Tumor Cell Dependence on Ran-GTP–Directed Mitosis

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Abstract

Deregulated cell division is a hallmark of cancer, but whether tumor cells become dependent on specific mitotic mechanisms is not known. Here, we show that the small GTPase Ran, a regulator of mitotic spindle formation, is differentially overexpressed in human cancer as compared with normal tissues, in vivo. Acute silencing of Ran in various tumor cell types causes aberrant mitotic spindle formation, mitochondrial dysfunction, and apoptosis. This pathway does not require p53, Bax, or Smac, but is controlled by survivin as a novel Ran target in cancer. Conversely, loss of Ran in normal cells is well tolerated and does not result in mitotic defects or loss of cell viability. Therefore, tumor cells can become dependent on Ran signaling for cell division, and targeting this pathway may provide a novel and selective anticancer strategy. [Cancer Res 2008;68(6):1826–33]

Introduction

With hundreds of deregulated and mutated genes, cancer cells exhibit extraordinary molecular complexity (1). Such subversion of fundamental cellular pathways poses formidable challenges to identify, at least in most cases (2), a single driving oncogenic lesion suitable for therapeutic intervention (3). On the other hand, the global changes associated with neoplastic transformation raise the possibility that critical cellular processes may be “qualitatively” different in tumor cells, relying on effector molecules that are mutated, amplified, or otherwise differently expressed in cancer, as opposed to normal tissues. In some cases, oncogenic pathways have been shown to provide such a dominant advantage for tumor growth that transformed cells become dependent on their signals, a process described as “oncogene addiction” (4). Although the extent by which this mechanism occurs in vivo and its implications for tumor maintenance remain to be established (4), pathways of oncogene dependence offer fresh therapeutic opportunities, which, at least in subsets of patients, have led to spectacular clinical responses and modest side effects (5).

Deregulated cell division is a pivotal cancer pathway, which results in aberrant cell proliferation, obliteration of cell cycle checkpoints, and propensity to aneuploidy (6). This requires a spatial-temporal assembly of a bipolar mitotic spindle (7), where microtubules nucleating from duplicated centrosomes or around mitotic chromosomes “search and capture” chromatids and ultimately segregate them between daughter cells (8). The small GTPase Ran plays a pivotal role in the process of spindle formation (9) by releasing target molecules (10, 11) that promote microtubule stability, especially TPX2 (12), from inhibition by importin α/β receptors (13). Conversely, inhibition of Ran signaling severely hampers mitosis, resulting in the appearance of flattened mitotic spindles, loss of microtubules, and gross chromosomal abnormalities (12). Some recent evidence suggests that this pathway may be important in cancer, as Ran target molecules seem to be overexpressed in transformed cells (14–16), and suppression of Ran levels in tumor cells has been associated with induction of cell death (11, 17).

In this study, we investigated the effect of Ran signaling on mitosis of normal versus tumor cells. We found that Ran is broadly overexpressed in cancer, as opposed to normal tissues, in vivo, and that this pathway becomes essential for cell division in transformed, but not normal, cells.

Materials and Methods

Cell culture and antibodies. Cervical carcinoma HeLa, colon adenocarcinoma HCT116, breast adenocarcinoma MCF-7 and MDA-MB-231, B lymphoblastoid Raji, lung adenocarcinoma H460 and H1975, epithelial carcinoma A431, prostate adenocarcinoma PC3, immortalized mammary epithelial MCF10A, normal human kidney epithelial HEK293, and primary fibroblast HFF, HGF, and WS-1 cells were obtained from the American Tissue Type Collection, and maintained in culture according the supplier’s specifications. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics, Inc. HCT116-p53−/−, HCT116-BAX−/−, and HCT116-Smac−/− cells were a kind gift from Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD). MCF-7 cells were transfected with a survivin cDNA, selected in G418-containing medium, and a clone stably expressing survivin (MCF-7 SVV) was established. Antibodies to survivin (Novus Biologicals), Ran (Novus Biologicals), Cell Signaling, Santa Cruz Biotechnology), α-tubulin (Sigma-Aldrich), TPX2 (Novus Biologicals), RCC1 (Santa Cruz Biotechnology), cytochrome c (BD Biosciences-Clontech), Smac (ProSci), Ran-GAP1 (Abcam), X-linked inhibitor of apoptosis (XIAP; BD Biosciences), caspase-3 (Cell Signaling), or β-actin (Sigma-Aldrich) were used.

Small interfering RNA transfections. Gene silencing by small interfering RNA (siRNA) was carried out with control nontargeted (VIII) or two independent Ran-directed siRNA oligonucleotides, Ran-1 (GAAAUUCGGUGGACUCGAGAUU) or Ran-2 (AGAUUGCUUAUUAAUCCAAUU; Dharmaco), used at 50 nMol. Subconfluent cultures were transfected by Oligofectamine (Invitrogen) and harvested after 48 to 96 h at 37°C.

Fluorescence microscopy and cell death analysis. Cells were permeabilized with 0.1% Triton X-100 for 10 min, blocked in 3% bovine serum albumin/0.2% Tween 20/PBS for 1 h at 22°C, and incubated with a primary antibody to α-tubulin or TPX2 (1:200) for 1 h at 22°C, followed by addition of Alexa 594–conjugated secondary reagent (1:1,000) of appropriate specificity (Molecular Probes). Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI; 6.5 μg/mL, Sigma). After washes, cells were analyzed under a Fluorescence microscope (Axioplan 2; Zeiss) equipped with a charge-coupled device camera (Axiocam, Zeiss). For analysis of cell death, normal or tumor cell types were transfected with nontargeted or Ran-directed siRNA and then analyzed for DNA content by propidium iodide staining and flow cytometry at various time intervals. In other experiments, transfected cells were harvested after 24 to 72 h and analyzed for nuclear morphology of apoptosis by DAPI staining and fluorescence microscopy, or, alternatively, simultaneously stained for DEVDase (caspase) activity.
and propidium iodide by multiparametric two-color flow cytometry, as described (18).

**Microarray meta-analysis.** Microarray data comparing various tumor tissues and their matched normal counterparts were analyzed for relative expression levels of Ran using Oncomine™ (19). Arrays were selected with a P value threshold of 0.001, outlier rank threshold 50, and raw data were normalized as follows: log 2 transformed, array median set to 0, array SD set to 1, and mean centered per study. Data were plotted as normalized expression units using Prism 4.0 (GraphPad Software). The number of patients per sample in the various cohorts was as follows: Graudens et al. (20), normal colon, 12; colorectal adenocarcinoma 18; Notterman et al. (21), normal colon, 18; colorectal adenocarcinoma, 18; Alon et al. (22), normal colon, 22; colorectal adenocarcinoma, 40; Tomlins et al. (23), benign prostate, 22; prostate adenocarcinoma, 30; Boer et al. (24), normal kidney, 129; clear cell renal cell carcinoma, 120; Dyrskjot et al. (25), normal bladder, 26; invasive transitional cell carcinoma, 14; Richardson et al. (26), normal breast, 7; breast adenocarcinoma, 40; Bhattacharjee et al. (27), normal lung, 17, lung squamous cell carcinoma, 21, lung adenocarcinoma, 139; Wachi et al. (28), normal lung, 5; lung squamous cell carcinoma, 5; Stearman et al. (29), normal lung, 19; lung adenocarcinoma, 20.

**Tissue procurement and immunohistochemistry.** Formalin-fixed, paraffin-embedded anonymous surgical specimens of two cases of human renal cell carcinoma, ovarian adenocarcinoma, and soft tissue sarcoma were obtained from the UMass Cancer Center Tissue Bank. Samples of large cell lymphoma and soft tissue sarcoma were collected from p53−/− mice at 4 to 6 mo of age. Tissue staining was carried out as described (18), with quenching of endogenous peroxidase and epitope heat retrieval. Slides were stained with a primary antibody to Ran or IgG using avidin-biotin-peroxidase technique (Histostain-plus, Zymed Laboratories) and 3,3′-diaminobenzidine as a chromogen.

**Results**

**Differential expression of Ran in cancer.** We began this study by analyzing the expression of Ran in normal or transformed human cultured cell lines. Ran was abundantly and ubiquitously found in all tumor cell types tested, including prostate adenocarcinoma PC3, B lymphoblastoid Raji, breast adenocarcinoma MCF-7 and MDA-MB-231, colon adenocarcinoma HCT116, and cervical carcinoma HeLa (Fig. 1A). In contrast, three primary normal human fibroblast cell types, WS-1, HGF, and HFF, expressed low levels of Ran (Fig. 1A). Next, we analyzed primary mouse tissues for Ran expression in vivo. Extracts of large cell lymphoma and soft tissue sarcoma isolated from p53−/− mice contained elevated levels of Ran (Fig. 1B). Except for testis, in which Ran was highly expressed, all other normal mouse tissues tested, including liver, kidney, lung, heart, and brain, had low expression of Ran (Fig. 1B). Consistent with this, Ran was strongly expressed in mouse soft tissue sarcoma, or lymphoma cells infiltrating the liver, whereas normal neighboring hepatocytes did not stain for Ran, and IgG was unreactive (Fig. 1C).

**Molecular profiling of Ran expression in human cancer.** In primary human cancers, Ran was intensely expressed in the tumor cell population of renal cell carcinoma, ovarian adenocarcinoma, and soft tissue sarcoma in vivo, whereas IgG gave no staining (Fig. 2A). Consistent with a differential distribution in tumors, Ran was abundantly present in kidney cancer but expressed at very low levels in normal kidney by Western blotting (Fig. 2B). To determine whether these changes occurred in larger patient cohorts and in multiple types of cancer, we next conducted a meta-analysis of Ran expression in various microarray data sets. Various patient cohorts with diagnosis of colon adenocarcinoma, prostate adenocarcinoma, renal cell carcinoma, bladder transitional cell carcinoma, breast adenocarcinoma, lung squamous cell carcinoma, and lung adenocarcinoma consistently revealed considerably higher levels of Ran expression in the tumor cell population, as compared with normal matched tissues (Fig. 2C). The differential Ran expression in cancer as compared with normal tissues (Fig. 2C) was highly statistically significant among the different patient series examined, with \( P = 5 \times 10^{-10} \) (colon cancer; ref. 20), \( P = 1.2 \times 10^{-6} \) (colon cancer; ref. 21), \( P = 1.1 \times 10^{-4} \) (colon cancer; ref. 22), \( P = 4 \times 10^{-4} \) (prostate cancer; ref. 23), \( P = 4.4 \times 10^{-4} \) (renal cell carcinoma; ref. 24), \( P = 5.5 \times 10^{-5} \) (bladder cancer; ref. 25), \( P = 3.9 \times 10^{-4} \) (breast adenocarcinoma; ref. 26), \( P = 1.8 \times 10^{-5} \) to \( 3.2 \times 10^{-5} \) (lung squamous cell carcinoma and lung adenocarcinoma, respectively; ref. 27), \( P = 4.1 \times 10^{-5} \) (lung squamous cell carcinoma; ref. 28), and \( 7.6 \times 10^{-4} \) (lung adenocarcinoma; ref. 29).

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Ran targeting induces aberrant mitoses and cell death in tumor cells. Because no pharmacologic inhibitors of Ran are presently available, we next used siRNA to target its expression in normal or tumor cell types. Transfection of HeLa cells with Ran-directed siRNA resulted in time-dependent decrease in Ran expression, which was maximal by 60 to 72 hours (Fig. 3A). In contrast, a nontargeted siRNA did not affect Ran levels (Fig. 3A). siRNA ablation of Ran in HeLa cells was associated with time-dependent decreased expression of survivin (Fig. 3B), consistent with its recent identification as a potential Ran target gene (30). Loss of survivin under these conditions also resulted in reduced expression of XIAP, in agreement with the ability of survivin to bind XIAP and enhance its stability in vivo (18), whereas Ran regulatory proteins, Ran-GAP1 or RCC1, were not affected (Fig. 3B). When analyzed by fluorescence microscopy, HeLa cells transfected with control siRNA exhibited normal mitotic spindles with a full complement of microtubules and strong reactivity for the Ran effector molecule, TPX2, on spindle poles and metaphase fibers (Fig. 3C). Conversely, Ran silencing in HeLa cells caused aberrant mitoses, characterized by flattened mitotic spindles, depletion of microtubules, chromosomal missegregation, and nearly complete loss of TPX2 from spindle microtubules (Fig. 3C). siRNA ablation of Ran in HeLa cells was associated with time-dependent appearance of a population with sub-G1 (i.e., hypodiploid) DNA content in a reaction maximal 60 to 72 hours after transfection, thus coinciding with peak Ran suppression (Fig. 3D).

Spectrum of anticancer activity of Ran targeting. To test the generality of Ran signaling for tumor cell viability, we next analyzed
a panel of tumor cell types for their response to Ran ablation. siRNA targeting comparably suppressed Ran levels in estrogen receptor–positive breast adenocarcinoma MCF-7 cells, as well as in immortalized, but not fully tumorigenic, MCF10A breast epithelial cells (Fig. 4A, left). Ran ablation in these cell types resulted in quantitatively comparable induction of cell death by hypodiploid DNA content and flow cytometry, whereas nontargeted siRNA had no effect (Fig. 4A, right). To confirm the specificity of Ran targeting, we next tested an independent Ran-directed dsRNA oligonucleotide (siRNA-2). Transfection of HeLa cells with Ran-directed siRNA-2 significantly suppressed Ran expression by Western blotting (Fig. 4B, left) and induced cell death 72 hours after transfection by DNA content analysis and flow cytometry (Fig. 4B, right). In contrast, a control siRNA had no effect on Ran levels or HeLa cell viability (Fig. 4B). In addition, transfection of disparate tumor cell types, including lung adenocarcinoma H460 and H1975, epithelial carcinoma A431, and estrogen receptor–negative breast adenocarcinoma MDA-MB-231, with Ran-directed siRNA resulted in significant suppression of Ran levels, as compared with control siRNA (Fig. 4C, left). Ran ablation in these cell types resulted in induction of cell death by nuclear morphology of apoptosis and fluorescence microscopy, whereas a nontargeted siRNA did not significantly affect tumor cell viability (Fig. 4C, right).

Requirements of tumor cell death induced by Ran targeting. We next further investigated the cell death pathway activated by Ran targeting in tumor cells. siRNA ablation of Ran in HeLa cells was associated with increased effector caspase activity (Fig. 5A), hypodiploid DNA content (Fig. 5B, top), time-dependent appearance of activated (i.e., cleaved) caspase-3 (Fig. 5B, bottom), and release of mitochondrial apoptogenic proteins Smac and cytochrome c in the cytosol (Fig. 5C). In contrast, a control siRNA had no effect on these hallmarks of apoptosis (Fig. 5A–C). A caspase inhibitor, z-VAD-fmk, completely reversed cell death (Fig. 5B, top), or caspase-3 cleavage (Fig. 5B, bottom), after Ran suppression, consistent with genuine induction of apoptosis under these conditions. To further elucidate the molecular requirements of this cell death response, we next ablated Ran expression in HCT116 cells lacking various apoptosis effectors and analyzed cell death responses. Suppression of Ran in wild-type (WT) HCT116 cells or isogenic cell types deficient in p53, proapoptotic Bcl-2 protein, Bax, or Smac resulted in quantitatively indistinguishable loss of cell viability by hypodiploid DNA content and flow cytometry (Fig. 5D). In contrast, a nontargeted siRNA had no effect on Ran expression or cell viability in the various HCT116 cells tested (Fig. 5D).

Differential sensitivity of normal cell types to Ran targeting. We next asked whether Ran targeting in normal cell types also resulted in mitotic defects and apoptosis. In sharp contrast with tumor cells, siRNA ablation of Ran in primary human WS-1 fibroblasts did not cause mitotic defects, and spindle formation in these cells was indistinguishable from control cultures transfected with nontargeted siRNA (Fig. 6A). Accordingly, siRNA ablation of Ran in various normal cell types, including WS-1, human kidney epithelial HEK293, or actively proliferating HUVECs, efficiently suppressed Ran expression (Fig. 6B, left), but had no significant effect on cell cycle progression or cell viability, as compared with control siRNA (Fig. 6B, right). Next, we wished to identify effector molecule(s) that may control the differential exploitation of Ran signaling in tumor versus normal cell types, and we focused on survivin for its potential role as a novel Ran target (30) and differential regulation of mitosis and apoptosis in cancer (31). For
these experiments, we established a new MCF-7 cell line stably transfected with survivin (MCF-7 SVV; Fig. 6C, left). Transfection of parental MCF-7 cells with Ran siRNA, but not control siRNA, resulted in a modest decrease of endogenous survivin levels, which was associated with mitotic spindle defects, chromosomal missegregation, and apoptosis (Fig. 6C, middle and right). Conversely, Ran silencing in MCF-7 SVV cells did not affect survivin levels (Fig. 6C, left), and these cells exhibited normal bipolar mitotic spindles with proper chromosomal alignment and complete reversal of cell death (Fig. 6C, middle and right).

Discussion

In this study, we have shown that the small GTPase Ran is abundantly expressed in many human cancers, but present at low levels in most normal tissues in vivo. Second, acute silencing of Ran in tumor cells triggered defects in mitotic spindle assembly, mitochondrial dysfunction, and loss of cell viability by apoptosis. Conversely, Ran ablation in various normal cell types, including actively proliferating endothelial cells, was well tolerated and did not elicit mitotic defects or cell death.

Among the regulators of cell division, Ran-GTP signaling has been recognized for a universal role in spindle assembly (9), largely centered on the delivery of target molecules, for instance TPX2 (12), which affect microtubule dynamics and promote spindle formation. However, emerging evidence suggests that this pathway may be preferentially exploited in cancer. Accordingly, several Ran effectors have been characterized as cancer genes, differentially expressed or mutated in transformed cells, and including an oncogenic kinase, Aurora A (32), a microtubule-associated protein overexpressed in certain cancers, HURP (14), and a DNA repair/checkpoint protein complex, BRCA1/BARD1, with critical roles in genomic integrity (33). The data presented here lend further credulity to the idea of a "cancer-specific" utilization of Ran signaling, with the demonstration that Ran is broadly overexpressed in cancer compared with normal tissues in vivo, and its pathway is selectively used by tumor cells to execute mitosis. Accordingly, Ran ablation in disparate tumor cell types caused extensive mitotic spindle defects, with mislocalization of TPX2 from microtubules; discharge of mitochondrial apoptotic proteins, cytochrome c, and Smac in the cytosol; and activation of caspase-dependent cell death, independently of apoptosis effectors, p53, Bax, and Smac.

Although interference with Ran signaling has been associated before with induction of tumor cell death (12, 17), the phenotype presented here did not involve a protracted S-phase arrest, and was...
comparably observed in multiple tumor cell types, regardless of their genetic makeup, and, in particular, independently of a mutant K-Ras allele, thus at variance with previous studies (17). Instead, a pivotal requirement of this cell death response was identified as an acute loss of survivin levels after Ran knockdown. In addition to other cancer genes identified as Ran effectors (14, 16, 33), recent data have shown that survivin, a regulator of mitosis and apoptosis in cancer (31), may also function as a Ran target. This involves binding of survivin to the Ran effector Crm1 in a pathway required for survivin nucleo-cytoplasmic shuttling and apoptosis inhibition (30) as well as formation of survivin-Ran physical complexes that contribute to mitotic spindle formation.2 Consistent with current models of survivin function (31), acute destabilization of survivin levels after Ran ablation may directly contribute to the dual phenotype of mitotic spindle abnormalities and activation of mitochondrial apoptosis observed here. Accordingly, forced expression of survivin was sufficient alone to completely rescue the appearance of cell division defects and cell death induced by Ran targeting in tumor cells.

Despite its evolutionary conservation in model organisms (12), our findings suggest that the Ran pathway is unexpectedly largely dispensable in normal cell types because ablation of Ran in quiescent or proliferating normal cells did not cause mitotic spindle defects or apoptosis. The basis for this differential sensitivity remains to be fully elucidated. However, one possibility is that the differential overexpression of Ran in cancer and the selective recruitment of cancer genes, including survivin, as Ran effectors may impart “qualitative” differences to this pathway in tumor cells. This may explain not only the relative insensitivity of normal cells to Ran ablation but also the exquisite dependence of transformed cells on Ran signaling for execution of mitosis and preservation of cell viability. The idea that tumor cells may become “addicted” (4) to a specific mitotic pathway, which can be relatively compensated for in normal tissues, is not entirely without precedent. Accordingly, loss or inhibition of cell division kinases, including cyclin-dependent kinase-4 (34), Polo (35), Aurora B (36), and Eg5 (37), has been shown to profoundly impair tumor cell mitosis, often resulting in apoptosis, while being well tolerated in normal cell types.

Why Ran signaling becomes a dominant pathway for tumor cell maintenance is currently unknown. However, deregulation of this pathway seems to be ideally positioned to promote chromosomal instability and aneuploidy, thus multiplying tumor diversity. This is consistent with a role of Ran and its effector molecules in chromosome positioning (38), loading of spindle checkpoint
proteins (39), and organization of kinetochore fibers (40), all mechanisms that are crucial for proper chromosome segregation. Supporting this model, the Ran targets TPX2 and Aurora A were recently identified in a chromosomal instability gene signature associated with poor outcome in various types of cancer (41), and gene profiling data in ovarian cancer (42) and experimental breast cancer (43) have consistently linked increased Ran levels to disease progression.

In summary, we have shown that most tumor cells, but not normal tissues, become dependent on Ran signaling for execution of mitosis. These findings should open concrete prospects for the development of a novel class of antimitotic agents (17), aimed at a pivotal cell division pathway that is quantitatively and qualitatively exploited in tumors, compared with normal tissues. Similar to other paradigms of “oncogene addiction” (5), potential antagonists of Ran signaling may exhibit effective anticancer activity by selectively disabling tumor cell mitosis while carrying minimal side effects for normal tissues in vivo.

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References


Figure 6. Insensitivity of normal cell types to Ran targeting. A, fluorescence microscopy. Primary WS-1 human fibroblasts were transfected with control or Ran-directed siRNA and analyzed by fluorescence microscopy. DNA was labeled with DAPI. B, analysis of cell death. WS-1 fibroblasts, normal human epithelial HEK293 cells, or HUVECs were transfected with control or Ran-directed siRNA and analyzed by Western blotting at the indicated time intervals (left) or for DNA content by propidium iodide staining and flow cytometry after 72 h (right). C, survivin rescue. MCF-7 or MCF-7 cells stably expressing survivin (MCF-7 SVV) were transfected with control or Ran-directed siRNA and analyzed by Western blotting (left), fluorescence staining (middle), or for DNA content by flow cytometry (right). DNA was stained with DAPI. B and C, the percentages of cells in sub-G1, G1, and G2-M peaks are indicated.
Kline-Smith SL, Walczak CE. Mitotic spindle assembly.


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