Project 1: **The role of Id proteins in determining the tumor initiating and metastatic properties of melanoma cells**

Principal Investigator:
Prof. Dr. Jonathan Sleeman, CBTM, Medical Faculty Mannheim, Heidelberg University
London Project Partner:
Dr. Caroline Hill, Cancer Research UK, London Research Institute

Short Summary
Cancer stem cells are thought to underpin the growth, metastasis and therapy resistance of tumors such as melanoma, through their tumor initiating properties. Building on unpublished observations, this project aims to substantiate the hypothesis that Id gene expression induced by 3D extracellular matrix (ECM) microenvironments plays a functional role in determining stemness and metastatic properties of melanoma cells. The role of integrins, TGF-β and BMPs in determining these properties will be determined. These data should identify new therapeutic targets.

3 State of the Art

3.1 State of knowledge in the field

The concept that the bulk of cells that make up a tumor, including melanomas, are derived from cancer stem cell (CSC) subpopulations is now widely accepted. CSCs are distinguished from other tumor cells by their ability to successfully seed new tumors when implanted in low numbers into experimental animals, and to recapitulate the morphology of the initial tumor. In contrast, the non-CSC population cannot initiate tumor growth *in vivo* even when implanted in high numbers. The potential significance of CSCs for cancer therapy is enormous. Current therapies appear to preferentially destroy the non-CSC population but do not efficiently kill CSCs, with the result that the tumor eventually regrows. Furthermore, as the CSC subpopulation represents by definition the only tumor cells that are able to initiate the growth of new tumors, then CSCs must play a central role in metastasis formation. Understanding the parameters that determine tumor-initiating properties should therefore identify targets for novel and efficient cancer therapies.

Tumor initiation *in vivo* is used to define CSCs. Numerous papers have shown that the take rate of tumors *in vivo* can be manipulated, for example by coinjecting tumor cells with matrigel. Indeed, single unsorted human melanoma cells in matrigel are capable of forming tumors in NOD/SCID Il2rg-/- mice (Quintana et al., 2008, *Nature*). These observations point to a critical role for the microenvironment, in particular the extracellular matrix, in determining tumor-initiating properties.

3.2 Preliminary work by the participants

We have a long track record in metastasis research. Recently we have begun exploring the role of CSCs in metastasis, partly in collaboration with Prof. Umansky (Project 13), with whom we have published work using the Ret murine melanoma model. In unpublished work we have investigated the role of ECM components in determining tumor-initiating properties *in vivo*. As few as 5 cells from the B16 or Ret murine melanoma cell lines were sufficient to initiate tumor growth when co-injected into syngeneic mice with matrigel. In contrast, tens of thousands of cells were required to initiate tumor growth in the absence of matrigel. In further experiments we found that co-injection of tumor cells with laminin or with collagen type I was also sufficient to elicit tumor growth from 5 cells. These data indicate that highly immunocompromised mice are not required for tumor initiation from just a few cells, and that several ECM components are able to initiate tumor growth from small numbers of melanoma cells, all of which are ligands for β1-containing integrins.

Microarray analysis revealed that Id1, Id3 and Smad6, archetypal TGF-β/BMP response genes, were uniquely upregulated (up to 40-fold) in response to 3D but not 2D ECM (matrigel, collagen or laminin), a finding confirmed using qPCR. Id genes are known to play a pivotal role in regulating tumor growth and determining stemness properties, while Smad6 counter-regulates TGF-β/BMP signalling. We also found that tumor cells that do not respond to 3D ECM by upregulating Id1 and Id3 do not show efficient tumor initiation when co-injected with matrigel *in vivo*. Collaborative work

with Prof. Utikal (Project 2) has shown that human melanoma cells can also respond to 3D matrix by upregulating Id1, Id3 and Smad6.

4. Project Plan

4.1 Specific Aims: (1) To test the hypothesis that Id1 and Id3 expression induced in 3D ECM microenvironments plays a functional role in determining the tumor initiating and metastatic properties of melanoma cells; **(2)** To determine whether β1-containing integrins mediate 3D ECM-mediated upregulation of Id1, Id3 and Smad6, and are required for efficient tumor initiation and metastasis formation *in vivo*; **(3)** To investigate whether TGF-β and/or BMP signalling mediates increased Id1, Id3 and Smad6 in 3D ECM microenvironments.

4.2 Experimental program

Aim 1: We will establish loss of function (shRNA, cannabidiol chemical inhibition) and gain of function (tet-inducible expression) for Id1 and Id3 (either alone or in combination) in B16 and Ret melanoma cells. We will then test the cells for their tumor initiating and metastatic ability *in vivo* in the presence (loss of function) or absence (gain of function) of matrigel. These data will demonstrate whether Id1 and Id3 induction in 3D ECM plays a role in specifying the tumor-initiating and metastatic properties of melanoma cells.

Aim 2: We will use loss of function (shRNA) and gain of function (constitutively active mutant) approaches to determine whether β1-containing integrins are involved in the induction of Id1, Id3 and Smad6 in response to 3D ECM microenvironments. If so, we will determine using B16 and Ret cells whether loss of β1 ablates efficient tumor initiation *in vivo* in the context of 3D ECM, and what effect it has on metastasis. Conversely, we will establish whether constitutively activated β1 integrin supports efficient tumor initiation *in vivo* even in the absence of a 3D ECM matrix, as well as whether it promotes metastasis formation.

Aim 3: We will determine which members of the TGF-β and BMP families and their receptors are expressed in B16 and Ret cells growing in 3D ECM environments. For those that are expressed, we will use shRNA to knockdown their expression, and/or use specific inhibitors of TGF-β and BMP signalling to block their activity, then determine whether this affects the ability of 3D ECM to induce Id1, Id3 and Smad6 expression, and to promote efficient tumor initiation *in vivo*. Effects on metastasis formation will also be determined. We will also investigate whether 3D ECM environments promote Id1, Id3 and Smad6 expression by sequestering TGF-β and BMP family members produced by the melanoma cells at locally high concentrations in the ECM around the cells. For all aims, the results of the experiments will be corroborated in human melanoma cells to demonstrate relevance to human disease.

4.3 Collaborations with other projects in the RTG: Collaborations with Prof. Utikal (Project 2) will include (i) isolation of CSC marker-enriched subpopulations from human melanoma samples and analysis of their Id protein expression (ii) analysis of Id expression in primary human melanomas and their metastases. Collaborations with Projects 5 and 7 comprise provision of antibodies, genetically modified mice, and expertise in techniques such as the analysis of cell interactions with hyaluronan and lymphangiogenesis.

5 References

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