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Activation of  $\text{Ca}^{2+}$ -Activated  $\text{K}^+$  Channels and Cell Migration by Hepatocyte Growth  
Factor/Scatter Factor in Madin-Darby Canine Kidney Cells

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A dissertation  
presented to  
the Faculty of the Department of Physiology, James H. Quillen College of Medicine  
East Tennessee State University

In partial fulfillment  
of the requirements for the degree  
Doctor in Philosophy

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by  
Min Jin  
December 2002

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Keywords:  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel, migration, HGF/SF, ERK, MEK

## ABSTRACT

### Activation of $\text{Ca}^{2+}$ -Activated $\text{K}^+$ Channels and Cell Migration by Hepatocyte Growth Factor/Scatter Factor in Madin-Darby Canine Kidney Cells

by

Min Jin

Hepatocyte Growth Factor/Scatter Factor (HGF/SF) stimulates migration of various cells and has been linked via Met tyrosine kinase signaling to transformation and the metastatic phenotype. HGF/SF–Met signaling also plays a role in malignancy. Migration of transformed MDCK-F cells depends on activation of a charybdotoxin (ChTX)-sensitive, volume-activated membrane  $\text{K}^+$  current. Patch-clamp electrophysiology and transwell migration assays were used to study the effects of HGF/SF on membrane  $\text{K}^+$  currents and cell migration in MDCK II cells. HGF/SF activated membrane  $\text{K}^+$  currents that increased over 24 hr, and these could be modulated by altering intracellular free calcium concentration  $[\text{Ca}^{2+}]_i$ . HGF/SF also significantly increased MDCK II cell migration. Specific  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel blockers, ChTX, iberiotoxin (IbTX), stichodactyla toxin (Stk) and clotrimazole (CLT) inhibited HGF/SF stimulation of membrane  $\text{K}^+$  currents and cell migration. This suggests that the activation of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels is necessary for HGF/SF stimulation of MDCK II cell migration. Furthermore, HGF/SF induced ERK phosphorylation, and addition of the MEK inhibitor PD98059 inhibited ERK phosphorylation, as well as HGF/SF stimulation of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  currents and cell migration in MDCK II cells. Taken together, HGF/SF induces phosphorylation of ERK, which plays a role in HGF/SF activation of  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels and enhancing cell migration in MDCK II cells.

## DEDICATION

I would like to dedicate this dissertation to my parents and brothers. Their love and support are with me wherever I go.

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## ABBREVIATIONS

BSA	bovine serum albumin
BFU	blood forming unit
BK	large conductance, Ca <sup>2+</sup> -activated K <sup>+</sup> channel
ChTX	charybdotoxin
CLT	clotrimazole
ERK	extracellular signal-regulated kinase
ERK-P	phosphorylated extracellular signal-regulated kinase
Gab	Grb2-associated binder
HGF/SF	hepatocyte growth factor/scatter factor
IbTX	iberiotoxin
IK	intermediate conductance, Ca <sup>2+</sup> -activated K <sup>+</sup> channel
IRS-like	insulin receptor substrate-like
kDa	kilodalton
mAbs	monoclonal neutralizing antibodies to HGF/SF
MAPK	mitogen-activated protein kinase
MLC	myosin light chain
MLCK	myosin light chain kinase
MSP	macrophage-stimulating protein
PBS	phosphate-buffered saline
PI 3-kinase	phosphatidylinositol 3-kinase
PLC	phospholipase C
RVD	regulatory cell volume decrease
RVI	regulatory cell volume increase
SH2	Src homology region 2
SHIP	SH2-domain-containing inositol 5 phosphatase
Stk	stichodactyla toxin
TBS	tris-buffer saline
TTBS	tris-buffer saline with 0.1% v/v Tween20

TGF $\beta$	transforming growth factor beta
TK	tyrosine kinase
tPA	tissue plasminogen activator
uPA	urokinase plasminogen activator

# CHAPTER 1

## INTRODUCTION

### HGF/SF

Hepatocyte growth factor/scatter factor (HGF/SF) is a multifunctional effector of cells expressing the Met tyrosine kinase receptor. HGF/SF was the first member of the plasminogen-related growth factor family, which resembles blood protease plasminogen in structure and mechanism of activation. Macrophage-stimulating protein (MSP) is the other member identified in this family so far. Expression of the HGF/SF gene is restricted to fibroblasts and other mesenchymal cells; whereas, expression of the c-Met gene is confined to cells that do not secrete the ligand.

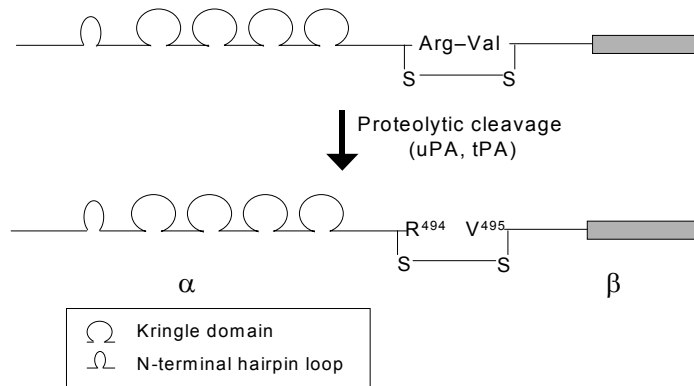
Inactive precursor of HGF/SF (proHGF/SF) is a single-chain peptide of 728 amino acids. Biologically active HGF/SF is produced by proteolytic cleavage of proHGF/SF between R<sup>494</sup> and V<sup>495</sup>. Mutations of R<sup>494</sup> generate an uncleavable HGF/SF, which can bind to HGF/SF receptor but cannot induce receptor phosphorylation (Hartmann et al. 1992). There is evidence that urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA) can convert proHGF/SF into the mature HGF/SF. Biologically active HGF/SF is a  $\alpha\beta$  heterodimer held together by a disulfide bond. The larger  $\alpha$  subunit (60 kDa) contains an N-terminal hairpin loop homologous to the activation peptide of plasminogen and four kringle domains which are triple-looped cysteine-rich motifs involved in protein-protein interaction. The smaller  $\beta$  subunit (34 kDa) resembles the catalytic domain of serine proteases (Birchmeier and Gherardi 1998). Fig.1A shows the schematic structure of HGF/SF. Studies on HGF/SF variants using progressive deletions and point mutations have mapped the receptor binding site within the first kringle and identified two groups of amino acids, E<sup>159</sup>, S<sup>161</sup>, E<sup>195</sup>, R<sup>197</sup> and D<sup>171</sup>, Q<sup>173</sup>, which appear to be essential for receptor binding (Lokker et al. 1992).

### HGF/SF Receptor, Met

HGF/SF receptor, Met, was originally isolated as the product of a human oncogene, *tpo-met*, which encodes an altered Met protein possessing constitutive, ligand-independent tyrosine kinase activity and transforming ability (Zhen et al. 1994). Met is a prototype of a tyrosine kinase family including the receptors encoded by the oncogenes *RON* and *SEA*. Met is a dimeric transmembrane polypeptide cleaved from a single polypeptide chain upon secretion. This receptor comprises an N-terminal  $\alpha$  chain (50 kDa) exposed at the cell surface and a C-terminal  $\beta$  chain (145 kDa) spanning the plasma membrane. The heavily glycosylated  $\alpha$  chain is disulfide linked to  $\beta$  chain. The  $\beta$  chain contains the tyrosine kinase (TK) domain, the tyrosine autophosphorylation sites and the multidocking site. The activation of the signal transduction downstream of Met is through the multidocking site, which comprises a short amino acid sequence motif (YVNVXXXVHV) located near the C-terminus of the  $\beta$  chain. Two tyrosine residues (Y1349 and Y1356) are located within the multidocking site and are phosphorylated upon ligand binding. Mutations of these tyrosine residues can abolish all receptor activity *in vitro* and *in vivo* (Park and Hayman 1999). Both  $\alpha$  and  $\beta$  chains of Met are required for high-affinity binding of HGF/SF. HGF/SF binding to Met induces receptor dimerization and phosphorylation of the Met receptor. Upon Met activation, multiple SH2 (Src homology region 2)-containing intracellular signal transducers and adaptor molecules bind to the phosphorylated tyrosine residues within the multidocking site, and the phosphorylation of these molecules results in the activation of down stream cascade, Fig. 1B. Several of the known transducers are Ras, p85 subunit of PI 3-kinase, PLC- $\gamma$ , Src-related tyrosine kinase, Grb-2 adaptor for SOS and Gab-1, an IRS-like multiadaptor protein, and a novel SH2-domain-containing inositol 5phosphatase (SHIP)-1 (Ponzetto et al. 1994; Weidner et al. 1996; Stefan et al. 2000). Recent *in vitro* studies have shown that cell motility response to HGF/SF requires the activation of both PI 3-kinase and Ras pathways (Ridley et al. 1995; Sachs et al. 1996), whereas Ras-MAPK pathway

through the Grb-SOS complex is necessary and sufficient for cell proliferation (Ponzetto et al. 1996).

A



B

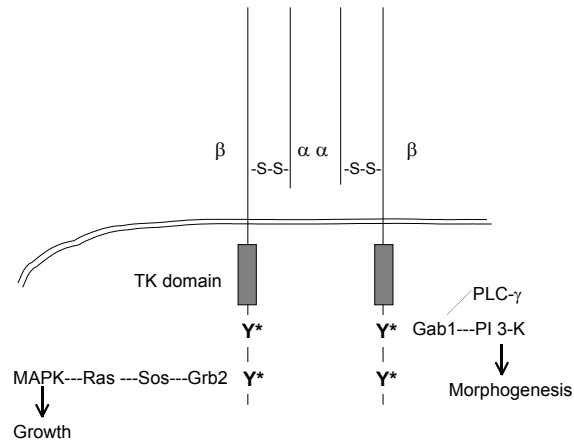


Figure 1 Schematic model of the proteolytic cleavage from proHGF/SF to HGF/SF and the Met dimerization and activation upon HGF/SF binding. A. Schematic model of the proteolytic cleavage from proHGF/SF to biologically active HGF/SF. The larger  $\alpha$  subunit contains an N-terminal hairpin loop and four kringles. The smaller  $\beta$  subunit resembles the catalytic domain of serine proteases. B. Schematic representation of Met dimerization and activation upon HGF/SF binding, and the main signaling molecules. The two phosphorylated tyrosines of the multidocking site (Y\*) are shown (see text for detailed description).



### Biological Activities of HGF/SF and Met

HGF/SF is secreted by mesenchymal cells, and it functions mainly through a paracrine mechanism on various cells expressing c-Met. HGF/SF is essential for normal embryological development of the nervous system, kidney, muscle, mammary gland, and liver because it plays a role in conversion from mesenchyme to epithelium (Matsumoto and Nakamura 1996, 1997). A germline point mutation in the multidocking site of c-Met causes a semi-lethal phenotype in mice, the newborns die from inefficient breathing, sucking and forelimb extension. This phenotype is due to the deficient migration of myoblast precursors from the cervical somites, resulting in the muscles of diaphragm, tongue, and forelimbs being underdeveloped. Full knock out mice die in utero from severe defects in liver and placenta. There also is evidence of an underdeveloped nervous system in the knock out mice; the sensory nerves in both limbs and thorax are severely reduced, revealing the role of HGF/SF-Met signaling in axonal development and survival (Maina et al. 1996; Stella and Comoglio 1999).

HGF/SF-Met signaling mediates various normal cellular processes in target cells, such as inducing cell proliferation, motility, morphological changes, and matrix invasion (Matsumoto and Nakamura 1996, 1997; Montesano et al. 1997). In addition to stimulating epithelial cell growth and survival, HGF/SF is a strong angiogenic factor for endothelial cells both *in vivo* and *in vitro*. HGF/SF also acts as an anti-apoptotic factor. Furthermore, HGF/SF is involved in bone remodeling that requires a tight coupling between osteoclasts and osteoblasts. HGF/SF is secreted by osteoclasts and binds to its receptors both on osteoclasts and osteoblasts. Secreted by monocytes and their precursors, HGF/SF induces erythroid hematopoietic precursors to grow and differentiate, thus stimulating the erythroid colonies (BFU) in blood (Stella and Comoglio 1999). HGF/SF signaling also is involved in the processes of tissue regeneration and wound healing (Matsumoto and Nakamura 1997).

This receptor-ligand pair also plays a role in malignancy. The coexpression of unaltered Met and HGF/SF molecules in the same cell, which generates an autocrine stimulatory loop, is

also oncogenic. Elevated levels of HGF/SF and/or over-expression of Met receptors are found in various types of tumors. HGF/SF-Met signaling has been shown to enhance the *in vitro* invasiveness and *in vivo* metastatic potential of tumor cells, such as ras-transformed NIH3T3, C127, and SK-LMS-1 cells (Jeffers et al. 1996; Jeffers et al. 1996a, 1996b).

Multiple signaling pathways downstream of HGF/SF-Met are involved in HGF/SF stimulation of cell migration and metastasis in various cell types. It has been shown that HGF/SF promotes cell invasion by increasing integrin avidity through Ras activation (Trusolino et al. 2000). PI 3-kinase activation stimulated by HGF/SF induces redistribution of junctional complex proteins, such as E-cadherin, desmoplakins and ZO-1, which in turn induces cell dissociation and scattering (Royal and Park 1995; Khwaja et al. 1998). HGF/SF also induces actin cytoskeleton remodeling, cell spreading and dissociation by activating Rho GTPase, PAK and Rho-kinase (Royal and Park 1995; Royal et al. 2000). PAK activity, through phosphorylation and inhibition of myosin light chain kinase (MLCK), promotes a decrease in actin-myosin filament assembly, allowing remodeling of actin cytoskeleton to form a lamellipodium (Royal and Park 1995; Sanders et al. 1999).

Furthermore, it is shown that HGF/SF can affect ion transport in normal and malignant cell types. HGF/SF-induced  $\text{Ca}^{2+}$  oscillation is observed in primary cultured rat hepatocytes (Kawanishi et al. 1995). It is also shown that HGF/SF induces sustained elevation of  $[\text{Ca}^{2+}]_i$  in rat ovarian surface epithelial cells in part by its ability to decrease cell contact, since homophilic N-cadherin binding between adhering cells plays an important role in maintaining  $\text{Ca}^{2+}$  homeostasis (Gulati and Peluso 1997; Peluso 1997). HGF/SF induces an oscillatory  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current in human gastric carcinoma SC-M1 cells, which is abolished when tetraethyl-ammonium chloride or a low  $\text{Ca}^{2+}$  solution is included in the recording pipette (Liu et al. 1998).

### Therapeutic Applications of HGF/SF and Met

HGF/SF expression is up regulated in liver injury and induces liver proliferation and regeneration. It has been shown that HGF/SF can recover hepatocytes from hyperaccumulation of lipids, preventing the onset of pathological ‘fatty liver’ (Tahara et al. 1999). It is also shown that HGF/SF gene therapy may prevent the cirrhotic liver (Ueki et al. 1999). HGF/SF stimulates compensatory growth in the remaining kidney in nephrectomized rats. This renotropic function of HGF/SF has been used to prevent renal failure following cisplatin treatment (Kawaida et al. 1994).

HGF/SF-Met signaling plays an important role in tumor growth and metastasis, which has drawn attention to develop drugs for cancer treatment. The intervention of HGF/SF-Met signaling inhibits cell invasiveness in vitro as well as tumor growth and metastasis in vivo in some tumors (Cao et al. 2001; Furge et al. 2001). Neutralizing monoclonal antibodies to HGF/SF used in this study has been shown to inhibit HGF/SF stimulation of scattering in MDCK cells and branching morphogenesis in SK-LMS-1 cells via blocking HGF/SF-Met signaling; more importantly, they also inhibit the growth of human glioblastoma multiforme xenografts in athymic *nu/nu* mice (Cao et al. 2001).

Recently, attempts have been made to engineer HGF/SF variants for cancer therapy. An HGF/SF MSP (macrophage-stimulating protein) chimera, Metron factor 1, prevents apoptosis and stimulates cell proliferation but is devoid of pro-invasive activity of HGF/SF (Michieli et al. 2002). HGF/NK4, a four-kringle competitive antagonist of HGF/SF, inhibits angiogenesis, as well as tumor growth and metastasis in mice (Kuba et al. 2000). Therefore, HGF/SF is a promising candidate for therapies of organ regeneration, as well as cancer.

### Cell Migration

Migration plays an important role in wound healing, tumor metastasis, and many other biological activities of various cells. Cell volume changes and actin cytoskeletal machinery

underlie cell migration. Migration involves substantial reorganization of the cytoskeleton both at the leading edge and the rear of the migrating cell. At the leading edge (lamellipodia or filopodia), polymerization of actin filaments prevails, resulting in the protrusion of lamellipodia or filopodia; whereas at the rear of the cells, actin filaments are depolymerized. The depolymerization is mediated by capping or escort proteins such as gelsolin, which are stimulated by  $\text{Ca}^{2+}$  (Stossel 1993; Witke et al. 1995). Bound to the capping or escort proteins, the actin monomers travel toward the leading edge, where they are reutilized for elongation of the actin filaments. At the rear of the cell, local cell swelling tends to occur because of actin depolymerization; however, regulatory volume decrease (RVD) and  $\text{Ca}^{2+}$  stimulus at the rear of the cell overcomes the effect of actin depolymerization, resulting in local osmotic shrinkage. Whereas, local osmotic swelling occurs at the leading edge of the cell as the regulatory volume increase (RVI) counteracts the effect of actin polymerization at the lamellipodium. Thus, polymerization at the leading edge and depolymerization at the rear of the cells contribute to the local volume changes that also facilitate cell migration (Lang et al. 1998). The matrix also exerts an opposite traction force on the migrating cell through cell-matrix attachment. Therefore, the protrusion of lamellipodia, contraction in the cell body, and cell-matrix interaction all play a role in cell migration.

It has been proposed that ion channels and transporters modulate cytoskeletal migration machinery by regulating  $[\text{Ca}^{2+}]_i$  and local cell volume in migrating cells (Schwab 2001).  $[\text{Ca}^{2+}]_i$  oscillation in the rear of the cell activates  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels, which results in local regulatory cell volume decrease (RVD) and promotes the retraction of a migrating cell.  $[\text{Ca}^{2+}]_i$  is also an important regulator for cytoskeletal proteins, it plays a role in triggering actin depolymerization in the rear end of migrating cells. In contrast, low  $[\text{Ca}^{2+}]_i$  in the front favors the protrusion of lamellipodium. Blocking  $\text{Ca}^{2+}$  channels decreases the rate of migration in granule cells (Komuro and Rakic 1998). At the lamellipodium,  $\text{Na}^+/\text{H}^+$  and  $\text{Na}^+-\text{K}^+-2\text{Cl}^-$  cotransporters favors local regulatory cell volume increase (RVI). Inhibition of  $\text{Na}^+-\text{K}^+-2\text{Cl}^-$

cotransporter or  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels prevents volume fluctuation and inhibits migration of alkali-transformed MDCK-F cells (Schwab et al. 1994). Glioma cell migration is inhibited by  $\text{Cl}^-$  and  $\text{K}^+$  channel blockers such as chlorotoxin, tetraethylammonium chloride, and tamoxifen (Soroceanu et al. 1999). There is evidence that differentiated intestinal epithelial cells after wounding exhibit increased migration partially by increasing voltage-gated  $\text{K}^+$  channel activity and elevation of cytosolic free  $\text{Ca}^{2+}$  concentration (Rao et al. 2002).

### MDCK-F Cells and $\text{K}^+$ channels

MDCK epithelial cells grow in colonies of tightly associated cells and have been used as an *in vitro* model for HGF/SF-induced scattering, migration, and tubulogenesis (Terauchi and Kitamura, 2000). Prolonged exposure to an alkaline culture medium transforms MDCK, which dramatically affects cell function and polarization (Schwab and Oberleithner 1996). Alkaline-transformed MDCK-F cells proliferate and migrate without contact inhibition, they also show remarkably altered electrophysiological properties, such as fluctuations of intracellular  $\text{Ca}^{2+}$  activity, membrane conductance and membrane potential (Schwab et al. 1995; Schwab and Oberleithner 1996).  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels underlie the oscillation of the membrane potential. Application of  $\text{K}^+$  channel blockers, such as barium, tetraethylammonium, 4-aminopyridine, and charybdotoxin markedly inhibits cell migration, pointing to the importance of  $\text{K}^+$  channels in this process (Schwab et al. 1994). Schwab et al. have demonstrated that the intermediate conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel, IK, modulates the migration of the alkaline-transformed MDCK-F cells by local volume regulation (Schwab et al. 1994).

### MAPK Signaling Pathway

It has been shown that MEK inhibitor PD98059 inhibits the scattering response and tubulogenesis in HGF/SF treated MDCK cells (Khwaja et al. 1998). Inhibition of MAP kinase pathway with PD98059 also inhibits HGF/SF induced cell migration in mICD-3 cells (cells

derived from the inner medullary collecting duct) (Karihaloo et al. 2001). HGF/SF stimulates MAPK signaling pathway in human hepatoma cells, and inhibition of ERK1/2 phosphorylation by MEK inhibitor PD98059 or inhibition of ERK1/2 kinase activity by nonsteroidal anti-inflammatory drugs aspirin and NS-398 suppresses HGF/SF-induced invasiveness of Hep2 human hepatoma cells (Abiru et al. 2002).

MAP kinase signaling pathway plays an important role in HGF/SF stimulation of biological activities such as cell migration, morphological changes, and invasiveness in various cell types. It has also been shown that TGF $\beta$ 1 stimulates large conductance, Ca<sup>2+</sup>-activated K<sup>+</sup> channel activity via activation of ERK MAP kinase in chick ciliary ganglions (Lhuillier and Dryer 2000). Therefore, we propose that MAP kinase signaling pathway plays a role in HGF/SF activation of Ca<sup>2+</sup>-activated K<sup>+</sup> channel and migration in MDCK II cells.

## CHAPTER 2

### MATERIAL AND METHODS

#### Cell Culture

Madin-Darby canine kidney epithelial (MDCK II) cells were maintained in 25-mm<sup>2</sup> T-flasks with Dulbecco's modified Eagle's medium/Ham's F-12 medium (Sigma, St. Louis, MO), which was supplemented with 5% fetal bovine serum (Atlanta Biologicals Inc., Norcross, GA), 100 I.U./ml penicillin, 100 µg/ml streptomycin (Sigma, St. Louis, MO). Cells were incubated in a 95% air/5% CO<sub>2</sub> in a water-jacketed tissue culture incubator. Medium was changed every other day, and cells were passaged every 5-7 days. The latter consisted of washing the cells twice with phosphate-buffered saline (PBS), incubating them 45-60 min with porcine trypsin (1.2 – 2.5 mg/ml PBS), followed by pelleting of the suspended cells, aspirating the trypsin solution, diluting with fresh complete medium, and replating the cells.

#### Reagents

Charybdotoxin, iberiotoxin, and stichodactyla toxin were obtained from Alomone Laboratories (Jerusalem, Israel), and Accurate Chemical & Scientific Corporation (Westbury, NY). Clotrimazole and PD98059 were obtained from Sigma (St. Louis, MO). Human HGF/SF was obtained as a gift from Dr. G. F. Vande Woude, Van Andel Research Institute, Grand Rapids, MI. HGF/SF concentrations are presented as scatter units/ml; five scatter units are equivalent to approximately 1 ng of protein.

#### Antibodies

Neutralizing monoclonal antibodies to HGF/SF were obtained as gifts from Dr. G. F. Vande Woude, Van Andel Research Institute, Grand Rapids, MI. Polyclonal antibodies to ERK and phosphorylated ERK were obtained from Cell Signaling Technology (Beverly, MA).

### Preparation of Microelectrodes

Borosilicate glass capillaries (1.2 mm OD, 0.68 ID, type EN-1, Garner Glass Co., Claremont, CA) were cleaned in a sonicator and dried in a convection oven at 90°C. Pipettes (3-8 MΩ in the bath solution) were fabricated from the capillaries with a Brown-Flaming horizontal micropipette puller (P-87, Sutter Instruments, San Rafael, CA). Whole-cell pipettes were coated to within 0.5 mm of the tip with polystyrene base coil dope (Polyweld 912, Amphenol, Wallingford, CT); cell-attached pipettes were coated with R6101 to reduce the noise (Dow Corning Corp., Midland, MI). The tips were heat-polished prior to use. A micromanipulator (MO-202, Narishige, Tokyo) fixed to the microscope was used to position pipettes.

### Whole-Cell and Cell-Attached Voltage Clamp Techniques

Cells were seeded on 4 × 4-mm sections of plastic coverslips for 24 hr before treatment with various agents. After treatment the coverslips were transferred to an acrylic chamber (Warner, New Haven, CT) on the stage of an inverted microscope (Olympus IMT-2, Melville, NY) equipped with Hoffman modulation contrast optics. The whole-cell and cell-attached configurations were obtained by standard patch clamp technique (Hamill et al. 1981). Membrane currents were measured with a patch clamp amplifier (Axopatch 1-D, Axon Instruments, Foster City, CA) with the lowpass, Bessel filtering (-3 dB) set at 2kHz. Whole-cell currents from the patch clamp amplifier were fed into a DMA-1 digital interface connected to a 486-SX computer equipped with Clampex software (Axon Instruments). Records were stored on an internal Jaz drive (Iomega, Roy, UT). Ag/AgCl half-cells constituted the electrodes, and an agar bridge (4% w/v in external gluconate solution) connected the reference electrode to the bath solution. The junction null was zeroed in the cell-attached mode prior to whole-cell access. Electrode series resistances were measured following whole-cell access, and were compensated prior to recording.



### Solutions Used for Patch Clamp

Cells for patch-clamp recording were superfused at room temperature with a standard external salt solution, and gluconate was used to substitute for the Cl<sup>-</sup> in the solution. For whole-cell patch clamp, pipettes were filled with standard internal solution, which was buffered with EGTA to reach a free Ca<sup>2+</sup> concentration of 28 nM, 1 μM or 7 μM according to G. Droogmans (<ftp://ftp.cc.kuleuven.ac.be/pub/droogmans/cabuf.zip>). For cell-attached patch clamp, pipettes were filled with standard external solution. The compositions of the solutions used through out these experiments were given in Table 1.

### Preparation of Transwell Membranes

Transwell polycarbonate membranes with a pore size of 8 μm were inserted into 24-well cluster plates (Costar Corporation, Cambridge, MA). The bottom side of each membrane was coated with 100 μl of fibronectin (25 μg/ml in sterile PBS; Sigma, St. Louis, MO). These were rinsed twice with PBS, once with 1% w/v bovine serum albumin (BSA; Sigma, St. Louis, MO) in PBS, and blocked with 1% BSA in PBS for 30 min at room temperature. The bottom side of each control membrane was treated with 100 μl BSA (1% w/v in PBS) for 1 hour at room temperature.

### Transwell Cell Migration Assay

800 μl of migration assay buffer (containing serum-free DMEM/F-12, 0.1% BSA, 100 I.U./ml penicillin, 100 μg/ml streptomycin) was added to the lower chamber of each well. Various agents and combinations were added to the lower chambers before moving the inserts into the wells. The final concentration of these drugs was computed by assuming diffusion equilibration with the 200 μl volume of medium added to the upper chambers. MDCK II cells were harvested, washed in PBS, and suspended in migration assay buffer at a cell density of

$3 \times 10^5$  cells/ml. 200  $\mu$ l aliquots of cells were plated on the upper membrane surface of each insert. The tissue culture plates were then returned to the tissue culture incubator for 8 hr.

#### Fixation, Staining and Counting of MDCK II Cells

After 8 hr incubation, inserts were rinsed, and cells on the upper membrane surface were gently removed using cotton swabs. Cells on the under surface were fixed in 100% methanol (10 min) at 4°C and stained for 15 min. with modified Harris hematoxylin solution (Sigma, St. Louis, MO). Ten randomly selected fields of 0.6 mm<sup>2</sup> were counted using an inverted microscope (Olympus IMT-2, Melville, NY) to determine the number of cells that migrated in the experimental groups and the untreated controls. Each experiment was repeated 3 times.

#### Protein Harvesting for Western Blotting

Cells were made quiescent by serum starvation for 24 hr followed by stimulation with HGF/SF (100 scatter units/ml). For ERK inhibition studies, cells were pre-incubated with 50  $\mu$ M PD98059 (Sigma, St. Louis, MO) for 30 minutes before the addition of HGF/SF. Cells were lysed for 20 minutes on ice with ice-cold lysis buffer, which contained 50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100 [pH=8.0], 1  $\mu$ g each of leupeptin, pepstatin, and antipain, 1 mM sodium orthovanadate, 1 mM PMSF, 1 mM EDTA, and 1 mM NaF (Sigma, St. Louis, MO). The extraction solutions were then centrifuged at 14,000  $\times$  g for 15 min at 4°C and the supernatants were saved, and the protein contents were determined by the BCA assay.

#### Western Blotting Analysis of ERK

A protein sample (20  $\mu$ g) from each extraction solution was separated on 12% SDS PAGE and transferred to nitrocellulose membranes. The membranes were placed in the blocking buffer (5% non-fat dry milk, 0.1% Tween20 in tris-buffered saline (TTBS)) for 1 hr, and

subsequently incubated at 4°C overnight with 1:1000 diluted primary anti-total ERK and anti-ERK-P antibodies, respectively (Cell Signaling Technology, Beverly, MA). The membranes were washed with TTBS for 15 minutes, 4 times, then incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1: 2000) for 1 hr at room temperature. After two washes in TTBS, two washes in tris-buffered saline (TBS), 15 minutes each, membranes were incubated with LumiGLO for 1 minute, and exposed to film. Densitometry was performed using Intelligent Quantifier (Bio Image, Ann Arbor, MI).

### Statistics

Results were given as mean  $\pm$  SE. Student t-test, one-way analysis of variance, multiple comparisons, and regression analysis were performed where applicable. Analysis of covariance and t-test were performed to compare slope conductance. Slope conductance was obtained from the regression analysis on the linear component (4 data points) of the I-V plots. Significant differences among means or slope conductance were determined at  $P < 0.05$ .

Table 1 Salt solutions used to fill bath and pipets.						
SOLUTIONS	Externa 1 (bath)	Externa 1 (hypo)	Externa 1 (hi K <sup>+</sup> )	Internal (7 μM [Ca <sup>2+</sup> ] <sub>i</sub> )	Internal (1 μM [Ca <sup>2+</sup> ] <sub>i</sub> )	Internal (28 nM [Ca <sup>2+</sup> ] <sub>i</sub> )
Na gluconate (mM)	135	95	0.4	-----	-----	-----
K gluconate (mM)	5.4	5.4	140	140	140	140
CaCl <sub>2</sub> (mM)	1.8	1.8	1.8	0.998	0.854	0.1
MgCl <sub>2</sub> (mM)	2	2	2	2	2	2
EGTA (mM)	-----	-----	-----	1	1	1
Glucose (mM)	1	10	10	-----	-----	-----
HEPES (mM)	1	5	5	5	5	5
PH	7.41	7.41	7.41	7.2	7.2	7.2

## CHAPTER 3

### RESULTS

#### HGF/SF Stimulates Membrane Currents in MDCK II Cells in A Time-Dependent Way

MDCK II cells formed discrete colonies of tightly juxtaposed cells when seeded on coverslips. The cells scattered by spreading and migrating during 8 and 24-hr stimulation by HGF/SF. These cells also were readily studied by patch clamp electrophysiology. Voltage ramps from  $-100$  to  $50$  mV (pipette potential) were applied in cell-attached configuration to MDCK II cells treated with different concentrations of HGF/SF (10, 50 100 scatter units/ml) for 24 hr. Fig. 2 shows HGF/SF stimulated a membrane  $K^+$  current over 24 hr, and the stimulation was more significant at higher dose of HGF/SF. There were also some inward currents activated at around  $50$  mV (pipette potential) with higher doses of HGF/SF, which could be sodium channels and need further study to characterize them.

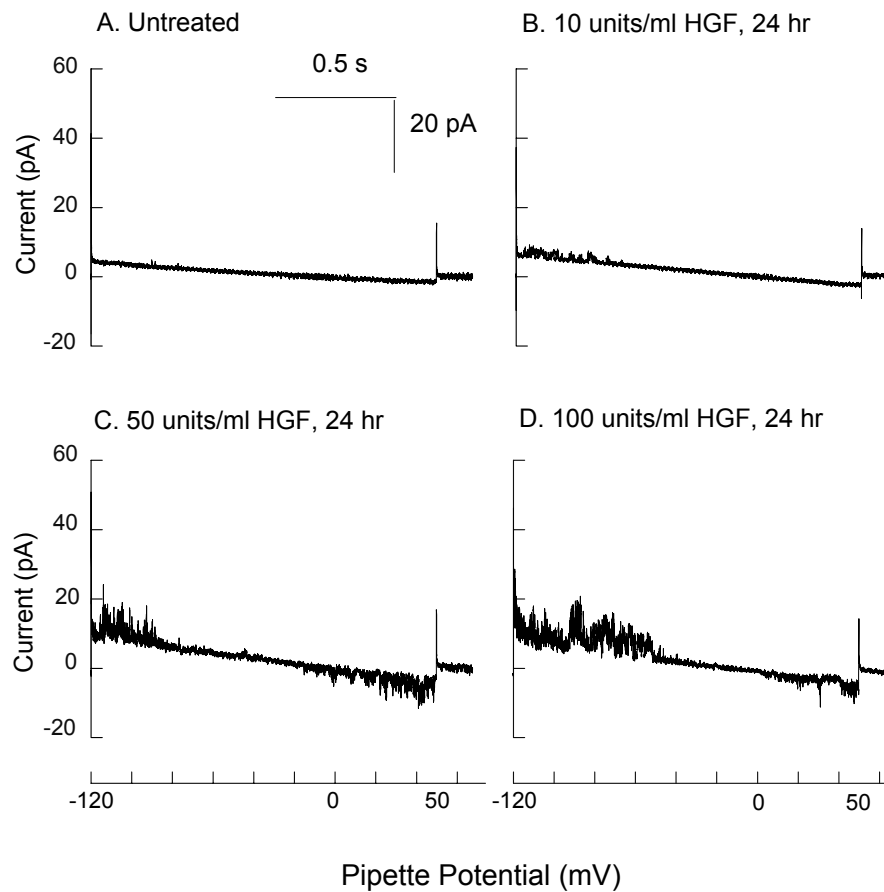


Figure 2 HGF/SF stimulates membrane  $K^+$  current in MDCK II cells in cell-attached configuration. Pipettes were filled with standard external solution with gluconate substitute for  $Cl^-$ . Cell-attached currents were recorded on voltage ramp from  $-120$  to  $50$  mV (pipette voltage) for a duration of  $1.4$  s. *A.* Cell-attached recording of an untreated MDCK II cell. *B.* Cell-attached recording of an MDCK II cell treated with  $10$  scatter units/ml HGF/SF,  $24$  hr. *C.* Cell-attached recording of an MDCK II cell treated with  $50$  scatter units/ml HGF/SF,  $24$  hr. *D.* Cell-attached recording of an MDCK II cell treated with  $100$  scatter units/ml HGF/SF,  $24$  hr.

Characteristics of Membrane Currents Stimulated by HGF/SF  
in MDCK II Cells

In order to understand the characteristics of these  $K^+$  channels, whole-cell recordings were performed with different free  $Ca^{2+}$  concentrations in internal solutions (pipette solution). The mean  $\pm$  SE value of pipette/membrane seal resistance was  $5.8 \pm 0.2$  G $\Omega$  (n=112). The corresponding measurements of whole-cell capacitance =  $16.8 \pm 0.7$  pF; the series access resistance =  $7.2 \pm 0.2$  M $\Omega$ ; and the whole-cell resistance =  $170 \pm$  M $\Omega$ .

Current-voltage (I-V) relationships that were obtained from data points taken at the end of similar voltage pulses yielded continuous increases in membrane  $K^+$  conductance at 4 and 24 hr of HGF/SF treatment compared with control cells, Fig. 3. Voltage ramps from  $-100$  to  $100$  mV were applied to an HGF/SF-treated cell (200 scatter units/ml for 24 hr), and the results are shown in Fig. 4. This was done first under control conditions of  $5.4$  mM external  $[K^+]$ ; then the voltage ramp was repeated after the cell was exposed to  $140$  mM external  $[K^+]$ , which was symmetrical with the pipette  $[K^+]$ ; and then after switching back to control solution. The increase in external  $[K^+]$  resulted in a shift in current reversal potential from  $\approx -60$  mV to  $\approx 5$  mV, along with an increase in slope conductance. These reversed after switching back to the control solution. Such responses of the HGF/SF-treated cell to the changes in external  $[K^+]$  were consistent with those expected of membrane  $K^+$  conductance.

To characterize the  $K^+$  channels activated by HGF/SF, intracellular free  $Ca^{2+}$  was buffered at  $28$  nM,  $1$   $\mu$ M or  $7$   $\mu$ M for whole-cell recordings. MDCK II cells were treated with  $200$  scatter units/ml HGF/SF (for 24 hr) for whole-cell recording with  $28$  nM and  $1$   $\mu$ M of  $[Ca^{2+}]_i$ ; whereas, the cells were treated with  $50$  scatter units/ml HGF/SF (for 24 hr) for whole-cell recordings with  $7$   $\mu$ M of  $[Ca^{2+}]_i$ . After obtaining the whole-cell currents recorded in the control bath solution, the control solution was changed to a solution with  $50$  nM ChTX. Slope conductance and reversal potentials ( $V_r$ ) recorded under various conditions were compared in

Table 2. ChTX (50 nM) had no effect on membrane  $K^+$  current at 28 nM or 1  $\mu$ M  $[Ca^{2+}]_i$  in untreated cells; however, it significantly inhibited membrane  $K^+$  current at 7  $\mu$ M  $[Ca^{2+}]_i$  in these cells. HGF/SF significantly increased slope conductance at 1  $\mu$ M  $[Ca^{2+}]_i$  (Fig. 6, *right*); it also stimulated membrane  $K^+$  currents at near zero and positive membrane potentials at 7  $\mu$ M  $[Ca^{2+}]_i$  (Fig. 8). ChTX (50 nM) significantly inhibited these increases stimulated by HGF/SF. However, at 28 nM  $[Ca^{2+}]_i$ , ChTX (50 nM) had no effect on membrane  $K^+$  currents in HGF/SF-treated cells. Therefore, HGF/SF stimulation on membrane  $K^+$  current is regulated by intracellular  $Ca^{2+}$  concentration.



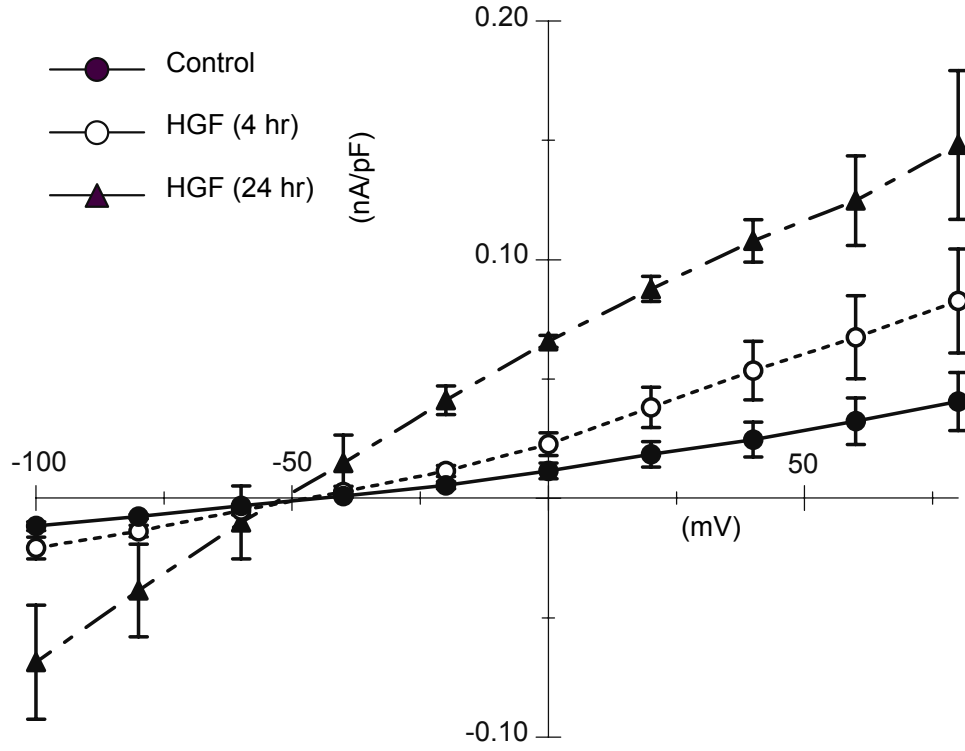


Figure 3 HGF/SF stimulates membrane  $K^+$  current in MDCK II cells after 4 and 24 hr treatment. HGF/SF concentration was at 200 scatter units/ml. Current-voltage (I-V) plot for whole-cell currents recorded in MDCK II cells during voltage pulses ranging from  $-100$  to  $80$  mV in consecutive,  $20$  mV increments. (Mean $\pm$ SE;  $n=3$  cells).

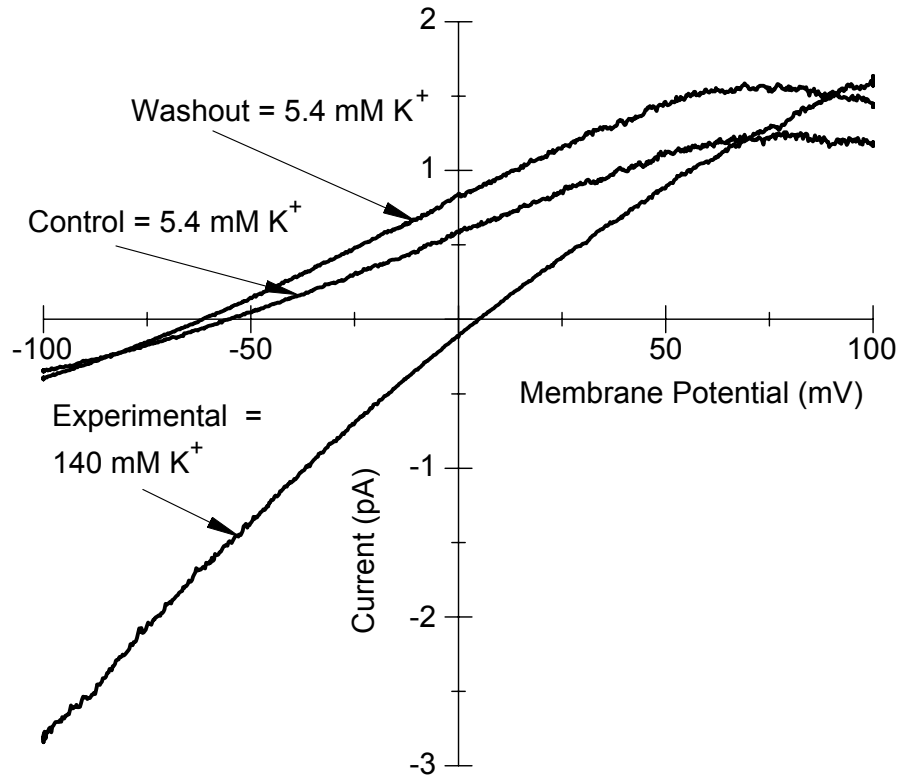


Figure 4 Whole-cell currents recorded in an MDCK II cell on application of voltage ramp. Voltage ramp was from  $-100$  to  $100$  mV ( $1.5$  s in duration). Control trace was recorded right after break-in, experimental trace was recorded shortly after exchange of extracellular solution with  $140$  mM  $K^+$  from a solution containing only  $5.4$  mM  $K^+$  (Control). Washout trace was recorded after switching back to control solution.

Table 2 Effects of ChTX (50 nM) on HGF/SF stimulation of membrane K <sup>+</sup> current at different intracellular [Ca <sup>2+</sup> ] <sub>i</sub> .					
[Ca <sup>2+</sup> ] <sub>i</sub>	bath solution	<u>untreated</u>		<u>HGF/SF-treated</u>	
		slope (nS/pF)	V <sub>r</sub> (mV)	slope	V <sub>r</sub>
28 nM <sup>a</sup>	control	0.24 ± 0.04 (n=4)	-55.9 ± 18.0 (n=4)	0.32 ± 0.08 (n=3)	-71.1 ± 10.9 (n=3)
	ChTX	0.23 ± 0.05 (n=4)	-60.6 ± 21.2 (n=4)	0.26 ± 0.09 (n=3)	-64.3 ± 14.5 (n=3)
1 μM <sup>a</sup>	control	0.38 ± 0.05 (n=7)	-47.6 ± 3.5 (n=7)	0.79 ± 0.15 (n=7); !	-52.1 ± 4.5 (n=7)
	ChTX	0.29 ± 0.06 (n=7)	-35.8 ± 4.1 (n=7)	0.30 ± 0.10 (n=7); *	-44.7 ± 3.8 (n=7)
7 μM <sup>b</sup>	control	0.90 ± 0.09 (n=5)	-100 ± 4.9 (n=5)	0.93 ± 0.16 (n=5)	-101.2 ± 3.5 (n=5)
	ChTX	0.37 ± 0.09 (n=5); *	-76.8 ± 7.6 (n=5); *	0.33 ± 0.08 (n=6); *	-70.8 ± 6.9 (n=6); *

a. For [Ca<sup>2+</sup>]<sub>i</sub> of 28 nM and 1 μM, HGF/SF-treated MDCK cells were treated with 200 scatter units/ml HGF/SF for 24 hr; b. for [Ca<sup>2+</sup>]<sub>i</sub> of 7 μM, HGF/SF-treated MDCK cells were treated with 50 scatter units/ml HGF/SF for 24 hr.

!Significantly different from the same measurement in untreated cells (P < 0.05, refer to Fig.6, *right*).

\*Significantly different from the same measurement in control solution (P < 0.05).

## K<sup>+</sup> Channel Blockers Inhibit HGF/SF-Stimulated Membrane K<sup>+</sup> Currents

([Ca<sup>2+</sup>]<sub>i</sub> = 1 μM) in MDCK II Cells

ChTX added at 50 nM for 5 min. inhibited membrane K<sup>+</sup> current in MDCK II cells that were treated with HGF/SF (200 scatter units/ml for 24 hr), Fig. 5. The I-V plots in Fig. 6 comprise summary results from repeated measurements of ChTX (50 nM) effects on untreated (*left*), HGF/SF treated MDCK II cells at 100 (*middle*) and 200 scatter units/ml (*right*). ChTX had no significant effect on slope conductance for K<sup>+</sup> currents in untreated MDCK II cells (ANCOVA,  $P > 0.05$ ,  $n = 7$  cells) or in cells treated for 24 hr with 100 scatter units/ml HGF/SF (ANCOVA,  $P > 0.05$ ,  $n = 3$  cells). HGF/SF at 200 scatter units/ml significantly increased slope conductance of membrane K<sup>+</sup> current, which was inhibited by added ChTX at 50 nM (ANCOVA,  $P < 0.05$ ,  $n = 7$  cells). IbTX also inhibited HGF/SF-stimulated membrane K<sup>+</sup> currents in MDCK II cells. IbTX at 100 nM significantly decreased slope conductance of membrane K<sup>+</sup> current (ANCOVA,  $P < 0.05$ ,  $n = 4$  cells), along with a modest shift in reversal potential toward 0 mV in MDCK II cells treated for 24 hr with 200 scatter units/ml of HGF/SF, Fig. 7. CLT at 1 μM didn't significantly inhibit HGF/SF-stimulated membrane K<sup>+</sup> current in MDCK II cells treated with HGF/SF (200 scatter units/ml, for 24 hr), the slope conductance in control bath solution was  $0.62 \pm 0.18$  (Mean  $\pm$  SE,  $n=3$  cells), the slope conductance in bath solution with 1 μM CLT was  $0.30 \pm 0.07$  (Mean  $\pm$  SE,  $n=3$  cells), paired t-test yielded a  $P > 0.05$ . Stk at 100 nM had no effect on HGF/SF stimulated membrane K<sup>+</sup> current in MDCK II cells treated with 200 scatter units/ml for 24 hr (data not shown).

MDCK II Cell Treated with HGF/SF (200 scatter units/ml) for 24 hr.

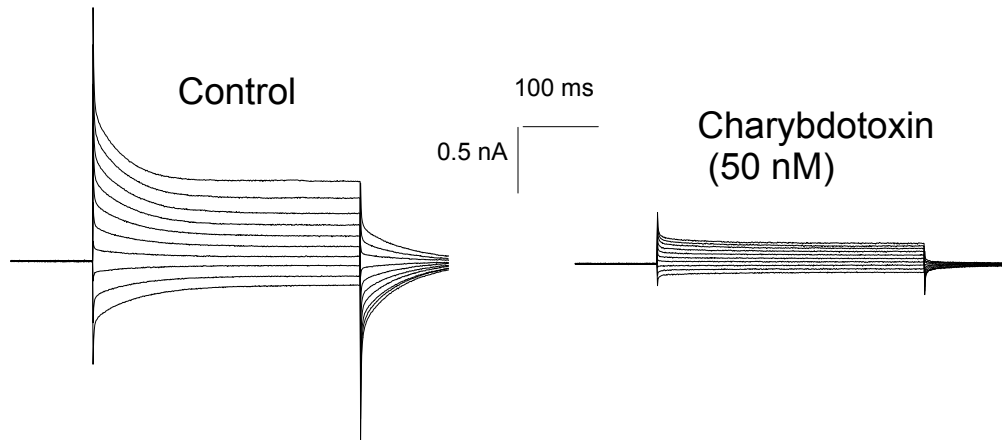


Figure 5 ChTX inhibits HGF/SF-stimulated membrane K<sup>+</sup> current in an MDCK II cell. Whole-cell currents were recorded during voltage pulses that ranged from -100 to 80 mV in consecutive, 20-mV increments. Holding potential = -50 mV. *Left.* MDCK II cell treated with 200 scatter units/ml HGF/SF for 24 hrs in standard external solution with gluconate. *Right.* The same cell 5 min after 50 nM ChTX in the external solution.

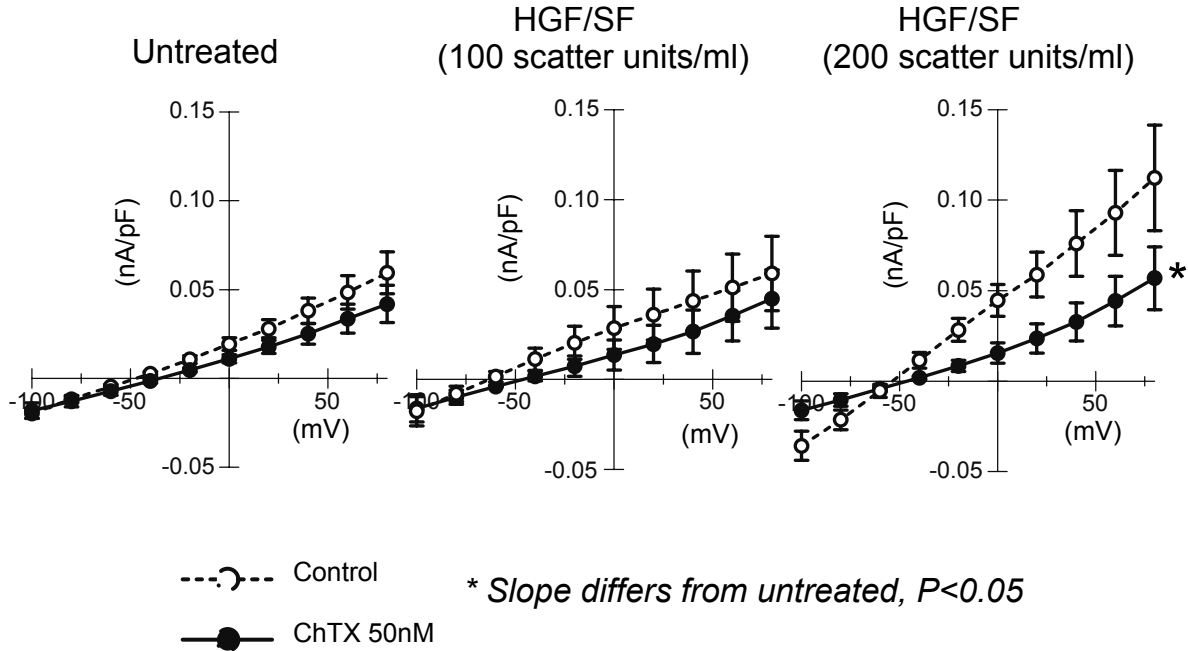


Figure 6 Inhibitory effect of ChTX on HGF/SF stimulation over 24 hr of membrane  $K^+$  current in MDCK II cells. Whole-cell currents were recorded during voltage pulses that ranged from -100 to 80 mV in consecutive, 20-mV increments. Holding potential = -50 mV. *Left.* Untreated MDCK II cells (Mean $\pm$ SE; n=7 cells). *Middle.* MDCK II cells treated with 100 scatter units/ml HGF/SF for 24 hr (Mean $\pm$ SE; n=3 cells). *Right.* MDCK II cells treated with 200 scatter units/ml HGF/SF for 24 hr (Mean $\pm$ SE; n=7 cells). \*There is significant difference between the slope conductance in control solution and in a solution with 50 nM ChTX.

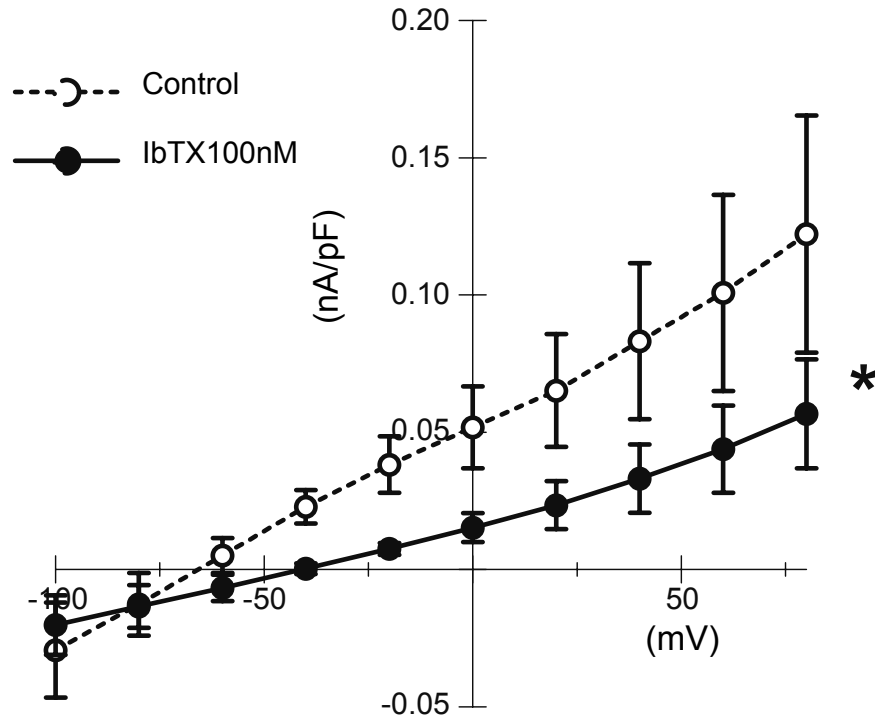


Figure 7 IbTX inhibits membrane  $K^+$  current in HGF/SF treated MDCK II cells. Whole-cell currents were recorded during voltage pulses that ranged from -100 to 80 mV in consecutive, 20-mV increments. Holding potential = -50 mV. (Mean $\pm$ SE; n=7 cells). \*There is significant difference between the slope conductance in control solution and in a solution with 100 nM IbTX.

## K<sup>+</sup> Channel Blockers Inhibit HGF/SF-Stimulated Membrane K<sup>+</sup> Currents

### ([Ca<sup>2+</sup>]<sub>i</sub> = 7 μM) in MDCK II Cells

To further characterize the HGF/SF stimulation of membrane K<sup>+</sup> current in MDCK II cells, the inhibitor experiments were repeated at 7 μM internal [Ca<sup>2+</sup>]<sub>i</sub>, a concentration that was sufficient for the activation of all types of Ca<sup>2+</sup>-activated K<sup>+</sup> channels (Hille 2001). Fig. 8 shows that HGF/SF (50 scatter units/ml) significantly stimulated membrane K<sup>+</sup> currents at near zero and positive membrane potentials in MDCK II cells (P<0.05, n=9 of untreated cells, n=9 of HGF/SF-treated cells). Representative whole-cell current traces are shown in Fig. 9A (insert) for comparison with those obtained from cells dialyzed with 1 μM [Ca<sup>2+</sup>]<sub>i</sub>, Fig. 4. Membrane currents were greater at all voltages compared with measurements made with 1 μM internal [Ca<sup>2+</sup>]<sub>i</sub>, and the corresponding I-V curves rectified inwardly, Fig. 9A-9D. K<sup>+</sup> channel inhibitors added for 5 min significantly reduced slope conductance of the whole-cell current in HGF/SF-stimulated cells, ChTX (Fig. 9A), IbTX (Fig. 9B), Stk (Fig. 9C) and CLT (Fig. 9D). All these inhibitors, except for CLT, also significantly reduced the reversal potential. ChTX (50 nM) also significantly reduced slope conductance and reversal potential of the whole-cell K<sup>+</sup> current in untreated MDCK II cells at 7 μM internal [Ca<sup>2+</sup>]<sub>i</sub> (not shown); however, CLT (1 μM) had no effect. Fig. 10 shows the difference in the currents recorded from the control solution, and a solution with a Ca<sup>2+</sup>-activated K<sup>+</sup> channel blocker by deducting the inhibited current from the control current in Fig. 9A-9D, respectively.



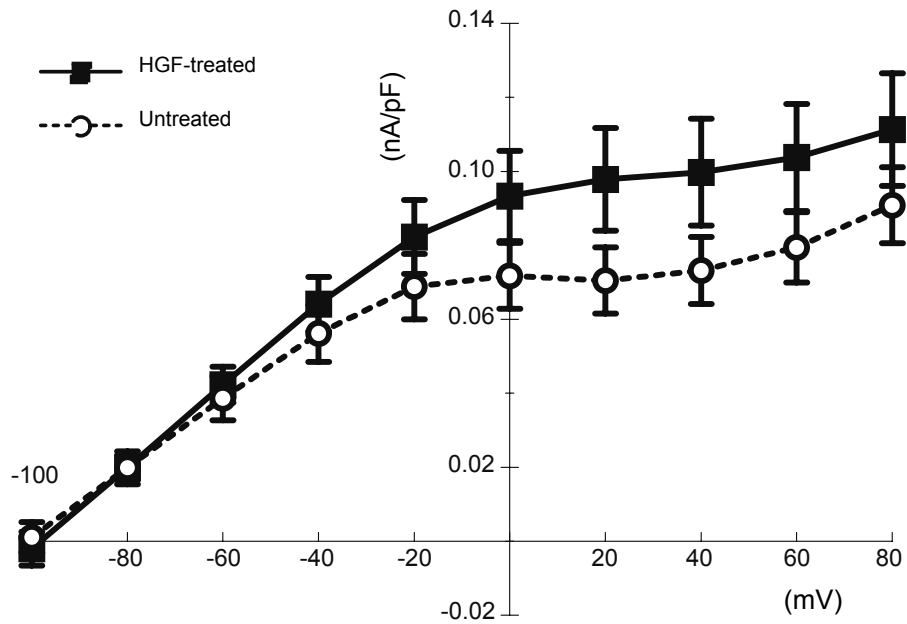


Figure 8 Current-voltage plots recorded from untreated and HGF/SF-treated MDCK II cells. Voltage steps were applied from  $-100$  to  $80$  mV in consecutive,  $20$  mV increments. HGF/SF treatment ( $50$  scatterunits/ml for  $24$  h) stimulated membrane current at near zero mV and at positive membrane potentials ( $n=9$  of untreated cells,  $n=9$  of HGF/SF treated cells).

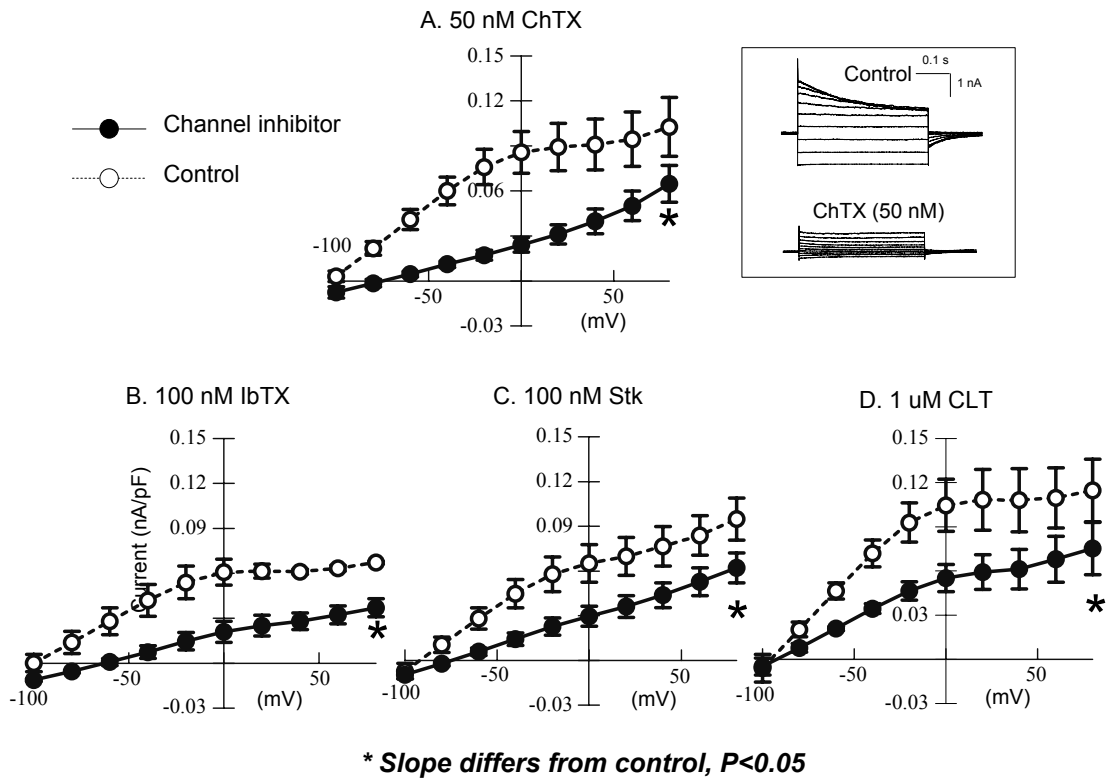


Figure 9  $K^+$  channel blockers inhibit HGF/SF stimulated membrane  $K^+$  current in MDCK II cells.  $[Ca^{2+}]_i$  was buffered to  $7 \mu M$ . HGF/SF concentration was at 50 scatter units/ml. Current-voltage (I-V) plot for whole-cell currents recorded in MDCK II cells during voltage pulses ranging from  $-100$  to  $80$  mV in consecutive,  $20$ -mV increments. *A.* Effect of  $50$  nM ChTX on HGF/SF treated MDCK II cells ( $n=5$ ). The insert shows the representative traces of whole-cell recording from an MDCK II cell under condition *A.* *B.* Effect of  $100$  nM IbTX on HGF/SF treated MDCK II cells ( $n=3$ ). *C.* Effect of  $100$  nM Stk on HGF/SF treated MDCK II cells ( $n=6$ ). *D.* Effect of  $1 \mu M$  CLT on HGF/SF treated MDCK II cells ( $n=4$ ). \*There was significant difference between the slope conductance in control solution. and in a solution with channel inhibitor. (Mean  $\pm$  SE;  $P < 0.05$ ).

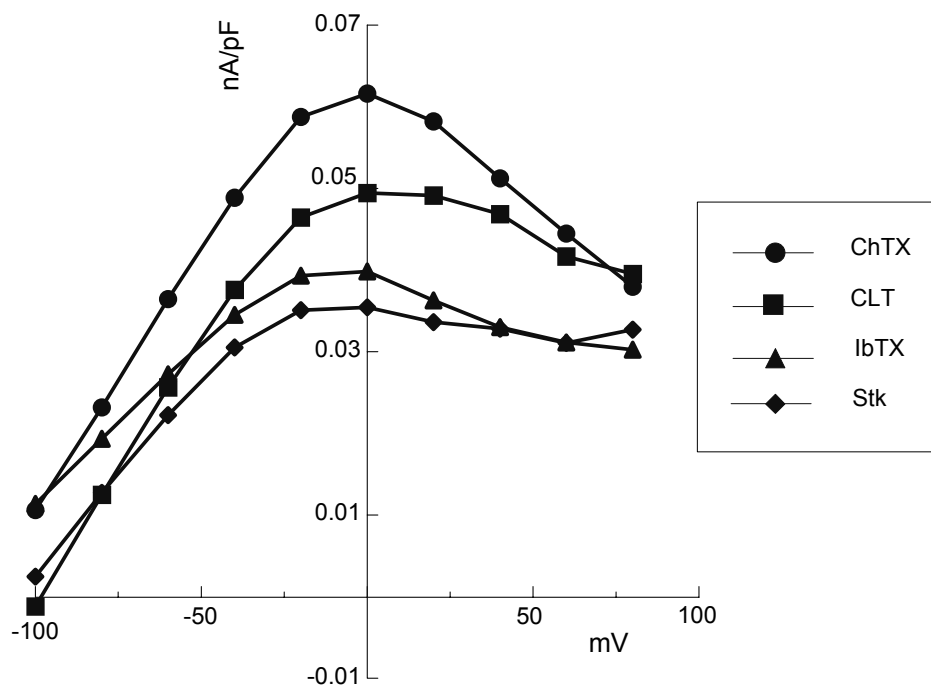


Figure 10 Difference currents recorded from control solution and a solution with a channel inhibitor. I-V curves were obtained by deducting the inhibited current from the control current in Fig. 9A-9D, respectively.

### Effects of HGF/SF and K<sup>+</sup> Channel Blockers on MDCK II Cell Migration

HGF/SF stimulated MDCK II cell migration in a dose-dependent manner, Fig. 11A. Maximum stimulation occurred at 50 scatter units/ml of HGF/SF (8 hr) with no further increases at higher concentrations. ChTX at 50 nM significantly inhibited stimulation of MDCK II cell migration by HGF/SF at 100 scatter units/ml (Mean  $\pm$  SE;  $P < 0.05$ ;  $n = 3$ ), Fig. 11B. Fig. 11C shows that CLT at 1  $\mu$ M and Stk at 100 nM both significantly inhibited MDCK II cell migration stimulated by HGF/SF at 100 scatter units/ml for 8 hr. The inhibition by ChTX increased with dose, Fig. 12. Here 25 scatter units/ml of HGF/SF stimulated MDCK II cell migration, and this was inhibited with increasing concentrations of ChTX. However, 100 nM ChTX was necessary for significant inhibition of this submaximal stimulation by HGF/SF. IbTX at 100 nM also significantly inhibited migration in MDCK II cells treated with 25 scatter units/ml HGF/SF (Mean  $\pm$  SE;  $P < 0.05$ ,  $n = 3$ ), Fig. 12.

HGF/SF has been shown to stimulate migration and metastasis of various normal and malignant cells. Transwell migration assays were used to quantify the effects of HGF/SF and other agents on MDCK II cell migration. Since a transwell migration assay took 8 hr, hemacytometer experiments were used to determine whether HGF/SF had any effect on cell proliferation during 8 hr, which may contribute to the cell count. Table 3 shows that there is no difference between untreated and HGF/SF-treated cell counts after 8 hr incubation, and HGF/SF doesn't affect cell growth during 8 hr migration assays.

Table 3 Hemacytometer experiments on MDCK II cells treated with or without HGF/SF (100 scatter units/ml) for 8 hr.		
	Cell count at time 0	Cell count after 8 hr
Control cells	$2 \times 10^5$ cells/petri dish	$(1.8 \pm 0.6) \times 10^5$ cells/petri dish
HGF/SF treated cells	$2 \times 10^5$ cells/petri dish	$(1.9 \pm 0.3) \times 10^5$ cells/petri dish

\*There was no significant difference between untreated and HGF/SF treated MDCK II cell counts (n=5 petri dishes) after 8 hr treatment (Mean  $\pm$  SE; P > 0.05).

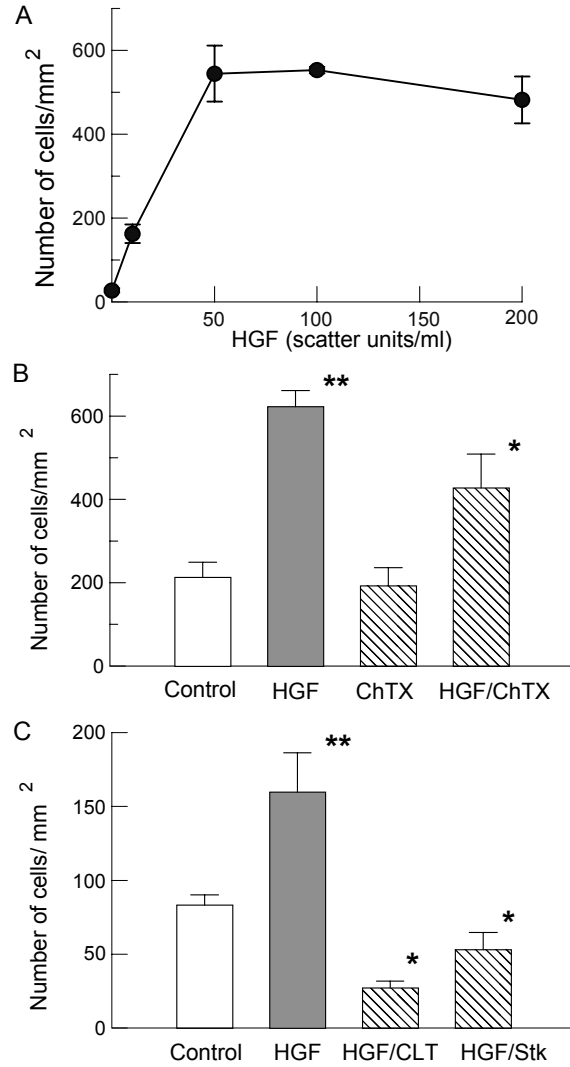


Figure 11 Effects of HGF/SF and K<sup>+</sup> channel blockers on MDCK II cell migration. *A.* HGF/SF stimulates MDCK II cell migration in transwell assays. MDCK II cells were treated with different concentrations of HGF/SF for 8 hr. *B.* ChTX inhibits HGF/SF-stimulated MDCK II cell migration. MDCK II cells were treated with 50 nM ChTX and/or 100 scatter units/ml HGF/SF for 8 hr. *C.* CLT and Stk inhibit HGF/SF-stimulated MDCK II cell migration. MDCK II cells were treated with 100 scatter units/ml HGF/SF alone, or with both HGF/SF and a channel inhibitor for 8 hr. \*Significantly different from HGF/SF treatment; \*\*significantly different from control (Mean  $\pm$  SE; n = 3 inserts).

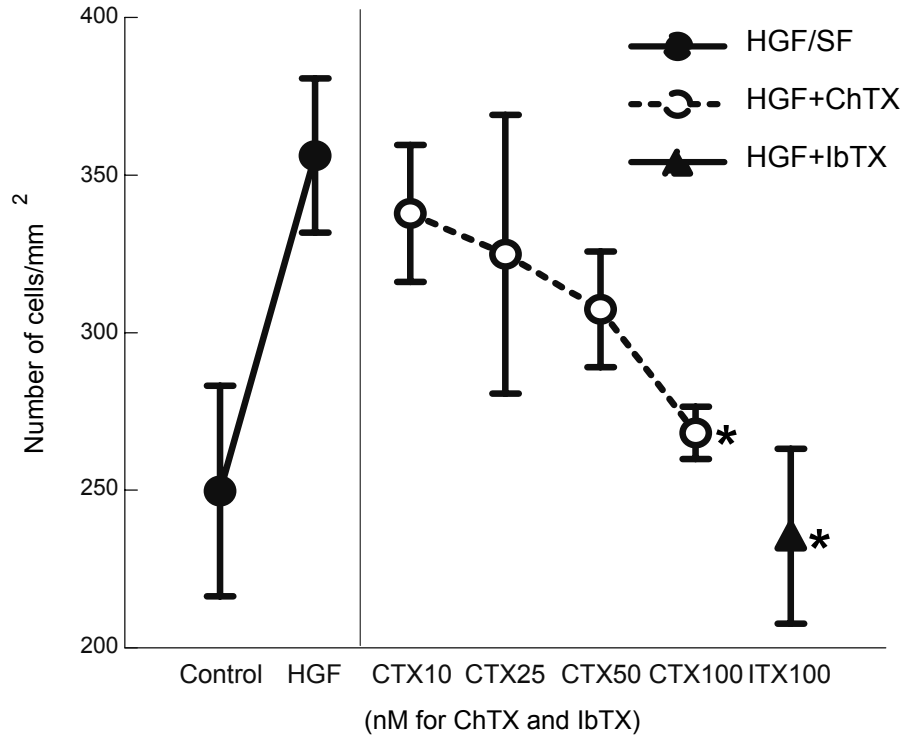


Figure 12 ChTX and IbTX inhibit MDCK II cell migration treated with 25 scatter units/ml HGF/SF. MDCK II cells were treated with HGF/SF and ChTX or IbTX for 8 hr in transwell migration assay. \*Statistically significant differences existed between cells treated with 25 scatter units/ml HGF alone and cells treated with HGF/SF plus 100 nm ChTX, or HGF/SF plus 100 nm IbTX. (Mean  $\pm$  SE; n = 3 inserts).

### ChTX Inhibits Hypotonic Stress-Induced Membrane K<sup>+</sup> Current in MDCK II Cells

MDCK II whole-cell currents were recorded first under normosmotic conditions (Fig. 13, *left*). Hypotonic stress, which was created by reducing external Na gluconate by 40 mM, activated inwardly rectifying K<sup>+</sup> currents that inactivated rapidly at positive voltages  $\geq 60$  mV (Fig. 13, *middle*). This current activation reversed on restoring the control solution (Fig. 13, *right*). Hypotonic stress increased the slope conductance of the membrane K<sup>+</sup> current, and the reversal potential shifted toward the E<sub>K</sub> of  $-85$  mV. ChTX at 50 nM completely inhibited these changes, Fig. 14A. However, IbTX (100 nM) failed to inhibit the hypotonic stress-activation of membrane K<sup>+</sup> current, Fig. 14B.



### Whole-Cell $K^+$ Currents in a MDCKII Cell

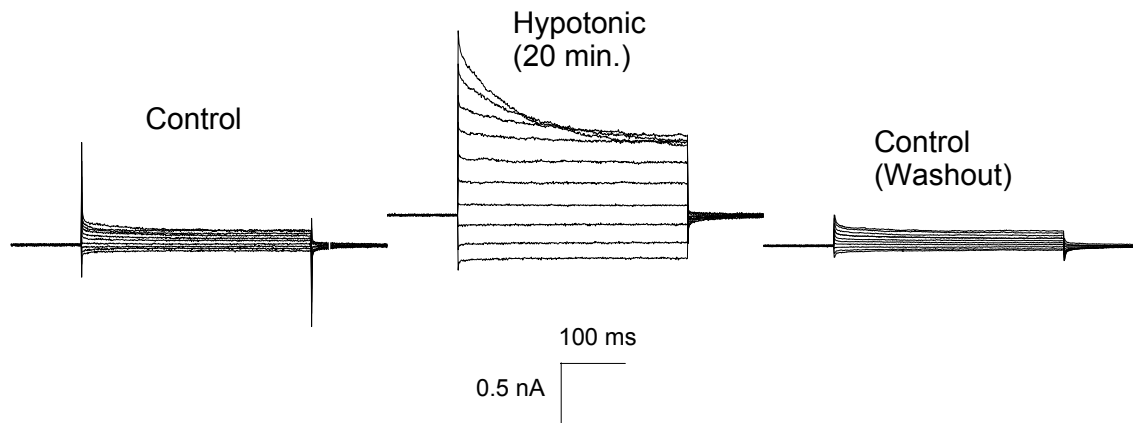


Figure 13 Hypotonic stress stimulates whole-cell currents in an MDCK II cell. Whole-cell traces were recorded during voltage pulses ranging from  $-100$  to  $80$  mV in consecutive,  $20$  mV increments. Control, hypotonic, and washout currents were recorded in the same cell. Hypotonic stress was created by reducing external Na gluconate concentration by  $40$  mM.

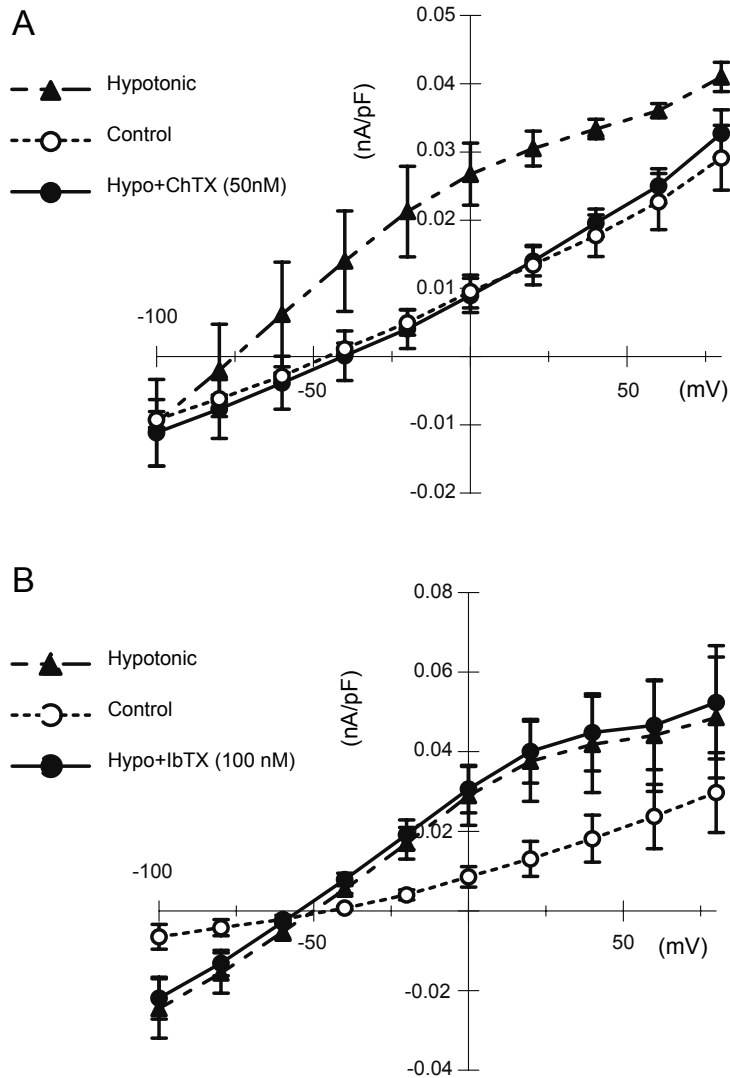
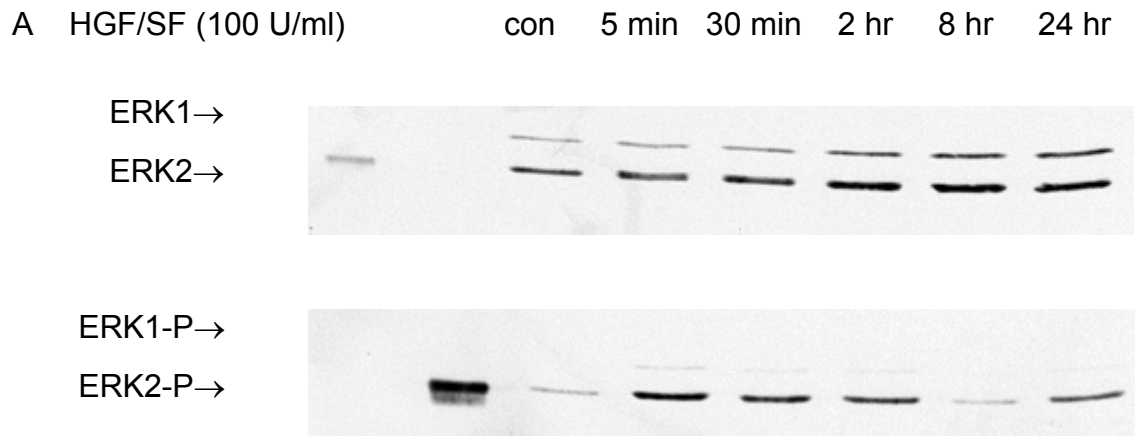


Figure 14 Effects of ChTX and IbTX on hypotonic stress-induced membrane  $K^+$  current in MDCK II cells. Current-voltage (I-V) plots for whole-cell currents recorded in MDCK II cells during voltage pulses ranging from  $-100$  to  $80$  mV in consecutive,  $20$  mV increments. A. Effect of ChTX ( $50$  nM) on hypotonic stress-induced membrane  $K^+$  current. B. Effect of IbTX ( $100$  nM) on hypotonic stress-induced membrane  $K^+$  current. (Mean  $\pm$  SE;  $n=3$  cells).

### HGF/SF Stimulates ERK Phosphorylation in MDCK II Cells

It has been shown that HGF/SF stimulates the MAPK signaling pathway, which then induces migration and morphological changes in various cell types. To demonstrate the effect of HGF/SF on MAPK signaling pathway in MDCK II cells, the cells were treated with 100 scatter units/ml HGF/SF from 5 min up to 24 hr, and western blotting analysis was performed on cell extracts. Two 12% SDS gels were loaded identically, and probed with anti-total ERK and anti-ERK-P, respectively, Fig. 15A. Lane 1 was loaded with control non-phospho-ERK protein, and lane 2 was loaded with control phospho-ERK protein. Two bands at 42 and 44 kDa were revealed, standing for ERK2 and ERK1, respectively. HGF/SF treatment transiently increased phosphorylation of ERK2, Fig.15A. Densitometry was performed to quantify the ratio of ERK-P to total ERK. HGF/SF treatment significantly ( $P < 0.05$ ) increased the ERK-P/ERK ratio at 5 min and 30 min, Fig. 15B. The stimulation diminished with further time, reaching the baseline level after 8 hr.



B

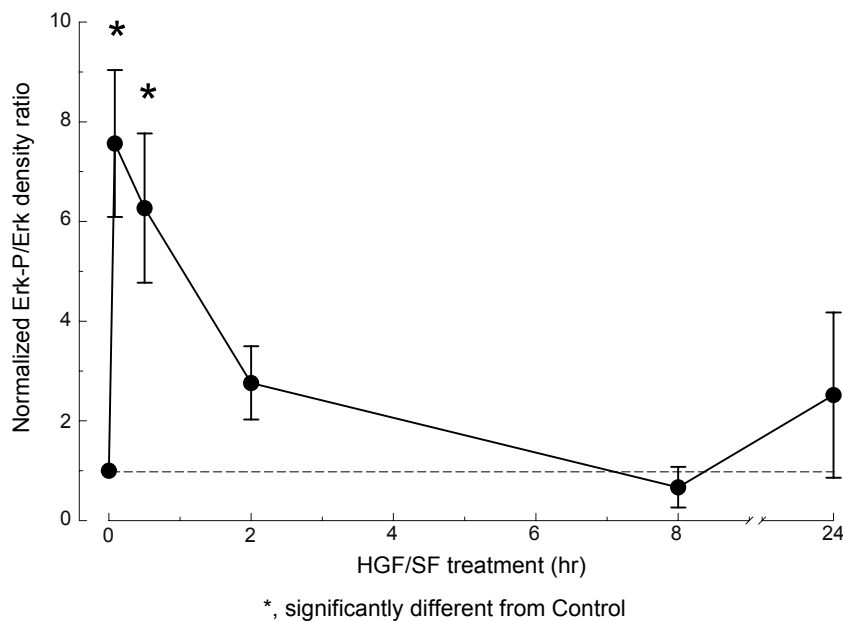


Figure 15 HGF/SF induces an immediate, but transient activation of phospho-ERK (ERK-P) in MDCK II cells. MDCK II cells were cultured in the presence of 100 scatter units/ml HGF/SF for various length of time as indicated. *A*. Representative blots of whole cell lysates separated by 12% SDS-PAGE and immunoblotted with anti-total ERK (upper panel) and anti-ERK-P (lower panel). *B*. Normalized ERK-P/total ERK ratios from three independent experiments as shown in *A*. (Mean  $\pm$  SE;  $P < 0.05$ ,  $n=3$ ). \*The ERK-P/ERK ratio of cells treated with HGF/SF for 5 min or 30 min was significant different from the control ratio.

MEK Inhibitor PD98059 Abolishes ERK Phosphorylation  
in Untreated and HGF/SF-Treated MDCK II cells

PD98059 is a potent inhibitor of MAPK signaling pathway because it inhibits MEK phosphorylation and the downstream cascade of MEK. Pretreating the MDCK II cells for 30 min. with the MEK inhibitor, PD98059 (50  $\mu$ M), completely blocked the HGF/SF stimulated increase in ERK phosphorylation, Fig. 16A. Fig. 16B shows that PD98059 abolished basal level ERK phosphorylation in untreated MDCK II cells, as well as HGF/SF-stimulated ERK phosphorylation in cells treated with HGF/SF for 5 min (Mean  $\pm$  SE;  $P < 0.05$ ,  $n=3$ ).

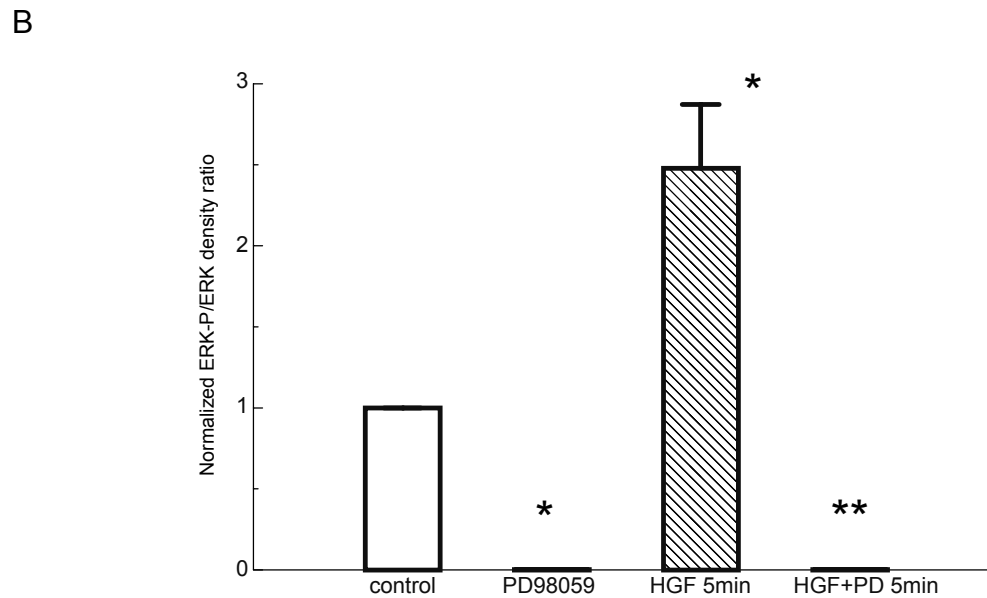
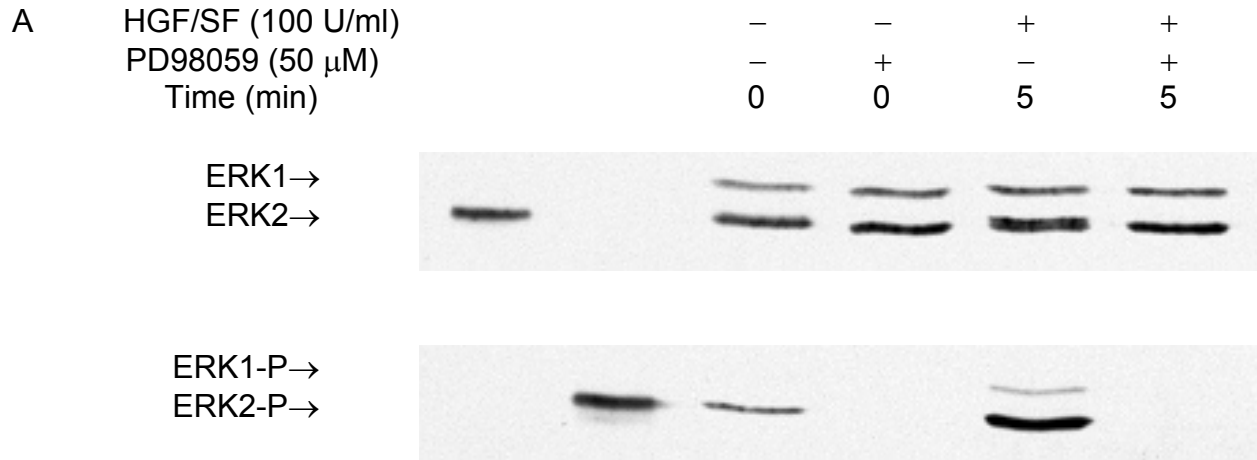


Figure 16 PD98059 abolishes ERK phosphorylation in untreated and HGF/SF-treated MDCK II cells. MDCK II cells were stimulated with HGF/SF (100-scatter units/ml) for 5 min with or without pre-treatment of PD98059 (50  $\mu$ M) for 30 min. Whole cell lysates were separated by 12% SDS-PAGE and immunoblotted with anti-total ERK (upper panel) and anti-ERK-P (lower panel). A. Representative blots. B. Normalized ERK-P/ total ERK ratios from three independent experiments as shown in A. \*Significantly different from control. \*\*Significant different from HGF/SF treatment (Mean  $\pm$  SE;  $P < 0.05$ ,  $n=3$ ).

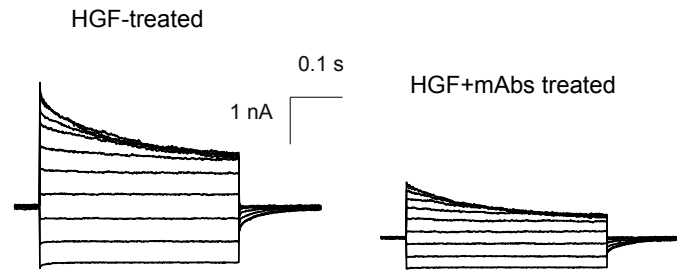
## Neutralizing Monoclonal Antibodies to HGF/SF Inhibit HGF/SF-Induced

### K<sup>+</sup> Currents in MDCK II Cells

We have shown previously that HGF/SF added for 24 hr stimulates Ca<sup>2+</sup>-activated K<sup>+</sup> currents in MDCK II cells. To determine whether the activation of membrane K<sup>+</sup> currents in HGF/SF-treated MDCK II cells is directly induced by HGF/SF, the neutralizing monoclonal antibodies to HGF/SF (mAbs) were added to the culture medium immediately before the addition of HGF/SF. Whole-cell patch clamp measurements were taken of MDCK II cells untreated, treated with HGF/SF (50 scatter units/ml) alone, or HGF/SF plus the mAbs (at a molar ratio of 1:20) for 24 hr. Fig. 17A shows the representative traces of whole-cell currents recorded in response to voltage steps applied from -100 to 80 mV on an HGF/SF-treated cell and a cell treated with HGF/SF and mAbs. Current-voltage (I-V) relationships, which were obtained from repeated measurements as shown in Fig. 17A, are shown in Fig. 17B. As reported previously (Fig. 2, 6, and 8), HGF/SF treatment for 24 hr significantly increased cell membrane K<sup>+</sup> conductance. Compared to MDCK II cells treated with HGF/SF alone, the cells treated with HGF/SF and mAbs had a significantly smaller slope conductance of membrane K<sup>+</sup> currents (Mean ± SE; P < 0.05, n=5 for both HGF/SF and HGF/SF + mAbs treated cells).

Untreated MDCK II cells were treated with or without mAbs to HGF/SF for 24 hr. The results are shown in Fig. 17B. The difference in either the slope conductance or V<sub>r</sub> between untreated and untreated + mAbs MDCK II cells were not significant (P > 0.05, n=9 of untreated cells, n=3 of untreated + mAbs cells). This suggests that neutralizing mAbs to HGF/SF did not have effects on membrane K<sup>+</sup> currents in untreated MDCK I cells.

### A. Whole-cell currents



### B. I-V plots

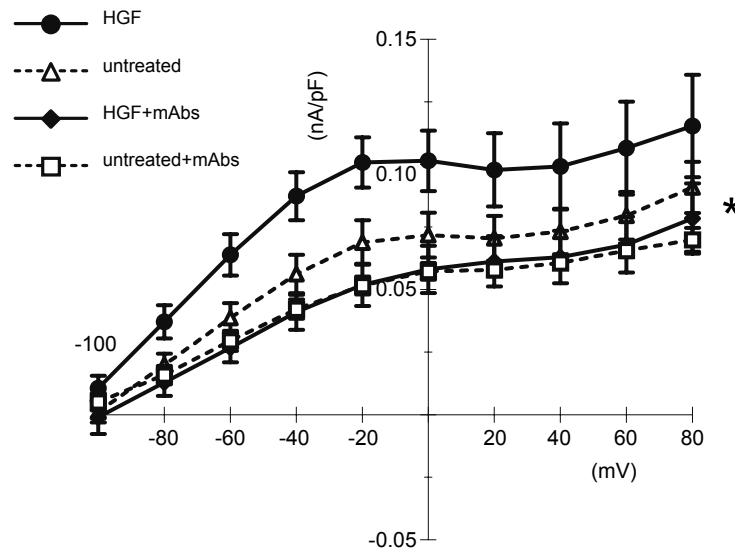


Figure 17 Neutralizing mAbs to HGF/SF block HGF/SF stimulation of membrane  $K^+$  current in MDCK II cells. MDCK II cells were treated with HGF/SF (50 scatter units/ml) alone, or HGF/SF+mAbs (at a molar ratio of 1:20) for 24 hr. *A.* Representative traces of whole-cell currents recorded in response to voltage steps applied from  $-100$  to  $80$  mV in consecutive,  $20$ -mV increments on an HGF/SF-treated cell (*left*) and a cell treated with HGF/SF and mAbs (*right*). *B.* Current-voltage (I-V) relationships obtained from repeated measurements of four treatments, untreated, untreated + mAbs, HGF/SF-treated, HGF/SF + mAbs treated. \*Significant difference between the slope conductance in HGF/SF-treated and HGF/SF + mAbs treated cells (Mean  $\pm$  SE;  $P < 0.05$ ,  $n = 5$ ). There was no significant difference in the slope conductance between untreated and untreated + mAbs MDCK II cells.



### MEK Inhibitor PD98059 Inhibits HGF/SF-Induced Membrane K<sup>+</sup> Currents in MDCK II Cells

MEK inhibitor PD98059 was used in the electrophysiological study to determine whether the MAPK signaling pathway was involved in HGF/SF stimulation of membrane K<sup>+</sup> currents in MDCK II cells. The representative traces of whole-cell currents recorded in response to voltage steps applied from -100 to 80 mV on an HGF/SF-treated (50 scatter units/ml) cell, a cell treated with HGF/SF + DMSO (vehicle control, 0.26 % v/v), and a cell treated with HGF/SF + PD98059 (50 μM) were shown in Fig. 18A. Fig. 18B shows the current-voltage (I-V) relationships, which were obtained from repeated measurements as shown in Fig. 18A. PD98059 (50 μM) significantly inhibited HGF/SF-stimulated K<sup>+</sup> current slope conductance in MDCK II cells (Mean ± SE; P<0.05, n=5 cells). DMSO (0.26% v/v) had no effect on HGF/SF-stimulated K<sup>+</sup> current slope conductance, (Mean ± SE; P>0.05, n=5 cells). Because PD98059 inhibited basal level ERK phosphorylation as well as HGF/SF-stimulated ERK phosphorylation in MDCK II cells, the effect of PD98059 on membrane K<sup>+</sup> currents was also studied in untreated MDCK II cells with added or without PD98059 (50 μM for 24 hr). In untreated MDCK II cells slope conductance was  $0.94 \pm 0.11$  nS/pF, and the reversal potential (V<sub>r</sub>) was  $-99.8 \pm 4.4$  mV (Mean ± SE, n=9 cells). Added PD98059 reduced slope conductance to  $0.26 \pm 0.01$  nS/pF and decreased V<sub>r</sub> to  $-74.3 \pm 9.8$  mV (Mean ± SE; P < 0.05, n=3 cells).

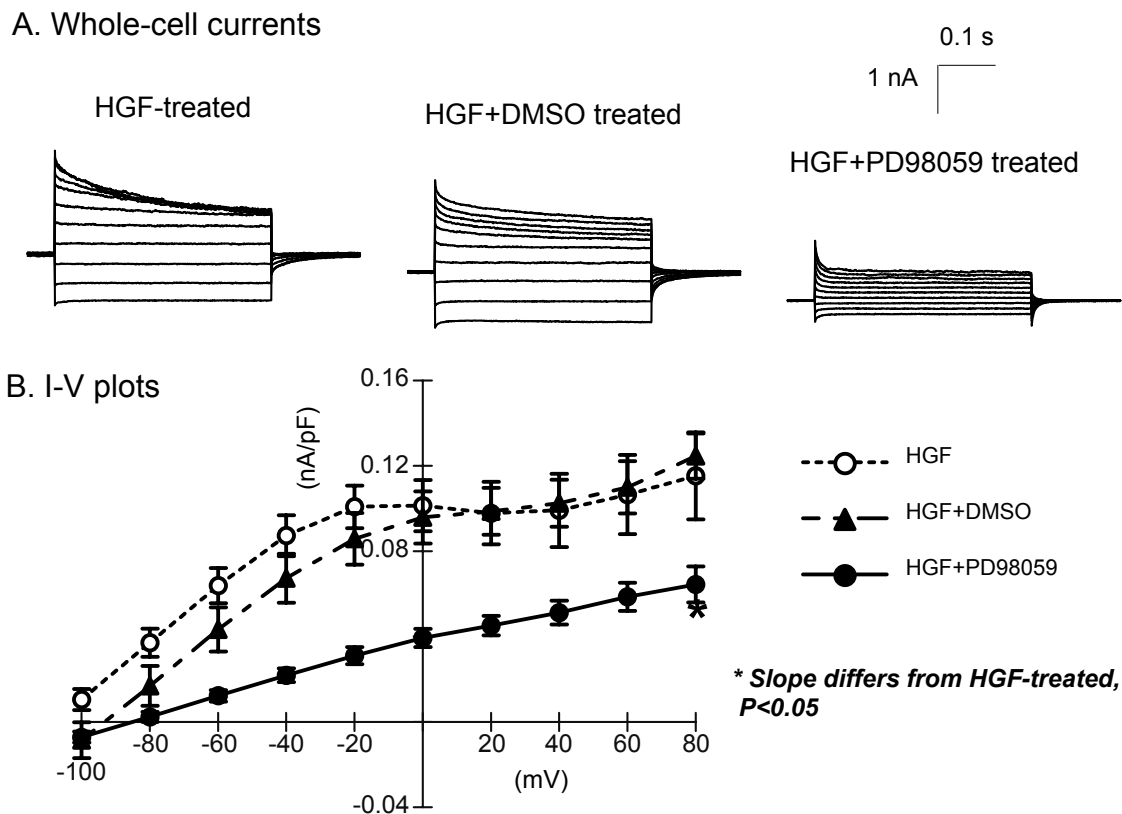


Figure 18 Effects of PD98059 and DMSO on membrane  $K^+$  current in HGF/SF-treated MDCK II cells. MDCK II cells were pre-treated with PD98059 (50  $\mu$ M) or DMSO (0.26% v/v) for 30 min before the addition of HGF/SF (50 scatter units/ml), and incubated for 24 hr. *A.*

Representative traces of whole-cell currents recorded in response to voltage steps applied from -100 to 80 mV in consecutive, 20-mV increments on an HGF/SF-treated cell (*left*), a cell treated with HGF/SF+DMSO (*middle*), and a cell treated with HGF/SF+PD98059 (*right*). *B.* Current-voltage (I-V) plot of whole-cell currents obtained from repeated measurements as shown in *A.*

\*Significant difference between the slope conductance in HGF/SF-treated cells and cells treated with HGF/SF+PD98059. (Mean  $\pm$  SE;  $P < 0.05$ ,  $n=5$  cells). There was no significant difference between cells treated with HGF/SF, and those treated with HGF/SF+DMSO. (Mean  $\pm$  SE;  $P > 0.05$ ,  $n=5$  cells).

### MEK Inhibitor PD98059 Inhibits HGF/SF-Induced MDCK II Cell Migration

To determine whether MEK inhibitor could affect HGF/SF stimulation of MDCK II cell migration, PD98059 (50  $\mu$ M) or DMSO (vehicle control, 0.26 % v/v) were added to the migration assay buffer 30 min before the addition of HGF/SF (100 scatter units/ml) in the transwell migration assay. Neutralizing mAbs to HGF/SF also were added to migration assay buffer immediately before the addition of HGF/SF (molar ratio of HGF/SF to mAbs = 1:20). Cells were then allowed to migrate for 8 hr. Fig. 19 shows the summary of the transwell migration assays. Neutralizing mAbs to HGF/SF had no effect on untreated cell migration (Mean  $\pm$  SE;  $P > 0.05$ ,  $n=3$ ), but they significantly inhibited HGF/SF-treated cell migration (Mean  $\pm$  SE;  $P < 0.05$ ,  $n=3$ ). PD98059 (50  $\mu$ M) significantly inhibited untreated cell migration, as well as markedly inhibiting HGF/SF-stimulated cell migration (Mean  $\pm$  SE;  $P < 0.05$ ,  $n=3$  inserts). DMSO alone (0.26 % v/v) also inhibited the HGF/SF-stimulated cell migration (Mean  $\pm$  SE;  $P < 0.05$ ,  $n=3$  inserts). However, this inhibition of HGF/SF-stimulated migration was not as great as that of PD98059.

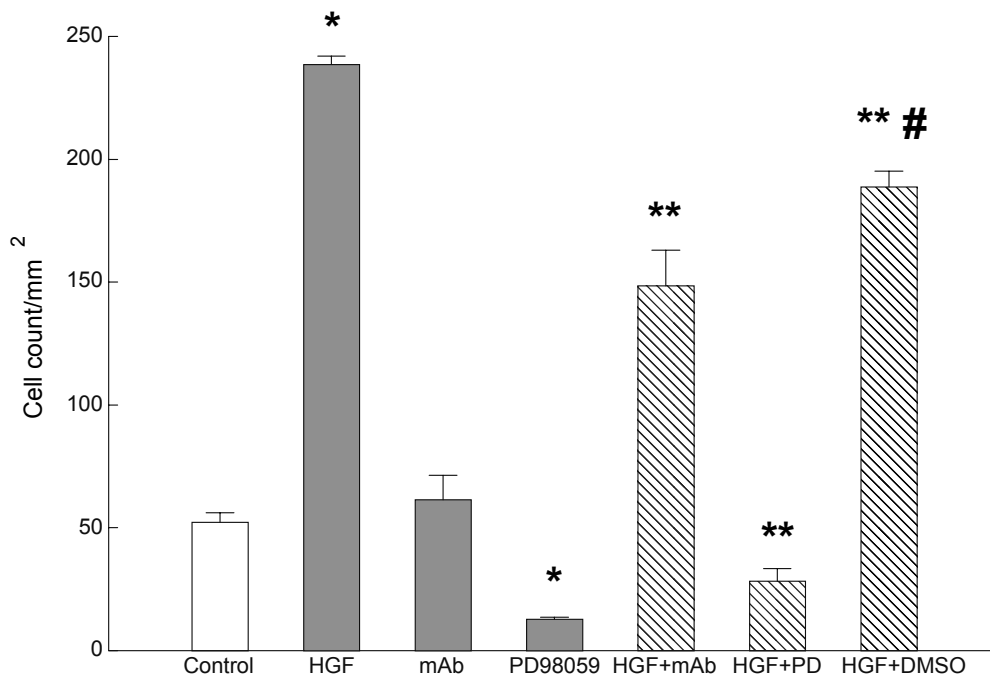


Figure 19 Effects of PD98059, DMSO, and neutralizing mAbs to HGF/SF on HGF/SF-stimulated MDCK II cell migration. PD98059 (50  $\mu$ M) and DMSO (0.26% v/v) were added to the migration assay buffer 30 min before the addition of HGF/SF (100 scatter units/ml). Neutralizing mAbs to HGF/SF (molar ratio of HGF/SF to mAbs=1:20) were added to the buffer right before the addition of HGF/SF. Cells were then allowed to migrate for 8 hr. (Mean  $\pm$  SE; n=3 inserts). \*Significantly different from control cell migration ( $P < 0.05$ , n = 3 inserts). \*\*Significantly different from HGF/SF-treated cell migration ( $P < 0.05$ , n = 3 inserts). #Significantly different from HGF/SF+PD98059 treated cell migration ( $P < 0.05$ , n = 3 inserts).

## CHAPTER 4

### DISCUSSION

This research has demonstrated that HGF/SF stimulates cell migration as well as membrane  $K^+$  currents in MDCK II cells. HGF/SF stimulation of the membrane  $K^+$  currents is regulated by intracellular free  $[Ca^{2+}]_i$ . The various agents that block  $Ca^{2+}$ -activated  $K^+$  channels, the peptides ChTX, IbTX, and Stk along with CLT, inhibit both HGF/SF-stimulated cell migration and membrane  $K^+$  current. Furthermore, we have observed that HGF/SF stimulates an immediate, but transient activation of ERK phosphorylation in MDCK II cells, and MEK inhibitor PD98059 abolishes ERK phosphorylation in untreated and HGF/SF treated cells. We also have shown that neutralizing mAbs to HGF/SF and PD98059 both inhibit HGF/SF stimulation of  $Ca^{2+}$ -activated  $K^+$  channel and cell migration. Therefore, we conclude that 1) the activation of  $Ca^{2+}$ -activated  $K^+$  channel activation is necessary for HGF/SF-induced cell migration; 2) MAPK ERK signaling pathway plays a role in HGF/SF-stimulated  $Ca^{2+}$ -activated  $K^+$  channel activation and cell migration in MDCK II cells.

The  $Ca^{2+}$ -activated  $K^+$  currents activated by HGF/SF in MDCK II cells inactivate rapidly, and, therefore, rectify inwardly, this resembles very much the intermediate conductance,  $Ca^{2+}$ -activated  $K^+$  channels (IK). Schwab and colleagues have demonstrated that the IK channel plays a role in the migration of alkaline-transformed MDCK-F cells (Schwab et al. 1993, 1994). Topical application of ChTX to the cell body inhibits MDCK-F cell migration (Schwab et al. 1997). It is proposed that migrating cells are polarized with  $K^+$  efflux dependent cell shrinkage occurring in the cell body to facilitate retreat, and osmotic swelling occurs in the lamellipodium providing membrane tension necessary to advance migration (Schwab 2001). Thus, our present findings extend previous work by elucidating the mechanisms through which HGF/SF affects the malignant transformation and the metastatic phenotype (Jeffers et al. 1996, 1996a, 1996b). We have shown that  $Ca^{2+}$ -activated  $K^+$  channel blockers, ChTX, IbTX, Stk, and

CLT, inhibit membrane  $K^+$  current and HGF/SF-stimulated cell migration. Thus, we proposed that the inhibition of these channel inhibitors on HGF/SF-induced cell migration may be through inhibition of local cell shrinkage, causing insufficient cell body contraction and retarded migration in MDCK II cells treated with HGF/SF.

The doses of HGF/SF that stimulate MDCK II cell migration agree well with the doses that stimulate outward-current channel activity using on-cell patch recording. Larger HGF/SF doses (200 scatter units/ml) are necessary to activate whole-cell  $K^+$  current when the cells are dialyzed with  $1\mu\text{M}$  free intracellular  $\text{Ca}^{2+}$ . However, HGF/SF at the concentration of 50 scatter units/ml effectively stimulate  $K^+$  current at positive membrane potential when the cells are dialyzed with  $7\mu\text{M}$  free intracellular  $\text{Ca}^{2+}$ . Moreover, the  $\text{Ca}^{2+}$ -activated  $K^+$  channel blockers that inhibit HGF/SF-stimulated MDCK II cell migration also inhibit these whole-cell  $K^+$  currents. Taken together these findings suggest interplay between intracellular  $\text{Ca}^{2+}$  activation of membrane  $K^+$  currents and the migration of MDCK II cells.

The intracellular  $[\text{Ca}^{2+}]_i$  needed for HGF/SF activation of membrane  $K^+$  current in MDCK II cells is high by physiological standards. Notwithstanding, the intermediate conductance, IK, and the high conductance, BK,  $\text{Ca}^{2+}$ -activated  $K^+$  channels are thought to be quite insensitive to  $[\text{Ca}^{2+}]_i$  with submicromolar amounts needed for IK and micromolar concentrations needed for BK (Hille 2001). We have no basis to favor either IK or BK in accounting for HGF/SF stimulation of either migration or membrane  $K^+$  current. Because the inhibitors considered to be quite specific for the respective channels inhibited both variables, we explain the relatively high  $[\text{Ca}^{2+}]_i$  needed for HGF/SF's stimulation of  $K^+$  current by the physiological kinetics of  $\text{Ca}^{2+}$ -activated  $K^+$  current, which includes the diverse parameters that regulate intracellular  $[\text{Ca}^{2+}]_i$  and the degree of colocalization of  $K_{(\text{Ca})}$  channels and the intracellular sites of elevated  $[\text{Ca}^{2+}]_i$ .

HGF/SF causes oscillations of calcium activity in hepatocytes (Baffy et al. 1992; Kaneko et al. 1992; Osada et al. 1992; Kawanishi et al. 1995) and oscillations of  $\text{Ca}^{2+}$ -activated  $K^+$

current in human gastric cancer cells (Liu et al. 1998). In this regard, migration of MDCK-F cells depends on a ChTX-sensitive oscillation of  $\text{Ca}^{2+}$ -sensitive 53-pS  $\text{K}^+$  channels (Schwab et al. 1994). Oscillations of intracellular  $[\text{Ca}^{2+}]_i$  trigger opening of these channels, which result in oscillations of membrane potential (Schwab and Oberliethner 1996). Moreover, migrating MDCK-F cells demonstrate a horizontal gradient for intracellular  $[\text{Ca}^{2+}]_i$  with oscillations of  $[\text{Ca}^{2+}]_i$  being greater in the cell body than in the lamellipodium (Schwab et al. 1997).

Gradients and oscillations of intracellular  $[\text{Ca}^{2+}]_i$  have been associated with local  $\text{Ca}^{2+}$  signaling called “puffs” in nonexcitable cells and “sparks” in excitable cells. These constitute cytoplasmic  $\text{Ca}^{2+}$  microdomains that control local, apical secretion in acinar cells (Kidd et al. 1999), neurotransmitter release (Neher 1998), or neuronal growth (Spitzer et al. 2000), or they may coalesce to form an intracellular  $\text{Ca}^{2+}$  wave that leads to myocyte contraction (Bootman et al. 2001). In smooth muscle  $\text{Ca}^{2+}$  sparks activate BK channels in the membrane microdomain that regulate membrane potential and voltage-gated channels (ZhuGe et al. 2000; Perez et al. 2001; Pozo et al. 2002). ZhuGe et al. have shown that in smooth muscle cells, BK channels are activated when exposed to a mean  $[\text{Ca}^{2+}]_i$  on the order of 10  $\mu\text{M}$  during a  $\text{Ca}^{2+}$  spark, also the BK channels are found to be at higher density at the spark site rather than uniformly distributed over the membrane (ZhuGe et al. 2002). In a similar manner, migrating MDCK-F cells are polarized cells that rely on  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  (IK) channels, which are more active in the rear of the cells (Schwab et al. 1995) and are associated with the larger of the  $\text{Ca}^{2+}$  transients (Schwab et al. 1997). Moreover, topical application of charybdotoxin to the cell body inhibits MDCK-F cell migration (Schwab et al. 1997) and results in cell swelling (Schneider et al. 2000); whereas, topical application of ionomycin to the cell body shrinks the cells (Schneider et al. 2000).

$\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels are critical to regulatory volume decrease (RVD) in many cell types (Pasantés-Morales and Morales-Mulia 2000). Both the  $\text{K}^+$  efflux-dependent cell shrinkage in rear of the cells and the osmotic swelling in the lamellipodium are required for cells to migrate (Schwab 2001). It has been proposed that ion channels and transporters modulate

cytoskeletal migration machinery by regulating  $[Ca^{2+}]_i$  and local cell volume of migrating cells (Schneider et al. 2000). Human melanoma cells that are devoid of the actin cross-linking protein ABP280 are unable to volume-regulate or activate  $K^+$  channels when exposed to a hypotonic stimulus. By transfecting with ABP280 these cells could migrate as well as regulate  $K^+$  channels (Cantiello et al. 1993). However, migration of rescued melanoma cells is inhibited when  $Ca^{2+}$ -activated  $K^+$  channels are blocked (Cantiello et al. 1993).

Whole-cell recordings under hypotonic stress (with  $1 \mu M [Ca^{2+}]_i$ ) and 50 scatter units/ml HGF/SF (with  $7 \mu M [Ca^{2+}]_i$ ) are both inwardly rectifying. Furthermore, ChTX inhibits hypotonic stress-activated  $K^+$  currents, as well as HGF/SF stimulated cell migration in MDCK II cells. This suggests that  $Ca^{2+}$ -activated  $K^+$  channels may play a role in HGF/SF-stimulated MDCK II cell migration via local cell volume regulation. However, IbTX inhibits HGF/SF stimulated MDCK II cell migration and membrane  $K^+$  current, but fails to inhibit hypotonic stress induced  $K^+$  current. This discrepancy may be explained that the subtypes of  $Ca^{2+}$ -activated  $K^+$  channels activated by HGF/SF are different from those activated by hypotonic stress, and the binding specificity of ChTX and IbTX to these subtype channels also vary. Some subtypes of  $Ca^{2+}$ -activated  $K^+$  channels may be more prevalent in HGF/SF stimulated situation than under hypotonic stress, whereas hypotonic stress may induce more complex and larger channel activation than HGF/SF. Our recording also shows that HGF/SF stimulates an inward current in MDCK II cells, which is likely to be  $Na^+$  current, further study is needed to characterize these channels, and it may be very helpful to understand the effect of HGF/SF on cell functions.

HGF/SF stimulates a transient activation of ERK phosphorylation, but the activation of membrane  $Ca^{2+}$ -activated  $K^+$  currents (over 24 hr) and cell migration (8 hr) are sustained for much longer time. This can be explained as follows. Firstly, it takes a long time for the cells to migrate through the pores and reach the bottom side of the inserts to be visualized under microscope in transwell migration assay, which accounts in part for the time length of 8 hours in



migration assay. Secondly, these data also suggest that the effects of HGF/SF on  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  current require the transcription and synthesis of new proteins, rather than through immediate, direct effects of the ligand. Western blotting using antibody to Slo  $\alpha$  subunit of large conductance,  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels (BK) shows that HGF/SF does not affect BK channel expression in MDCK II cells (data not shown). It has also been shown that TGF $\beta$ 1 stimulates  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels by making new proteins that regulate channel activation rather than channel proteins (Lhuillier and Dryer 2000). Hypotonic stress induces an immediately activation (within 5 min) of inwardly rectifying  $\text{K}^+$  current in MDCK II cells, which is significantly inhibited by ChTX (50 nM) in the bath solution. This indicates that the  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels are already in the cell and respond to the hypotonic stress quickly. Therefore, we propose that HGF/SF stimulates the synthesis of new proteins that regulate  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels functions, rather than  $\text{K}^+$  channel proteins. The new proteins synthesized probably interact with channels, modulate channel activity (via intracellular  $\text{Ca}^{2+}$  oscillation, channel sensitivity to  $\text{Ca}^{2+}$ , etc.), or recruit channels already in the cytoplasm of the plasma membrane.

MAPK signaling pathway has been shown to play an important role in HGF/SF stimulation of scattering, migration and invasiveness in MDCK cells and other cell types. A constitutively active ERK1 mutant and a stably dedifferentiated derivative (MDCK-C7F, which expresses increased ERK2 activity) of MDCK-C7 cells assume elongated fibroblastoid shape and high invasiveness (Montesano et al. 1999). We have shown that HGF/SF stimulates ERK2 phosphorylation, thus increases the ratio of ERK-P/total ERK in MDCK II cells. Nevertheless, Schramek et al. have demonstrated that ERK1 protein expression and serum-induced ERK1 activation are greatly reduced in alkali-dedifferentiated MDCK-C7F cells; however, the ERK2 phosphorylation was substantially increased in these cells. This indicates an association between the changes of ERK1 and ERK2. The inhibition of ERK1 expression and increased stimulation of ERK2 might be involved in alkaline stress-induced dedifferentiation and/or growth – inhibition of MDCK-C7F cells (Schramek et al. 1997). The specific MEK inhibitor PD98059

inhibits the phosphorylation of MEK, thus inhibits the activation of ERK and other downstream reactions in the cascade. We also demonstrated that PD98059 inhibits  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels and cell migration induced by HGF/SF, which suggests that MAPK signaling pathway plays a role in HGF/SF stimulation of channel activation and cell migration.

Besides the inhibition on  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel activation by HGF/SF, PD98059 may also inhibit cell migration via cell-cell and cell-matrix interaction. It has been shown that that activation of ERK phosphorylation is required for HGF/SF-stimulated scattering by causing increased adherens junction proteins, and PD98059 inhibits both (Lu et al. 1998; Potempa and Ridley 1998). ERK phosphorylation also has direct effects on cytoskeletal organization and cell motility; it activates myosin light chain kinase (MLCK), which in turn regulates MLC and myosin II formation in FG carcinoma cells (Klemke et al. 1997). Taken together, MAP ERK signaling pathway modulates HGF/SF-stimulated cell migration by regulating local cell volume via  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel, cell-cell, and cell-matrix interaction and direct effect on cytoskeletal machine. Figure 18 shows the schematic model of the role of MAP ERK relating  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels and cell migration in MDCK II cells.

In summary, HGF/SF is a multifunctional effector that stimulates morphological changes, dissociation, scatter and migration of epithelial and other cell types. ChTX, IbTX, Stk, and CLT all inhibit HGF/SF-stimulated membrane  $\text{K}^+$  current and migration in MDCK II cells. Therefore, we propose that  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels play a role in HGF/SF-stimulated MDCK II cell migration, and MAPK ERK signaling pathway is in part the cellular mechanisms relating  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels and cell migration to HGF/SF in MDCK II cells. Met and/or HGF/SF expression and over-expression have been observed in a variety of tumors. HGF/SF-stimulated cell migration plays an important role in tumor invasiveness and metastasis. Therefore, the dependence of HGF/SF-stimulated migration on  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels may provide a target on the cell surface for therapeutic intervention for diseases in which cell migration contributes to the pathology.

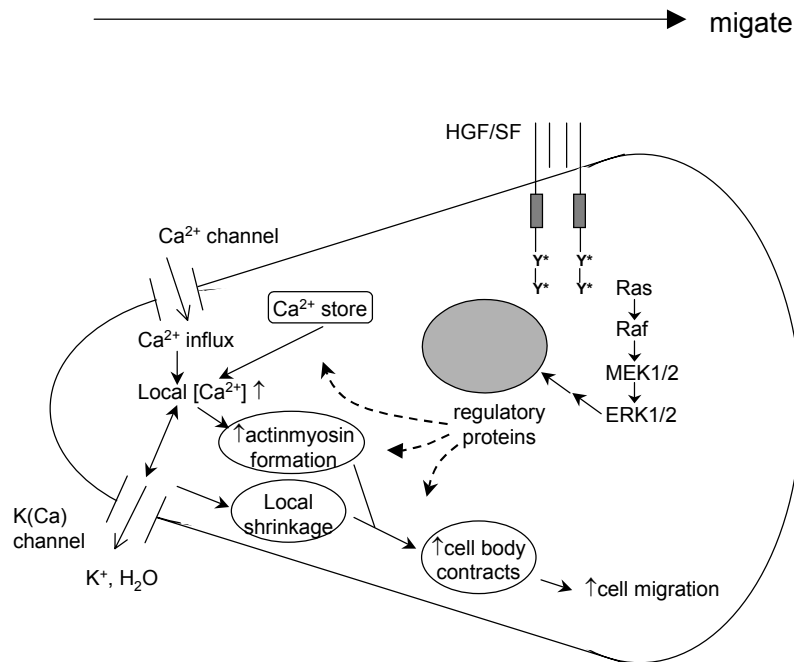


Figure 20 Schematic model of the role of MAPK/ERK relating  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel activation and cell migration in HGF/SF-treated MDCK II cells. Upon HGF/SF binding, Met receptor are dimerized and activated. Ras is activated through Grb-Sos as indicated in Fig. 2, then downstream Raf, MEK1/2, ERK1/2 are activated. ERK activation signals to the nucleus and new regulatory proteins are synthesized, which then regulate  $\text{Ca}^{2+}$  channel, as well as  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel.  $\text{Ca}^{2+}$  channel activation induces actinmyosin formation by elevating local  $[\text{Ca}^{2+}]$ . The activities of  $\text{Ca}^{2+}$  channel and  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel have two-way effects on each other.  $\text{K}^+$  and  $\text{H}_2\text{O}$  leave the rear of the cell upon  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel activation, thus causing local shrinkage at the cell body. Both local cell shrinkage and actinmyosin formation then facilitate cell migration by inducing the rear of the cell to contract.

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