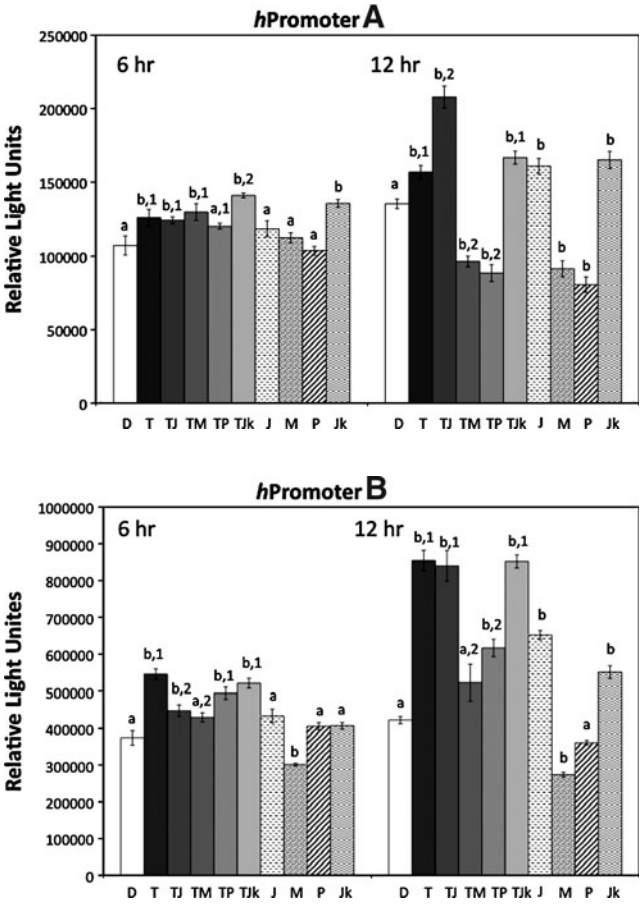


FIG. 5. Increase in *h*promoter A and *h*promoter B activity in response to TNF-alpha. NIH3T3 cells stably transfected with *h*promoter A p2178 and *h*promoter B p1981 reporter constructs were treated for 6 and 12 h with 5 ng/mL TNF-alpha only (T) and the following inhibitors with (solid bars) and without (patterned bars) TNF-alpha: NF-kappaB inhibitor (JSH-23, 30 μM) (TJ and J), MEK1/2 inhibitor (U0126, 10 μM) (TM and M), p38 inhibitor (SB203850, 10 μM) (TP and P), Jun N-terminal kinase inhibitor (SP600125, 20 μM) (TJk and Jk). Control cells were treated with DMSO (D) (open bar). Cells were collected and assayed for luciferase activity, expressed as relative light units. Bars are ± S.E.M. N = 4. For statistical analyses, letters are pair-wise comparisons of each value to the DMSO control. Numbers are statistical pair-wise comparisons between TNF-alpha plus inhibitor and the TNF-alpha only values. A different letter or number indicates significance ($p < 0.05$).

affected. Using our stable lines of NIH3T3 containing the *WNT5A* *h*promoter A p2178 and *h*promoter B p1981 luciferase reporter constructs, the effect of TNF-alpha on promoter activity was analyzed. The cells were treated with 5 ng/mL TNFalpha and inhibitors of NF-kappaB, p38, MEK1/2 (hence ERK), and JNK for 6 and 12 h (Fig. 5). Luciferase activity increased in TNF-alpha-treated cells at 6 h for both *h*promoter B and *h*promoter A, but less for hpromoter A. At 12 h, there was a significant increase in activity of both *h*promoters A and B, as measured by luciferase activity. The *h*promoter B showed a greater increase of ~1.5- to 2.25-fold at 12 h, compared to 1.2- to 1.5-fold for the *h*promoter A. These results indicate that TNF-alpha increases activity of both *h*promoters A and B, but the effect is greater for the *h*promoter B.

The effects of the various inhibitors are shown in Figure 5. At 6 h, inhibitors of NF-kappaB, MEK1/2, and p38 had no affect on the slight increase in *h*promoter A activity due to TNF-alpha treatment. A small, but significant increase in luciferase above the DMSO control and TNF-alpha-treated cells was detected in the TNF-alpha plus JNK inhibitor-treated cells. This is likely due to an effect of JNK independent of TNF-alpha as the JNK inhibitor, alone, increased activity. At 12 h, *h*promoter A activity was increased by TNF-alpha; however, the NF-kappaB inhibitor increased activity in comparison to both the control- and TNF-alpha-treated cells. The MEK1/2 and p38 inhibitors reduced activity to below DMSO control levels, with and without TNF-alpha. Again, the JNK inhibitor increased activity alone, but had no effect on TNF-alpha-treated cells. These results indicate that for the *h*promoter A, the TNF-alpha-induced increase in activity at 12 h involves MEK1/2 and p38, but that these kinases also contribute to the level of constitutive *h*promoter A activity in untreated cells. The increase in reporter activity in cells treated with TNF-alpha plus NF-kappaB inhibitor and NF-kappaB inhibitor only, suggest that NF-kappaB has a negative effect on promoter A activity, at least at 12 h.

At 6 h for the *h*promoter B, both the NF-kappaB and MEK1/2 inhibitors decreased activity, relative to the TNF-alpha-treated cells. P38 and JNK inhibitors had no effect on the TNF-alpha increase in activity. NF-kappaB, p38, and JNK inhibitors, alone, did not alter activity, whereas the MEK1/2 inhibitor slightly, but significantly decreased activity relative to the DMSO control. At 12 h, only the MEK1/2 and p38 inhibitors significantly reduced activity of the *h*promoter B relative to the TNF-alpha-treated cells, whereas the NF-kappaB and JNK inhibitors had no effect. The NF-kappaB and JNK inhibitors, alone, increased activity, whereas the MEK1/2 inhibitor decreased activity. The p38 inhibitor, alone, slightly decreased activity, but not significantly. These results suggest that NF-kappaB is involved in the early response (6 h) of *h*promoter B to TNF-alpha, but at 12 h, p38 is more important. MEK1/2 kinase likely has a role in both the TNF-alpha increase in *h*promoter B activity and the constitutive level of *h*promoter B activity.

Endogenous Wnt5a *promoters affected by TNF-alpha*

To determine if the genomic promoters A and B are also responding to TNF-alpha stimulation, NIH3T3 cells were treated with TNF-alpha for 6 h and RNA isolated. qRT-PCR analysis was conducted using *m*promoter A- and B-specific TaqMan primer–probes and commercial cyclin D1 primer–probes (Fig. 6). Cyclin D1 transcription is known to increase in TNF-alpha-stimulated cells. A relative increase in all three target sequences was detected at 6 h. *m*Promoter A transcripts increased by 1.24×, whereas *m*promoter B increased by1.43× and cyclin D1 transcript levels by 1.5×. Treatment with the NF-kappaB inhibitor, JSH-23, had no effect on *m*promoter A transcript levels in TNF-alpha-treated cells, but caused a decrease in *m*promoter B and cyclin D1 transcripts. These results suggest that NF-kappaB has a role in TNF-alpha stimulation of *m*promoter B and cyclin D1 activity at 6 h in mouse cells. The data in Figure 6 also confirms that at 6 h MEK1/2 has a positive effect on *m*promoter B and cyclin D1 transcription. A p38 inhibitor reduced *m*promoter B transcript levels, but increased levels for cyclin D1 transcripts. These results suggest that MEK1/2 and p38 have a role in the level of *m*promoter B activity.

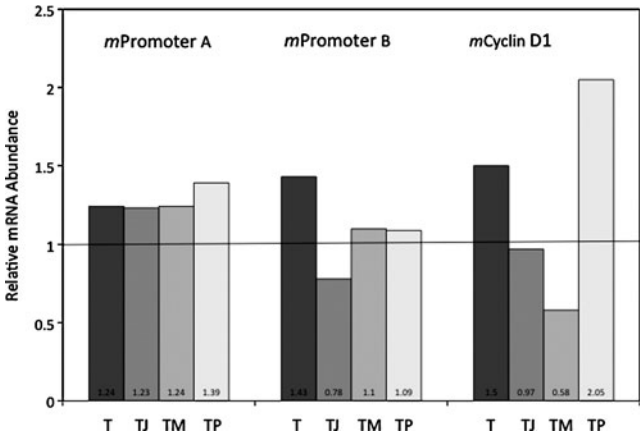


FIG. 6. qRT-PCR analysis of endogenous *m*promoter A, *m*promoter B, and *m*cyclin D1-specific transcripts in TNF-alpha-treated cells. NIH3T3 cells were treated for 6 h with 5 ng/mL TNF-alpha (T), with and without a NF-kappaB inhibitor (JSH-23, 30 μM) (TJ), MEK1/2 inhibitor (U0126, 10 μM) (TM), and p38 inhibitor (SB203850, 10 μM) (TP). Control cells were treated with DMSO (D). The relative level of *m*promoter A, *m*promoter B, and *m*cyclin D1 transcripts were determined in comparison to the DMSO-treated cells, using GAPDH as the internal control.

Discussion

The human *WNT5A* upstream sequences from the alternative transcription start sites termed *h*promoter A and *h*promoter B were individually cloned and analyzed to determine relative levels of transcriptional activity and to map potential regulatory sequences by deletion analysis. Our results suggest that *h*promoters A and B have similar transcriptional potential when assayed separately, but that the *h*promoter A is more active in human and mouse fibroblast cells. Deletion analysis of the *h*promoter A and B upstream sequences indicate that both positive and negative acting regulatory sequences are located within the ~2000 bp of upstream sequence of both *h*promoter A and B and that 356 to 425 bp of upstream sequence is sufficient for a high level of expression. The relative expression levels varied only two-

to fourfold for the different constructs, indicating that any removed regulatory sequences have only a minor effect on *WNT5A* activity at least in NIH3T3 and Caco-2 cells. These data support the conclusion that *WNT5A* is constitutively expressed in these cell types and that its regulation involves subtle modulations in transcriptional activity. Alternatively, it is possible that *WNT5A* regulation is primarily centered on the most proximal promoter region. This may involve *h*promoter B intron sequences, which are included on the reporter construct. *h*Promoter A intron 1 sequences are not required for activity of the promoter as they were not included in the constructs, although possible regulatory sequences may be located within these sequences.

The patterns of expression for the different constructs were similar for the *h*promoter A in both a fibroblastic (NIH3T3) and epithelial cell (Caco2). In contrast, the *h*promoter B displayed a distinctive pattern of expression in Caco-2 in comparison to NIH3T3. We found that in Caco-2 cells removal of *h*promoter B upstream sequences lead to a continual increase in expression. In NIH3T3, the levels of expression were similar for the different constructs. This finding suggests that negative regulatory sequences are being removed between the 1981- and 356-bp region of *h*promoter B that are functional in Caco-2 cells, but not in NIH3T3 cells. The different expression patterns of the *h*promoter B constructs in Caco-2 are likely a consequence of cell-specific regulatory factors. In general, this finding suggests that *h*promoters A and B are differentially regulated.

Figure 2 displays potential transcription factor binding sites. These sites were selected for analysis based on their previous involvement in *WNT5A* expression and link to TNF-alpha and MAPK signaling. *h*promoters A and B include multiple binding sites within the sequences contained in the shortest construct (p420 and p356). Common to both are c-Myb and CUTL1 sites. Wnt5a was found to be a target of CUTL1 and overexpression of CUTL1 increased total *WNT5A* transcripts and protein levels (Ripka *et al.*, 2007). It is not known if both *h*promoters A and B are affected by CUTL1 and which of the CUTL1 sites are functional. The *h*promoter B harbors overlapping sites for Pax-2, AP-1, and c-Fos within its unique first intron. There is support for the involvement of Pax-2 in *WNT5A* transcription; however, the identified binding site was located 100-kb upstream of the promoter A (Tamimi *et al.*, 2008). Pax-2 is unlikely to be an essential transcription factor for the *h*promoter A, as a consensus Pax-2 binding site is lacking from the p420 construct. STAT3 has been implicated, but not proven to be involved in *WNT5A* regulation and a putative STAT3 site was identified in exon 4, common to both promoter A and B transcripts (Katoh and Katoh, 2007). No STAT3 sites were identified in the promoter A and B sequences we analyzed. The involvement of these and other transcription factors in promoter A and B activities will require overexpressing and RNAi knockdown of the transcription factors, and then quantifying the levels of promoter A- and B-derived transcripts using our costume-designed TaqMan primer–probes. One difficulty is the possibility that the activity levels of promoters A and B are dependent on the combinational inputs of a diverse group of transcription factors and that the contribution of anyone factor is limited.

Collectively, our results using stable lines of NIH3T3 cells containing the *WNT5A* *h*promoter A and B luciferase reporter constructs and qRT-PCT analysis of endogenous *m*promoter A and B transcripts support the conclusion that activation of the TNF-alpha signaling pathway increases both promoter A and B activity, but that promoter B is more strongly affected. We used inhibitors to block the activity of downstream effectors of TNF signaling, including the transcription factor NF-kappaB, and the kinases MEK 1/2, p38, and JNK. The results indicate that at 6 h, NF-kappaB is responsible for some of the increased promoter B activity, but not for promoter A. At 12 h, the NF-kappaB inhibitor (without TNF-alpha treatment) increased *h*promoters A and B activities. This finding suggests that NF-kappaB is active in NIH3T3 cells not treated with any inducer of NF-kappaB and that it is having a negative effect on *h*promoters A and B. Potential NF-kappaB binding sites were identified in the human promoter A and B sequences contained in the p2178 and p1981 reporter vectors (Fig. 2). The NF-kappaB site in promoter B is the same as the one identified by Katoh and Katoh (2009). In promoter A, the NF-kappaB site nearest the beginning of the transcription start is the same as the site identified by Ge *et al.* (2011). In that study, an increase in *WNT5A* mRNA and protein levels was detected in primary condylar chondrocyte cells treated with interleukin-1β (an inducer of TNF signaling pathway), which was reduced by an inhibitor of NF-kappaB activation. Moreover, using ChIP analysis, Ge *et al.*, provided evidence that NF-kappaB binding is enriched at this sequence. In another study, *WNT5A* transcript levels increased in bone marrow stromal cells treated with TNF-alpha for 48 h and this induction was decreased by treatment with an inhibitor of NF-kappaB (Rauner *et al.*, 2011). In both studies, however, total *WNT5A* transcripts were measured, thus it is not possible to know if the detected increase was due to *h*promoter A- or B-derived transcripts.

We also found that the kinases p38 and ERK contribute to the activity levels of promoters A and B, particularly at 12 h. For promoter A, activity levels were reduced by the p38 and MEK1/2 kinase inhibitors to the same level as in cells treated with and without TNF-alpha. For promoter B, the kinase inhibitors reduced promoter activity in both TNF-alpha-treated and untreated cells, but activity was greater in the TNF-alpha-treated cells. Hence, for both promoters A and B, the p38 and MEK1/2 kinases appear to be involved in the constitutive activity of the promoters and likely have some role in TNF-alpha stimulation of activity. This appears to be the first report of p38 involvement in WNT5A transcriptional regulation. WNT5A stimulation of the noncanonical Ca^{2+} pathway has been shown to result in p38 kinase activation (Ma and Wang, 2007). This and our finding suggest a positive feedback loop with p38 kinase leading to increased *WNT5A* expression, and then WNT5A receptor binding leading to further p38 kinase activation.

There are only a few studies linking MAPK signaling pathway to *WNT5A* transcriptional regulation. In one, SW480 cells (human colon cancer cell line) were subjected to amino acid deprivation to induce the MAPK pathway (Wang and Chen, 2009). An increase in ERK1/2 phosphorylation in these cells was associated with a decrease in *WNT5A* mRNA levels. These data are contrary to our findings as we observed a decrease in *Wnt5a* transcription (of both promoter A and B transcripts) when cells, with and without TNF-alpha, are treated with the MEK1/2 inhibitor. Consistent with our findings, Rauner *et al.* (2011), measured an increase in *Wnt5a* transcript levels due to stimulation with TNF-

alpha, which was reduced by treatment with a MAPK inhibitor. In neither of these studies were the individual promoter A- and B-derived transcripts analyzed.

Our finding that the Wnt5a alternative promoters A and B are differentially regulated is not unexpected. Alternative promoter usage giving rise to multiple transcripts appears to be a common feature of mammalian genes; it is estimated that 50% of mammalian genes have alternative promoters (Kimura *et al.*, 2006; Baek *et al.*, 2007). The idea that alternative promoters provide for more complex differential gene regulation is supported by numerous studies. For example, the two promoters of the gene coding for the transcription factor, Runx1, are differentially utilized during hematopoiesis, allowing for fine control of overall Runx1 levels at the appropriate developmental stage (Bee *et al.*, 2010). Three alternative promoters have been identified in the gene coding for acetyl-CoA carboxylase β, which show unique tissue-specific activity in both human and rat (Oh *et al.*, 2005). And, the human microsomal epoxide hydrolase gene (EPHX1) has an alternative promoter that is utilized only for liver-specific expression of the gene (Liang *et al.*, 2005). Alternative promoter usage has also been associated with human disease. In one study, the five alternative promoters of the aromatase (CYP19) gene were found to have distinct patterns of activity in nonsmall cell lung cancer cells, in comparison to the surrounding normal tissue (Demura *et al.*, 2011). And, a genome-wide analysis of genes associated with disease susceptibility showed a positive association with the presence of alternative promoters (Liu, 2010). The misregulation of *WNT5A* in numerous cancers involves both increases and decreases in transcript levels, depending on the cancer type. It is likely that this altered expression involves differential utilization of the *WNT5A* alternative promoters, including promoters A and B. Distinct promoter A and B expression patterns are likely to occur in normal cells as well. In both cases, the expected outcome would be a change in the ratio of WNT5A protein isoforms A and B. We are currently using our promoter A and B TaqMan primer–probes to analyze the levels of promoter-specific transcripts in different cancer cells and analyzing the functional distinctions, if any, between protein isoforms A and B.

Conclusion

We provide evidence for differential regulation of two of the *Wnt5a* alternative transcription start sites in different cell types and as a consequence of TNF signaling pathway activation. The promoter B was found to be more sensitive to TNF-alpha. The transcription factor NF-kappaB and the MEK1/2 and p38 kinases have distinct roles in determining the activity levels of promoters A and B. These findings indicate a mechanism of more complex and subtle regulation of *Wnt5a* expression involving its alternative promoters. Further studies are necessary to determine if and to what degree the amounts of promoter-specific transcripts vary during development, in different cell types, and during cellular transformation.

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Disclosure Statement

No competing financial interests exist.

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