PATHOBIOLOGY IN FOCUS

Embryonic signaling in melanoma: potential for diagnosis and therapy

Luigi Strizzi^{1,2}, Katharine M Hardy¹, Gina T Kirsammer¹, Pedram Gerami^{2,3} and Mary JC Hendrix^{1,2}

As the frequency of melanoma diagnosis increases, current treatment strategies are still struggling to significantly impact patient survival. Some promise has been shown in treating certain melanomas by targeting activated signaling pathways resulting from specific mutations in proteins, such as BRAF and NRAS. Recently, the identification of embryonic signaling pathways in melanoma has helped us better understand certain biological characteristics, such as cellular heterogeneity and phenotypic plasticity, and has provided novel insight pertinent to diagnosis and therapy. For instance, our studies have shown that the TGF- β family member, Nodal, is expressed in melanoma and is responsible, at least in part, for tumor cell plasticity and aggressiveness. Since the majority of normal adult tissues do not express Nodal, we reason that this embryonic morphogen could be used to identify and target aggressive melanoma cells. We have also identified that molecular cross-talk between the Notch and Nodal pathways may represent a mechanism responsible for the over-expression of Nodal in melanoma. Further exploitation of the relationship between embryonic signaling pathways and cancer pathogenesis could lead to novel approaches for diagnosis and therapy in cancers, such as melanoma.

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INTRODUCTION

The incidence and mortality rates of melanoma have been increasing over the last few decades. The 2010 American Cancer Society reports the highest lifetime risk of developing melanoma at approximately 1 in 50 for Caucasians. Overall, it is the sixth most common cancer in men and seventh most common in women. While melanoma-related deaths also continue to increase, some significant breakthroughs in our understanding of the pathobiology of melanoma have resulted in promising new therapies.

Well-known risk factors for developing melanoma include prolonged exposure to ultraviolet radiation (UVR), increased number of melanocytic nevi, a family history of melanoma or a history of previous melanoma.¹ Somatic mutations are common in melanoma. Epidemiologists have long recognized that melanomas occurring on the head and neck of elderly patients are strongly associated with chronic UVR exposure from the sun. In fact, ~17% of these melanomas harbor KIT mutations, whereas melanomas occurring on the trunk, which are strongly correlated to intense, intermittent bouts of UVR exposure, have frequent mutations in BRAF or

NRAS. Acral melanomas that have little to no relation to sun exposure also have KIT mutations in ~23% of cases.² Of note is the recent case-control study, demonstrating the strong correlation between exposure to artificial UVR associated with indoor tanning and risk of melanoma—regardless of age when indoor tanning began.³ This provides clear evidence in support of classifying UVR as a human carcinogen.⁴

Recent research progress has helped categorize melanomas into subgroups with similar clinical, epidemiologic and molecular features, which may be susceptible to distinct targeted therapy, as will be discussed herein. Several models have been proposed describing melanoma as a linear process evolving from hyperplastic and dysplastic nevi to early and late stage melanoma. Clinicopathological data indicate a more complex reality with overlap of both biological and sometimes even morphological characteristics such that the diagnosis of benign dysplastic nevi and melanoma based on morphology alone can be difficult—even for experienced pathologists.⁵ Recent studies have suggested methods for improving the diagnosis of melanoma. For instance, multiple

¹Children's Memorial Research Center, Chicago, IL, USA; ²Robert H. Lurie Comprehensive Cancer Center, Northwestern University Feinberg School of Medicine, Chicago, IL, USA and ³Department of Dermatology, Northwestern University Feinberg School of Medicine, Chicago, IL, USA
Correspondence: Dr L Strizzi, MD, PhD, Children's Memorial Research Center, Robert H Lurie Comprehensive Cancer Center, Northwestern University Feinberg School of Medicine, 2300 Children's Plaza, Box 222, Chicago, IL 60614, USA.
E-mail: lstrizzi@childrensmemorial.org

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tissue marker arrays⁶ or fluorescence *in situ* hybridization DNA probes⁷ have claimed improved rates of discrimination of benign lesions from melanoma. Important prognostic factors, such as the depth of invasion of melanoma, are used to predict clinical outcome, with deeper (or thicker) lesions associated with reductions in overall patient survival.⁸ Other factors, including the presence of tumor ulceration or increased mitotic rates, also negatively affect prognosis.⁸

Current treatment strategies for advanced stage melanoma employing cytotoxic agents are often accompanied by serious side effects and associated with relatively low percentages of objective response rates.⁹ Generally, anti-cancer drugs appear to have some initial effect usually due to killing of the majority of cancer cells sensitive to the chemotherapeutic agent. However, as will be discussed further, increased signaling of cell survival pathways, enhanced DNA repair mechanisms or mutations of molecular targets in melanoma cells often lead to resistance to therapy. Similarly, molecular redundancy and cross-talk between multiple signaling pathways appear to undermine the efficacy in instances of targeted molecular biotherapy. Efforts continue at improving detection and quantification of chemoresistance, not only to determine the molecular mechanisms involved in drug resistance in melanoma, but also to help select those patients most likely to present resistance to standard treatment regimens and who may otherwise benefit from a more aggressive or alternative approach. Studies continue to increase our understanding of the biological processes of melanoma and offer the potential for improving current methods for diagnosis and therapy.

PLASTICITY AND MELANOMA

Aggressive melanoma tumor cells have been shown to express a plastic, multipotent phenotype similar to embryonic stem cells.¹⁰ However, unlike normal embryonic progenitors, these tumor cells lack major regulatory check points, resulting in the aberrant activation of embryonic signaling pathways, which underlies their plastic phenotype, unregulated growth, and aggressive behavior. Particularly noteworthy are the phenotypes expressed by melanoma cells that are associated with endothelial cells and human embryonic stem cells (hESCs). The respective molecular signatures associated with these phenotypes have been shown to demonstrate functional plasticity as well.¹¹ For example, in aggressive melanoma cells, an endothelial-like phenotype can exist and is characterized by the expression of vascular endothelial cadherin and the ability to engage in vasculogenic mimicry.¹¹ Vasculogenic mimicry contributes to the formation of an extravascular, fluid-conducting network in aggressive melanoma that represents an alternative mechanism for perfusing tumors. This can accompany co-option of preexisting vessels, mosaic vessels, angiogenesis, vasculogenesis and intussusceptive microvascular growth.¹² Most importantly, experimental studies have revealed the transendothelial functionality of human metastatic melanoma cells as they express anti-coagulant factors important for maintaining

patency of the vasculogenic mimicry perfusion network,¹³ in addition to participating in the neovascularization of circulation-deficient tissues.¹⁴ Indeed, vasculogenic mimicry is only one example of the plastic phenotype associated with aggressive melanoma cells, but this endothelial-like phenotype has been reported in many different tumor types¹⁵ as well as during normal tissue development.¹² It is tempting, therefore, to speculate that the formation of an extravascular perfusion pathway in aggressive tumors recapitulates an embryonic event—implemented by normal progenitor cells and cancer cells.

SIGNIFICANCE OF STEM CELL CHARACTERISTICS IN MELANOMA

Melanoma cells undeniably share features of plasticity and unlimited self-renewal with stem cells. Two features in particular, the ability to initiate new tumors and the potential to generate heterogeneous subpopulations of daughter cells, invite speculation as to the extent of behavioral overlap between melanoma cells and stem cells. Whether regulatory mechanisms, particularly those that might cue melanoma cells to adopt a more benign fate, may be shared as well is also of debate.

Unlike many solid tumors that appear to grow and derive heterogeneous daughter cells from an isolable population of cancer stem cells (CSCs), melanoma cells appear to reversibly generate stem-like cells through ‘phenotype switching’. By this process, melanoma cells can readily convert between a migratory, stem-like state that may be quiescent and resistant to therapy, and a proliferative state that creates tumor bulk, but cannot migrate or seed new tumors. Evidence for the phenotype switching model includes a recent study wherein a high proportion (at least 25%) of melanoma cells isolated from melanoma patients were able to initiate new tumors.¹⁶ In the same study, commonly used CSC markers did not identify a tumor-initiating melanoma cell. Finally, genes essential for seeding new tumors display dynamic transcription patterns in melanoma, turning on and off in different melanoma subpopulations, making it difficult to isolate a stable population of tumor-initiating cells.^{15,17} In many ways, these changes mimic the developmental fate of melanocyte stem cells, which are able to migrate and adopt new fates upon encountering the proper niche.

Intriguingly, and similar to normal development, phenotypic changes in tumor cells appear to be instigated by changes in the microenvironment that may cue tumor cells to switch from a more stem-like fate to a proliferative one, as in the generation of new metastases, or vice versa as tumors acquire the ability to migrate. As discussed later, unlike normal stem cells, tumor cells may bear mutations in key signaling pathways that prevent their normal response to environmental cues and lead to a dynamic, pro-tumor, reciprocal signaling relationship with the microenvironment. Understanding the basis of phenotype switching in melanoma will be critically important for designing therapies,

as any cell left behind may have the potential to relapse. Furthermore, treatment options must consider changes in the microenvironment that may activate signaling pathways important for the switch to a dormant, treatment-resistant, more stem-like fate.

SIGNAL TRANSDUCTION IN MELANOMA

While the mechanisms underlying melanoma growth are highly complex and generally not well delineated, a number of signaling pathways have so far been identified as important in melanoma disease progression and/or metastasis. One pathway currently receiving significant attention as a potential target for melanoma therapy is the mitogen-associated protein kinase (MAPK) pathway.^{18,19} MAPK signaling is normally activated by receptor tyrosine kinases that trigger a series of intracellular phosphorylation events, which converge on extracellular signal-related kinase (ERK). Activated ERK protein translocates to the nucleus where it phosphorylates and activates transcription factors that promote the transcription of genes including those involved in cell proliferation, survival and migration.¹⁹

A large proportion of melanomas are known to exhibit hyperactivated MAPK. The constitutive activation of MAPK signaling appears to be most typically associated with activating mutations in one of two upstream signaling molecules, NRAS and BRAF, but these are rarely found in the same tumor. The most common mutation is a single amino-acid substitution in the BRAF protein (V600E) that mimics an active protein conformation. This mutation is also present at high frequency in benign and atypical nevi, suggesting that activated BRAF alone is not sufficient to promote melanoma. However, BRAF mutations are more common in advanced stage than early stage lesions, and recent clinical trials with new targeted BRAF inhibitors (eg GSK2118436 and PLX4032) are showing promise in the treatment of patients with metastatic melanoma harboring the V600E, or, less common, V600K or V600D BRAF mutations.^{20,21} Additional clinical trials are utilizing pharmacological inhibitors to target molecules downstream of BRAF or NRAS, such as MEK (eg AZD6244 and GSK1120212) or phosphoinositide 3-kinase (PI3K) (eg GDC0941 and XL147).^{20,21}

One major issue with targeting the MAPK pathway with single-target inhibitors is the acquisition of drug resistance. Certainly, treatment of melanomas harboring BRAF mutations with selective BRAF inhibitor is ineffective in some patients and short lived in others.^{20,21} Studies in melanoma cell lines carrying the V600E BRAF mutation suggest a spectrum of mechanisms for the acquisition of resistance to BRAF or MEK inhibitors, resulting in the reactivation of MAPK signaling or activation of alternate signaling pathways. Such events may include a switch from BRAF to CRAF dependency,^{22,23} alternative receptor tyrosine kinase upregulation or acquired NRAS mutations,²⁴ CCND1 amplification,²⁵ basal or acquired AKT activation,^{26,27} or COT-mediated, RAF-independent MAPK activation.²³ Alterations in other

molecules may also contribute to melanoma progression, including c-KIT, MITE, PTEN, p53, β -catenin, BIM or CDKN2A (p16^{INK4A} and p14^{ARF}), which function in pathways regulating cell-cycle progression or apoptosis.^{18,19,27}

Cross-talk with the PI3K pathway that promotes cell proliferation and survival is certainly an important factor in some melanoma cases. NRAS can stimulate PI3K signaling as well as MAPK signaling, so activating NRAS mutations typically upregulate both AKT and ERK activity.²⁰ PTEN, a direct inhibitor of the PI3K signaling cascade, is often deleted or mutated in melanoma and is frequently coincident with BRAF mutations, causing deregulation of PI3K signaling and a resulting upregulation of AKT activity. Furthermore, AKT itself is amplified in some melanomas.^{26,27} As such, simultaneous targeting of both the MAPK/ERK and PI3K/AKT pathways may be necessary for efficacious melanoma treatment options.^{20,21} Certainly, in three-dimensional *in vitro* cultures, the growth and invasion of metastatic melanoma cell lines could be blocked by simultaneous inhibition of the MAPK and PI3K pathways, but not by targeting either one alone.²⁸

Additional pathways have been described to contribute to melanoma progression and/or metastatic behavior, underscoring the complexity of the signaling network that underlies melanoma development. One such pathway is Notch signaling. While expression of multiple Notch receptors is upregulated in melanoma compared with normal melanocytes, research has so far predominantly focused on Notch1 and, most recently, Notch4.^{19,29,30} Notch1 has been shown to exhibit oncogenic behavior, and can transform normal melanocytes and early-stage melanoma cell lines to a more aggressive phenotype both *in vitro* and *in vivo*, via mechanisms that include stabilization of β -catenin, and/or regulation of the MAPK/ERK and PI3K/AKT pathways.²⁹ Importantly, Notch1 had little effect on metastatic cell lines, suggesting it primarily functions in early transformation events. Notch4, on the other hand, is expressed in aggressive, metastatic cell lines but not in poorly aggressive cells, and expression correlates with advanced disease in human tissue samples.³⁰ Inhibition of Notch4 signaling in metastatic melanoma cell lines blocks cell growth, promotes cell death and limits aggressive cell behavior *in vitro*, at least partially via regulation of the expression of Nodal, a growth factor belonging to the TGF- β superfamily. Nodal, an embryonic morphogen not typically expressed in normal adult tissues, contributes to metastatic melanoma cell plasticity and tumorigenesis.¹⁰ Cross-talk between the Notch and Nodal pathways may offer other potential targets for melanoma therapy (Figure 1).

RE-EMERGENCE OF EMBRYONIC SIGNALING IN MELANOMA AND POTENTIAL FOR DIAGNOSIS AND TARGETED THERAPY

Previous studies have shown that it is possible to reprogram the melanoma tumorigenic phenotype by exposing melanoma cells to factor(s) present in the embryonic micro-

Figure 1 The Nodal signaling pathway in melanoma. **(a)** Classic Nodal signaling involves binding to a heteromeric complex composed of Cripto-1 and Activin type I (Alk4/7) and Type II (ActRIIB) serine threonine kinase receptors. Nodal activation of the Type I/Type II receptor complex induces phosphorylation and activation of Smad2/3/4. The activated Smad2/3/4 complex translocates to the nucleus where it activates the transcription of target genes, including Nodal itself. **(b)** Notch signaling involves the cleavage of the Notch intracellular domain (NICD) by activated γ -secretase (g-SC). NICD is then capable of binding to a multimeric activator complex (AC) and translocate to the nucleus where it regulates the expression of target genes. Recently, we have shown that Notch4 can positively regulate Nodal expression in aggressive human melanoma cells. **(c)** Nodal has been shown to control plasticity of melanoma cells for example during vasculogenic mimicry where melanoma cells acquire endothelial-like characteristics and organize to form vascular-like networks. Nodal expression in melanoma has also been associated with progression of melanoma from radial growth phase to vertical growth phase to metastatic spread.

environment.¹⁰ This suggests that melanoma cells may share some characteristics with stem cells that allow them to respond to cues from the embryonic microenvironment. In fact, melanoma cells can be reprogrammed when injected into the developing chick,¹⁰ suggesting that they are responsive to factors specifically expressed in the embryonic microenvironment. We have shown that expression of Nodal is reactivated in melanoma cells.³¹ Nodal commonly generates its signal by binding to and activating a heteromeric complex composed of the EGF-like glycoprotein co-receptor Cripto-1, and types I and II activin-like kinase receptors (Alk4/7 and ActRIIB, respectively).³² This ultimately leads to activation and nuclear translocation of the Smad 2/3/4

complex where it regulates expression of genes, such as those involved in stem cell maintenance and cellular plasticity, in addition to inducing its own expression (Figure 1). It is possible that a putative melanoma stem cell-like population may respond to the embryonic microenvironment via regulation of component(s) of the Nodal signaling pathway.

Work from our laboratory has described detectable levels of Nodal in melanoma cells in contrast to normal melanocytes, in which Nodal was not detected.³¹ The belief that Nodal expression is largely restricted to very early progenitor and reproductive cell types, and re-emerges during tumorigenesis is supported by online data banks, such as the Sage Anatomic Viewer of the Cancer Genome Anatomy Project (<http://cgap.nci.nih.gov/SAGE/AnatomicViewer>), which shows that Nodal expression is restricted to embryonic tissues, hESCs and cancer cells. In addition to being expressed in tumor cells, our studies indicate that Nodal expression positively correlates with melanoma tumor progression towards a metastatic phenotype.³³ In fact, immunohistochemical analyses have shown that Nodal protein is absent in normal skin and only rarely seen in poorly invasive radial growth phase (RGP) melanomas. This is in contrast to invasive vertical growth phase (VGP) melanomas and melanoma metastases where Nodal expression is detectable in up to 60% of cases.³³ A recent study has confirmed our initial findings of increased Nodal expression in progressive melanoma.³⁴ Collectively, these expression studies indicate that Nodal may prove to be a useful biomarker of melanoma progression from non-aggressive RGP to more aggressive VGP and metastasis.

Our work has also shown that downregulation of Nodal reduces melanoma plasticity and aggressive behavior. For instance, treatment of human metastatic C8161 melanoma cells with the compound SB431542, a general inhibitor of TGF- β /Activin/Nodal signaling, resulted in a reduction in tumor cell invasion through a defined extracellular matrix.³³ More specifically, treatment of C8161 cells with an anti-Nodal morpholino resulted in a dramatic decrease in Nodal protein expression and decreased tumorigenesis in Nude mice.³³ Using immunohistochemical staining for Ki67 as a measure of proliferation and terminal deoxynucleotidyl transferase biotin-dUTP nick-end labeling as a measure of apoptosis, we determined that inhibition of Nodal expression with an anti-Nodal morpholino decreases proliferation and increases apoptosis in orthotopic melanoma tumors.³¹

While Nodal is expressed in melanoma cells, Lefty, the natural inhibitor of Nodal, is not. This suggests that deregulation of Nodal expression in cancer may be the result of the absence of expression of this Nodal antagonist, which has been confirmed by our observation of complete methylation of Lefty in these tumor cells, resulting in its silencing.³⁵ We have shown that hESC-derived Lefty inhibits the expression of Nodal in melanoma and breast carcinoma cells as well as blocking cell clonogenicity *in vitro*.³¹ We also examined the effects of hESC-derived Lefty on palpable tumors formed by

C8161 cells injected orthotopically into Nude mice. We demonstrated that the tumors formed from Nodal-expressing human metastatic melanoma cells exhibited a decrease in tumor cell proliferation and increase in apoptosis when injected with hESC-derived Lefty, but not recombinant Lefty. The difference between the effects of recombinant Lefty and hESC-derived Lefty on melanoma cells appears to be related to differences in post-translational modification including the glycosylation state of hESC-derived Lefty.³¹ Most noteworthy, when C8161 metastatic melanoma cells were injected retroorbitally in Nude mice and allowed to colonize to the lung, mice injected intraperitoneally with a function blocking anti-Nodal antibody showed less evidence of macroscopic tumor cell colonies on the surface of the lung. This was compared with mice injected with an isotype control antibody whose tumor cell lung colonies were very evident macroscopically.³⁶ Moreover, melanoma cells in the lungs of mice treated with anti-Nodal antibody more often showed signs of cellular distress, such as cytoplasmic swelling and vacuolization, and apoptosis compared with mice treated with control IgG. More recently, we have shown that interference with Notch4 signaling via blocking antibodies or specific anti-Notch4 siRNAs leads to abrogation of Nodal expression and function in aggressive melanoma cells, suggesting the possibility of introducing Notch inhibitors to target Nodal in melanoma.³⁰ These findings implicate Nodal not only as a diagnostic or prognostic marker but also as a potential new therapeutic target.

FUTURE PERSPECTIVE

The plastic phenotype of aggressive melanoma has presented a significant challenge in the detection and targeting of tumor cells exhibiting stem cell-like characteristics. As the molecular signaling pathways underlying tumor cell plasticity become more transparent, our understanding of how to suppress this elusive phenotype will be enhanced. Indeed, we are making progress in identifying critical embryonic pathways, such as the Nodal signaling pathway, that re-emerge in aggressive tumor cells—in the absence of regulatory check points. Recognizing the importance of Nodal in the maintenance of hESC pluripotency, it is tempting to speculate that Nodal may represent a master plasticity gene in advanced forms of melanoma (and other cancers). Because Nodal is not expressed by the majority of normal adult tissues, and is overexpressed by aggressive tumor cells, it may represent a valuable new therapeutic target. Equally noteworthy is the molecular cross-talk between Notch (another stem cell signaling molecule) and Nodal, particularly with respect to the regulation of Nodal expression by Notch4. The implications of this significant new finding suggest that the small subpopulations of melanoma cells co-expressing Notch4 and Nodal may better define the stem cell phenotype of the most aggressive melanoma cells. Further studies are required to rigorously address this premise, and possibly add another viable target to the armamentarium of inhibiting aggressive

melanoma. Noteworthy additional options for developing and testing new therapies also include combinatorial multi-target treatments—to take advantage of molecularly based approaches to suppress the aggressive melanoma phenotype in individual patients.³⁷ Collectively, we have learned a great deal from studies that focus our attention on the convergence of embryonic and tumorigenic signaling pathways. At this intersection of normal development and carcinogenesis reside the clues to suppressing cancer formation and progression.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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