Title
Misclocalized scaffolding by the Na-H exchanger NHE1 inhibits fibronectin production and assembly

Author
Karydis, Anastasios

Publication Date
2009

Peer reviewed|Thesis/dissertation

Mislocalized Scaffolding by the Na-H exchanger NHE1 Inhibits Fibronectin Production and Assembly

by

Anastasios Karydis

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Oral and Craniofacial Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

To my father, George,

to Demetra, whom we miss,

and to the rest of my family,

for their love & support.

Misclocalized scaffolding by the Na-H exchanger NHE1 inhibits fibronectin production and assembly

Anastasios Karydis

Abstract

Secretion and assembly of the extracellular matrix protein fibrobnectin (FN) regulates a plethora of normal cell and tissue functions, including development, cell growth, differentiation and cell migration, and is dysregulated in diseases such as fibrosis, diabetes, and cancer. My thesis work revealed that mislocalized scaffolding by the plasma membrane Na-H exchanger NHE1 dominantly suppresses FN expression, secretion, and assembly, and inhibits cleavage of latent to active TGF-β. By catalyzing an electroneutral exchange of extracellular Na^+ and intracellular H^+, NHE1 has recognized functions in intracellular pH and osmotic homeostasis. Recent evidence indicates NHE1 also functions as a plasma membrane anchor for the actin cytoskeleton by binding directly to ERM (ezrin, moesin, radixin) proteins and a PI(4,5)P2-binding scaffold, which are independent of ion translocation by NHE1. Endogenous NHE1 localizes to the distal margin of membrane protrusions in lamellipodia, however a mutantNHE1-KRA2, lacking binding sites for PI(4,5)P2 and the ERM proteins ezrin, radixin and moesin, is mislocalized and distributed uniformly along the plasma membrane. Fibroblasts expressing NHE1-KRA2 but not wild-type NHE1 have impaired FN expression, secretion, and assembly, and reduced active but not latent TGF-β. We found that

FN production is not regulated by changes in intracellular pH, nor is it attenuated in NHE1-deficient cells, indicating FN expression and TGF-β activation are independent of NHE1 activity. However, treating NHE1-KRA2 fibroblasts with recombinant TGF-β restores FN secretion and assembly. These data suggest that scaffolding by NHE1-KRA2 sequesters and mislocalizes signals necessary for FN synthesis and TGF-β activation. Although the precise signals sequestered by NHE1-KRA2 remain unknown, these findings suggest that NHE1-KRA2 could be a valuable tool for obtaining a mechanistic understanding of how FN production and TGF-β activation are regulated, and more speculatively for therapeutic control of increased FN production in pathological conditions such as fibrosis and inflammation.

Table of Contents

List of Figures

List of Tables

List of Abbreviations

FN: fibronectin

ECM: extracellular matrix

NHE1: Na-H exchanger isoform 1

FA: focal adhesion

PI(4,5)P2: Phosphatidylinositol 4,5 bisphosphate

ERM: ezrin/moesin/radixin

PH_i: intracellular pH

TGF-β: transforming growth factor-β

TGF-β-R1: transforming growth factor-β receptor type I

MMP: matrix metalloproteinase

PA-I: plasminogen activator inhibitor-1

1. Introduction

The extracellular matrix (ECM) provides a scaffold with positional and environmental information that is essential for tissue organization and function and for many cell processes and behaviors. By providing support and anchorage for cells that bind to its components, the ECM separates tissues from one another and forms amazingly diverse structures. Variations in the type and amount of ECM components can create diverse tissues including tendons, bands of fibrous connective tissue with great tensile strength; the cornea, the outer tough fibrous transparent layer that covers the pupil and the iris; and teeth and bones, calcified hard structures. By binding to cell surface receptors that anchor actin filaments, the ECM also determines organization of the cytoskeleton and cytoskeleton-dependent adhesive and protrusive structures. The ECM also acts as a reservoir for growth factors and hormones, which are usually bound within ECM components in inactive form that are released by protease cleavage. With structural components and bound proteins, the ECM provides signaling cues that affect intracellular signaling pathways and determine cell processes, including survival, development, proliferation, adhesion and migration (Lodish *et al.*, 1999). Understanding the components of the ECM are regulated and organized is key to understanding the complex dynamics of cell and tissue functions.

The ECM components are produced intracellularly and secreted in the extracellular space where they are organized in networks. The most abundant

ECM components include collagens that provide strength and resilience, proteoglycans like heparan sulfate that are highly hydrated and cushion cells, hyaluronic acid that resists compression and facilitates cell migration, and proteins with structural and functional roles including fibronectin (FN), elastin and laminins that bind to cell surface receptors and to ECM components. Elastin gives elasticity to tissues allowing them to stretch and return to their original state. Laminins are major components of the basal laminae that support the epithelium and separate it from the underlying connective tissue. FN creates a network where cell surface receptors and other ECM components bind and plays an important role in development, wound healing and blood clotting. A better understanding of how FN is regulated will provide important information on its physiological and pathological processes.

1.1. Fibronectin

Function in normal and pathological processes

Fibronectin, a high molecular weight glycoprotein of 250 ~kDa, is a major component of the ECM that surrounds cells in most tissues. There are two predominant forms of FN: plasma and cellular (Figure 1). The plasma soluble FN (Figure 1A) forms dimers, is synthesized predominantly by hepatocytes and is

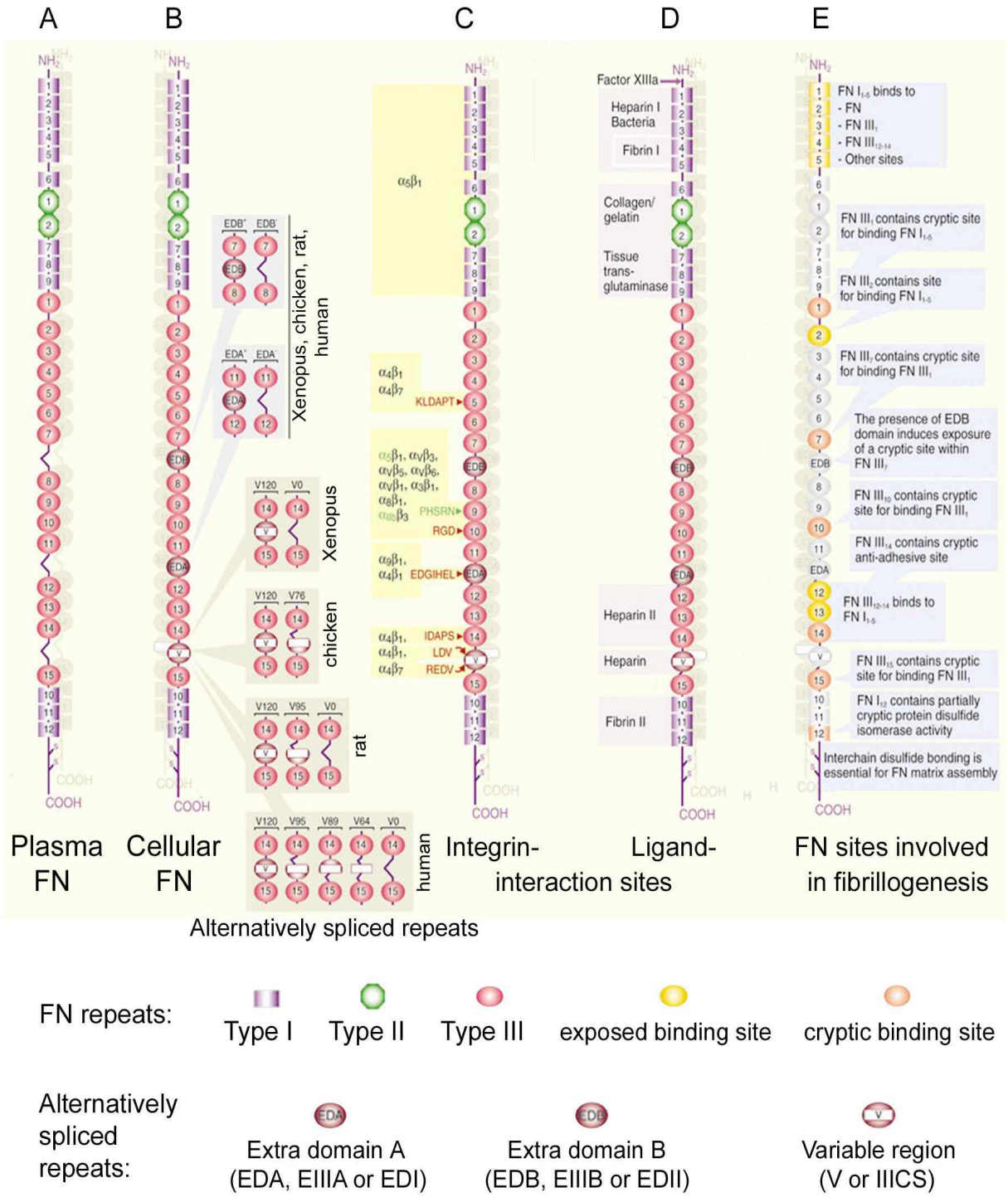

A
B
C
D
E
Xenopus, chicken, rat, human
Xenopus
chicken
rat
human
Factor XIIIa
Heparin I
Bacteria
Fibrin I
Collagen/
gelatin
Tissue
trans-
glutaminase
Heparin II
Heparin
Fibrin II
KLDAPT
PHSRN
RGD
EDGIHEL
IDAPS
LDV
REDV
FN I1-5 binds to
- FN
- FN III1
- FN III12-14
- Other sites
FN III1 contains cryptic site for binding FN I1-5
FN III2 contains site for binding FN I1-5
FN III7 contains cryptic site for binding FN III1
The presence of EDB domain induces exposure of a cryptic site within FN III7
FN III10 contains cryptic site for binding FN III1
FN III14 contains cryptic anti-adhesive site
FN III12-14 binds to FN I1-5
FN III15 contains cryptic site for binding FN III1
FN I12 contains partially cryptic protein disulfide isomerase activity
Interchain disulfide bonding is essential for FN matrix assembly
Plasma FN
Cellular FN
Integrin-interaction sites
Ligand-interaction sites
FN sites involved in fibrillogenesis
Alternatively spliced repeats
FN repeats:
Type I
Type II
Type III
exposed binding site
cryptic binding site
Alternatively spliced repeats:
Extra domain A (EDA, EIIIA or EDI)
Extra domain B (EDB, EIIIB or EDII)
Variable region (V or IIICS)

Figure 1.

Figure 1.

Diagram of the fibronectin (FN) molecule: (A) Plasma and (B) cellular FN monomers, consisting of three types of repeating units (FN repeats), type I, II and III. Alternative splicing generates multiple FN variants and occurs within the type III repeats (extra domain EDB between repeats III7-III8, extra domain EDA between repeats III11-III12, and variable region V between repeats III14-III15). (C) FN binding sites for members of the integrin receptor family. (D) FN binding sites for other biologically important molecules, including heparin, collagen, gelatin and fibrin. (E) FN self-association sites involved in fibrillogenesis. Some of these self-association sites are exposed, while others are cryptic and become accessible after conformational changes by cell-driven mechanical stretching of the FN molecule. (modified, from Pankov and Yamada, 2002)

secreted into the bloodstream reaching a fairly high concentration (approximately 300 μg/ml). The cellular FN (Figure 1B), usually in a multimeric cross-linked form, is secreted by fibroblasts, chondrocytes, myoblasts and epithelial cells and assembled as fibrils in the ECM (Tamkun and Hynes, 1983; Hynes, 1990). Secreted cellular FN initially forms dimers through interchain disulfide bonds (S-S) at the C-terminus and subsequently assembles into a fibrillar extracellular network that depends on FN binding to cell surface adhesion receptors of the integrin family.

FN production and assembly play critical roles in normal development, tissue morphogenesis and wound healing, cell differentiation, adhesion, migration and thrombus formation (Grinnell, 1984; Hynes, 1990; Yost, 1992; Clark *et al*., 1997; Sottile *et al*., 1998; Wylie, 1999; Cho and Mosher, 2006). The critical role of FN in development is highlighted by the early embryonic lethal phenotype of homozygous mice with engineered FN gene deletion (George *et al*., 1993). FN-null embryos have a greatly shortened anterior-posterior axis, a bent neural tube, severe defects in mesoderm-derived tissues, including absence of somites and a notochord, and impaired formation of heart and embryonic vessels (Francis *et al*., 2002). Mesodermal defects are attributed to loss of FN-dependent mesenchymal cell migration, proliferation and differentiation. Consistent with these findings, inhibition of FN assembly in Xenopus embryos impairs the direction of mesoderm cell migration in early stages of development (Winklbauer and Nagel, 1991), and inhibition of FN-cell interaction results in defects in amphibian gastrulation and

mesoderm architecture, and inhibits avian neural crest cell migration (Boucaut *et al*., 1984; Darribère and Schwarzbauer, 2000).

Aberrant FN production is associated with a number of pathological conditions. Increased FN synthesis occurs in hepatic fibrosis and leads to hepatic dysfunction and portal hypertension. The increased accumulation of FN, collagen, and other proteoglycans is a result of abnormal inflammatory reaction and abnormal wound healing after chronic exposure to infectious or toxic agents (Kershenobich Stalnikowitz and Weissbrod, 2003; Rockey, 2006). Excessive synthesis of FN and collagens I and III occurs also in pulmonary fibrosis and leads to respiratory insufficiency (Crouch, 1990; Hetzel *et al*., 2005; Muro *et al*., 2008). Increased FN deposition is observed during demyelinating diseases in the central nervous system, as in multiple sclerosis, and is implicated in the microglial activation that initiates and promotes tissue damage (Milner *et al*., 2007). FN synthesis is also increased in diabetic nephropathy, retinopathy, and macroangiopathy (Andresen *et al*., 1998; Hohenadel and van der Woude, 2004). In contrast, decreased FN production and reduced cell adhesion is observed in some cancer cells, including human breast cancer (Labat-Robert *et al*., 1980) and human cervical cancer (Larizza *et al*., 1982). Moreover, increased expression of FN in cancer cells is associated with decreased metastatic activity and explains the low degree of metastatic potential of basal cell carcinoma (Peltonen *et al*., 1988), where secreted FN is deposited in the tumor's periphery, compared with other carcinomas. Increased FN expression is suggested as an indicator of better prognosis in invasive breast carcinomas and increasing FN synthesis lowers the

risk for metastasis and prolongs survival (Christensen *et al*., 1988), and may be used as a prognostic factor (Takei *et al*., 1995). In addition, a stronger link has been reported between tumorigenicity and short immature FN fibrils, as opposed to longer branched FN fibrils (Der and Stanbridge, 1980; Stanbridge *et al*., 1982), indicating also a role for FN assembly and organization in disease. Imbalances in FN synthesis, abundance and assembly are associated with numerous diseases and highlight the critical role of FN regulation.

Regulated expression

The FN gene belongs to the broad superfamily of early response genes that rapidly increase their expression within two hours after quiescent cells are stimulated with serum or growth factors. In addition to FN, early response genes include the transcription factors *c-fos*, *c-jun* and *c-myc*, actin, and integrin receptors (Blatti *et al*., 1988; Ryseck *et al*., 1989). The early expression of FN after cell stimulation underlines the role of FN as an early regulator in cell differentiation, development, growth and migration.

FN expression is regulated at the promoter, transcription, splicing and secretion levels and is tissue-specific, and development- and age-related (Schwarzbauer, 1991). The unique FN gene is ~ 75 kb, contains ~ 50 exons and is transcribed from a single promoter to a single primary transcript. Regulation of the FN promoter relies on a number of different regulatory elements. The human

(Dean *et al*., 1987), rat (Patel *et al*., 1986) and mouse (Polly and Nicholson, 1993) FN promoters have been cloned and share extensive homology for many regulatory elements. A cAMP responsive element (CRE) at position –170 is necessary for increased FN expression with serum (Dean *et al*., 1990). Two more CRE sites are located at –260 and –450 regions and are conserved in human and rat FN gene promoter (Bowlus *et al*., 1991; Schwarzbauer, 1991). The human FN promoter also includes a TATA box at –25 region and other regulatory regions upstream, including a CCAAT box at –150, promoter specific transcription factor SP1boxes at –102 and –45 regions, and *Arabidopsis* APETALA2 AP2 sites at –120 and –67. The early growth response transcription factor Egr-1 binds to GC-rich sequences in the –105 to +14 region of the human promoter and regulates cell growth, differentiation and development (Liu *et al*., 2000). Members of the Nuclear Factor κB (NF-κB) family of transcription factors are also key regulators in immune and inflammatory responses, although paradoxically some members activate and inhibit FN expression. The NF-κB p65/p50 variant binds to the responsive element at –41 and inhibits FN expression while NF-κB p65 binds to the responsive regulatory element at position –1180 and activates the FN transcription (Lee *et al*., 2000; Lee *et al*., 2002).

Regulation of FN transcription is induced by a myriad of intrinsic and extrinsic signals. The FN gene is activated by serum, cAMP, growth factors, including epidermal growth factor (EGF), platelet-derived growth factor (PDGF), and transforming growth factor beta (TGF-β) (Blatti *et al*., 1988; Dean *et al*., 1988), and cytokines (Baeuerle and Baichwal, 1997). FN induction by growth

factors is thought to be mediated by the transcription factor Egr-1 (Liu *et al*., 2000) and increased transcription by cytokines is likely mediated by the transcription factor NF-κB (Lee *et al*., 2002). FN gene expression is also sensitive to cell density and cell shape changes, increasing with higher cell density and smaller cell area (Perkinson *et al*., 1996). Consistent with decreased FN in cancers, viral oncogenes inducing cell transformation attenuate FN transcription (Nakajima *et al*., 1992; Nakamura *et al*., 1992).

TGF-β peptides are recognized as important inducers of FN transcription. TGF-βs are multifunctional proteins with potent and direct effects on many cell types and they regulate fibroblast gene expression and promote FN deposition. There are three structurally similar isoforms of TGF-β (TGF-β1, β2 and β3) encoded by three distinct genes (Schiller *et al*., 2004). The three isoforms bind to the same plasma membrane type I and II dimeric receptors and have similar cellular targets, although they have tissue specific expression and are controlled by distinct promoters (Letterio and Roberts, 1998). TGF-β1 is the most prevalent isoform and is found almost ubiquitously, while the other isoforms are expressed in a limited number of cells and tissues. TGF-βs regulate in a wide range of sometimes contradictory biological processes, including stimulation of mesenchymal cell proliferation, regulation of ECM deposition and degradation, maintenance of tissue architecture, inhibition of growth, induction of apoptosis, inhibition of epithelial cell growth, regulation of inflammation and of the immune response, and wound repair (O'Kane and Ferguson, 1997; Fleisch *et al*., 2006; Wahl, 2007). The distinct roles of the TGF-β isoforms are indicated by knockout

experiments in mice revealing that each isoform plays an important but independent role in embryonic development. Half of the TGF-β1 null mice die *in utero* due to defective vasculogenesis and hematopoiesis (Dickson *et al.*, 1995). The remaining mice die two-four weeks after birth by a wasting syndrome associated with multifocal inflammation and massive lymphocyte and macrophage infiltration primarily in the heart and lungs (Kulkarni *et al.*, 1993). It is possible though that the variability in embryonic lethality of TGF-$\beta1^{-/-}$ mice is due to their mixed genetic background and modifier genes may be involved in modulation of the TGF-$\beta1^{-/-}$ phenotype. TGF-β2 knockout mice exhibit perinatal mortality and a wide range of developmental defects, including cardiac, pulmonary, craniofacial, spinal and urogenital defects (Sanford *et al.*, 1997). TGF-β3 null animals exhibit defective epithelial-mesenchymal interactions that cause cleft palate and abnormal lung development (Proetzel *et al.*, 1995). The distinct phenotypes of the TGF-β knockout animals highlight the non-compensatory and independent functions of the three isoforms.

Regulation of FN splicing is responsible for the distinct functions of the FN variants in FN secretion, cell adhesion and assembly. FN comprises three repeated homology units of 40, 60 and 90 amino acids, known as type I, II and III repeats, respectively. Although there is only a single FN gene (Kornblihtt *et al.*, 1983; Schwarzbauer *et al.*, 1983), three alternatively spliced segments (EDA, EDB and the V region) generate subunit diversity (Figure 1B). The single FN gene is responsible for approximately 20 different variants in humans through alternative splicing (Schwarzbauer, 1991). FN diversity can also be generated by the

combination of different FN subunits that are assembled into dimers. The alternatively spliced segment EDA is located within the III_{11}-III_{12} repeats, the EDB within the III_6-III_7 repeats and V or IIICS region within the III_{14}-III_{15} repeats. The extra domain A (also known EDA, EDI or EIIIA) is distinctive of cellular FN (Kornblihtt *et al.*, 1984), is overexpressed in proliferating tissues, such as the developing embryo (Norton and Hynes, 1987) and by fibroblasts and macrophages at the site of cutaneous wound (Brown *et al.*, 1993), indicating a role in wound healing. The extra domain B (also known EDB, EDII or EIIIB) is also expressed during embryonic development, at sites of wound healing and after transformation with RNA and DNA tumor viruses (Mosher, 1989; Hynes, 1990), and is stimulated by TGF-β1 (Borsi *et al.*, 1990). The V (for variable) region, also known as IIICS region (to denote that it is a type III repeat Connecting Segment), has five human variants and is necessary for FN dimer secretion (Schwarzbauer *et al.*, 1989), as FN V-0 homodimers do not reach the cell surface and get degraded intracellularly. The V region regulates protein trafficking and dimer secretion.

Adhesion and FN assembly

Regulation of FN assembly depends on FN binding to cell surface receptors and other ECM molecules (Figure 1C and 1D). The type I, II and III FN repeats are organized into domains that have distinct binding activities. FN binding to integrins is mediated by two major FN domains that both locate at the type III repeats. The main cell-binding domain contains the RGD (Arg-Gly-Asp) sequence

and is located in repeat III_{10}. The RGD sequence is present in all FN variants and is recognized by different cell types mainly through the integrin $\alpha_5\beta_1$. The second domain is located in the adjacent III_9 FN repeat and contains the PHSRN sequence. It is also described as the synergy site because, although the PHSRN sequence has no binding activity by itself, it acts synergistically with the flanking RGD sequence and causes a ~100-fold increase in cell adhesion to the RGD site by $\alpha_5\beta_1$ integrin (Obara *et al.*, 1988; Nagai *et al.*, 1991; Aota *et al.*, 1994). Both the RGD and synergy sites along with the heparin-binding domain of the V region appear to cooperate for maximal effect on cell adhesion. The second major binding domain, also located in the V region, contains the LDV (Leu-Asp-Val) sequence, is conserved in human, rat, bovine and avian FN, and is recognized by $\alpha_4\beta_1$ integrin present in lymphocytes, monocytes, and neural crest and melanoma cells (Humphries *et al.*, 1988; Guan and Hynes, 1990; Mould *et al.*, 1990; Komoriya *et al.*, 1991). Also known as the CS1 site, the LDV sequence is located within the alternatively spliced V region and promotes RGD-independent FN binding and assembly. The FN carboxy-terminal region therefore provides critical binding sites necessary for cell adhesion.

FN binding sites for other FN molecules are also required for FN assembly (Figure 1E), while binding sites for other matrix components facilitate ECM organization. The amino-terminal region of FN contains several binding sites mainly for other ECM molecules, including fibrin, gelatin, heparin, collagen and other FN units, while the FN carboxy-terminal region includes more binding sites for heparin, heparan sulfate and other FN molecules (Heremans *et al.*, 1990;

Mosher, 1993; Barkalow and Schwarzbauer, 1991; Sechler *et al*., 1998). The type I and II repeats predominantly mediate associations with FN and other ECM molecules. During FN assembly, these interactions stabilize the matrix, posing FN as a central player in this functional extracellular network. Sites involved in cell attachment reside within the type III repeats.

FN assembly into fibrils is a regulated, stepwise process that includes initiation, elongation and stabilization of the FN matrix. It involves the binding of the FN molecule to integrins and other FN molecules through multiple FN domains. Initially, secreted FN is a soluble, inactive dimer, folded into a conformation that does not undergo fibril assembly (Williams *et al*., 1982; Erickson and Carell, 1983; Johnson *et al*., 1999) due to its compact structure that retains many binding sites cryptic (Figure 1E). FN binding to integrins leads to partial unfolding and conformational changes of the FN molecule that expose cryptic binding sites and allow FN self association and assembly initiation. Initiation depends on interactions between the FN cell-binding domains and integrin receptors, usually $\alpha_5\beta_1$ integrin binding to the RGD site. The RGD sequence in repeat III_{10} and the synergy site in III_9 co-operate for maximal $\alpha_5\beta_1$ integrin-mediated matrix assembly. Cell-associated FN is initially distributed diffusely over the cell surface. The initiation of FN assembly leads to formation of short detergent-soluble fibrils. During the initiation stage, FN self-association induces integrin clustering by binding to multiple integrins, and integrin binding immobilizes the dimeric FN. The integrin-FN clusters create a relatively high local concentration of FN that further assists the FN association with other FN

molecules and promotes the FN fibril growth phase. Immobilization of FN induces conformational changes that expose FN-binding sites and activate the protein to bind more FN dimers. Dimeric FN forms short deoxycholate-soluble fibrils. The incorporation of additional FN dimers generates fibril elongation and conversion of fibrils into a detergent insoluble form. As assembly progresses, the FN fibrils are subsequently converted into a dense, stable network containing longer detergent-insoluble fibrils (McKeown-Longo and Mosher, 1983; Sechler *et al*., 1997; Pankov *et al*., 2000; Wierzbicka-Patynowski and Schwarzbauer, 2003).

1.2. Integrins: fibronectin receptors and focal adhesions

Integrins are heterodimeric adhesion receptors formed by the non-covalent association of α and β subunits. Each subunit is a glycoprotein with a relatively large exracellular domain and a short cytoplasmic tail. Integrin extracellular domains bind to diverse ECM ligands to mediate cell-substrate and cell-cell adhesion. The integrin cytoplasmic tail translates ligand binding to a dynamic cellular process tethering the receptor to the cytoskeleton and interacting with multiple signaling molecules (Critchley, 2000; Calderwood, 2004). Integrins are present in all metazoans and play a critical role in development, adhesion and migration (Hynes, 1992). In mammals, twenty-four different heterodimers form from 18 α subunits and 8 β subunits. Each heterodimer binds to specific cell surface, ECM or soluble ligands and has distinct functions.

The interaction of the cells with FN and other ECM components mediates the formation of focal adhesions (FAs) by clustering integrin receptors, which recruits signaling molecules at the integrin cytoplasmic tail and assembles bundled actin filaments. FAs in cultured cells are initially formed at the leading edge of the cell in the lamellipodia and consist of an ECM component, integrins and some adapter proteins such as talin and paxillin bound to integrin's cytoplasmic tail. These immature FAs are often also called focal contacts. As FAs mature, they recruit additional adapter and signaling proteins such as focal adhesion kinase (FAK), zyxin and proteins that bind the actin cytoskeleton including vinculin, α-actinin and filamin that like talin link the integrins with the actin-based cytoskeleton. Focal adhesions are dynamic protein complexes that connect the ECM to the cytoskeleton and transmit mechanical forces and regulatory signals (Schoenwaelder and Burridge, 1999). At the leading edge of migrating cells, focal adhesions undergo rapid cycles of assembly and turnover, creating and disrupting, respectively, sites of traction necessary for forward movement of the cell body. Hence, FAs have considerable functional diversity, including cell anchoring, cell motility, and signal transduction.

Integrin $\alpha_5\beta_1$ is considered the major cell-surface receptor that supports FN assembly, although other integrins have also been identified in RGD-independent FN assembly pathways. Integrins $\alpha_4\beta_1$, $\alpha_v\beta_3$ and $\alpha_{IIb}\beta_3$ have all been reported to be able to promote and initiate FN assembly in various cell models (Wu *et al.*, 1995; Wennerberg *et al.*, 1996; Yang and Hynes, 1996; Wu, 1997; Sechler *et al.*, 2000). FN assembly into fibrils is mediated by a series of events involving both

the actin cytoskeleton and the integrin receptors. While integrin binding to its ligand is required for integrin activation and matrix assembly (Clark *et al*., 2004), additional post-occupancy events involving the β integrin subunit cytoplasmic domain interaction with the actin cytoskeleton are necessary as well (Wu *et al*., 1995; Hughes *et al*., 1996; Magnusson and Mosher, 1998). The β integrin cytoplasmic tail is linked to F-actin by direct binding of the actin-binding proteins talin, α-actinin, filamin and by indirect binding through vinculin (Critchley *et al*., 1999). Rho-A mediated contractility generates the tension that exposes cryptic FN binding sites within the FN molecule that are required for FN assembly (Hocking *et al*., 1994, Zhong *et al*., 1998, Sechler *et al*., 2001). Disruption of the actin cytoskeleton with cytochalasin D or truncation of the β cytoplasmic domain abolishes FN matrix formation independently of the integrin activity status (Wu *et al*., 1995). Signaling networks downstream of integrins involving active Src and PI 3-kinase also regulate early stages in FN matrix assembly because cells lacking Src family kinases or pharmacological inhibition of PI 3-kinase attenuates FN matrix assembly in SYF fibroblasts and CHO cells respectively (Wierzbicka-Patynowski and Schwarzbauer, 2002). However, despite integrin activation and signaling being necessary for FN matrix assembly, integrins do not regulate FN production (Wu *et al*., 1995; Feral *et al*., 2007).

1.3. Na-H exchanger NHE1

Established role in ion transport

The plasma membrane Na-H exchanger NHE1 plays a central role in intracellular pH (pH_i) and cell volume homeostasis by catalyzing an electorneutral exchange of extracellular Na^+ and intracellular H^+. The NHE gene family is evolutionarily conserved in metazoans and includes nine isoforms with tissue-specific expression and functions (Putney *et al*., 2002). The ubiquitously expressed NHE1 isoform in mammals is a resident plasma membrane protein, in contrast with NHE2 and NHE3 isoforms that recycle between the plasma membrane and endosomes and have limited expression in epithelia. NHE1 (Figure 2) contains two structural and functional domains: the NH2-terminal domain or ion translocation domain that includes 12 transmembrane α-helices and is sufficient for basal exchange activity, and the relatively long (~300 amino acids) COOH terminal cytoplasmic domain or regulatory domain that confers regulation of exchanger activity by growth factors, hormones, integrins and osmotic stress (Meima *et al*., 2007). Reduced pH_i (Aronson *et al*., 1982), hosmotic challenge by hypertonic media (Grinstein *et al*., 1992), treatment with the epidermal growth factor EGF (Liaw *et al*., 1998) and the platelet-derived growth factor PDGF (Di Sario *et al*., 1999) and integrin activation by plating cells on FN (Ingber *et al*., 1990) or treating with insoluble FN (Schwartz *et al*., 1991) all stimulate NHE1 activity, while somatostatin (Barber *et*

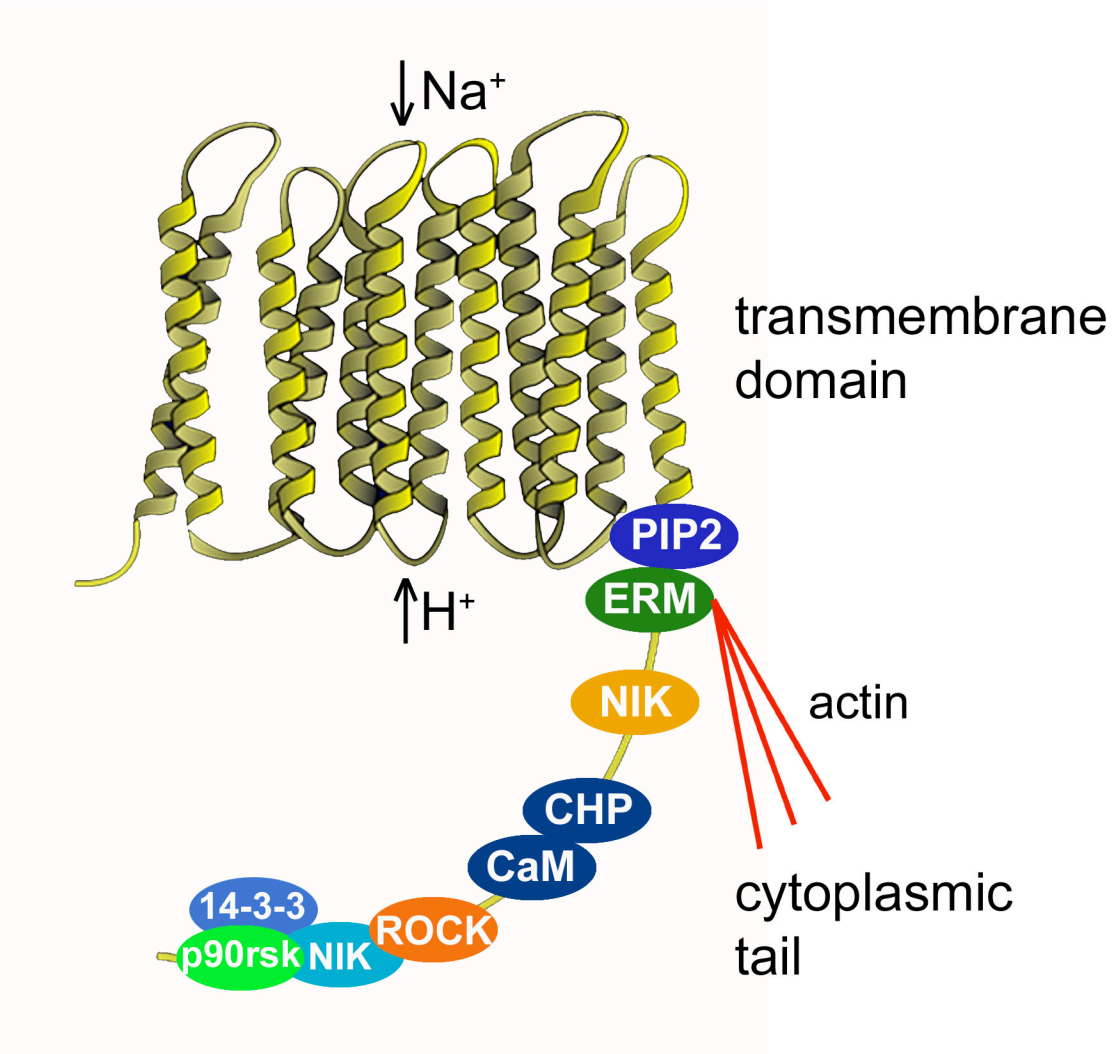


Figure 2.

Na-H exchanger (NHE1) topology and structural features of actin anchoring and scaffolding signaling molecules

al., 1989) and dopamine (Ganz *et al*., 1990) inhibit NHE1 activity. NHE1 has an established permissive role in cell proliferation and promotes cell survival (Putney *et al*., 2002) and is necessary for cell migration, polarity, membrane protrusion and adhesion (Denker and Barber, 2002; Denker and Barber, 2002b; Patel and Barber, 2005). Recent evidence indicates these functions depend not only on ion translocation by NHE1 but also on newly identified functions in cytoskeletal anchoring and scaffolding of signaling complexes by NHE1.

Role in cytoskeletal anchoring and scaffolding signaling complexes

Emerging evidence indicates that many ion transport proteins, including channels, pumps and exchangers, are directly or indirectly anchored to actin filaments. Actin anchoring by NHE1 is mediated by binding of charged Lys-Arg residues in the COOH terminal juxtamembrane domain to ERM proteins ezrin, radixin, and moesin (Denker *et al*., 2000), which belong to the protein 4.1 superfamily. The NH_2-terminal FERM domain of protein 4.1 and ERM proteins binds integral membrane proteins and their COOH-terminus binds actin filaments. ERM binding to NHE1 is also necessary for dynamic reorganization of the actin-based cytoskeleton. ERM binding to NHE1 spatially restricts the clustered localization of NHE1 at the distal margin of membrane protrusions in the lamellipodia of fibroblasts, and data suggest that localization of NHE1 at the leading edge of migrating cells is necessary for polarity, actin filament assembly, and efficient directed movement (Denker and Barber, 2002; Patel and Barber, 2005; Frantz *et*

al., 2007).

In addition to binding ERM proteins the COOH terminal cytoplasmic regulatory domain of NHE1 directly interacts with at least 10 different signaling molecules (Figure 2). A current view is that NHE1 functions as a scaffolding platform for the assembly of signaling complexes (Meima *et al*., 2007). In addition to NHE1, other ion transport proteins have recently been shown to scaffold macromolecular complexes, including TRP channels in photoreceptor cells (Popescu *et al*., 2006), and potassium channels in neurons (Kaczmarek, 2006). The juxtamembrane region of the NHE1 COOH regulatory domain binds phosphatidylinositol(4,5)bisphosphate (PI(4,5)P2) (Aharanovitz *et al*., 2000), ERM proteins (Denker *et al*., 2000), and the calcineurin homologous protein CHP – a ubiquitously expressed essential cofactor for NHE1 activity (Lin and Barber, 1996; Mishima *et al*., 2007). The middle region of the COOH terminus includes high and low-affinity binding sites for calmodulin (Bertrand *et al*., 1994) and a binding site for the Ste20-like Nck-interacting kinase (NIK) (Yann *et al*., 2001; Baumgartner *et al*., 2006). NIK phosphorylates serine residues in the distal COOH terminus of NHE1 - a region termed the phosphorylation domain. The extracellular signal-regulated kinase (ERK) downstream effector kinase p90Rsk (Takahashi *et al*., 1999), p38 mitogen-activated protein kinase (Khaled *et al*., 2001) and Rho-activated kinase (ROCK) (Tominaga *et al*., 1998) also phosphorylate serines in this domain. Phosphorylation of NHE1 at Ser703 by p90Rsk promotes binding of the adaptor protein 14-3-3 after serum-dependent activation (Lehoux *et al*., 2001).

The role of NHE1 as a scaffolding platform for signaling molecules is likely both dependent and independent of its ion transporter activity. A reciprocal action between ion transport proteins and the cytoskeleton is proposed, where ERM binding localizes ion transport proteins, and in turn, localized activity of ion transport proteins regulates the assembly and organization of actin filaments to promote actin-dependent processes (Denker and Barber, 2002b). Changes in intracellular pH and cell signaling are gaining attention based on emerging data indicating that the activities or binding affinities of selective proteins are pH sensitive (Srivastava *et al.*, 2007). In contrast, transport-independent scaffolding by NHE1 could facilitate signal relay through the forced proximity of functionally interacting proteins. Localized scaffolding by NHE1 permits ERM proteins to bind PI(4,5)P2 and be phosphorylated in order to adopt an open conformation for binding transmembrane proteins and actin. ERM proteins are also directly phosphorylated by NIK (Baumgartner *et al.*, 2006) and ROCK (Matsui *et al.*, 1998), which also assemble with NHE1. Additionally, localized scaffolding of the calcium binding proteins CHP and CaM could facilitate shared actions in calcium-dependent signaling pathways (Lin and Barber, 1996). Transport-dependent scaffolding is predicted to function in pH-dependent signaling processes such as cell proliferation and migration by localized changes in the protonation state of selective pH-sensitive proteins (Putney and Barber, 2003, Meima, 2007; Srivastava *et al.*, 2007).

In fibroblasts, ERM binding and ion transport activity by NHE1 are both necessary for dynamic remodeling of FA and for directed migration. Both the

assembly of focal adhesions and actin stress fibers by the activation of integrins and Rho are impaired in fibroblasts lacking NHE1, but are restored in fibroblasts expressing wild-type NHE1 (Vexler *et al.*, 1996; Tominaga and Barber, 1998; Tominaga *et al.*, 1998; Denker *et al.*, 2000). Fibroblasts expressing NHE1 with mutations that disrupt ERM binding but not ion translocation activity have decreased assembly of FAs and attenuated assembly of actin stress fibers (Denker *et al.*, 2000; Denker and Barber, 2002). In contrast, fibroblasts expressing a mutant NHE1 that lacks ion translocation have decreased assembly of actin filaments and decreased turnover of FAs in response to migratory cues (Srivastava *et al.*, 2008; Frantz *et al.*, 2008). Both ERM binding and ion translocation by NHE1 are necessary for efficient polarized migration of fibroblasts (Denker and Barber, 2002).

NHE1 fibroblast cell model

To investigate the effects of NHE1 on cell adhesion and regulation of FAs, we used NHE1-deficient PS120 fibroblasts without and with stable expression of WT or mutant NHE1 (Figure 3A):

- PS120 NHE1-deficient cells generated from CCL39 hamster lung fibroblasts that express only the Na-H exchanger isoform NHE1.

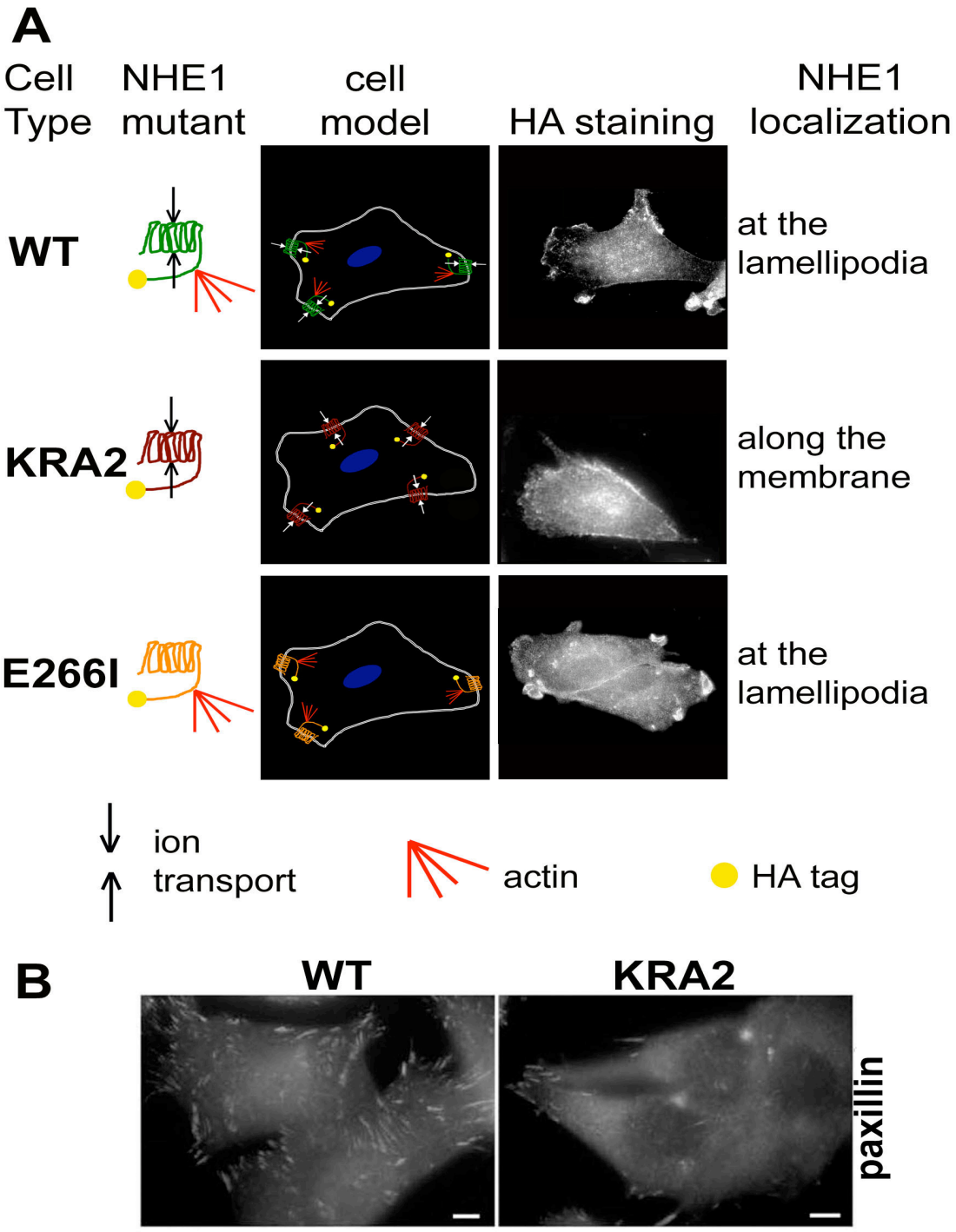

A
Cell Type
NHE1 mutant
cell model
HA staining
NHE1 localization
WT
at the lamellipodia
KRA2
along the membrane
E266I
at the lamellipodia
ion transport
actin
HA tag
B
WT
KRA2
paxillin

Figure 3.

Figure 3.

(A) Fibroblast cell model of WT NHE1 and NHE1 mutants. Schematic diagram of NHE1 indicating positions of the HA-epitope tag, the WT-NHE1 that retains ion translocation activity and ERM/PI(4,5)P2 binding, the KRA2-NHE1 with alanines substituted for lysine and arginine residues that abolishes ERM/PI(4,5)P2 binding and actin cytoskeleton anchorage but retains ion translocation, and E266I-NHE1 that abolishes ion translocation but retains actin cytoskeletal anchorage. Through the ERM/actin cytoskeleton binding WT-NHE1 and E266I-NHE1 localize at the lamellipodia of WT and E266I cells respectively, while KRA2-NHE1 is evenly distributed along the plasma membrane as shown by immunolabaling for the HA epitope in WT, KRA2 and E266I fibroblasts. (B) Fibroblasts expressing mislocalized NHE1-KRA2 have decreased abundance and size of FA complexes. Paxillin immunolabeling reveals decreased abundance and size of FA complexes in KRA2 cells compared with WT cells. Bar, 5 mm.

- WT cells: PS120 NHE1-deficient fibroblasts stably expressing WT NHE1,
- KRA2 cells: PS120 fibroblasts expressing a mutant NHE1 with impaired PI(4,5)P2 and ERM binding that contains alanine substitutions of lysine and arginine residues in the COOH-terminal juxtamembrane domain, and
- E266I cells: PS120 cells expressing a mutant ion translocation-defective NHE1 containing an isoleucine substitution for glutamine 266.

Wild type NHE1, either endogenous in CCL39 fibroblasts (Sardet *et al.*, 1990; Grinstein *et al.*, 1993) or recombinant expressed in NHE1-deficient PS120 fibroblasts localizes to the distal margin of membrane protrusions in the lamellipodia. In E266I cells NHE1 is also localized at the leading edge of the cell because ERM protein binding is retained. In contrast, in KRA2 cells NHE1 is not clustered in lamellipodia but is uniformly distributed along the plasma membrane (Figure 3A). Moreover, the assembly of focal complexes (Figure 3B) and actin stress fibers is impaired in KRA2 cells, and wound closure is significantly delayed compared with WT in would healing assays (Denker and Barber, 2002).

The secretion of ECM proteins was tested as a possible explanation for the impaired focal complex assembly and wound closure in KRA2 fibroblasts. My thesis work reveals that KRA2 fibroblasts expressing a mutant NHE1 that lacks PI(4,5)P2 and ERM protein binding have markedly decreased FN production. The next question was whether FN production is impaired in KRA2 cells because H^+ efflux is decreased and mislocalized or because KRA2-NHE1 is mislocalized, since KRA2-NHE1 is uniformly distributed along the plasma membrane instead of

clustering in lamellipodia. Decreased FN production is due to mislocalized scaffolding by NHE1 and not mislocalized H+ efflux. Because FN expression is regulated by TGF-β, I next asked whether TGF-β activation and downstream signaling of TGF-β receptors is impaired in KRA2 cells. TGF-β activation but not downstream signaling by the TGF-β Type 1 Receptor to SMAD3 is decreased in fibroblasts expressing mislocalized NHE1 and FN production is restored by exogenous TGF-β. These data suggest that mislocalized NHE1 sequesters and mislocalizes signals necessary for FN synthesis and TGF-β activation.

2. Methods

Cell culture

NHE1-deficient PS120 fibroblasts derived from hamster lung CCL39 fibroblasts that express NHE1 (Pouyssegur *et al.*, 1984) were obtained from J. Pouyssegur (INSERM, Nice, France). Wild type and mutant NHE1 containing an HA-epitope at the COOH terminus and stably expressed in PS120 cells (WT, E266I, and KRA2) were previously described (Denker *et al.*, 2000). PS120 cells stably expressing the System N1 transporter (SN1 cells) are previously described (Chaudry *et al.*, 1999) (description of all cell lines, Table 1). All cell types above were maintained in DME-H21 medium containing 25mM $NaHCO_3$ and supplemented with 5% FBS and 1% Pen-Strep (growth medium) at 5% CO_2. Mink lung epithelial cells (MLEC) were cultured in DME-H21 medium containing 25mM $NaHCO_3$ and supplemented with 10% FBS, 1% Pen-Strep and 250 μg/ml Geneticin (G-418) (Gibco BRL, Grand Island, NY) at 5% CO_2.

Constructs

The mammalian expression vector pCMV-WT-NHE1-HA was previously described (Orlowski, 1993) and was used to generate two mutant constructs (pCMV-E266I-NHE1-HA where the glutamate 266 was replaced by isoleucine;

Table 1. Cell lines used in this study

Cell line	Description	Reference
CCL39	clonal hamster lung fibroblasts with endogenous NHE1	Pouyssegur *et al.*, 1984
PS120	NHE1-deficient cells derived from CCL39 fibroblasts	Pouyssegur *et al.*, 1984
WT	PS120 cells stably expressing wild-type NHE1-HA	Denker *et al.*, 2000
KRA2	PS120 cells stably expressing KRA2-NHE1-HA, which contains alanine substitutions for COOH-terminal juxtamembrane lysine and arginine resiudes and does not bind PI(4,5)P2 or ERM proteins but does retain H+ efflux	Denker *et al.*, 2000
E266I	PS120 cells stably expressing E266I-NHE1-HA, where glutamate 266 was substituted by isoleucine; does not have H+ efflux but does retain PI(4,5)P2 and ERM binding	Denker *et al.*, 2000
K2E	KRA2 cells stably co-expressing E266I-NHE1-Myc	This study
CK	CCL39 cells stably co-expressing KRA2-NHE1-HA	This study
SN1	PS120 cells stably expressing the System N1 gluamine- H+ transporter	Chaudry *et al.*, 1999

and pCMV-KRA2-NHE1-HA where lysine and arginine in the 553–564 region where replaced by alanine, as previously described (Denker *et al*. 2000). All constructs above contain the sequence of the HA epitope (YPYDVPDYA) at the extreme carboxyl terminus of the rat NHE1 sequence; this epitope was preserved in the relevant stable cell lines: WT-NHE1, KRA2-NHE1 and E266I-NHE1.

The Myc epitope (LDEESILKQE) was added by PCR and replaced the HA epitope of the pCMV-E266I-NHE1-HA vector (forward primer sequence 5'AAG CTT GGA TCC GAT ATC ATG CTA AGG TGG TCT GGC ATC TGG3'; backward primer sequence 5'GCG GCC GC CTA CAG ATC TTC TTC AGA AAT AAG TTT TTG TTC GCC CTG CCC TTT GGG GAT GAA AGG3'). The new NHE1-E266I-Myc construct was inserted in pCDNA3.1 Hygro (+) and stably expressed in KRA2 cells (named K2E cells) by using Lipofectamine™ 2000 for transfection (Invitrogen, Carlsbad, CA). The NHE1-KRA2-HA construct of the pCMV-KRA2-NHE1-HA vector was stably expressed in the parental CCL39 fibroblasts (named CK cells) by using Lipofectamine™ 2000 for transfection (Invitrogen). (For description of all cell lines, Table 1).

Immunolabeling

Unless otherwise indicated, cells were grown for 48 hours on glass coverslips, washed twice with PBS, fixed with 4% Parafolmadehyde in PBS for 20 min,

washed twice with PBS, permeabilized with 0.1% Tx-100 in PBS for 10 min, then blocked for 5' to 15' with 10% Fetal Bovine Serum FBS in PBS. Incubation for 1 hour with the primary antibody was followed by three washes with PBS and incubation for 45 min with the secondary antibody.

Primary antibodies and dilutions included anti-paxillin mAb (1:100, Zymed Laboratories Inc., South San Francisco, CA), anti-FN rbAb (1:100, Sigma, Saint Louis, MI), anti-β1 integrin rbAb (9EG7; 1:100, BD Pharmingen, San Diego, CA), anti-HA mAb (12CA5; 1:100; Roche Molecular Biochemicals, Mannheim, Germany), anti-Myc mAb (9B11; 1:200; Cell Signaling Technology Inc., Danvers, MA).

For live-cell staining of extracellular FN, cells were incubated at 4°C with the antibodies for FN for 1 hour and then fixed as described above without permeabilization. For β1 integrin labeling, the Avidin/Biotin/Streptavidin Blocking Kit was used (Vector Laboratories, Burlingame, CA): the cells were incubated with Avidin D for 15 min, rinsed with PBS and then incubated with Biotin blocking solution for 15 min before the incubation with 9EG7.

Secondary antibodies were conjugated to FITC or Texas-red (Invitrogen, Carlsbad, CA), and were used at 1:200. The avidin conjugate was used for β1 integrin labeling.

To visualize nuclei, cells were incubated for 5 min with Hoechst 33342 (1:10,000, Invitrogen). Immunolabeling was visualized using a Zeiss Axiovert 35 and images were collected with a Spot RT cooled CCD.

FN induction with TGF-β1: FN production and assembly was induced by treatment with 0.5, 1, 2 and 5ng/ml of rhTGF-β1 (PeproTech) on WT and KRA2 cells grown on coverslips. Cells were treated with the corresponding rhTGF-β1 concentrations six hours after plating. Control cultures were treated with 10 μM of specific inhibitor of TGF-β1 Receptor Kinase activity, SB-431542 (Tocris Bioscience, Ellisville, MI; gift from R. Derynck, University of California San Francisco) that inhibits FN synthesis (Laping *et al*. 2002). Cells on coverslips were processed 48 hours after plating for FN immunolabeling, as described above.

Collagen expression

Collagen expression was determined by reverse transcription-polymerase chain reaction (RT-PCR) analysis for hamster Collagen a1 (V) chain. Total RNA extracted using the RNeasy kit (Qiagen, Valencia, CA) and 2 mg were reverse transcribed using random decamer primers and amplified with PCR Supermix High Fidelity (Invitrogen) for 30 cycles at 94°C, 56°C and 72°C. Oligos included: F2 forward primer 5' TAC CCT GGA AGA CAA GGG CC 3' and R2 reverse primer 5' TCC TGG AGG GCC AGT CTT GC 3', which yielded a 322-bp product, and F3 forward primer 5' ATG GTG AAC CTG GAC AGA CG 3' and R3 reverse primer 5' TCC TTT GAG TCC AGG GAG TC 3', which yielded a 329-bp product. Primers against 18S RNA were used as a control.

Immunoblotting

Immunoblotting for secreted FN was performed after chloroform-methanol precipitation of cell culture medium (conditioned medium) from cell cultures 48 hours after plating. Briefly, the conditioned medium was mixed with an equal volume of methanol and 1/4 volume of chloroform. The mix was vortexed for 2 min, then centrifuged at 10,000 g for 5 min. The upper phase was discarded, leaving the interface intact, and the remaining mixture was washed with 300 µl methanol and centrifuged for 5 min. Pellets were resuspended in 1x Laemmli buffer according to the concentration of the corresponding cell lysate. Proteins were separated by SDS-PAGE, transferred to PVDF membranes, and processed for immunoblotting as described previously (Denker *et al.*, 2000). Membranes containing conditioned medium samples were probed with anti-FN rbAb (1:2000, Sigma-Aldrich, St; Louis, MO) and membranes containing cell lysate samples were probed with anti-β-actin (C4; 1:5,000; Millipore/Chemicon, Temecula, CA).

Immunoblotting for NHE1 and the TGF-β receptor type I (TGF-β-RI) was performed using NHE1-HA immune-precipitated complexes. Cells plated in 100 mm dishes and maintained in growth medium for 48 h were washed 3X in PBS and lysed in a modified RIPA buffer (50 mM Tris-HCl, 135 mM NaCl, 3 mM KCl, 1 mM EDTA, 1% NP-40, and 0.1 TIU/ml aprotinin, 2 µg/ml leupeptin and 1 mM PMSF). Equal amounts of protein from post-nuclear supernatants (850 *g* for 3 min) were incubated for two hours with Sepharose-conjugate anti-HA antibodies (Roche Molecular Biochemicals). Immune complexes were recovered by

centrifugation at 10,000 *g* for 3 min, washed 3X and released from the beads by boiling for 5 min in 1x Laemmli sample buffer. Immunoprecipitated proteins separated by SDS-PAGE and transferred to PVDF membranes were probed with antibodies to NHE1 (1:2000; Millipore/Chemicon), Myc (9B11; 1:1000), HA (12CA5; 1:1000), or TGF-β-RI (1:500; vendor and location). Proteins in total cell lysates were immunoblotted using antibodies to β-actin (C4; 1:5,000; Millipore/Chemicon).

Bound antibody was detected by using the enhanced chemiluminescence system (Amersham, Piscataway, NJ).

Northern blotting

Cells were plated in 100 mm culture dishes to achieve 95%, 50% and 25% confluency after 48 h. Total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA). RNA (20 μg) was separated on 1.0% agarose gels containing formaldehyde and transferred to a nylon membrane (Hybond-N+, Amersham Pharmacia). A FN DNA fragment (FN N29; provided by C. Damsky, University of California, San Francisco) was used as a template to prepare radiolabeled probe by using the Rad Prime DNA system (Invitrogen) according to the manufacturer's directions. Hybridization was performed under stringent conditions with wash buffer 1x SSC (Roche), 0.1% SDS at 65°C for two hours. GAPDH was used to confirm equal loading of samples.

Luciferase assays

FN promoter assay: The pGLFN105 promoter vector (Promega, Madison, WI) containing the FN promoter region between -105 and +14 was provided by D. Mercola (Sidney Kimmel Cancer Center, San Diego, CA) and previously described (Liu *et al*., 2000). pRK5-betagal plasmid containing beta-galactosidase under the control of a human CMV promoter was provided by R. Derynck (University of California, San Francisco) and previously described (Feng *et al*., 1995). Cells were transfected by using Transfast according to the manufacturer's recommendations (Promega). For each transfection the total quantity of transfected plasmid DNA pGLFN105 and pRK5 was kept constant. Forty-eight hours after transfection, cells were harvested and lysed in Reporter Lysis Buffer (Promega) prior to analysis of reporter activity using a luciferase assay system (Pharmingen) and β-galactosidase detection kit (Tropix). Luciferase was detected using a luminometer (Molecular Devices, Sunnyvale, CA), luciferase activity was reported as relative light units (RLU) and results were normalized for transfection efficiency by beta-galactosidase activity.

TGF-β activity assay: Mink lung epithelial cells (MLEC) were used, stably expressing a construct containing a truncated plasminogen activator inhibitor-1 (PAI-1) promoter fused to the firefly luciferase reporter gene, and previously described (Abe *et al*., 1994). The cell lines tested (WT, KRA2 and PS120) were cultured as mentioned above for 24 hours to reach 90% confluency and the

medium from these cultures (conditioned medium) was used on the MLEC reporter cell line. Conditioned medium collected from the indicated cells maintained for 18 h in DMEM supplemented with 0.2% FBS was directly applied to MLEC cells for determining secreted active TGF-β or heated at 80° C for 10 min before applying for determining secreted latent TGF-β. In parallel cultures, MLEC cells were incubated in DMEM supplemented with rhTGF-β1 (0.1 to 5000 ng/ml; PeproTech). Cell extracts were prepared and assayed for luciferase activity using the Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase units obtained from conditioned medium samples were normalized to cell number and secreted TGF-β was quantified relative to luciferase units from samples supplemented with rhTGF-β1.

All assays were performed in triplicates and were repeated at least three times. Error bars represent the standard deviation of the sample.

FITC-FN labeling, assembly assays and FN induction with TGF-β1

Fibronectin assembly was determined by plating cells on glass coverslips coated with 15 μg/ml or 50 μg/ml of exogenous FITC-labeled bovine FN, or by adding exogenous FITC-labeled FN in the medium (McKeown-Longo and Mosher, 1985). Bovine plasma FN (Calbiochem-Novabiochem Corp, La Jolla CA) was labeled using the FluoReporter® FITC protein labeling kit (Molecular Probes Inc. Invitrogen) according to the manufacturer's directions. Controls included adding

medium without cells to FITC-FN coated coverslips. Cells were maintained for 48 hours, unless otherwise indicated, and then washed with PBS, fixed, and visualized as described for immunolabeling. FN assembly was similar either cells were plated on FITC-FN or the FITC-FN existed in the medium. Assembly on data shown was on pre-coated glass coverslips with FITC-labeled FN, unless otherwise indicated.

Integrin expression and FITC-FN binding

β1 integrin expression by biotinylation: To determine β1 integrin expression, cells were biotinylated on ice for 90 min, lysed in RIPA buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1mM EDTA, 1% NP-40, 1 mM Pefablock, 1 μg/ml Leupeptin, 1 μg/ml Aprotinin, 1 mM Na-orthovanadate), and the lysate was incubated with an antibody directed against β1 integrin (9EG7, 1:100) for 60 min, followed by incubation for 1 hour with protein A-Sepharose beads. Eluted proteins were probed for biotin with streptavidin-HRP.

β1 integrin expression by flow cytometry analysis: Integrin expression was confirmed by flow cytometry analysis. One million cells per sample were incubated in 1 ml PBS with 3% PBS-based dissociation buffer (Gibco BRL) and 2% FBS. collected in culture medium, washed twice with PBS and kept in suspension in PBS containing 2% FBS. After incubating cells with anti-β1 integrin antibodies in PBS at 4°C for 1 hour, cells were washed with PBS and

incubated with Alexa Fluor® 488 (A488) anti-rabbit (Invitrogen; gift from T. Wittmann, University of California, San Francisco) at 4°C for 30 min. Cells were washed in PBS and analyzed on a FACS Vantage SE cell sorter (Becton Dickinson Inc., San Jose CA). Flow cytometry data were analyzed using CellQuest Pro 4.0.1 software (Becton Dickinson Inc.).

FN binding assays: β1 integrin binding to FN, cells in suspension were mixed with the indicated concentrations of FITC-labeled FN. After incubating cells with the labeled protein for 60 min, FITC-positive cells were analyzed as described above.

Intracellular pH Measurements

NHE1 activity and intracellular pH were determined in cells plated at 25 X 10^5 per well in 12-well plates and loaded with the fluorescent pH-sensitive dye 2,7-biscarboxyethyl-5(6)-carboxyfluorescein (BCECF; Invitrogen, Carlsbad, CA) by modifications of previously described methods (Denker *et al.*, 2000). Ratios of BCECF fluorescence at Ex 490/Em 530 and Ex 440/Em 530 were acquired using a SpectraMax M5 plate reader (Molecular Dynamics, Sunnyvale, CA). Fluorescence ratios were converted to pH_i by calibrating each experiment with 10 μM nigericin (Invitrogen) in 105 mM KCl. NHE1 activity was determined by measuring the rate of pH_i recovery (dpH_i/dt) from an acid load by evaluating the derivative of the slope of the time-dependent pH_i recovery at intervals of 0.05 pH units.

Statistical Analysis

All experiments were performed at least three times. Data was analyzed with Prism 4 GraphPad Software (GraphPad Software, Inc., La Jolla, CA). Statistical significance between tested cell lines and consecutive experiments was evaluated with two-way analysis of variance. The statistical significance level between cell lines was determined by unpaired two-tail t-test analysis, unless otherwise indicated. Blots were quantified with the ImageJ 1.38x Software, National Institutes of Health, USA.

Error bars in graphs represent the standard error of the mean of the samples.

3. Results

FN expression and transcription are decreased in cells with mislocalized NHE1

ERM and PI(4,5)P2 binding by NHE1 is necessary for focal adhesion formation and directed cell migration. In KRA2 fibroblasts expressing NHE1 with mutations in charged clusters of lysine and arginine residues substituted by alanine in the juxtamembrane region of the COOH-terminus that constitute binding sites for ERM proteins (Denker *et al.*, 2000) and for $PI(4,5)P_2$ (Aharonovitz *et al.*, 2000) NHE1 is not clustered in lamellipodia but is uniformly distributed along the plasma membrane (Figure 3A) and FA complexes are fewer and smaller (Figure 3B). The migratory rate of fibroblasts expressing mutant NHE1 that does not bind to ERM and PI(4,5)P2 is also impaired in wound assays. Wound closure is significantly delayed in the fibroblasts expressing the mislocalized mutant NHE1, compared with the WT cells (Denker and Barber, 2002). In asking why FA complexes and migration are attenuated in KRA2 cells, extracellular matrix proteins were examined. More specifically, the expression of collagen and FN was tested.

Immunolabeling extracellular FN in live confluent fibroblasts on ice without permeabilization shows a marked decrease in secreted FN in KRA2 cells compared with WT cells (Figure 4A) or CCL39 fibroblasts (Figure 5A). KRA2

cells have decreased total FN (both extracellular and intracellular) (Figure 4B), determined by immunolabeling of permeabilized cells, and decreased secreted FN, determined by immunoblotting the culture medium (Figure 4C). Decreased total FN is not a clonal variation and is observed in all cell clones generated from separate transfection experiments and stably expressing mislocalized mutant NHE1-KRA2 (Figure 5B).

After establishing that FN secretion is decreased in KRA2 cells, FN transcription was tested. FN transcription has been reported to be sensitive to cell density and cell shape, increasing with increased cell density or decreased cell area (Perkinson *et al*., 1996). To test whether cell density is playing a role in FN transcription, cells at 25, 50 and 95% confluency were tested. Northern blot analysis reveals a significant decrease in FN mRNA in KRA2 cells compared with WT cells at all three densities (Figure 6A and B) ($p<0.001$; $n=3$), while cell density does not affect FN transcription in either WT or KRA2 cells (Figure 6C) ($p>0.05$; $n=3$). FN promoter activity also is significantly decreased in the KRA2 cells compared with WT cells, determined by expression of the pGLFN105 promoter vector containing a fragment (-105 and +14) of the human FN promoter (Figure 6D) ($p < 0.05$, $n = 3$). These data indicate that in KRA2 cells FN production is decreased at the level of transcription and promoter activity.

Collagen V Expression is similar in KRA2 and WT cells as shown by RT-PCR analysis (Figure 6E). Although available antibodies to collagen I, II, III and

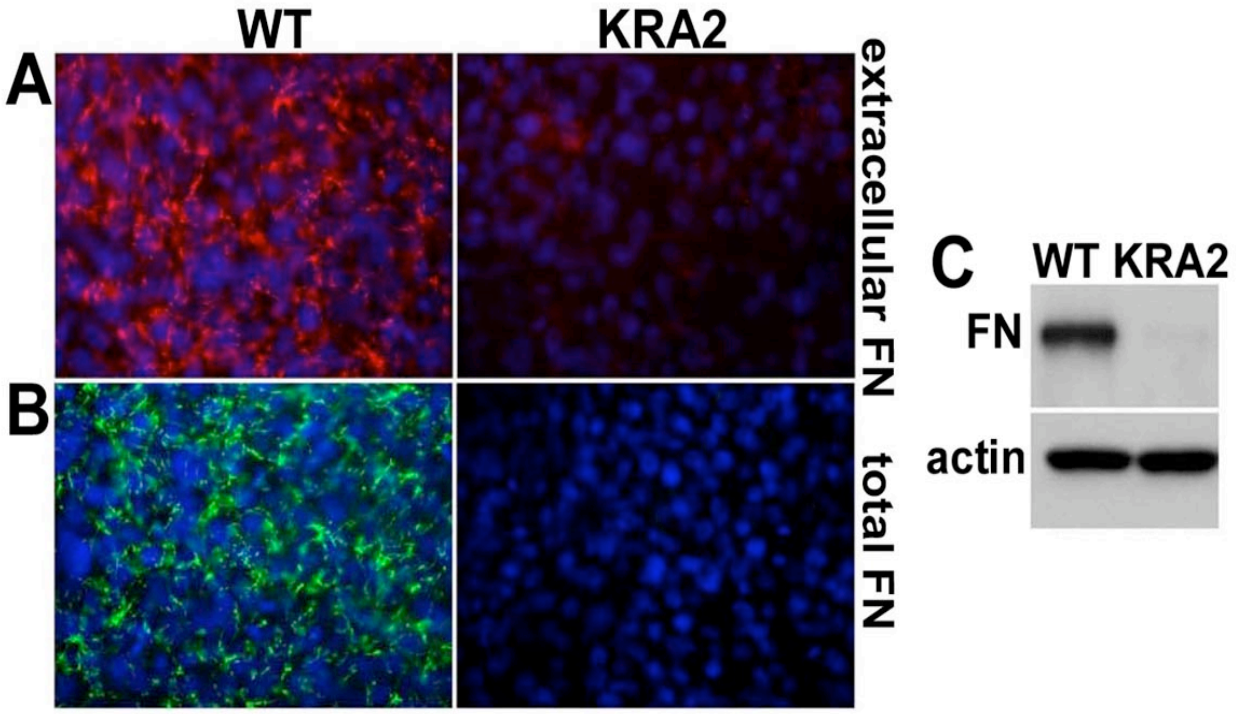


Figure 4.

Fibronectin expression is decreased in fibroblasts expressing mislocalized NHE1-KRA2. (A) Live-cell immunolabeling with FN antibodies reveals a marked decrease in secreted FN in KRA2 cells compared with WT cells (red: extracellular FN, blue: Hoechst staining for nuclei). (B) Total FN in permeabilized cells is decreased in KRA2 cells compared with WT cells (green: total FN, blue: Hoechst staining for nuclei). (C) Immunoblotting culture medium indicates secreted FN is attenuated in KRA2 cells compared with the WT cells. Immunoblotting for actin in cell lysates was used to confirm equivalent amount of total protein used to measure secreted FN.

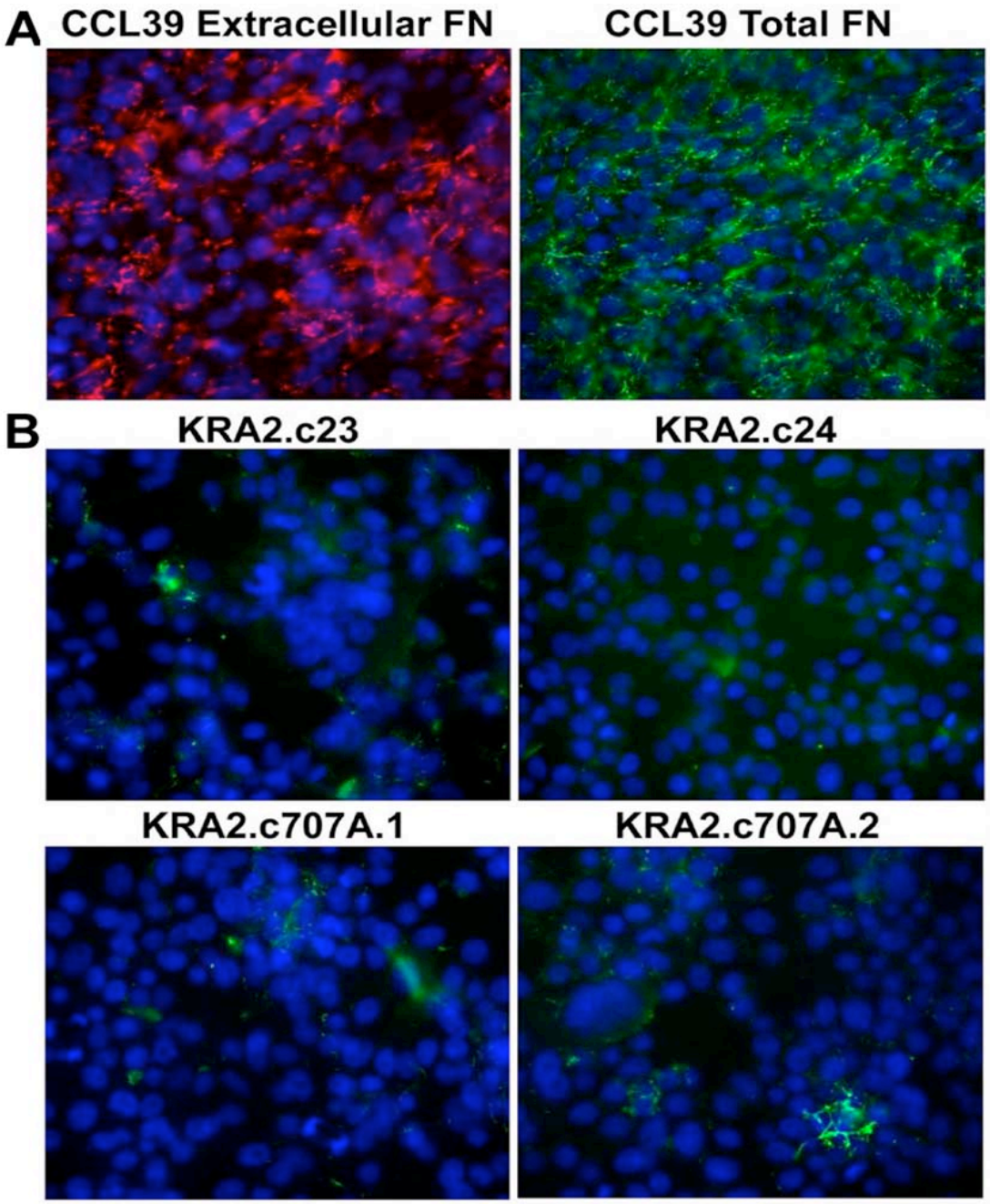

A
CCL39 Extracellular FN
CCL39 Total FN
B
KRA2.c23
KRA2.c24
KRA2.c707A.1
KRA2.c707A.2

Figure 5.

Figure 5.

FN production is attenuated in KRA2 cell clones compared with CCL39 cells. (A) Immunolabeling of secreted (left) and total (right) fibronectin in NHE1-expressing CCL39 fibroblasts, which are parental cells for NHE1-deficient PS120 cells. (left, live cell labeling, red: extracellular FN, blue: Hoechst staining for nuclei) (right, fixed cell labeling, green: total FN, blue: Hoechst staining for nuclei). (B) Immunolabeling of total FN in the indicated KRA2 clones confirms that attenuated FN production is not a clonal variation (green: total FN, blue: Hoechst staining for nuclei).

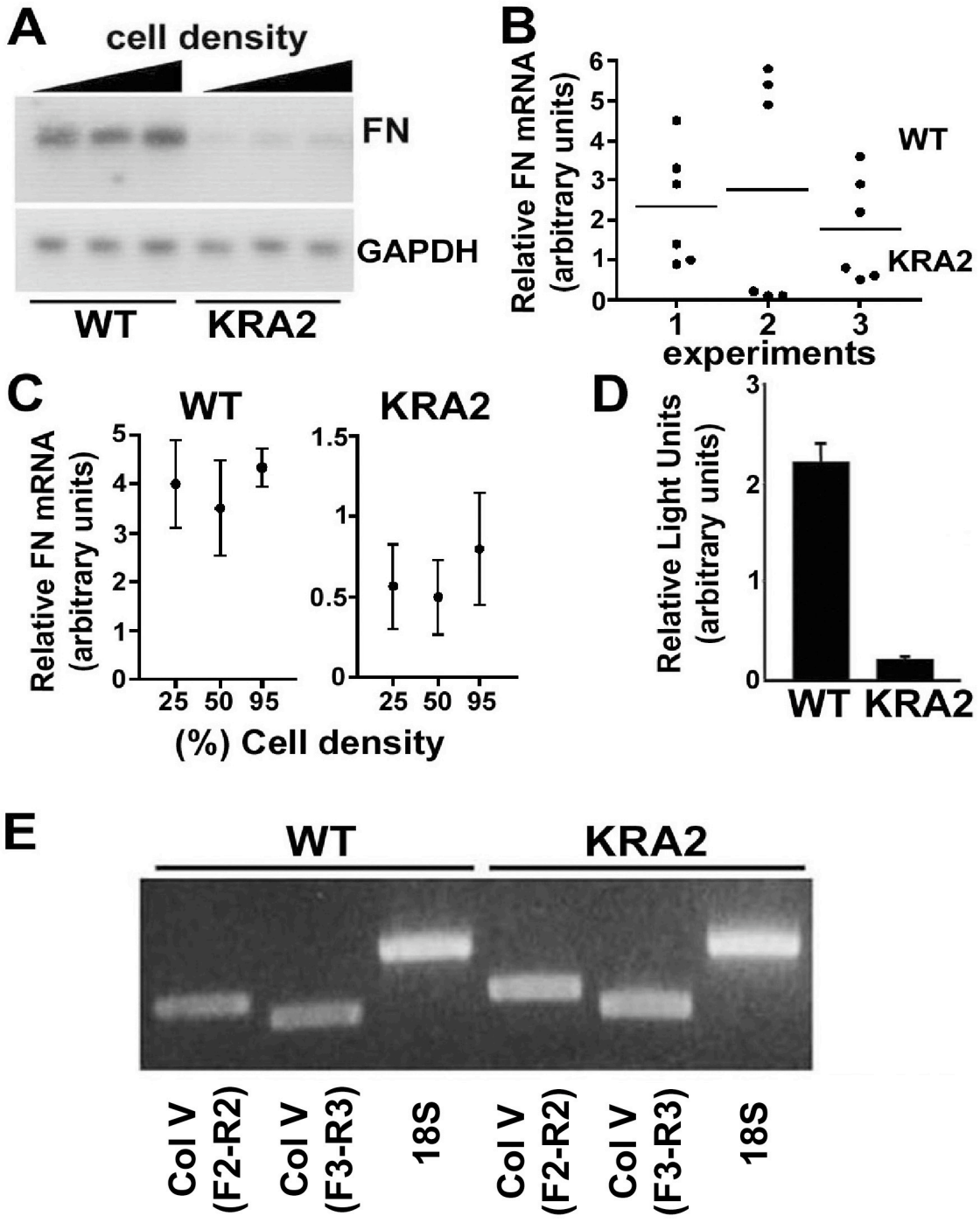

A
cell density
FN
GAPDH
WT
KRA2
B
Relative FN mRNA (arbitrary units)
WT
KRA2
experiments
C
WT
KRA2
Relative FN mRNA (arbitrary units)
25 50 95
25 50 95
(%) Cell density
D
Relative Light Units (arbitrary units)
WT KRA2
E
WT
KRA2
Col V (F2-R2)
Col V (F3-R3)
18S
Col V (F2-R2)
Col V (F3-R3)
18S

Figure 6.

Figure 6.

Fibronectin transcription is decreased in fibroblasts expressing mislocalized NHE1-KRA2. (A and B) Northern blot analysis shows attenuated FN mRNA in KRA2 cells compared with WT cells, with GAPDH used as a loading control. Data are representative of blots from mRNA isolated from 3 separate experiments and cell preparations ($p < 0.01$, $n = 3$). (C) FN transcription is not affected within each cell line by cell density and was similar for 95%, 50% and 25% confluency (WT: $p > 0.05$, $n = 3$; KRA2: $p > 0.05$, $n = 3$). Data represent means ± sem. (D) FN promoter activity, determined using a fragment of the human FN promoter between -105 to +14, is markedly decreased in the KRA2 cells compared with WT cells ($p < 0.05$, $n=3$). Data represent means ± sem. (E) RT-PCR showing transcripts for hamster collagen a1 (V) chain in WT and KRA2 fibroblasts. Bands indicated are products using different collagen primers (F2 to R2 and F3 to R3), and products of control primers for 18S RNA. Data are representative of two independent cell preparations.

V did not immunolabel hamster WT and KRA2 fibroblasts, RT-PCR indicated both cell types express collagen V. Primers designed from the mouse collagen I for RT-PCR did not yield any results for the hamster WT and KRA2 fibroblasts.

Fibronectin assembly but not integrin expression or FN binding is impaired in KRA2 fibroblasts

In addition to having decreased FN production and secretion, KRA2 cells are unable to assemble exogenous FN into a fibrillar matrix. When plated for 48 h on FITC-labeled FN (15 μg/ml) or with soluble FITC-labeled FN in the medium (15 μg/ml), WT cells but not KRA2 cells assemble a fibrillar matrix (Figure 7A and B). Cell-dependent assembly of FN is confirmed in control incubations without cells (Figure 7A, right panel). KRA2 cells also do not assemble a fibrillar matrix with 30 or 50 μg/ml of exogenous FITC-labeled FN (data not shown), indicating impaired matrix assembly is independent of the exogenous FN concentration. Because fibronectin assembly is dependent on integrin activation (Wu *et al*., 1995; Wennerberg *et al*., 1996; Sechler *et al*., 2000), impairment of integrins activation is considered as a possible cause of the impaired FN production and assembly, and integrin activation might induce either FN assembly or production or both in KRA2 cells. By activating integrins with $MnCl_2$ and integrin activating 9EG7 antibodies, FN assembly is partially restored in KRA2 cells. After plating KRA2

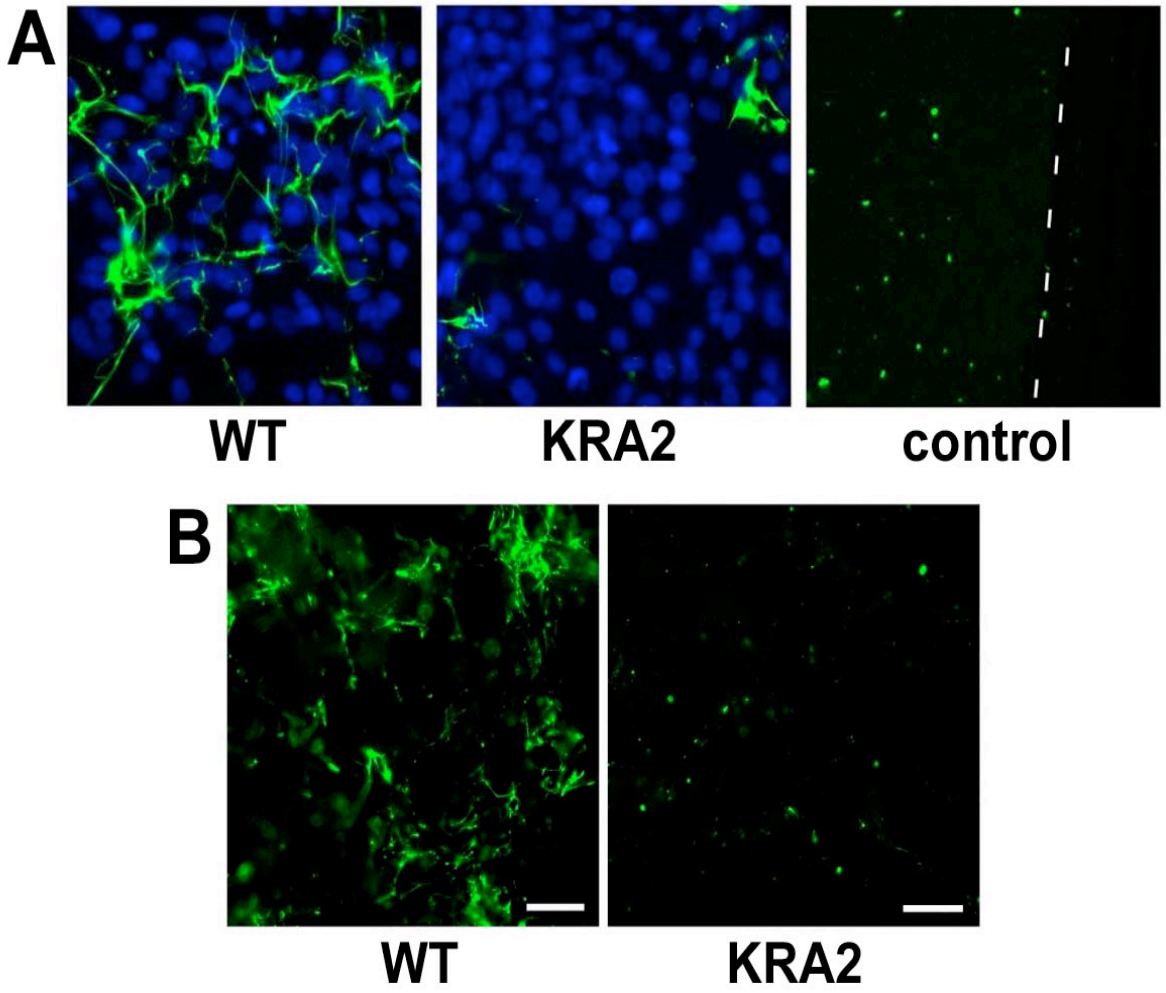


Figure 7.

Figure 7.

Fibronectin assembly is impaired in KRA2 fibroblasts. (A) WT cells but not KRA2 cells plated for 48 h assemble a matrix of exogenous FITC-labeled FN. FITC-FN coated coverslips without cells are included as a control to confirm cell-dependent FN assembly (right panel). The right end of the FITC-FN coated coverslip was scraped with a pipette tip (across the dotted line). (B) KRA2 cells do not assemble a matrix of exogenous soluble FN. Fluorescence images of KRA2 and WT cells maintained for 48 h in medium containing FITC-labeled FN (15 μg/ml) (Bar, 20 μm).

cells on FITC-FN for 24 h, very limited matrix assembly is seen with the addition of $MnCl_2$ (Figure 8, two left columns) (or with the addition of 0.5 μg/ml 9EG7 antibody, data not shown). More assembled FN is seen with the combination of $MnCl_2$ and 9EG7 antibodies compared with $MnCl_2$ or 9EG7 alone, however, assembly is still less than that seen with WT cells (Figure 8). Addition of $MnCl_2$ and/or 9EG7 does not affect endogenous FN synthesis, as shown by immunolabeling of permeabilized WT and KRA2 cells for total FN (Figure 8, two right columns).

Three approaches were used to show that expression of β1 integrin is similar in WT and KRA2 cells: immunolabeling live, unpermeabilized cells with β1 antibodies (Figure 9A), β1antibodies were used to immunoprecipitate lysates of biotinylated cells and immunoblotted immune complexes with streptavidin-HRP (Figure 9B), and flow cytometry analysis of dissociated cells labeled with β1 antibodies (Figure 9C and Figure 10) ($p > 0.05$, $n = 3$). There also is no difference in total cell binding of FITC-labeled bovine FN quantified by flow cytometry for WT and KRA2 cells between 45 to 450 nM FN (Figure 11A and C) ($p > 0.05$, $n = 5$). Specificity of FITC-FN binding is confirmed by blocking binding to WT cells with increasing concentrations of a FN RGD fragment (Figure 11B). These data suggest that β1 expression and FN binding are not impaired in KRA2 cells, although attenuated integrin avidity, which cannot be directly measured in fibroblasts, might contribute to the inability of KRA2 cells to assemble a FN

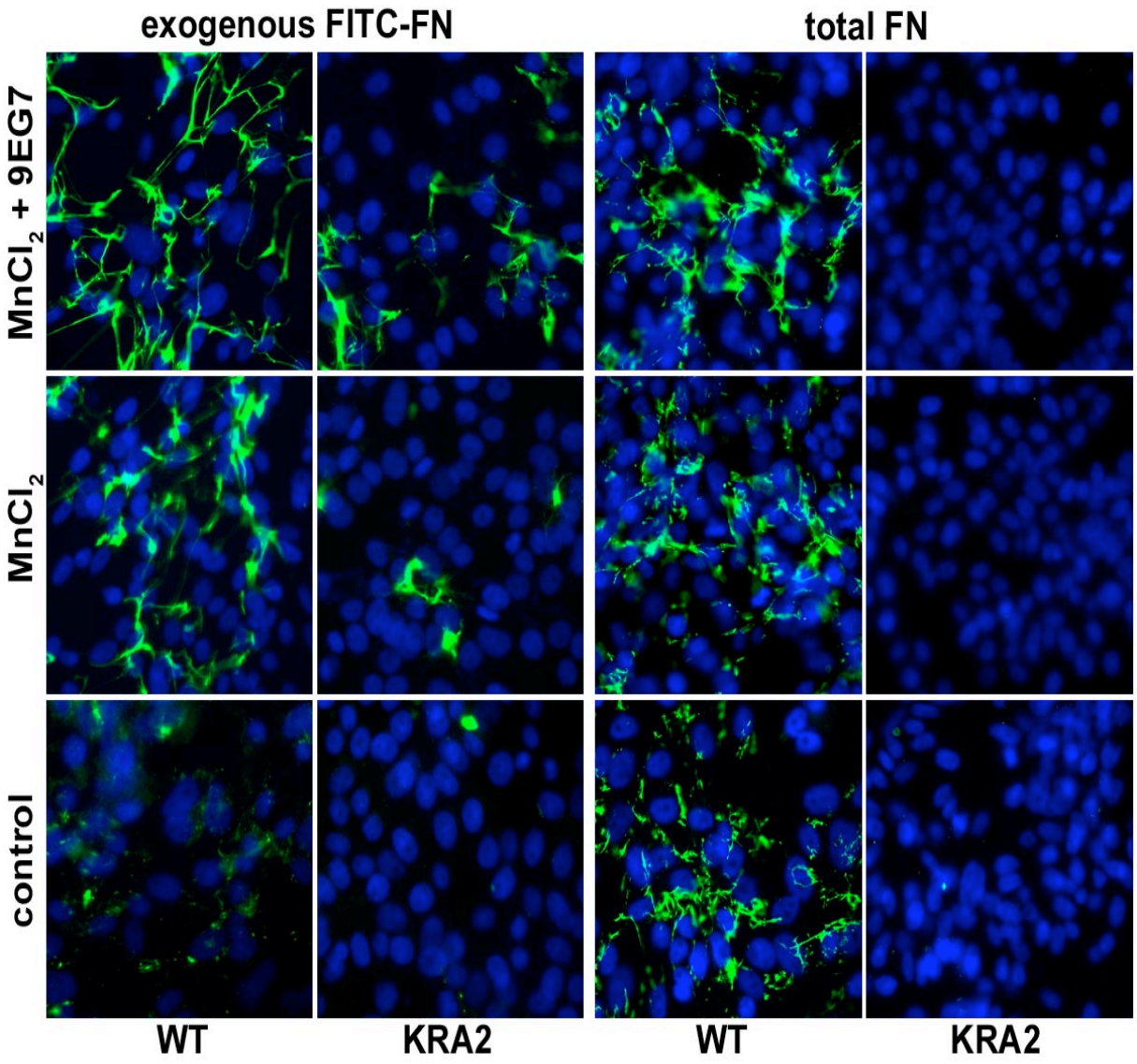

exogenous FITC-FN
total FN
MnCl2 + 9EG7
MnCl2
control
WT
KRA2
WT
KRA2

Figure 8.

Figure 8.

Impairment of FN assembly but not FN production in KRA2 cells is partially rescued by integrin activation. (A) Exogenous FITC-labeled FN assembly in WT and KRA2 fibroblasts at 24 hours after integrin activation with 0.05 mM $MnCl_2$ alone or with 0.5 μg/ml integrin activating antibody 9EG7. $MnCl_2$ plus 9EG7 accelerates FN assembly in WT cells and partially restores FN matrix assembly in KRA2 cells (green: exogenously added FITC-labeled FN, blue: Hoechst/nuclei). (B) Immunolabeling of total FN in WT and KRA2 cells 48 hours after integrin activation with 0.05 mM $MnCl_2$ alone or with 0.5 μg/ml integrin activating antibody 9EG7. (green: total FN, blue: Hoechst/nuclei).

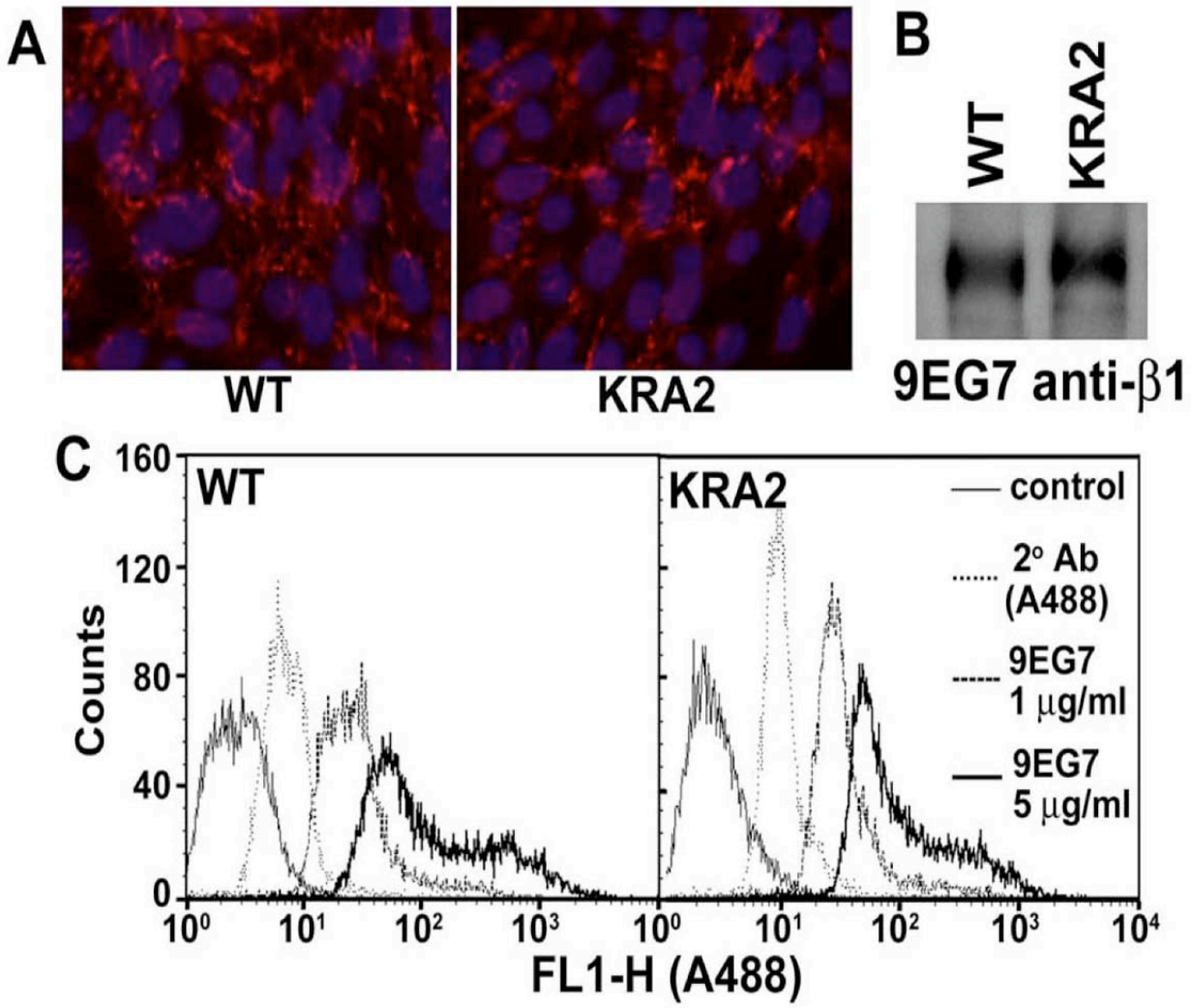

A
WT
KRA2
B
WT
KRA2
9EG7 anti-β1
C
WT
KRA2
control
2° Ab (A488)
9EG7 1 μg/ml
9EG7 5 μg/ml
Counts
160
120
80
40
0
10⁰
10¹
10²
10³
10⁴
FL1-H (A488)

Figure 9.

Figure 9.

β1 integrin expression is similar in WT and KRA2 fibroblasts. (A) Live-cell immunolabeling the surface of WT and KRA2 fibroblasts with β1 integrin antibodies (red: extracelllular β1 integrin, blue: Hoechst staining for nuclei). (B) Cell surface expression of β1 integrin in WT and KRA2 cells determined after biotinylation, immunoprecipitation with 9EG7 anti-β1 antibodies, and immunoblotting the immune complex with streptavidin-HRP. (C) Cell surface expression of β1 integrin in WT and KRA2 cells determined by flow-cytometry analysis using the indicated concentrations of anti-β1 9EG7 antibodies. Secondary antibody (A488) alone was used to show non-specific binding.

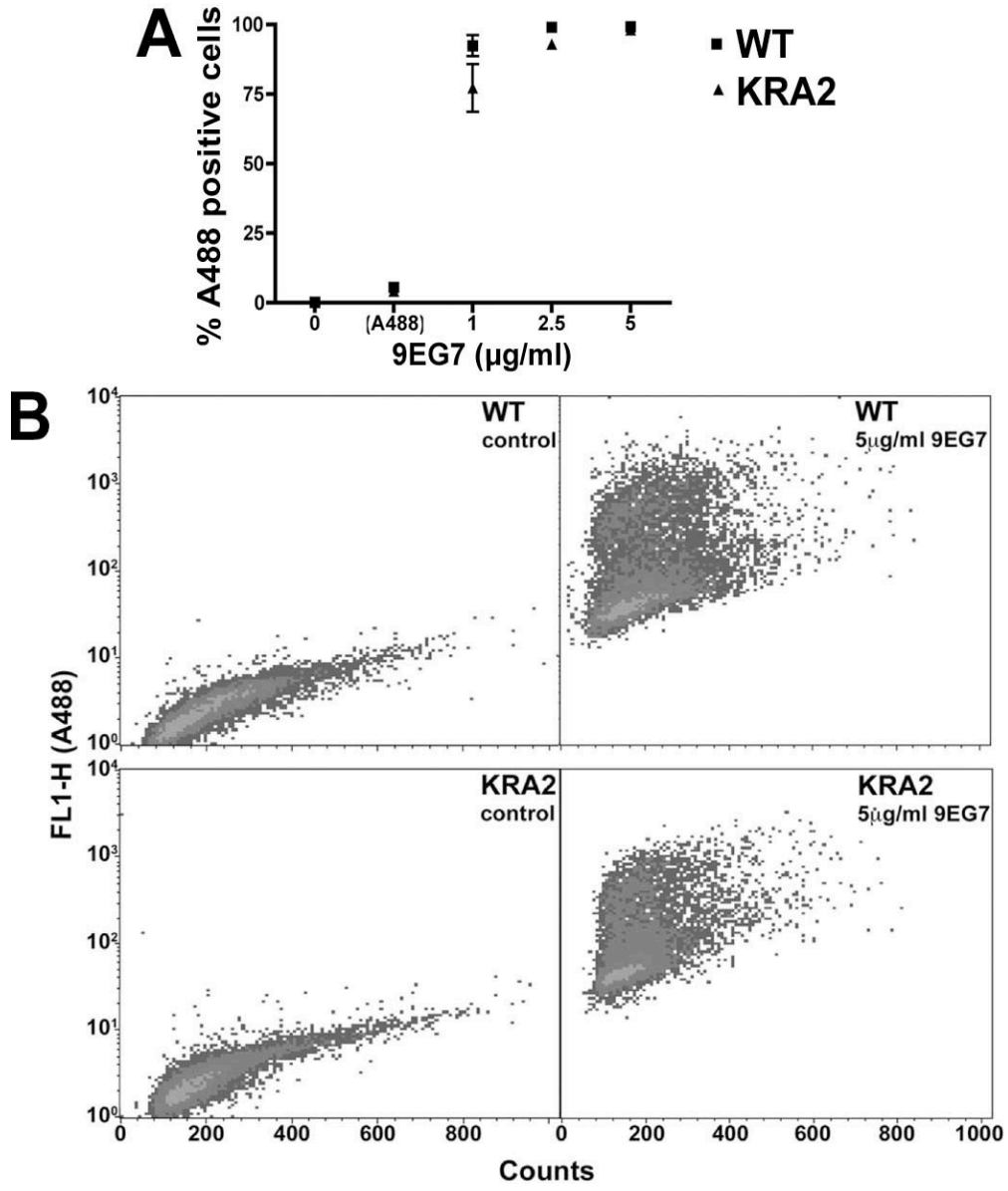

A
% A488 positive cells
100
75
50
25
0
0
(A488)
1
2.5
5
9EG7 (µg/ml)
WT
KRA2
B
WT
control
WT
5µg/ml 9EG7
KRA2
control
KRA2
5µg/ml 9EG7
FL1-H (A488)
Counts
0
200
400
600
800
1000

Figure 10.

Figure 10.

β1 integrin expression is not decreased in KRA2 cells compared with WT cells. (A) Cell surface expression of β1 integrin with flow-cytometry analysis using the indicated concentrations of anti-β1 9EG7 antibodies. Secondary antibody (A488) alone was used to show non-specific binding ($p > 0.05$, $n=3$). Data are means ± sem of 3 separate binding assays. (B) Representative data from flow-cytometry analysis showing surface expression of β1 integrin in WT and KRA2 cells at 0 and 5 μg/ml anti-β1 9EG7 antibody.

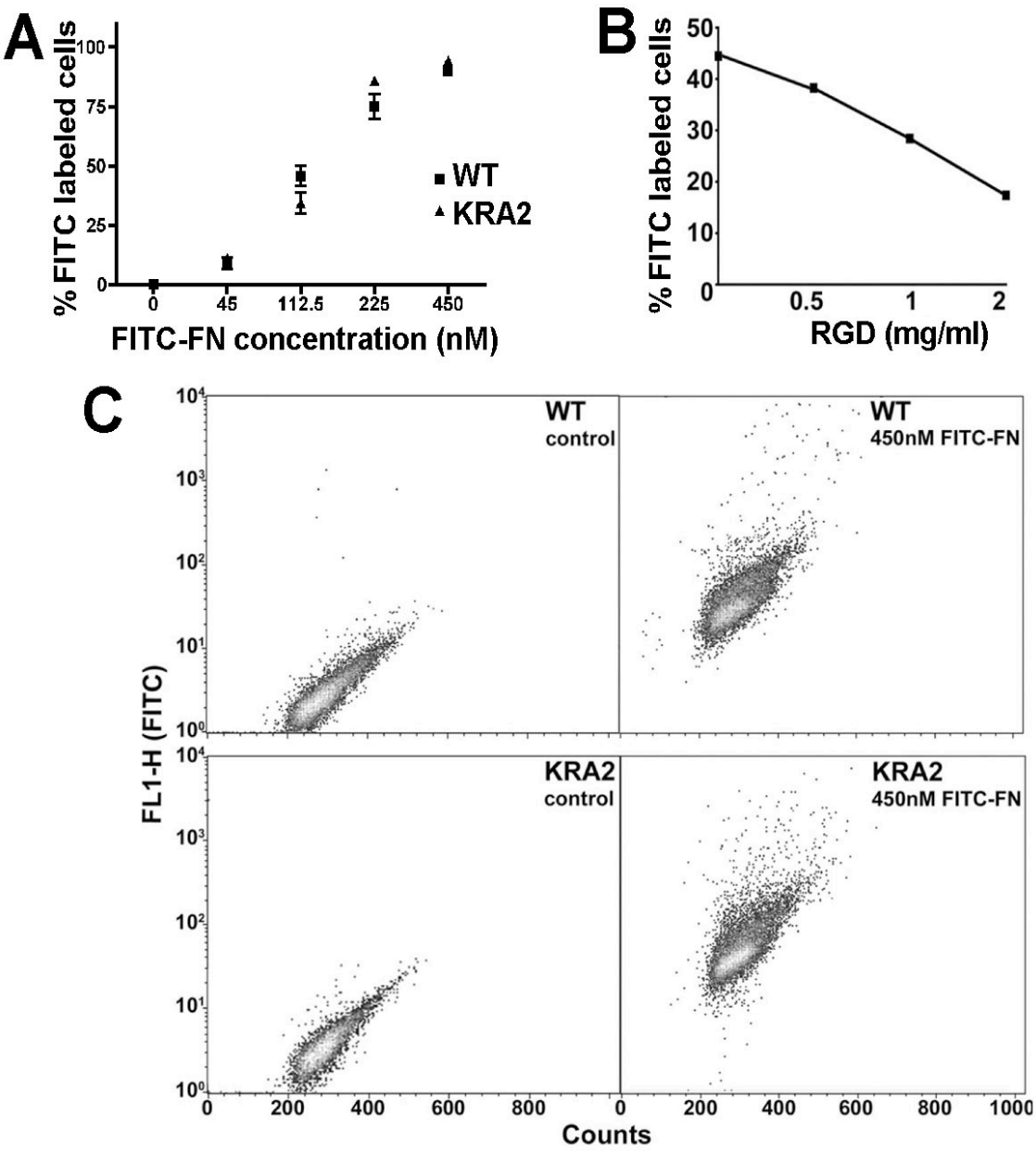

A
% FITC labeled cells
100
75
50
25
0
0
45
112.5
225
450
FITC-FN concentration (nM)
WT
KRA2
B
% FITC labeled cells
50
40
30
20
10
0
0.5
1
2
RGD (mg/ml)
C
WT
control
WT
450nM FITC-FN
KRA2
control
KRA2
450nM FITC-FN
FL1-H (FITC)
Counts
0
200
400
600
800
1000

Figure 11.

Figure 11.

β1 integrin affinity for FN is similar in WT and KRA2 cells. (A) Binding of FITC-FN determined by flow-cytometry analysis indicates no difference between WT and KRA2 cells ($p > 0.05$, n=5). Data are means ± sem of 5 separate binding assays. (B) Integrin affinity to 112.5 nM FITC-FN is inhibited by incubation with increasing concentrations of an integrin antagonist RGD fragment. Data are means of 3 separate binding assays. (C) Representative data from flow-cytometry analysis showing FN binding of WT and KRA2 cells with FITC-labeled FN at 0 and 450 nM.

matrix. However, integrin activity is not necessary for FN biosynthesis (Wu *et al.*, 1995; Feral *et al.*, 2007), suggesting that decreased FN transcription in KRA2 cells is independent of possible impaired integrin signaling. Moreover, these data indicate that decreased FN production in KRA2 cells does not attenuate β1 integrin expression.

H^+ efflux by NHE1 is not necessary for FN production or assembly

The next question was whether FN production and assembly are impaired in KRA2 cells because H^+ efflux is decreased or mislocalized. Although KRA2 cells have regulated NHE1-dependent H^+ efflux, NHE1 activity is attenuated compared with that in WT cells (Aharonovitz *et al.*, 2000). However, live cell immunolabeling reveals that extracellular FN matrix abundance and organization is not impaired in cells lacking H^+ efflux by NHE1 (Figure 12). This is confirmed in NHE1-deficient PS120 cells and in PS120 cells stably expressing a mutant inactive NHE1-E266I (E266I cells) (Denker *et al.*, 2000). These data also suggest that decreased steady-state intracellular pH (pH_i) does not inhibit FN production because although the steady-state pH_i of KRA2 cells is ~7.2 and less than the ~ 7.4 pH_i of WT cells, the pH_i of PS120 and E266I cells is ~ 7.0 (Denker *et al.*, 2000; Frantz *et al.*, 2007).

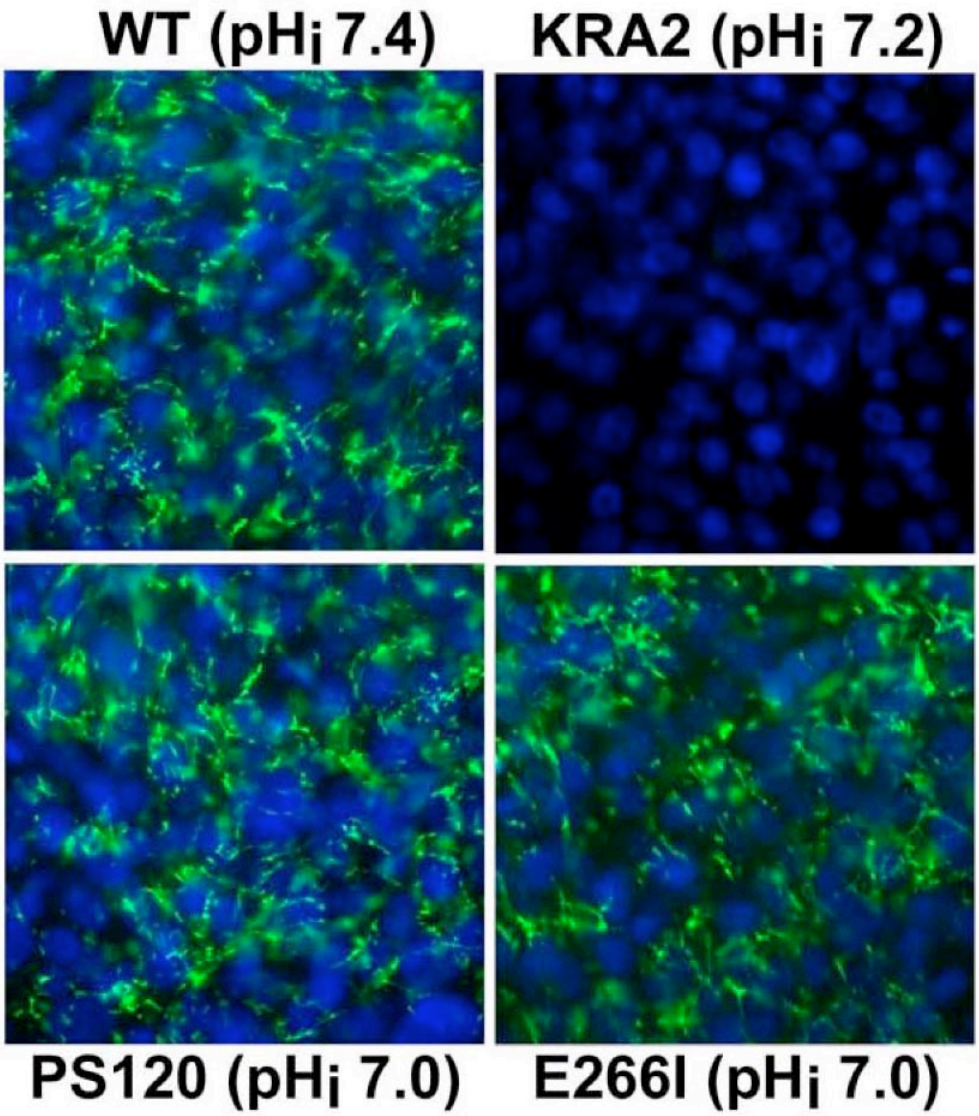


Figure 12.

Figure 12.

pH_i is not a determinant for FN production or assembly. NHE1-deficient PS120 cells and PS120 cells stably expressing mutant inactive NHE1-E266I lacking H^+ efflux have a FN matrix similar to the WT cells. Steady-state pH_i is given for indicated cell types. (green: extracelllular FN, blue: Hoechst staining for nuclei). Steady-state pH_i is indicated for each incubation condition. Data are representative of 3 separate cell preparations.

Although NHE1-E266I is inactive, it retains binding to ERM proteins and PI(4,5)P2 and localizes like wild-type NHE1 at the distal margin of lamellipodia (Denker *et al.*, 2000). Therefore, it is speculated that perhaps FN production is attenuated in KRA2 cells because H^+ efflux is mislocalized. To test this prediction PS120 cells were used that stably express the system N1 transporter (SN1 cells). SN1 is a plasma membrane amino acid transporter expressed in the central nervous system that couples uptake of extracellular glutamine with efflux of intracellular H^+ efflux. When expressed in PS120 cells, SN1 is uniformly localized along the plasma membrane and increased H^+ efflux can be driven acutely by increasing the concentration of extracellular glutamine (Chaudry *et al.*, 1999). However, extracellular FN secretion by SN1 cells is similar to that by WT cells at extracellular glutamine concentrations of 25 μM, in which SN1 is quiescent and pH_i is 7.01, at 400 μM, which increases H^+ efflux by SN1 resulting in pH_i of 7.3, or when maintained in DMEM growth medium that contains 4 mM of glutamine and pH_i is 7.45 (Figure 13). FN secretion by WT cells is independent of extracellular glutamine concentration. These data confirm that FN production and matrix assembly is independent of pH_i regulation.

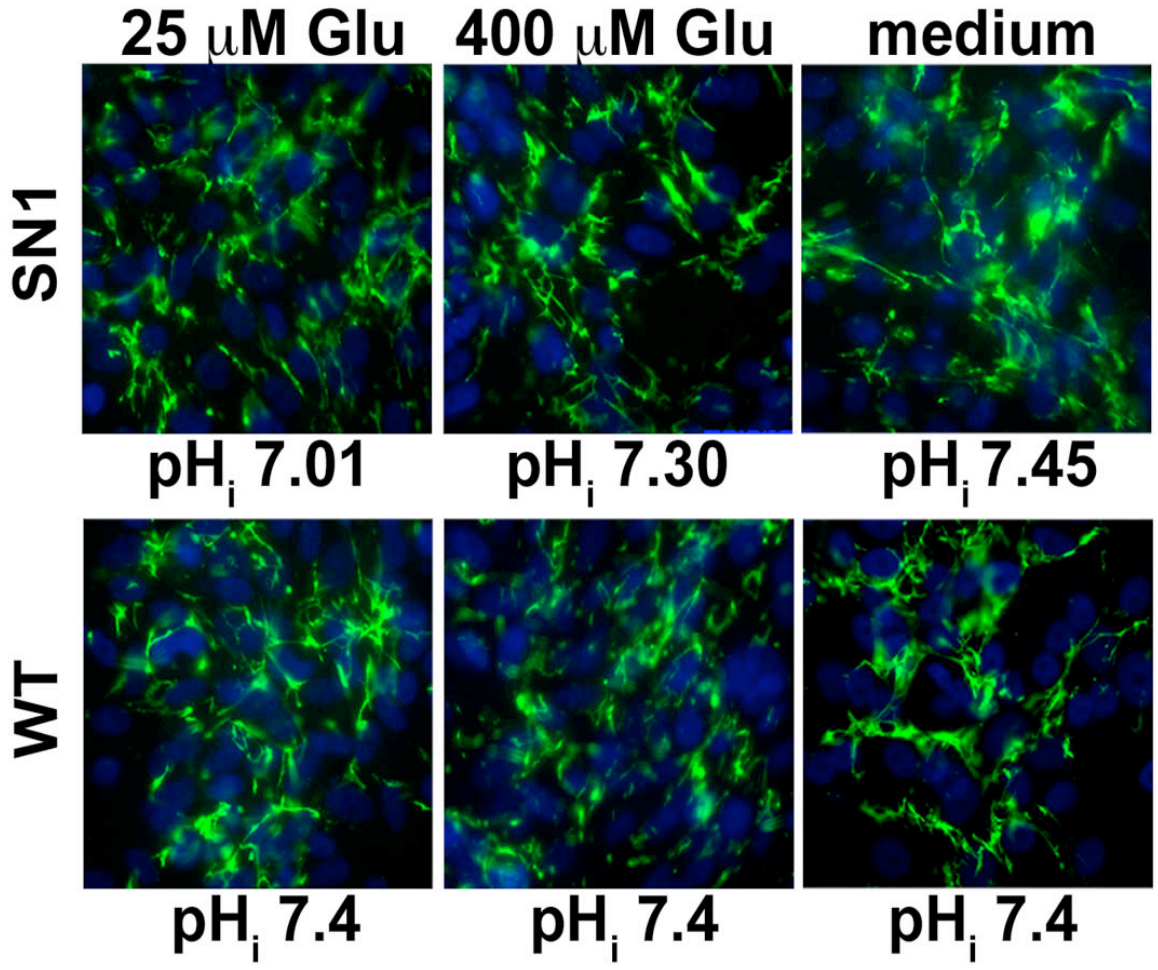


Figure 13.

Mislocalized H^+ efflux by NHE1 is not necessary for FN production or assembly. Live-cell FN immunolabeling of WT cells and SN1 cells (PS120 fibroblasts stably expressing the SN1 amino acid transporter) maintained in glutamine-free DMEM supplemented with 25 mM and 400 μM extracellular glutamine or in growth medium, which contains 4 mM glutamine (green: extracelllular FN, blue: Hoechst staining for nuclei). Steady-state pH_i is indicated for each incubation condition. Data are representative of 3 separate cell preparations.

Localized scaffolding by NHE1 restores FN production in KRA2 cells

Attenuated FN production and assembly in KRA2 cells is not determined by decreased or mislocalized H^+ efflux, and NHE1 expression is not necessary for FN production. In addition to catalyzing Na^+ and H^+ exchange, NHE1 is recognized as a scaffolding platform through its direct binding of more than ten signaling proteins. NHE1-KRA2 might act in a dominant negative manner, perhaps by sequestering and mislocalizing a signaling molecule necessary for FN production. To test this prediction NHE1 with normal localization in membrane protrusions was co-expressed in KRA2 cells to observe whether FN production could be restored. To confirm independence of H^+ efflux NHE1-E266I-Myc was stably co-expressed in KRA2 cells (K2E cells). Expression of E266I-NHE1-Myc is confirmed by immunoblotting and has no effect on the abundance of NHE1-KRA2-HA expression (Figure 14A). Immunolabeling confirms the clustered localization of E266I-NHE1-Myc in K2E cells at membrane protrusions (Figure 14B) while the HA-NHE1-KRA2 is evenly distributed along the membrane, similar in the K2E and the KRA2 cells (Figure 14C). Immunolabeling unpermeabilized cells for extracellular FN (Figure 14D) and permeabilized cells for total FN (data not shown) and immunoblotting for secreted FN in the medium (Figure 14E) indicate that FN production and assembly are restored in K2E cells compared with KRA2 cells and is similar to FN production in E266I cells.

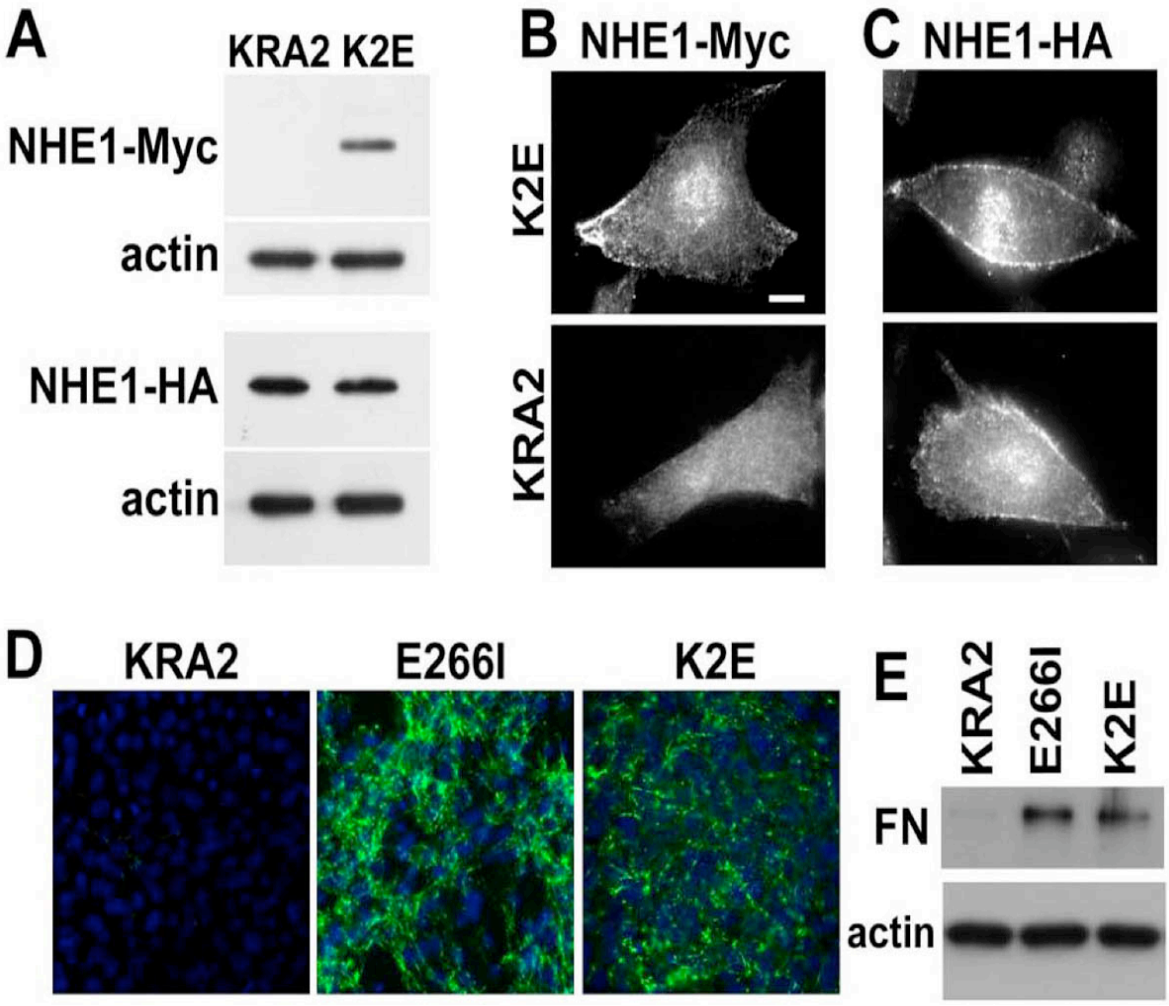

A
KRA2 K2E
NHE1-Myc
actin
NHE1-HA
actin
B NHE1-Myc
K2E
KRA2
C NHE1-HA
D
KRA2
E266I
K2E
E
KRA2
E266I
K2E
FN
actin

Figure 14.

Figure 14.

FN production in KRA2 cells is restored by co-expression of NHE1-E266I localized at membrane protrusions in K2E. (A) Expression and (B and C) localization of NHE1-E266I-Myc and NHE1-KRA2-HA in the indicated cells determined by immunoblotting and immunolabeling, respectively, with antibodies to Myc and HA. Immunoblotting with antibodies to actin was used as a loading control. (B and C, Bar, 5 μm) (D) Live-cell immunolabeling of FN in KRA cells, E266I cells and K2E cells co-expressing NHE1-KRA2 and NHE1-E266I indicate co-expression of NHE1-E266I restores FN production and matrix assembly (green: extracelllular FN, blue: Hoechst staining for nuclei). (E) Immunoblot of secreted FN in KRA2, E266I and K2E cells confirms K2E cells have restored FN matrix compared with KRA2 cells.

There was no clonal variation in FN production, which was seen in three different selected clones from two different transfection experiments (Figure 15). FN production is restored probably because scaffolding by NHE1-E266I might suppress a dominant negative action of NHE1-KRA2.

Mislocalized scaffolding by NHE1 does not impair FN production in CCL39 parental cells

To test whether mislocalized scaffolding by NHE1 might decrease the FN production in CCL39 cells, the mislocalized NHE1-KRA2 was co-expressed in CCL39 parental fibroblasts with endogenous NHE1 (CK cells). Fourteen CK clones were generated by two independent experiments (clones CK 2.1 - 2.8 and 3.1 - 3.6). The expression of NHE1-KRA2-HA in the indicated CK cell clones, as determined by immunoblotting with antibodies to HA, is variable (Figure 16A). Immunoblotting for actin is used as a loading control. The variablility of the expression of NHE1-KRA2 in the CK cells does not translate to any obvious decrease in the FN production, as shown by live-cell immunolabeling of FN in CK cells co-expressing NHE1-KRA2-HA with WT-NHE1 (Figure 16B). These data indicate that the localized scaffolding by the endogenous NHE1 in the CK cells may outweigh the effect of the mislocalized scaffolding of the co-expressed KRA2-NHE1 in CCL39 fibroblasts.

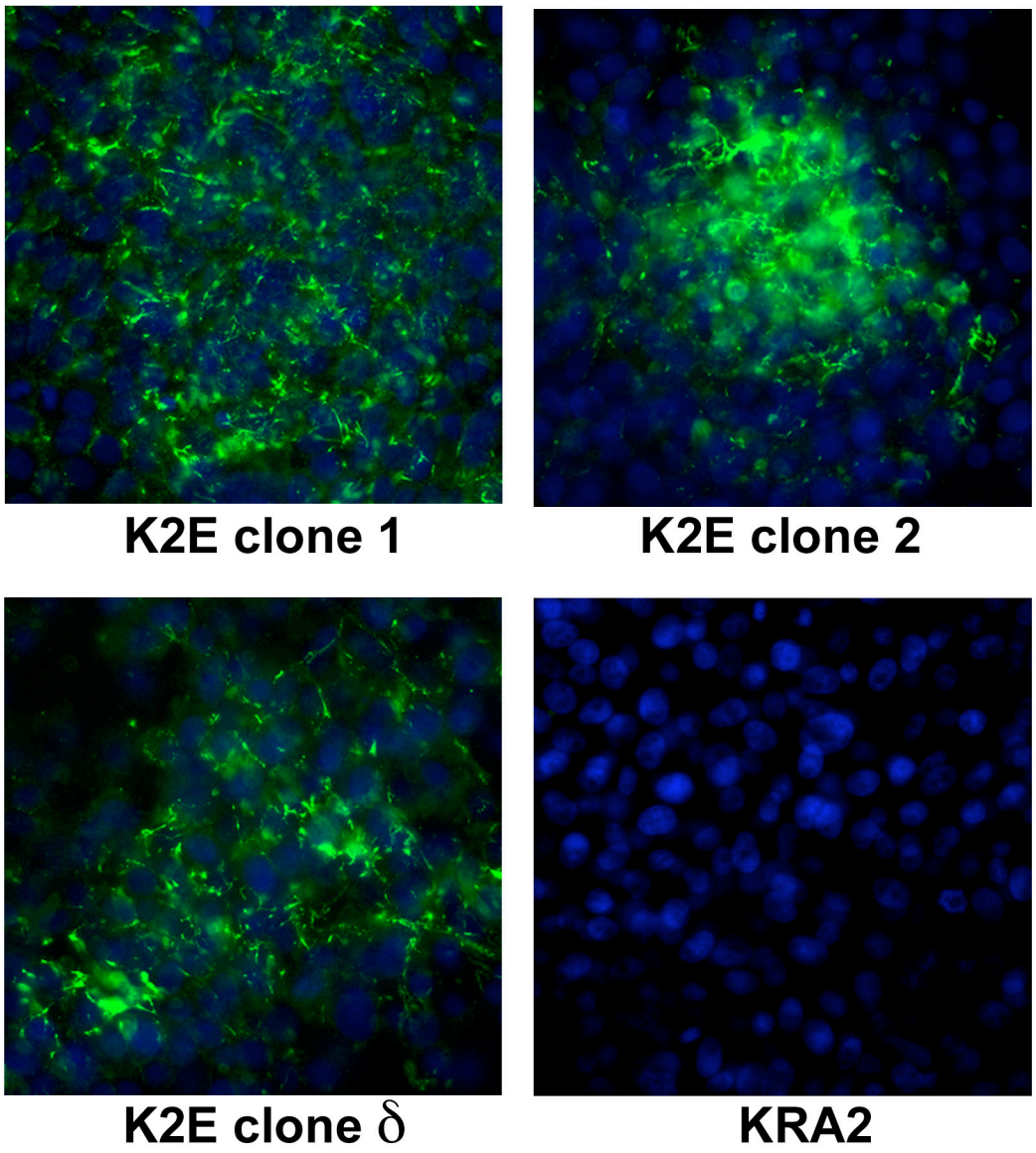


Figure 15.

The restored FN production in KRA2 cells by co-expression of NHE1-E266I is not a clonal variation (green: extracellular FN, blue: Hoechst staining for nuclei).

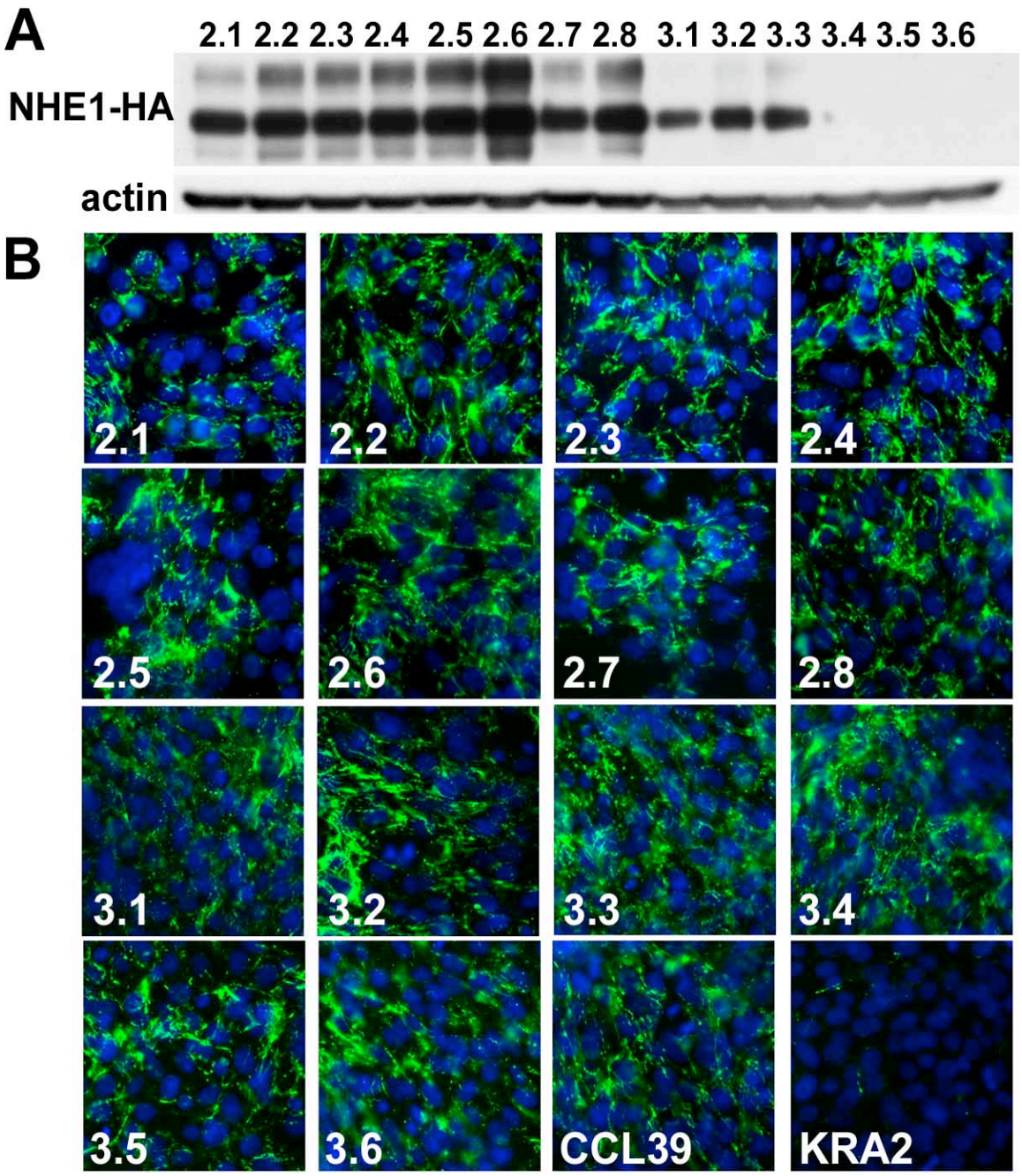

A
2.1 2.2 2.3 2.4 2.5 2.6 2.7 2.8 3.1 3.2 3.3 3.4 3.5 3.6
NHE1-HA
actin
B
2.1
2.2
2.3
2.4
2.5
2.6
2.7
2.8
3.1
3.2
3.3
3.4
3.5
3.6
CCL39
KRA2

Figure 16.

Figure 16.

FN production in CCL39 cells is not impaired by co-expression of mislocalized NHE1-KRA2 in CK cells. (A) Expression of NHE1-KRA2-HA in the indicated CK cell clones determined by immunoblotting with antibodies to HA. Immunoblotting for actin was used as a loading control. (B) Live-cell immunolabeling of FN in CK cells co-expressing NHE1-KRA2-HA with WT-NHE1 indicates no effect in FN secretion (green: extracelllular FN, blue: nuclei). CK clones 2.1 - 2.8 and 3.1 - 3.6 were generated in two independent experiments.

KRA2 cells have delayed autophosphorylation of FAK

Several cell processes are impaired in KRA2 cells, including bundling of actin filaments into stress fibers and assembly of FA complexes (Denker *et al*. 2000; Figure 3B). However, because these processes are also impaired in NHE1-deficient PS120 cells (Denker et al, 2000; Denker and Barber, 2002) that express and assemble FN (Figure 12), they likely do not determine attenuated FN biosynthesis in KRA2 cells. Autophosphorylation of focal adhesion kinase (FAK)-Y397 with plating on FN is delayed in KRA2 cells compared with WT cells (Figure 17B) ($p > 0.05$, $n = 7$), however this delay is also seen in PS120 cells and in E266I cells (Figure 17A). Additional signaling mechanisms associated with regulating FN production, including expression of the transcription factor CREB, phosphorylation of extracellular signaling kinase ERK, and increased glycolytic flux, are attenuated in E226I cells (Putney and Barber, 2004; Meima and Barber, unpublished observations) and are therefore unlikely causes of decreased FN synthesis in KRA2 cells.

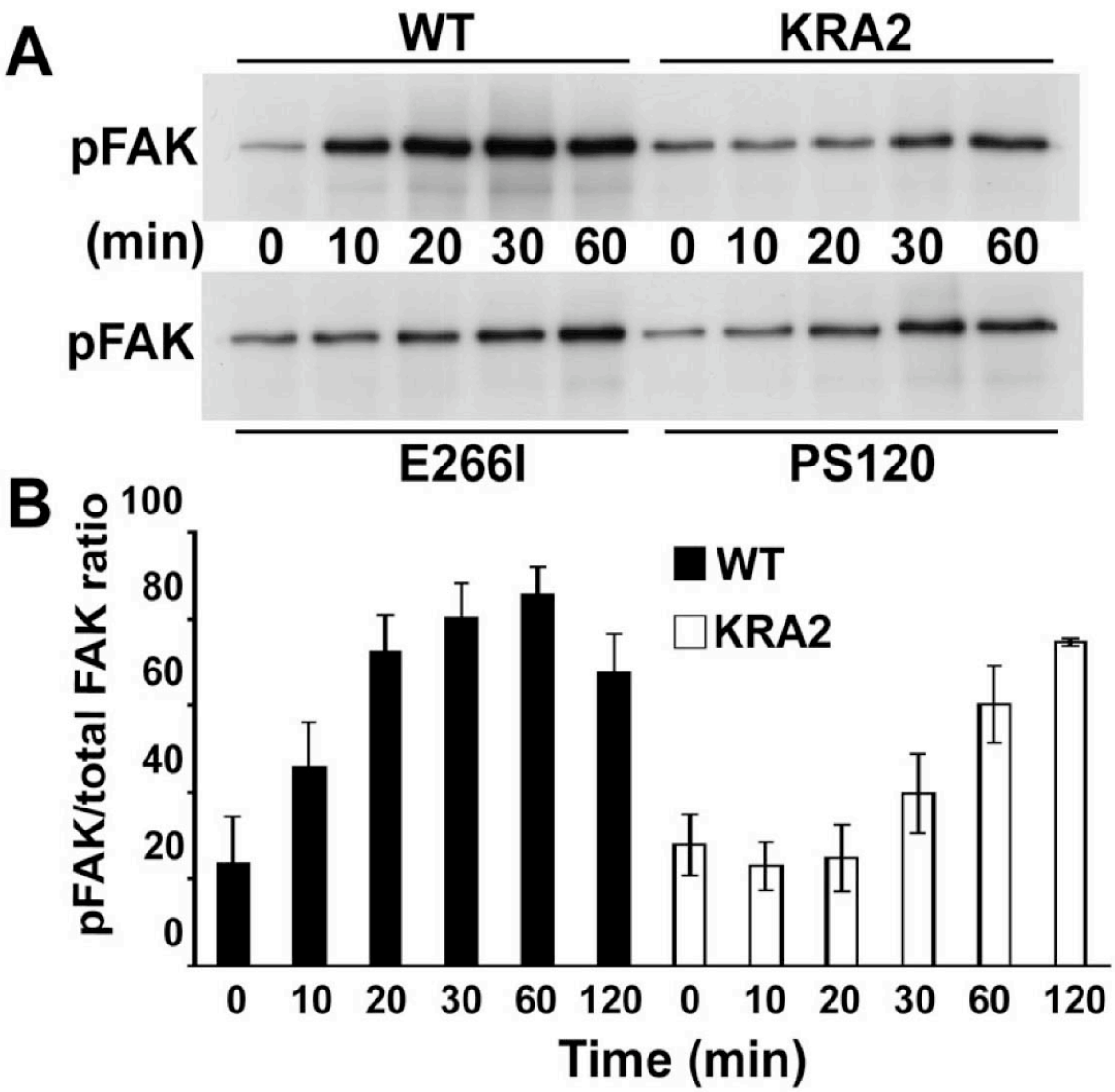

A
WT
KRA2
pFAK
(min)
0 10 20 30 60 0 10 20 30 60
pFAK
E266I
PS120
B
pFAK/total FAK ratio
100
80
60
40
20
0
WT
KRA2
0 10 20 30 60 120 0 10 20 30 60 120
Time (min)

Figure 17.

Figure 17.

KRA2 cells have delayed autophosphorylation of FAK. (A) Time-dependent increase in autophosphorylation of FAK after plating cells on FN (10 mg/ml) determined by immunoblotting cell lysates with antibodies to phosphorylated FAK-Y397 is delayed in KRA2, E266I, and PS120 cells compared with the WT cells ($p > 0.05$, $n = 7$). Data are means ± sem of 7 separate experiments.

(B) Abundance of FAK autophosphorylation relative to total FAK, determined by immunoblotting, after plating WT and KRA2 cells for the indicated times on FN ($p > 0.05$, $n = 7$). Data are means ± sem of 7 separate cell preparations.

Active TGF-β is decreased, and exogenous TGF-β restores FN production and assembly in KRA2 cells

Because cytokines, particularly TFG-β, stimulate FN expression (Blobe *et al*., 2000), we asked whether FN production could be restored in KRA2 cells with exogenous TFG-β. Two approaches were used to test whether TGF-β signaling is involved in the impairment of FN production in KRA2 fibroblasts: the first approach by asking whether inhibiting TGF-β could impair the FN production in WT cells and the second by asking whether FN production could be restored in KRA2 cells treated with active TFG-β. Treating WT cells with the pharmacological inhibitor of TGF-β-Type I receptor kinase activity SB-431542 (Laping *et al*., 2006) inhibits FN production, as shown by immunolabeling for extracellular FN of live unpermeabilized WT and KRA2 cells treated for 48 h with the TGF-β-RI inhibitor SB-431542 (10 mM) in the absence and presence of TGF-β (5 ng/ml) (Figure 18). These data indicate that FN production in WT cells is TGF-β-dependent, and therefore impaired TGF-β signaling and/or activity may be responsible for the attenuated FN production in fibroblasts expressing the mislocalized KRA2-NHE1.

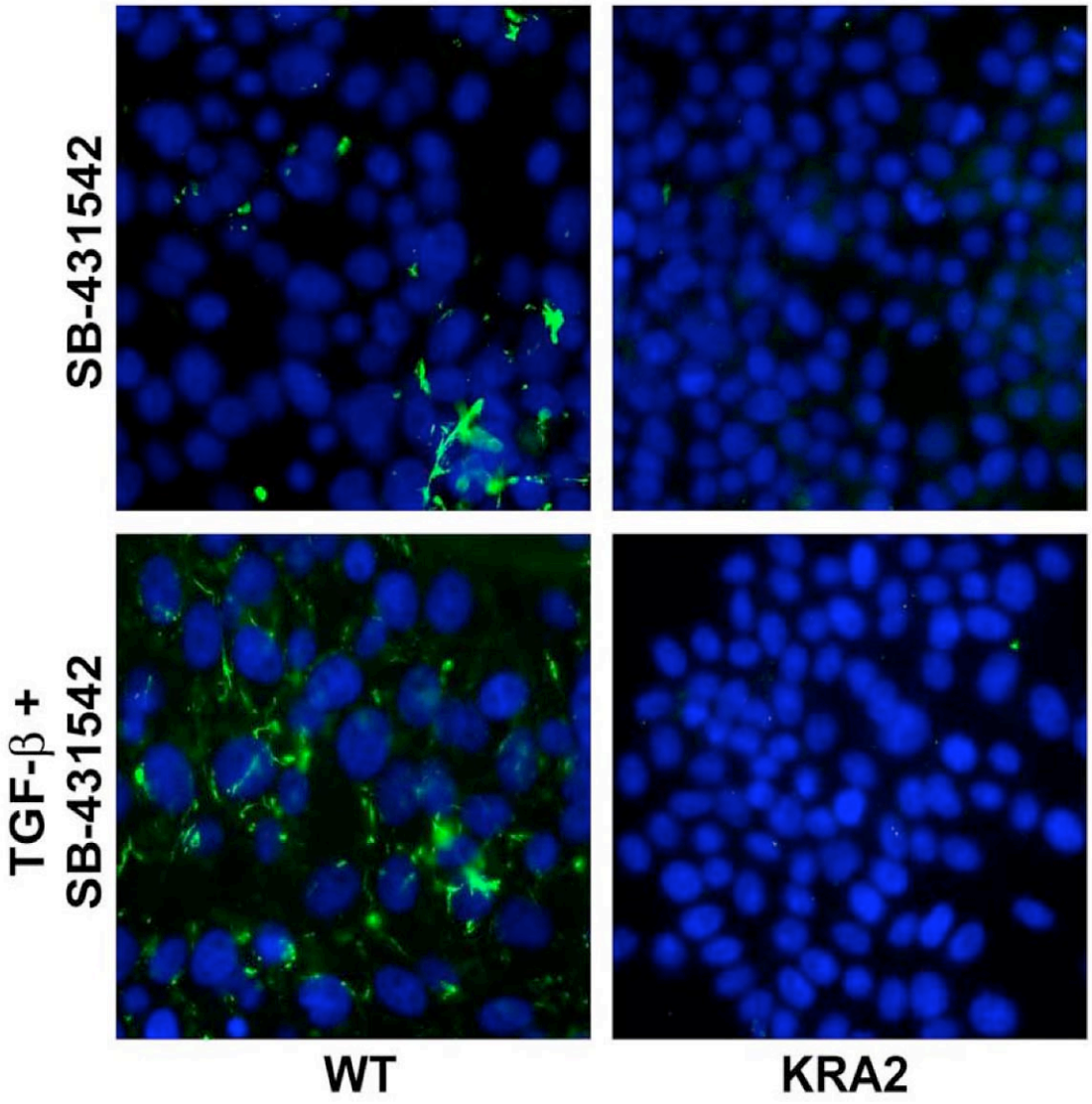


Figure 18.

Pharmacological inhibition of TGF -β-RI inhibits FN production. Immunolabeling for extracellular FN of live WT and KRA2 cells treated with the TGF-β-RI inhibitor SB-431542 (10 mM) in the absence and presence of TGF-β. (green: extracellular FN, blue: Hoechst staining for nuclei).

To test whether FN production can be restored in KRA2 cells with TFG-β, cells were treated with exogenous TFG-β (5 ng/ml) for 48 h. The TFG-β treatment restores FN production and assembly by KRA2 cells and appears to increase fibrillogenesis in WT cells (Figure 19A), as shown by immunolabeling of unpermeabilized cells for extracellular FN. These data confirm that FN production in WT and KRA2 cells is TGF-β-dependent and indicate that impaired TGF-β activation or signaling may be responsible for the attenuated FN production.

To test whether the level of active TGF-β might be attenuated in KRA2 cells, a luciferase assay was used with MLEC cells expressing a truncated PAI-1 promoter that is activated by TGF-β (Abe *et al.,* 1993). Secreted active TGF-β in conditioned medium of KRA2 cells is significantly less compared with WT cells ($p < 0.001$, $n = 6$) or PS120 cells ($p < 0.01$, $n = 6$ cell preparations[1]) but similar in WT and PS120 cells ($p > 0.1$, $n = 6$) (Figure 19B, left panel). However, secreted total or latent TGF-β, determined in boiled samples of conditioned medium, is not significantly different in KRA2 cells compared with WT or PS120 cells ($p > 0.1$; $n = 4$) (Figure 19B, right panel). Activation of latent TGF-β is regulated by a number of conditions including proteolytic cleavage of LLC and by transmission of cell traction forces to latent TGF-β bound to integrins and the ECM (reviewed by Wipff and Hinz, 2008), and whether decreased activation by KRA2 cells is determined by lack of FN production or by other factors such as

[1] Experiments were repeated by the mentor's lab

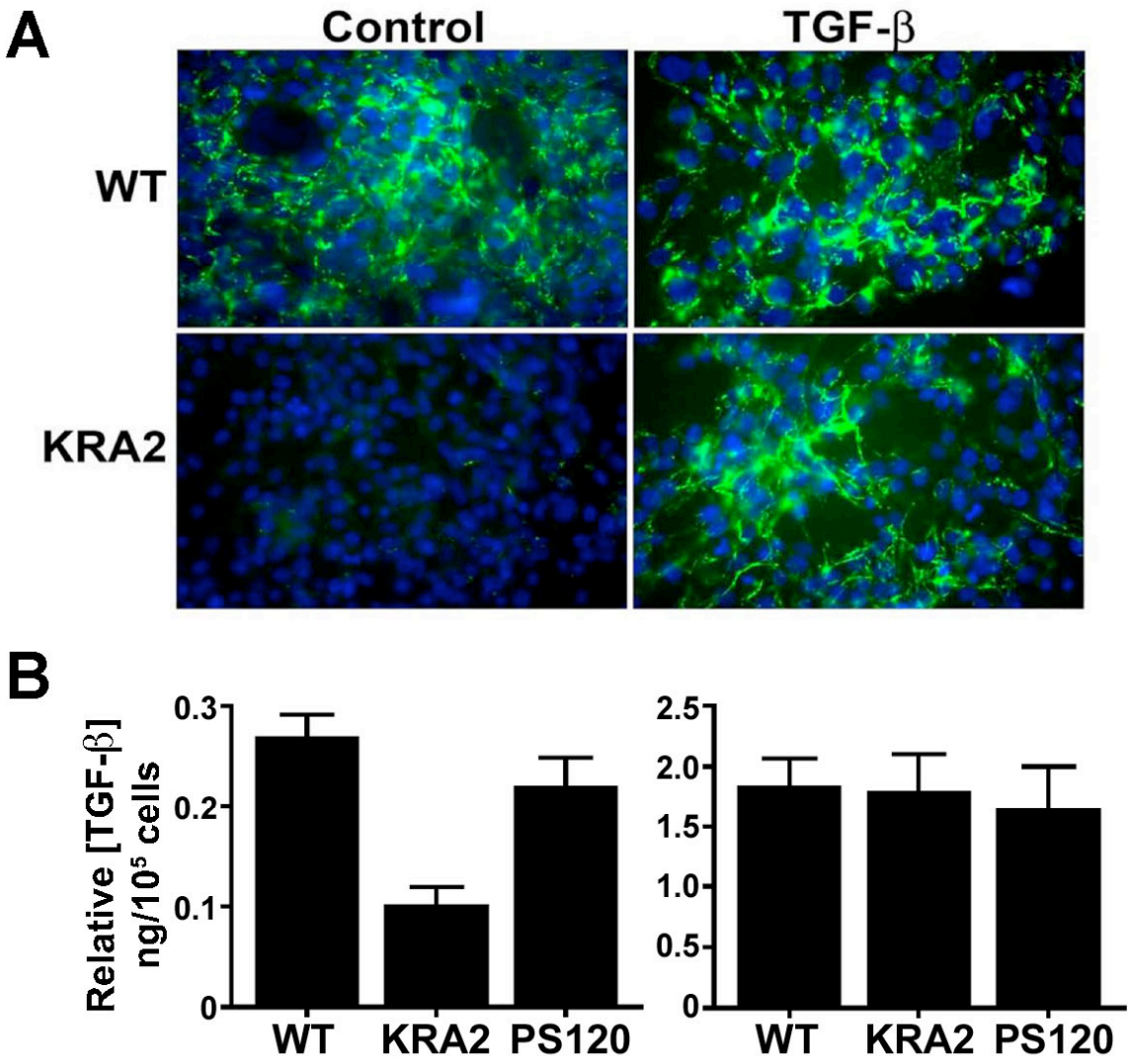

A
Control
TGF-β
WT
KRA2
B
Relative [TGF-β] ng/10⁵ cells
0.3
0.2
0.1
0
WT
KRA2
PS120
2.5
2.0
1.5
1.0
0.5
0
WT
KRA2
PS120

Figure 19.

Figure 19.

TGF-β1 restores FN production and assembly by KRA2 cells. (A) FN production and assembly, determined by immunolabeling for extracellular FN, is similar in WT and KRA2 cells treated with 5 ng/ml of rhTGF-β1 for 48 h. Data are representative of 4 separate cell preparations. (B) Active (left panel) and total (right panel) TGF-β determined by luciferase assays using MLEC cells expressing a truncated plasminogen activator inhibitor-1 (PAI-1) promoter and treated with conditioned medium from WT, KRA2 and PS120 cells. The relative TGF-β concentration (ng/ml/10^5 cells) is quantified by luciferase assays of MLEC cells treated with control concentrations of recombinant human TGF-β1 (0, 0.1, 0.5 and 2ng/ml) and is expressed in. Active TGF-β is significantly reduced in KRA2 cells compared with WT ($p < 0.001$, $n = 6$) and PS120 cells ($p < 0.01$, $n = 6$cell preparations) but similar in WT and PS120 cells ($p > 0.1$, $n = 6$). but total (active and latent) TGF-β, as determined in boiled samples of conditioned medium, is not significantly different for KRA2 cells compared with WT or PS120 cells ($p > 0.1$, $n = 4$ cell preparations). Data are expressed as means ± sem of relative TGF-β concentration normalized per 10^5 cells.

decreased abundance or activity of extracellular proteases remains to be determined.

A hypothesis that would explain the reduced TGF-β activity in KRA2 cells is that mislocalized NHE1 interferes with TGF-β signaling, as NHE1 (Bullis *et al*., 2002; Bourguignon *et al*., 2004) and TGF-β-R1 (Razani *et al*., 2001) co-sediment in caveolin-enriched plasma membrane fractions. Endogenous TGF-β-R1 co-precipitates with wild-type and mutant NHE1-E266I and NHE1-KRA2 (Figure 20A), suggesting the proteins co-localize in a membrane microdomain. However, increased phosphorylation of SMAD3, a downstream effector of TGF-β-R1 activation, is similar in KRA2 and WT cells treated with exogenous TGF-β (Figure 20B). These data suggest that active TGF-β but not intracellular signaling in response to TGF-β is impaired in KRA2 cells.

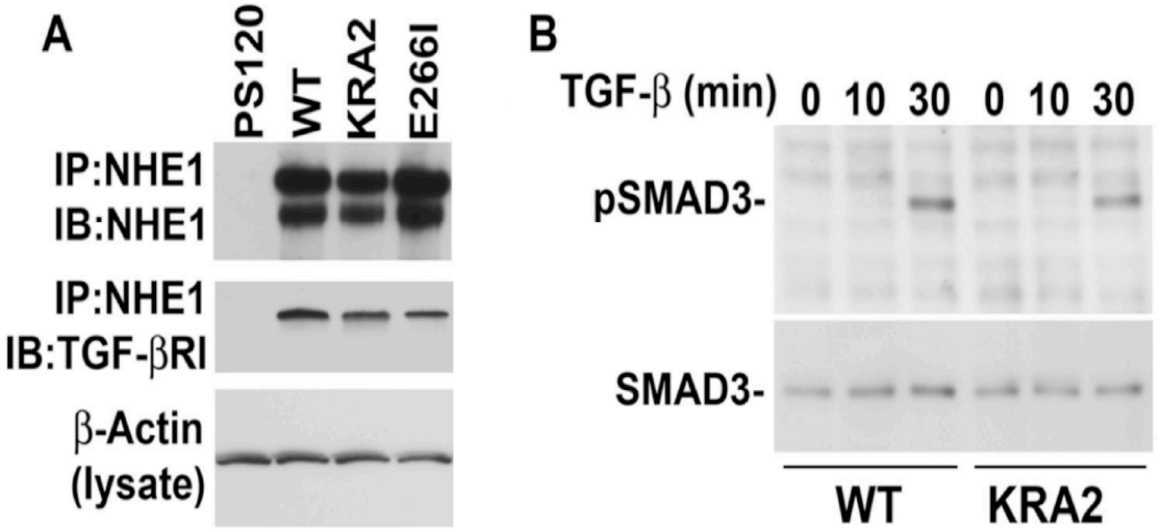

A
PS120
WT
KRA2
E266I
IP:NHE1
IB:NHE1
IP:NHE1
IB:TGF-βRI
β-Actin
(lysate)
B
TGF-β (min) 0 10 30 0 10 30
pSMAD3-
SMAD3-
WT
KRA2

Figure 20.

Figure 20.

TGF-β-RI co-precipitates with NHE1 but TGF-b signaling is not inhibited and SMAD3 phosphorylation is not affected in KRA2 compared with WT cells. (A) TGF-β-RI co-precipitates with WT and mutant NHE1-HA. Equal amounts of protein in lysates from the indicated cell types were incubated with antibodies to HA and immune complexes were immunoblotted with antibodies to TGF-β-RI. Aliquots of lysates were immunoblotted for actin to confirm equivalent amount of protein used for immunoprecipitation. Data are representative of 2 separate cell preparations. (B) Time-dependent increase in phosphorylated SMAD3 (pSMAD3) is similar in WT and KRA2 cells treated with 5ng/ml of TGF-β1. Immunoblotting for total SMAD3 indicates similar abundance in WT and KRA2 cells in the absence and presence of TGF-β.

4. Discussion

Localized scaffolding by NHE1 is necessary for establishing polarity and directionality in migrating fibroblasts and the mislocalized NHE1 in fibroblasts expressing the mutant NHE1 that lacks binding sites for PI(4,5)P2 and the ERM proteins ezrin, radixin and moesin attenuates the FA assembly and impairs cell migration in wounding assays. The current data now reveal that the localized scaffolding by NHE1 also regulates FN production in fibroblasts. Mislocalized scaffolding by PI(4,5)P2/ERM binding mutant KRA2-NHE1 inhibits the FN secretion, expression, transcription and promoter activity in hamster fibroblasts. Mislocalized NHE1 also impairs the exogenous FN assembly but does not affect integrin expression or affinity to the ligand. Ion exchange activity and pH_i regulation by NHE1 are not necessary for FN production or assembly. Co-expression of the mislocalized NHE1 with the H^+ efflux deficient NHE1 restores the FN production and assembly. Moreover, exogenous TGF-β restores the FN phenotype (both production and assembly) in fibroblasts with mislocalized NHE1, which present decreased active TGF-β but similar total (active and latent) TGF-β compared with the WT-NHE1 fibroblasts. These findings suggest a previously unrecognized link between NHE1 and FN and indicate that localized NHE1 at the lamellipodia, but not ion transport activity, is important for the FN production and assembly most likely through a TGF-β-dependent mechanism.

The assembly of a FN matrix requires a number of recognized signaling mechanisms, including activation of integrins and integrin-regulated kinases (reviewed in Wierzbicka-Patynowski and Schwarzbauer, 2003), and the low molecular weight GTPase Rho, the Rho kinase ROCK, and Rho-regulated myosin contractility (Zhong et al., 1998; Zhang *et al.*, 1999; Yoneda et al., 2007). However, less is known about signals regulating FN synthesis. FN gene expression is increased by serum, growth factors, cytokines, and viruses, and is attenuated by many oncogenes (Kornblihtt et al., 1996), although these regulatory mechanisms primarily occur in pathogenic conditions, including fibrosis, inflammation, or malignant transformation. Our understanding of physiological regulation of FN production remains limited.

Previous reports indicate a functional interaction between integrin signaling and NHE1. Localized NHE1 regulates integrin-dependent attachment and spreading (Tominaga and Barber, 1998) through a reciprocal regulation with integrins. NHE1-dependent cell spreading is likely due to its ability to promote integrin clustering and recruitment of focal adhesion proteins. NHE1 is necessary for integrin-induced increases in FAK activity (Tominaga and Barber, 1998) and FN induction of the early response gene c-*jun* (Dike and Ingber, 1996). Additionally, activation and clustering of integrin receptors by FN binding stimulates NHE1 activity (Schwartz, *et al.*, 1991; Tominaga and Barber, 1998). These reports confirm that there is extensive cross-talk between integrins and NHE1.

Even though β1 integrin expression and affinity to the ligand is not affected by mislocalizing NHE1 in the current fibroblast cell model, integrin clustering may be affected and could be responsible for the FN phenotype. Certainly, ligand occupancy has been reported to stimulate integrin clustering (Erb *et al.*, 1997, Li *et al.*, 2003) and propagation of intracellular signals (Humphries *et al.*, 2003). The FN ligand's absence in KRA2 fibroblasts may by itself impair integrin clustering. If some integrin clustering by initial binding to soluble secreted FN is required to provide positive feedback and downstream signaling important for FN regulation, then necessary interactions with intracellular regulatory proteins may also be disrupted and impaired in KRA2 cells. However, although integrin function is necessary for assembly of secreted FN, it has not been reported to be necessary for FN production.

KRA2 cells are unable to assemble an exogenous FN matrix, indicating an additional defect that is independent of decreased FN production. This defect is likely also due to a dominant-negative effect of mislocalized NHE1-KRA2 because matrix assembly is not impaired in E226I or PS120 cells, is partially restored in KRA2 cells treated with $MnCl_2$ and integrin activating antibodies, and is mostly restored in KRA2 cells co-expressing NHE1-E266I or treated with TGF-β. Assembly of FN into a fibrillar matrix requires tension generated by interaction of clustered FA proteins tethered to the actin cytoskeleton (Wierzbicka-Patynowski and Schwarzbauer, 2002). NHE1-KRA2 might dominantly mislocalize cytoskeletal proteins such as spectrin, and cytoskeletal anchoring proteins such as ankyrin. Like ERM proteins, ankyrin binds directly to wild-type

NHE1 (Denker and Barber, unpublished findings), although whether it binds to NHE1-KRA2 is unknown. Hence, mislocalized scaffolding by NHE1-KRA2 could dominantly inhibit FN assembly through changes in cytoskeletal contractility or additionally by mislocalizing focal adhesion regulators such as ROCK, which directly phosphorylates NHE1 (Tominaga *et al*., 1998).

The cytoskeleton plays also an important role in FN regulation. Disruption of actin microfilaments blocks the induction of FN gene expression by serum (Michaelson *et al*., 2002) and F-actin disrupting agents or inhibition of RhoA prevent matrix assembly (Christopher *et al*., 1997, Zhang *et al*., 1997, Zhong *et al*., 1998), highlighting the crucial role of actin cytoskeleton in FN expression and assembly. Rho-A mediated contractility may generate the tension required for the exposure of self-assembly and adhesion sites that are cryptic within the soluble unbound FN molecule (Hocking *et al*., 1994, Zhong *et al*., 1998, Sechler *et al*., 2001). Actin filament binding by the COOH-terminus of ERM proteins bound to NHE1 could regulate the assembly or stability of FA through the cytoskeleton. Moreover, FAK activity is necessary for FN matrix assembly (Wierzbicka-Patynowski and Schwarzbauer, 2002), but it has not been reported to regulate FN synthesis.

The impaired FN assembly (and possibly production too) may be a result of the cytoskeleton changes in KRA2 cells. The cytoplasmic domain of NHE1 directly binds the NH2-terminal FERM (4.1, ezrin, radixin, moesin) domain of ERM proteins (Denker *et al*., 2000). FA-associated proteins talin and FAK contain FERM domains that might associate with NHE1, and this association

might be disrupted in KRA2 cells. Bundling of actin filaments into stress fibers and FA are also attenuated in cells expressing a mutant NHE1 that lacks ERM/PI(4,5)P2 protein binding (Denker *et al.*, 2000). However, actin stress fibers and FA assembly, but not FN expression and assembly, are attenuated in PS120 cells (Tominaga and Barber, 1998; Denker *et al.*, 2000). Moreover, autophosphorylation of FAK-Y397 is delayed and attenuated in KRA2 cells plated on FN, but E266I cells expressing the Na^+/H^+ ion translocation defective exchanger, and NHE1-deficient PS120 cells that have FN production similar to WT cells also have delayed and attenuated FAK autophosphorylation. The above data suggest that the FA impairment and the actin cytoskeleton disruption are not responsible for the impaired FN phenotype.

Ion transport activity and pHi homeostasis by NHE1 are not necessary for FA assembly or FN production. E266I cells form abundant FA (Denker *et al.*, 2000; Srivastava *et al.*, 2008) and the current data indicate normal FN production and assembly in E266I cells and in SN1 cells maintained in 25 μM glutamine, which induces a lower pHi compared with KRA2 and WT cells. Together these findings indicate that NHE1 ion translocation activity is not critical for FN production.

NHE1 as a scaffold protein at the lamellipodia with a dominant-negative effect

The NHE1 cytoplasmic domain at the COOH-terminus acts as a scaffold with binding sites for $PI(4,5)P_2$, the actin-binding proteins ezrin, radixin, moesin (ERM family of proteins) through their FERM (4.1, ezrin, radixin, moesin) domain (Baumgartner *et al.*, 2004; Meima *et al.*, 2007) and α-actinin via charged clusters of the COOH-tail (Meima and Barber, data not published). Moreover, the talin head possesses a FERM domain (Calderwood *et al.*, 2002) and might bind to NHE1. Talin binds to integrin β tails and is essential for β1 and β3 integrin activation (Tadokoro *et al.*, 2003). NHE1 is normally restricted to membrane protrusions or lamellipodia retaining these proteins in specialized membrane domains. The ERM and $PI(4,5)P_2$ binding deficient KRA2-NHE1 is uniformly distributed at the plasma membrane, mislocalizing also its binding partners which may regulate signaling cues important for FN production. We reported that fibroblasts expressing the mislocalized NHE1-KRA2 have fewer and smaller FA compared with fibroblasts expressing wild-type NHE1. Although wild-type NHE1 does not localize to FA, its function as a scaffolding platform might promote the recruitment of FA-associated proteins to membrane protrusions, including talin, FAK, and vinuclin. The mislocalized NHE1 scaffolding sequesters and mislocalizes FA proteins that may be necessary for FN production.

Mislocalized NHE1 may divert signaling cues from growth factors that are necessary for FN production. The platelet-derived growth factor (PDGF) and the

epidermal growth factor EGF activate NHE1 and regulate its function (Ma *et al.*, 1994; Di Sario *et al.*, 1999; Liaw *et al.*, 1998). FN gene is rapidly induced by serum and growth factors (Ryseck *et al.*, 1989) and mislocalized downstream growth factor signaling may be critical for the FN phenotype in KRA2 fibroblasts. Moreover, mislocalization of ROCK in KRA2 may play a role in the impaired FN assembly, since ROCK I or II downregulation blocks FN assembly, and cortical myosin II-driven contractility, but not stress fibers, may be critical for this (Yoneda *et al.*, 2007). However, the impaired FN production and assembly could be due to a combination of the possibilities described above, one determining FN production and another determining FN assembly.

NHE1-KRA2 might also act in a dominant-negative manner for impaired FN synthesis by mislocalizing cytoskeletal or other proteins such as spectrin and ankyrin. Although NHE1-KRA2 does not bind ERM proteins it remains enriched in P100 particulate fractions (Barber Lab, unpublished findings), suggesting it retains cytoskeletal association. Like ERM proteins, ankyrin binds directly to wild-type NHE1 (Denker and Barber, unpublished findings), although whether it binds to NHE1-KRA2 is unknown. Additional factors that could be affected by mislocalized scaffolding of NHE1 include changes in membrane tension and cytoskeletal contractility, or mislocalized signaling proteins such as ROCK, which directly phosphorylates NHE1 (Tominaga *et al.*, 1998).

The current data suggest that mislocalized NHE1-KRA2 might have a dominant-negative function in sequestering and hence mislocalizing signals necessary for FN production and assembly. This possibility is supported by the

ability of co-expressed NHE1-E266I to restore FN production in cells expressing NHE1-KRA2. NHE1-E266I was co-expressed instead of wild-type NHE1 to confirm pH_i-independent effects on FN synthesis. When NHE1-KRA2 was co-expressed in the parental fibroblasts CCL39 to test whether variable amounts of expressed NHE1-KRA2 protein affects the FN production of the parental fibroblasts in a dose-dependent manner, no obvious dose-related effect on FN production or assembly was observed in the generated clones with variable NHE1-KRA2 expression. These data indicate that the localized scaffolding by NHE1 in the CK cells is sufficient to sequester enough signaling cues at the lamellipodia for FN production.

Mislocalized NHE1 impairs TGF-β activation

TGF-β signaling has an established role in promoting extracellular matrix deposition, maintenance of tissue architecture, and wound repair (O'Kane and Ferguson, 1997; Letterio and Roberts, 1998; Fleisch *et al.*, 2006; Wahl, 2007). In addition to its other functions, TGF-β is also a key mediator in the pathogenesis of cardiac hypertrophy by inducing interstitial fibrosis, and in the matrix deposition in the infarct by upregulating collagen and FN synthesis (Bujak and Frangogiannis, 2007). NHE1 has also been reported to play a role in matrix deposition in the pathogenesis of cardiac hypertrophy and myocardial infarction. NHE1 activity increases in hypertrophic myocardium, but the hypertrophy is prevented by NHE1 inhibition (Perez *et al.*, 1995; Ennis *et al.*, 2003) suggesting a

role of NHE1 in matrix regulation and a possible cross-talk with TGF-β. Moreover, inhibition of NHE1, which is the cardiac specific NHE isoform, attenuates matrix deposition in myocardial infarction-induced hypertrophy (Yoshida and Karmazyn, 2000; Kusumoto *et al*., 2001). Although NHE1 activity is known to be regulated by growth factors and integrins (Meima *et al*., 2007), no link has been reported between NHE1, ECM deposition and TGF-β activation. In addition, the current understanding of the role of TGF-β in ECM regulation is incomplete, partially because of the intricate mechanisms of TGF-β activation.

Activation of TGF-β is a complex and tightly regulated process. All TGF-β isoforms are synthesized in a latent inactive pro-TGF-β form, consisting of TGF-β that is covalently linked to the latency associated protein (LAP). While LAP is cleaved from TGF-β intracellularly, it retains high affinity for TGF-β and assembles into the non-covalent inactive small latent complex (SLC) that comprises of a LAP homodimer and the active TGF-β molecule. TGF-β is usually secreted as part of a large latent complex (LLC), with a covalent disulfide bond between SLC and a family member of the latent TGF-β1-binding proteins (LTBPs). LTBPs belong to the superfamily of fibrillin-like ECM proteins and bind to other ECM molecules including FN (Taipale *et al*., 1994), vitronectin (Schoppet *et al*., 2002) and fibrillin-1 (ten Dijke and Arthur, 2007; reviewed in Wipff and Hinz, 2008; and Jenkins, 2008) and can thus sequester TGF-β in the ECM and regulate its distribution and availability. Latent TGF-β is unable to associate with its receptors and its activity is regulated by various mechanisms, primarily by conversion of latent TGF-β to active TGF-β by proteolytic cleavage

of the latent complex (Annes *et al*., 2003). Cleavage of LLC and release of active TGF-β is achieved by matrix metalloproteinases (MMPs), including membrane-type-1(MT-1) MMP (Mu *et al*., 2002), bone morphogenetic protein-1 (BMP-1), MMP-2 (Ge and Greenspan, 2006), MMP-3 (Maeda *et al*., 2002), MMP-9 (Yu and Stamenkovich, 2000) and MMP-13 (D'Angelo *et al*., 2001), thrombin, plasmin (Taipale *et al*., 1992), elastase (Taipale *et al*., 1995), chymase (Lindstedt *et al*., 2001) and tryptase (Berger *et al*., 2003). Latent TGF-β is also activated by integrins and the ECM, by transmission of cell traction forces that are translated in biochemical signals and growth factor activation. Integrins $\alpha_v\beta_3$, $\alpha_v\beta_5$ (Wipff *et al*., 2007), $\alpha_v\beta_6$ (Jenkins *et al*., 2006), $\alpha_v\beta_8$ (Mu *et al*., 2002), β_1 (Wipff *et al*., 2007) have been reported to participate in activating the TGF-β through a mechanism that involves the cytoskeleton, cell contraction and a mature ECM.

In addition to decreased FN synthesis, KRA2 cells with mislocalized scaffolding by NHE1 have decreased activation of TGF-β, but secretion of latent TGF-β is not impaired. Although treating KRA2 cells with exogenous TGF-β restored FN synthesis and assembly, the complex functional interplay between growth factors and FN makes it difficult to understand the primary defect in KRA2 cells. One possibility is that decreased FN production is the primary defect that secondarily inhibits TGF-β activation. The latent TGF-β is bound to FN (Fontana *et al*., 2005) and integrins (Wipff *et al*., 2007), which regulate TGF-β activation and the absence of a mature FN matrix impairs the activation of TGF-β. Alternatively, decreased TGF-β activation could be the primary defect that secondarily suppresses FN expression. Activation of TGF-β is regulated by

proteases, low extracellular pH, and binding to integrins and FN (Annes *et al.*, 2003; Fontana *et al.*, 2005); Jenkins *et al.*, 2006; Wipff *et al.*, 2007; Wipff and Hinz, 2008). Although TGF-β activation was not reduced in NHE1-deficient PS120 cells, mislocalized H^+ efflux by NHE1-KRA2 might inhibit a pH-dependent cleavage of latent TGF-β. TGF-β-RI co-precipitates in an immune complex with the WT-NHE1, as well as with the mislocalized KRA2-NHE1, and the deficient ion transporter E266I-NHE1. It is unknown whether TGF-β-RI binds directly NHE1 but most likely co-localizes with NHE1 in a plasma membrane "signalosome" microdomain and mislocalization of this complex inhibits FN production and possibly TGF-β activity regulation. FN has been reported to be necessary for TGF-β activation that requires cell adhesion of latent-TGF-β via $\alpha_v\beta_6$ and binding of LTBP to FN (Fontana *et al.*, 2005). However, KRA2 cells retain TGF-β signaling, as indicated by similar abundance of pSMAD3 in WT and KRA2 cells with exogenous TGF-β treatment. The hyaluronan receptor CD44 may also be a link between NHE1 and TGF-β signaling. CD44 co-precipitates with NHE1 (Bourguignon *et al.*, 2004) and TGF-β-RI (Ito *et al.*, 2004) and regulates the function of both proteins.

NHE1 is necessary for the organization of the actin stress fibers and may have a critical role in regulating contractile forces necessary for both FN assembly and extracellular TGF-β regulation. The assembly of actin cytoskeleton and FAs and the activation of integrins and Rho are impaired in fibroblasts lacking NHE1, but are restored in fibroblasts expressing wild-type NHE1 (Vexler *et al.*, 1996; Tominaga and Barber, 1998; Tominaga *et al.*, 1998; Denker *et al.*, 2000). KRA2

fibrobasts expressing the mutant KRA2-NHE1 with disrupted ERM binding also show attenuated assembly of focal complexes and actin stress fibers (Denker *et al*., 2000; Denker and Barber, 2002) and may be unable to exert the necessary contractile forces for the exposure of cryptic sites in FN (Zhong *et al*., 1998) when cultured with exogenously added FN. In addition, cell contraction and mechanical tension are pivotal for the activation of extracellular latent TGF-β, which requires the resistance of functional stress fibers (Wipff *et al*., 2007) and the impaired cytoskeleton in KRA2 cells may also inhibit the transmission of forces through integrins necessary for TGF-β activation. A unifying hypothesis that links the findings in KRA2 fibroblasts is that NHE1 is critical for transduction of tension necessary for TGF-β activation and FN matrix assembly by integrins.

5. Significance and future directions

My thesis work revealed that scaffolding by NHE1, but not ion translocation, regulates the FN production and assembly, the TGF-β activation, and provides insight on how FN may be regulated. The critical importance of FN matrix in morphogenesis and tissue remodeling underscores the importance of understanding how production of FN is regulated in physiologic conditions and how this is affected during disease. The current findings indicate that NHE1-KRA2 could be a valuable tool for obtaining a mechanistic understanding on FN biosynthesis and more speculatively for therapeutic control of increased or decreased FN production. Such information is necessary since FN production changes in several pathological conditions, including hepatic fibrosis, inflammation and malignant transformation.

An important next step will be to determine whether NHE1-KRA2 impairs TGF-β activation indirectly via the absence of a mature organized FN matrix or through the inhibition of a latent TGF-β protease, or NHE1-KRA2 impairs primarily TGF-β activation, which then attenuates FN synthesis and assembly. Reduced TGF-β activity in KRA2 fibroblasts may be a mere result of the lack of assembled FN, as $FN^{-/-}$ fibroblasts fail to activate latent TGF-β and deficient FN assembly also suppresses TGF-β activation (Fontana *et al.*, 2005). Further investigation of TGF-β acivation in KRA2 fibroblasts plated on mature assembled FN matrix from WT fibroblasts would determine whether the impaired FN is the result of reduced levels of TGF-β activation or vice versa.

Localized NHE1 scaffolding recruits FA proteins at the lamellipodia and may direct growth factor signaling critical for FN production and assembly as part of a plasma membrane "signalosome" microdomain. However NHE1-deficient PS120 cells have FN production and assembly similar to WT-NHE1 cells, indicating that NHE1 is not necessary for FN production, but it may modify and regulate TGF-β activity, probably by sequestering a factor necessary for FN biosynthesis and/or TGF-β activation. Further investigation is necessary to reveal how mislocalized scaffolding by NHE1 attenuates active TGF-β and FN production. If inhibition of FN assembly in WT fibroblasts with integrin-function blocking antibodies or the saturation of the integrin receptors with an RGD fragment also suppresses TGF-β activation, then the attenuated FN matrix in KRA2 fibroblasts is responsible for the reduced TGF-β activity. Otherwise, if the impaired FN assembly in WT fibroblasts has no effect on the activation of TGF-β, then NHE1 localization at the lamellipodia, in a plasma membrane "signalosome" microdomain, is sufficient for activation of TGF-β. In addition, NHE1-KRA2 may impair inside-out signaling necessary for conversion of the inactive latent TGF-β to active TGF-β by inhibiting the proteases involved in the cleavage of the latent complex and the liberation of active TGF-β. The mislocalized scaffolding by NHE1 might also impair one or more of the proteases involved in TGF-β activation, including MT-1 MMP, MMP-2, MMP-3, MMP-9, MMP-13.

Egr-1 is another candidate whose regulation may be affected by mislocalized NHE1-KRA2 and may be responsible for the decreased FN production in KRA2 cells. Egr-1 is a 59 kDa transcription factor that has

significant tumor suppressor properties, and regulates TGF-β gene in epithelial growth (Baron *et al*., 2006). The promoter of the human TGF-β1 gene contains at least two Egr1 binding sites both of which can lead independently to the initiation of transcription (Dey *et al*., 1994; Liu *et al*., 1996). FN is a direct target gene of Egr1 via Egr1-response elements in the proximal region of the promoter (Liu *et al*., 2000) and the impaired FN promoter activity in KRA2 cells may be due to reduced Egr1 in the mutant fibroblasts expressing the mislocalized KRA2-NHE1. NHE1-KRA2 may impair both TGF-β and FN regulation by independently regulating the transcription factor Egr-1 that controls both FN and TGF-β transcription. The above findings suggest that NHE1 with its established function as a scaffolding protein and a spatial integrator of signaling cues could participate in the regulation of FN and the modification of TGF-β activity, and could be used to expand our understanding in FN and TGF-β regulation.

6. Conclusions

The mislocalized scaffolding by NHE1 dominantly suppresses FN secretion, expression, transcription and promoter activity in hamster fibroblasts. Mislocalized NHE1 also impairs the exogenous FN assembly but does not affect integrin expression or affinity to the ligand. Moreover, ion exchange activity and pH_i regulation by NHE1 are not necessary for FN production or assembly. Co-expression of the mislocalized NHE1 with the H^+ efflux deficient NHE1 restores the FN production and assembly.

Exogenous TGF-β restores the FN phenotype (impaired production and assembly) in fibroblasts expressing mislocalized NHE1, and mislocalized scaffolding by NHE1 inhibits activation of TGF-β. It remains to be determined whether the FN production is the primary defect in fibroblasts with mislocalized NHE1 that secondarily inhibits TGF-β activation, or, alternatively, misocalized scaffolding by NHE1 inhibits TGF-β activation that secondarily suppresses FN production.

These findings suggest that scaffolding by NHE1-KRA2 sequesters and mislocalizes signals necessary for FN synthesis and assembly, and TGF-β activation. Although the precise signals sequestered by NHE1-KRA2 remain unknown, these data suggest that NHE1-KRA2 could be a valuable tool for

obtaining a mechanistic understanding of how FN production and TGF-β activation are regulated.

References

Abe M, Harpel JC, Metz CN, Nunes I, Loskutoff DJ, Rifkin DB. An assay for Transforming Growth Factor-β using cells transfected with a Plasminogen Activator Inhibitor-1 Promoter-Luciferase construct. Analytical Biochem 1994, 216: 276-284

Aharonovitz O, Zaun HC, Balla T, York JD, Orlowski J, Grinstein S. Intracellular pH regulation by Na(+)/H(+) exchange requires phosphatidylinositol 4,5-bisphosphate. J Cell Biol 2000, 150:213-224

Allen-HoffmannBL, Schlosser SJ, Brondyk WH, Fahl WE. Fibronectin levels are enhanced in human fibroblasts overexpressing the c-sis protooncogene. J Biol Chem 1990, 265:5219-5225

Andresen JL, Rasmussen LM, Ledet T. Diabetic macroangiopathy and atherosclerosis. Diabetes. 1996, 45 Suppl 3:S91-4

Annes JP, Munger JS, Rifkin DB. Making sense of latent TGF-beta activation. J Cell Sci 2003,116:217-224

Aota S, Nomizu M, Yamada KM. The short amino acid sequence Pro-His-Ser-Arg-Asn in human fibronectin enhances cell-adhesive function. J Biol Chem 1994, 269:24756–24761

Aronson PS, Nee J, Suhm MA. Modifier role of internal HC in activating the Na+-H+ exchanger in renal microvillus membrane vesicles. Nature 1982, 299:161–63

Barber DL, McGuire ME, Ganz MB. Beta-adrenergic and somatostatin receptors regulate Na-H exchange independent of cAMP. J Biol Chem 1989, 264:21038–42

Barkalow FJB, Schwarzbauer JE. Localization of the major heparin binding site in fibronectin. J Biol Chem 1991, 266:7812-7818

Baron N, Norman D, Willis A, Campbell ID. Structure of the fibronectin type I module. Nature 1990, 34:642-646

Baron V, Adamson ED, Calogero A, Ragona G, Mercola D. The transcription factor Egr1 is a direct regulator of multiple tumor suppressors including TGFbeta1, PTEN, p53, and fibronectin. Cancer Gene Ther 2006, 13:115-124

Baeuerle PA, Baichwal VR. NF-kB as a frequent target for immunosuppressive and anti-inflammatory molecules. Adv Immunol 1997, 65:111–137

Baumgartner M, Sillman AL, Blackwood EM, Srivastava J, Madson N, Schilling JW, Wright JH, Barber DL. The Nck-interacting kinase phosphorylates ERM proteins for formation of lamellipodium by growth factors. Proc Natl Acad Sci USA 2006, 103:13391-13396

Berger P, Girodet PO, Begueret H, Ousova O, Perng DW, Marthan R, Walls AF, Tunon de Lara JM. Tryptase-stimulated human airway smooth muscle cells induce cytokine synthesis and mast cell chemotaxis. FASEB J 2003, 17:2139–214

Bertrand B, Wakabayashi S, Ikeda T, Pouyssegur J, Shigekawa M. The Na+/H+ exchanger isoform 1 (NHE1) is a novel member of the calmodulin-binding

proteins. Identification and characterization of calmodulin-binding sites. J Biol Chem 1994, 269:13703–13709

Blatti SP, Foster DN, Ranganathan G, Moses HL, Getz MJ. Induction of fibronectin gene transcription and mRNA is a primary response to growth factor stimulation in AKR-2B cells. Proc Natl Acad Sci USA 1988, 85:1119-1123

Blobe GC, Schiemann WP, Lodish HF. Role of transforming growth factor beta in human disease. N Engl J Med 2000, 342: 1350–1358

Borsi L, Castellani P, Risso AM, Leprini A, Zardi L. Transforming growth factor-beta regulates the splicing pattern of fibronectin messenger RNA precursor. FEBS Lett 1990, 261:175-178

Boucaut JC, Darribère T, Li SD, Boulekbache H, Yamada KM, Thiery JP. Evidence for the role of fibronectin in amphibian gastrulation. J Embryol Exp Morphol 1985, 89(Suppl):211-227

Bourguignon LY, Singleton PA, Diedrich F, Stern R, Gilad E. CD44 interaction with Na+-H+ exchanger (NHE1) creates acidic microenvironments leading to hyaluronidase-2 and cathepsin B activation and breast tumor cell invasion. J Biol Chem 2004, 279:26991-7007

Bowlus CL, Mc Quillan JJ, Dean DC. Characterization of three different elements in the 5'-flanking region of the fibronectin gene which mediate a transcriptional response to cAMP. J Biol Chem 1991, 266:1122-1127

Brown LF, Dubin D, Lavigne L, Logan B, Dvorak HF, Van de Water L. Macrophages and fibroblasts express embryonic fibronectins during cutaneous wound healing. Am J Pathol 1993, 142:793-801

Bujak M, Frangogiannisa NG. The role of TGF-β Signaling in Myocardial Infarction and Cardiac Remodeling. Cardiovasc Res 2007, 74: 184–195

Bullis BL, Li X, Singh DN, Berthiaume LG, Fliegel L. Properties of the Na+/H+ exchanger protein. Detergent-resistant aggregation and membrane microdistribution. Eur J Biochem 2002, 269:4887-95

Calderwood DA. Integrin activation. J Cell Sci 2004, 117:657-666

Chaudry FA, Reimer RJ, Krizaj D, Barber D, Storm-Mathisen J, Copenhagen DR, Edwards RH. Molecular Analysis of System N suggests novel physiological roles in Nitrogen Metabolism and Synaptic Transmission. Cell 1999, 99:769-780

Cho J, Mosher DF. Role of fibronectin assembly in platelet thrombus formation. J. Thromb Haemost 2006, 4:1461-1469

Christensen L, Nielsen M, Andersen J, Clemmensen I. Stromal fibronectin staining pattern and metastasizing ability of human breast carcinoma. Cancer Res. 1988, 48:6227-6233

Clark RAF, McCoy GA, Folkvord JM, McPherson JM. TGF-β1 stimulates cultured human fibroblasts to proliferate and produce tissue-like fibroplasias: A fibronectin matrix-dependent event. J Cell Physiol 1997, 170:69-80

Critchley DR. Focal adhesions – the cytoskeletal connection. Curr Opin Cell Biol 2000, 12:133-139

D'Angelo M, Billings PC, Pacifici M, Leboy PS, Kirsch T. Authentic matrix vesicles contain active metalloproteases (MMP). A role for matrix vesicle-associated MMP-13 in activation of transforming growth factor-beta. J Biol Chem 2001, 276:11347–11353

Darribère T, Schwarzbauer JE. Fibronectin matrix composition and organization can regulate cell migration during amphibian development. Mech Develop 2000, 92:239-250

Dean DC, Bowlus CL, Bourgeois S. Cloning and analysis of the promotor region of the human fibronectin gene. PNAS USA 1987, 84:1876-1880

Dean DC, Newby RF, Bourgeois S. Regulation of fibronectin biosynthesis by dexamethasone, transforming growth factor beta, and cAMP in human cell lines. J Cell Biol 1988, 106:2159-2170

Dean DC, McQuillian JJ., Weintraub S. Serum stimulation of fibronectin gene expression appears to result from rapid serum-induced binding of nuclear proteins to a cAMP response element. J Biol Chem 1990, 265:3522-3527

Denker SP, Huang DC, Orlowski J, Furthmayr J, Barber DL. Direct binding of the Na-H exchanger NHE1 to ERM proteins regulates the cortical cytoskeleton and cell shape independently of H^+ translocation. Mol Cell 2000, 6:1425-1436

Denker SP, Barber DL. Cell migration requires both ion translocation and cytoskeletal anchoring by the Na-H exchanger NHE1. J Cell Biol 2002, 159:1087-1096

Denker SP, Barber DL. Ion transport proteins anchor and regulate the

cytoskeleton. Curr Opin Cell Biol 2002b, 14:214-220

Der CJ, Stanbridge EJ. Alterations in the extracellular matrix organization associated with the reexpression of tumorigenicity in human cell hybrids. Int J Cancer 1980, 26:451-459

Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature 2003, 425:577–584

Dey BR, Sukhatme VP, Roberts AB, Sporn MB, Rauscher FJ, Kim SJ. Repression of the transforming growth factor-beta 1 gene by the Wilms' tumor suppressor WT1 gene product. Mol Endocrinol 1994, 8:595–602

Dickson MC, Martin JS, Cousins FM, Kulkarni AB, Karlsson S, Akhurst RJ. Defective haematopoiesis and vasculogenesis in transforming growth factor-beta 1 knock out mice. Development 1995, 121:1845–1854

Dike LE, Ingber DE. Integrin-dependent induction of early growth response genes in capillary endothelial cells. J Cell Sci 1996, 109:2855-63

Di Sario A, Bendia E, Svegliati Baroni G, Ridolfi F, Bolognini L, Feliciangeli G, Jezequel AM, Orlandi F, Benedetti A. Intracellular pathways mediating Na+/H+ exchange activation by platelet-derived growth factor in rat hepatic stellate cells. Gastroenterology 1999, 116:1155-66

Ennis IL, Escudero EM, Console GM, Camihort G, Dumm CG, Seidler RW, Camilión de Hurtado MC, Cingolani HE. Regression of isoproterenol-induced cardiac hypertrophy by Na+/H+ exchanger inhibition. Hypertension 2003, 41:1324-9

Erickson HP, Carell NA. Fibronectin in extended and compact conformations.

Electron microscopy and sedimentation analysis. J Biol Chem 1983, 258:14539-14544

Feng XH, Filvaroff EH, Derynck R. Transforming growth factor-beta (TGF-beta)-induced down-regulation of cyclin A expression requires a functional TGF-beta receptor complex. Characterization of chimeric and truncated type I and type II receptors. J Biol Chem 1995, 270:24237-24245

Féral CC, Zijlstra A, Tkachenko E, Prager G, Gardel ML, Slepak M, Ginsberg MH. CD98hc (SLC3A2) participates in fibronectin matrix assembly by mediating integrin signaling. J Cell Biol 2007, 178:701-11

Fleisch MC, Maxwell CA, Barcellos-Hoff M-H. The pleiotropic roles of transforming growth factor beta in homeostasis and carcinogenesis of endocrine organs. Endocrinology 2006, 13:379–400

Fontana L, Chen Y, Prijatelj P, Sakai T, Fassler R, Sakai LY, Rifkin DB. Fibronectin is required for integrin alphavbeta6-mediated activation of latent TGF-beta complexes containing LTBP-1. FASEB J 2005, 19:1798–1808

Frantz C, Barreiro G, Dominguez L, Chen X, Eddy R, Condeelis J, Kelly M, Jacobson M, Barber D. Cofilin is a pH sensor for actin free barbed end formation. J Cell Biol 2008, 183:865-879

Frantz C, Karydis A, Nalbant P, Hahn KM, Barber DL. Positive feedback between Cdc42 activity and H+ efflux by the Na-H exchanger NHE1 for polarity of migrating cells. J Cell Biol 2007, 179:403-410

Ganz MB, Pachter JA, Barber DL. Multiple receptors coupled to adenylate cyclase regulate Na-H exchange independent of cAMP. J Biol Chem 1990,

265:8989–92

Ge G, Greenspan DS. BMP1 controls TGF{beta}1 activation via cleavage of latent TGF{beta}-binding protein. J Cell Biol 2006, 175:111–120

George EL, Georges-Labouesse EN, Patel-King RS, Rayburn H, Hynes RO. Defects in mesoderm, neural tube and vascular development in mouse embryos lacking fibronectin. Development 1993, 119:1079-1091

Grady W, Myeroff L, Swinler S, Rajput A, Thiagalingam S, Lutterbaugh J, Neumann A, Brattain M, Chang J, Kim S, Kinzler K, Vogelstein B, Willson J, Markowitz S. Mutational inactivation of transforming growth factor beta receptor type II in microsatellite stable colon cancers. Cancer Res 1999, 50:320–324

Grinnell F. Fibronectin and wound healing. J Cell Biochem 1984, 26:107-116

Grinstein S, Woodside M, Sardet C, Pouyssegur J, Rotin D. Activation of the Na+/H+ antiporter during cell volume regulation. Evidence for a phosphorylation-independent mechanism. J Biol Chem 1992, 267:23823–28

Grinstein S, Woodside M, Waddell TK, Downey GP, Orlowski J, Pouyssegur J, Wong DC, Foskett JK. Focal localization of the NHE-1 isoform of the Na+/H+ antiport: assessment of effects on intracellular pH. EMBO J 1993, 12:5209-18

Guan JL, Hynes RO. Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor alpha 4 beta 1. Cell 1990, 60:53-61

Hay ED. Cell biology of extracellular matrix. Plenum Press, 1991, New York.

Heremans A, De Cock B, Cassiman JJ, Van Den Berghe H, David G. The core protein of the matrix-associated heparan sulfate proteoglycan binds to fibronectin. J Biol Chem 1990, 265:8716-8724

Hooley R, Yu CY, Symons M, Barber DL. . Gα13 stimulates Na+H+ exchange through distinct Cdc42-dependent and RhoA-dependent pathways. J Biol Chem 1996, 271:6152–6158

Hynes RO. Fibronectins. Springer-Verlag New York Inc. 1990, New York

Hynes RO. Integrins: versatility, modulation and signaling in cell adhesion. Cell 1992, 69:11-25

Humphries MJ, Akiyama SK, Komoriya A, Olden K, Yamada KM. Neurite extension of chicken peripheral nervous system neurons on fibronectin: relative importance of specific adhesion sites in the central cell-binding domain and the alternatively spliced type III connecting segment. J Cell Biol 1988, 106:1289-1297

Humphries MJ, Olden K, Yamada KM. Fibronectin and cancer: Implication of cell adhesion to fibronectin for tumor matastasis. In: Fibronectin in Health and Disease. Ed. Steven E Carsons. CRC Press Inc. 1989, Boca Raton. pp: 161-199

Ingber DE, Prusty D, Frangioni JV, Cragoe EJ Jr, Lechene C, Schwartz MA. Control of intracellular pH and growth by fibronectin in capillary endothelial cells. J Cell Biol 1990, 110:1803-1811

Ito T, Williams JD, Fraser D, Phillips AO. Hyaluronan attenuates transforming growth factor- beta1-mediated signaling in renal proximal tubular epithelial cells. Am J Pathol 2004, 164:1979-1988

Jenkins RG, Su X, Su G, Scotton CJ, Camerer E, Laurent GJ, Davis GE, Chambers RC, Matthay MA, Sheppard D.. Ligation of protease-activated receptor 1 enhances alpha(v)beta6 integrin-dependent TGF-beta activation and promotes acute lung injury. J Clin Invest 2006, 116: 1606–1614

Jenkins G. The role of proteases in transforming growth factor-beta activation. Int J Biochem Cell Biol 2008, 40:1068-78

Johnson KJ, Sage H, Briscoe G, Erickson HP. The compact conformation of fibronectin is determined by intramolecular ionic interactions. J Biol Chem 1999, 274:15473-15479

Kaczmarek LK. Non-conducting functions of voltage-gated ion channels. Nat Rev Neurosci 2006, 7:761-771

Khaled AR, Moor AN, Li A, Kim K, Ferris DK, Muegge K, Fisher RJ, Fliegel L, Durum SK. Trophic factor withdrawal: p38 mitogen-activated protein kinase activates NHE1, which induces intracellular alkalinization. Mol Cell Biol 2001, 21:7545-7557

Komoriya A, Green LJ, Mervic M, Yamada SS, Yamada KM, Humphries MJ. The minimal essential sequence for a major cell type-specific adhesion site (CS1) within the alternatively spliced type III connecting segment domain of fibronectin is leucine-aspartic acid-valine. J Biol Chem 1991, 266:15075-15079

Kornblihtt AR, Vibe-Pedersen K, Baralle FE. Isolation and characterization of cDNA clones for human and bovine fibronectins. PNAS USA 1983, 80:3218-3222

Kornblihtt AR, Vibe-Pedersen K, Baralle FE. Human fibronectin: cell specific alternative mRNA splicing generates polypeptide chains differing in the number of internal repeats. Nucleic Acids Res 1984, 12:5853-5868

Kornblihtt AR, Pesce CG, Alonso CR, Cramer P, Srebrow A, Werbajh S, Muro AF. The fibronectin gene as a model for splicing and transcription studies. FASEB J 1996, 10:248-257

Kulkarni AB, Huh CG, Becker D, Geiser A, Lyght M, Flanders KC, Flanders KC, Roberts AB, Sporn MB, Ward JM, Karlsson S. Transforming growth factor beta 1 null mutation in mice causes excessive inflammatory response and early death. Proc Natl Acad Sci USA 1993, 90:770–774

Kusumoto K, Haist JV, Karmazyn M. Na(+)/H(+) exchange inhibition reduces hypertrophy and heart failure after myocardial infarction in rats. Am J Physiol Heart Circ Physiol 2001, 280:H738-45

Labat-Robert J, Birembaut P, Adnet JJ, Mercantini F, Robert L. Loss of fibronectin in human breast cancer. Cell Biol Int Rep 1980, 4:609-16

Laping NJ, Grygielko E, Mathur A, Butter S, Bomberger J, Tweed C, Martin W, Fornwald J, Lehr R, Harling J, Gaster L, Callahan JF, Olson BA. Inhibition of transforming growth factor (TGF)-beta1-induced extracellular matrix with a novel inhibitor of the TGF-beta type I receptor kinase activity: SB-431542. Mol Pharmacol 2002, 62:58-64

Larizza L, Tenchini ML, Mottura A, De Carli L, Colombi M, Barlati S. Expression of transformation markers and suppression of tumorigenicity in human cell hybrids. Eur J Cancer Clin Oncol 1982, 18:845-851

Lee BH, Kim MS, Rhew JH, Park RW, de Crombrugghe B, Kim IS. Transcriptional regulation of fibronectin gene by phorbol myristate acetate in hepatoma cells: a negative role for NF-kappaB. J Cell Biochem 2000, 76:437-51

Lee B-H, Park S-Y, Kang K-B, Park R-W, Kim I-S. NF-κB activates fibronectin expression in rat hepatocytes. Biochem Biophys Res Commun 2002, 297:1218-1224

Lehoux S, Abe J, Florian JA, Berk BC. 14-3-3 binding to Na+/H+ exchanger isoform-1 is associated with serum-dependent activation of Na+/H+ exchange. J Biol Chem 2001, 276:15794-15800

Letterio JJ, Roberts AB. Regulation of immune responses by TGF-beta. Annu Rev Immunol 1998,16:137–161

Liaw YS, Yang PC, Yu CJ, Kuo SH, Luh KT, Lin YJ, Wu ML. PKC activation is required by EGF-stimulated Na+-H+ exchanger in human pleural mesothelial cells. Am J Physiol 1998, 274:L665–72

Lin X, Barber DL. A calcineurin homologous protein inhibits GTPase-stimulated Na-H exchange. Proc Natl Acad Sci U S A 1996, 93:12631-12636

Lindstedt KA, Wang Y, Shiota N, Saarinen J, Hyytiainen M, Kokkonen JO, Keski-Oja J, Kovanen PT. Activation of paracrine TGF-beta1 signaling upon stimulation and degranulation of rat serosal mast cells: a novel function for

chymase. FASEB J 2001, 15:1377–1388

Liu C, Adamson E, Mercola D. Transcription factor EGR-1 suppresses the growth and transformation of human HT-1080 fibrosarcoma cells by induction of transforming growth factor beta 1. Proc Natl Acad Sci U S A 1996, 93:11831–11836

Liu C, Yao J, Mercola D, Adamson E. The transcription factor EGR-1 directly transactivates the fibronectin gene and enhances attachment of human glioblastoma cell line U251. J Biol Chem 2000, 275:20315-23

Lodish H, Berk A, Zipursky SL, Matsudaira P, Baltimore D, Darnell J. Chapter 22. Integrating Cells into Tissues. In: Molecular Cell Biology. 4th Ed. WH Freeman and Co. New York, New York pp: 969-998

Ma YH, Reusch HP, Wilson E, Escobedo JA, Fantl WJ, Williams LT, Ives HE. Activation of Na+/H+ exchange by platelet-derived. J Biol Chem 1994, 269: 30734-39

Maeda S, Dean DD, Gomez R, Schwartz Z, Boyan BD. The first stage of transforming growth factor beta1 activation is release of the large latent complex from the extracellular matrix of growth plate chondrocytes by matrix vesicle stromelysin-1 (MMP-3). Calcif Tissue Int 2002, 70:54–65

Markowitz S, Wang J, Myeroff L, Parsons R, Sun L, Lutterbaugh J, Fan R, Zborowska E, Kinzler K, Vogelstein B. Inactivation of the type II TGF-beta receptor in colon cancer cells with microsatellite instability. Science 1995, 268:1336–1338

Massague J. How cells read TGF-beta signals. Nat Rev Mol Cell Biol 2000,

1:169–78

Matsui T, Maeda M, Doi Y, Yonemura S, Amano M, Kaibuchi K, Tsukita S, Tsukita S. Rho-kinase phosphorylates COOH-termina threonines of ezrin/radixin/moesin (ERM) proteins and regulates their head-to-tail association. J Cell Biol 1998, 140:647-657

McKeown-Longo PJ, Mosher DF. Binding of plasma fibronectin to cell layers of human skin fibroblasts. J Cell Biol 1983, 97:466-472

McKeown-Longo PJ, Mosher DF. Interaction of the 70,000-mol-wt amino-terminal fragment of fibronectin with the matrix-assembly receptor of fibroblasts. J Cell Biol 1985, 100: 364-374

Meima ME, Mackley JR, Barber DL. Beyond ion translocation: structural functions of the sodium-hydrogen exchanger isoform-1. Curr Opin Nephourol Hypertens 2007, 16:365-72

Milner R, Crocker SJ, Hung S, Wang X, Frausto RF, del Zoppo GJ. Fibronectin- and vitronectin-induced microglial activation and matrixmetalloproteinase-9 expression is mediated by integrins $\alpha_5\beta_1$ and $\alpha_v\beta_5$. J Immunol 2007, 178:8158-8167

Mishima M, Wakabayashi S, Kojima C. Solution structure of the cytoplasmic region of Na+/H+ exchanger 1 complexed with essential cofactor calcineurin B homologous protein 1. J Biol Chem 2007, 282:2741-2751

Mosher DF. Assembly of fibronectin into extracellular matrix. Curr Opin Struct Biol 1993, 3:214-222

Mould AP, Wheldon LA, Komoriya A, Wayner EA, Yamada KM, Humphries MJ. Affinity chromatographic isolation of the melanoma adhesion receptor for the IIICS region of fibronectin and its identification as the integrin alpha 4 beta 1. J Biol Chem 1990, 265:4020-4024

Mu D, Cambier S, Fjellbirkeland L, Baron JL, Munger JS, Kawakatsu H, Sheppard D, Broaddus VC, Nishimura SL. The integrin alpha(v)beta8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-beta1. J Cell Biol 2002, 157: 493–507

Muro AF, Moretti FA, Moore BB, Yan M, Atrasz RG, Wilke CA, Flaherty KR, Martinez FJ, Tsui JL, Sheppard D, Baralle FE, Toews GB, White ES. An essential role for fibronectin extra type III domain A in pulmonary fibrosis. Am J Respir Crit Care Med 2008, 177:638-645

NakajimaT, Nakamura T, Tsunoda S, Nakada S, Oda K. E1A-responsive elements for repression of rat fibronectin gene transcription. Mol Cell Biol 1992, 12:2837–2846

Nakamura T, Nakajima T, Tsunoda S, Nakada S, Oda K, Tsurui H, Wada A. Induction of E1A-responsive negative factors for transcription of the fibronectin gene in adenovirus E1-transformed rat cells. J Virol 1992, 66:6436-6450

Nagai T, Yamakawa N, Aota S, Yamada SS, Akiyama SK, Olden K, Yamada KM. Monoclonal antibody characterization of two distant sites required for function of the central cell-binding domain of fibronectin in cell adhesion, cell migration, and matrix assembly. J Cell Biol 1991, 1141295-1305

Norton PA, Hynes RO. Alternative splicing of chicken fibronectin in embryos and in normal and transformed cells. Mol Cell Biol 1987, 7:4297-4307

O'Kane S, Ferguson MWJ. Transforming growth factor [beta]s and wound healing. Int J Biochem Cell Biol 1997, 29:63–78

Obara M, Kang MS, Yamada KM. Site-directed mutagenesis of the cell binding domain of the human fibronectin: separable, synergistic sites mediate adhesive function. Cell 1988, 53:649-657

Ohashi T, Kiehart DP, Erickson HP. Dynamics and elasticity of the fibronectin matrix in living cell culture visualized by fibronectin-green fluorescent protein. PNAS USA 1999, 96:2153-2158

Orlowski J. Heterologous expression and functional proper ties of amiloride high affinity (NHE-1) and low affinity (NHE-3) iso forms of the rat Na/H exchanger. J Biol Chem 1993, 268: 16369–16377

Orlowski J, Grinstein S. Na +/H+ exchangers of mammalian cells. J Biol Chem 1997, 272:22373–76

Pankov R, Cukierman E, Katz BZ, Matsumoto K, Lin DC, Lin S, Hahn C, Yamada KM. Integrin dynamics and matrix assembly: tensin dependent translocation of alpha(5)beta(1) integrins promotes early fibronectin fibrillogenesis. J Cell Biol 2000, 148:1075-1090

Pankov R, Yamada KM. Fibronectin at a glance. J Cell Science 2002, 115:3861-3863

Patel RS, Odermatt E, Schwarzbauer JE, Hynes RO. Organization of the fibronectin gene provides evidence for exon shuffling during evolution. EMBO J 1987, 6:2565-2572

Patel H, Barber DL. A developmentally regulated Na-H exchanger in Dictyostelium discoideum is necessary for cell polarity during chemotaxis. J Cell Biol 2005, 169:321-329

Peltonen J, Jaakkola S, Lask G, Virtanen I, Uitto J. Fibronectin gene expression by epithelial tumor cells in basal cell carcinoma: an immunocytochemical and in situ hybridization study. J Invest Dermatol 1988, 91:289-293

Pérez NG, Alvarez BV, Camilión de Hurtado MC, Cingolani HE. pHi regulation in myocardium of the spontaneously hypertensive rat. Compensated enhanced activity of the Na(+)-H+ exchanger. Circ Res 1995, 77:1192-200

Perkinson RA, Kuo BA, Norton PA. Modulation of transcription of the rat fibronectin gene by cell density. J Cell Biochem 1996, 63:74-85

Peters JH, Spom LA, Ginsberg MH, Wagner DD. Human endothelial cells synthesize, process and secrete fibronectin molecules bearing an alternatively spliced type III homology (EDI). Blood 1990, 75:1801-1808

Piek E, Heldin CH, Ten Dijke P. Specificity, diversity, and regulation in TGF-beta superfamily signaling. FASEB J 1999, 13:2105–24

Polly P, Nicholson RC. Sequence of the mouse fibronectin-encoding gene promoter region. Gene 1993, 137:353-354

Popescu DC, Ham A-JL, Shieh B-H. Scaffolding protein INAD regulates deactivation of vision by promoting phosphorylation of transient receptor

potential by eye protein kinase C in Drosophila. J Neurosci 2006, 26:8570-8577

Pouyssegur J, Sardet C, Franchi A, Allemain G, Paris A. A specific mutation abolishing Na+/H+ antiport activity in hamster fibroblasts precludes growth at neutral and acidic pH. Proc Natl Acad Sci USA 1984, 81:4833-4837

Proetzel G, Pawlowski SA, Wiles MV, Yin M, Boivin GP, Howles PN, Ding J, Ferguson MW, Doetschman T. Transforming growth factor-beta 3 is required for secondary palate fusion. Nat Genet 1995, 11:409-414

Putney LK, Denker SP, Barber DL. The changing face of the Na+/H+ exchanger, NHE1: structure, regulation and cellular actions. Annu Rev Pharmacol Toxicol 2002, 42:527–52

Putney LK, Barber DL. Na-H exchange dependent increase in intracellular pH times G_2/M entry and transition. J Biol Chem 2003, 278:44645-44649

Roberts A, Wakefield L. The two faces of transforming growth factor beta in carcinogenesis. Proc Natl Acad Sci USA 2003, 100:8621–8623

Rockey DC. Hepatic fibrosis, stellate cells, and portal hypertension. Clin Liver Dis 2006, 10:459-479

Ruoslahti E, Pierschbacher MD. New perspectives in cell adhesion: RGD and integrins. Science 1987, 238:491-497

Ryseck RP, MacDonald-Bravo H, Zerial M, Bravo R. Coordinate induction of fibronectin, fibronectin receptor, tropomyosin, and actin genes in serum-stimulated fibroblasts. Exp Cell Res 1989, 180:537-545

Sanford LP, Ormsby I, Gittenberger-de Groot AC, Sariola H, Friedman R, Boivin GP, Cardell EL, Doetschman T. TGFbeta2 knockout mice have multiple developmental defects that are non-overlapping with other TGFbeta knockout phenotypes. Development 1997,124:2659–2670

Sardet C, Counillon L, Franchi A, Pouysségur J. Growth factors induce phosphorylation of the Na+/H+ antiporter, glycoprotein of 110 kDa. Science 1990 , 247:723-726

Schiller M, Javelaud D, Mauviel A. TGF-beta-induced SMAD signaling and gene regulation: consequences for extracellular matrix remodeling and wound healing. J Dermatol Sci 2004, 35:83–92

Schoppet M, Chavakis T, Al-Fakhri N, Kanse SM, Preissner KT. Molecular interactions and functional interference between vitronectin and transforming growth factor-beta. Lab Invest 2002, 82:37–46

Schwartz MA, Lechene C, Ingber DE. Insoluble fibronectin activates the Na/H antiporter by clustering and immobilizing integrin $\alpha_5\beta_1$, independent of cell shape. Proc Natl Acad Sci 1991, 88:7849-7853

Schwartz MA, Schaller MD, Ginsberg MH. Integrins: emerging paradigms of signal transduction. Annu Revu Cell Dev Biol 1995, 11:549-599

Schwarzbauer JE, Tamkun JW, Lemischka IR, Hynes RO. Three different fibronectin mRNAs arise by alternative splicing within the coding region. Cell 1983, 35:421-431

Schwarzbauer JE. The fibronectin gene. In: Extracellular Matrix Genes. 1990, Edited by Sandell L.J., Boyd C.D. Academic Press, San Diego, 1990; pp 195-219

Schwarzbauer JE. Fibronectin: from gene to protein. Cur Op Cell Biol 1991, 3:786-791

Schwarzbauer JE, Sechler JL. Fibronectin fibrillogenesis: a paradigm for extracellular matrix assembly. Curr Opin Cell Biol 1999; 11:622-627

Schwarzbauer JE, Spencer CS, Wilson CL. Selective secretion of alternatively spliced fibronectin variants. J Cell Biol 1989, 109:3445-3453

Sechler JL, Corbett SA, Scwarzbauer JE. Modulatory roles for integin activation and the synergy site of fibronectin during matrix assembly. Mol Biol Cell 1997, 8:2563-2573

Sechler JL, Corbett SA, Wenk MB, Schwarzbauer JE. Modulation of cell-extracellular matrix interactions. Ann NY Acad Sci 1998, 857:143-154

Sechler JL, Schwarzbauer JE. Control of cell cycle progression by fibronectin matrix architecture. J Biol Chem 1998, 273:25533-25536

Shi Y, Massague J. Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 2003, 113:685–700

Stanbridge EJ, Der CJ, Doersen CJ, Nishimi RY, Peehl DM, Weissman BE, Wilkinson JE. Human cell hybrids: analysis of transformation and tumorigenicity. Science 1982, 215:252-259

Sottile J, Hocking DC, Swiatek PJ. Fibronectin matrix assembly enhances adhesion-dependent cell growth. J Cell Sci 1998, 111:2933-43

Srivastava J, Barber DL, Jacobson MP. Intracellular pH sensors: design principles and functional significance. Physiology 2007, 22:30-39

Srivastava J, Barreiro G, Groscurth S, Gringas AR, Goult BT, Critchley DR, Kelly MJS, Jacobson MP, Barber DL. Structural model and functional significance of pH-dependent talin-actin binding for focal adhesion remodeling. Proc Natl Acad Sci 2008, 105:14436-41

Taipale J, Koli K, Keski-Oja J. Release of transforming growth factor-beta 1 from the pericellular matrix of cultured fibroblasts and fibrosarcoma cells by plasmin and thrombin. J Biol Chem 1992, 267:25378–25384

Taipale J, Miyazono K, Heldin CH, Keski-Oja J. Latent transforming growth factor-beta 1 associates to fibroblast extracellular matrix via latent TGF-beta binding protein. J Cell Biol 1994, 124: 171–181

Taipale J, Lohi J, Saarinen J, Kovanen PT, Keski-Oja J. Human mast cell chymase and leukocyte elastase release latent transforming growth factor-beta 1 from the extracellular matrix of cultured human epithelial and endothelial cells. J Biol Chem 1995, 270:4689–4696

Takahashi E, Abe J, Gallis B, Aebersold R, Sprin DJ, Krebs EG, Berk BC. p90(RSK) is a serum-stimulated Na+/H+ exchanger isoform-1 kinase: regulatory phosphorylation of serine 703 of Na+/H+ exchanger isoform-1. J Biol Chem 1999, 274:20206-20214

Takei H, Iino Y, Horiguchi J, Yokoe T. Immunohistochemical fibronectin staining pattern and prognosis in invasive breast carcinoma. Oncology 1995, 52:106-111

Tamkun JW, Hynes RO. Plasma fibronectin is synthesized and secreted by hepatocytes. J Biol Chem 1983, 258:4641-4647

Tang B, Vu M, Booker T, Santner SJ, Miller FR, Anver MR, Wakefield LM. TGF-beta switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. J Clin Invest 2003, 112:1116–1124

Ten Dijke P, Arthur HM. Extracellular control of TGFbeta signalling in vascular development and disease. Nat Rev Mol Cell Biol 2007, 8:857–869

Tominaga T, Barber DL. Na-H exchange acts downstream of RhoA to regulate integrin-induced cell adhesion and spreading. Mol Biol Cell 1998; 9:2287-2303

Tominaga T, Ishizaki T, Narumiya S, Barber DL. p160ROCK mediates RhoA activation of Na-H exchange. EMBO J 1998, 17:4712-4722

Velling T, Risteli J, Wennerberg K, Mosher DF, Johansson S. Polymerization of type I and III collagens is dependent on fibronectin and enhanced by integrins $\alpha_{11}\beta_1$ and $\alpha_2\beta_1$. J Biol Chem 2002, 277:37377-37381

Vexler ZS, Symons M, Barber DL. Activation of Na+-H+ exchange is necessary for RhoA-induced stress fiber formation. J Biol Chem 1996, 271: 22281–22284

Wahl SM. Transforming growth factor-[beta]: innately bipolar. Curr Opin Immunol 2007, 19:55–62

Wakabayashi S, Fafournoux P, Sardet C, Pouyssegur J. The Na/H antiporter cytoplasmic domain mediates growth factor signals and controls “H+-sensing”. Proc Natl Acad Sci USA.1992, 89:2424–2428

Wennerberg K, Lohikangas L, Gullberg D, Pfaff M, Johansson S, Fassler R. Fibronectin fibrillogenesis: a paradigm for extracellular matrix assembly, β1 integrin-dependent and -independernt polymerization of fibronectin. J Cell Biol 1996, 132:227-238

Wierzbicka-Patynowski I, Schwarzbauer JE. Regulatory role for Src and Phosphatidylinositol 3-kainase in initiation of Fibronectin Matrix assembly. J Biol Chem 2002, 277:19703-19708

Wierzbicka-Patynowski I, Schwarzbauer JE. The ins and outs of fibronectin matrix assembly. J Cell Sci 2003, 116:3269-3276

Winklbauer R, Nagel N. Directional mesoderm cell migration in the Xenopus gastrula. Dev Biol 1991, 148:573-589

Williams EC, Janmey PA, Ferry JD, Mosher DF. Conformational states of fibronectin. Effects of pH, ionic strength and collagen-binding. J Biol Chem 1982, 257:14973-14978

Wipff P-J, Rifkin DB, Meister J-J, Hinz B. Myofibroblast contraction activates TGFb1 from the extracellular matrix. J Cell Biol 2007, 179:1311-1323

Wipff PJ, Hinz B. Integrins and the activation of latent transforming growth factor b1 – An intimate relationship. Eur J Cell Biol 2008, 87:601–615

Wu C, Kevins V, O'Toole TE, McDonald JA, Ginsberg MH. Integrin activation and cytoskeletal interaction are critical steps in the assembly of a fibronectin matrix. Cell 1995, 83:715-724

Wu C. Roles of integrins in fibronectin matrix assembly. Histol Histopathol 1997, 12:233-240

Wylie C. Germ cells. Cell 1999, 96:165-174

Yang JT, Hynes RO. Fibronectin receptor functions in embryonic cells deficient in $\alpha_5\beta_1$ integrin can be replaced by αv integrins. Mol Biol Cell 1996, 7:1737-1748

Yoshida H, Karmazyn M. Na(+)/H(+) exchange inhibition attenuates hypertrophy and heart failure in 1-wk postinfarction rat myocardium. Am J Physiol Heart Circ Physiol 2000 , 278:H300-4

Yost HJ. Regulation of vertebrate left-right asymmetries by extracellular matrix. Nature 1992, 14;158-61

Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. Genes Dev 2000, 14:163–176

Zhang Q, Magnusson MK, Mosher DF. Lysophosphatidic acid and microtubule-destabilizing agents stimulate fibronectin matrix assembly thourough Rho-dependent actin stress fiber formation and cell contraction. Mol Biol Cell 1997, 8:1415-1425

Zhang Q, Peyruchaud O, French KJ, Magnusson MK, Mosher DF. Sphingosine1-Phosphate Stimulates Fibronectin Matrix Assembly Through a Rho-Dependent Signal Pathway. Blood 1999, 93:2984-2990

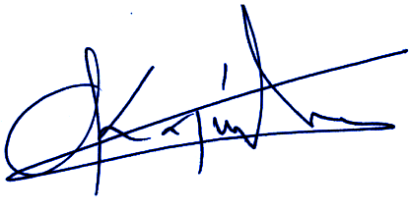
Zhong C, Chourzanowska-Wodnicka M, Brown J, Shaub A, Belkin AM, Burridge K. Rho-mediated contractility exposes a cryptic site in fibronectin and induces matrix fibronectin assembly. J Cell Biol 1998, 141:539-551

Publishing Agreement

It is the policy of the University to encourage the distribution of all theses, dissertations, and manuscripts. Copies of all UCSF theses, dissertations, and manuscripts will be routed to the library via the Graduate Division. The library will make all theses, dissertations, and manuscripts accessible to the public and will preserve these to the best of their abilities, in perpetuity.

Please sign the following statement:

I hereby grant permission to the Graduate Division of the University of California, San Francisco to release copies of my thesis, dissertation,or manuscript to the Campus Library to provide access and preservation, in whole or in part, in perpetuity.



March 2nd, 2009

Author Signature Date