Voltage-Gated Na\(^+\) Channel \textit{SCN5A} Is a Key Regulator of a Gene Transcriptional Network That Controls Colon Cancer Invasion

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**Abstract**

Voltage-gated Na\(^+\) channels (VGSC) have been implicated in the metastatic potential of human breast, prostate, and lung cancer cells. Specifically, the \textit{SCN5A} gene encoding the VGSC isotype Na\(_{1,5}\) has been defined as a key driver of human cancer cell invasion. In this study, we examined the expression and function of VGSCs in a panel of colon cancer cell lines by electrophysiologic recordings. Na\(^+\) channel activity and invasive potential were inhibited pharmacologically by tetrodotoxin or genetically by small interfering RNAs (siRNA) specifically targeting \textit{SCN5A}. Clinical relevance was established by immunohistochemistry of patient biopsies, with strong Na\(_{1,5}\) protein staining found in colon cancer specimens but little to no staining in matched-paired normal colon tissues. We explored the mechanism of VGSC-mediated invasive potential on the basis of reported links between VGSC activity and gene expression in excitable cells. Probabilistic modeling of loss-of-function screens and microarray data established an unequivocal role of VGSC \textit{SCN5A} as a high level regulator of a colon cancer invasion network, involving genes that encompass Wnt signaling, cell migration, ectoderm development, response to biotic stimulus, steroid metabolic process, and cell cycle control. siRNA-mediated knockdown of predicted downstream network components caused a loss of invasive behavior, demonstrating network connectivity and its function in driving colon cancer invasion. *Cancer Res; 70(17); OF1–11. ©2010 AACR.*

**Introduction**

An increasing body of evidence is accumulating on the importance and functional contribution of ion channels, signaling molecules involved in ion transport, enzyme activity, secretion, and intercellular communication in cancer (1–4). Voltage-gated Na\(^+\) channels (VGSC) are most abundant in excitable cells such as neurons and cardiomyocytes, where they are responsible for the depolarization phase of the action potential and are important for neurite extension and neurotransmitter release (5, 6). VGSC activation can lead to increased Na\(^+\) influx, resulting in alterations in both intracellular Ca\(^{2+}\) concentration and pH and additional changes in normal cellular homeostasis. Interestingly, a study performed several decades ago indicated that tumor samples had higher intracellular concentrations of Na\(^+\) compared with normal tissues, and this phenomenon was postulated to be connected with oncogenesis (7). More recent studies now implicate VGSCs in the invasive potential of prostate (8), breast (9), and lung (10) cancer cells. However, it is unclear the exact mechanism(s) by which VGSC genes and/or their functional expression confer an oncogenic advantage to cancer cells. One study has shown that the activity of the VGSC \textit{\alpha}-subunit Na\(_{1,5}\), encoded by the \textit{SCN5A} gene and normally associated with human cardiac tissue, increases the invasiveness of human breast cancer cells possibly by providing favorable conditions for proteolytic activity on extracellular matrix proteins (11). In another study investigating melanoma cells, Na\(_{1,8}\) (a paralog of the Na\(_{1,5}\) isoform) has been shown to facilitate podosome formation (12).

Meta-analysis of gene expression profiling data from colon cancer patient samples and oncogenic transformation of fibroblasts suggests that genes typically associated with neuronal or excitable cells, including ion channels and intracellular signaling molecules, may be "commandeered" as part of the process of cancer progression (13, 14). In addition to expression of ion channel genes, a number of neuronal molecular markers have also been described in other transformed tissues, such as small-cell lung cancer (15). Little is known...
about the transcriptional regulation of VGSC genes or the downstream genes regulated by VGSCs in the context of cancer progression. For example, there have been no systematic studies examining VGSC activity together with changes in gene expression and invasion potential of cancer cells. Ion channel activity stimulates a variety of intracellular signaling pathways (16, 17), and the functional state of ion channels is known to affect gene expression in neuronal and skeletal muscle cells (18, 19).

The regulatory connections among genes involved in tumor cell invasion remain rudimentary, and characterization of a gene network in the invasiveness pathway is vital to fully appreciate the molecular mechanisms underlying metastasis (20). Such gene-gene network interactions can be reconstructed from the transcriptional consequences of RNA interference–mediated knockdown of network components (21). The goal of this study was to establish whether functional expression of VGSCs in colon cancer cells contributes to invasion potential through transcriptional regulation of downstream invasion/migration genes.

Materials and Methods

Immunohistochemistry

Fresh-frozen or paraffin-embedded colon tissues were cut into 10-μm sections and processed using DAKO Cytomation Envision-System HRP [3,3'-diaminobenzidine (DAB)] kit according to the manufacturer's instructions (Dako). Sections were incubated with anti-Na,1,5 polyclonal antibody (1:100; Alomone Labs) for 3 hours, followed by 1 hour with HRP-labeled polymer–conjugated antirabbit secondary antibody. Sections were counterstained with diiate Mayer's hematoxylin (Dako). Quantification of DAB staining was performed as described previously (22). All incubations were done at room temperature. Sections from each specimen were fixed in methanol and stained with H&E, carcinoembryonic antigen, and Mak6 to confirm tissue integrity. Experiments were approved by the George Washington University Medical Center Institutional Review Board.

Cell culture and small interfering RNA transfections

Human colon cancer cells, HT29, SW620, SW480, HEK293, and Caco-2, were obtained directly from the American Type Culture Collection (ATCC) and used within 6 months of receipt. ATCC authenticates cell lines through short tandem repeat profiling, morphology analysis, karyotyping, and isoenzyme analysis [ATCC cell line verification test recommendations, technical bulletin no. 8 (2007)]. Cells were maintained in DMEM supplemented with 10% fetal bovine serum at 37°C and 5% CO2. Cells were cultured for 24 hours to 30% confluence in 35-mm dishes for electrophysiologic studies. The whole-cell patch-clamp technique was used to record voltage-activated currents from individual cells. The electrode solution contained 140 mmol/L CsCl, 2 mmol/L MgCl2, 0.1 mmol/L CaCl2, 1.1 mmol/L EGTA, and 10 mmol/L HEPES (pH 7.2). The extracellular solution contained 140 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L MgCl2, 2.5 mmol/L CaCl2, 10 mmol/L HEPES, and 11 mmol/L glucose (pH 7.4) with and without the indicated concentrations of tetrodotoxin (TTX). Currents were recorded using an Axopatch 200B amplifier, low-pass filtered at 5 kHz, digitized at 10 kHz using a Digidata 1320A interface, and acquired using pCLAMP8 software (all from Molecular Devices).

RNA isolation, microarrays, and quantitative real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) and RNeasy kit (Qiagen) according to the manufacturers' instructions. Gene expression profiling and statistical analysis were performed as previously described (13, 14, 20). The hybridization data and associated normalization information can be accessed from the Gene Expression Omnibus database under the series accession number GSE11848 and associated platform accession number GPL6978. For validation of gene knockdowns, quantitative real-time PCR (qRT-PCR) was performed as described previously (23). Housekeeping genes PPA1 and EIF4X (GenBank accession numbers NM_021129 and NM_001412, respectively) were used for normalization. Quantification and normalization of relative gene expression were accomplished using the comparative threshold cycle method or ΔΔCT (20). Primer sequences are provided in Supplementary Table S1.

Matrigel assay

Colon cancer cells (2.5 × 104 to 105) were seeded in the top well of a Matrigel coated invasion chamber (BD Biosciences) in DMEM containing 0.1% serum. For differentiated Caco-2 cells, cultures were maintained for 10 days after cells reached confluence prior to seeding. The bottom well was filled with 750 μL of DMEM containing 10% serum as chemosattractant. After ~48 hours, noninvading cells were scraped from the upper side using a cotton swab. Invading cells on the bottom of the insert were fixed and stained with Diff-Quik Stain (IMEB, Inc.) and counted under a light microscope. The total number of invading cells was counted for each insert.

Inference of signaling network

Network inferences by factor graph nested effects modeling (FG-NEM) was performed as previously described (23). Briefly, the method takes as input a matrix of expression level changes for a set of “effect” genes (E-genes) exhibiting at
least a minimum level of variance across gene knockdowns. Each column of the matrix represents the expression of E-genes under the knockdown of a particular signaling gene (S-gene). By searching for probabilistic nested relationships among the set of expression changes observed for the E-genes, the procedure returns network interactions among the S-genes. Additional details for FG-NEM, network bootstrap confidence determination, and network frontier expansion can be found in Supplementary Materials and Methods.

**Results and Discussion**

**SCN5A is functionally expressed in colon cancer cell lines**

Quantitative RT-PCR was performed on an assortment of cell lines to assess expression of VGSC isoforms (see Supplementary Table S2). The set of colon cancer cell lines SW620, SW480, and HT29 expressed multiple isoforms, with the most abundant generally being SCN5A, a TTX-resistant isoform. The protein expression and localization of Na\(_{\text{v}}\)1,5 in colon cancer cells were confirmed immunocytochemically using an antibody against Na\(_{\text{v}}\)1,5. Punctate expression of Na\(_{\text{v}}\)1,5 protein at the plasma membrane was observed in all three cell lines with no nuclear localization (Fig. 1A). To our knowledge, this is the first study showing expression of this isoform in colon cancer cells.

To show functional expression of the VGSCs in HT29, SW480, and SW620 cells, Na\(^+\) currents were recorded by whole-cell patch clamp technique (Fig. 1B). Top traces represent currents elicited by depolarizing cells from −80 mV to between −50 and 0 mV at 10-mV increments. Bottom traces represent currents elicited by stepping from −80 mV to between 10 and 60 mV in 10-mV increments. The average maximum current densities for HT29, SW480, and SW620 were 7.2 ± 2.5 (n = 15), 5.3 ± 1.6 (n = 16), and 17.9 ± 2.2 pA/pF (n = 36), respectively, compared with 57 ± 8.9 pA/pF (n = 20) for SK-N-SH neuroblastoma cells. Average current-voltage relationship plots show similar electrophysiologic characteristics among the colon cancer cell lines with an average threshold of activation at ~−40 mV and peak current amplitude between 0 and 10 mV (Fig. 1C). The activation and inactivation curves in SW620 cells for example are very similar to those recorded previously from cardiac myocytes (24), and it seems that a small fraction of current (window current) is available at steady state between −60 and −20 mV (Supplementary Fig. S1A). VGSC currents were partially inhibited by 10 μmol/L TTX treatment (Fig. 1D) and concentration-response relationships in HT29, SW480, and SW620 cells indicate IC\(_{50}\) values of 4, 8, and 2 μmol/L, respectively (Supplementary Fig. S2A), suggesting functional expression of Na\(_{\text{v}}\)1,5, which has been reported to have an IC\(_{50}\) value of 4, 8, and 2 μmol/L (25). The absence of involvement of voltage-gated calcium channels (VGCC) in the recorded inward currents was confirmed by the lack of measurable current in colon cancer cells, but not in neuronal PC12 cells, in the presence of 10 mmol/L Ba\(^{2+}\) (substituting for Ca\(^{2+}\)) and 100 μmol/L TTX (Supplementary Fig. S3A–C). Moreover, the dihydropyridine VGCC blocker nimodipine failed to influence invasion by colon cancer cells (Supplementary Fig. S3D).

**SCN5A functionally participates in the invasive potential of colon cancer cells**

The contribution of Na\(_{\text{v}}\)1,5 channels to the invasive potential of colon cancer cells was tested in a Matrigel assay following pharmacologic and genetic knockdown of channel activity. In the presence of 30 μmol/L TTX, representing a concentration 4–to-15-fold in excess of the IC\(_{50}\) for Na\(^+\) current inhibition and leading to >70% loss of Na\(^+\) current activity (Supplementary Fig. S2A), the total number of invading cells was significantly reduced compared with vehicle control for all three cell lines (Fig. 2A). In the presence of an siRNA specifically targeting the SCN5A transcript, a significant reduction was observed in the total number of invading cells compared with cells treated with a nonsense control siRNA (Fig. 2B). A loss of channel activity by gene knockdown was confirmed with whole-cell patch clamp electrophysiology, where at least a 65% decrease in maximum current density was observed in all three lines (see Supplementary Fig. S2B for SW620 cells). Taken as a whole, our findings strongly indicate that the invasive potential of colon cancer cells is linked to the function of Na\(_{\text{v}}\)1,5. The fact that neither pharmacologic nor genetic knockdown of SCN5A inhibited cell invasion completely may be explained by the presence of other mechanisms that contribute to cell invasion, by the existence of additional functional isoforms (such as Na\(_{\text{v}}\)1,8, encoded by SCN10A, the most TTX-resistant isoform; see Supplementary Table S2), or simply by incomplete gene knockdown. It is clear from this study and others that a variety of VGSC isoforms are operational in different cancer cell types. The combination of electrophysiologic, molecular, and Matrigel invasion assays has established a definitive role for the TTX-resistant Na\(_{\text{v}}\)1,5 in breast (9) and colon (present study; but see discussion below pertaining to different Na\(_{\text{v}}\)1,5 splice forms for breast and colon cancers), the TTX-sensitive Na\(_{\text{v}}\)1,7 in prostate (26), and the TTX-resistant Na\(_{\text{v}}\)1,5 and sensitive isoforms (Na\(_{\text{v}}\)1,6 and Na\(_{\text{v}}\)1,7) in non–small-cell lung cancer cell invasiveness (10).

To further address the relationship between VGSC expression and the transformation/differentiation status of cells, we analyzed SCN5A expression and invasion potential in differentiated and undifferentiated Caco-2 colon carcinoma cells. Caco-2 cells undergo differentiation in culture, a process that is completed ~10 days postconfluency (27). SCN5A expression and invasion potential were highest in undifferentiated Caco-2 cells compared with differentiated cells (Supplementary Fig. S4A). We also analyzed Na\(_{\text{v}}\)1,5 expression and invasion potential in low- versus high-passage HEK293 cells, where the latter has been shown to be tumorigenic (28). High-passage cells had both significantly higher maximum current density and invasion potential compared with low-passage cells (see Supplementary Fig. S4B). Taken together, these data further implicate SCN5A in an invasive phenotype.

**Na\(_{\text{v}}\)1,5 expression is restricted to the luminal surface of human colon cancer samples**

To further confirm the significance of SCN5A in colon cancer, we assessed the protein expression and localization of Na\(_{\text{v}}\)1,5 in a panel of human colon cancer specimens by immunohistochemistry. Na\(_{\text{v}}\)1,5 immunoreactivity was mainly...
Figure 1. Nav1.5 VGSCs are functionally expressed in colon cancer cells. A, representative merged images showing Nav1.5 immunoreactivity in SW620, SW480, and HT29 colon cancer cells. Anti-Na$_1$1.5 conjugated to Alexa 488 in green and 4',6-diamidino-2-phenylindole nuclear staining in blue. Images are representative of at least three independent experiments (bar, 10 μm). B, superimposed Na$^+$ currents elicited by depolarizing from −80 mV to between −50 and 0 mV (top traces) and between 10 and 60 mV (bottom traces) in 10-mV increments. C, current-voltage (I-V) relationship of Na$^+$ currents elicited by depolarizing from a holding potential of −80 mV to between −70 and 60 mV in 10-mV voltage steps. Current amplitudes were normalized to maximum peak current recorded from each cell. Data are averages of 36, 16, and 15 recordings from SW620, SW480, and HT29 colon cancer cells, respectively. Vertical lines, SEM; error bars for SW620 and SW480 recordings are within the data symbols. D, TTX-mediated inhibition of current after bath application of 10 μmol/L TTX. Current is restored after subsequent wash.
confined to the plasma membrane with minimal staining in the cytoplasm. Immunostaining revealed distinct expression of this isoform in malignant cells on the luminal surface (Fig. 3A). In contrast, normal-matched control samples showed little or no staining of colon epithelial cells (Fig. 3A). Automated digital selection (22) of DAB-labeled tissues revealed a substantial and significantly higher percentage of Na,1.5-positive areas in cancer samples compared with their normal-matched controls (Fig. 3B and C). These data should be viewed in light of the report of Barshack and colleagues (29) which showed expression of VGSCs in normal colonic epithelial using a pan anti-Na, antibody (although isoform content remains to be delineated). Our findings suggest that functional expression of the Na,1.5 isoform may be selectively repressed in normal colon and becomes aberrantly overexpressed in colon cancer.

Of interest is the finding that the neonatal, but not the adult, splice variant form of Na,1.5 in primary breast cancer specimens strongly correlates with lymph node metastasis (3, 9). Analogously, expression of the neonatal form is associated in vitro with strong invasive behavior in the highly metastatic breast cancer cell line MDA-MB-231, whereas the weakly metastatic lines MDA-MB-468 and MCF-7 do not readily invade Matrigel nor do they express the neonatal variant (9). Consequently, we investigated the nature of the Na,1.5 variant expressed in both clinical specimens and the three colon cancer cell lines. Quantitative RT-PCR analysis revealed the presence of only the adult variant in colon cancer specimens and cell lines (data not shown), including SW620, which is derived from a lymph node metastasis (30). In conclusion, our findings suggest that the adult variant of Na,1.5 may be critical for colon cancer invasiveness, whereas the fetal variant seems to be essential for the breast cancer metastatic behavior.

Mapping of a Na,1.5-regulated colon cancer invasion transcriptional network

A major goal of this study is to identify a structured gene network participating in colon cancer invasion and to determine if VGSCs participate in the regulation of such a network. It is plausible that recruitment of Na,1.5 expression in colon cancers may facilitate the regulation of downstream genes involved in invasive potential, given that VGSC activity is directly associated with gene expression changes in neuronal cells (18). Previously, we had mapped a rudimentary colon cancer invasion network in HT29 cells composed of different tiers or levels of invasion genes (20). Tier 1 contained the genes ADAM21, CCR9, and CD53, whereas tier 2 contained GLS, RPL32, KRT20, DHX32, and the transcription factor TFDP1 (ref. 20; gene names and corresponding gene symbols can be found in Supplementary Table S1). A drawback to this early map was a lack of connectivity among the eight colon cancer invasion genes owing to the sequential nature of our earlier mapping strategy.

More recently, we have developed a probabilistic computational approach termed FG-NEM to identify a richer set of connections among S-genes (23). FG-NEM iteratively processes data from loss-of-function screens (targeted gene knockdown

Figure 2. Na,1.5 contributes to the invasive potential of colon cancer cells. A, the total number of invading SW620, SW480, and HT29 colon cancer cells was significantly reduced with 30 μmol/L TTX compared with vehicle control. B, siRNA-mediated knockdown of SCN5A significantly reduced the invasion potential of colon cancer cells compared with cells treated with a nonsense siRNA used as a control. Columns, mean from at least three independent experiments; bars, SEM. *, P < 0.05, compared with control (two-sided, unpaired t test).
Figure 3. Na,1.5 staining is significantly higher in colon cancer tissues compared with normal-matched colon tissues. A, Na,1.5 immunoreactivity is confined primarily to the plasma membrane of malignant cells in the luminal surface (brown staining in the periphery). Images are representative of at least three independent experiments from each of seven patients (C1–C7). Sections C1 to C4 were prepared from fresh-frozen tissue specimens and C5 to C7 from formalin-fixed paraffin-embedded tissue specimens. B, DAB+ stained areas selected from 24-bit BN image using preset threshold on ImageJ image processing software. C, quantification of DAB+ stained areas is displayed as percent positive pixels divided by the total number of pixels. Columns, mean; bars, SEM. *, P < 0.05, compared with normal (two-sided, paired t test).
by siRNA and screening for loss of invasion) and gene expression experiments profiling the downstream transcriptional effects resulting from each knockdown. A network is constructed among the knocked down genes based on their downstream effects. FG-NEM also attaches new E-genes to the network by identifying the most likely attachment points. We refer to the collection of attached E-genes as the “network frontier.” The modeling is iterative and repeats itself as E-genes are chosen from the frontier for knockdown, loss-of-function screening, and transcriptional profiling.

FG-NEM was applied to the original microarray data derived from the knockdowns of eight invasion genes (20) along with expression data from the knockdown of SCN5A in HT29 cells (this study). A total of 54 E-genes were identified and 15 were chosen for individual gene knockdown in HT29 cells (Fig. 4A). The choice of E-genes was based, in part, on the potential role of these genes in cancer invasion. For example, invasion requires degradation of the extracellular matrix, a feat accomplished by disintegrin and metalloproteinase domain–containing proteins, such as ADAM9, and matrix metalloproteinases (17, 31). UBE2L6 functions as part of the ubiquitin-proteasome pathway for protein degradation, and this pathway is intricately tied to the expression and/or activation of matrix metalloproteinases (32). Lastly, the guanine nucleotide binding protein GNAI3 has been associated with the control of cell motility and is thought to be involved in direction detection (33), whereas the serine/threonine kinase STK24 is a homologue of the yeast protein Ste20p, which is involved in mitogen-activated protein kinase (MAPK) signaling for the invasive growth of yeast (34).

Two different siRNAs were tested for each of the 15 E-genes [as was the case for the original eight invasion genes (20) and SCN5A], whenever feasible, to ensure specificity (Fig. 4B). Successful gene knockdown was defined as a

![Figure 4](cancerres.aacrjournals.org)
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≥50% reduction in mRNA levels as determined by qRT-PCR. Knockdown of 13 of the 15 tested E-genes led to a significant reduction in the total number of invading HT29 cells compared with cells treated with a nonsense control siRNA (Fig. 4B). It should be noted, however, that the less efficient knockdown of a particular gene (e.g., ADAM9) may contribute to the limited effect of the gene on invasion. The 13 E-genes empirically linked to loss-of-invasion were "promoted" to S-gene status. Expression profiling after individual knockdown of the 13 S-genes was performed for a second round of FG-NEM analysis to identify additional downstream regulatory connections, thus allowing reconstruction and expansion of the invasion network.

The second round of FG-NEM analysis on the 22 S-genes (i.e., the 8 original invasion genes, SCN5A, and the 13 downstream genes from the first round of FG-NEM) identified 1,114 downstream E-genes (Fig. 5A). The resulting network was fully connected and spanned all 22 S-genes (Fig. 5B). Confidence levels in network features were assessed by bootstrapping the data and inferring new networks for each bootstrap sample (Fig. 5C). Across virtually all bootstrap iterations, SCN5A was placed upstream of all other S-genes.

The invasion network could be delineated into five subdomains with SCN5A, STK24, and KRT20/RPL32 as gene entry points (Fig. 5B). It is intriguing that the SCN5A gene was situated as a highly confident entry point in the invasion network. Its upstream position implicates VGSCs as potential transducers of the invasion machinery, connecting membrane electrophysiology with metastatic behavior. Lending further support to this idea are studies revealing that VGSCs are commonly mutated in both glioblastoma and colon cancer (35, 36). Of particular interest, at least one of the mutants harbors a predicted gain-of-function mutation in the conserved voltage sensor, and such mutations are typically associated with ion channelopathies leading to sustained cationic leak (37, 38). Each network subdomain was associated with a distinct functional theme, namely, integral membrane proteins and proteases, Wnt signaling regulation, calcium signaling, MAPK signaling, and membrane remodeling and secretion (Fig. 5B).

Functional enrichment of the frontier

FG-NEM was used to expand the frontier of the 22 S-gene invasion network by predicting where new E-genes might attach. We identified 1,752 E-genes attached in the frontier at a 5% false discovery rate (FDR) level. Gene Ontology (GO) enrichment analysis was performed on the E-genes at specific attachment points of the frontier (boxes in Fig. 5B). Seven main GO categories were found to be representative of the frontier at an FDR of 5%, specifically ectoderm development (24 E-genes), carboxylic acid transport (22 E-genes), negative regulation of mitotic cell cycle (9 E-genes), response to biotic stimulus (17 E-genes), steroid metabolic process (28 E-genes), cell migration (30 E-genes), and regulation of Wnt signaling (5 E-genes; see the complete list provided in Supplementary Table S3). Moreover, the network frontier seems to be significantly enriched with colon cancer–specific genes based on the meta-analysis of Oncomine data sets describing the interrogation of different cancers including colon cancer (see Supplementary Tables S4 and S5). Nine of the frontier E-genes have previously been shown to be mutated in colon cancer (i.e., LAMA4, FNI, SMA3, COL4A1, APOB, LAMC1, TGF3, APBB2, and KRT20 refs. 35, 36), and seven from this subset are predicted to participate in a protein-protein interaction network (39). In a separate study, two of our frontier E-genes, PPL and IFT116, are predicted to participate in a protein interaction network in late-stage human colorectal cancer (40). IFT116, located in the “response to biotic stimulus” category, exhibited the highest attachment score of any E-gene in our network, and this gene has recently been proposed to be a molecular marker for human colorectal tumors (41). Furthermore, elevated expression of IFT116 in head and neck squamous cell cancer (42) and in gastric cancer (43) leads to increased levels of invasive behavior; conversely, suppressing IFT116 decreases invasive behavior.

The cell migration category contains a number of E-genes associated with colon cancer invasion. In particular, the neuronal cell adhesion molecule (NRCAM) gene had the highest attachment score. NRCAM has been previously identified in our laboratory as a gene regulated by osteopontin and CD44 signaling in Ras-transformed NIH3T3 cells, promoting invasive behavior (14). Both osteopontin and CD44 are molecular markers for metastatic colon carcinomas (44). Increased NRCAM expression has also been shown to be mediated by the Wnt signaling pathway, enhancing the cell motility and tumorigenesis of colon cancer cells (45). The attachment of NRCAM to the Wnt signaling region of our network is consistent with this earlier finding. Another cell migration E-gene with a high attachment score, CCDC88A (also known as KIAA1212, GIV, or GIRDIN), is predicted to be inhibited by the invasion network. CCDC88A is an established binding partner of our S-gene GNAI3 (33), and GNAI3 has been shown to be essential for leading-edge pseudopod formation and cell migration (33).

Among the E-genes associated with the GO category steroid metabolic process, the colon cancer marker gene INSIG2 had the strongest connection to the invasion network and has recently been shown to promote invasive behavior when ectopically expressed in HCT116 colon cancer cells (46). Within the regulation of Wnt signaling category, PPP2R1A had the highest attachment score. Protein phosphatase 2A (PP2A) is a heterotrimeric serine-threonine phosphatase composed of structural subunit A (i.e., encoded by PPP2R1A or PPP2R1B), regulatory subunit B, and catalytic subunit C. PP2A activity has been shown to regulate Wnt and phosphatidylinositol 3-kinase signaling (47). Mutations in both structural subunit A genes have been found in several cancers including breast, lung, and colon, and studies suggest that PP2A functions as a tumor suppressor (47, 48).

The most significantly enriched GO term was Ectoderm Development. FG-NEM predicts that the invasion network represses some members of this set and activates others, mostly collagens, laminins, keratins, and regulators of these structural genes. The gene CTGF, which encodes the protein connective tissue growth factor, had the highest attachment score.

Figure 5. Network interactions predicted from E-gene expression under S-gene knockdown. A, expression values of selected E-genes. Each row shows the log-ratio expression of a single E-gene under various targeted siRNA-mediated knockdowns relative to a nonsense siRNA control. B, inferred S-gene network and frontier. Nodes represent S-genes (ovals), E-genes (gray boxes), and GO categories (white boxes). Arrows indicate activation, and tees indicate repression. Mixed arrow/tee line endings indicate GO set enrichment among both activated and inhibited E-genes. For simplicity, only direct interactions are shown. C, S-gene interaction confidence. Each pixel in the heatmap corresponds to the proportion of times an S-gene interaction was recovered across bootstrap iterations. Upstream S-genes are labeled on the right, and downstream on the top. Rows show upstream and columns show downstream bootstrap proportion.
score in this GO term and is predicted to be inhibited by the invasion network. CTGF produces an extracellular matrix protein and in liver has been proposed as a master regulator of the epithelial-mesenchymal transition (49).

Validation of network frontier

To validate the involvement of predicted downstream E-genes in the invasion network, the invasion potential of HT29 cells was assessed following individual siRNA-mediated knockdown of NRCAM, IFITM1, INSIG2, CTGF, and PPP2R1A, typifying high attachment score genes from five different regions (Fig. 5B). NRCAM, IFITM1, and INSIG2 represent activated genes in the invasion network. Consequently, one might predict that suppression of these genes would lead to a loss of invasion potential. A significant loss of invasion indeed occurred with knockdowns of NRCAM, IFITM1, or INSIG2 compared with nonsense siRNA control (Fig. 6). Conversely, PPP2R1A and CTGF were predicted to suppress invasion based on their upstream inhibitory connections (Fig. 5B). Alternatively, these inhibitory connections may represent counter-regulatory measures. A significant enhancement of the invasive potential indeed occurred on knockdown of PPP2R1A (Fig. 6). The knockdown of CTGF was unsuccessful with five tested siRNAs based on qRT-PCR results. However, the specific combination of two siRNAs resulted in a 53% knockdown and a corresponding loss of invasion potential (Fig. 6). The combined results of siRNA knockdown and Matrigel invasion assays strongly support the regulatory connections proposed in the invasion network.

In conclusion, we have reconstructed a network of gene interactions implicated in invasive colon cancer. Genes previously associated with colon cancer and those never before tied to colon cancer have been linked operationally. Our data highlight the transcriptional changes that occur with functional VGSC expression and support the hypothesis that ion channel activity leads to gene expression changes that favor an invasion phenotype. Both activation and inactivation gene links have been defined in the invasion network as would be expected in the regulation of a complex network. It should be noted that future studies are warranted to define precisely how ion channel activity leads to downstream transcriptional effects in colon cancer. A number of recent studies have associated the use of local anesthetics (blockers of VGSCs) during surgical resection of cancers with decreased reoccurrence and metastasis (50). These findings are intriguing in light of the positioning of SCN5A as an early entry point in the invasion network and immunohistologic validation of aberrant upregulation of Na,1.5 protein in clinical colon cancer specimens. Our study implicates the VGSC Na,1.5 subunit (adult splice variant) and its network constituents as potential targets for the development of new therapies for hindering colon cancer progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Anastas Popratiloff for assistance with confocal microscopy.

Grant Support

NIH grants CA120316 and 1S10RR025565-01 (N.H. Lee), PhRMA Foundation Award (C.D. House), and NSF Career grant DBI-0845783 (J.M. Stuart).

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Received 04/05/2010; revised 06/15/2010; accepted 06/28/2010; published OnlineFirst 07/22/2010.

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Figure 6. Validation of predicted downstream network interactions. Significant changes in invasion occur with siRNA-mediated knockdown of frontier E-genes predicted to be connected to the invasion network. Knockdowns were determined to be >50% by qRT-PCR. Columns, mean from three independent experiments for each siRNA; bars, SEM. *, P < 0.05, compared with siNonsense control (ANOVA, post hoc Tukey).
Voltage-Gated Na\textsuperscript{+} Channel SCN5A Is a Key Regulator of a Gene Transcriptional Network That Controls Colon Cancer Invasion


_Cancer Res_ Published OnlineFirst July 22, 2010.

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