Exercise: Invasion and metastasis

1) Memorization and deduction:

a) Name 3 general roles of integrins in cancer:

Integrins primarily enable 1. cell adhesion, 2. cell migration and 3. cell survival. Treatment with FAK inhibitors indicate that factors downstream of integrins directly or indirectly may also promote cell proliferation, but this is not considered to be one of their "general roles".

b) What increases the versatility of integrins to mediate such diverse functions in various cell and tissue environments?

A large number of possible *heterodimers* of α and β chains provides a versatile arsenal to bind diverse substrates and intracellular signal transduction components.

c) Tumor invasiveness critically depends on extracellular proteolysis that is exquisitely regulated in space and time. What "barriers" exist in normal tissues to prevent that this dangerous process happens spontaneously?

1. ECM remodeling requires cleavage of multiple substrates by *multiple interdependent* proteases: Just one protease alone cannot break down all these factors. Therefore, the probability of accidental breakdown is close to zero.

2. The *spatial confinement* of such proteolysis: All of these proteases are made as zymogens => their activation requires spatially localized proteolytic *cascades* so that all players must come together at the right time and at the right place, e.g. during infection, blood clotting and wound healing, and for tissue morphogenesis during normal embryonic development.

3. Numerous endogenous protease inhibitors further increase the threshold to inadvertently activate such protease cascades in the wrong place at the wrong time.

2) Former exam MCQ:

Which one of the following statements about metastatic cancer cells is ***false***:

A. They form metastases independently of known metastasis-specific driver mutations
B. They tend to upregulate stem cell-like gene signatures compared to non-metastatic cells in the same tumor
C. Binding to blood platelets facilitates their survival
D. A complete EMT is essential for the formation of macrometastases
E. They may be recognized in sentinel lymph nodes by the immune system

The process of EMT:

A. increases cell adhesion to the extracellular matrix
B. induces the degradation of basement membrane
C. correlates with increased invasion and metastasis
D. is essential for cancer cells to resist chemotherapy
E. is required for colonization

3) Tumor cell heterogeneity

a) The ability to metastasize is only acquired by a subset of cells within a given tumor. What observations and experiments support the existence of such a "hierarchy" among the cancer cells within a given tumor?

1. Morphological heterogeneity within a solid tumor: *Invasive front* tends to be morphologically quite different (less differentiated, more mesenchymal, less cell-cell adhesion).

2. When cells from a given human tumor are dissociated and grafted at limited dilution into immunodeficient recipient mice, their potential to initiate a new tumor varies. Tumor-initiating potential tends to be increased in subpopulations with stem-like traits and/or mesenchymal gene expression signatures. A similar functional heterogeneity is usually observed also when cells from the resulting tumors are sorted again: This supports the notion that metastatic potential is likely a metastable trait (cell plasticity).

In other words: The cells within the primary tumor that are competent to metastasize express more proinvasive and prometastatic gene expression signatures (EMT-like) than those that are not. By contrast, an advantage of some clones over others in tumor initiation or metastasis could never be attributed to specific mutations or to heterogeneity in the mutational load, even though such intratumoral genetic heterogeneity is common.

b) What explanation(s) have been offered to explain why "colonization" is not more efficient? In other words, why do micrometastatic cells only rarely give rise to macrometastases? i) One likely possibility is that only a minority of micrometastases can re-acquire an epithelial phenotype, or that this epigenetic process takes a long time.

ii) In addition, the majority of micrometastases can be kept in check by the co-evolving immunity against tumor neoantigens that are induced by pre-existing and newly accumulating passenger mutations. Heterogeneity among immune infiltrates in different metastatic sites of the same patients indicates that successful colonization also correlates with impaired immune surveillance.

Note that these two scenarios are not mutually exclusive.

iii) An earlier hypothesis proposed that colonization may require additional new mutations. However, genome sequencing of large cohorts of metastases and their primary tumors revealed no evidence that colonization is associated with any specific new driver mutations.

4) Data interpretation: Role of Id1 in metastatic lung colonization.

The final step of the metastatic cascade is called colonization. To test how this rate-limiting step is regulated, researchers introduced a doxycycline-regulated TRE-Id1 transgene into Ras-transformed human mammary epithelial cells (HMLER) that were engineered to stably express SV40T and a GFP marker, together with either Twist or Snail. 24 hours after injection of such engineered HMLER cells into the tail vein of immunodeficient mice, the hosts were treated with or without doxycycline (**Fig. 1A**), followed by analysis of GFP+ foci 3 weeks later as a readout of metastatic growth (**Fig. 1B-D**).

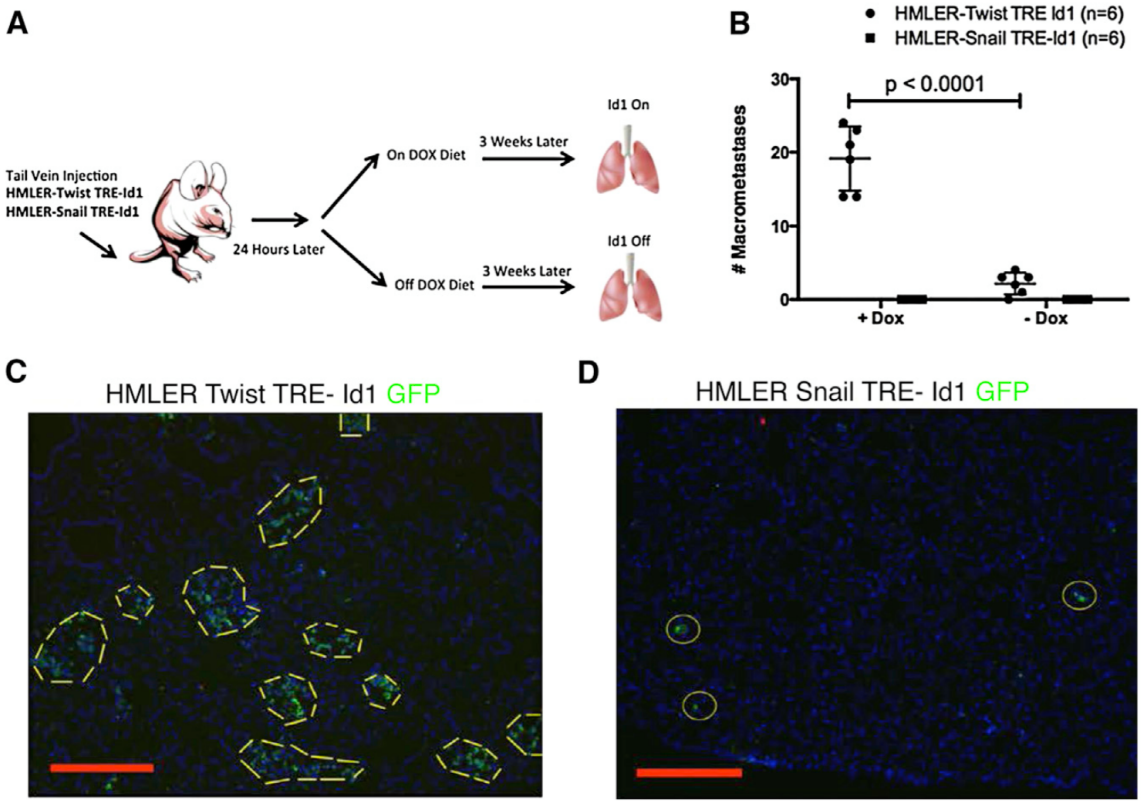


Figure 1. Influence of inducible Id1 on lung colonization by breast cancer cells that were forced to undergo EMT in vitro. *(A) Experimental strategy. (B) Quantification of the number of macrometastatic outgrowths under the indicated experimental conditions. (C, D) Representative images of GFP+ clusters of metastatic cells expressing the EMT transcription factors Twist (C) or Snail, respectively (D). Source: Stankic et al., Cell Rep. 5: 1228-1242 (2013)*

i) What was the rationale for transducing the HMLER cells with Twist or Snail, and what effect of Id1 on the resulting cells can you observe here *in vivo*?

Twist or Snail were expressed to force the epithelial HMLER cells to undergo EMT. This allows to test whether increased EMT is *sufficient* for colonization. On the other hand, by inducing Id1 expression to block the function of Twist after micrometastasis formation, they were able to test whether colonization (i.e. the outgrowth of macrometastases) increases if the EMT is reversible.

ii) What system was used here to activate the tetracycline-regulatory element (TRE) that drives the expression of transgenic Id1: The tet transactivator protein (Tet-OFF), or rather the reverse tet transactivator rtTA (Tet-ON)?

The fact that addition of doxycycline *induced* the TRE-Id1 transgene implies that expression was regulated by rtTA (rtTA binds DNA in the *presence* of dox).

By contrast, the tTA protein (a fusion of the transactivation domain of VP16 to the DNA binding domain of prokaryotic tet repressor) would activate transcription from a TRE only in the *absence* of dox.

iii) Twist and Snail are key regulators of EMT during development. They have also been shown to promote metastasis in breast cancer models. Here, the authors wanted to coexpress them with or without inducible Id1. To do so, why did the authors administer doxycycline only 24 hours *after* the grafting of tumor cells? Why not at time zero?

During the first 24 hours, the injected cells were allowed to extravasate in mesenchymal state from microvessels into adjacent parenchymal tissue: During that phase, the mesenchymal state was induced and maintained by the constitutively expressed Snail or Twist transgenes, respectively. Doxycycline was administered only *after* tumor cell extravasation to test whether Id1 may specifically facilitate tumor re-epithelialization (MET) and colonization (i.e. the outgrowth of macrometastases from micrometastases).

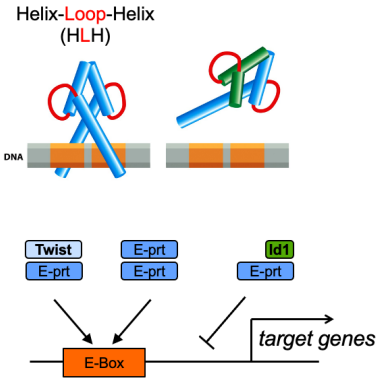
iv) What did they learn about the mechanism of colonization from the fact that the resulting GFP+ metastases in panel C were much bigger than those in panel D?

Panels B to D show that induction of Id1 was necessary and sufficient for lung colonization by Twist-expressing cells. In sharp contrast, no such effect of Id1 was seen in HMEL cells expressing Snail, confirming that Id1 likely acts by specifically depleting Twist of its partner of the E-protein family, rather than by a non-specific effect.

Background information slide:

Today's exercise:
Reversal of Twist-induced EMT by Id1

- Twist belongs to the family of bHLH transcription factors
- To bind DNA, Twist must dimerize with E-protein
- Alternatively, E-proteins bind with high affinity to Id
- Since Id1,2,3,4 lack a DNA binding domain, they function like natural 'dominant negative' inhibitors of E-proteins:



v) Which metastases would you predict to stain positive for E-cadherin: Those in panel C or rather those in D, or both? Explain your answer.

C. Explanation: The model that we saw in the lecture predicts that Id1 should increase the colonization by promoting an MET program (as determined by increased E-cadherin staining) that reverts the Twist-induced EMT. If macrometastatic outgrowth ('colonization') in C is mediated by MET, the cells by definition must have reverted to an epithelial state that is marked by the re-appearance of adherens junctions (these AJs are formed by E-cadherin).

vi) In the lecture on replicative immortality, we heard that Id1 can inhibit the induction of p16INK4A. Why did the authors here not consider a potential effect of Id1 expression on cellular senescence? Hint: What is SV40T?

They could exclude senescence because the HMLE cell grafts were already immortalized by SV40 large T antigen (which binds and inhibits both RB1 and p53 and thus suppresses cellular senescence).

vii) How can you exclude the possibility that Id1 here increased metastatic colonization only indirectly by facilitating tumor cells *invasiveness*? Hint: How important is *invasion* in their animal model of experimental metastasis?

They used tail vein injections: i.e., this model bypasses the need for any invasiveness since the cancer cells are directly injected into the circulation.