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Trophinin in cell adhesion and signal transduction

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1. ABSTRACT

The process of human embryo implantation is mediated not only by evolutionarily conserved mechanisms but by activities unique to humans. Among the latter, evidence suggests that the cell adhesion molecule trophinin plays a unique role in human embryo implantation. Here, we describe characteristics of trophinin protein and of the trophinin-associated proteins bystin and tastin. We then describe trophinin-mediated signal transduction in trophectoderm cells during human embryo implantation and events related to human sperm tail motility. We also report dual roles for trophinin in human cancers in terms of promoting malignancy in some tumor types and suppressing it in others.

2. INTRODUCTION

Processes associated with mammalian embryo implantation differ substantially among mammalian species (1). Consequently, some mechanisms of embryo implantation are unique to humans. However, since it is not possible to analyze human embryo implantation directly, researchers often investigate human cell lines cultured *in vitro*. Trophinin was discovered using such an *in vitro* model system consisting of trophoblastic HT-H and endometrial epithelial SNG-M cells (2). Although it is not feasible to test trophinin activity during human embryo implantation *in vivo*, increasing evidence obtained during the last 15 years supports the hypothesis that trophinin functions in this process (3-10). In addition, recent findings

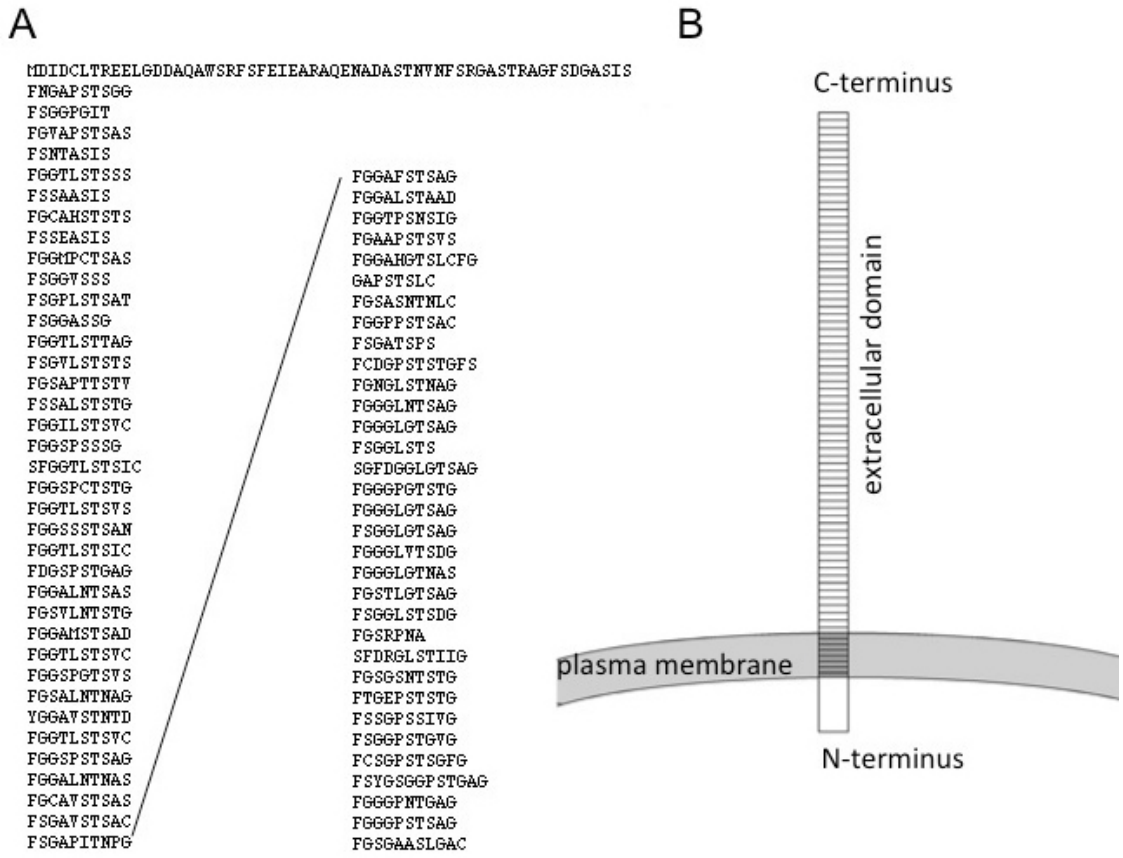

Figure 1. Structure of human trophinin protein. (A) Peptide sequence of human trophinin. Majority of the peptide is made of decapeptide repeats. (B) Proposed topology of trophinin protein, with decapeptide repeats as outer cellular and N-terminal region in the cytoplasm.

relevant to trophinin activity in sperm tail (11) and in several human cancers (12-15) suggest multiple roles of trophinin in signal transduction.

3. STRUCTURE AND FUNCTION OF TROPHININ

To establish an alternative means of investigating molecular mechanisms potentially underlying human embryo implantation (2, 16), we chose embryonal teratocarcinoma HT-H cells as a model to represent implantation stage trophectoderm cells. Teratocarcinomas are composed of undifferentiated stem cells and variously differentiated cell types (17). Since stem cells in these tumors can differentiate into several cell types, they are considered pluripotent, and tumors containing these cells are known as embryonal carcinomas (ECs). Both human and mouse EC lines exhibit characteristics of early embryonic cells (17-19). Human EC lines have the tendency to differentiate into trophoblastic cells (20). HT-H cells spontaneously differentiate into syncytiotrophoblasts *in vitro*, secreting the trophoblastic marker, human chorionic gonadotrophin (hCG) (16). Trophoblastic HT-H cells adhere and grow as a monolayer on tissue culture dishes, and when trypsinized and added onto a human endometrial adenocarcinoma SNG-M cell monolayer, HT-H cells instantly adhere to the monolayer (2). Both HT-H and SNG-M cells also adhere to themselves, suggesting that they express a homophilic adhesion molecule. Neither HT-H cells nor SNG-M cells adhere to epithelial cells derived from other cell types, such as colon, lung, and liver. These observations suggest the existence of a trophoblast/endometrial cell-type specific apical cell adhesion molecule in both HT-H and SNG-M cells.

To identify such a factor in trophoblastic cells and endometrial epithelia, we employed expression cloning and identified trophinin solely by apical cell adhesion activity (2, 4). COS cells, which normally do not adhere to SNG-M cells, were transfected with a cDNA library constructed from HT-H cells in a mammalian expression vector, and COS cells adhering to SNG-M cells were selected. Isolation of constructs mediating cell adhesion activity revealed two cDNAs, designated trophinin and tastin. When COS cells were co-transfected with vectors each encoding trophinin and tastin, COS cells adhered to SNG-M cells (2). Trophinin was determined to be a cell adhesion molecule, while tastin was found to be a cytoplasmic protein required for trophinin to exhibit cell adhesion activity in COS cells.

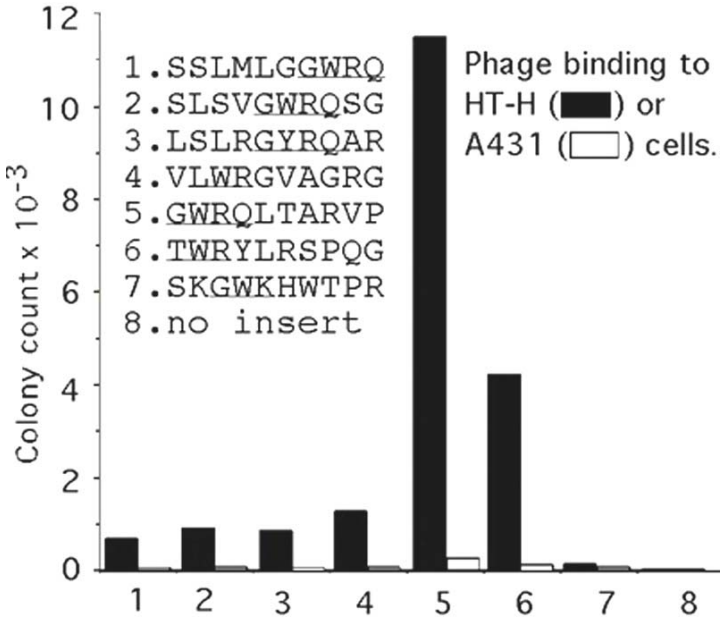

Figure 2. Binding of individual phage clones from screening of a 10-mer peptide phage library for binding to HT-H cells. The screen identified a series of peptides with the consensus sequence GWRQ. Note that the strongest binder is a representative of peptides that contain the GWRQ motif at the N-terminus.

Human trophinin is a 69 kDa protein exhibiting unique decapeptide repeats (2) (Figure 1A). Trophinin protein exhibits a cytoplasmic N-terminal domain followed by the repeats. Although trophinin does not have a leader peptide characteristic of conventional plasma membrane proteins processed via the sorting pathway, experimental evidence indicates that trophinin is an intrinsic membrane protein (2, 7). The existence of several hydrophobic domains initially suggested that this protein traverses the lipid bilayer multiple times (2). Since most cell adhesion molecules generally extend from the plasma membrane, a more plausible possibility is that the trophinin protein is a single transmembrane domain protein utilizing the first hydrophobic decapeptide repeats near the N-terminus to span the membrane (Figure 1B). The remaining C-terminal decapeptide repeats may be extracellular. The proposed topology is yet to be validated.

Nonetheless, this model predicts that the extracellular decapeptide repeats must provide a structural basis for trophinin's function as homophilic cell adhesion molecule. While trophinin repeats are predicted to form a beta-sheet, no information relevant to its three-dimensional structure is available to date. Unlike many cell adhesion molecules that require calcium for adhesion, adhesion by trophinin is independent of divalent cations (2). Furthermore, trophinin-mediated cell adhesion occurs in paraformaldehyde-fixed cells (2). When a peptide-display phage library was screened on a monolayer of fixed HT-H cells as a target in the presence of EDTA, identified phage clones of linear 10-mer (X_{10}) peptides exhibited the consensus sequence GWRQ (Figure 2). Clones with GWRQ at the N-terminus were stronger binders than those with this sequence in the middle (7). Both GWRQ peptide-displayed phage and chemically synthesized GWRQ peptide bound trophinin and triggered signals leading HT-H cells to proliferate and invade (7, 8). Since the GWRQ sequence does not exist in trophinin, we speculate that one part of the trophinin repeats assumes a unique three-dimensional structure mimicked by GWRQ, while another portion of the repeats conforms to the GWRQ acceptor pocket.

Trophinins bind each other when they are presented *in trans* at the respective apical cell surface of trophectoderm cells and endometrial epithelial cells. Other well-characterized homophilic cell adhesion molecules, such as cadherins, also bind one another *in trans* (21). Cadherin-mediated cell adhesion requires insertion of the conserved N-terminal W2 domain or GW peptide from one molecule into the hydrophobic core of another cadherin protein. This feature of the W2 domain and the W2 acceptor pocket (21) parallels the proposed trophinin structure, which is mimicked by GWRQ and its acceptor pocket in trophinin decapeptide repeats.

4. TROPHININ-INTERACTING PROTEINS

COS cells transfected with trophinin cDNA alone do not adhere to SNG-M cells; instead, trophinin-mediated cell adhesion requires the proline-rich cytoplasmic protein, tastin (also known as trophinin-associated protein *TROAP* product). Since the discovery of trophinin and tastin, another cytoplasmic protein, bystin, has been found to serve as a protein bridging trophinin and tastin (22). Bystin was initially overlooked because, unlike trophinin and tastin, which have a limited expression pattern in primate cells, the *BYSL* gene encoding bystin is expressed in all cell

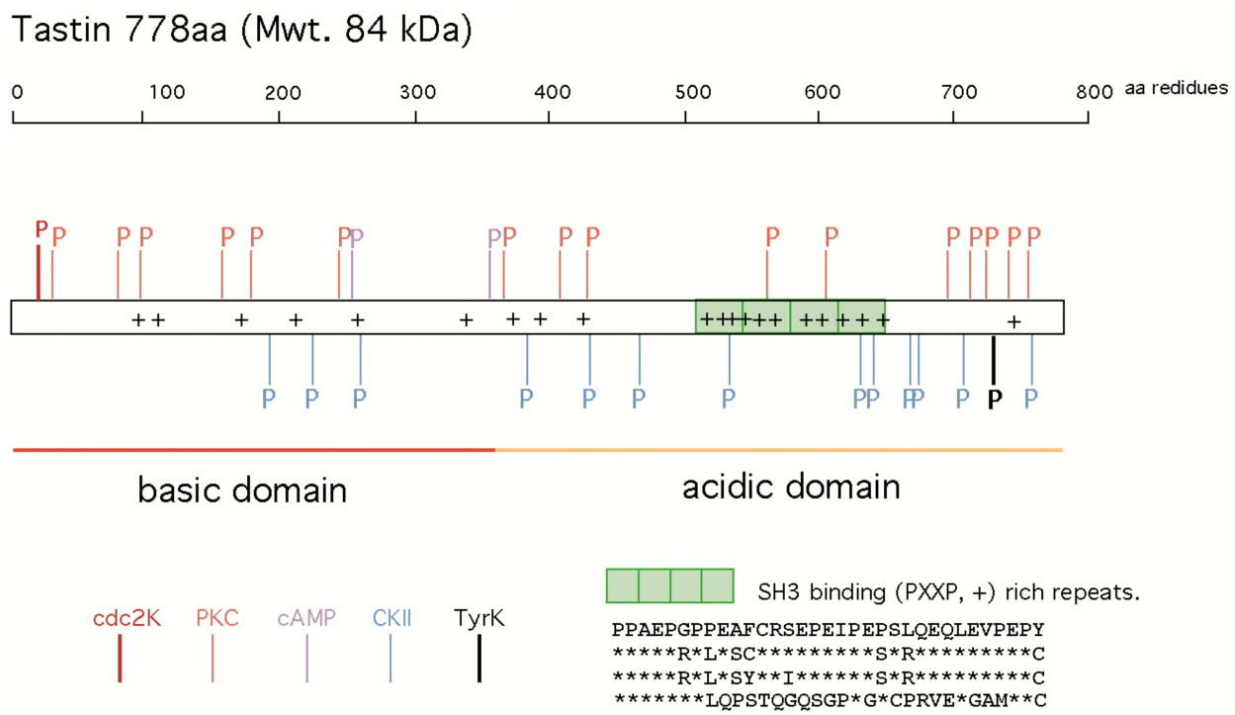

Figure 3. Structure of tastin. Illustration was made based on Nadano *et al.* (23), and Yang *et al* (37). Human tastin is an 84 kDa protein rich in proline residues (16% of the total amino acid residues). Tastin contains many PXXP (shown by +), SH3 (*src* homology 3)-binding motief, including four PXXP-rich domains (shown by green). Thus tastin may bind to SH3 proteins such as c-Src. Tastin also contains many potential phosphorylation sites for cyclin-dependent kinase-2 (CDC-2), protein kinase C (PKC), cAMP kinase (cAMP), casin kinase II (CKII) and tyrosine kinases (TyrK).

types, including COS cells. *In vitro* and *in vivo* assays have confirmed interactions between trophinin, bystin and tastin in the cytoplasm (7, 22). This complex is thought to be fixed to the cytokeratin network in trophectoderm cells and endometrial epithelial cells (22). In other cell types, this complex may be linked to microtubules, as tastin binds to microtubule-associated dynein (ATPase) (11, 23).

Although the *TRO* and *TROAP* genes are unique to mammals, *BYSL* is conserved across a wide range of eukaryotes, including yeast, nematodes, insects, snakes, and mammals (24-28). Amino acid sequences of bystins from yeast, fly, and humans are highly homologous, particularly in the C-terminal region, although human bystin cannot compensate for loss of the yeast homologue *ENP1* (for essential nuclear protein 1) in yeast. *ENP1* is required for vegetative growth in budding yeast (24). In yeast, Enp1 protein was found in the nucleus and concentrated in the nucleolus. A yeast temperature-sensitive *ENP1*-deficient mutant is defective in processing 18S ribosomal RNA, suggesting a function in pre-ribosomal RNA processing (25). Biochemical and cell biological analysis of bystin in human cancer cells and mouse embryos indicates that bystin functions in both biogenesis of the 40S ribosome and in cell growth (29, 30). B*ysl* gene knockdown experiments show that bystin is required for survival of pre-implantation stage mouse embryos (30), and bystin null mouse embryos survive only to the epiblast stage and die soon after implantation (31). These findings indicate that bystin plays an essential role in eukaryote survival.

A publicly available database shows that bystin mRNA levels are relatively low in normal human tissues but increase in various tumors (32, 33). Other microarray analyses of human breast tumor specimens identify bystin as part of a "proliferation cluster" (34). The embryonic lethality of the *Bysl* null mouse and bystin expression seen in the epiblast support the hypothesis that bystin is essential for growth of mammalian stem cells in epiblast cells, which must proliferate rapidly after implantation. Bystin expression in the epiblast is also consistent with a report showing that *Bysl* is one of 216 genes commonly expressed in mouse embryonic, neural and hematopoietic stem cells (35). Furthermore, *BYSL* was identified as the highest scored gene co-regulated by *MYC (36)*, suggesting that its expression is closely associated with human malignancies.

Tastin is a proline-rich cytoplasmic protein with a basic N-terminus and an acidic C-terminus (Figure 3) (2, 23, 37). In the C-terminus, tastin contains four 33-amino-acid repeats predicted to function as an SH3 ligand (2). Tastin may function in some malignancies by activating SH3 domain proteins in specific tumor types. Publicly available databases of various human cancers show that tastin mRNA is overexpressed in prostate cancer (38) and bladder carcinoma, but not in other human cancers. Previous studies showed that tastin associates with

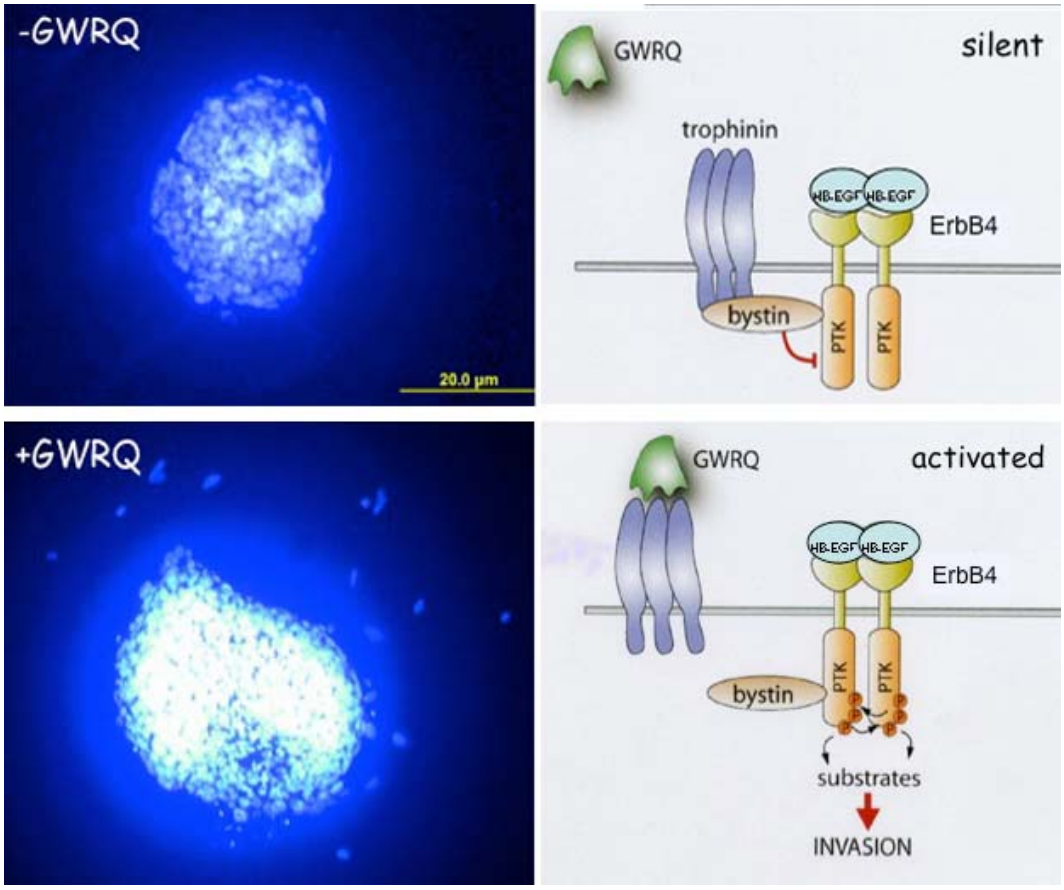

Figure 4. In silent trophectoderm cells (upper panels), trophinin arrests ErbB4 through bystin. Under this condition, ErbB4 is inactive for protein kinase. On the other hand, upon trophinin-mediated cell adhesion (lower panels), bystin dissociates from trophinin and ErbB4 is activated. GWRQ peptide can mimic trophinin-mediated cell adhsion. Modified from Sugihara *et al.* (7).

microtubules and plays a role in mitosis (23, 37). Tastin's restricted expression pattern may facilitate design of specific reagents to diagnose and treat tastin-expressing human cancers.

In normal human tissues other than those at the embryo implantation site, tastin is primarily expressed in brain and testis. In mature sperm, tastin likely regulates sperm motility by interacting with microtubule-associated dynein ATPase (11).

5. SIGNALS TRIGGERED BY TROPHININ

In trophoblastic cells, trophinin's cytoplasmic N-terminus binds to bystin, which binds to the cytoplasmic domain of ErbB4 (7). When the ErbB4 ligand HB-EGF binds to ErbB4 on trophinin-expressing human trophoblastic cells, ErbB4 is not activated (7, 8). By contrast, when trophinin-mediated cell adhesion occurs, bystin is released from trophinin, allowing activation of ErbB4 protein tyrosine kinase. Thus trophinin-mediated cell adhesion acts as a molecular switch for trophoblast activation, promotion of invasion and proliferation (Figure 4) (7, 8). This mechanism explains the observation that, at the human embryo implantation site, trophectoderm cells adhering to uterine epithelia grow and invade, while those at non-adhesive sites remain silent (39).

Several reports suggest that endometrial epithelial cells undergo apoptosis upon adhesion of the blastocyst (40-43). Since human trophectoderm cells express Fas ligand, and endometrial epithelial cells express Fas, the Fas/FasL system has been proposed to mediate implantation-dependent apoptosis of endometrial cells (41, 44). Fas/FasL activity is linked to activation of mitogen-activated protein kinases (MAPK) and c-Jun N-terminal kinase (JNK) in endometrial epithelial cells (43, 44). Recently we found that trophinin-mediated cell adhesion induces apoptosis of endometrial epithelial cells through a Fas/FasL-independent mechanism (45), suggesting that multiple mechanisms govern adhesion-dependent apoptosis of endometrial epithelial cells in human embryo implantation.

In addition to cells involved in embryo implantation, the TRO gene is highly expressed in male and female germ cells (46). We found that trophinin, bystin and tastin proteins are localized in the tail of human sperm

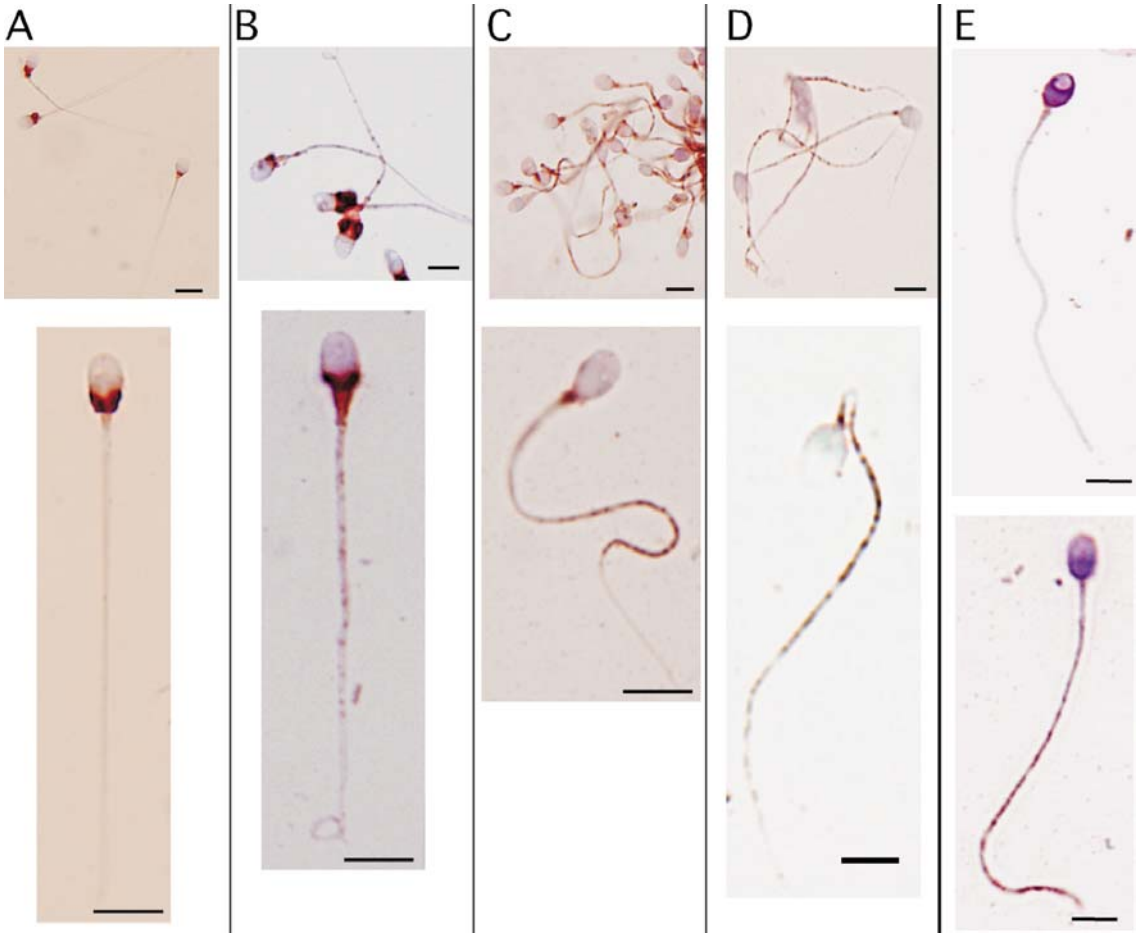

Figure 5. Immunohistochemistry of human sperm. (A) Intact human sperm stained for trophinin. (B) Human sperm stained for trophinin, after mild acid hydrolysis. (C and D) Fixed sperm were permeabilized by methanol and stained for bystin (C) and tastin (D). (E) Fixed and acid-treated sperm were incubated with control phage (E, upper) or with GWRQ peptide-displaying phage (E, lower). Phage was detected by anti-phage antibody. Scale bars= 0.2 mm. Modified from Hatakeyama *et al.* (11).

(Figure 5) (11). When trophinin-binding peptide GWRQ was added to human sperm, sperm motility was enhanced. This enhancement was associated with rapid ATP consumption, suggesting that conformational changes in the trophinin of extracellular domain are transmitted to the cytoplasm of the sperm tail to activate ATPase. We hypothesize that GWRQ binding to trophinin dissociates bystin and tastin from trophinin, activating dynein ATPase (Figure 6). Trophinin may also regulate sperm motility by suppressing dynein ATPase, so that ATP produced in sperm cells by oxidative phosphorylation or glycolysis (47) is not preserved. Storage of ATP during the movement of sperm through the uterus and oviduct is necessary to achieve the hyperactivation required for oocyte penetration

6. TROPHININ, BYSTIN AND TASTIN IN HUMAN CANCER

Bystin is overexpressed in many tumors, and tastin is overexpressed in a specific subset of cancers. As far as we have investigated, trophinin expression is positively correlated with cancer cell invasiness in testis (48), colon (12), and lung (14), whereas it is negatively correlated with malignancy of ovarian cancer cells (13). The trophoblastic marker hCG is elevated in cancers in the testis, bladder, cervix, lung and naso-pharynx (49), whereas hCG is negatively correlated with malignancy in breast cancer (50) and ovarian cancer (51). In those cancers in which hCG and trophinin expression correlate with malignancy, trophinin apparently promotes cell motility as seen in testicular cancer and colon cancer (12, 48).

Discrepancies in the functional correlation between trophinin expression and malignancy may be related to pathways affected by trophinin activity in each cell type: in trophectoderm cells trophinin regulates the HB-EGF/ErbB4 pathway (7, 8), whereas in endometrial epithelial cells, trophinin is linked to apoptosis pathways (45).

Many studies note the resemblance of highly invasive and proliferative trophoblasts to malignant tumor

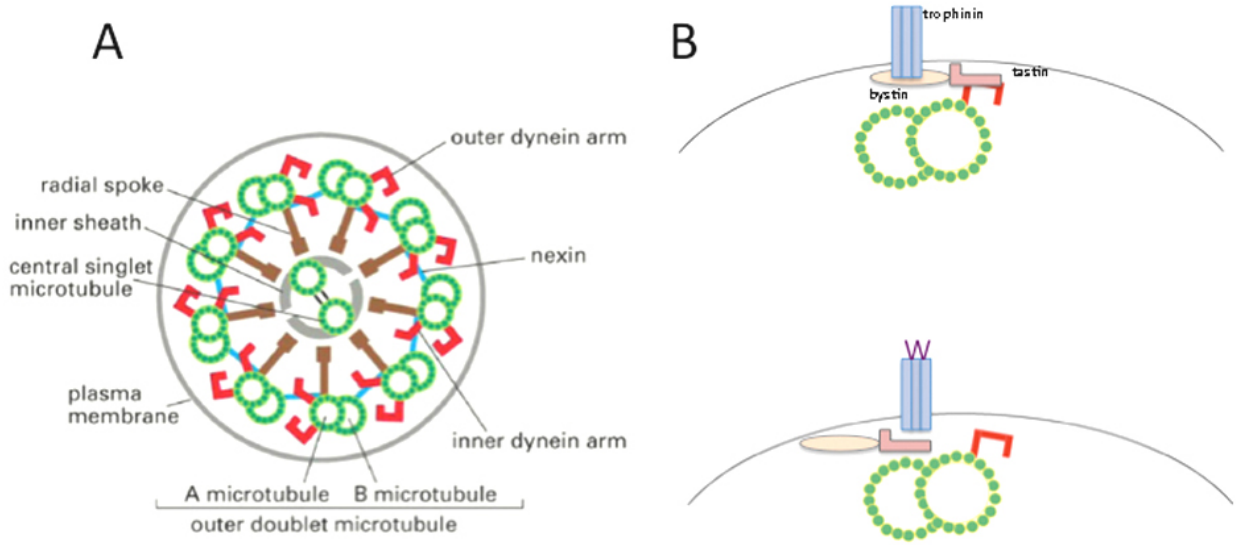

Figure 6. Proposed action of GWRQ peptide on trophinin in sperm tail. (A) Sperm tail is made of microtubule bundles, to which motor dynein ATPase are associated. (B) In the absence of GWRQ, trophinin, bystin and tastin complex binds tightly with dynein, arresting dynein ATPase from activation. (B) When GWRQ binds to trophinin outer cellular domain, bystin dissociate from trophinin. This may release ATPase from the tastin arrest, so that dynein hydrolyzes ATP and functions as motor.

cells (49, 52-56). As the role of the trophinin, bystin and tastin complex in human embryo implantation is unique to humans, their roles may also be unique to human cancers.

7. PERSPECTIVES

Humans reproduce a limited number of offspring, requiring "selection" of optimal implantation sites. Numerous mechanisms and factors govern processes of human embryo implantation. All may ensure strict quality control during the initial adhesion between the trophectoderm and endometrial epithelia (10, 45). Trophoblastic cancers including testicular cancer and endometrial cancers are aggressive forms of human cancer, but these cancers are also unique to humans. As the processes of trophinin-mediated cell adhesion and signal transduction are unique to humans, further understanding of these processes in human embryo implantation could help our understanding of characteristics of uniquely human cancers.

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