Dissecting pathways contributing to oncogenic transformation by the tyrosine kinase receptor TrkB

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CHAPTER 6

General discussion

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As cancer is a highly complex disease, the development of novel drug therapies will require unraveling of its underlying mechanisms. In this thesis, I describe our identification of the MAPK-Twist-Snail-Zeb1-E-cadherin pathway as a critical component of the oncogenic functions of TrkB. In this way, we shed further light on the mechanism by which TrkB promotes cancer. Furthermore, recently, two processes important for cancer development, EMT and senescence, have been linked. In this respect, we have shown that Twist and Zeb1 are regulated differently between EMT and senescence. This implies that these transcription factors can have a double impact on cancer progression, by simultaneously promoting EMT and inhibiting senescence. Further elucidation of the mechanism by which these processes are intertwined may eventually lead to successful new cancer therapies.

Critical pathway for TrkB oncogenic functions
Cancer is the most common cause of death of people in the world. It is a complex disease comprising multiple different steps ranging from primary tumor initiation to the outgrowth of secondary tumors at distant sites. Most cancer patients do not die from the primary tumor, but rather from metastases. Metastasis comprises many different steps that are relayed by distinct biochemical and cell biological events (Geiger & Peeper, 2009). Therefore, it is important to unravel its mechanism and identify as many critical pathways as possible, in order to strategically develop new anti-cancer agents that could prevent metastasis.

Previously, we have identified the TrkB oncogene as an important metastasis regulator (Douma et al., 2004). Importantly, TrkB may be overexpressed in several cancers and inhibiting agents are currently under development (Thiele et al., 2009). With the work described in this thesis, we aimed to unravel the underlying mechanism of TrkB-induced metastasis. We identified the MAPK-Twist-Snail-Zeb1-E-cadherin pathway to be critical for TrkB these functions. Some players within this pathway may be potential drug targets. Indeed, several MEK inhibitors are currently being tested in the clinic for their antitumor activity (Adjei et al., 2008; Haura et al. 2010). Although the critical factors that we identified are mostly transcription factors, which are very difficult to target, we know that blocking one of these transcription factors, either directly or indirectly, would be sufficient to block metastasis.

Do all players act in a single pathway?
Besides the regulation of Twist and Snail via Zeb1 described in chapters 2 & 3, it is known that both Twist and Snail can directly bind the E-boxes within the *CDH1* promoter, thereby inhibiting its transcription (Cano et al., 2000; Batlle et al., 2000; Vesuna et al., 2008). This suggests that E-cadherin repression does not always occur via Zeb1, consistent with the model presented in chapter 3 (see Figure 7, chapter 3). This could imply that Twist and Snail can have dual effects, by acting via Zeb1 and directly on E-cadherin. This is the case, for example, in hepatocellular carci-

noma, in which Snail and Twist overexpression occurs frequently. In this tumor type, combined overexpression of these factors is associated with a decreased cancer-free interval and overall survival. These effects are corroborated by *in vitro* studies, in which co-expression of Twist and Snail has additive effects on E-cadherin regulation, migration and metastasis (Yang et al., 2009).

While multiple transcription factors are involved in EMT and metastasis, it is striking to note that, as we show in the chapters 2 & 3, silencing of a single transcription factor is sufficient to block TrkB-induced EMT and metastasis. One wonders, therefore, whether all E-cadherin repressors, including Slug (Snai2), Zeb2 (SIP1) and E12/E47, are equally involved in TrkB-induced EMT and metastasis. Our observations show that Zeb2 is also induced by TrkB and that it is required for TrkB-induced EMT and anoikis resistance (unpublished observations, M.A.S., D.S.P.). However, TrkB fails to induce Slug and E12/E47 (chapter 2 and unpublished results, M.A.S., T.R.G., D.S.P.). This suggests that not all E-cadherin repressors contribute to TrkB-induced EMT and metastasis.

We have observed a significant overlap in the target genes (whether direct or indirect) of Twist, Snail and Zeb1, which is not for the first time. For example, an overlap in profiles of Snail, Slug and E47 is observed in MDCK cells overexpressing either gene (Moreno-Bueno et al., 2006). Of course, in different cell systems, pathways can be differently regulated. This means that the Twist-Snail-Zeb1 pathway might not be the only pathway critical for EMT and metastasis, but that at least in our cell system, TrkB-induced EMT and metastasis is regulated via this pathway.

EMT versus senescence: all the same players?
We have shown in chapter 5 that BRaf^{E600} and Ras^{V12} activate the MAP kinase pathway, independently of the cellular context analyzed. However, both Twist and Zeb1 are differently regulated in EMT and senescence. It is, therefore, still an open question as to how ERK regulates Twist and Zeb1. Furthermore, it would be interesting to find out why activation of MEK leads to induction of Twist and Zeb1 in one setting and to downregulation in the other. Since the epithelial cells and fibroblasts used in our study differ not only in their origins but also in the way they were immortalized, it could be the number of deficiencies in the cells that makes the difference. It is conceivable that ERK needs the collaborating action of another cancer-promoting agent in order to induce Twist or Zeb1 transcription. This would be in line with the notion that tumor cells require multiple mutations. A single oncogene cannot cause cancer but often acts cytostatically or pro-apoptotically; oncogene collaboration is required to bypass these events and allow for the development of a fully malignant tumor.

Another question arising is whether these transcription factors act on the same target genes in EMT and senescence. During EMT, Twist and Zeb1 mainly act through E-cadherin, by repressing its transcription. But Twist and Zeb1 have more targets.

For example, Twist can induce fibronectin and N-cadherin (Alexander et al., 2006; Yang et al., 2007). Conceivably, one or more of these targets has a role in senescence beyond regulating EMT. However, further examination of this hypothesis is required to make definitive statements. A second possibility is that Twist and/or Zeb1 act on senescence players, for example p16^{INK4A}. The *CDKN2A* promoter consists of several E-boxes (Zheng et al., 2004), suggesting that it is a potential Twist or Zeb1 target. Although there is no evidence to date that p16^{INK4A} is involved in the regulation of EMT, p16^{INK4A} overexpression induces anoikis and causes loss of tumorigenicity. This is regulated via integrin α5, a fibronectin receptor, as was illustrated by the observation that inhibition of the α5 chain of fibronectin almost completely blocked p16^{INK4A}-induced anoikis (Plath et al., 2000).

Until now, we have not detected any discrepancy between EMT induction and anoikis resistance: all cells that have undergone EMT were anoikis-resistant. Most likely, anoikis resistance and EMT are coupled, perhaps implyin that p16^{INK4A} can block EMT. However, at least the epithelial cell lines that we have used in chapter 5 had lost p16^{INK4A} expression. This leaves open the possibility that in primary epithelial cells, p16^{INK4A} could play a role in this setting. The other option is that p16^{INK4A} is important for senescence only and that another Twist or Zeb1 target contributes to EMT. Further examination of this will required to dissect the complete mechanism of the connection between EMT and senescence.

E-cadherin regulators, real oncogenes?
Knowing that Twist and Zeb1 can both promote EMT and block senescence, these transcription factors are likely to have a double impact on cancer progression (Figure 1). But does this finding make Twist and Zeb1 genuine oncogenes? Twist is also involved the N-MYC-induced transformation of *Ink4a-ARF$^{-/-}$* MEFs (Valsesia-Wittmann et al., 2004) and it enhances transformation induced by E1A and Ras^{V12} (Maestro et al., 1999). Besides these findings, Twist protects cells from apoptosis (Maestro et al., 1999). Both processes contribute to cancer progression, therefore implying a general role of Twist in cancer progression. Zeb1, on the other hand, is critical for TrkB-induced anoikis resistance and metastasis. Therefore it is likely that Zeb1 can also prevent apoptosis. Although it seems that these transcription factors have only a cancer-promoting effect (at least in susceptible settings), for other transcription factors this is more complex. For example, although Snail has generally a cancer-promoting effect, in certain settings it has a tumor-suppressive function, by inducing cell cycle arrest (Vega et al., 2004). The same holds true for SIP1, which can inhibit cyclin D1 in specific settings (Mejlvang et al., 2007). This implies that the regulation of the transcription factors is tightly regulated and can differ in certain settings. This notwithstanding, targeting Twist or Zeb1 may have a strong impact on cancer cells, and further elucidation of the pathway could reveal novel therapeutic targets that have also a double impact.

Chapter 6
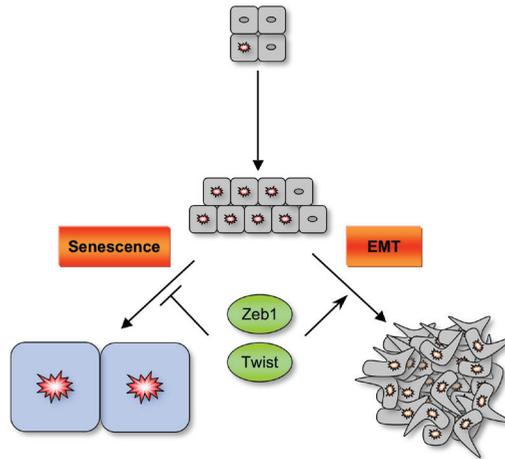
Figure 1: Role of Twist and Zeb1 in cancer progression. Upon the acquisition of one or more oncogenic mutations, incipient cancer cells may expand and form a premalignant lesion. Tumor expansion is limited by several constraints, including (oncogenes-induced) cellular senescence and the need to acquire an invasive phenotype. In the context of oncogenic mutations, transcription factors like Twist and Zeb1 can simultaneously suppress the senescence response and induce an epithelial-mesenchymal transition (EMT), both contributing to malignant progression. Figure from Smit MA and Peeper DS (2008) *Cancer Cell* **14**: 5-7.

EMT and cancer progression

It is generally believed that EMT is involved in the first steps of invasion (Thiery, 2002). The increased migratory phenotype of the cells induced by EMT is required for the invasion of tumor cells into surrounding tissue and the blood. But EMT could also be involved in driving tumor growth, since it can endow cells with stem cell properties (Mani et al., 2008). The important EMT regulators Twist, Snail and Zeb1 are all connected to such features. For example, while Twist or Snail overexpression induces stem cell properties (Mani et al., 2008), Zeb1 can repress expression of stemness-inhibiting miRNAs (Wellner et al., 2009). In the group of Weinberg, the induction of EMT was used to screen for inhibitors specifically killing cancer stem cells, thereby aiming to find new therapies. They found salinomycin to inhibit mammary tumor growth *in vivo* (Gupta et al., 2009). This implies that EMT is playing a role in stem cell formation, arguing that EMT represents a rate-limiting event for tumor growth. Another recent observation suggests that EMT is involved in tumor initiation. Senescence, which prevents tumor formation, can be bypassed by overexpression of Twist. This senescence bypass is accompanied by EMT (Ansieau et al., 2008). It is for these reasons that EMT could play a role in tumor initiation (Weinberg, 2008).

Concluding remarks

We have identified one important pathway critical for TrkB oncogenic functions. This sheds further light on how TrkB promotes metastasis. Furthermore, the finding that two independent processes, EMT and senescence, are connected, provide novel insight in the mechanism of cancer progression and metastasis. Further elucidation of this connection may eventually reveal novel anti-cancer targets.

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