Cancer early dissemination: cancerous epithelial-mesenchymal transdifferentiation and transforming growth factor β signaling

Hisataka Sabe

Department of Molecular Biology, Hokkaido University Graduate School of Medicine, Sapporo 060-8638

Running title: cancer early dissemination and the EMT signaling

連絡先：
北海道大学大学院医学研究科
札幌市北区北１５条西７丁目
佐邊壽孝
Te: 011-706-5044
Fax: 011-706-7865
E-mail: sabeh@med.hokudai.ac.jp

Contrary to the long believed hypothesis, it is now evident that breast cancer cells can disseminate from the early phases of the oncogenesis; and that such early disseminated cells sometimes survive at the sites of dissemination and may outgrow after a long latency of years and decades. For cancer cells to leave their origin, they must at least transiently loosen their adhesion with adjacent epithelial cells and stroma, and become motile while avoiding anoikis. Such processes resemble epithelial-mesenchymal transdifferentiation (EMT), which normally takes place in situations such as embryogenesis and wound healing. Interestingly, the occurrence of an EMT-like process in breast cancer cells has been implicated in the generation of cancer stem-like cells, in which TGFβ1 signaling often plays core roles. Here, I discuss the current knowledge regarding cancerous EMT and its signaling pathways with the aim to consider the possible mechanisms of early dissemination, and also the generation of cancer stem-like cells in mammary tumor.

Key words: cancer stem cells, EMT, p53, TGFβ, tumor dissemination

Abbreviations: EMT, epithelial-mesenchymal transdifferentiation; ER, estrogen receptor; PRKD, Protein kinase D; RTK, receptor tyrosine kinases.

(Text)

The concept of 'systemic metastatic dissemination of cancer cells from the early phases of their oncogenesis', which is referred to here as 'cancer early dissemination' for short, was established by pioneer works of C. A. Klein and colleagues. In their cohort studies of breast cancer patients, they provided the first evidence that breast cancer cells can disseminate into the bone marrow in a far less progressed genetic state than previously thought, and hence discussed that genomic aberrations that make them metastatic (*i.e.*, outgrowing from the sites of dissemination) might be acquired after this step (*1*). This notion was confirmed experimentally using mouse models for human breast cancer (*2*). Metastasis is the greatest threat of cancer. According to the model of 'cancer early dissemination', it is easy to understand the very late appearance of metastatic lesions, long after the resection of a primary tumor as well as its regional lymph nodes, as C. A. Klein has mentioned. Early disseminated tumor cells will acquire genome alterations independent of the primary site after leaving, that allow them to grow out. Thus, this model may also imply that therapies based on characters of the primary tumors have some limitations when treating metastatic lesions and recurrent cancers, that have originated from early disseminated cells. This model also shed a new light on 'cancer of unknown primary (CUP)', which comprises up to 7% of cancer patients bearing metastases, whose primary tumor remains unrecognized.

Mechanisms of cancer early dissemination are still largely unidentified. From a cell biological point of view, however, early dissemination is a phenomenon rather easy to understand because full-transformation is not necessary to move out (Fig. 1). Obviously, because of the lack of the full transformation, early disseminated cells do not outgrow immediately at their sites of dissemination, but instead sometimes become dormant, as has been demonstrated using untransformed mammary epithelial cells (*3*). Cancer is a tissue-specific disease, and studies discussed in this review are mostly carried out with breast cancers, while some parts of the intracellular mechanisms and signaling have yet to be confirmed in this cancer.

Cancerous EMT

EMT is a normal cellular process of epithelial cells in which their E-cadherin is downregulated and certain integrins are upregulated in order to move out of the

epithelial cell layer, described originally by E. Hay (*4*). EMT occurring in normal cells involves many changes including an alteration in the expression of intermediate filaments, from cytokeratins to vimentin. However, cancer cells often express both cytokeratins and vimentin simultaneously (*5*), indicating that the EMT-like process which occurred in most cancer cells is not normal. Here I will refer to the EMT-like processes of cancer cells as 'cancerous EMT'.

Loss of E-cadherin: not simply the disruption of cell-cell adhesions resulting in cells to move-out

A long-standing question is whether the loss of E-cadherin-mediated cell-cell adhesion is enough to render cells to move-out. On the other hand, it is well known that E-cadherin associates with β-catenin, which is bifunctional and acts as a transcriptional co-activator, such as of Tcf/Lef in the nucleus. The current prevailing model suggests that association of E-cadherin with β-catenin suppresses β-catenin from entering into the nucleus to prevent transformation. A question is whether this is true.

Onder *et al.* (*6*) have addressed this issue by the use of HMLER cells, which are generated by immortalization of human primary normal mammary epithelial cells by introducing hTERT and SV40 large T antigen, followed by the introduction of H-Ras to make them tumorigenic (*7*). Although HMLER cells are highly tumorigenic, they are not metastatic on their own. Onder *et al.* have shown that the disruption of cell-cell adhesions by a dominant-negative form of E-cadherin is unable to facilitate their metastasis (*6*). On the other hand, loss of E-cadherin expression by shRNA renders HMLER cells to be invasive, anoikis resistant, and highly metastatic. Similar results has been shown by others (*8*). Gene expression profiles indicated that HMLER cells undergo EMT upon loss of E-cadherin expression. However, Onder *et al.* also show that the nuclear accumulation of β-catenin is not sufficient, although necessary, to induce the metastatic properties. Therefore, the loss of E-cadherin expression, but not simply the loss of E-cadherin-mediated cell-cell adhesions, appears to be a key for cancer cells to move out. Twist was originally found as an inducer of mesoderm formation in *Drosophila* (*9*); and is expressed upon the onset of EMT to suppress gene transcription including the *CDH1* (E-cadherin) gene in mammals (*10*). Twist is induced upon the loss of E-cadherin expression in HMLER cells (*6*). Twist may well

contribute to cancer malignancy, perhaps even independently of the suppression of E-cadherin expression (also see later).

TGFβ1 signaling is the most frequent factor inducing mesenchymal and stem-like properties of breast cancer cells

TGFβ is one of the EMT-inducing factors. Under physiological conditions, TGFβ exhibits biphasic roles in both the promotion and inhibition of branching morphogenesis of mammary gland ducts, depending on their concentration and cellular context (*11*). In cancer, TGFβ signaling also appears to be biphasic: it acts as a tumor suppressor during early oncogenesis, while in later stages it is often a tumor promoter (*12,13*).

Among the different signaling pathways, TGFβ signaling appears to be most frequently involved in inducing the mesenchymal and cancer stem cell-like property of primary human breast cancers. The gene expression profile of CD44+/CD24-/low breast cancer cells resembles that of stem cells (*14*). Moreover, CD44+ breast cancer cells are negative for estrogen receptor (ER) even in some ER+ cancers and are refractory to current adjuvant hormonal therapy (*15*). In these cancers, ER is expressed in CD24+ cells. Polyak and colleagues have studied gene expression and genetic profiles of cancerous and normal breast tissues of humans, and identified that direct interaction networks built around the lin-/CD44+/CD24-/low breast cancer cell-specific genes is centered around TGFβ1 (*15*). Correlation of high expression of gene signatures specific for CD44+ cancer cells, as well as for the TGFβ pathway of these cells, with metastasis-free survival of breast cancer patients was also demonstrated. Interestingly, CD24+/CD44- cancer cells may also exhibit abnormal localization of E-cadherin and nuclear accumulation of β-catenin, while their *TGFβR2* gene expression is epigenetically silenced. Apparently paradoxically, moreover, CD24+ cancer cells are dramatically higher in distant metastases regardless of their sites, while positivity for CD44 expression is not consistently different among metastatic sites (*15*). The reason for this remains unclear.

Consistent with above results primarily based on clinical samples, Weinberg and colleagues have shown *in vitro* that TGFβ1 induces EMT, and this EMT conversion induces cancer stem cell properties of HMLE cells (*16*). HMLE cells are generated by

immortalizing human primary normal mammary epithelial cells (HMECs) by introducing hTERT and SV40 large T antigen. This group has previously shown that Twist is expressed at high levels in invasive lobular and ductal carcinomas, and that Twist appears to be one of the master regulators of the mesenchymal morphogenesis and the metastatic activities (*10*). Expression of Twist alone can induce mesenchymal and stem cell-like properties in HMLEs. They also described that the *CDH1* gene is not the sole target of Twist (*16*). In contrast to HMLEs, in the case of HMECs expression of Twist is not sufficient: Twist expression only partially induces EMT, and the significant but incomplete decrease of E-cadherin and the modest increase of vimentin (*17*). However, co-expression of H-RasV12 (or ErbB2) with Twist1 (or Twist2) in HMECs triggers complete EMT and induces invasive properties (*17*). Twist can override oncogene-induced premature senescence during EMT, most likely by abrogating p53- and Rb-dependent pathways (*17*). Taken together, HMLER cells (HMLEs expressing Ras) are non-mesenchymal and non-invasive as far as their *CDH1* gene expression is not suppressed and hence Twist is not expressed, while HMLE/Twist and HMECs/Ras/Twist cells are mesenchymal and invasive. It should be remembered that SV40LT, used in HMLE cells, interferes with the normal function of p53 and Rb. From these data, oncogenic Ras signaling and abrogating the normal function of p53 and /or Rb seem to be involved in processes that make mammary epithelial cells able to undergo EMT and become cancer stem cell-like, in response to TGFβ or Twist.

Balancing proliferative and cytostatic programs: roles of wild type p53

p53 functions both in the nucleus and the cytoplasm, and its mutation is very common in cancer (*18*). Upon accumulation in the nucleus, wild type p53 participates in transcriptional regulation of a number of genes via specific DNA elements, in which each set of expressed gene products is thought to be engaged either in the control of cell cycle arrest, differentiation, senescence or apoptosis. Wild type p53 thereby exhibits cytostatic and tumor suppressive properties. Wild type p53 also appears to be important in the regulation of polarity of cell division in mammary stem cells, and its loss is coupled with the symmetric division of cancer stem cells and may hence contribute to tumor growth (*19*).

Ras/MAPK pathways, activated downstream of growth factor receptor

tyrosine kinases (RTKs), generally exert growth-promoting effects in normal cells, while these pathways may well act towards tumor suppression dependent on wild type p53 (*20,21*). Moreover, RTK signaling crosstalks with TGFβ signaling, affecting TGFβ-induced growth arrest of mammalian cells, as well as mesoderm development in vertebrate embryos (*22*). Piccolo and colleagues (*23*) have shown that wild type p53 is phosphorylated at Ser6/Ser9 by casein kinase 1 ε/δ, which are activated downstream of Ras, and this phosphorylation induces the association of p53 with Smads to promote cytostasis in vertebrate cells, as well as to confine mesoderm specification in *Xenopus* embryos. Therefore, this appears to be a mechanism to compromise the growth promoting effects elicited by RTKs with the cytostatic signaling by TGFβ, in order to limit neoplastic transformation.

Conversion of TGFβ1 signaling to be pro-malignant: roles of oncogenic p53 and Ras

Mutations of p53 often gain oncogenic activities, rather than simply abrogating its tumor suppression function (*24*). Human pathological analyses have indicated that mutant-p53 expressing tumors are often very aggressive and associated to poor-prognosis, especially in Her2-positive and basal-like breast cancers (*25,26*). The malignant future of mutant p53 was confirmed experimentally by generating mutant-p53 knock-in mice: tumors emerged in these mice exhibited very aggressive and metastatic that were never detected in tumors developing in a p53 null background (*27,28*). Like p53, mutations of Ras can also be oncogenic. In breast cancer, however, Ras mutations are relatively rare (*29*), while overexpression of non-mutated Ras as well as hyper-activation of RTKs signaling such as EGFR are frequent events (*30-32*). In a mouse model, mutant p53 and oncogenic Ras are cooperative in enhancing malignant cancer development (*33,34*).

Given that TGFβ1 signaling is the most frequent among the different types of signaling involved in the mesenchymal and stem cell-like properties of human breast cancers, while on the other hand it can crosstalk with wild type p53 and Ras to be cytostatic, an important question is how TGFβ1 signaling becomes able to induce such malignant properties in cancer cells. In other words, what kinds of cancerous contexts convert TGFβ1 signaling to induce EMT without stopping cell-cycle progression? Oft

et al. have shown that oncogenic H-Ras signaling cooperates with TGFβ1 signaling, especially with activated Smad2, to induce mesenchymal and invasive/metastatic properties of already proliferative breast, colon and skin cancers (*35-37*). Piccolo and colleagues have demonstrated a way by which mutant p53 enhances TGFβ-induced invasive and metastatic activities (*38*). In this pathway, oncogenic mutant p53 forms a complex with Smads, when mutant p53 is phosphorylated by casein kinase 1 ε/δ activated under Ras signaling, and this mutant-p53/Smads protein complex then binds to p63, a p53 family-member. p63 can oppose TGFβ-induced metastasis. Incorporation of p63 into the mutant-p53/Smads complex antagonizes the transcriptional functions of p63, and hence appears to enhance TGFβ-induced metastasis. They identified that the expression of *Sharp-1* and *Cyclin G2* genes are downstream of p63, that are essential to antagonize TGFβ induced invasive/metastatic activities. Decreased expression of *Sharp-1* or *Cyclin G2* genes was validated clinically with relation to a significant high probability to develop recurrence of breast cancers (*38*). Of note, *Sharp-1* and *Cyclin G2* are not contained in the previously known gene signature for breast cancer metastasis (*38*).

Molecular machineries required for cancerous EMT

Sharp-1 and Cyclin G2, as well as p53 and Smads, are all basically engaged in the regulation of gene expression, while they each regulate groups of genes rather than a certain single gene. Moreover, their target genes are not fixed intrinsically, but are altered depending on cellular contexts and other transcriptional factors they may work with. Therefore, while understanding the status of such factors that regulate gene expression and genome status is very important, attempting their understanding may sometimes be frustrating, like seeing shadow pictures. Another aspect towards the substantial understanding would be to understand the molecular machineries that actually accomplish the properties of cancer malignancy. Below I briefly discuss the molecular machineries that execute cancerous EMT and the moving out.

Core events for EMT are the activation of integrin and the inactivation of E-cadherin. Integrins are activated in two ways. One is the conformational change, which has already been discussed extensively in other reviews (*39*). Another aspect is their *de novo* synthesis and the recycling. It is well known, for example, that

transcription of the β1integrin gene is induced during TGFβ1-induced EMT of mammary epithelial cells (*40*), although the transcriptional control is not discussed here. On the other hand, integrin actively recycles between the plasma membrane and early endosomes, recycling which has been demonstrated to constitute a basic machinery driving cell migration (*41*). Moreover, functions of integrin in cancer cells include phagocytosis of degraded ECM components, which also involves endocytosis.

Arf6 is primarily involved in the recycling of plasma membrane components (*42*), and its signaling greatly contributes to invasive and metastatic activities of many breast cancer cells (*43-46*). Arf6 colocalizes with β1 integrin at endosomes and is implicated in serum-induced recycling-back of β1 integrin (*47,48*). β1integrin forms a complex with several Arf GTPase-related proteins, to be under the control of Arf6. It has been shown that ACAP1, a GTPase activating protein for Arf6, forms a complex with β1 integrin, and this complex formation is regulated by Akt to be dependent on external stimulations (*49*). In breast cancer, β1 integrin associates with AMAP1 (Onodera, Y. and H.S., unpublished observations), an effector for Arf6 (*44*). Protein kinase D (PRKD) regulates intracellular traffic as well as cell proliferation and apoptosis (*50*), and is an essential component of invadopodia (*51*, Onodera, Y. & H.S., unpublished observations). Yeaman et al. (*52*) have shown that PRKD1/2 is involved in basolateral protein sorting, including β1 integrin and E-cadherin. Norman and colleagues have shown that PRKD1 binds to the β3 integrin subunit, and this binding is involved in the platelet derived growth factor-induced recycling-back of αvβ3 integrin (*53,54*). Our results indicate that β1 integrin binds to PRKD1, and PRKD1 then binds to AMAP1, and through this complex β1 integrin becomes under the control of Arf6 as well as RTKs in invasion of breast cancer cells (Onodera, Y. and H.S., unpublished observations). Interestingly, p53 oncogenic mutation has been shown to also promote integrin recycling and invasion, by inhibiting p63 (*55*).

Rab GTPases are also important. The above mentioned PRKD1-mediated recycling-back of β3 integrin appears to be dependent on Rab4, but not Rab11 (*53,54*). On the other hand, Rab11, but not Rab4, has been shown to be involved in the endosomal colocalization of β1 integrin with Arf6, and its recycling (*48*). Moreover, Rab11 is involved in the trafficking of β4 integrin in hypoxia-induced invasion of some

breast cancer cells (*56*). Rab GTPases consist of more than 80 isoforms in humans, while Arf6 is the sole member among the Arf-family GTPases to be involved in recycling. The primary functions of Rabs appear to be the sorting of cargos and fate determination of endosomes, which are accomplished by interaction of Rabs with their cognate binding partners. Comprehensive identification of Rabs involved in integrin trafficking, as well as identification of their binding partners are necessary for the fine understanding of the mechanisms regulating the intracellular traffic of integrins, essential for EMT and cell motility.

Inactivation of E-cadherin is also accomplished in two different ways. One is suppression of its gene transcription, as already mentioned. The other is the regulation of endocytosis, in which again, Arf6 plays an important role. It has been shown that expression of an inactive form of Arf6 blocks hepatocyte growth factor-induced endocytosis of E-cadherin-based junctional components, while expression of a constitutively active form of Arf6 causes disassembly of adherens junctions (*57*). The results of siRNA-mediated knockdown of GEP100 and of *Arf6*-/- mice also support this notion (*58,59*). Moreover, activation of Arf6 by GEP100 disrupts E-cadherin adhesion, while activation of Arf6 by other GEFs does not (*45*). We have moreover shown that Git1 is responsible for the inactivation of Arf6 at the plasma membrane in order to prevent E-cadherin from being endocytosed (*60*). In this mechanism, Git1 was found to be activated by EphA2, whose dysfunction is frequently observed in malignant cancers. We have moreover shown that EPB41L5 is induced during EMT and is essential for EMT conversion, by facilitating E-cadherin endocytosis (*61*).

Perspective

In this review, I have discussed mainly the intracellular signaling of breast cancer cells, which most frequently arises from luminal epithelial cells. However, it should be noted that the mammary gland is composed of several components, including luminal epithelial cells, myoepithelial cells and the basement membrane. Stromal cells, such as fibroblasts, are also present around the ducts; and they may all participate in the regulation of the mammary gland. In particular, myoepithelial cells have recently been recognized as regulators of normal mammary gland development and function (*62*),

besides their contractile role in expelling milk from the ducts. Interestingly, myoepithelial cells act to suppress tumor growth, invasion and angiogenesis (*63,64*), while stromal fibroblasts act to enhance tumor malignancy (*65*). Such functions of myoepithelilal cells and fibroblasts have been shown to be effective even in the absence of detectable genome alterations in the tumor epithelial cells. In these events, TGFβ is again involved as a key player (*65*).

As discussed, oncogenic mutation of p53 and activation of Ras (or aberrant activation of RTK) appear to be prominent events during the processes that result in breast cancer cells to be reactive to TGFβ and undergo EMT, and sometimes also become stem cell-like cells. Oncogenic mutation of p53 as well as RTK signaling may also be a key in activating the GEP100-Arf6-AMAP1 pathway in response to TGFβ stimulation of breast cancer cells (Hashimoto, A., Hashimoto S., and H.S., unpublished observations: also see Fig. 2), that induces EMT-like properties by activating β1 integrin and inactivating E-cadherin adhesion. TGFβ is produced in adults such as upon wounding and inflammation in order to facilitate wound repair while suppressing some immune responses; and can be produced both by breast cancer cells and the stroma cells. Taken together, an important point in understanding the processes initiating cancer early dissemination would be the identification of what conditions (or even genome status and alterations) trigger production of TGFβ in what types of cells within the microenvironment wherein precancerous epithelial cells already have p53 mutations, and aberrant Ras- or RTK-signaling. Another important aspect is the clarification of whether p53 mutations and excess Ras signaling are really the minimal requirements for luminal epithelial cells to undergo EMT in response to TGFβ. It may also be interesting to examine whether certain types of p53 mutations are more prone than other mutations of p53 in inducing cancerous EMT.

REFERENCES

1. Schmidt-Kittler, O., Ragg, T., Daskalakis, A., Granzow, M., Ahr, A., Blankenstein, T.J., Kaufmann, M., Diebold, J., Arnholdt, H., Muller, P., Bischoff, J., Harich, D., Schlimok, G., Riethmuller, G., Eils, R., and Klein, CA. (2003) From latent disseminated cells to overt metastasis: genetic analysis of systemic breast cancer progression. *Proc. Natl. Acad. Sci. USA* **100**, 7737-7742
2. Husemann, Y., Geigl., J.B., Schubert, F., Musiani, P., Meyer, M., Burghart, E., Forni, G., Elis, R., Ferm, T., Riethmuller, G., and Klein, C. (2008) Systemic spread is an early step in breast cancer. *Cancer Cell* **13**, 58-68
3. Podsypanina, K., Du, Y.C., Jechlinger, M., Beverly, L.J., Hambardzumyan, D., and Varmus, H. (2008) Seeding and propagation of untransformed mouse mammary cells in the lung. *Science* **321**, 1841-1844
4. Hay, E.D. (1995) An overview of epithelio-mesenchymal transformation. *Acta Anat (Basel)* **154**, 8-20
5. Thomas, P.A., Kirschmann, D.A., Cerhan, J.R., Folberg, R., Seftor, E.A., Sellers, T.A., and Hendrix, M.J. (1999) Association between keratin and vimentin expression, malignant phenotype, and survival in postmenopausal breast cancer patients. *Clin Cancer Res* **5**, 2698-2703
6. Onder, T.T., Gupta, P.B., Mani, S.A., Yang, J., Lander, E.S., and Weinberg, R.A. (2008) Loss of E-cadherin promotes metastasis via multiple downstream transcriptional pathways. *Cancer Res* **68**, 3645-3654
7. Elenbaas, B., Spirio, L., Koerner, F., Fleming, M.D., Zimonjic, D.B., Donaher, J.L., Popescu, N.C., Hahn, W.C., and Weinberg, R.A. (2001) Human breast cancer cells generated by oncogenic transformation of primary mammary epithelial cells. *Genes Dev* **15**, 50-65
8. Wong, A.S., and Gumbiner, B.M. (2003) Adhesion-independent mechanism for suppression of tumor cell invasion by E-cadherin. *J Cell Biol* **161**, 1191-1203
9. Thisse, B., el Messal, M., and Perrin-Schmitt, F. (1987) The twist gene: isolation of a Drosophila zygotic gene necessary for the establishment of dorsoventral pattern. *Nucleic Acids Res* **15**,3439-3453
10. Yang, J., Mani, S.A., Donaher, J.L., Ramaswamy, S., Itzykson, R.A., Come, C., Savagner, P., Gitelman, I., Richardson, A., and Weinberg, R.A. (2004) Twist, a

master regulator of morphogenesis, plays an essential role in tumor metastasis. *Cell* **117**, 927-939

11. Pollard, JW. (2001) Tumour-stromal interactions. Transforming growth factor-β isoforms and hepatocyte growth factor/scatter factor in mammary gland ductal morphogenesis. *Breast Cancer Res* **3**, 230-237
12. Bierie, B., and Moses, H.L. (2006) TGF-β and cancer. *Cytokine Growth Factor Rev* **17**, 29-40
13. Padua, D., and Massagué, J. (2009) Roles of TGFβ in metastasis. *Cell Res* **19**, 89-102
14. Al-Hajj, M., Wicha, MS., Benito-Hernandez, A., Morrison, S.J., and Clarke, M.F. Prospective identification of tumorigenic breast cancer cells. (2003) *Proc Natl Acad Sci USA* **100**, 3983-3988
15. Shipitsin, M., Campbell, L.L., Argani, P., Weremowicz, S., Bloushtain-Qimron, N., Yao, J., Nikolskaya, T., Serebryiskaya, T., Beroukhim, R., Hu, M., Halushka, M.K., Sukumar, S., Parker, L.M., Anderson, K.S., Harris, L.N., Garber, J.E., Richardson, A.L., Schnitt, S.J., Nikolsky, Y., Gelman, R.S., and Polyak, K. (2007) Molecular definition of breast tumor heterogeneity. *Cancer Cell* **11**, 259-273
16. Mani, S.A., Guo, W., Liao, M.J., Eaton, E.N., Ayyanan, A., Zhou, A.Y., Brooks, M., Reinhard, F., Zhang, C.C., Shipitsin, M., Campbell, L.L., Polyak, K., Brisken, C., Yang, J., and Weinberg, R.A. (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. *Cell*. **133**, 704-715
17. Ansieau, S., Bastid, J., Doreau, A., Morel, A.P., Bouchet, B.P., Thomas, C., Fauvet, F., Puisieux, I., Doglioni, C., Piccinin, S., Maestro, R., Voeltzel, T., Selmi, A., Valsesia-Wittmann, S., Caron de Fromentel, C., and Puisieux, A. (2008) Induction of EMT by twist proteins as a collateral effect of tumor-promoting inactivation of premature senescence. *Cancer Cell* **14**, 79-89
18. Goldstein, I., Marcel, V., Olivier, M., Oren, M., Rotter, V., and Hainaut, P. (2011) Understanding wild-type and mutant p53 activities in human cancer: new landmarks on the way to targeted therapies. *Cancer Gene Ther* **18**, 2-11
19. Cicalese, A., Bonizzi, G., Pasi, C.E., Faretta, M., Ronzoni, S., Giulini, B., Brisken, C., Minucci, S., Di Fiore, P.P., and Pelicci, P.G. (2009) The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells. *Cell* **138**, 1083-1095

20. Lowe, S.W., Cepero, E., and Evan, G. (2004) Intrinsic tumour suppression. *Nature* **432**, 307-315
21. Roper, E., Weinberg, W., Watt, F.M., and Land, H. (2001) p19ARF-independent induction of p53 and cell cycle arrest by Raf in murine keratinocytes. *EMBO Rep* **2**, 145-150
22. Attisano, L., and Wrana, J.L. (2002) Signal transduction by the TGF-β superfamily. *Science* **296**, 1646-1647
23. Cordenonsi, M., Montagner, M., Adorno, M., Zacchigna, L., Martello, G., Mamidi, A., Soligo, S., Dupont, S., and Piccolo, S. (2007) Integration of TGF-β and Ras/MAPK signaling through p53 phosphorylation. *Science* **315**, 840-843
24. Brosh, R., and Rotter, V. (2009) When mutants gain new powers: news from the mutant p53 field. *Nat Rev Cancer* **9**, 701-713
25. Sørlie, T., Perou, C.M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M.B., van de Rijn, M., Jeffrey, S.S., Thorsen, T., Quist, H., Matese, J.C., Brown, P.O., Botstein, D., Eystein Lønning, P., and Børresen-Dale, A.L. (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proc Natl Acad Sci USA* **98**, 10869-1087
26. Soussi, T., and Béroud, C. (2001) Assessing TP53 status in human tumours to evaluate clinical outcome. *Nat Rev Cancer* **1**, 233-240
27. Lang, G.A., Iwakuma, T., Suh, Y.A., Liu, G., Rao, V.A., Parant, J.M., Valentin-Vega, Y.A., Terzian, T., Caldwell, L.C., Strong, L.C., El-Naggar, A.K., and Lozano, G. (2004) Gain of function of a p53 hot spot mutation in a mouse model of Li-Fraumeni syndrome. *Cell* **119**, 861-872
28. Olive KP, Tuveson DA, Ruhe ZC, Yin B, Willis NA, Bronson RT, Crowley D, and Jacks T. (2004) Mutant p53 gain of function in two mouse models of Li-Fraumeni syndrome. *Cell* **119**, 847-860
29. Kraus, M.H., Yuasa, Y., and Aaronson, S.A. (1984) A position 12-activated H-ras oncogene in all HS578T mammary carcinosarcoma cells but not normal mammary cells of the same patient. *Proc Natl Acad Sci USA* **81**, 5384-5388
30. Hand, P.H., Thor, A., Wunderlich, D., Muraro, R., Caruso, A., and Schlom, J. (1984) Monoclonal antibodies of predefined specificity detect activated ras gene expression in human mammary and colon carcinomas. *Proc Natl Acad Sci USA* **81**, 5227-5231

31. Slamon, D.J., deKernion, J.B., Verma, I.M., and Cline, M.J. (1984) Expression of cellular oncogenes in human malignancies. *Science* **224**, 256-262
32. Clark, G.J., and Der, C.J. (1995) Aberrant function of the Ras signal transduction pathway in human breast cancer. *Breast Cancer Res Treat* **35**, 133-144
33. Hingorani, S.R., Wang, L., Multani, A.S., Combs, C., Deramaudt, T.B., Hruban, R.H., Rustgi, A.K., Chang, S., and Tuveson, D.A. (2005) Trp53R172H and KrasG12D cooperate to promote chromosomal instability and widely metastatic pancreatic ductal adenocarcinoma in mice. *Cancer Cell* **7**, 469-483
34. Caulin, C., Nguyen, T., Lang, G.A., Goepfert, T.M., Brinkley, B.R., Cai, W.W., Lozano, G., and Roop, D.R. (2007) An inducible mouse model for skin cancer reveals distinct roles for gain- and loss-of-function p53 mutations. *J Clin Invest* **117**, 1893-1901
35. Oft, M., Peli, J., Rudaz, C., Schwarz, H., Beug, H., and Reichmann, E. (1996) TGF-β1 and Ha-Ras collaborate in modulating the phenotypic plasticity and invasiveness of epithelial tumor cells. *Genes Dev* **10**, 2462-2477
36. Oft, M., Heider, K.H., and Beug, H. (1998) TGFβ signaling is necessary for carcinoma cell invasiveness and metastasis. *Curr Biol* **8**, 1243-1252
37. Oft, M., Akhurst, R.J., and Balmain, A. (2002) Metastasis is driven by sequential elevation of H-ras and Smad2 levels. *Nat Cell Biol* **4**, 487-494
38. Adorno, M., Cordenonsi, M., Montagner, M., Dupont, S., Wong, C., Hann, B., Solari, A., Bobisse, S., Rondina, M.B., Guzzardo, V., Parenti, A.R., Rosato, A., Bicciato, S., Balmain, A., and Piccolo, S. (2009) A Mutant-p53/Smad complex opposes p63 to empower TGFβ-induced metastasis. *Cell* **137**, 87-98
39. Takagi, J., and Springer, T.A. (2002) Integrin activation and structural rearrangement. *Immunol Rev* **186**, 141-163
40. Miettinen, P.J., Ebner, R., Lopez, A.R., and Derynck, R. (1994) TGF-β induced transdifferentiation of mammary epithelial cells to mesenchymal cells: involvement of type I receptors. *J Cell Biol* **127**, 2021-2036
41. Bretscher, M.S., and Aguado-Velasco, C. (1998) Membrane traffic during cell locomotion. *Curr Opin Cell Biol* **10**, 537-541
42. Donaldson, J.G. (2003) Multiple roles for Arf6: sorting, structuring, and signaling at the plasma membrane. *J Biol Chem* **278**, 41573-41576
43. Hashimoto, S., Onodera, Y., Hashimoto, A., Tanaka, M., Hamaguchi, M., Yamada,

A., and Sabe, H. (2004) Requirement for Arf6 in breast cancer invasive activities. *Proc Natl Acad Sci USA* **101**, 6647-6652

44. Onodera, Y., Hashimoto, S., Hashimoto, A., Morishige, M., Mazaki, Y., Yamada, A., Ogawa, E., Adachi, M., Sakurai, T., Manabe, T., Wada, H., Matsuura, N., and Sabe, H. (2005) Expression of AMAP1, an ArfGAP, provides novel targets to inhibit breast cancer invasive activities. *EMBO J* **24**, 963-973
45. Morishige, M., Hashimoto, S., Ogawa, E., Toda, Y., Kotani, H., Hirose, M., Wei, S., Hashimoto, A., Yamada, A., Yano, H., Mazaki, Y., Kodama, H., Nio, Y., Manabe, T., Wada, H., Kobayashi, H., and Sabe, H. (2008) GEP100 links epidermal growth factor receptor signalling to Arf6 activation to induce breast cancer invasion. *Nat Cell Biol* **10,** 85-92
46. Sabe, H., Hashimoto, S., Morishige, M., Ogawa, E., Hashimoto, A., Nam, J.M., Miura, K., Yano, H., and Onodera, Y. (2009) The EGFR-GEP100-Arf6-AMAP1 signaling pathway specific to breast cancer invasion and metastasis. *Traffic* **10**, 982-993
47. Brown, F.D., Rozelle, A.L., Yin, H.L., Balla, T., and Donaldson, J.G. (2001) Phosphatidylinositol 4,5-bisphosphate and Arf6-regulated membrane traffic. J Cell Biol 154, 1007-1017
48. Powelka, A.M., Sun, J., Li, J., Gao, M., Shaw, L.M., Sonnenberg, A., and Hsu, V.W. (2004) Stimulation-dependent recycling of integrin β1 regulated by ARF6 and Rab11. *Traffic* **5**, 20-36
49. Dai, J., Li, J., Bos, E., Porcionatto, M., Premont, R.T., Bourgoin, S., Peters, P.J., and Hsu, V.W. (2004) ACAP1 promotes endocytic recycling by recognizing recycling sorting signals. *Dev Cell* **7**, 771-77
50. Van Lint, J., Rykx, A., Maeda, Y., Vantus, T., Sturany, S., Malhotra, V., Vandenheede, J.R., and Seufferlein, T. (2002) Protein kinase D: an intracellular traffic regulator on the move. *Trends Cell Biol* **12**, 193-200
51. Bowden, E.T., Barth, M., Thomas, D., Glazer, R.I., and Mueller, S.C. (1999) An invasion-related complex of cortactin, paxillin and PKCμ associates with invadopodia at sites of extracellular matrix degradation. *Oncogene* **18**, 4440-4449
52. Yeaman, C., Ayala, M.I., Wright, J.R., Bard, F., Bossard, C., Ang, A., Maeda, Y., Seufferlein, T., Mellman, I., Nelson, W.J., and Malhotra, V. (2004) Protein kinase D regulates basolateral membrane protein exit from trans-Golgi network. *Nat Cell*

Biol **6**, 106-112

53. Roberts, M., Barry, S., Woods, A., van der Sluijs, P., and Norman, J. (2001) PDGF-regulated rab4-dependent recycling of αvβ3 integrin from early endosomes is necessary for cell adhesion and spreading. *Curr Biol* **11**, 1392-1402
54. Woods, A.J., White, D.P., Caswell, P.T., and Norman, J.C. (2004) PKD1/PKCm promotes αvβ3 integrin recycling and delivery to nascent focal adhesions. *EMBO J* **23**, 2531-2543
55. Muller, P.A., Caswell, P.T., Doyle, B., Iwanicki, M.P., Tan, E.H., Karim, S., Lukashchuk, N., Gillespie, D.A., Ludwig, R.L., Gosselin, P., Cromer, A., Brugge, J.S., Sansom, O.J., Norman, J.C., and Vousden. K,H. (2009) Mutant p53 drives invasion by promoting integrin recycling. *Cell* **139**, 1327-1341
56. Yoon, S.O., Shin, S., and Mercurio, A.M. (2005) Hypoxia stimulates carcinoma invasion by stabilizing microtubules and promoting the Rab11 trafficking of the α6β4 integrin. *Cancer Res* **65**, 2761-2769
57. Palacios, F., Price, L., Schweitzer, J., Collard, J.G., and D'Souza-Schorey, C. (2001) An essential role for ARF6-regulated membrane traffic in adherens junction turnover and epithelial cell migration. *EMBO J* **20**, 4973-4986
58. Hiroi, T., Someya, A., Thompson, W., Moss, J., and Vaughan, M. (2006) GEP100/BRAG2: activator of ADP-ribosylation factor 6 for regulation of cell adhesion and actin cytoskeleton via E-cadherin and α-catenin. *Proc Natl Acad Sci USA* **103**, 10672-10677
59. Suzuki, T., Kanai, Y., Hara, T., Sasaki, J., Sasaki, T., Kohara, M., Maehama, T., Taya, C., Shitara, H., Yonekawa, H., Frohman, M.A., Yokozeki, T., and Kanaho, Y. (2006) Crucial role of the small GTPase ARF6 in hepatic cord formation during liver development. *Mol Cell Biol* **26**, 6149-6156
60. Miura, K., Nam, J.M., Kojima, C., Mochizuki, N., and Sabe, H. (2009) EphA2 engages Git1 to suppress Arf6 activity modulating epithelial cell-cell contacts. *Mol Biol Cell* **20**, 1949-1959
61. Hirano, M., Hashimoto, S., Yonemura, S., Sabe, H., and Aizawa, S. (2008) EPB41L5 functions to post-transcriptionally regulate cadherin and integrin during epithelial-mesenchymal transition. *J Cell Biol* **182**, 1217-1230
62. Lakhani, S., and Bissell, M. (2005) Introduction: the role of myoepithelial cells in integration of form and function in the mammary gland. *J Mammary Gland Biol*

Neoplasia **10**, 197-198

63. Barsky, S.H., and Karlin, N.J. (2005) Myoepithelial cells: autocrine and paracrine suppressors of breast cancer progression. *J Mammary Gland Biol Neoplasia* **10**, 249-260
64. Polyak, K., and Hu, M. (2005) Do myoepithelial cells hold the key for breast tumor progression? *J Mammary Gland Biol Neoplasia* **10**, 231-247
65. Hu, M., Yao, J., Carroll, D.K., Weremowicz, S., Chen, H., Carrasco, D., Richardson, A., Violette, S., Nikolskaya, T., Nikolsky, Y., Bauerlein, E.L., Hahn, W.C., Gelman, R.S., Allred, C., Bissell, M.J., Schnitt, S., and Polyak, K. (2008) Regulation of *in situ* to invasive breast carcinoma transition. *Cancer Cell* **13**, 394-406

Legends to Figs.

Fig.1. Full transformation is not necessary to move out. Genes, as well as their mutations involved in oncogenesis are overlapped with those regulating cell motile phenotypes. Therefore, cells under oncogenesis may thereby acquire motile phenotypes that are enough to move out from their original place, even if they are not fully transformed.

Fig. 2. Our current model as to how GEP100-Arf6-AMAP1 pathway is involved in cancerous EMT and perhaps also 'early dissemination' of primary tumors of the human breast. An ourtstanding question of this model is to clarify detailed mechanisms as to how TGFβ1 stimultation activates RTKs in cells under oncogenesis, that are linked to the GEP100-Arf6-AMAP1 pathway.

Fig. 1

Human Mammary Tumors

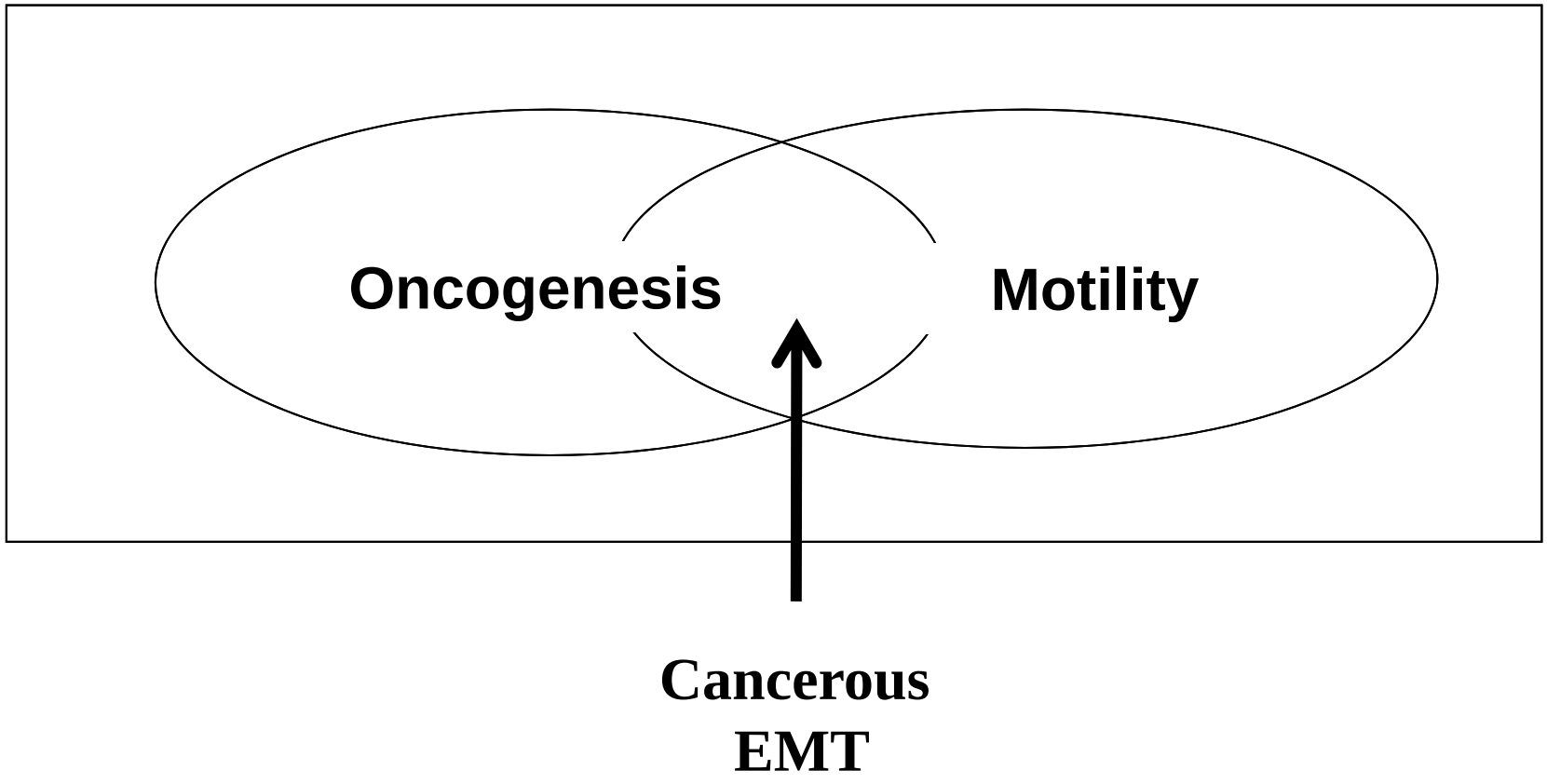
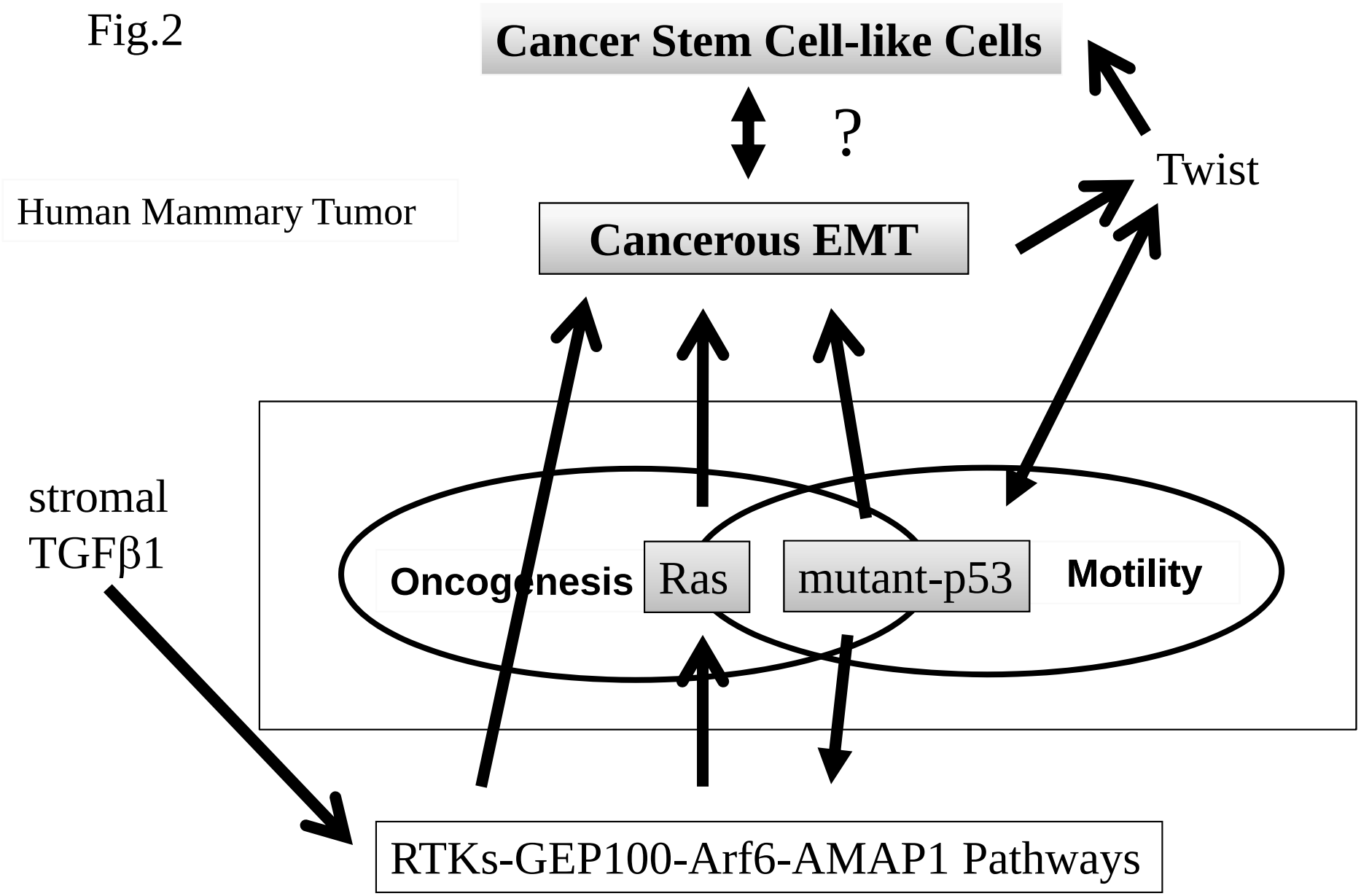


Fig.2


Cancer Stem Cell-like Cells
?
Twist
Human Mammary Tumor
Cancerous EMT
stromal
TGFβ1
Oncogenesis
Ras
mutant-p53
Motility
RTKs-GEP100-Arf6-AMAP1 Pathways