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## Stromelysin-1 and mesothelin are differentially regulated by Wnt-5a and Wnt-1 in C57mg mouse mammary epithelial cells

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### Abstract

**Background:** The Wnt signal transduction pathway is important in a wide variety of developmental processes as well as in the genesis of human cancer. Vertebrate Wnt pathways can be functionally separated into two classes, the canonical Wnt/beta-catenin pathway and the non-canonical Wnt/Ca<sup>2+</sup> pathway. Supporting differences in Wnt signaling, gain of function of Wnt-1 in C57mg mouse mammary epithelial cells leads to their morphological transformation while loss of function of Wnt-5a leads to the same transformation. Many downstream target genes of the Wnt/beta-catenin pathway have been identified. In contrast, little is known about the Wnt/Ca<sup>2+</sup> pathway and whether it regulates gene expression.

**Results:** To test the hypothesis that a specific cell line can respond to distinct Wnts with different patterns of gene expression, we over-expressed Wnt-5a and Rfz-2 in C57mg mammary epithelial cells and compared this cell line to C57mg cells over-expressing Wnt-1. These Wnts were chosen since previous studies suggest that C57mg cells respond differently to these Wnts, and since these Wnts can activate different signaling pathways in other systems. Using DNA microarray analysis, we identified several genes that are regulated by Wnt-5a and Rfz-2 as well as by Wnt-1. We then focused on two genes previously linked to various cancers, mesothelin and stromelysin-1, which are respectively up-regulated by Wnt-1 and Wnt-5a in C57mg cells.

**Conclusion:** Different Wnts have distinct effects on gene expression in a single cell line.

### Background

Wnt ligands are secreted glycoproteins that function in intracellular signaling pathways to regulate a variety of developmental processes including cell growth, cell differentiation, cell polarity, and apoptosis. Mis-regulation of Wnt signaling leads to the development of several human cancers, including colon carcinomas and melanoma [1]. Canonical Wnt signaling initiates in the binding of a Wnt ligand to its cell surface receptor, members of the frizzled gene family, along with the co-receptor LRP5/6 [2,3], leading to changes in the activities of cytoplasmic ef-

factors resulting in the stabilization of beta-catenin protein. Beta-catenin then accumulates in the nucleus where it interacts with its transcriptional co-activators, members of the Lymphoid Enhancer Factor/T Cell Factor (LEF/TCF) family of high mobility group DNA binding proteins to regulate gene expression [1]. Many downstream gene targets of the canonical Wnt/beta-catenin pathway have been identified <http://www.stke.org>.

There is a large family of Wnt ligands and not all Wnts are thought to function in the same pathway. Wnts can be

operationally separated into two classes; the first being a class that transforms C57mg mouse mammary epithelial cells and also promotes duplication of the dorso-ventral axis when over-expressed in *Xenopus* embryos. Wnts in this functional class activate the canonical Wnt/beta-catenin pathway (Wnt-1, -3A, and -8). The second functional group of Wnts does not transform C57mg cells nor promote axis duplication. Instead, when over-expressed in frog or fish embryos, these Wnts perturb the movements of gastrulation. Wnts in this class have been shown to signal in a non-canonical Wnt signaling pathway, the Wnt/Ca<sup>2+</sup> pathway (Wnt-5a, -4, -11). The Wnt/Ca<sup>2+</sup> pathway has been shown to increase intracellular Ca<sup>2+</sup> levels in zebrafish embryos [4] and to activate PKC and CamKII in *Xenopus* embryos [5,6] as well as in human melanomas [7]. Moreover, it may activate Cdc42 [8] and JNK [9]. It is important to emphasize that whether Wnts activate distinct responses and likely pathways is context-dependent, and thus in other contexts a given Wnt may activate another pathway.

Vertebrate Wnts signal through frizzled receptors that in some experimental contexts (e.g., without co-expressing LRP5/6) preferentially activate the Wnt/beta-catenin or Wnt/Ca<sup>2+</sup> pathways [5]. Interestingly, Wnt-5a may couple to either pathway depending on which frizzled receptor is present. When co-expressed with human frizzled-5, Wnt-5a can induce a secondary axis in *Xenopus* [10]. In other assays, however, Wnt-5a along with rat frizzled 2 (Rfz-2) increases intracellular Ca<sup>2+</sup> levels and activates PKC and CamKII [4–6]. The activation of Wnt/Ca<sup>2+</sup> signaling by Wnt-5a and Rfz-2 seems to be antagonistic to the canonical Wnt signaling pathway. Specifically, Wnt-5a expression inhibits ectopic Wnt-8 induction of secondary axis formation in *Xenopus* embryos [11]. Supportingly, the endogenous expression of Wnt-5a maintains C57mg cells in a normal growth state since anti-sense Wnt-5a mimics Wnt-1 transformation of C57mg cells [12]. This data suggests that Wnt-1 and Wnt-5a work in an opposing manner in some cellular contexts. This potential role of Wnt-5a as a tumor suppressor is further supported by the evidence that ectopic Wnt-5a in human uroepithelial cells prevents tumorigenesis when injected into athymic nude mice [13].

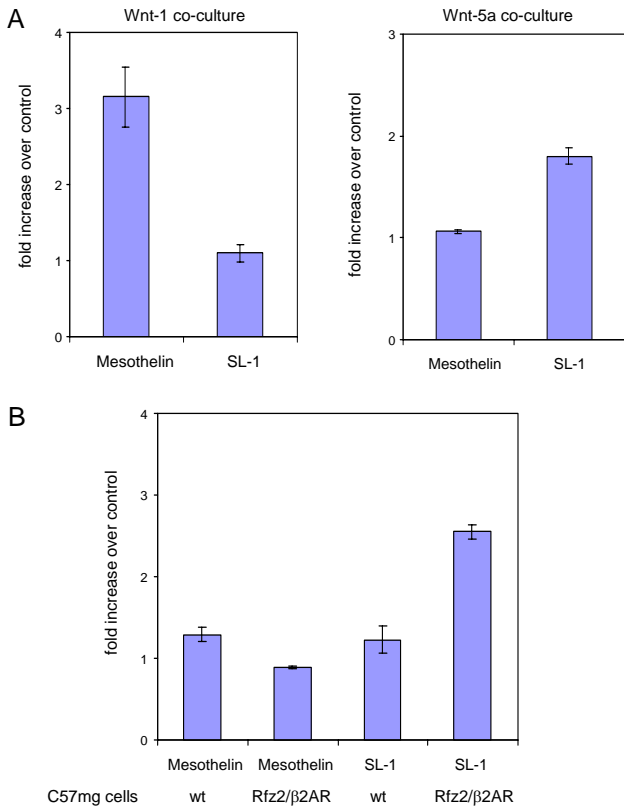
Much is still unknown about how Wnt-5a signals and, importantly, whether it regulates any genes. The ability of dnWnt-11 to block expression of some genes in *Xenopus* [6] and the ability of Wnt-5a to activate the Ca<sup>2+</sup> sensitive transcription factor nuclear factor of activated T cells (NFAT) [14], support the likelihood that Wnt-5a can regulate gene expression through a beta-catenin-independent mechanism. We sought to identify genes regulated by Wnt-5a and compare whether these were different from

canonical Wnt-1 gene targets using DNA microarray analysis.

## Results

To identify downstream genes regulated by Wnt-5a we created C57mg cells that stably express both Wnt-5a and Rfz-2, a potential ligand and receptor pair that in some assays function in the same signaling pathway. We overexpressed Rfz-2 in addition to Wnt-5a in C57mg cells in order to augment the activation of noncanonical Wnt signaling. Both have been shown to elevate intracellular Ca<sup>2+</sup> levels and to activate PKC and CamKII [4–6]. In addition Wnt-5a and Rfz-2 have been shown to synergize in their induction of CamKII [6]. Wnt-1/C57mg cells were also used for comparison, in an effort to identify genes preferentially regulated by Wnt-5a. C57mg cells appeared to respond to ectopic expression of both Wnt-5a and Rfz-2 in C57mg cells since the cells exhibited a moderate reduction in growth rate in comparison to wildtype (wt) C57mg cells as well as Wnt-1/C57mg cells (data not shown). Phenotypically the Wnt-5a/Rfz-2/C57mg cells were more spread out and the overall size of the cells increased compared to wt C57mg cells or Wnt-1/C57mg cells (data not shown). In contrast, ectopic expression of Wnt-1 induced the expected morphological transformation of C57mg cells (data not shown). Previous reports of ectopic expression of different Wnts in C57mg cells demonstrated Wnt-1 to be highly transforming whereas Wnt-5a failed to induce transformation [15,16]. By identifying downstream targets of Wnt-5a and Rfz-2, we wanted to determine which genes are responsible for these phenotypic changes as well as which genes might be targets of noncanonical Wnt signaling.

RNA isolated from wt C57mg cells, C57mg cells stably transformed by Wnt-5a and Rfz-2 retroviruses, and C57mg cells stably transformed by Wnt-1 retrovirus were used for DNA microarray analysis. Biotinylated cRNA probes synthesized from the RNA were hybridized to Affymetrix mouse GeneChips Mu11k subA and subB arrays. Over 11,000 oligonucleotide probe sets were analysed including known genes and ESTs. Two independent sets of experiments were performed that initiated from the growth of cells. Probe sets that underwent a 2-fold or greater change in the Wnt-ligand expressing cells compared to control cells were selected for further analysis. Over 90 genes that repeated in both experiments appeared to change in the Wnt-expressing cells. Of those, 50 genes were chosen for validation by an independent method (quantitative RT-PCR). A list of some of these genes, with the observed fold-changes determined by DNA microarray analysis and quantitative RT-PCR, is shown (Table 1; list of primers shown in Table 2). Genes are separated by functional categories with the greatest number of genes having roles in cell adhesion, transcriptional regulation,



**Figure 1**  
**Induction of mesothelin and SL-1 expression by Wnt-1 or Wnt-5a signaling.** A. RT-PCR was performed on RNA obtained from 3 separate experiments in which C57mg cells were co-cultured with 293T cells expressing Wnt-1, Wnt-5a, or CS2+ as control. The amounts of mesothelin and SL-1 mRNAs were normalized to HPRT and plotted as fold increase relative to C57mg cells co-culture with 293T cells expressing CS2+. B. C57mg cells (wt) or C57mg cells stably expressing the Rfz-2/β<sub>2</sub>AR chimeric receptor were treated with the β<sub>2</sub>AR agonist isoproterenol or the antagonist propranolol as control for 8 hours prior to harvesting for RNA extraction and RT-PCR analysis. The amounts of mesothelin and SL-1 mRNAs were normalized to HPRT and plotted as fold increase relative to propranolol treated cells. Mean levels were determined from 3 separate experiments.

and signaling. The list of genes shows those that are specific to Wnt-5a/Rfz-2, differential between Wnt-5a/Rfz-2 and Wnt-1, specific to Wnt-1, and common to both Wnt-1 and Wnt-5a/Rfz-2. Previously identified genes regulated by Wnt signaling in this list include fibronectin [17], IL-6 [18], BMP-4 [19], groucho related protein [20], and Krox-

20 [21,22]. Since Wnt-5a may couple to more than one frizzled receptor, different Wnt signaling pathways may be activated by Wnt-5a depending on the endogenous expression of various frizzled receptors [6,10]. Thus, it is possible that the gene expression pattern we identified may be due to the activation of both canonical and non-canonical Wnt signaling pathways. We compared the Wnt-5a/Rfz-2 expression profile to the Wnt-1 expression profile to rule out genes common in both pathways and to identify those genes specific to Wnt-5a and Rfz-2.

We next wanted to examine whether any of these genes were regulated in a similar manner if we used alternative methods for expressing Wnt-1 or Wnt-5a. In particular, we wanted to activate the function of these Wnts for short durations rather than relying on stable expression. The first approach was to co-culture C57mg cells with cells secreting Wnt ligand. For the co-culture experiments, C57mg cells were grown together with Wnt-1 or Wnt-5a expressing 293T cells for 24 hours. RNA was isolated and quantitative RT-PCR was performed using primers to the list of genes from Table 1. The majority of genes had little if any background in the PCR reactions from the human 293T cell RNA (data not shown). One gene, mesothelin, exhibited a 3-fold induction in C57mg cells treated with the Wnt-1 but not the Wnt-5a secreting 293T cells (Figure 1, panel A). This fold change was similar to the data obtained from stable expression with Wnt-1 in C57mg cells (see Table 1). Mesothelin is a cell-surface antigen of unknown function that is expressed in the mesothelium. It is synthesized as a 69 kDa precursor that is proteolytically processed into a 40 kDa membrane bound form that is glycosylphosphatidylinositol (GPI)-linked and a 32 kDa secreted form. The secreted form has been referred to as megakaryocyte potentiating factor (MPF) for its ability to stimulate the megakaryocyte colony forming activity of murine IL-3 in mouse bone marrow cell culture [23,24]. Mesothelin expression is induced in a variety of cancers including ovarian, pancreatic, lung, and cervical cancer [25,26]. The soluble portion of mesothelin has drawn attention as a potential serum tumor marker since it is highly over-expressed in patients with ovarian carcinomas and pancreatic adenocarcinomas [27,28].

Stromelysin-1 (SL-1) was induced almost 2-fold in C57mg cells co-cultured with Wnt-5a secreting 293T cells but not with Wnt-1 secreting 293T cells (Figure 1, panel A). A similar level of induction was also observed with Wnt-5a/293T cells co-cultured with Rfz-2/C57mg cells (data not shown), suggesting that the induction of SL-1 is due to expression of Wnt-5a and not only to Rfz-2. These data are similar to the ~4 fold induction of SL-1 in Wnt-5a/Rfz-2/C57mg cells (Table 1). SL-1 is a matrix metalloproteinase that can alter ECM-mediated signaling events during development [29–31]. Both SL-1 and Wnt-5a have

**Table 1: Genes differentially regulated by Wnt-5a/Rfz-2 and Wnt-1 in C57mg cells.**

Functional Category of Genes	GenBank acc. no.	Avg. fold changes			
		Wnt-5a Rfz-2 Microarray	Wnt-5a Rfz-2 Quant. RT-PCR	Wnt-1 Microarray	Wnt-1 Quant. RT-PCR
nd, not determined					
<b>Cell Growth/Differentiation</b>					
BMP-4	L47480	-3	-2.4	1.4	1.2
<b>Cell Adhesion</b>					
plasminogen activator inhibitor-1	M33960	-2.8	-2.4	2.1	1.2
osteoblast specific factor 2	D13664	-2.4	-1.6	1.4	1.1
Flt4 ligand	U58112	3.3	2	1	1.2
fibronectin	ET62894	2	nd	-1.8	nd
HTK ligand	L38847	-2.1	-1.9	2.5	3.2
mesothelin	D86370	-4	-2.9	8.4	2.8
stromelysin 1	X66402	3.8	4.4	-1.6	-2.5
<b>Transcriptional Regulator</b>					
groucho-related gene 1 protein	U61362	-2.1	-1.6	1.4	-1.4
retinoic acid receptor-alpha	X57528	-2.3	-2	1	-1.3
Id4 helix-loop-helix protein	X75018	-4.1	-3.6	1.6	-1.1
neural-restrictive silencer factor	U13878	-3.5	1.1	1.6	1.8
bcl-3	M90397	1.9	2.2	-2	-1.4
Krox-20	X06746	-3.4	-1.7	6.2	1.5
<b>Inflammation</b>					
PTX3	X83601	-2.5	-2.1	1.6	1.6
bradykinin B1 subtype receptor	U47281	-2.5	4	2.7	3.2
<b>Signaling</b>					
Rho-associated protein kinase	U58513	-1.2	nd	-4.9	nd
guanine nucleotide-binding protein	W83658	1.6	1.7	-4.4	-4.4
signaling molecule (ATTP)	U97571	-1.3	1.5	-3.5	-2.7
son of sevenless 1	Z11574	2.5	2.9	-2.6	-2
SHPS-1	D87967	-2.7	-1.4	3	-1.7
TRAF1	L35302	2.6	1.5	2	1.7
<b>Cell surface receptor</b>					
interleukin 2 receptor gamma	U21795	2	2.7	-1.4	1.9
transferrin receptor	X57349	-1.6	-1.6	-2.3	-1.9
<b>Cytokine</b>					
interleukin-6	X54542	2.1	-1.1	1.6	1
<b>Cell Cycle</b>					
Ki-67 antigen	ET62993	-1.45	-1.1	-6.5	-5.8
<b>Axon Guidance</b>					
semaphorin F	X97817	2.5	3.1	-2	-4
DM-GRASP	L25274	3.3	1.8	-2.8	-2.6

Acc., accession; Avg., average; Quant., quantitative; BMP-4, bone morphogenetic protein-4; HTK ligand, hepatoma transmembrane kinase ligand; PTX3, pentraxin 3; SHPS-1, src homology protein substrate-1; TRAF1, TNF receptor associated factor-1. Shown is a list of genes that are differentially regulated by Wnt-5a/Rfz2 and Wnt-1 in C57mg cells identified by DNA microarray analysis. The values determined by DNA microarray analysis were validated using quantitative RT-PCR. Average fold changes from DNA microarray analysis and quantitative RT-PCR were determined from two independent experiments and from three independent experiments performed in triplicate respectively.

roles in mammary gland development and are elevated in certain breast cancers [32–35], although Wnt-5a is reduced in other types of breast cancer [36].

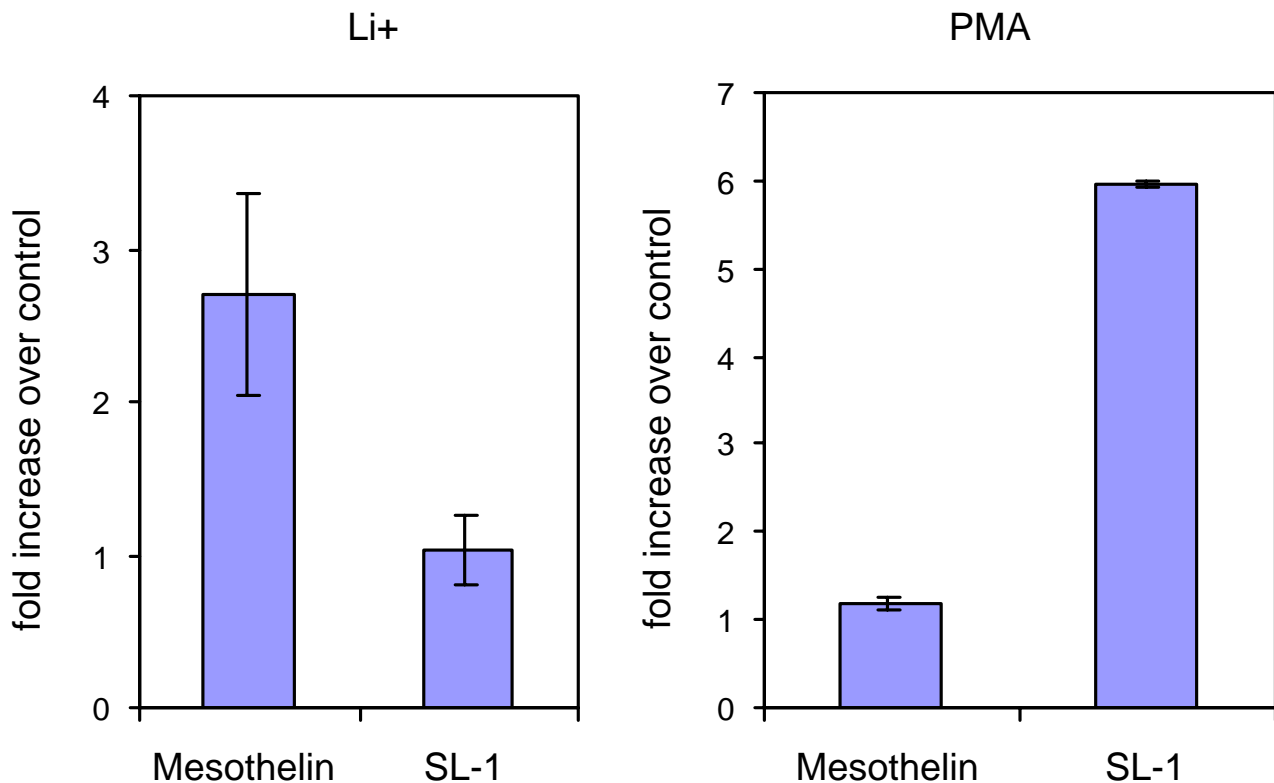
Since Wnt-5a may activate both vertebrate Wnt pathways we turned to an approach that more specifically would transiently activate the Wnt/Ca<sup>2+</sup> signaling pathway. This approach involves the use of a drug-inducible chimeric receptor consisting of the extracellular and transmembrane

spanning portions derived from the hamster  $\beta_2$  adrenergic receptor ( $\beta_2$ AR) and the intracellular domains derived from Rfz-2. This receptor has previously been shown to elevate intracellular Ca<sup>2+</sup> and activate CamKII in a ligand-dependent manner [6,37,38]. C57mg cells stably transformed with Rfz-2/ $\beta_2$ AR retrovirus or wt C57mg cells were treated overnight with propranolol, a  $\beta_2$ AR antagonist, to maintain the chimeric receptors in an inactivated state. Subsequently, the  $\beta_2$ AR agonist isoproterenol was added

**Table 2: Primers used in quantitative RT-PCR.**

HPRT:	forward primer	5'-TGGAAAGAATGTCTTGATTGTTGAA-3'
	reverse primer	5'-AGCTTGCAACCTTAACCATTTTG-3'
BMP-4:	forward primer	5'-GCTTGAGTACCCGGAGCGT-3'
	reverse primer	5'-TGTTCTTCGTGATGGAAACTCCT-3'
Plasminogen activator inhibitor-1:	forward primer	5'-GGCCTCCAAAGACCGGA-3'
	reverse primer	5'-CAGCATAGCCAGCACCGA-3'
Osteoblast specific factor 2:	forward primer	5'-AAGGAAAAGGGTCATACACGTACTTC-3'
	reverse primer	5'-TGCGAATGTCAGAATCCAGGT-3'
Flt4 ligand:	forward primer	5'-CAGCTGCGGAAAGGCG-3'
	reverse primer	5'-TTTACTGTCCCCTGCTCTGG-3'
HTK ligand:	forward primer	5'-GCAGGAGACACCGCAAACA-3'
	reverse primer	5'-GTGGCCAGTGTGCTGAGAGA-3'
Mesothelin:	forward primer	5'-GCAGTCAGGGAGGTTCTGAGG-3'
	reverse primer	5'-GGTGGAGACTGACCACTTCGA-3'
Stromelysin-1:	forward primer	5'-TGGCCATCTTTCCAA-3'
	reverse primer	5'-CCCAGAAGTGAATTCCTTAAAAATG-3'
Groucho related protein I	forward primer	5'-CCCTATGCCCTCATTGGA-3'
	reverse primer	5'-CCGTGGTTGAGGGTGTGAT-3'
Retinoic acid receptor-alpha:	forward primer	5'-AGCACCAGCTTCCAGTCAGTG-3'
	reverse primer	5'-CTGCTGCTCTGGGTCTCGA-3'
Id4:	forward primer	5'-GAACAAGCAGGGTGACAGCA-3'
	reverse primer	5'-CCGCACACCTGGCCAT-3'
Neural-restrictive silencer factor:	forward primer	5'-CGGAAGACAAATGCAGGAGTTC-3'
	reverse primer	5'-TTCGGCTTCGTAAGGCA-3'
Bcl3:	forward primer	5'-GCACGGCGCCAACGT-3'
	reverse primer	5'-GCCAGACGCAGAATGCAGA-3'
Krox-20:	forward primer	5'-GCGGGAGATGGCATGATC-3'
	reverse primer	5'-GAAGCTACTCGGATACGGGAGA-3'
PTX3:	forward primer	5'-TGGACAACGAAATAGACAATGGA-3'
	reverse primer	5'-CTGGCGGCAGTCGCAT-3'
Bradykinin B1 receptor:	forward primer	5'-TCAACTGGCCCTTTGGAAGT-3'
	reverse primer	5'-AACAGGTTGGCCTTGATGACC-3'
Guanine nucleotide-binding protein:	forward primer	5'-CTGGTAGAACAGCTGAAGATGGAAG-3'
	reverse primer	5'-ACAGTAGGCCATCAAGTCAGCA-3'
ATTP signaling molecule:	forward primer	5'-TGCTCCACCCATTTGCTTCT-3'
	reverse primer	5'-TGGCATCCACATGTTTTCCA-3'
Son of sevenless I:	forward primer	5'-CCTGAAAGCCCTCCCTTGT-3'
	reverse primer	5'-AGATGTAATGGTGAGCTTGAGAAAAC-3'
SHPS-1:	forward primer	5'-GGTCAACATCTTCCACACGGTT-3'
	reverse primer	5'-TTTGTGTCCTGGATCTGGGTTA-3'
TRAF1:	forward primer	5'-TGTGTGGCCGGACTGTCA-3'
	reverse primer	5'-AGCGCAGGCACAACCTGTAAC-3'
IL-2 receptor gamma:	forward primer	5'-TTGGTGCAGTACCGGAGCA-3'
	reverse primer	5'-GCAGGGAGAATCTAGTTTCATGA-3'
Transferrin receptor:	forward primer	5'-GACGCTTTGGGTGCTGGT-3'
	reverse primer	5'-GGCAAGTTTCAACAGAAGACCTGT-3'
IL-6:	forward primer	5'-TTACACATGTTCTCTGGGAAATCG-3'
	reverse primer	5'-AAGTGCATCATCGTTGTTTCATACA-3'
Ki-67 antigen:	forward primer	5'-TTTCAGGTCTCTGGAAGCAGTCA-3'
	reverse primer	5'-ATCTCCATAATTGCTTTGATTGCA-3'
Semaphorin F:	forward primer	5'-AGTGGTGAGCTCTATGCTGCAA-3'
	reverse primer	5'-CCCAGACTTCGGTAAATGGC-3'
DM-GRASP:	forward primer	5'-GCAAACTGATTGTGGGAATTG-3'
	reverse primer	5'-CAGTAGACGACACCAGCAACG-3'

Shown is a list of forward and reverse primers specific to the murine genes used in quantitative RT-PCR.



**Figure 2**

**Effect of Li<sup>+</sup> or PMA on mesothelin and SL-1 expression in C57mg cells.** For Li<sup>+</sup> treatment, C57mg cells were treated with 10 mM LiCl or 10 mM KCl as control for 6 hours. For PMA treatment, C57mg cells were treated with 1  $\mu$ M PMA or ethanol as control for 6 hours. RNA was extracted and RT-PCR analysis was performed using primers specific to mesothelin or SL-1 mRNAs. mRNA levels were normalized to HPRT and fold increase shown is relative to KCl treatment in the case of Li<sup>+</sup> and to ethanol treatment in the case of PMA. Mean levels were determined from 3 independent experiments.

for 8 hours at which point RNA was isolated and quantitative RT-PCR was performed. Supporting the co-culture data discussed above, SL-1 exhibited almost a 3-fold induction with isoproterenol treated Rfz-2/ $\beta_2$ AR expressing C57mg cells but not wt C57mg cells treated with isoproterenol (Figure 1, panel B).

SL-1 appears to be up-regulated by Wnt-5a and not Wnt-1 (Table 1 and Figure 1, panel A). Conversely, mesothelin appears to be up-regulated specifically by Wnt-1 and not Wnt-5a since mesothelin was not induced by co-culturing with Wnt-5a secreting cells or by activation of the chimeric Rfz-2/ $\beta_2$ AR receptor (Figure 1, panels A and B). To our knowledge this is the first report to show that two genes

are differentially regulated by two different Wnts within the same cell type.

In order to examine the mechanism by which Wnt-1 or Wnt-5a regulates mesothelin or SL-1, respectively, C57mg cells were treated with either LiCl or phorbol myristic acetate (PMA). LiCl has previously been shown to mimic activation of Wnt-1 signaling by stabilizing beta-catenin through its inhibition of glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ), a kinase that phosphorylates beta-catenin and promotes its degradation via the ubiquitin proteasome pathway [39]. C57mg cells treated with LiCl exhibited elevated levels of mesothelin but not SL-1 (Figure 2, left panel). On the other hand, PMA treatment of C57mg cells

elevated SL-1 transcript levels 6-fold but not mesothelin (Figure 2, right panel). PMA, a phorbol ester, activates PKC and has previously been shown to activate SL-1 [40]. It appears that mesothelin is activated by Wnt-1, which can be mimicked by LiCl, and that SL-1 is activated by Wnt-5a in a beta-catenin-independent manner, which can be mimicked by activating PKC.

## Discussion

We used DNA microarray analysis to identify genes regulated by Wnt-1 and Wnt-5a in C57mg cells. C57mg cells were chosen since they become transformed by ectopic expression of Wnt-1 and by loss of function of Wnt-5a [12,15,16]. In addition, Wnt-5a/Rfz-2 C57mg cells exhibited phenotypic differences from wt C57mg cells (see results). We focused on two genes that showed differential regulation by Wnt-1 and Wnt-5a since their regulation was reproducible using several methods to activate the Wnt signaling pathways. The first, mesothelin, was up-regulated by Wnt-1 both by stable expression of Wnt-1 in C57mg cells and by co-culturing C57mg cells with Wnt-1 secreting cells. Similarly, mesothelin expression was induced by Li<sup>+</sup>, an inhibitor of GSK-3 $\beta$ , that mimics Wnt-1. Mesothelin is markedly over-expressed in a variety of cancers derived from tissues of the mesothelium, including lung, cervix, and pancreas. Interestingly, mesothelin expression is induced in lung, ovarian, and pancreatic carcinomas where constitutive activation of Wnt signaling is caused by stabilizing mutations in beta-catenin [27,28,41–44]. The induction of mesothelin in these carcinomas may be due to misregulation of Wnt signaling as are other targets of the Wnt pathway, c-myc and cyclin D1. Mutations in different effectors of Wnt signaling have been identified in a variety of cancers. Interestingly, Wnt-5a down-regulated mesothelin expression, perhaps through antagonism of endogenous Wnt/beta-catenin signaling.

Second, SL-1 was up-regulated by Wnt-5a in C57mg cells. Three lines of evidence demonstrate that SL-1 induction is associated with activation of Wnt-5a signaling. First, C57mg cells stably expressing Wnt-5a and Rfz-2 elevate SL-1 levels. Second C57mg cells co-cultured with Wnt-5a secreting cells up-regulate SL-1. Third, activation of chimeric Rfz-2 receptors stably expressed in C57mg cells augment SL-1 expression. To further examine how SL-1 is up-regulated by Wnt-5a, we tested whether SL-1 induction could be mediated by downstream effectors of Wnt/Ca<sup>2+</sup> signaling such as PKC. Activation of PKC by PMA treatment of C57mg cells did indeed elevate SL-1 levels significantly. SL-1 is an important modulator of cell adhesion through its function as a matrix metalloproteinase that degrades the ECM. SL-1 induction in p2S cells, an untransformed mouse mammary epithelial cell line, triggered the disappearance of E-cadherin and beta-catenin

from cell-cell junctions and reduced the expression of beta-catenin [35]. Wnt-5a and SL-1 may play similar roles in inhibiting cadherin-mediated cell-adhesion, as Wnt-5a decreases Ca<sup>2+</sup>-dependent cell adhesion similar to a dominant negative cadherin [11]. The regulation of SL-1 by Wnt-5a may be important in developmental processes such as gastrulation where Wnt-5a affects the morphogenetic movement of tissues [45].

Whether mesothelin and SL-1 are direct targets of Wnt-1 and Wnt-5a, respectively, and whether alterations in their expression are responsible for the observed phenotypic changes in Wnt-5a/Rfz-2/C57mg cells, has yet to be determined. Whether they are direct or indirect does not alter the observation that the expression of both change in an unexpected manner in response to these two Wnts. Much less is understood about Wnt-5a signaling pathway(s) and how Wnt-5a signals are transmitted to the nucleus to affect gene expression compared to the canonical Wnt/beta-catenin signaling pathway. A recent report demonstrated that Wnt-5a and Rfz-2 can induce the nuclear localization of the transcription factor NF-AT [14]. Their results show that NF-AT is a downstream target of the Wnt/Ca<sup>2+</sup> pathway and antagonizes the Wnt/beta-catenin pathway in *Xenopus* embryos. The SL-1 promoter has putative NF-AT binding sites but whether these are important for Wnt-5a regulation requires further testing. Nevertheless, our data clearly demonstrate that different Wnts have distinct effects on gene expression in a single cell line.

## Conclusions

Using DNA microarray analysis we identified several genes that are differentially regulated by Wnt-1 and Wnt-5a signaling within the same cell type, C57mg. We focused on two genes, mesothelin and SL-1, whose regulation by Wnts was reproducible using a variety of methods to activate the Wnt signaling pathways. Mesothelin, a potential tumor serum marker, was up-regulated by Wnt-1 and down-regulated by Wnt-5a. SL-1, a matrix metalloproteinase, was up-regulated by Wnt-5a only, showing that Wnt-5a can indeed affect gene expression. Our data indicate that different Wnts can stimulate distinct sets of genes.

## Methods

### Cell Culture, retroviral infections, and DNA transfections

C57mg cells were grown in DMEM supplemented with 10% FBS and 10  $\mu$ g/ml of insulin. 293T cells were grown in DMEM supplemented with 10% FBS. The Wnt-5a/Rfz-2 expressing C57mg cell line and the Rfz-2/ $\beta_2$ AR expressing C57mg cell line were established by retroviral infections. The coding region for *Xenopus* Wnt-5a including the myc epitope tag in pSP64T [45] was digested with BglII, treated with klenow enzyme and ligated into the retroviral vector PMI-hCD5 [46] digested with NotI and

treated with Klenow enzyme. The Rfz-2 coding sequence from plasmid CS2+ [47] was amplified by PCR using the following primers: 5'-AAAAGCGGCCGCGAGTGGGGGCGGCCGCGCC-3' and 5'-AAAAGTCGACGCGCCAGCAGCGAGACCGC-3'. The PCR product was cloned into the NotI and SalI sites in PMI-hCD2 [46]. The coding sequence for the Rfz-2/ $\beta_2$ AR chimeric receptor in CS2+ [6,37] was digested with EcoRI and XhoI and cloned into PMI-hCD2 at the EcoRI and SalI sites. Generation of the retrovirus, infection of the C57mg cell line, and enrichment of infected cells was performed as described [46,48]. Cell populations enriched to >95% were used for RNA isolation. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) as directed by the manufacturer. Wnt-1 expressing C57mg cells were a kind gift from G. Shackelford. For co-culture experiments, 293T cells were transfected with either Xenopus Wnt-5a [45], mouse Wnt-1 [49], or CS2+ as control using the lipofectamine PLUS reagents (GIBCO BRL) as directed by the manufacturer. 24 hours after transfection the Wnt-expressing 293T cells were transferred to culture dishes seeded with C57mg cells. Both cell types were plated at a 1:1 ratio. Cells were co-cultured for 24 hours and RNA was isolated. For induction of the Rfz-2/ $\beta_2$ AR chimeric receptor, chimeric receptor expressing C57mg cells or wt C57mg cells were treated with 10  $\mu$ M propranolol overnight and then with 10  $\mu$ M isoproterenol for 8 hours. Control cells were treated with 10  $\mu$ M propranolol only. For Li+ and PMA stimulation experiments, C57mg cells were incubated with 10 mM LiCl, 10 mM KCl as a control, or 1  $\mu$ M PMA for 6 hours. All treatments were followed by RNA extraction.

#### Oligonucleotide Array

Total RNA was extracted using the RNeasy kit (Qiagen) according to the manufacturer's protocols. Transcript profiling with Affymetrix GeneChips (Affymetrix, Santa Clara, CA) was performed using the Mu11KsubA and subB chips containing ~11,000 probe sets. Briefly, 15  $\mu$ g of total RNA were reverse-transcribed with an oligo(dT) primer coupled to a T7 RNA polymerase binding site. Biotinylated complementary RNA (cRNA) was then synthesized from the resulting complementary DNA (cDNA) with the use of T7 polymerase. 30  $\mu$ g of biotinylated cRNA were then randomly sheared (to an approximate length of 50 nucleotides). Hybridization to the arrays and first-pass analysis of the scanned data were performed at the PAN Facility (Stanford University, Palo Alto, California).

#### Reverse Transcriptase-PCR (RT-PCR) Analysis

Confirmation of gene expression was performed using quantitative RT-PCR. cDNA was created from total RNA using the ThermoScript RT-PCR System (Invitrogen) with an oligo(dT)<sub>20</sub> primer. Minus RT controls were also prepared similarly. 2  $\mu$ g RNA were included in each reaction in a total volume of 20  $\mu$ l. The reaction was performed at

55°C for 1 hour and terminated by incubating at 85°C for 5 minutes. The reaction was diluted 7.5-fold in water and 2.5  $\mu$ l were added to the PCR reaction to yield a total volume of 25  $\mu$ l. Since nonspecific amplification was obtained in testing some of the primers sets with the -RT controls, optimization of the primer concentration was performed as per the SYBR green PCR protocol (Applied Biosystems). Three concentrations of each of the forward and reverse primers were used, 50 nM, 300 nM, and 900 nM, to obtain nine different combinations. The amplification reaction was performed using the following thermocycler conditions: 94°C for 5 min followed by 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C 30 s. +RT, -RT, and no template controls were tested for the primer concentration optimization and samples were taken out at 20, 25, 30 and 35 cycles to run on 2% agarose gels. The combination of forward and reverse primer concentrations was selected based on the presence of a single band only in the +RT sample. Using the optimized primer concentrations, the PCR reaction was performed with the SYBR Green PCR Master Mix (Applied Biosystems) and the ABI Prism 7700 Sequence Detector (Applied Biosystems). The amplification reaction consisted of the following: 50°C for 2 min, denaturation at 95°C for 10 minutes, and 40 thermal cycles of 95°C for 15 s and 60°C for 1 min. PCR quantification was performed in triplicate. Fold induction was obtained by using the  $\Delta\Delta C_t$  method in which all samples are first normalized to the level of mouse hypoxanthine phosphoribosyltransferase (HPRT) in each sample. Relative normalized units were then compared between the experimental sample and its control. The primer sequences are shown in Table 2. To determine the specificity of the primer sets to the murine mesothelin and SL-1 targets genes, we performed PCR using the primers sets with cDNA from human 293T cells, and found no amplification.

#### Author's contributions

MP performed the experiments. RTM supervised the work. All authors read and approved the final manuscript.

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MP is an Associate, and RTM an Investigator, of the HHMI.

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