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Phenotypic changes in a squamous cell carcinoma cell line induced by an effector kinase of a small G protein Rap2

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ABSTRACT

Purpose: Traf2- and Nck-interacting kinase (TNIK) is one of the effector kinases of the Ras-like GTPase Rap2, and belongs to the STE20 group of mitogen-activated protein kinase kinase kinase kinases (MAP4Ks). The purpose of this study was to explore the roles of TNIK in the phenotype of cancer cells. **Results:** Derivatives of a cutaneous squamous cell carcinoma (SCC) cell line, Pam212, in which expression of hemagglutinin-tagged TNIK (HA-TNIK) can be induced by modulating the concentration of a tetracycline derivative in culture medium were established by using a retrovirus-mediated gene transduction system. Morphological analyses revealed that HA-TNIK regulated intercellular adhesion and epithelial cell shape. A strong induction of HA-TNIK disrupted intercellular adhesion and abolished the epithelial cell shape. Moderately induced HA-TNIK co-localized with E-cadherin, a principal component of intercellular adherens junctions (AJs), and facilitated cellular detachment during hepatocyte growth factor-induced cell scattering and wound-healing cell migration assays. Moderately induced HA-TNIK also retarded Ca^{2+}-dependent re-assembly of AJs that had been pre-disassembled by transient depletion of Ca^{2+} in culture medium. **Conclusion:** These results complement our preceding work using a proteomic approach (Kuninaka et al. "Proteomic changes in a squamous cell carcinoma cell line induced by an effector kinase of a small G protein Rap2" Ryukyu Med. J. 26: 135-145, 2007) on roles of TNIK in SCC cells. *Ryukyu Med. J., 32(3,4)89～94, 2013*

Key words: cancer cell phenotype, Rap2, MAP4K, skin cancer

INTRODUCTION

Rap2 is a member of the Ras family of small GTPases[1)]. Ras regulates mainly Erk, the classical mitogen-activated protein kinase (MAPK), by directly interacting with its downstream effector kinase Raf. Because of one residue difference (Ser39 vs. Phe39) between the effector-interacting core regions of Ras and Rap2, Rap2 interacts with effectors that do not interact with Ras[2-4)]. We identified three Rap2-specific effectors, MAPK kinase kinase kinase 4 (MAP4K4)[2)], Traf2- and Nck-interacting kinase (TNIK)[3)], and Misshapen/NIKs-related kinase (MINK)[4)], which all belong to the STE20 group of MAP4Ks that act upstream of c-Jun N-terminal kinase (JNK), a stress-related MAPK[5)].

TNIK's roles in physiological or pathological cellular processes are still not completely known. To address this issue, we took a proteomic approach in an earlier study[6)]. We examined the effect of over-expressed TNIK on the protein expression profile in a squamous cell carcinoma cell line, Pam212, which arose from mouse epidermal keratinocytes[7)]. The results revealed downregulation of three proteins that were under

study as candidate proteins for cancer prognostic markers and target proteins for cancer chemotherapeutic drug development: Rho guanine nucleotide dissociation inhibitor α (Rho GDIα), stomatin-like protein 2, and ornithine aminotransferase. Although clinically relevant to some extent, these results left the phenotypic effect of TNIK unclear. In the present study, we used a cell biological approach to address this issue.

MATERIALS and METHODS

Pam212-derived clones

Pam212 cells were from Dr. Stuart H. Yuspa (National Institutes of Health, Bethesda, MD). Procedures for generating Pam212-derived lines that are induced to express hemagglutinin-tagged TNIK (HA-TNIK) in the absence of tetracycline (Rev-Tet-Off System, BD Biosciences), and selection of inducible clones have been described in detail[6].

Induction of HA-TNIK expression

Cells were maintained in Dulbecco's Minimal Essential Medium (DMEM) containing 10% fetal bovine serum (FBS) in the presence of 1 μg/ml of the tetracycline-derivative doxycycline (Dox) to shut off induction, or grown for 3 days in the absence of Dox for induction as described[6].

Phenotypic analyses

For phenotypic analyses including HA-TNIK induction, hepatocyte growth factor (HGF)-induced scattering and Ca^{2+} switch assays, Western blotting and immunofluorescence laser scanning confocal microscopy (LSCM) were performed as we described previously[2-4, 8, 9], using antibodies against HA-tag (Roche), phospho-Erk (Cell Signaling), total Erk (Cell Signaling) and E-cadherin (Santa Cruz), and 40 ng/ml HGF (R & D Systems). Actin was visualized using rhodamine-phalloidin (Cytoskeleton, Inc) in LSCM.

For the *in vitro* wound-healing assay, cell monolayers were scratched with a pipette tip to create a cell-free area ("wound") as previously described[10].

In the Ca^{2+} switch assay, cells were incubated in 2 mM Ca^{2+} in serum-free DMEM for 1 h, followed by incubation in 2 μM Ca^{2+} (DMEM with 5 mM EGTA) for 1 h, to switch medium Ca^{2+} concentration as previously described[11, 12]. Cells were then recovered in DMEM containing 10% FBS for 1.5 h.

RESULTS

In our preceding work[6], strong induction of HA-TNIK expression was attained in clones A and B when the cells were grown in the absence of Dox ("Dox−"). However, incomplete suppression was also observed in the presence of Dox ("Dox+")[6]. As this "leaky expression" in Dox− cells could complicate analyses, in this study we additionally selected clone C, which exhibited moderate induction and was free from leaky expression (Fig. 1A).

Clone C differed phenotypically from clones A and B under both Dox+ and Dox− conditions (Fig. 1B). Dox+ clone A grew with loose cell-cell contacts and poor epithelial appearance, while Dox+ clone C grew as tightly packed colonies of cells with typical cobblestone epithelial appearance (Fig. 1B). Dox− clone A cells were scattered and the cells resembled migrating mesenchymal cells, while Dox− clone C still maintained colonies in the absence of the cell scattering factor HGF (Dox−/HGF− cells). Notably, however, Dox− clone C in the presence of HGF (Dox−/HGF+ cells) often exhibited scattering in response to HGF, unlike Pam212[13]. Pam212 variants that overexpress the HGF receptor exhibit scattering[13], and HGF-induced migration of cultured keratinocytes correlates with Erk activation[14]. Thus we examined HGF-induced Erk activation (Fig. 1C). However, no marked differences were observed among Pam212, Dox− clone C and Dox+ clone C.

Clone C was further examined using immunofluorescence LSCM. At the junctional level (Fig. 2 left), the adherens junction (AJ) was marked by E-cadherin and the "circumferential actin belt"[12] in Dox+/HGF−, Dox+/HGF+ and Dox−/HGF− cells. In Dox−/HGF− cells, the HA-TNIK signal was prominent at the boundaries of cells inside colonies, but not outer free edges of peripheral cells. In the Dox−/HGF+ situation, HA-TNIK was distributed throughout the plasma membrane in partially scattered colonies, similarly to E-cadherin.

At the basal level (Fig. 2 right) of Dox+/

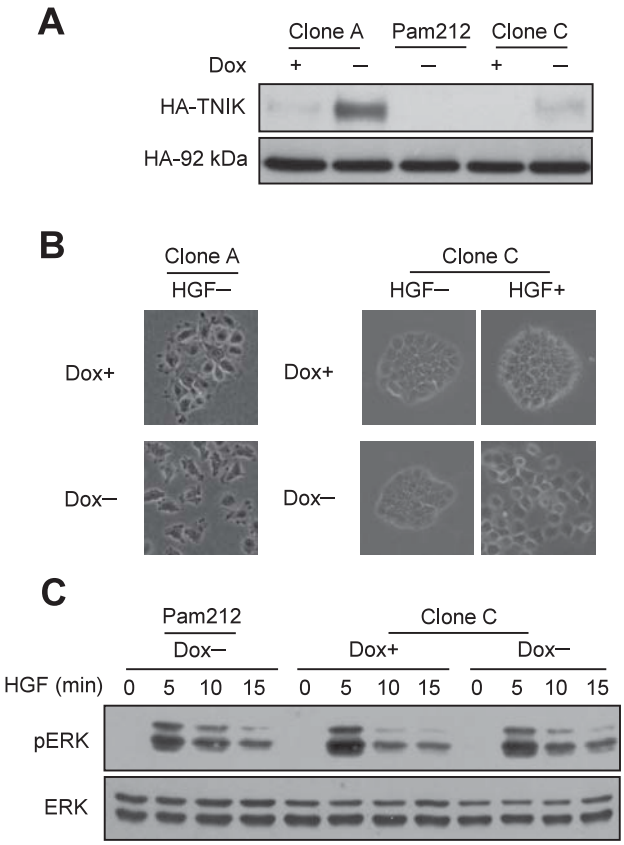
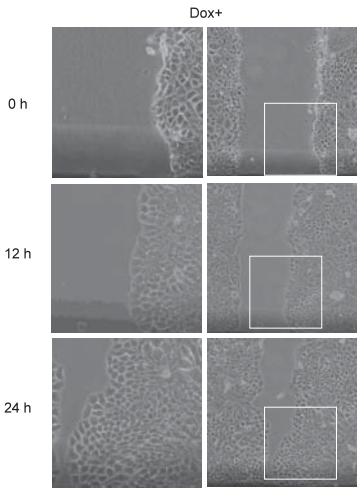


Fig. 1



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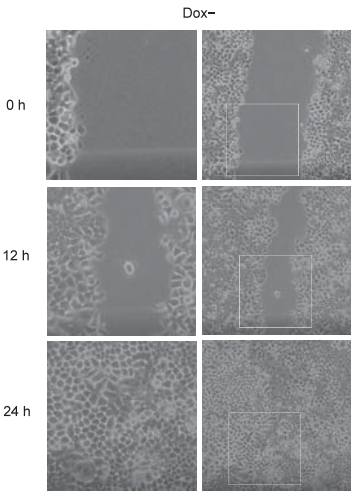

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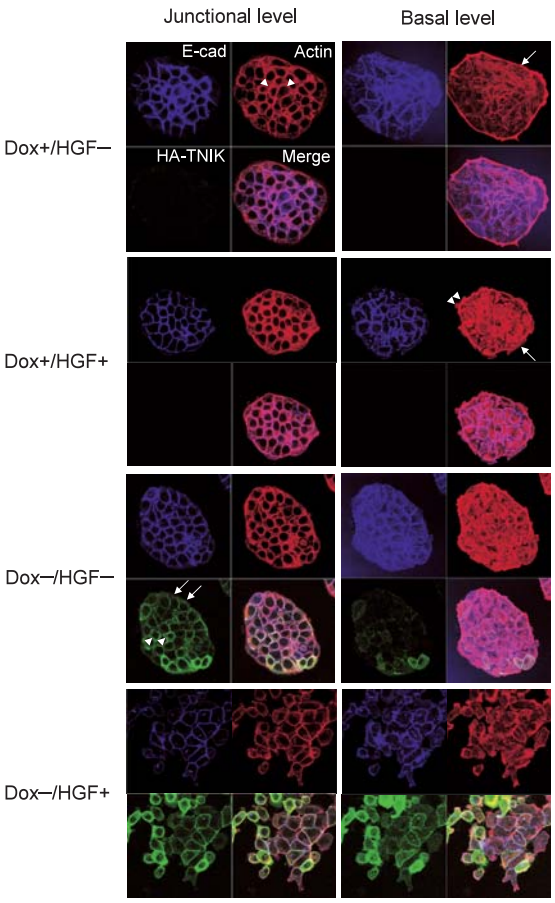

Fig. 2

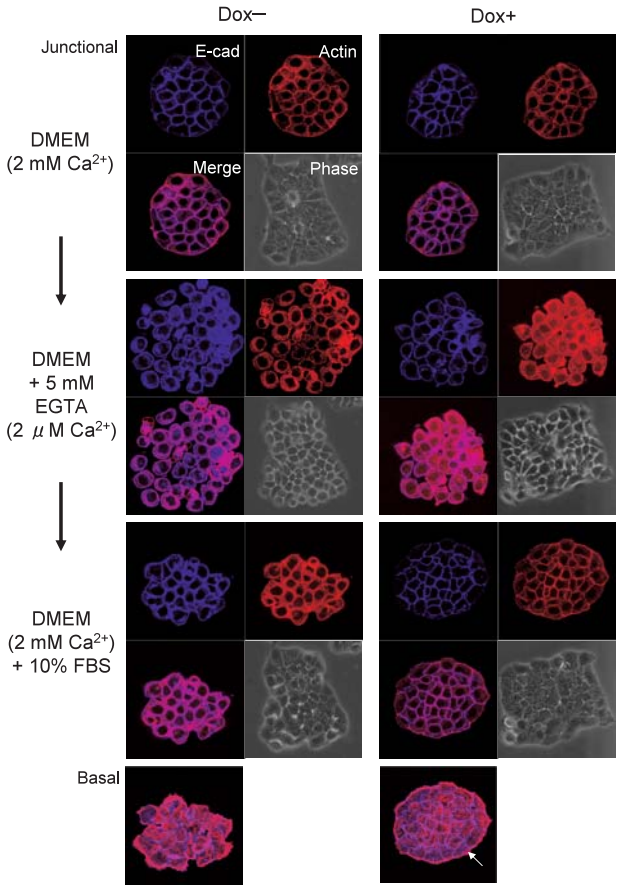

Fig. 5

Fig. 1 **Characterization of cell lines** (A) The indicated cells were grown in the presence (+) or absence (−) of Dox for 3 days and subjected to Western blotting with an anti-HA antibody for HA-TNIK detection. A 92-kDa constant nonspecific band was used as a loading control. (B) Clones A and C were grown as in (A). Clone C was grown for one more day under the indicated combinations of Dox and HGF. Shown are phase contrast micrographs. Micrographs of Dox− clone C colonies represent the same single colony before and after HGF exposure. (C) Pam212 and clone C cells were grown as in (A), followed by stimulation with HGF for the indicated periods of time, and then subjected to Western blotting with antibodies against phospho-ERK (pERK) and total ERK.

Fig. 2 **Immunofluorescence LSCM analysis of clone C during HGF-induced scattering assay** Colonies of clone C at the indicated conditions were examined at junctional and basal levels. Arrowheads and an arrow in Dox+/HGF− panels indicate the circumferential actin belts and the peripheral actin bundle, respectively. Arrowheads and an arrow in Dox+/HGF+ panels indicate the membrane ruffles and the lamellipodium, respectively. Arrowheads and arrows in Dox−/HGF− panels indicate the cell boundaries inside the colony and the outer free edges of the colony peripheral cells, respectively. E-cad, E-cadherin.

Fig. 3 **Wound healing assay of Dox+ clone C cells** Phase-contrast micrographs of the wound at the indicated times are shown. Left panels are larger images of the areas indicated by white squares in the right panels.

Fig. 4 **Wound healing assay of Dox− clone C cells** Phase-contrast micrographs of the wound are shown as in Fig. 3.

Fig. 5 **Ca^{2+} switch assay of Dox− and Dox+ clone C cells** Immunofluorescence LSCM and phase-contrast micrographs of the clone C colonies at the indicated conditions are shown. Phase-contrast micrographs represent the same Dox− or Dox+ colonies. Junctional level LSCM images are shown except for the last condition, where basal level images are also shown. An arrow in the basal level image of the Dox+ colony indicates the peripheral actin bundle surrounding the colony. E-cad, E-cadherin; Phase, Phase-contrast.

HGF−, Dox+/HGF+ and Dox−/HGF− colonies where cells attached firmly to the substratum, cell boundaries were marked by E-cadherin only. Actin stress fibers were seen inside boundaries. In Dox+/HGF− colonies, prominent "peripheral actin bundles"[15] were observed running contiguously along all outer edges of the peripheral cells. In Dox+/HGF+ colonies, many peripheral cells showed formation of "membrane ruffles"[15] or "lamellipodia", where peripheral actin bundles were disrupted[15]. In Dox−/HGF− colonies, HA-TNIK was not prominent at the boundaries. In the Dox−/HGF+ situation, scattering cells extended lamellipodia, and stress fibers were barely detectable, reminiscent of HGF-exposed MDCK cells[15]. Distributions of HA-TNIK and E-cadherin were similar to that seen at the junctional level.

We next examined clone C in wound healing *in vitro*. Wound in a Dox+ monolayer never healed within 24 h (Fig. 3), like that in Pam212 cell monolayer[10]. At 12 h after wounding, the wound edge cells became flattened, exhibited membrane ruffling and lamellipodia formation, and elongated toward the wound. However, even at 24 h they had not detached from neighboring cells to migrate into the wound. In contrast, the wound in the Dox− monolayer always healed within 24 h (Fig. 4). At 12 h, the wound edge cells became flattened, extended large lamellipodia, and elongated, and some detached from neighboring cells.

Finally, we examined clone C in a "Ca^{2+} switch" assay (Fig. 5). When the Ca^{2+} concentration of the medium was reduced from 2 mM to 2 μM, cells gradually detached from each other, and E-cadherin and actin signals became like shrunk rings, with some portion of them appearing together on perinuclear vesicles, as has been described in other epithelial cells[11, 12]. After the cells were fed with fresh medium, they re-attached, re-forming new junctions. Dox− cells exhibited retarded recovery as clearly indicated by the lack of peripheral actin bundles at the basal level.

DISCUSSION

HA-TNIK-expressing clone C (Dox− cells) scattered in response to HGF (Fig. 1B). This response is distinct from those of Pam212[13] and Dox+ cells, which did not scatter, but is similar to those of other epithelial cell models including MDCK cells[15]. HA-TNIK did not upregulate the HGF receptor, as HGF-induced Erk activation was not enhanced in Dox− cells. Thus, scattering resulted from changes caused by HA-TNIK in other signaling pathways. It is also possible that the scattering resulted from other HA-TNIK-induced changes unrelated to HGF signaling.

In the LSCM analysis of Dox−/HGF− colonies (Fig. 2), HA-TNIK co-localized at the junctional level with circumferential actin belts, but not co-localized at the basal level with other actin structures such as peripheral actin bundles or stress fibers. Furthermore, HA-TNIK co-localized with E-cadherin at the junctional level but not at the basal level. These results suggest that HA-TNIK was only present around AJs, unlike E-cadherin, which distributes throughout lateral cell-cell contacts. In the Dox − /HGF+ situation, HA-TNIK distributed throughout the plasma membrane in scattering cells at both junctional and basal levels, likely because the AJs did not longer existed and because migrating cells were flattened. It should be noted, however, that we cannot conclude that HA-TNIK was in the AJ protein complex, owing to the limited microscopic resolution and the lack of biochemical data supporting it. HA-TNIK could even be in the protein complex forming the tight junction (TJ), which localizes just apical to AJs[16]. In Dox+/HGF+ colonies, peripheral cells showed formation of membrane ruffles and lamellipodia, and peripheral actin bundles maintaining colony edge integrity were disrupted. Nevertheless, peripheral cells did not migrate out, suggesting that they were ready to scatter but failed to detach from inner cells.

In the wound-healing assay, Dox+ wound edge cells also appeared ready to migrate into the wound but failed to detach from neighboring cells (Fig. 3). In contrast, Dox− wound edge cells successfully detached from neighboring cells (Fig. 4), suggesting that HA-TNIK augmented healing by facilitating intercellular detachment.

Finally, in the Ca^{2+} switch assay (Fig. 5), Dox− cells had not completely recovered even at 1.5 h after returning to standard culture conditions, with the internalized E-cadherin still being detected in a perinuclear staining pattern. During the Ca^{2+} switch assay in MDCK cells, internalized E-cadherin was detected biochemically in the intercellular vesicle fractions in which endosomal proteins EEA1 and Rab11 were enriched[11]. As EEA1 and Rab11 are enriched in early and recycling endosomes, respectively[17], these perinuclear E-cadherin molecules likely underwent endocytosis but remained in recycling endosomes before being recycled back to the plasma membrane. This is consistent with a view that AJ integrity is maintained through proper turnover of E-cadherin[16]. Taken together with our finding that Rap2 localizes to recycling endosomes and regulates TNIK[8], it is interesting to hypothesize that TNIK negatively regulates AJ integrity by affecting the function of recycling endosomes. This hypothesis is also consistent with phenotypes found in HGF-mediated scattering and wound healing, if we assume that cells fail to detach from each other in the absence of TNIK because of enhanced stability of AJs.

In addition, the balanced activities of the Rho family of small GTPases, Rho, Rac and Cdc42 are required to establish and maintain epithelial intercellular junctions[16]. Our preceding study found downregulation of Rho GDI α in Dox− cells[6]. Rho GDI α knockout mice exhibited disruption of renal tubular epithelial cells and epithelia of seminiferous tubules in the testis[18]. As Rho GDI α suppresses activities of RhoA, Rac1 and Cdc42, the authors speculated that activated Rho family signaling resulted in disassembly of TJs and AJs in the kidneys and testes. Thus, HA-TNIK in Dox− cells may negatively regulate intercellular junction integrity through Rho GDI α downregulation.

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