Review Article

Receptor tyrosine kinases and their activation in melanoma

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Summary

Receptor tyrosine kinases (RTKs) and their downstream signalling pathways have long been hypothesized to play key roles in melanoma development. A decade ago, evidence was derived largely from animal models, RTK expression studies and detection of activated RAS isoforms in a small fraction of melanomas. Predictions that overexpression of specific RTKs implied increased kinase activity and that some RTKs would show activating mutations in melanoma were largely untested. However, technological advances including rapid gene sequencing, siRNA methods and phospho-RTK arrays now give a more complete picture. Mutated forms of RTK genes including *KIT, ERBB4,* the *EPH* and *FGFR* families and others are known in melanoma. Additional over- or underexpressed RTKs and also protein tyrosine phosphatases (PTPs) have been reported, and activities measured. Complex interactions between RTKs and PTPs are implicated in the abnormal signalling driving aberrant growth and survival in malignant melanocytes, and indeed in normal melanocytic signalling including the response to ultraviolet radiation. Kinases are considered druggable targets, so characterisation of global RTK activity in melanoma should assist the rational development of tyrosine kinase inhibitors for clinical use.

Introduction

Tyrosine phosphorylation is a key component of signal transduction pathways regulating normal mammalian cellular functions (Blume-Jensen and Hunter, 2001). Phosphotyrosine-based signalling can mediate normal growth, survival, differentiation, attachment and migration (Hunter, 2009), while in oncogenesis it contributes to each of the six phenotypic changes described as hallmarks of cancer (Hanahan and Weinberg, 2000). Malignant melanoma in particular contains more tyrosine phosphate than pigmented naevi and normal melanocytes (McArdle et al., 2005; Zakut et al., 1993), signifying elevated protein tyrosine kinase and/or decreased protein tyrosine phosphatase activity (McArdle et al., 2001). Melanoma is highly resistant to conventional chemotherapy; thus less-toxic, targeted treatments are needed. **Receptor tyrosine kinases (RTKs)** form the largest family of oncogenes, and tyrosine kinase inhibitors have now offered remarkable therapeutic successes in some cancers. Nonetheless, encouraging preclinical data have not always held up in the clinical setting, which may reflect deficiencies in our understanding of RTK function and dysfunction. In melanoma, while such understanding has grown substantially since this topic was previously reviewed (Easty and Bennett, 2000), some common RTKs driving oncogenesis and progression may still remain to be identified.

There is diverse evidence for abnormalities of both tyrosine kinases and phosphatases in melanoma, which will be reviewed here with principal focus on RTKs. First we will introduce both families and their signalling pathways. Secondly we will review studies of altered RTK signalling and RTKs themselves in melanoma, as criteria for rational therapeutic targets, by (a) sequencing (the tyrosine kinome), (b) expression, (c) activity (including some unpublished data) and (d) functional assays. Finally, we will focus on specific RTKs as putative new targets for therapy.

Protein tyrosine kinases (PTKs)

Ninety PTKs have been identified in humans. They catalyse transfer of the γ-phosphate of ATP to the hydroxyl group of a tyrosine residue. 58 of them are plasma membrane receptors (RTKs), and 32 are nonreceptor or cytoplasmic PTKs, also involved in signal transduction, usually from receptors (Hunter, 2009; Lemmon and Schlessinger, 2010; Robinson et al., 2000). Vertebrate PTKs fall into 20 families of receptors and 10 families of nonreceptors, which have been reviewed in detail for humans and other vertebrates (Manning et al., 2002; Robinson et al., 2000). Remarkably, at least 51 (57%) of the 90 PTKs have been implicated in cancer, by mutation, overexpression or underexpression (Bennasroune et al., 2004; Blume-Jensen and Hunter, 2001).

RTKs are transmembrane proteins with conserved intracellular catalytic domains and extracellular ligand-binding domains. The extracellular domain is the most variable,

combining characteristic modules that vary with the ligand. RTKs have generally been considered as mutually independent, but heterogeneous interactions have recently been identified between RTKs of different classes (cross-talk) in signal transduction. EGFR and ERBB2 are examples of central players in networks of coactivated RTKs (Stommel et al., 2007; Xu and Huang, 2010). Cross-talk has been reported for PDGFR, MET, EPHA2, IGF1R and RON (Morgillo et al., 2006; Peace et al., 2003), see also references in Xu and Huang (2010). This is a putative mechanism for acquired resistance against targeted drugs. For example, overexpression of EGFR correlates with elevated IGF1R expression in non-small cell lung cancer (NSCLC) cells (Morgillo et al., 2006). A previous report described heterodimerization of EGFR and IGF1R in NSCLC cells; interestingly, heterodimers increased on treatment with the EGFR inhibitor erlotinib   (Morgillo et al., 2006). IGF1R/EGFR heterodimers mediated increases in survivin and EGFR protein synthesis, while inhibition of IGF1R activity induced apoptosis and decreased resistance to erlotinib in NSCLC cells. Hence the authors suggested combination of IGF1R inhibitors with EGFR inhibitors currently used in NSCLC (Morgillo et al., 2006).

Protein tyrosine phosphatases (PTPs)

Although PTPs have received less attention than the kinases to date, they are increasingly believed to play important roles in cancer development and progression. Human PTPs comprise a large multigene family of 107 members, with further diversity conferred by alternative splicing and post-translational modifications (Tonks, 2006). They are divided into four classes according to their catalytic domain sequences, which are Cys-based in three of the classes and Asp-based in the fourth (Alonso et al., 2004). Class I is the only large class of PTPs, with 99 members comprising 38 classical (tyrosine-only) and 61 dual-specificity (tyrosine and serine-threonine) PTPs. The classical PTPs comprise 21 receptors and 17 non-receptors. Dual-specificity PTPs include PTEN (also with lipid phosphatase activity), an important melanoma suppressor gene; others are not considered here (Alonso et al., 2004; Tonks, 2006). Since they oppose tyrosine kinase actions, PTPs have been anticipated to function as tumour-suppressors. On the other hand, global PTP activity is elevated in melanoma compared to normal melanocytes (McArdle et al., 2003), and some PTPs show increased activity in tumours and behave as transforming oncogenes in experimental assays (Ostman et al., 2006), as discussed later.

Signal transduction pathways from RTKs

Overview

A summary of the main signalling pathways from RTKs is shown in Figure 1, as reviewed by Weinberg (2006), with some melanocyte-specific elements, namely transcription factor MITF and its upstream and downstream components. Following ligand binding, receptors dimerize or oligomerize, allowing autophosphorylation and substrate phosphorylation on tyrosine. Signalling requires the recruitment of adaptor proteins and intracellular kinases, which physically bind tyrosine phosphates on the activated RTK via either SH2 (SRC-homology 2) domains or another phosphotyrosine binding (PTB) domain (Seger et al., 2008). Bound components vary with the specific RTK (Weinberg, 2006), but typically include GRB2 (which recruits exchange factors for RAS GTPases, activating the RAS family); phosphoinositide 3-kinase (PI3K), phospholipase Cγ (PLCγ) and the SRC family. These activate the various signalling pathways in Figure 1, which are frequently overactive in cancer (Hunter, 2009; Weinberg, 2006). Figure 1 also includes the important nonreceptor PTK, focal adhesion kinase (FAK), which transduces signals from integrins (matrix receptors) and some G-protein-coupled receptors including endothelin receptors (Weinberg, 2006). FAK recruits SRC-family PTKs, and signals through all the main RTK pathways.

Finally, emerging evidence suggests surprisingly that membrane receptors such as EGFR family members, FGFR1, MET, VEGFR and IGF1R can shuttle into the nucleus where they still transduce signals [review: Wang et al. (2010)]. The EGFR has been implicated in transcriptional regulation (Wang and Hung, 2009), and in keratinocytes ultraviolet irradiation results in nuclear translocation of EGFR (Xu et al., 2009).

RAS pathways

RAS proteins mediate at least three downstream signalling pathways (Figure 1). The best-studied is the RAF-ERK pathway, in which a RAF-family member (e.g. BRAF) is recruited by binding to activated RAS, leading to a kinase cascade through MEK1 and 2 (serine/threonine kinases) and mitogen-activated protein kinases 1 and 3 (MAPK1, 3), also known as ERK2 and 1. Phosphorylations by ERKs upregulate protein synthesis through ribosomal S6 protein kinase (RSK), and cell proliferation through AP1 and MYC as shown (Figure 1). Secondly, RAS also activates signalling through RALGDS and RAL. This lesser known pathway has been implicated in tumour progression including melanoma (Mishra et al., 2010) and also as a target of the important tumour-suppressive phosphatase PP2A (Sablina et al., 2007). Thirdly, activated RAS binds the p110 subunit of PI3K, which is required for PI3K activation by some RTKs (Gupta et al., 2007).

PI3K and PLCγ pathways, and others

Other RTKs do not require RAS activation to activate PI3K, but can activate PI3K by direct binding of its p85 subunit. PI3K generates phosphatidylinositol 3,4,5-trisphosphate (PIP3), which recruits protein kinase B/AKT to the membrane, allowing phosphorylation and activation by PDK1. AKT is a powerful promoter of cell growth (protein synthesis) through mTOR, and cell survival through several intermediates, as shown. AKT can also stimulate proliferation by inhibiting the important regulatory kinase GSK3β, which represses β-catenin and AP1 pathways. PLCγ acts through protein kinase C (PKC) to stimulate proliferation and remodel cell shape to a more fibroblast-like, migratory form (EMT or epithelial-mesenchymal transition), as reviewed previously (Bennett, 2008).

An intermediate specific to melanocytes and melanoma is microphthalmia-associated transcription factor, splice-variant M (MITF-M, or MITF for short). MITF, impacted by PTK signalling through at least two pathways (Figure 1), is considered a master regulator of differentiation in melanocytic cells, promoting transcription of pigmentary enzymes and other cell-specific components. MITF upregulates the pro-survival protein BCL2 (Levy et al., 2006), and several positive or negative regulators of cell proliferation, including the RTK KIT which is crucial in normal melanocyte development and maintenance (Bennett, 2008; Hou et al., 2000; Levy et al., 2006). The JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway may also be activated (Lopez-Bergami et al., 2008). An immunohistochemical study detected phosphorylated STAT3 in melanoma but not benign nevi or melanocytes (Messina et al., 2008). Finally, STAT3 may also play a central role in melanoma metastasis (Kong et al., 2008).

Abnormalities of RTKs and their signalling in melanoma: overview

RTK pathways have long been known as drivers of melanoma development. Important early data were derived from animal models: receptor kinase *Xmrk* (an EGFR homologue) was the first known nonviral oncogene, identified in the *Xiphophorus* fish melanoma model [review: (Adam et al., 1993)]. RTKs overexpressed or activated in transgenic mice also resulted in melanoma development, including RET (Iwamoto et al., 1991), and MET via expression of its ligand hepatocyte growth factor from the metallothionein promoter (highly active in melanocytes) (Otsuka et al., 1998). Reduced growth-factor requirements of advanced human melanoma cells compared to melanocytes suggested the presence of abnormal phosphotyrosine signalling here too (Halaban et al., 1988). Use of degenerate primers to amplify expressed PTKs indeed revealed a complex pattern of expression and differences between melanocytes and melanomas (Easty et al., 1993; Lee et al., 1993). Much new information has been acquired since then about specific abnormalities in RTK expression

and signalling in melanoma, as will now be discussed.

Abnormalities of RTK downstream pathways in melanoma

Although our main focus will be on RTKs themselves, known alterations in downstream signalling components will first be summarized briefly.

General downstream pathways

MAPK signalling is overactive in nearly all melanomas. This occurs most commonly through mutually exclusive activating mutations of *BRAF* (47%) or *NRAS* (21%) (Bennett, 2008; Forbes et al., 2010) with some activating mutations of RTKs (more below). It has attracted much interest, with the development of small molecule inhibitors including farnesyl transferase inhibitors for RAS, and BRAF inhibitors such as (initially) sorafenib. In preclinical studies, sorafenib inhibited growth of human melanoma cell lines and xenografts. However, single-agent sorafenib has been of limited value in clinical trials, and a combination of sorafenib with chemotherapeutic agents appears more promising (Egberts et al., 2008; Eisen et al., 2006). The great majority of activating *BRAF* mutations in melanoma are V600E mutations, and a new inhibitor PLX4032 with high specificity for this mutant protein is a promising new treatment for patients with melanomas that express it. At doses over 240 mg twice daily, 37/48 (77%) patients with metastatic melanoma carrying $BRAF^{V600E}$ showed objective responses, including 3 complete responses (Flaherty et al., 2010), an unprecedented level of response for this disease. A notable but treatable side-effect was the development of squamous cell carcinomas. Alternative BRAF inhibitors are also under development [e.g. (Zambon et al., 2010)].

Physiological RTK signaling pathways contain multiple negative feedback loops. Within the RAF/MEK/ERK pathway, negative feedback occurs via Sprouty and dual specificity phosphatases (DUSP). Increased RAF/MEK/ERK pathway signals leads to inhibition of CBL, SOS, ERK and PLK3, by DUSP4/6 and SPRY2/4 (Pratilas et al., 2009; Pratilas and Solit, 2010). Inhibition of BRAF will thus result in a loss of negative feedback and (by this and other routes), increased flux into parallel signaling pathways (Halaban et al., 2010).

Hyperactivation of the PI3K/AKT pathway can confer resistance to chemotherapy. This can occur through various routes, the most frequent known in melanoma being the inactivation of PTEN by mutation or deletion (in 30% of melanomas), while activating mutations of the p110α subunit of PI3K and amplifications of AKT3 are also reported [review: (Bennett, 2008)]. Interestingly the translational regulator and tumour suppressor 4E-BP1 was recently reported to be a common target of AKT and MEK signalling, and a potential therapeutic target for cancers with activation of both these pathways (She et al., 2010) (Figure 1).

MITF expression is increased in some melanomas, the gene being amplified in 15% to 20% of metastatic melanomas, correlating with decreased patient survival (Garraway et al., 2005; Levy et al., 2006); however MITF expression is reduced in a set of melanomas that have high levels of transcription factor BRN2, associated with a more invasive, possibly melanoblast-like phenotype (Goodall et al., 2008). Combined expression of MITF and BRAF^{V600E} was reported to transform human melanocytes expressing mutant CDK4, activated p53 and TERT (Garraway et al., 2005). RTKs known to upregulate MITF activity include KIT in melanocytes (Hemesath et al., 1998) and TYRO3 in melanoma (Zhu et al., 2009). More generally the ERK pathway increases the expression of the more mitogenic short MITF-M splice variant, which is specifically upregulated in some metastatic melanomas (Primot et al., 2010). In turn, MITF-M increases transcription of mitogenic RTKs KIT (Hou et al., 2000) and MET (McGill et al., 2006), completing potential positive feedback loops.

Signalling pathways downstream of IGF1R

Much current work indicates an important interaction between IGF1R and BRAF^{V600E} signalling. Specific inhibition of IGF1R by the cyclolignan picropodophyllin resulted in decreased ERK1/2 activity and induced apoptosis even in melanoma cells with *BRAF^{V600E}* mutations, indicating the importance of an alternative, non-BRAF pathway in MAPK activation (Karasic et al., 2010).

The role of Insulin-like growth factor binding protein 7 (IGFBP7) in melanoma genesis remains uncertain. IGFBP7 binds IGF1, repressing binding to the IGF1R. An initial report concluded that expression of exogenous BRAF^{V600E} in melanocytes resulted in IGFBP7 secretion and mediation of senescence and apoptosis. In human melanomas with *BRAF^{V600E}*, *IGFBP7* was epigenetically silenced; moreover, addition of recombinant IGFBP7 inhibited growth of melanoma in xenografts (Wajapeyee et al., 2008). This idea was challenged when a second group described repression (rather than induction) of IGFBP7 following expression of BRAF^{V600E} (Scurr et al., 2010). These disparate findings may reflect differences in culture conditions (Scurr et al., 2010); this seems unlikely however, since the first group have now duplicated their earlier findings in the absence of antibiotic selection (Wajapeyee et al., 2010).

PTP alterations in melanoma

PTPs are not included in Figure 1 and their interactions are still being elucidated, but they rate as signalling components in these pathways, and some knowledge is emerging on their abnormalities in melanoma. An emerging trend has been the identification of reduced PTP gene expression in some melanomas. As another general point of interest for skin cancer, ultraviolet irradiation generates reactive oxygen species within cells, which can react with cysteine residues in the active site of PTPs and impair activity. This may amplify PTK

activity, by prolonging tyrosine phosphorylation of both PTKs and their substrates (Xu et al., 2006).

PTPRK and PTPRU in melanoma

Following RT-PCR with degenerate primers to clone PTPs expressed in melanocytes and melanoma, two members of the 2B receptor subfamily, PTPRK (or κ) and PTPπ (HUGO name: PTPRU) were confirmed by northern analysis to be absent or down-regulated in most melanomas (McArdle et al., 2001). Interestingly, *PTPRK* maps to 6q22.2-2.3, a common site for chromosome rearrangement in melanoma (Zhang et al., 1998). Mutated PTPRK was also identified as the source of a peptide recognized by T cells in a melanoma patient (Novellino et al., 2003). The authors showed deletion of *PTPRK* in several advanced melanomas, and that PTPRK co-localizes with and dephosphorylates β-catenin, resulting in reduced nuclear accumulation of β-catenin with reduced transcriptional activity and decreased expression of targets MYC and cyclin D1. Furthermore, engineered expression of PTPRK in melanoma cells reduced proliferation and migration. This provides a potential mechanism for tumour suppression by PTPRK (Novellino et al., 2008). Meanwhile, in keratinocytes, PTPRK specifically dephosphorylates EGFR and its knockdown increases EGFR phosphorylation (Xu et al., 2005). Hence PTPRK counters intrinsic EGFR kinase activity and inactivation of PTPRK is a likely mechanism for activation of EGFR by UV-irradiation (Xu et al., 2009).

PTPRD in melanoma

Homologous deletions of *PTPRD* have been described in several tumour types including melanoma (Forbes et al., 2010; Stark and Hayward, 2007). In addition, 10 somatic mutations were identified in 7/57 melanomas (12%). Its location at 9p23-4 has led to suggestions that it may be the predicted additional melanoma suppressor gene near *CDKN2A* (Solomon et al., 2008). Lentiviral re-expression of wild-type (but not mutant) PTPRD in null melanoma and glioblastoma cells resulted in suppression of growth, and apoptosis, supporting its identity as a tumour suppressor. PTPRD appears to be a homophilic cell adhesion molecule and thus a candidate mediator of contact inhibition (Solomon et al., 2008). PTPRD was also found to dephosphorylate the proto-oncoprotein STAT3 in glioblastoma cells (Chan and Heguy, 2009). STAT3 appears to be a central component of signal transduction: activity is increased on phosphorylation by receptor (EGFR) and cytoplasmic (JAKs) tyrosine kinases, and inhibited by tyrosine phosphatases such as PTPRD and PTPRT. It remains unclear whether EGFR inhibitors would be effective inhibitors of growth in PTPRD-null melanomas (Chan and Heguy, 2009).

Alterations specific to RTKs in melanoma

RTKs and criteria for rational therapeutic targets

We will now consider alterations specific to RTKs. The most valuable therapeutic targets will be those providing critical transforming signals that drive tumour growth and survival, where inactivation results in selective apoptosis and tumour regression. This dependence has been termed "oncogene addiction" (Weinstein and Joe, 2008). Oncogene addiction is seen for several tyrosine kinases, such as BCR-ABL in chronic myeloid leukaemia, targeted by imatinib (Druker, 2004; George et al., 2004). Targeted therapy has been most effective when directed against tumours expressing highly activated kinases, such as BCR-ABL in acute myeloid leukaemia and EGFR in NSCLC. Pointers indicating a good RTK therapeutic target in a cancer type include reports of: (1) high expression, (2) activating mutations, (3) tyrosine phosphorylation (indicating RTK activation), and (4) inhibition of RTK activity in functional studies, with apoptosis and growth arrest. In addition, the target should lack a vital role in normal adult physiology (Bauman et al., 2007; George, 2002). Here we review findings concerning RTK sequence, expression, activation and function in melanoma. Five RTKs that are, or may become therapeutic targets are then considered in further detail.

Sequence variants of RTKs in melanoma

Until around 2007, there was surprisingly little evidence for driver mutations within RTKs in melanoma, although these were long expected, since RTK activation would upregulate all the pathways in Figure 1. Downregulation or loss of the vital melanocytic RTK, KIT, had been reported in most cutaneous melanomas, as reviewed previously (Easty and Bennett, 2000). However activating *KIT* mutations have now been identified in some melanoma subtypes (mucosal, acral and non-sun-exposed) in which *BRAF* and *NRAS* mutations are rare; here, KIT is still expressed (Ashida et al., 2009; Curtin et al., 2006). *FGFR1* activating mutations were likewise reported in two melanomas lacking activated *RAF* or *RAS* (Thomas et al., 2007).

Technological advances have now allowed a comprehensive cataloguing of somatic mutations within the large PTK gene family (kinome), in cancers including human melanomas and cell lines derived from them. (Note: "mutation" in these studies designates any nonconservative sequence alteration not seen in the germline, and may have included some changes not affecting function). All 86 PTK genes were sequenced in established tumour cell lines including 53 melanoma lines, and made available as a database of tyrosine kinome variants (Ruhe et al., 2007). In general there was a low incidence of these somatic mutations; still, mutations were identified within 19 RTKs including *MET, TYRO3, EPHA2, EPHB2, EPHB6* and *NTRK1-3* (summarized in Table 1). Mutated cytoplasmic PTKs included *FAK* and *PTK2B*, both in the FAK subfamily (Ruhe et al., 2007). In a second large-

scale study, the 86 PTK genes were sequenced either partially or fully from 79 melanoma cultures (Prickett et al., 2009). Again they described somatic mutations in 19 PTK genes, but largely different from those seen by Ruhe et al. (2007). Mutations in receptors are listed in Table 1. They include notably frequent somatic mutations in *ERBB4* (19%); also *EPHB2, EPHB6* and *VEGFR1* (9-10% each). Cytoplasmic PTKs with mutations included *PTK2B* and *FAK* again; also *FER, PTK6*, and *PTK7*.

The reason for the largely different findings between these two studies is unclear. Both groups studied metastatic, not primary melanomas. However, Ruhe et al. analysed established cell lines, comparing them to unmatched nontumorigenic cell lines and normal tissues, while Prickett et al. used fresh, low-passage cultures, compared to matched normal genomic DNA from patients' peripheral blood. Ruhe et al. sequenced all exons in full, while Prickett et al. sequenced the kinase domain of all PTKs, and then all exons of the 19 PTKs that showed kinase-domain mutations. Differences may also reflect the overall diversity of cancers and a paucity of very common PTK alterations. Some of the mutations are likely to play a role in melanoma progression (drivers); others may represent passengers (random, neutral changes). Functional studies of ERBB4 are discussed below. Mutations of *PTK2B* were found in a substantial fraction (10%) of melanomas. Identified in both studies, PTK2B is a member of the FAK subfamily. It binds to a GTPase regulator associated with FAK, and the SH2 domain of GRB2 (Schaller, 2010). Separate studies of specific RTK genes in melanoma describe mutations in *MET, KIT* and *FGFR2*, the last being inactivating mutations reminiscent of the common losses of KIT already mentioned (summarized in Table 1). A linking theme may be that where there is downstream oncogenic activation, of proteins such as BRAF, RAS or MITF, then upstream receptors may become redundant, or even inhibitory by pathway overactivation. Otherwise RTKs may themselves become oncogenically activated and/or amplified.

Altered RTK expression in melanoma

Significant increases in expression of a given RTK in melanoma compared to melanocytes suggest that the kinase may promote tumour progression. RTK expression has been extensively studied in melanomas and precursor lesions. Previous studies used northern and immunoblotting analysis and immunohistochemistry. These were reviewed previously, when 12 RTKs and 4 nonreceptor PTKs to date had been reported to be overexpressed or ectopically expressed relative to normal melanocytes. Some of the most common were EPHB3 (94% of melanoma lines), EPHA2 (93%) and FGFR4 (64%) (Easty and Bennett, 2000). More recently, microarrays and RT-PCR analysis of candidate genes have been extensively applied and permit further meta-analysis of melanoma RTK expression (Table 1). Microarray data in the *Oncomine* database identify apparently increased expression in uncultured melanomas of mRNAs for ERBB3 (3-5 fold increase) and IGF1R (2-fold increase)

compared to normal skin, and TRKC (18-fold increase) compared to benign naevi. Expression of several RTK mRNAs (MSPR, NTRK2/TRKB and EPHA4) was lower in melanoma compared to naevi (Oncomine: https://www.oncomine.org/resource/login.html). Note: "abnormal" gene expression in melanoma would be expected to mean different from that in the normal cellular counterpart, melanocytes. Since melanocytes comprise only a small fraction of cells in normal skin, some studies (as found in Oncomine) compare melanoma with whole skin, which may give misleading results such as suggesting that melanocyte-specific genes are "overexpressed" in melanoma. Naevi provide a better control than skin, but still have various differences from melanocytes. Thus these comparisons should be interpreted with caution unless confirmed by other methods. Table 1 summarises reported changes in RTK levels.

RTK activity and abnormalities in melanoma

Studies of RTK mRNA or protein abundance may not always reflect activation. More recently, phospho-arrays have been used to analyze RTK activity in melanoma (Margaryan et al., 2009). These employ arrayed antibodies specific for the phosphorylated (usually the active) form of a protein kinase, permitting simultaneous semi-quantitative analysis of receptor activity in cell or tissue extracts (Margaryan et al., 2009). Differing signal intensities on a phospho-RTK array may arise from: (1) differences in abundance, via differing transcription, translation or degradation; (2) activating or inactivating mutations in RTKs, (3) the presence of cognate ligands (exogenous or endogenous) and (4) activity of PTPs or other interacting components. We used Proteome ProfilerTM Arrays (R&D Systems) to assess phosphorylation of 42 RTKs in 17 melanocytic cell lines of various degrees of malignancy. (See Supplementary Information for Materials and Methods). Non-small cell lung cancer (HCC827) cells containing amplified, kinase-activated EGFR (36 copies) were used as a positive control and yielded an intense signal for EGFR as reported previously (Engelman et al., 2007).

Each cell line was tested on arrays in duplicate assays, with consistently reproducible results. Representative arrays are included as supplementary data. Supplementary Figure 1 indicates grid orientation on the Proteome ProfilerTM Array. We analysed cells from various stages of melanoma progression, including: normal melanocytes (Supplementary Figure 2), radial growth phase melanoma (Supplementary Figure 3), vertical growth phase melanoma (with data from co-isogenic melanoma cell lines, Supplementary Figure 4), and metastatic melanoma (Supplementary Figure 5). Although the Proteome ProfilerTM Arrays (R&D Systems) have been widely used to assay kinase activity within other tumours (Ball et al., 2007; Eckstein et al., 2008; Engelman et al., 2007; Stommel et al., 2007), data presented here remain preliminary and will require further validation by specific RTK

immunoprecipitation and immunoblotting with anti-phosphotyrosine antibodies (MS in preparation).

A complex pattern of 25/42 activated RTKs was detected in melanocytic cells (Figure 2; summary in Table 1). Phosphorylation signal intensity for melanocytic cells was low to moderate: typically 30 fold less than the amplified EGFR control. Normal melanocytes showed some variation in activated RTKs, but EGFR, TYRO3, KIT, TIE1 and EPHB2 were consistently active in both lines. The KIT ligand SCF is an additive to the culture medium, EGF and macrophage stimulating protein (MSP, the RON ligand) are present in serum, and it seems that ligands for TYRO3 (bovine protein S, see below) and for TIE1 must also be present. (Note that all cells were grown with serum.) TIE1 and TIE2 have been identified as receptors for angiopoietins (Seegar et al., 2010), vasculogenic regulators expressed by various cells including some tumours, but it is unknown whether they are present in normal melanocytes. TYRO3 is discussed later. Phosphorylation of EPHB6 was seen in a proportion of lines. EPHB6 lacks kinase activity, so this may reflect heterodimerization with an active RTK, with cross-phosphorylation of EPHB6.

None of 15 melanomas showed KIT activity. This included SGM2, grown in the presence of SCF, so that differences between cell lines are unlikely to reflect solely differences in composition of culture medium. TIE1 activity was lost in a proportion of melanoma lines, though without an obvious relation to progression. EGFR and TYRO3 activity were maintained in all melanomas and EPHB2 in all but one, suggesting a positive role. A number of RTKs were activated in at least 2 of the 15 melanoma lines but not in melanocytes, namely ERBB3 (4 lines), FGFR2 (6), FGFR3 (9), INSR (6), PDGFRA (2), FLT3 (2), VEGFR2 (10), VEGFR3 (2) and EPHA4 (2). Three isogenic lines showed similar patterns: WM793 (poorly tumorigenic parental cells), WM793P1 (more tumorigenic derivative) and 1205Lu (metastatic derivative), with a few differences which may have been related to increasing malignancy but further study would be needed. Our initial expectation was that RTK arrays might identify kinases over- or underactive in advanced melanoma cell lines. However no clear pattern of changing RTK activation emerged in early versus advanced melanoma in this study. Nor was any single RTK up- or downregulated in all melanomas. However at the receptor family level, upregulated activity was seen in 12/15 melanomas for an FGF receptor and 11/15 for a VEGF receptor. The latter may be due to increased receptor expression, from mRNA studies (Table 1).

Functions and alterations of specific RTKs in melanoma

As discussed, upregulated RTKs, which may mediate biological functions including growth, survival, migration and angiogenesis, may represent suitable targets for cancer therapy, but this depends on any change in level or activity being a driver (active in carcinogenesis)

rather than a passenger. This can be determined by tests of biological function by manipulation of expression levels. Various methods have been used for RTK manipulation, providing evidence that a number of RTKs are indeed drivers of melanoma development. These findings are summarized in Table 2, and RTKs of particular interest will now be discussed individually.

ERBB kinases in melanoma

The EGFR family (subclass I) of RTKs comprises EGFR, ERBB2, ERBB3 and ERBB4 (Manning et al., 2002), which recognize more than 11 structurally related ligands. ERBB2 however has no identified cognate ligand, functioning as a co-receptor for other family members. ERBB3 lacks kinase activity but can activate RTK pathways through ERBB2. In general, signalling from the homodimers is weaker than heterodimeric signalling (as with ERBB2/ERBB3).   When anti-phosphotyrosine affinity chromatography and mass spectroscopy were used to analyse signalling networks following EGFR activation, diverse proteins were identified, including adapters GRB2, SHC, SCK, and NSP2, and additional kinases including focal adhesion kinase (FAK), PTK2B, YES, EPHA2, and EPHB4 (Thelemann et al., 2005).

Inhibition of EGFR by the drug gefitinib partially decreased proliferation of human melanoma cells, associated with a reduced tyrosine phosphorylation of EGFR, ERBB2 and ERBB3 (Djerf et al., 2009). However, treatment with another EGFR inhibitor, erlotinib, did not affect melanoma cell proliferation although there was inhibition of MAPK and AKT signalling pathways (Schicher et al., 2009). Moreover, in a Phase II clinical study, erlotinib (150 mg daily) was preliminarily reported to have minimal or no single-agent activity against metastatic melanoma (Wyman et al., 2006). A recent study also found that increased ERBB3 expression is a marker of poor prognosis in melanoma, with frequent high expression in melanoma and no expression in normal melanocytes (consistent with the above phosphoarray findings). Moreover, downregulation of ERBB3 in melanoma cells resulted in decreased proliferation, migration and invasion, together with decreased chemoresistance to DTIC (Reschke et al., 2008).

As mentioned, global sequence analysis of PTKs revealed *ERBB4* mutations in 19% of 79 human melanomas (Prickett et al., 2009). ERBB4 was highly phosphorylated in these lines compared to cells with wild-type receptor. Functional analysis of seven missense mutations in *ERBB4* indicated elevated kinase activity and increased capacity to transform NIH3T3 cells. Finally, inhibition of ERBB4 activity by two methods, short hairpin (shRNA) knockdown and lapatinib (pan-ERBB inhibitor), each decreased the growth of melanoma cells expressing mutant ERBB4. Decreased phosphorylation of ERBB4 following lapatinib may have resulted from either decreased transphosphorylation by EGFR/ERBB2 or direct effects upon ERBB4 (Prickett et al., 2009).

EPHA2 in melanoma

During development, EPH receptors can mediate signals for cell-cell repulsion (or adhesion), and direct growth cone and neural crest cell migration (Poliakov et al., 2004). Hence it is not unexpected that they may play a role in melanoma progression. Overexpression of EPHA2 and its ligand ephrin A1 have been widely reported in melanomas and the cell lines derived from them (Easty et al., 1995a; Easty et al., 1999; Easty and Bennett, 2000; Hendrix et al., 2003; Kinch and Carles-Kinch, 2003; Straume and Akslen, 2002). Increased EPHA2 expression was associated with melanoma thickness, increased cell proliferation (Straume and Akslen, 2002) and vasculogenic mimicry (Margaryan et al., 2009). A recent global screen in melanocytes found increased EPHA2 transcription in response to UV radiation; interestingly, in EPHA2-null mouse embryo fibroblasts there was resistance to UV-induced apoptosis (Zhang et al., 2008). The authors suggest a model whereby EPHA2 induces signals for both apoptosis and survival/proliferation, but in melanoma pro-apoptotic signals are abrogated through additional mutations.

Two new mechanisms have recently been proposed in melanoma metastasis: vasculogenic mimicry and mesenchymal-amoeboid transition. Folberg et al. (1993) identified a system of matrix-rich vascular networks in melanoma tissues, significantly associated with decreased survival. When highly aggressive melanoma cells were cultured *in vitro* on a 3-dimensional matrix, the actual melanoma cells formed similar vessel-like networks (termed vasculogenic mimicry), and it was hypothesized that these structures might provide channels for tumour perfusion and metastases. Expression of EPHA2 (together with FAK) correlated with vasculogenic mimicry in melanoma cells (Margaryan et al., 2009).

Two interesting functional studies provided direct evidence for the role of EPHA2 in melanoma progression. Parri et al. (2009) found exogenous EPHA2 to promote melanoma invasion by inducing mesenchymal-amoeboid transition (MAT). Here melanoma cells become rounded (amoeboid-like) and progress through stromal barriers with no requirement for proteolysis of the extracellular matrix. The authors suggested this mechanism may promote lymphatic metastasis. Separately, knockdown of EPHA2 in a highly metastatic melanoma cell line resulted in growth-inhibitory phosphorylations of CHK2 and p53, decreased invasion and vasculogenic mimicry, and suppressed tumour growth in xenografts (Margaryan et al., 2009).

EPHA2 signalling appears complex. There is some evidence that EPHA2 is constitutively activated when non-phosphorylated (Pasquale, 2008; Walker-Daniels et al., 2003), although this may have been due to RTK clustering during immunoprecipitation. This may explain why we did not detect phosphorylated EPHA2 on phospho-arrays (Figure 2), even though EPHA2 mRNA and protein appear upregulated in most melanomas (Table

1), suggesting functionality. EPHA2 is likewise highly expressed but poorly phosphorylated in mammary cancer (Walker-Daniels et al., 2003). Overexpressed EPHA2 in mammary epithelial cells remained poorly phosphorylated, yet resulted in transformation and growth in soft agar. However, more recent evidence indicates that tyrosine phosphorylation of EPHA2 upon binding of ephrin-A1 ligand is important for kinase activity in endothelial cells (Fang et al., 2008). The tumour microenvironment in vivo may supply an EPHA2 ligand or stimulate its autocrine secretion.

Interestingly, EPHA2 has been reported to associate with and enhance signalling via members of the EGFR gene family (Pasquale, 2008); this effect may be independent of tyrosine phosphorylation of EPHA2. Studies in a breast cancer cell line (MDA-MB-231) containing abundant hypophosphorylated EPHA2 indicate that phosphorylation of EPHA2 is regulated by tyrosine phosphatase LMW-PTP, a known oncogene. Expression of a catalytically inactive mutant (LMW-PTP129A) in these cells resulted in decreased levels of EPHA2. Moreover, the oncogenic activity of LMW-PTP was inhibited by antisense EPHA2. It is unknown however whether LMW-PTP is present in melanoma.

KIT in melanoma

In most melanoma lines, KIT expression is downregulated or lost compared to normal melanocytes (Lassam and Bickford, 1992; Natali et al., 1992) (Table 1). The ligand stem cell factor (SCF) inhibited growth of KIT-expressing melanoma cells (Zakut et al., 1993). Moreover, expression of KIT in A375M human melanoma cells resulted in apoptosis in the presence of SCF, and decreased tumorigenicity and metastatic potential in xenografts (Huang *et al.*, 1996). All these findings suggested that *KIT* behaved as a tumour suppressor.

More recently however, several groups have shown recurrent activating KIT mutations as well as copy-number increases in mucosal, acral and CSD (arising in chronically sun-damaged skin) melanomas (Curtin et al., 2006; Garrido and Bastian, 2010). Within these subtypes of melanoma, BRAF and NRAS activations appear rarely (Curtin et al., 2006). Hence it appears that oncogenic RTK activation is mutually exclusive with mutational activation of downstream signalling, with the caveat that not all known components of downstream signalling have been sequenced for mutations as yet. Pre-clinical studies then indicated that a subset of melanoma cell lines overexpressed both cyclin-dependent kinase 4 (CDK4) and KIT. Interestingly, these cells were resistant to BRAF inhibitors but sensitive to the PTK inhibitor imatinib (Smalley et al., 2008).

Initial clinical trials of imatinib for metastatic melanoma were disappointing. There was no objective response or disease progression at 6 months in groups of 16 and 26 patients (Ugurel et al., 2005; Wyman et al., 2006), despite positivity for KIT protein in most melanomas in the first group. However, an anecdotal report described a major response in one patient with a mucosal melanoma containing mutant *KIT* (Lutzky et al., 2008).

Importantly, expression of mutant *KIT* in acral and mucosal melanoma was then found to correlate with imatinib sensitivity (Jiang et al., 2008). Recent results of another phase II trial of single-agent imatinib have been reported. Patients enrolled had metastatic melanoma expressing 2/3 RTKs of KIT, PDGFR and ABL. One patient (with overexpression of KIT in an acral melanoma) had marked improvement at 6 weeks and a partial response for 12 months (Kim et al., 2008). A complete response was reported in another patient with stage IV mucosal melanoma expressing mutant KIT. Resection and irradiation were followed by the multi-kinase inhibitor sorafenib with the alkylating agent temozolomide ((Quintás-Cardama et al., 2008).

Finally, a recent study screened 32 patients with metastatic acral or mucosal melanoma for *KIT* mutations, finding mutations in mucosal (38%) and acral (6%) melanomas. Three patients receiving imatinib and one patient receiving sorafenib showed initial responses, but with subsequent development of brain metastases in three patients (Handolias et al., 2010), apparently consistent with failure of Imatinib to penetrate the blood-brain barrier (Garrido and Bastian, 2010). However, imatinib may be useful in a subset of patients with acral and mucosal melanoma containing *KIT* mutations.

TYRO3 in melanoma

Interestingly TYRO-3 was recently identified as a positive regulator of MITF expression (Figure 1), in a pathway via SOX10. A genome-wide screen of 16,000 cDNAs used an MITF reporter to identify gene products which upregulate MITF expression in B16 mouse melanoma cells (Zhu et al., 2009). Interestingly, overexpression of TYRO3 also rescued primary melanocytes from induction of senescence by mutant BRAF V600E. Moreover, stable knockdown of TYRO3 in melanoma cell lines, via shRNA targeting, resulted in decreased proliferation and an increased sensitivity to chemotherapeutic agents cisplatin and docetaxel but not temozolomide, and knockdown also markedly reduced tumorigenic potential of a xenograft model in nude mice. Thus, TYRO3, which can also signal through AKT, may be a driver of melanoma and a suitable target for pharmacological intervention (Zhu et al., 2009).

The same authors used real-time quantitative RT-PCR to report a 3-fold increased expression of TYRO3 in 20/40 melanoma tissue samples compared to normal skin (Zhu et al., 2009). This is not an ideal comparison though because, as already discussed, TYRO3 expression in normal melanocytes or naevi was not assessed. However two early studies used RT-PCR to clone TYRO3 from normal melanocytes (Easty et al., 1993; Lee et al., 1993). A small-scale northern blotting analysis showed some melanoma lines to express similar amounts of TYRO3 to normal melanocytes, while others had no detectable message (Easty et al., 1993). Phospho-array analysis was perhaps more sensitive, showing similar TYRO3 activity in all studied melanoma lines as well as normal melanocytes (Figure 2).

TYRO3 ligands include plasma proteins Growth Arrest-Specific 6 (GAS6) and Protein S; oddly, human protein S fails to activate TYRO3, although bovine protein S does activate human TYRO3 (Godowski et al., 1995; Nyberg et al., 1997). Since melanoma cell lines were cultured with bovine serum prior to analysis on phospho-arrays, this is a potential source of activating ligand. It currently seems unclear whether this growth-promoting RTK is maintained unchanged or oncogenically activated in some melanomas, and hence whether it would be specific enough as a therapeutic target. Immunostaining of TYRO3 in normal melanocytes in skin versus melanomas would clarify the picture, as would further mRNA analyses or studies of the effect of small-molecule inhibitors of TYRO3 upon the growth of melanoma xenografts or of cultured melanoma cells and melanocytes.

Perspectives from other tumours

Here we discuss the putative role of PTK inhibitors in melanoma, relative to findings from other tumours. Several studies show multiple active RTKs within single cancers, including lung, glioblastoma and melanoma (Engelman et al., 2007; Margaryan et al., 2009; Stommel et al., 2007). Phospho-RTK array studies of glioblastoma multiforme showed activated RTKs typically including EGFR, ERBB4, insulin receptor and CSFR (Stommel et al., 2007). The authors hypothesized that unresponsiveness of cancer to a single PTK inhibitor can result from such multiple signalling mechanisms. Interestingly, they found that a combination of three specific PTK inhibitors, or siRNAs, was required for significant inhibition of survival in glioblastoma cells (Huang et al., 2007; Stommel et al., 2007). Redundant RTK signalling pathways in melanoma seem consistent with the lack of objective response to specific PTK inhibitors in some clinical trials (Eisen et al., 2006; Sosman and Puzanov, 2006). The identification of useful combinations of PTK inhibitors for melanoma therapy would require a systematic approach, whereby pairs of RTKs are inhibited in turn across a panel of tumours. An important question concerns the number of RTKs that can be simultaneously inhibited in a tumour, with an expected increase in off-target effects (Socinski, 2008).

Even in tumours with a good initial response to a PTK inhibitor, the effect is typically relatively short-lived, since resistance develops and the patient relapses. The inactivation of essential kinases selects for the development of resistance. Potential resistance mechanisms include: (a) genomic amplification and up-regulation of target RTK expression, (b) presence of secondary mutations conferring insensitivity to the PTK inhibitor, and (c) activation of new RTKs. Recent studies have described "kinase switching" in cancer cells treated with kinase inhibitors. Hence NSCLC cells become resistant to the effects of gefitinib via amplification of MET (Engelman et al., 2007; Engelman and Settleman, 2008). Likewise

GIST tumours, initially sensitive to imatinib, became resistant following activation of AXL (Mahadevan et al., 2007).

Emerging principles

Metastatic melanoma is resistant to cytotoxic chemotherapy and management of advanced (stage IV) melanoma remains palliative.   Advanced melanoma remains exceptionally resistant to apoptosis, with frequent constitutive activation of PI3K and MAPK pathways (Soengas and Lowe, 2003) among other anti-apoptotic changes (Bennett, 2008).   This review reconfirms the heterogeneous nature of RTKs in melanoma, with co-activation of multiple kinases. The concept of oncogene addiction has been described however, where signalling from one activated oncogene is required for malignancy (Weinstein and Joe, 2008).   Such dominant oncogenes are ideal targets, and identification of these will be critical for therapeutic advances in melanoma. The introduction of tyrosine kinase inhibitors into the clinic offers a rational approach to cancer therapy (Bennasroune et al., 2004).

Much interest surrounds recent reports of a new model for melanoma biology, where a slow-cycling population of JARID1B-expressing cells (putative stem cells) is required for continuous growth (Roesch et al., 2010). Studies in breast cancer suggest the presence of cancer stem cells expressing ERBB2, consistent perhaps with the good response to herceptin seen in some patients (Korkaya et al., 2008). It would be of interest to determine RTK expression in JARID1B positive and negative cell populations in melanoma.

Important questions concerning RTK biology remain unanswered. Is it better to target RTKs or components of their downstream pathways, such as PI3K, BRAF, and MAPK?   In cells containing multiple activated RTKs, how many receptors can or should be simultaneously inhibited? What period of time is required for treatment of tumours with PTK inhibitors? It was initially expected that melanomas containing mutations within RAS or BRAF would be insensitive to inhibition of RTK activity.   However, melanoma cells expressing mutant BRAF remain susceptible to down-regulation of IGF1R (Yeh et al., 2006). Current data indicate an important role for IGF signalling in melanoma cell survival (Kanter-Lewensohn et al., 1998; Karasic et al., 2010) and interactions between IGF1R and BRAF^{V600E} signaling have been described (Wajapeyee et al., 2010). Hence, a combined approach of both BRAF and specific RTK inhibitors may be a useful approach. With imatinib and PLX4032, the first targeted therapies for melanoma are in clinical practice. Selection of patients for entry into specific clinical trials is likely to improve response rates. The hope is that further PTK inhibitors, perhaps including inhibitors of kinases highlighted here, will become effective therapeutic agents.

Acknowledgements

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Table 1. RTKs in melanoma: expression changes, mutation, and activation in cultured cells

Family	RTK	Expression [a]	Mutation [b]	Phospho-Array [c]
AXL	AXL		(+)[d]	+
	TYRO3	↑R[e], ↓N[f]	(+)[d]	100
	MER			+
DDR	DDR2	↑N[g,f]		
EGFR	EGFR	↑I[h]	5%[d]	100
	ERBB2			53
	ERBB3	↑M, ↑I[i]	(+)[d]	↑26
	ERBB4		19%[j]	87
EPH	EPHA1			0
	EPHA2	↑N[k], ↑W[g,l], ↑I[g,m], ↑R[l]	(+)[d]	0
	EPHA3		(+)[d]	0
	EPHA4	↓W[n], ↓M	(+)[d]	+
	EPHA6		6.3%[j]	0
	EPHA7			0
	EPHB2		8.9%[j]; 5%[d]	93
	EPHB3	↑N[f] (94%)		
	EPHB4			0
	EPHB6	↓I[o], ↓R[o], ↓M	8.6%[j], 5%[d]	66
FGFR	FGFR1	↑R, ↑I[p,q]	(+)[r] 5%[d]	0
	FGFR2		10%[s]	33
	FGFR3			↑60
	FGFR4	↑N[g,f]		0
INSR	INSR			33
	IGF1R	↑I[t], ↑M		47
MET	MET (HGFR)	↑I, ↑W[u]	(+)[u], 5%[d]	0[v]
	MST1R (RON, MSP-R)		(+)[d]	33
MUSK	MUSK			0
PDGFR	PDGFRA	↑I[w] ↓M	5%[j]	+
	PDGFRB			0
	KIT	↓W[x,y], ↓N[x,z] ↑I[q,aa]	29%[bb], 18%[aa]	0
	FLT3 (FLK2)		(+)[d]	+
	CSF1R (MCSFR, FMS)	↑I[cc]		0
PTK7	PTK7 (CCK4)	↓N[n,f]		
RET	RET			+
ROR1	ROR1		(+)[d]	26
	ROR2			0
RYK	RYK		(+)[d]	
TIE	TIE1	↑N[g,f]	7.6%[j]	33
	TEK (TIE2)		(+)[d]	+
TRK	NTRK1 (TRKA)		2.5%[j], 5%[d]	+

Family	RTK	Expression [a]	Mutation [b]	Phospho-Array [c]
VEGFR	**NTRK2 (TRKB)**	↓ M	(+)[d]	0
	NTRK3 (TRKC)	↑ M	(+)[d]	0
	FLT1 VEGFR1)	↑ M[k]	10%[j]; (+)[d]	0
	KDR (VEGFR2)	↑ M[k], ↑N[f]	5%[d]	↑66
	FLT4 (VEGFR3)	↑ M[k]		+

HUGO terms are used as primary names, with common synonyms shown.

[a]Various methods have been used to determine RTK expression in melanoma cell lines, melanocytes and uncultured melanomas. Key: (I) immunohistochemistry, (M) microarray database / publicly available gene expression data (Oncomine, https://www.oncomine.org/resource/login.html), (N) northern blotting analysis, (R) RT-PCR and (W) immunoblotting. (↑) increase, (↓) decrease.

[b]Two large studies sequenced global RTKs in melanoma cell lines (Ruhe et al., 2007), and uncultured melanomas (Prickett et al., 2009). (+) indicates mutations detected in a small number of samples (<2%).

[c]Phospho-array analysis (this paper), where score indicates percentage of melanoma cell lines (grown with serum) containing an activated RTK; (+) indicates activity in less than 15% of samples, and 0 indicates no detectable activity; ↑ indicates activity in melanoma but not in normal melanocytes.

[d]Ruhe et al. (2007), [e]Zhu et al. (2009), [f]Easty and Bennett (2000), [g]Easty et al. (1995b; 1999), [h]de Wit et al. (1992; Sparrow and Heenan (1999), [i]Reschke et al. (2008), [j]Prickett et al. (2009), [k]Mehnert et al. (2010), [l]Margaryan et al. (2009), [m]Straume and Akslen (2002), [n]Easty et al. (1997), [o]Hafner et al. (2003), [p]FGFR1 was positive within stromal cells and at much lower levels in melanoma (Xerri et al., 1996), [q]Giehl et al. (2007), [r]Thomas et al., (2007), [s]Gartside et al. (2009) found loss-of-function mutations in FGFR2 in 10% of uncultured melanomas and melanoma cell lines, [t]Kanter-Lewensohn et al. (1998), [u]Puri et al. (2007), [v]Puri et al. (2007) found tyrosine residue 1003 of MET was phosphorylated in 21% of human melanomas, [w]Barnhill et al. (1996), [x]Lassam and Bickford (1992), [y]Natali et al. (1992), [z]Easty et al. (1993), [aa]Ashida et al. (2009), [bb]Curtin et al. (2006), [cc]Storga et al., (1992).

Table 2. Functional studies of RTKs in melanoma

RTK	Induced Change	Method	Outcome	Inferred Function	Reference
EGFR	↓	P	↓ Proliferation		(Djerf et al., 2009)
ERBB3	↓	si, Ab	↓ Proliferation ↓ Invasion		(Reschke et al., 2008)
ERBB4	↓	P, sh	↓ Proliferation	Oncogene	(Prickett et al., 2009)
EPHA2	↓	Si	↓ Proliferation, ↓VM, ↓Invasion	Oncogene	(Margaryan et al., 2009)
FGFR1	↓	An, DN	Apoptosis, ↓ Proliferation		(Valesky et al., 2002; Wang and Becker, 1997)
IGF1R	↓	An, Ab, si	Apoptosis, ↓ Proliferation		(Kanter-Lewensohn et al., 1998; Resnicoff et al., 1994; Yeh et al., 2006)
KIT	↑	WT	↓Tumorigenicity ↓Metastasis in xenografts	Tumour suppressor	(Huang et al., 1996)
KIT	↓	P	↓ Proliferation	Oncogene	(Ashida et al., 2009; Jiang et al., 2008)
MET	↓	P, si	Apoptosis, ↓ Proliferation ↑ Differentiation		(Puri et al., 2007)
PDGFRA	↑	WT	Apoptosis, Cell cycle arrest	Tumour suppressor	(Faraone et al., 2009)
TYRO3	↓	Sh	↓ Proliferation, ↓Chemoresistance ↓Tumorigenicity	Oncogene	(Zhu et al., 2009)

Various methods have been used to modulate RTK activity in melanomas and melanoma cell lines. Key: (↑) increased and (↓) decreased RTK expression. (An) antisense targeting, (Ab) antibody blocking of ligand binding, (P) pharmacological inhibition, (si) small interfering RNA, (sh) (short hairpin RNA, (DN) dominant negative RTK expression, (WT) wild-type RTK expression, (VM) vasculogenic mimicry.

Figure legends

Figure 1. Summary of signalling pathways from RTKs, with emphasis on those with known importance in melanoma cells. Arrows: upregulation; T-bars: downregulation. Red indicates pro-tumorigenic signaling and blue, tumor-suppressive signaling. Elements that are not clearly one or the other are shown in white (the MITF pathway can be either, depending on accompanying abnormalities). Rectangles indicate transcription factors. Dashed line: exact pathway unclear. Items often represent protein families rather than one protein (e.g. RTK, RAS, AKT, SRC); but TYRO3 is included individually with its MITF-specific signalling. Abbreviations mostly follow HUGO/NCBI conventions and can be found in the OMIM or Entrez Gene databases; however some exceptions have been made where the gene symbol is not familiar as a protein name, or not appropriate, as follows. 4EBP1, eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1); GPCR, G-protein coupled receptors; MTOR, mammalian target of rapamycin (FRAP1); PKC, protein kinase C family (PRKCA, -B etc); PLCγ, phospholipase Cγ (PLCG1). MAPK1 and 3 are also called ERK2 and -1, while STK11 is also called LKB1. For further explanation see text.

Figure 2. Summarized activity of 42 RTKs in 17 melanocytic cell lines. These were normal melanocytes (Nohm-1, 830c), RGP melanoma (WM35, WM1650, SGM2), VGP melanoma (ME1402, WM9, WM1341D, WM98.1, WM793, WM793P1), metastatic melanoma (1205LU, WM852, WM239A, WM1158, DX3, A375P) and a control NSCLC cell line (HCC827). All cells were grown in the presence of serum, which may have been the source of ligands for some RTKs. Signals with intensity greater than 2x SD above the mean intensity of 10 negative controls were scored as positive. Red shading indicates high, and green shading low or undetectable kinase activity.
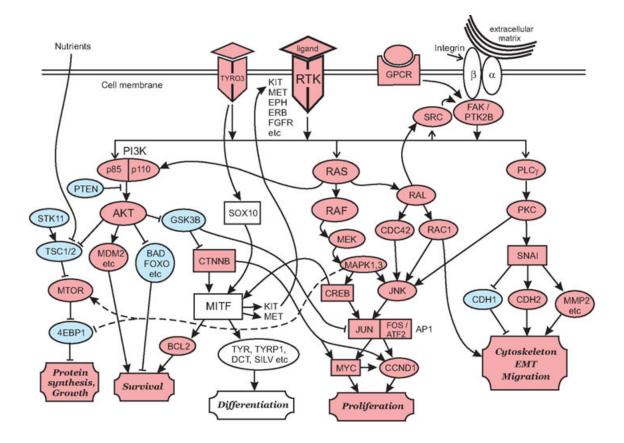
Figure 2

	Normal		RGP			VGP						Metastatic						
	830c	Nohm-1	SGM2	WM35	WM1650	ME1402	WM9	WM1341D	WM98.1	WM793	WM793P1	1205Lu	WM852	WM239A	WM1158	DX3	A375P	HCC827
EGFR	G	R	G	R	G	R	G	G	R	R	R	R	G	R	R	R	G	R
ERBB2	G	R	G	R	G	R	G	G	R	G	G	R	G	G	R	G	G	R
ERBB3	G	G	G	R	G	G	G	R	G	R	R	G	G	R	G	G	G	R
ERBB4	R	G	R	R	R	G	R	R	G	R	R	G	G	G	R	R	R	G
FGFR1	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
FGFR2	G	G	G	G	G	G	R	G	G	G	G	G	G	R	G	G	G	R
FGFR3	G	G	R	R	R	R	G	G	R	R	R	R	G	R	R	R	G	R
FGFR4	G	G	G	G	G	G	G	G	G	G	G	G	G	G	R	G	G	G
INSR	G	G	G	R	R	G	G	R	G	G	G	G	G	G	G	R	G	R
IGF1R	G	R	G	R	R	G	R	R	G	R	R	R	G	G	R	R	G	R
AXL	G	G	G	G	G	G	G	G	G	G	G	G	G	G	R	G	G	G
TYRO3	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	G	R
MER	R	G	G	G	G	G	G	G	G	R	G	G	G	G	G	G	G	R
MET	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	R
MST1R	R	G	G	G	R	G	G	R	G	R	G	G	G	G	R	G	G	R
PDGFRA	G	G	G	G	G	G	G	G	G	R	G	R	G	G	G	G	G	G
PDGFRB	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
KIT	R	R	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
FLT3	G	G	G	G	G	G	G	R	G	G	G	R	G	G	G	G	G	G
CSF1R	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
RET	G	G	G	G	G	G	G	G	G	G	R	G	G	G	G	G	G	R
ROR1	G	R	G	R	G	G	G	R	G	R	G	G	G	R	G	G	G	G
ROR2	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
TIE1	R	R	G	G	G	R	G	G	R	G	G	R	R	R	G	G	G	G
TEK	R	G	G	R	R	G	G	G	R	G	G	R	G	R	G	G	G	G
NTRK1	G	R	G	R	R	G	G	R	G	G	G	G	G	G	G	G	G	G
NTRK2	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
NTRK3	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
FLT1	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
KDR	G	G	R	R	G	R	G	R	G	R	R	G	G	R	R	G	R	G
VEGFR3	G	G	R	G	R	G	G	G	G	G	G	G	G	G	G	G	G	G
MUSK	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
EPHA1	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
EPHA2	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
EPHA3	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
EPHA4	G	G	G	G	R	G	G	G	G	G	G	G	G	R	G	G	G	G
EPHA6	R	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
EPHA7	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
EPHB1	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
EPHB2	R	R	R	R	R	R	R	R	R	R	R	R	G	R	R	R	R	G
EPHB4	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G	G
EPHB6	R	G	R	R	R	G	R	R	G	R	G	R	R	R	G	R	R	G