Mechanical force modulates global gene expression and β-catenin signaling in colon cancer cells

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Summary
At various stages during embryogenesis and cancer cells are exposed to tension, compression and shear stress; forces that can regulate cell proliferation and differentiation. In the present study, we show that shear stress blocks cell cycle progression in colon cancer cells and regulates the expression of genes linked to the Wnt/β-catenin, mitogen-activated protein kinase (MAPK) and NFκB pathways. The shear-stress-induced increase of the secreted Wnt inhibitor DKK1 requires p38 and activation of NFκB requires IκB kinase-β. Activation of β-catenin, important in Wnt signaling and the cause of most colon cancers, is inhibited by shear stress through a pathway involving laminin-5, α6β4 integrin, phosphoinositide 3-kinase (PI 3-kinase) and Rac1 coupled with changes in the distribution of dephosphorylated β-catenin. These data show that colon cancer cells respond to fluid shear stress by activation of specific signal transduction pathways and genetic regulatory circuits to affect cell proliferation, and indicate that the response of colon cancers to mechanical forces such as fluid shear stress should be taken into account in the management of the disease.

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Key words: β-catenin, Shear stress, Laminin, α6β4 integrin, Rac, PI 3-kinase, Cell cycle

Introduction
The effects of mechanical force induced by fluid shear stress on the activity of several signal transduction pathways in endothelial cells is well documented (for a review, see Resnick and Gimbrone, Jr, 1995). Mechanical forces, such as those generated by fluid shear stress, are sensed at the cell surface (at adhesion points) by β1 and β3 integrins, and transduced through the cell by kinase cascades that ultimately result in changes in gene expression (for reviews, see Shyy and Chien, 2002; Alenghat and Ingber, 2002). Although most studies that investigate the effects of shear stress use endothelial cells, it is clear that many epithelia are regularly exposed to shear stress during normal activity in vivo. For example, epithelial cells in the gastrointestinal tract are continually subjected to peristalsis and shearing forces as gut contents move and as cells move along the crypt-villus unit (Jeffrey et al., 2003). In addition, cancers of the intestinal tract are exposed to differing degrees of fluid shear stress as they grow into the lumen of the gut. Laminar flow and the resulting shear stresses within the intestinal epithelium are required for its normal function in uptake and transport, and some studies suggest a role for gut motility and pressure changes in regulating the differentiation and proliferation of enterocytes (Basson, 2003).

Activation of the Wnt/β-catenin pathway, either by mutation of adenomatous polyposis coli (APC) or β-catenin itself, is the cause of most colon cancers (for a review, see Polakis, 1999). In the absence of Wnt signaling a complex consisting of APC, axin, protein phosphatase 2A (PP2A) and glycogen synthase kinase-3 phosphorylates β-catenin, marking it for degradation by the ubiquitin-proteosome pathway and thereby preventing activation of T-cell factor (TCF)-regulated genes. In Drosophila and in mouse osteocytes, the Wnt/β-catenin pathway is activated by twisting or stretching forces (Farge, 2003; Norvell et al., 2004). However, no studies have investigated the effects of mechanical forces on β-catenin signaling in cells in which the Wnt pathway is already activated, such as colon cancer cells. In other studies we used short-term laminar flow assays as a tool to investigate the role of integrins in cell-rolling behavior and to measure cadherin-mediated adhesion strength (Byers et al., 1995; Tozeren et al., 1994). We now show that cell cycle progression in colon cancer cells is regulated by mechanical force induced by fluid shear flow. We used shear stresses of 4-35 dyn/cm², a range normally found in the intestinal epithelium during peristalsis (Jeffrey et al., 2003). Our studies show that fluid shear stress activates the small G-protein Rac1 and the mitogen-activated protein kinase (MAPK) substrate p38 and regulates the expression of genes linked to the Wnt/β-catenin, MAPK and nuclear factor-κB (NFκB) pathways. We further demonstrate that α6β4 integrin
functions as a mechanosensor of the flow-induced shear stress, and that a decrease in β-catenin signaling is mediated by phosphoinositide 3-kinase (PI 3-kinase) and the small G-protein Rac1. Remarkably, even though shear stress does not affect total β-catenin levels, it has a dramatic effect on the localization of activated, dephosphorylated β-catenin.

**Results**

Laminar shear stress regulates the expression of genes linked to the Wnt/β-catenin, MAPK/JNK and NFκB pathways in colon cancer cells

SW480 cells were exposed to 15 dyn/cm² shear stress for 12 hours or grown under the same conditions in the absence of shear stress. Total RNA was isolated, amplified and subjected to microarray analysis. Several shear stress-regulated genes could be linked directly or indirectly to the Wnt/β-catenin (Table 1 and supplementary material).

Other shear stress-regulated genes could be linked either directly or indirectly to the MAPK-c-Jun NH2-terminal kinase (JNK) or NFκB pathways (Table 1 and supplementary material). Change in the expression of several genes was confirmed by real-time PCR (supplementary material Table S2).

Differential regulation of NFκB, p38, Rac1 and Wnt/β-catenin by fluid shear stress in colon cancer cells

Fluid shear stress activates MAPKs, including JNK and extracellular signal-regulated kinases (ERKs), in vascular endothelial cells (for a review, see Shyy and Chien, 2002). In keeping with the known constitutive activation of certain MAPK pathways in colon cancer cells we found that pERK and pJNK levels were very high in SW480 cells even when grown under static conditions (Fig. 1A). A slight increase in pERK and pJNK was observed following exposure to shear stress. p38 phosphorylation was clearly and consistently increased transiently by shear stress in SW480 and HT29 cells (Fig. 1B), consistent with an earlier study that showed that Rac1 was rapidly converted to its GTP-bound form by shear stress (Fig. 1C).

Dickkopf-1 (DKK1) is a secreted protein that potently antagonizes Wnt signaling but may also have functions independent of its role in Wnt signaling (Holmen et al., 2005). Our microarray data, qPCR and western blot shows that DKK1 expression is robustly increased by shear stress in SW480 cells (Fig. 1D). DKK1 mRNA is also regulated by shear stress in HT29 colon cancer cells (Fig. 1E). Consistent with the activation of p38 by shear stress, we found that only the p38 inhibitor sb202192 but not inhibition of JNK/ERK1 inhibited the shear-mediated increase in DKK1 expression.

In endothelial cells, shear stress-induced NFκB activation depends on the activation of the IkκB kinase (IKK) complex (Bhullar et al., 1998). Our expression data analysis also points to activation of the NFκB pathway by shear in SW480 cells. Consistent with this, exposure to shear stress increased NFκB reporter activity threefold and was reversed in cells expressing dominant-negative (DN) IKKβ but not in cells expressing DN IKKα (Fig. 1F).

**Table 1. Laminar shear stress regulates the expression of genes linked to the Wnt/β-catenin, MAPK/JNK and NFκB pathways in colon cancer cells**

| Gene ID | Symbol | Fold-change | WNT | MAPK | NFκB |
|---------|---------|-------------|-----|------|------|
| NM_002546.1 | TNFRSF11B | −3.56±0.534 | Up | Up | Up |
| AF016535.1 | MDR1 | −2.74±0.649 | P-glycoprotein (ABCB1) (Yamada et al., 2000) | Up | Up | – |
| U57059.1 | TNFRSF10A | −2.43±0.699 | TNF superfamily, member 10/TRAIL (Wang et al., 2003) | Up | Down | Up |
| NM_021151.4 | PSA | −2.39±0.623 | Phosphoserine aminotransferase (Ojala et al., 2002) | Up in colon cancer | – | – |
| U04897.1 | RORA | −2.14±0.408 | Nuclear receptor RORα1 (Delerive et al., 2001) | Interacts with p300 | Antagonist | – |
| S99110.1 | HOXA1 | −1.98±0.145 | Homeobox A1 (Calvo et al., 2000) | Up | – | – |
| NM_001731 | BTG1 | −1.92±0.45 | B-cell translocation gene 1 (Saka et al., 2000) | Regulated by brachyury | – | – |
| AF153882.1 | RIL | 1.99±0.230 | Reversion-induced LIM protein (Fu et al., 2000) | – | Up | – |
| NM_005562.1 | LAMC2 | 2.04±0.281 | Laminin, gamma 2 (Huubek et al., 2001) | Up | Up | – |
| NM_002228.2 | JUN | 2.07±0.350 | v-jun 17 oncogene homolog (Mann et al., 1999) | Up | Up | – |
| U16996.1 | DUSP5 | 2.14±0.403 | Dual specificity phosphatase 5 (Kovanen et al., 2003) | Up in colon cancer | – | – |
| NP-054690 | GADD45b | 2.23±0.296 | Growth arrest and DNA-damage-inducible 45b | Down | Antagonist | – |
| U08839.1 | PLAU | 2.28±0.338 | Plasminogen activator (Shibata et al., 2000) | Up | – | – |
| NM_003550 | MAD2L1 | 2.42±0.146 | MAD2 mitotic arrest deficient-1 (Matinez et al., 2003) | Down | – | – |
| NM_021913.1 | AXL | 2.84±0.738 | AXL receptor tyrosine kinase (Goruppi et al., 1996) | – | Activator | – |
| BG251266 | FOSL1 | 3.67±1.39 | FOS-like antigen 1 (Mann et al., 1999) | Up | – | – |
| U37546.1 | MBHC | 3.71±1.207 | IAP homolog C (MBHC) (Pryhuber et al., 2002) | Up | – | – |
| NM_012242 | DKK1 | 5.54±2.56 | Dickkopf homolog 1 (Ma et al., 2002) | Antagonist | Up | – |
| NM_001554 | CYR61 | 6.03±3.27 | Cysteine-rich, angiogenic inducer, 61 (Han et al., 2003) | – | Up | – |

Rac1 is a crucial upstream mediator of MAPK pathways and is a particularly important regulator of p38 (Tzima et al., 2002). We next tested the ability of Rac1 to interact with p38 following short periods of shear stress (Benard et al., 1999). We modified the Rac assay to increase sensitivity (see Materials and methods). The modified PBD pull-down assay showed that Rac1 was rapidly converted to its GTP-bound form by shear stress (Fig. 1C).

Laminar shear stress negatively regulates β-catenin signaling

The expression of most β-catenin target genes (MDR1, PSA, BTG1, HOX1, osteoprotegerin) was decreased by shear stress, pointing to potential inhibition in Wnt-pathway activity, whereas the expression of other genes, such as JUN, FOSL1 and laminin γ2, was increased by shear stress. However, even though these genes are nominal targets of β-catenin/TCF they are also regulated by other pathways, including the MAPK-JNK pathway. It is likely that the overall change in gene expression in response to mechanical forces is a result of the combined action of several signal transduction
pathways, including the Wnt/β-catenin, MAPK and NFκB pathways. To directly test the effects of mechanical strain on the Wnt/β-catenin pathway we examined the effects of laminar shear stress on the activity of β-catenin/TCF-sensitive promoters.

Laminar flow shear stress experiments were performed with SW480 colon cancer cells transiently transfected with a β-catenin/TCF luciferase reporter (TOPFLASH). A similar reporter construct (FOPFLASH), which has mutated TCF/lymphoid enhancer factor (LEF) binding sites, was used as a negative control. Many experiments were also performed using a different reporter set, OT and OF, with identical results. After 12 hours of exposure the activity of TOPFLASH and the TCF-sensitive cyclin D1 promoter reporter decreased as shear stress increased from 0 to 35 dyn/cm² (Fig. 2A,B). Similar results were obtained in SW480 cells grown at high and low cell density (Fig. 2C). The SW480 cells used in our studies do not express E-cadherin at any density (data not shown). All other studies were performed at low cell densities to minimize the effects of cell-cell adhesion. Exposure to shear stress of 25 dyn/cm² resulted in a maximum decrease in β-catenin signaling. Because SW480 cells contain a mutant APC gene, which results in elevated β-catenin protein levels and signaling, these results show that the shear stress regulation of β-catenin signaling is independent of APC-regulated β-catenin degradation. Consistent with this, no changes were observed in the total amount of β-catenin before and after shear flow (inset in Fig. 2A). Shear flow experiments were also performed in β-catenin-transfected A1N4 human mammary epithelial cells that contain functional APC. After 12 hours of exposure to shear flow, β-catenin signaling decreased to 22% of control values (Fig. 2D). To investigate the time dependence of shear flow modulation on β-catenin signaling, SW480 cells were exposed to 15 dyn/cm² of shear stress for 0-24 hours. β-catenin signaling decreased significantly following 12 hours of flow, reaching a maximum after 24 hours (Fig. 2E). To ensure that the changes in β-catenin signaling were reversible following return to static conditions, SW480 cells were exposed to 15 dyn/cm² of shear stress for 12 hours, which point the shear flow was stopped and the cells were incubated in static culture for 0 to 25 hours. β-catenin signaling returned to pre-shear levels after 25 hours of static incubation, demonstrating that the effects of shear stress are not a result of changes in cell viability (Fig. 2F).

Fluid shear stress causes SW480 cell cycle arrest in G1

Laminar shear stress inhibits vascular endothelial cell proliferation and blocks the cell cycle at the G0/G1 phase (Akimoto et al., 2000). By contrast, increased luminal pressure and cyclic strain stimulates the proliferation of colonic cells (Walsh et al., 2004). As normal intestinal cells and colon cancer cells are
exposed to fluid shear stress as well as changes in pressure and strain, we explored the effect of shear stress on the cell cycle in the colon cancer cell line SW480. Fluorescence-activated cell sorting (FACS) analysis of SW480 cells exposed to continuous shear flow (15 dyn/cm²) for 24 hours showed that 61% of cells exposed to shear flow remained in the G1 phase of the cell cycle, compared with 37% of cells not exposed to shear stress (Fig. 2G,H). This degree of G1 block is highly significant considering the short duration of the experiment, and is consistent with the effects of fluid shear stress on the cell cycle in endothelial cells and with the effects of β-catenin on G1/S progression (Orford et al., 1999).

Laminin and fibronectin affect fluid shear stress modulation of β-catenin signaling

Although the experiments described above were performed by incubating cells on glass without added extracellular matrix (ECM), our microarray results demonstrate that shear stress increased the expression of the β3 and γ2 chains of laminin-5 but not other ECM components, such as fibronectin or collagen. Thus, during the course of the experiments it was clear that cells do produce endogenous ECM components, particularly laminin. To investigate the effects of ECM on shear flow-regulated β-catenin signaling, SW480 cells were incubated on glass slides coated with laminin or fibronectin (0-50 μg/ml). In cells not exposed to shear flow, β-catenin signaling was unaffected by laminin or fibronectin (Fig. 3A). Laminin significantly potentiated the effects of shear flow on β-catenin signaling when compared with glass alone, whereas fibronectin attenuated it (P<0.01, Student’s t-test; Fig. 3B). These data indicate that integrin cell adhesion receptors, most likely those that interact with laminin, mediate the shear flow-induced modulation of β-catenin signaling.

α6β4 integrin functions as a mechanosensor for fluid shear stress modulation of β-catenin signaling

SW480 cells express α1, α2, α3, α5, α6, β1 and β4 integrins and do not express α4 or β3 integrins (Schramm et al., 2000). α5β1 is predominantly a fibronectin receptor and α6β1 and α6β4 are laminin receptors. In colon cancer cells such as SW480 cells that express α6, β1 and β4 integrins, α6β4 is the favored heterodimer (Tozzeren et al., 1994). SW480 cells were incubated in suspension with anti-α5,-α6,-β1 and -β4 integrin-blocking antibodies for 2 hours prior to use in laminar flow assays (Fig. 3). Incubation of cells with anti-α6 or anti-β4 integrin antibody completely reversed shear flow-induced reduction of β-catenin signaling without any obvious effect on cell morphology or detachment in response to flow. By contrast, incubation with anti-α5 and anti-β1 integrin antibody did not significantly reverse signaling (Fig. 3C). However, incubation of cells with anti-β1 integrin antibody did
result in several cells (~30% reduction in renilla values) detaching following the onset of shear flow. β-catenin signaling in cells treated with the same antibodies but not exposed to shear stress was unaffected (Fig. 3D). These results indicate that α6β4, but not α5β1 or any other β1-integrin, functions as a mechanosensor of mechanical force-mediated effects on β-catenin in SW480 colon carcinoma cells. The data are consistent with the expression array data showing that the α6β4 ligand laminin-5 is increased following shear stress.

Fluid shear stress modulation of β-catenin signaling requires the activity of PI 3-kinase and Rac1

We next wanted to identify intracellular mediators or components of the signaling cascade linking α6β4 integrin to β-catenin nuclear signaling. Preliminary experiments ruled out a role for glycogen synthase kinase 3 (GSK-3) and the Wnt inhibitor DKK (not shown). Kinases that are known to be components of integrin-mediated kinase signaling cascades include focal adhesion kinase (FAK), integrin-linked kinase (ILK), IKK, PI 3-kinase, Rac and NFκB-inducing kinase (NIK) (for a review, see Shyy and Chien, 2002). Shear flow regulation of β-catenin signaling was unaffected by the FAK inhibitor FRNK, DN IKKα, DN IKKβ, kinase-dead (KD) ILK or KD NIK (Fig. 3E). In other studies (to be presented elsewhere) we show that DN IKKα or DN IKKβ, at concentrations less than those used in the present study, completely inhibits TNFα regulation of β-catenin signaling in SW480 cells. Thus, although the IKK pathway is activated by shear in SW480 cells, and mediates the NFκB response, it is not involved in the observed changes in β-catenin signaling.

PI 3-kinase activity plays a role in cell growth, proliferation, survival and differentiation, and is stimulated by exposure to shear flow in endothelial cells (Shyy and Chien, 2002). Because PI 3-kinase can be activated by α6β4 integrin, we considered it a candidate transducer of the shear stress signal sensed by α6β4 integrin (Hintermann et al., 2001). In addition, α6β4 integrin activation of PI 3-kinase promotes carcinoma cell invasion, a process that exposes the cell to mechanical strain (Shaw et al., 1997). The effects of shear stress on β-catenin signaling were significantly (P<0.01, Student’s t-test) attenuated in cells transfected with a DN mutant of the p85 regulatory subunit of PI3-kinase (Fig. 3F). DN p85 also slightly increased β-catenin signaling in SW480 cells not exposed to shear stress. Experiments were also performed with a membrane-targeted constitutively active (CA) catalytic subunit of PI3-kinase (p105 CAAX). Transfection of cells with p105 CAAX decreased β-catenin signaling in cells in static culture and potentiated the effects of shear flow. By contrast, transfection of cells with KD p105 CAAX partly reversed the effects of shear flow on β-catenin signaling but did not significantly affect signaling in the absence of shear stress (Fig. 3F). Collectively, these data
Mechanical force modulates gene expression

indicate that PI 3-kinase is involved in a shear flow-regulated β-catenin signaling pathway but that it is probably not completely responsible for transmitting the α6β4 integrin signal. Although the serine/threonine kinase Akt is a well-known downstream target of PI 3-kinase, expression of DN Akt in SW480 cells did not affect β-catenin signaling under static or shear conditions (Fig. 3F).

Rac1 is a crucial mediator of the integrin-specific control of the cell cycle in endothelial cells, an effect that depends on PI 3-kinase but is independent of Akt (Mettouchi et al., 2001; Rodriguez-Viciana et al., 1997). Activation of Rac1 is required for the differentiation and migration of enterocytes along the crypt-villus unit in the developing mouse intestine (Stappenbeck and Gordon, 2000). Rac1 is activated in SW480 and HT29 cells following exposure to shear stress, and the shear stress-induced decrease in β-catenin signaling was blocked in cells transfected with DN Rac1, demonstrating that Rac1 is a component of the shear flow-regulated β-catenin signaling pathway (Fig. 3F).

Fluid shear stress regulates the level and localization of dephosphorylated activated β-catenin

Our microarray data show that cyclin D1 mRNA levels are unaffected by shear stress. Consistent with this, cyclin D1 protein level and localization was the same before and after shear stress (Fig. 4A,B). Although the total amount of β-catenin in SW480 cells is not affected by shear stress, it is possible that the distribution of a small pool of activated dephosphorylated β-catenin in these cells is altered (van Noort et al., 2002). β-catenin was distributed in both the cytoplasm and nucleus of cells not exposed to shear (Fig. 4C). Following 12 hours of shear stress, no reduction in total β-catenin staining was observed (Fig. 4D). In most cells nuclear staining of β-catenin was more intense in cells exposed to shear stress. In SW480 cells dephosphorylated activated β-

![Image](Fig. 4. Shear flow negatively regulates the level and localization of activated β-catenin. Localization of cyclin D1 in control cells (A) and cells exposed to shear stress (B). Localization of β-catenin in control cells (C) and cells exposed to shear stress (D). Localization of dephosphorylated activated β-catenin in control cells (E) and cells exposed to shear stress (F). Lanes 1-4 of the inset in F show a western blot of dephosphorylated β-catenin in the cytoplasmic (lanes 1, 3) and NP-40 pools (lanes 2, 4) before (lanes 1, 2) and after (lanes 3, 4) shear stress. The numbers above the lanes indicate the densitometric ratio of activated β-catenin (static:shear).

![Image](Fig. 5. (A,B) Localization of β-catenin phosphorylated on residues 33, 37 and 41 in control cells (A) and in cells exposed to shear stress (B). (C,D) Localization of β-catenin phosphorylated on residues 41 and 45 in control cells (C) and in cells exposed to shear stress (D). (E,F) Cells were transfected with β-catenin mutated on residues 33 and 37 and with TOPFLASH (E) or with a –163 cyclin D1 reporter (F) and exposed to shear stress for 16 hours. (G) HCT116 cells, which express β-catenin missing serine 45, were transfected with TOPFLASH and exposed to shear stress for 12 hours.)
catenin is present in the cytoplasm and is particularly concentrated in the nuclei (Fig. 4E). The nuclear and cytoplasmic staining of phosphorylated β-catenin was markedly reduced in cells exposed to fluid shear stress (Fig. 4F). The image in Fig. 4F is somewhat overexposed so that the cells could be imaged. The cytoplasmic but not NP-40-soluble pool of phosphorylated β-catenin was reduced more than twofold (inset in Fig. 4F). Note that in Fig. 2A (inset) we showed that total β-catenin levels were unchanged by shear stress. We next investigated the localization of phosphorylated forms of β-catenin using antibodies specific to β-catenin phosphorylated on S33, S37, T41 or S45 (Fig. 5A,B). Staining for the 33/37/41 phosphorylated form of β-catenin was low in control SW480 cells and was predominantly localized in the cytoplasm and was absent from the nuclei; however, in cells exposed to shear stress, staining was more intense and was clearly localized to both the nucleus and cytoplasm. Similar results were observed using an antibody specific for the S45 phosphorylated form of β-catenin (Fig. 5B). We were unable to use these antibodies for western blot analysis and could not verify whether the actual level of phosphorylated β-catenin was affected by shear stress. However, if the level and localization of activated β-catenin are important for regulation by shear stress, a form of β-catenin that cannot be phosphorylated on serine residues 33 and 37 or 45 should confer resistance to shear stress. To test this we transfected SW480 cells with the S33A/S37A double mutant β-catenin. Transient transfection of this mutant form of β-catenin significantly reversed the effects of shear stress on the activity of TOPFLASH and the TCF/β-catenin-regulated cyclin D1 reporter (Shtutman et al., 2002) (Fig. 5C). To further test the role of N-terminal serine residues we used another colon cancer cell line, HCT116, which has normal APC but expresses one allele of β-catenin in which serine 45 is deleted (Sekine et al., 2002). In contrast to SW480 cells and β-catenin-transfected A1N4 cells, β-catenin signaling activity in HCT116 cells was unaffected by shear stress (Fig. 5C). These data indicate that shear stress modifies the localization or degradation or phosphorylation status of a small pool of activated β-catenin.

Discussion

In the present study we show that mechanical forces imparted as a result of laminar flow block the cell cycle at G1 and regulate the MAPK, Wnt/β-catenin and NFκB pathways as well as the expression of specific genes in colon cancer cells. We go on to show that the shear stress is sensed and transduced by a pathway that includes laminin-5, α6β4 integrin, PI 3-kinase and Rac1 to selectively regulate the level of activated β-catenin, the single most important mediator of colon cancer in humans.

Activation of NFκB, p38 and Rac1

NFκB reporter activity and nuclear DNA binding is markedly increased by fluid flow-induced shear stress in endothelial cells (Lan et al., 1994). The effects of shear stress on NFκB activity require rapid integrin-mediated activation of the IKK complex (Bhullar et al., 1998). Inflammation of the intestinal epithelium and activation of NFκB are known to be important in diseases such as colitis and colon cancer (Andresen et al., 2005; Scaife et al., 2002). Shear stress induced a significant increase in NFκB reporter activity in SW480 cells that was prevented by expression of DN IKKβ. Interestingly, in a colitis-associated cancer model, deletion of IKKβ in intestinal epithelial cells leads to a dramatic decrease in tumor incidence (Greten et al., 2004).

In endothelial cells, activation of the ERK and JNK pathways is involved in the differential effect of shear stress (Jo et al., 1997). However, in cancer cells these pathways are often constitutively active, as is the case in the SW480 and HT29 cells used in our experiments (Steinmetz et al., 2004). Although shear stress slightly increased ERK and JNK activation in SW480 cells, it actually decreased JNK activation in HT29 cells. Thus, any additional activation of ERK or JNK pathways by shear stress in SW480 cells is not likely to be responsible for the observed changes in gene expression, although it is possible that the high constitutive level of ERK activation is required for the effect of shear stress to be manifest.

p38, the third branch of the MAPK family, has been implicated in cell shape changes and migration (Hedges et al., 1999; Rousseau et al., 1997). In particular, the p38/heat shock protein (HSP) 25/27 complex is thought to play a crucial role in actin dynamics. p38 is activated by shear stress in endothelial cells and its function is related to their reorientation (Azuma et al., 2001). Activation of p38 by shear stress in colon cancer cells may also result in reorganization of the actin cytoskeleton and is certainly responsible for the changes in the expression of DKK1 that we observed. One particularly important regulator of p38 is Rac1 (Han et al., 2003). Activation of Rac1 is also required for the differentiation and migration of enterocytes along the crypt-villus unit in the developing mouse intestine (Stappenbeck and Gordon, 2000). Our data show a rapid transient increase in the amount of GTP-bound Rac1 following shear stress, suggesting that shear stress activation of Rac1 may be involved in p38 activation and the observed changes in gene expression.

Genes regulated by shear stress in colon cancer cells

Remarkably, our microarray analyses identified only 68 shear stress-sensitive genes in colon cancer cells. Of these, 18 can be clearly linked to the Wnt/β-catenin/TCF and/or MAPK-JNK and/or NFκB pathways. Some of these – for example, the multi-drug resistance gene (MDR1, P-glycoprotein), JUN, FOSL1 (FRA1), osteoprotegerin and laminin γ2 – are known direct targets of β-catenin/TCF (Yamada et al., 2000; Mann et al., 1999; Olsen et al., 2000; Glass et al., 2005). Others genes regulated by Wnt and/or β-catenin and/or APC but which are not known to be direct targets include homeobox A1 (Calvo et al., 2000), plasminogen activator receptor (Mann et al., 1999), MADJ1 (increased by APC) (see Fultz and Gerner, 2002) and BTG1 (regulated by the Wnt target gene, brachyury) (Saka et al., 2000; Yamaguchi et al., 1999). Phosphoserine amino transferase (PSA) is included in this category because it is highly upregulated in colon cancers (Ojala et al., 2002). The expression of dickkopf 1 (DKK1), a well-known negative regulator of Wnt signaling, was increased by shear stress (Mao et al., 2002). GADD45b, which inhibits β-catenin dephosphorylation and β-catenin signaling, was also increased by shear stress (Hildesheim et al., 2004). Cyclin D1 and c-Myc expression was not altered by shear stress. Expression of the cellular inhibitor of the apoptosis protein 2 gene is regulated...
by tumor necrosis factor (TNF) and other stimuli that activate NFκB (Pryhuber et al., 2000). The Apo-2L/TRAIL gene is a well-known member of the TNF family and osteoprotegerin is a decoy receptor for the TNF receptor family member RANK, which is a target for p38 and TCF/β-catenin (Wang and El Deiry, 2003; Glass et al., 2005; Tazoe et al., 2003). JUN, FOSL1, laminin γ2 and MDRI are targets of MAPK-JNK as well as β-catenin/TCF pathways (Olsen et al., 2000; Kang et al., 2000). Dkk1 is regulated by JUN and CYR61 is regulated by p38 and Wnt/β-catenin (Grotewold and Ruther, 2002; Brigstock, 2003; Si et al., 2006). Shear stress increased the expression of both chains of laminin-5, consistent with our demonstrated role for the laminin-5 receptor, α6β4 integrin, in the sensing of mechanical strain. The multi-drug resistance gene (ABC1, MDRI, P-glycoprotein) is arguably the most important and sensitive of the direct β-catenin/TCF target genes in neoplastic progression (Yamada et al., 2000). Mdr1 expression is reduced by shear stress, pointing to the possibility that mechanical forces may alter the drug-resistance profile of colon cancers. By contrast, DKK1, an inhibitor of canonical Wnt signaling, is markedly increased following shear stress, an effect that is mediated by activation of p38.

Shear stress and cell cycle progression

Our results show that fluid shear stress blocks the cell cycle in G1 in SW480 cells. By contrast, increased luminal pressure and cyclic strain stimulates the proliferation of another colon cancer cell, Caco2 (Walsh et al., 2004). It is possible that the contrasting effects of different types of mechanical forces on SW480 and Caco2 cells is a cell-type-dependent phenomenon. However, shear stress also blocks the cell cycle in endothelial cells and we favor the idea that different classes of physical manipulations lead to regulation of different pathways and phenotypes.

A role for PI 3-kinase and Rac1 in coupling α6β4 integrin to changes in β-catenin signaling

Integrins are transducers of shear stress in several cell types and also control progression through the G1 phase of the endothelial cell cycle (Mettouchi et al., 2001). Our results demonstrate that α6β4 integrin (laminin receptor), and not α5β1 integrin (fibronectin receptor) or other β1 integrins, is a mechanosensor of shear flow regulation of β-catenin signaling in colon cancer cells. α6β4 integrin plays a major role in migration and invasion of carcinoma cells and is also important in wound healing (for a review, see Mercurio et al., 2001). Our data show that Rac1 and PI 3-kinase are components of a shear flow-induced β-catenin signaling pathway. Furthermore, although Akt is activated by shear stress it is not involved in the transmission of the shear stress signal to regulate β-catenin signaling in SW480 cells. Activation of Akt increases β-catenin signaling in other systems (Monick et al., 2001). β-catenin signaling is decreased much more in cells exposed to shear flow and plated on laminin compared with cells plated on fibronectin. This is consistent with the demonstration that Rac1 is activated to a much greater extent by ligand binding to laminin than to fibronectin (Gu et al., 2001). Similarly, PI 3-kinase is activated more effectively by α6β4 integrin than by any other integrin, particularly with respect to cell migration (reviewed by Mercurio et al., 2001). In addition, α6β4 integrin stimulation of PI 3-kinase activates Rac, which is central to cell migration (reviewed by Mercurio et al., 2001). Remarkably, activation of Rac1 is required for the differentiation and migration of enterocytes along the crypt-villus unit in the developing mouse intestine (Stappenbeck and Gordon, 2000). Interestingly, and consistent with our data, the ability of α6β4 integrin and PI 3-kinase to activate Rac1 is independent of Akt (Shaw et al., 1997). Thus, the cell migration signaling pathway is similar to the shear flow-induced regulation of β-catenin signaling. It is reasonable to speculate that the forces exerted by the α6β4 integrins on the ECM in response to shear flow mimic those transmitted to the ECM during cell migration in epithelial cell wound healing (reviewed by Mercurio et al., 2001).

How does shear stress selectively affect activated β-catenin?

In SW480 cells shear stress does not affect total β-catenin protein levels and β-catenin staining in the nucleus was more intense after shear stress. In our experiments epithelial cells were plated at sub-confluent densities, minimizing a role for cell-cell contact and the E-cadherin-adherens junction complex in shear stress regulation of β-catenin signaling. In addition, our SW480 cells do not express E-cadherin. However, like shear stress, exogenous expression of E-cadherin can inhibit β-catenin/TCF signaling in SW480 cells in the absence of discernable changes in cytoplasmic or nuclear β-catenin (Gottardi et al., 2001). These data led the authors to speculate that most of the β-catenin in SW480 cells is refractory to cadherin and TCF binding and that a small pool of transcriptionally active β-catenin can be modulated by E-cadherin. Our data show that such a pool of activated β-catenin does indeed exist in these cells and that it is selectively targeted by shear stress. It is not clear how shear stress-regulated activation of α6β4 integrin, PI 3-kinase and Rac1 can selectively deplete this pool of active β-catenin. However, a constitutively active β-catenin mutant (S33, 37A) was refractory to shear stress and we did observe a corresponding increase in the levels of β-catenin phosphorylated on these residues and on S45 following 12 hours of shear stress. In addition, in contrast to APC-mutant SW480 colon cancer cells, HCT116 colon cancer cells that harbor a β-catenin allele missing S45 are resistant to the effects of shear stress. The present work, and other published studies, suggest a model in which α6β4 integrin, at points of adhesion to laminin, senses shear stress and couples the signal to an intracellular pathway that involves PI 3-kinase and Rac1. Consistent with this, autocrine laminin-5 production and ligation of α6β4 integrin in breast cancer cells also results in Rac and NFκB activation (Zahir et al., 2003). Rac1 activation is coupled directly or indirectly to changes in the localization of activated β-catenin, which ultimately alters gene expression and cell growth.

Our data, taken together with the pioneering studies of Basson and co-workers (Walsh et al., 2004), demonstrate that colon cancer cells exhibit marked changes in signal transduction, gene expression and proliferation as a result of exposure to mechanical forces (Basson and Coppola, 2002). It is well accepted that hemodynamic shear stress is a fundamental determinant of vascular remodeling and atherogenesis, but the notion that mechanical forces are natural regulators of gut remodeling is not. Cells in the gastrointestinal tract are continually subjected to peristalsis and shearing forces
as gut contents move and as cells move up from the crypts of Lieberkuhn. The importance of gut motility on intestinal cell proliferation and turnover is emphasized by the clinical observation that marked mucosal changes occur in patients who are not fed orally, even though they are receiving adequate parenteral nutrition (Groos et al., 2003). Notwithstanding the caveat that our data were obtained with colon cancer cells, it is reasonable to propose that, as in the endothelium, shear stress is an important regulator of gut development, differentiation, function and pathology. Shear stress-mediated alterations in MAPK, Wnt/catenin and NFκB pathways are also likely to be important in the onset and progression of intestinal cancers.

Materials and Methods
Cell culture, transfections and reporter assays
SW480 and HT29 cells are APC-mutant human colorectal cancer cells that contain elevated β-catenin. HCT116 cells are human colorectal cancer cells with normal APC but mutated β-catenin. SW480 and HT29 cells were maintained at 37°C in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal bovine serum (FBS). A1N4 cells are a non-transformed human mammary epithelial cell line that are grown in improved minimal essential medium, supplemented with 0.5% FBS, 0.5% hydrocortisone, 5 μg/ml insulin and 10 ng/ml epidermal growth factor. Transfections were performed by calcium-phosphate precipitation. 24 hours after seeding, the cells were transfected with 1 μg/ml of plasmid and 10 ng/ml of the constitutively expressed pCMV-renilla luciferase (Promega). TFCL/LEF TOPFLASH and its control FOPFLASH reporters were provided by Marc van de Watering (University Medical Center, Utrecht University, The Netherlands) (Korinek et al., 1997); the –163 cyclin D1 luciferase reporter; –163 ALEF cyclin D1 luciferase reporter; NFPB reporter; DN IKK ε; DN IKK β; NK; constitutively active membrane bound PI 3-kine (p110α-CAAAX); DN PI 3-kine (p110α-CAAKKD); DN PI 3-kine regulatory subunit (DPN85) and DN Rac1 (pBSTR1/N17mycRac1) were described previously (Albanese et al., 2003; Lamberti et al., 2001). The FAK inhibitor FRNK was provided by Ed Rosfold. Georgetown University, Washington, DC. KD ILK was provided by Shoukat Dedhar, University of British Columbia, Canada. To generate a DN Akt mutant we made a KD form lacking both autophosphorylation and transphosphorylation sites. The ATP-binding site mutand site (Akt) (K179M) cloned in pLb is kinase inactive (Li et al., 1999). To convert this to a DN Akt inhibitor we first mutated T 308 A to using the K179M mutant as the target. The QuickChange site-directed mutagenesis kit (Stratagene) was used with the following primers: sense 5′-CGGCGTTCGCGAAGGCCCTTCATAGTGCAACC-3′; antisense 5′-GGTGCCACTATGAAGGGCTTCTGCGGAACGCCG-3′. Successful mutation on T 308 generated a new Stu site, allowing us to detect positive clones for subsequent sequencing. A K179M/T308A mutant clone was further mutated on S 473 to A using the same method. The primers for the S473A mutation were: sense 5′-CAGTTCTCTGAGGTCTGTCCTGCGAC3′; antisense 5′-GCTGGCCGAGTGGAGGCAATGCGGC-3′. Successful mutation on S 473 to A generated an additional Bgl site. The final mutant (K179M/T308A/S473A) was sequenced using an ABI sequencer. The controls for each experimental plasmid were the appropriate empty vector. All results are normalized to the renilla luciferase activity for control of transfection efficiency and variations in cell number.

Exposure of cells to shear stress and cell-cycle analysis
Cells were seeded at a density of 0.8 × 10^6 cells on 38 × 76 mm slides. 12-14 hours after seeding, the cells were either kept as static controls or subjected to a laminar flow for 24 hours and compared with cells not exposed to shear. After 24 hours, cells were harvested, washed several times in PBS, and centrifuged at 300 g for 5 minutes. 0.1 ml of 360 mg/ml sodium sulfite buffer was added to the cell suspension in preparation for FACS analysis.

RNA isolation and oligonucleotide microarray
SW480 cells were exposed to 15 dyn/cm^2 shear stress for 12 hours or grown under the same conditions in the absence of shear stress. Total RNA was isolated using Trizol (Invitrogen) combined with RNAeasy (Qiagen) according to the manufacturer’s instructions. Total RNA was amplified and hybridized to HG-U133A GeneChips according to the Affymetrix protocol (GeneChip Eukaryotic Small Sample Target Labeling Assay Version II). Raw expression values, representing the average difference in hybridization intensity between oligonucleotides containing single-base-pair mismatches, were measured. Experiments were repeated three times.

Multidimensional scaling and cluster analysis
Data generated after scanning were subjected to comparison analysis to select new changes. Comparisons were made between static and shear stress-treated SW480 cells and a change call list was generated. To assess the effects of shear stress itself, change call lists from three different experiments were further subjected to comparison analyses and a new change call list in which genes consistently increased or decreased in all three replicates were generated. The data selected after this comparison analysis were further filtered based on absolute analysis using the Mann-Whitney U-test and detection calls for 72 genes were selected for multidimensional scaling and hierarchical clustering. Multidimensional scaling coordinates were calculated with Matlab software. Distances between samples treated with shear stress or not were calculated using the Pearson correlation coefficient: Dissim = 1 – Pearson correlation coefficient. To visualize expression of the 72 genes that were selected, an intra- and inter-sample pairs hierarchical-clustering was performed using Cluster 3.0 (Stanford University, Palo Alto, CA). A gene list corresponding to clusters was generated using the Data Mining Tool from Affymetrix.

Data visualization
We calculated the three-dimensional projections of multidimensional gene expression microarray data sets using discriminant component analysis (DCA) (Wang et al., 2000; Wang et al., 2003). DCA uses Fisher separability-based multidimensional scaling and projects high-dimensional data into three-dimensional data space (Wang et al., 1998; Wang et al., 2000). The reconstruction error was computed by summing the variance spanned by the three top components divided by the total variance spanned by all gene dimensions. Individual gene expression profiles were generated using Treeview (see supplementary material Fig. S4a). A three-dimensional DCA projection of a 75-dimensional data set for three shear stress-treated and three control experiments is shown in supplementary material Fig. S4b, and demonstrates that the shear stress-regulated genes occupied discrete regions of gene expression space that was separable from control cells. For further analysis, genes with an expression ratio (static: shear) of 1.8 above were considered positively regulated by shear stress, whereas those that had a ratio of –1.8 or below were considered negatively regulated (see supplementary material Table S1). Three genes satisfied the threshold values in two of three experiments, Hs00155479; PLAUR, Hs00182181; Rai3, Hs00173681; ITGB4, Hs00174009. Primers and probes were bought from PE Applied Biosystems (Assay-On-Demand System). GeneChips were hybridized to HG-U133A GeneChips according to the Affymetrix protocol (GeneChip Eukaryotic Small Sample Target Labeling Assay Version II). Raw expression values, representing the average difference in hybridization intensity between oligonucleotides containing single-base-pair mismatches, were measured. Experiments were repeated three times.

Real-time PCR
Total RNA was prepared as described above. cDNA was synthesized from total RNA using TaqMan® reverse transcription reagents (Applied Biosystems; cat no. N808-0234) in a 20 μl reaction volume. 500 ng total RNA was incubated at 25°C for 10 minutes, reverse transcribed at 48°C for 30 minutes and inactivated at 95°C for 5 minutes. TaqMan amplification reactions were set up in a reaction volume of 20 μl supplied in a TaqMan PCR Core Reagent kit (Applied Biosystems). PCR primers and probes were bought from PE Applied Biosystems (Assay-On-Demand primer and probes: GAPDH, Hs09999905; DKK1, Hs00183740; CYR61, Hs00155479; ABCB1, Hs00184491; CDK5R1, hs00243655; GADD45b, Hs00169587; PLAUR, Hs00182181; Rai3, Hs00173681; ITGB4, Hs00174009). Amplification data was collected by the ABI Prism 7900 Sequence Detection System and analyzed using the Sequence Detection System software developed by Applied Biosystems.

Treatment with kinase inhibitors
Sp600125 is a cell permeable, selective and reversible inhibitor of JNK (IC₅₀, 40 nM for JNK-1 and JNK-2 and 90 nM for JNK-3), which inhibits over 300-fold greater selectivity for JNK compared with ERK1 and p38. SB202190 is a selective antagonist of p38MAPK/ERK2. Cells were pre-incubated with 25 μM of the inhibitor for 1 hour before exposure to 15 dyn/cm² shear stress.

Modified Rac1 activation assay
We found that even though SW480 cells expressed high levels of Rac1, we had difficulty detecting activated Rac1, even after treatment with growth factors known to result in strong Rac1 activation. Like other small G-proteins, GFP-bound Rac1
can be rapidly converted to its GDP-bound form following activation and cell lysis. This would reduce its interaction with PBD and result in artificially reduced measurements of rac activation. To test this we lysed cells in the presence of the non-hydrolysable form of GTP (GTPγS). We reasoned that GTPγS should inhibit the conversion of GTP-Rac1 to GDP-Rac1 and might increase assay sensitivity. Remarkably, inclusion of GTPγS in the lysis buffer increased the sensitivity of the assay many-fold.

Western blot and immunocytochemistry
NP-40 and cytoplasmic lysates were made as described previously (Orford et al., 1997). Western blotting was performed using β-catenin monoclonal antibody (1:1000; Transduction Laboratories), or monoclonal anti-dephosphorylated β-catenin (1:750; Upstate Biotechnologies) or phospho-specific polyclonal anti-ERK (1:2000, anti-JNK (1:2000) and anti-p38 (1:1000; all from Transduction Laboratories) and HRP-goat anti-rabbit serum (1:2000; Upstate Biotechnologies). Immunofluorescent staining was performed on cells under static and shear stress conditions using monoclonal anti-β-catenin (1:1000) or with monoclonal anti-actin (1:300) as described previously (Orford et al., 1999). On some occasions western blots were quantitated by densitometry using ImageJ.

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