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Developmental Pathways Activated in Melanocytes and Melanoma

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Abstract

Cutaneous malignant melanomas originate primarily within epidermal melanocytic cells. Melanoma cells share many characteristics with melanocyte precursors, suggesting that melanoma cells utilize the developmental programs of their normal counterpart for their own progression. The pigmentation system provides an advantageous model to assess survival pathway interactions in the melanocytic lineage, as genetic alterations controlling melanocyte development can be easily detectable by coat color phenotype that do not affect the viability of an animal. By integrating combinatorial gene knockout approaches, cell-based assays and immunohistochemical observations, recent studies have illustrated several genes and pathways that play important roles both in melanocyte specification and maintenance and in melanoma formation and progression. We are reviewing those genes and pathways to understand the connection between normal and cancerous development and to reveal therapeutic potential of targeting developmental pathways for melanoma therapy.

Introduction

Melanocytes are pigment-producing cells, which are derived from neural crest cells and located at the basal layer of the epidermis, hair bulb, eyes, ears, and meninges.During embryonic development, multipotent trunk neural crest cells migrate from the neural plate to the epidermis and dermis, undergoing lineage specification to form melanocyte precursors - melanoblasts, which eventually generate differentiated melanocytes. Taking advantage of transgenic studies using mouse models as well as other species, a number of genes involved in cell lineage specification and melanocyte development have been identified and characterized. Cutaneous melanoma is the deadliest form of skin cancer, which arises from normal melanocytes or their precursors. Although remarkable advances in melanoma therapy were made recently with the approval of several new drugs against MAPK (mitogen-activated protein kinase) pathway, none of them are regarded as inducing cures in

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terms of targeted therapy. It has been known that tumor cells utilize the properties of their normal counterpart and the progenitors for their own progression. Indeed, the molecular and cellular mechanisms involved in proliferation and migration of melanoblasts during development and of melanoma cells during tumor progression are often closely related. The goal of this brief review is to dissect the signaling pathways operating during melanocyte development and melanoma tumorigenesis, thus providing complementary information.

Normal melanocyte development

Melanocytes are derived from neural crest cells (NCCs), which are highly migratory embryonic cells. After gastrulation, the neural crest is first induced at the border of the neural plate and non-neural ectoderm, and then delaminates from the region between the dorsal neural tube and overlying ectoderm upon neural tube closure. NCCs are initially multipotent but gradually become lineage-restricted in developmental potential, which is determined by where they migrate and settle. NCCs can give rise to a number of differentiated cell and tissue types including sensory neurons and glial cells, melanocytes, craniofacial cartilage and bone, and smooth muscle (Cichorek et al., 2013).

The development of a multipotent neural crest stem cell into a mature melanocyte involves the generation of melanoblasts (Mbs) - the precursors of pigmented melanocytes – from a bipotential glial-melanocyte lineage progenitor. After emerging from the neural crest, Mbs begin their journey to the skin by invading the dorsolateral pathway between the somite and the ectoderm. In the hair-covered body skin of mouse, Mbs travel through the epidermis to arrive at newly forming hair follicles. Upon localized within the follicles, Mbs segregate into two distinct subpopulations: differentiated melanocytes that reside within the hair matrix and contribute to hair pigmentation and melanocyte stem cells (MSCs) that localize in the bulge at the lower permanent portion of the hair follicle, and give rise differentiated melanocyte population for subsequent hair growth cycles. In contrast, in human skin, where hair follicles are relatively sparse, melanocytes also reside in the basal layer of interfollicular epidermis which is close to the dermal-epidermal junction, and respond to environmental cues from surrounding keratinocytes or others for differentiation / pigmentation (Cichorek et al., 2013; Bandarchi et al., 2013).

Melanoma progression

Malignant melanoma is an aggressive form of skin cancer and its incidence is increasing worldwide. Early-stage melanomas can be successfully treated mainly through surgical excision of the primary tumor lesion. However, advanced stage melanomas are difficult to treat once the disease has spread beyond the primary site to distant organs as most patients eventually develop resistance to currently available therapies.

The transformation of normal melanocytes into melanoma cells is often considered as a multistep process. The horizontal or radial growth phase is the first step towards the invasive phenotype, in which melanocytes undergo alterations that offer a proliferative and survival advantage (Herlyn et al., 1985). It is followed by a vertical growth phase, in which tumor cells deeply invade into the dermis/hypodermis. Metastatic melanoma cells may eventually break through the endothelium and travel to distant sites (Herlyn et al., 1985).

There exist some dominant genetic altering events in melanoma tumoregenesis. Constitutively activating BRAF and NRAS mutations are found in nearly 50% and 20% of melanomas, respectively. These mutations appear to be somatically acquired, as wild-type BRAF and NRAS are detected in normal tissues from melanoma patients. The most common T1799A point mutation in *BRAF* gene causes the V600E amino acid substitution, resulting in a 500-fold increase in inherent BRAF kinase activity that enhances cell division and survival (Wan et al., 2004). Notably, many human nevi harbor BRAFV600E or NRAS mutations, which suggests that *BRAF* and *NRAS* mutations are critical but not sufficient for melanoma formation (Pollock et al., 2003; Charbel et al., 2014). Another common genetic mutation in melanoma is the deletion of the CDKN2A locus, which encodes two tumor suppressor proteins, $p16^{INK4a}$ and $p14^{ARF}$. While $p16^{INK4a}$ is an inhibitor of the cyclin-dependent kinases CDK4 and CDK6 and prevents cell cycle progression, $p14^{ARF}$ functions as a positive regulator of p53. Deletions of the *CDKN2A* locus have been found in up to 50% of melanomas (Curtin et al., 2005).

Correlating with the mutational status in melanoma, RAS-RAF-MAPK signaling pathway is heavily upregulated in melanoma patients. Activation of PI3K/Akt signaling pathway also plays a significant role in melanoma development, frequently in the setting of concurrent activation of the MAPK signaling. On the other hand, deletion of CDKN2A and the associated signaling alterations also contributes to melanoma progression. Owing to space limitations and the focus of this review on pathways important for animal development, we are not going to provide a complete survey of the field, especially exciting progresses related to MAPK, PI3k and CDKN2A pathways.

Developmental pathways in melanocyte and melanoma development

Many genes involved in melanocyte development have also been implicated in melanoma progression. Several genetic pathways are highlighted as follows that regulate various steps in melanocyte development as well as melanoma progression.

Notch

The Notch signaling pathway is evolutionarily conserved in most multicellular organisms. Mammals possess four different notch receptors, Notch1-4, which are single-pass transmembrane receptor proteins. The Notch signaling cascade is triggered upon binding of membrane-bound ligand - Jagged or Delta - to the heterodimeric receptor through cell-cell interactions. Once activated, the intracellular region of the Notch receptor is cleaved through two sequential proteolytic events to release the active intracellular domain of Notch (NIC), which is subsequently translocated into the nucleus to generate a transactivation complex with the RBP-J transcription factor (Pinnix and Herlyn, 2007). The resulting transcriptional activation complex promotes transcription of various target genes, including members of the Hairy / Enhancer of Split (Hes) gene and Hairy / E(spl)-related with YRPW (Hey) gene families (Figure 1). These nuclear proteins antagonize the expression of lineage-specifying genes such as Ascl1, MyoD, Atoh1 and E2A, thus maintaining cells in undifferentiated state (Pinnix and Herlyn, 2007; Bedogni, 2014).

Notch proteins during melanocyte development

Using transgenic mouse models, a series of studies have shown that Notch signaling plays a vital role in the maintenance of Mbs as well as MSCs of the skin (Moriyama et al., 2006; Schouwey et al., 2007; Kumano et al., 2008) (Table 1A). To assess the role of Notch signaling in Mbs, Nishikawa and colleagues conditionally ablated the *RBP-J* gene in the melanocyte lineage using the *Tyr-Cre: RBP-J*$^{f/f}$ mouse model (Moriyama et al., 2006). The initial hair pigmentation defects along with premature hair graying in subsequent hair cycles were observed in these mice, which suggests a key role of Notch signaling in the maintenance of both embryonic Mbs and MSCs. This phenotype was reminiscent of the effect caused by pharmacologic inhibition of Notch signaling pathway using a γ-secretase inhibitor (GSI), DAPT, in which apoptosis of Mbs was initiated. Hair derived from DAPT-treated skin organ cultures was unpigmented. The function of Notch in melanocyte development was further supported by the generation of *RBP-Jκ*, *Notch1*, and *Notch2* conditional knockout mice (Schouwey et al., 2007). Conditional deletion of *Notch1* and *Notch2* alleles in the melanocyte lineage results in hair graying in a dose-dependent manner. Dispersed gray hair was detectable when two *Notch* alleles are floxed in *Tyr:Cre/°; Notch1*$^{flox/+}$*; Notch2*$^{flox/+}$, Tyr:Cre/°; Notch1$^{+/+}$; Notch2$^{flox/flox}$ and Tyr:Cre/°; Notch1$^{flox/flox}$; Notch2$^{+/+}$ mice, and pigmentation of hair was almost completely lost in the absence of both Notch1 and Notch2 (*Tyr:Cre/°; Notch1*$^{flox/flox}$*; Notch2*$^{flox/flox}$ mice). Interestingly, while epidermal melanocytes are eliminated in the melanocyte-specific Notch1 and Notch2 double-deficient mice, dermal and choroidal melanocytes are left unaffected. In another study, Notch signaling was manipulated through the oral administration of a GSI YO01027 to mice (Kumano et al., 2008). A gradual increase in gray spots was observed in YO01027-treated mice, which remained stable for at least 20 weeks after discontinuing the GSI. Furthermore, YO01027 treatment led to loss of melanocyte progenitors in the bulge/sub-bulge region of hair follicles. To further dissect the role of Notch signaling in various steps of melanocyte-lineage postnatal development, an *in vivo* tracking technique using *Dct-lacZ* reporter transgene was applied to directly visualize the entire set of melanocyte lineage cells in individual hair follicles (Aubin-Houzelstein et al., 2008). This technique allowed the researchers to reveal several unrecognized roles of Notch signaling in the melanocyte lineage, which include maintaining the immature status of Mbs, controlling proper location of Mbs, and preventing migration of differentiated melanocyte to ectopic locations outside the hair matrix.

Notch signaling in melanoma

The role of Notch signaling in melanoma progression has been investigated by a number of laboratories. While the expression levels of the Notch receptors are in general low or undetectable in mature melanocytes, increased expression of Notch receptors has often been found in human melanoma lesions and melanoma cell lines (Nickoloff et al., 2003; Hoek et al., 2004; Balint et al., 2005; Pinnix et al., 2009; unpublished data) (Table 1A). Notably, a gradually elevated expression pattern can be observed from nevi, primary melanoma to metastatic melanoma (Hendrix et al., 2002; Hoek et al., 2004; Balint et al., 2005; unpublished data). As all four different Notch receptors appear to be up-regulated in melanoma lesions (Nickoloff et al., 2003), a GSI was used to simultaneously inhibit all

Notch receptor pathways (Qin et al., 2004). When proliferating melanocytes or melanoma cells were exposed to GSI treatment, the melanocytes were arrested at the G2/M growth phase, but the melanoma cells underwent prompt apoptosis, which was mediated by up-regulation of BH3-only members Bim and Noxa. This finding is promising for therapy, as the pro-apoptotic response was selectively induced in melanoma cells and not in normal melanocytes.

Among the Notch family members, Notch1 is the best studied in melanoma. In addition to contributing to melanoma survival, Notch1 activation confers a metastatic phenotype to primary melanoma cells *in vivo* (Balint et al., 2005; Liu et al., 2006). Constitutive activation of Notch1 signaling by ectopic expression of the Notch1 intracellular domain enabled primary melanoma cells to present higher survival capacities when cultured as three-dimensional spheroids and gain metastatic potential *in vivo*. Such oncogenic effect is associated with beta-catenin signaling, as its expression was up-regulated following Notch1 activation and suppression of beta-catenin expression reversed Notch1-induced tumor growth and metastasis. Activation of Notch1 signaling is also capable of regulating the activities of the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)-Akt pathways in human melanoma cells. Moreover, Notch1 activation leads to an increase in tumor cell adhesion and up-regulation of N-cadherin expression. Another study highlighted the interactions between Notch1 signaling and the microenvironment that is required for melanomagenesis (Bedogni et al., 2008). Bedogni et al. has shown that Notch1 functions as an important effector of both hypoxia and Akt during melanoma development. Lack of Notch1 function sensitizes melanoma cells to hypoxia-induced cell death through apoptosis and inhibits cell proliferation partially through down-regulating cyclin D1, which collectively results in reduction in melanoma growth.

Although most of the studies indicating a role of Notch signaling in melanoma are focused on the investigation of Notch1 function, it is noteworthy that roles of the other Notch receptors in melanoma progression is emerging. Notch4 has been shown to play a vital role both in promoting cell proliferation and in regulating the aggressive phenotype of melanoma cell lines (Hardy et al., 2010). Notch4 also plays a role in vasculogenic mimicry and anchorage-independent growth in aggressive melanomas, which is due in part to Notch4 regulation of Nodal, a TGFβ superfamily member participating in embryonic stem cell maintenance. Both Notch1 and Notch2 are involved in proliferation and invasion in uveal melanoma (Asnaghi et al., 2012). Uveal melanoma cell lines with very high expression of Notch targets were sensitive to GSI treatment, which can be rescued by constitutive activation of Notch1 and Notch2. While suppression of canonical Notch activity using short hairpin RNA targeting Notch2 was also able to reduce tumor growth and invasion, constitutive Notch activation promoted uveal melanoma cell growth. The function of Notch3 in melanoma development is intriguing. In one study, a screen was performed to identify important mediators of melanoma-endothelial communication and Notch3 was found to be upregulated in melanoma cells co-cultured with endothelial cells, which activates Notch signaling in melanoma cells (Howard et al., 2013). Activated Notch3 signaling is associated with enhanced melanoma cell migration without affecting tumor cell growth. However, another study identified a novel function of Notch3 in senescence regulation and tumor

suppression (Cui et al., 2013). Expression of Notch3 was elevated in human cells undergoing senescence induced by various stimuli. While down-regulation of Notch3 led to a delayed onset of senescence, ectopic expression of Notch3 was sufficient to promote senescence and p21 expression. A significant reduction of Notch3 expression was observed in melanoma samples compared with normal tissues, and restoration of Notch3 expression led to activation of senescence and inhibition of cell proliferation. This apparent discrepancy reflects the complexity of Notch signaling in melanoma biology, which requires further investigations to elucidate Notch functions in various biological contexts.

Wnt

The Wnt signaling pathways are highly conserved from Drosophila to humans and are involved in a variety of cellular functions ranging from embryonic development, adult homeostasis to tumor progression. The Wnt family contains at least 19 members, which are secreted lipid-modified and cysteine-rich signaling glycoproteins with 350-400 amino acids in length. So far, three Wnt signaling pathways have been characterized: the canonical Wnt/beta-catenin pathway, the noncanonical Wnt/Calcium pathway, and the noncanonical planar cell polarity (PCP) pathway (Lim and Nusse, 2013; Lucero et al., 2010). All three Wnt signaling pathways are activated by the binding of a Wnt-protein ligand to target cells via a Frizzled (FZD) family receptor, which subsequently recruits Dishevelled (DVL) to the plasma membrane and induces a cascade of intracellular signaling events. The canonical Wnt pathway regulates transcription of a broad spectrum of target genes, the noncanonical PCP pathway leads to the regulation of cytoskeleton rearrangement, and the noncanonical Wnt/calcium pathway controls calcium levels inside the cell (Lim and Nusse, 2013; Lucero et al., 2010).

1) Canonical Wnt signaling

The signature of Wnt/beta-catenin signaling is β-catenin stabilization. Without Wnt ligand binding, beta-catenin level is restricted in the cytoplasm by a destruction multiprotein complex containing adenomatous polyposis coli (APC), Axin and glycogen synthase kinase 3 beta (GSK3β). β-catenin is phosphorylated by GSK3β, which leads to the ubiquitination of the protein and its degradation by the proteasome. Upon Wnt ligand binding to FZD and LDL receptor-related proteins, the function of the destruction complex is disrupted. Axin is translocated to the plasma membrane and forms a new complex with DVL, and GSK3β is phosphorylated and inactivated (Lucero et al., 2010; Larue and Delmas, 2006). As a consequence, β-catenin is allowed to accumulate and translocate to the nucleus, and subsequently induces a cellular signaling response via gene transduction along with the TCF/LEF transcription factors (Figure 2).

Wnt/β-catenin signaling in melanocyte development—Wnt1, 3a and 8 are the three main Wnt ligands involved in the canonical Wnt/β-catenin pathway. One of the earliest implications that Wnt signaling might be important for cell fate decision towards Mbs comes from the observation that Mbs are not present in Wnt1/Wnt3a double knockout mice (Ikeya et al., 1997) (Table 1B). Subsequent studies in zebrafish suggest that Wnt signaling modulates a lineage switch between pigment-cell fate and neural and glial cell fates through

regulation of β-catenin (Dorsky et al., 1998). Overexpression of β-catenin in NCCs promotes pigment cell formation as well as inhibits the formation of glial derivatives. Loss-of-function approaches were also made to study the function of β-catenin. Conditional inactivation of β-catenin led to a loss of Mbs (Hari et al., 2002). Using a transgenic mouse model, overexpression of Wnt1 in neural crest explant cultures led to expansion of Mbs in number and differentiation of Mbs to pigmented melanocytes, suggesting that Wnt signaling plays essential roles in directing melanocyte lineage specification (Dunn et al., 2000).

Wnt/β-catenin signaling in melanoma—The function of canonical Wnt signaling has been controversial in melanoma. There are currently not many studies in which Wnt proteins on their own have been directly implicated in human tumors. Instead, in many cases, other members such as β-catenin, axin and APC of this pathway are involved in tumorigenesis. An earlier study examined Wnt ligand production in nevi and melanoma, and reported the presence of high levels of Wnt2, Wnt5a, Wnt7b and Wnt10b in all nevi tested (Pham et al., 2003). However, there was no clear correlation between pattern of Wnt expression and depth of invasion in melanoma. You et al found that Wnt2 expression level was high in melanoma cell lines, and antibodies against Wnt2 inhibited β-catenin signaling and suppressed tumor growth in an *in vivo* xenograft model by inducing apoptosis (You et al., 2004). In contrast, in another study, B16 melanoma cells expressing Wnt3a exhibited decreased tumor size and decreased metastasis when implanted into mice (Chien et al., 2009). A genome-wide transcriptional profiling was also performed in lentiviral-transduced B16F1 cells to determine genes upregulated by the expression of Wnt3a, but not Wnt5a. In this analysis, genes that are implicated in melanocyte differentiation were identified, such as *MITF*, *Trpm1*, *Met*, *SOX9*, and *Kit*, several of which are down-regulated with melanoma progression. These findings suggest that Wnt3a may drive melanoma cells toward a more differentiated melanocytic cell fate.

Recently, an interesting study has suggested that Wnt signaling in melanoma cells regulates their interaction with the microenvironment by affecting their ability to recruit blood and lymph vessels (Niederleithner et al., 2012). Cell culture supernatants (SNs) of Wnt1(+) and Wnt1(−) melanoma were added to endothelial spheroids. SNs of Wnt1(−) melanoma cells, but not those of Wnt1(+) cells, induced lymphatic sprouts. Subsequent tests revealed that Wnt1 negatively regulates lymphangiogenesis by suppressing melanoma-derived VEGF-C expression.

The role of β-catenin signaling in melanoma development is intriguing. β-catenin has been shown to play dual roles in both promoting and preventing melanoma progression. Activation of β-catenin signaling seems to be involved in melanomagenesis. One study found that constitutive activation of Wnt/β-catenin signaling enhances cell growth of murine melanoma cells *in vitro* (Widlund et al., 2002). Genetic studies in transgenic mice have implicated this pathway as a promoter of melanoma development. One group has shown that co-expression of stabilized nuclear β-catenin with oncogenic NRas promotes melanoma formation *in vivo* and increased immortalization of melanocytes *in vitro* (Delmas et al., 2007). Along this line, by utilizing a conditional mouse model of melanoma that is based on melanocyte-specific Pten loss and the $Braf^{V600E}$ activating mutation, Damsky et al revealed the central role of β-catenin as a mediator of tumor progression and metastasis (Damsky et

al., 2011). In this Pten/Braf mouse model, increasing or decreasing β-catenin levels leads to enhanced or repressed metastasis formation, respectively. Consistent with this, multiple immunohistochemistry studies of human melanomas revealed that melanocytic nevi and many primary melanoma tumors have elevated levels of nuclear β-catenin. Interestingly, in contrast to this, in more malignant and metastatic melanomas, nuclear β-catenin levels appear to decrease (Kageshita et al., 2001; Maelandsmo et al., 2003; Bachmann et al., 2005; Chien et al., 2009). And down-regulation of Wnt/β-catenin signaling in patient tumors is associated with decreased patient survival. Recently, Biechele et al have shown that suppression of endogenous beta-catenin may be achieved through constitutive activation of BRAF by the V600E mutation (Biechele et al., 2012). These findings suggest that the consequences of Wnt/β-catenin signaling activation are complex and likely dependent on the biological context. While Wnt/catenin signaling may be required for the initiation of melanoma development or melanocyte transformation, additional research is needed to determine the consequences of down-regulation of this pathway in already established melanoma tumors.

2) Noncanonical Wnt signaling

The noncanonical PCP pathway and the noncanonical Wnt/calcium pathway do not involve β-catenin. The noncanonical PCP pathway uses NRH1, RYK, PTK7 or ROR2, with LRP-5/6 as its co-receptor. After a Wnt ligand binds FZD and the co-receptor, DVL is recruited and forms a complex with Dishevelled-associated activator of morphogenesis 1(DAAM1), which subsequently activates the small G-protein Rho and Rho-associated kinase (ROCK) to regulate cytoskeleton dynamics. DVL can also form a complex with Rac, which activates JNK and also leads to actin rearrangement. In the noncanonical Wnt/calcium pathway, upon Wnt ligand binding, FZD directly interfaces with a trimeric G-protein, which leads to the activation of either phospholipase C (PLC) orphosphodiesterase (PDE) to positively or negatively regulate calcium release, respectively.

Wnt5a is the best characterized Wnt ligand regarding its role in melanoma. Wnt5a was found to be upregulated in about half of the melanoma cell lines (Iozzo et al., 1995), and it was more highly expressed in late-stage aggressive melanomas (Bittner et al., 2000). As indicated by immunohistochemical analysis, detection of high Wnt5a in tumors was subsequently correlated with poor patient survival (Da Forno et al., 2008). Studies on Wnt5a in melanoma have focused largely on the role of this ligand in promoting cell motility and invasion in melanoma (Weeraratna et al., 2002; Dissanayake et al., 2007). Interestingly, while Wnt5a signals via the noncanonical pathway, it inhibits the Wnt/β-catenin pathway in a manner that is independent of GSK3β, but through the activation of the ubiquitin ligase SIAH2 (L. Topol et al., 2003). However, Grossman et al. showed that in a subset of melanoma cell lines, Wnt5a can cooperate with canonical FZD and LRP6 receptors to increase β-catenin signaling (Grossman et al., 2013). Thus, signaling of Wnt5a can be receptor-dependent in different contexts: in cases when ROR2 is predominant, Wnt5a might transduce the signal to degrade β-catenin; when LRP6 is dominant, Wnt5a might signal to activate β-catenin (Webster and Weeraratna, 2013).

Endothelins

Endothelins are a family of vasoactive peptides (Edn1, Edn2, Edn3) that bind to the G-protein coupled receptors Ednra or Ednrb (Sakurai et al., 1992). Ednra binds Edn1 and Edn2 with similar affinity and Edn3 with 1000-2000 fold lower affinity, whereas Ednrb binds all three isopeptides with similar affinity (Sakurai et al., 1992). Binding of endothelins to either receptor leads to a cascade of signaling transduction events via heterotrimeric G-proteins (Figure 3). In melanocytes, upon binding of Edn1 and Edn3 to Ednrb, a number of pathways are activated including protein kinase C (PKC), mitogen-activated protein kinase (MAPK), Raf-1 and p90 ribosomal S6 kinase (Bohm et al., 1995; Imokawa et al., 1996).

Endothelins and melanocyte development

Endothelins play significant roles in neural crest and melanocyte development. Edn3 and Ednrb were first found to play a major role during the development of melanocytes from NCCs using genetic knockout mouse models (Baynash et al., 1994; Hosoda et al., 1994). Deletions or mutations of Ednrb or Edn3 in mice resulted in an almost complete loss of melanocytes (Table 1B). A series of subsequent studies dissected the roles of endothelins in different stages of melanocyte development.

Genetic disruption of the Ednrb gene caused a drastic reduction in the number of Mbs by embryonic day 12.5 (E12.5), which suggests that Ednrb signaling affects melanocyte development prior to or at E12.5 (Lee et al., 2003). Shin et al further determined the time window of E10-E12.5 for the function of Endrb in pigmentation by temporally manipulating the expression of the Ednrb gene through a tet-inducible system (Shin et al., 1999). As this time frame corresponds to the period when Mbs have entered the migrating stage, it suggests that Ednrb is required for the survival and migration of Mbs, but not for the earlier stages of Mb specification.

Several *in vitro* studies in both avian and mouse models have provided evidence that endothelins also function in promoting proliferation and self-renewal of neural crest precursors as well as the committed Mbs (Reid et al., 1996; Lahav et al., 1998; Dupin et al., 2000; Real et al., 2006). In quail, initial addition of Edn3 promotes NCC proliferation via the activation of Ednrb. In contrast, prolonged addition of Edn3 induces melanocyte differentiation via activation of Ednrb2 (Lahav et al., 1998). Edn3 treatment was able to stimulate proliferation of clonal cultures of pigmented melanocytes (Dupin et al., 2000), promote their de-differentiation into cells that restore the expression of early NCC markers such as Sox10, FoxD3, Pax3 and Slug (Real et al., 2006), and even led them to trans-differentiate into glial marker-positive cells (Dupin et al., 2000). A role of Edn3 as a proliferation promoter for committed melanocyte precursors was revealed by exposing murine NCC cultures to Edn1 or Edn3, which led to an increase of Mb number via enhanced cell proliferation (Reid et al., 1996).

In vitro studies as well as *in vivo* observations suggest the function of Ednrb signaling in melanocyte differentiation following the initial specification of NCCs into Mbs. In the avian model, although inhibition of differentiation was observed upon the initial exposure of NCCs to Edn3, prolonged exposure to Edn3 was found to induce melanocyte differentiation

as shown by an increase in the population of pigmented cells (Lahav et al., 1998). Along this line, although Dct-positive cells are present in murine NCC cultures in the absence of Edn3, these cells fail to produce pigment unless they are treated with endothelins or PKC activators (Opdecamp et al., 1998).

Collectively, these studies imply that the endothelin signaling pathway is involved in various aspects of melanocyte biology, from the very early developmental stages to the final differentiation steps. Future investigations are needed to elucidate the molecular mechanisms of how endothelins are mechanistically regulated to fulfill their multiple functions in melanocyte development.

Endothelins and melanoma

Ednrb is upregulated in most melanoma cell lines (Bittner et al., 2000; Ross et al., 2000) (Table 1B). Ednrb is considered an indicator for melanoma progression, as its expression is enhanced in metastatic melanoma compared to primary tumors (Demunter et al., 2001). Consistent with the increased Ednrb level, aberrant Edn3 expression was observed in metastatic melanoma cells in cell culture and tissue biopsies. And Edn3 treatment enhanced the survival of these cells *in vitro* (Tang et al., 2008).

Edn1 and Edn3 can function through Ednrb to alter tumor–host interactions that lead to the progression of cutaneous melanoma. Edn1 and Edn3 induced the down-regulation of E-cadherin and associated catenin adhesion proteins in melanoma cells. On the other hand, Edn1 and Edn3 also upregulated N-cadherin in human melanoma cell lines, which promotes release from keratinocyte control on melanoma cells and the formation of heterotypic cell–cell interactions (Bagnato et al., 2004; reviewed in Li et al., 2002). Edn1 and Edn3 treatments also resulted in the impairment of intercellular communication through phosphorylation of connexin 43, a gap junctional protein (Bagnato et al., 2004). Edn1 and Edn3 were also found to induce the expression of the transcription factor HIF-1α, which in turn leads to the up-regulation of vascular endothelial growth factor (VEGF), a correlation with poor prognosis in human melanomas (Spinella et al., 2007).

Ednrb-mediated signaling may drive melanoma cell invasiveness in various ways, including by up-regulating *SNAIL* expression at the transcriptional level (Bagnato et al., 2004). Ednrb signaling has also been shown to cooperate with *BRAF* activation. Edn1 partially rescued the defect in colony formation and cell proliferation in melanoma cells with the knockdown of oncogenic BRAFV600E (Christensen and Guldberg, 2005).

As shown by several studies, the Ednrb antagonist BQ788 as well as the Ednrb small interfering RNA (siRNA) are able to block the function of endothelins in melanoma growth and progression (Bagnato et al., 2004; Spinella et al., 2007). BQ788 was found to inhibit the growth of human melanoma cell lines, enhance pigmentation of the cell and alter cell morphology to dendritic shape that resembles mature melanocytes. In addition to promoting cell death in culture, this inhibitor was also capable of reducing human melanoma tumor growth in nude mice. Another Ednrb inhibitor, the small molecule A-192621, was also found to significantly inhibit melanoma cell growth in nude mice (Bagnato et al., 2004).

SOX Proteins

The SOX family consists of approximately 20 transcription factors that all share a similar high-mobility-group (HMG) domain (Schepers et al., 2002). SOX proteins mediate sequence-specific DNA binding through their HMG domain (Harley et al., 1994). In mammals, SOX proteins can be divided into nine groups of: A, B1, B2 and C-H (Schepers et al., 2002). The SOXE group includes SOX9 and SOX10, which are developmental regulators of melanogenesis. Here, we will focus on the functions of SOX9 and SOX10 in melanocyte development and melanoma.

SOX proteins in melanocyte development

Despite the similarities between SOX9 and SOX10 and their overlapping expression patterns, the necessity of each during embryogenesis varies. While SOX9 plays an essential role in neural crest generation as well as gonad and chondrocyte development, SOX10 is required for the maintenance and differentiation of NCCs as they become migratory (reviewed in Guth and Wegner, 2008). As loss of SOX9 or SOX10 is associated with an almost complete lack of NCCs and even causes early embryonic lethality in mice, their functions throughout Mb development have not been fully determined. However, through loss-of-function studies using animals carrying SOX10 mutations, a critical role for Sox10 in the establishment of the melanocyte lineage has been indicated by the exhibited pigmentary defects of these animals (Kellerer et al., 2006). On the other hand, evidence from human cell culture suggests that after the establishment of the Mb, SOX9 plays a similar role in melanocyte differentiation in the adult organism (Cook et al., 2005; Passeron et al., 2007). The expression level of SOX9 is upregulated as Mbs transition to a more melanocytic state (Cook et al., 2005). In contrast, SOX10 is highly expressed in Mbs, but is downregulated as Mbs differentiate into melanocytes (Cook et al., 2005) (Table 1B).

Sox proteins in melanoma progression

Some recent studies have focused on assessing potential roles for SOX9 and SOX10 in melanomagenesis. Based on expression levels, it seems that there exists an antagonistic relationship between SOX9 and SOX10 in melanoma development (Table 1B). SOX10 is present in 31% of melanocytic nevi, 43% of primary melanoma and 50% of metastatic melanoma (Bakos et al., 2009; Passeron et al., 2009). Conversely, SOX9 expression is observed in a high percentage of nevi (75–100% and 83.9% by individual studies), but decreased moderately with progression of melanoma (Passeron et al., 2009; Rao et al., 2010). Consistent with its down-regulation in more aggressive melanoma, overexpression of SOX9 in melanoma cell lines inhibited tumorigenicity both in mice and in a human *ex vivo* model of melanoma (Passeron et al., 2009). In contrast, as indicated by several studies, SOX10 appears to be oncogenic. It was reported that SOX10 represents a promising target for the treatment of congenital nevi and melanoma in patients (Shakhova et al., 2012). In this study, a mouse model for giant congenital naevi was established, which showed that nevi and melanomas prominently express SOX10. Strikingly, SOX10 haploinsufficiency counteracts Nras (Q61K)-driven congenital nevus and melanoma formation without affecting the physiological functions of neural crest derivatives in the skin. In another study, melanoma cell lines required wild-type SOX10 expression for proliferation and SOX10

haploinsufficiency reduced melanoma initiation in a transgenic mouse model (Cronin et al., 2013). Stable SOX10 knockdown in human melanoma cells resulted in arrested cell growth, altered cellular morphology and induction of cellular senescence (Cronin et al., 2013). Inhibition of SOX10 was also found to cause a significant reduction in invasive capacity both in human melanoma cell lines and the chick embryo model (Graf et al., 2014). Interestingly, SOX10 appears to play a role in reversible and adaptive resistance to BRAF(V600E) inhibition in melanoma. SOX10 suppression caused activation of TGF-β signaling, thus leading to up-regulation of EGFR and PDGFRB, which confer resistance to BRAF and MEK inhibitors (Sun et al., 2014).

These studies suggest that while high levels of SOX10 are correlated with a proliferative and transformative cell phenotype, higher SOX9 expression results in a less proliferative, more differentiated cell phenotype. More investigations are needed to elucidate whether SOX9 and SOX10 function independently or in combination during melanoma progression.

MITF

Microphthalmia-associted transcription factor (MITF) is a melanocyte lineage-specific gene that particularly determines melanocyte development and maintenance. Loss of MITF results in an almost complete absence of melanocytes in mice (Lister et al., 1999). MITF is required for Mb survival as well as melanocyte specification (Hornyak et al., 2001; Widlund et al., 2003). It is regulated transcriptionally by several other genes to support melanocyte development. β-catenin / LEF binding sites are found in the *MITF* promoter, which also contains multiple target sites for other transcription factors, such as SOX10, PAX3 and the cyclic-AMP responsive factor CREB (Takeda et al., 2000; Bondurand et al., 2000; Potterf et al. 2000). In summary, a variety of signals are converged to regulate *MITF* expression and activity, which in turn specify melanocyte fate.

MITF is overexpressed in many melanomas and it is thought to be an important player in melanoma biology (Garraway et al., 2005). MITF is proposed to function via a “lineage addiction” mechanism, whereby it controls many aspects of the phenotypic expression of the melanocytic lineage and drives cell transition to a non-invasive stage.

Conclusions and future perspectives

The studies described above have shown that genes and signaling pathways essential to the proliferation and migration of Mbs during melanocyte development often contribute to tumor progression during melanoma formation. Clinical challenges in treating melanoma still remain, and effective treatments for patients with metastatic melanoma are still lacking. Considering the extensive effects of Notch, Wnt, Endothelin and SOX10 in melanoma development, manipulation of these pathways would definitely have great therapeutic value. Indeed, there exist several antagonists targeting some molecules of those pathways. However, therapeutic efficacy is still lacking when applying them in the clinics. Therefore, future directions include discovering the right proteins and pathways to target in melanoma, and developing therapeutic approaches that more efficiently treat this disease.

Studies in model systems can be used to address these issues. Genetic screens have been important in connecting novel genes to an interesting phenotype and can be used to identify potential enhancers or suppressors of melanoma. RNAi screens are also powerful tools for loss-of-function genetic analysis in cells and model animals to identify novel genes and pathways involved in melanoma progression. Chemical screens can be applied to identify compounds that may target melanoma or prevent melanoma formation. Though significant achievements have been made in understanding melanoma formation, further study of the genes and pathways involved in melanocyte development will be needed to develop better clinical therapies for melanoma cure.

Acknowledgments

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Highlights

- Melanocyte development and melanoma progression share similarities.
- Developmental genes critical for melanocyte and melanoma development are described.
- Several genetic pathways are highlighted including Notch, Wnt, Endothelins and Sox.

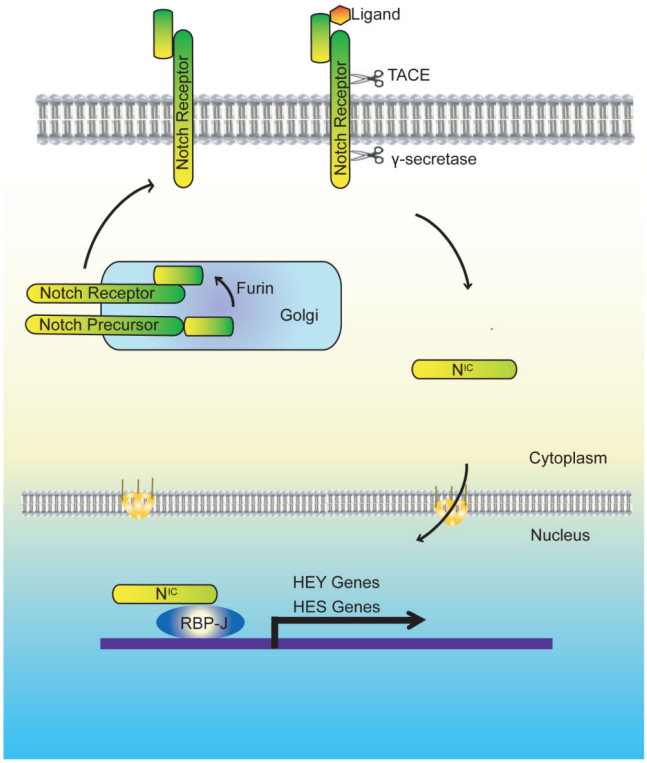

Figure 1. Overview of Notch signaling pathway

The Notch receptor is translated as a full-length protein but it is cleaved in the trans-Golgi network into two subunits by furin-like convertases. After transport to the membrane, these subunits form the heterodimeric Notch receptor. The Notch signaling cascade is initiated upon binding of membrane-bound ligand (Jagged 1,2 and DSL 1,3,4) to the Notch receptors. Upon ligand binding two sequential proteolytic events occur to liberate active Notch Intracel- lular (NIC). The first cleavage is mediated by a metalloprotease, TNF-a-converting enzyme (TACE). The final cleavage is mediated by a γ-secretase complex. Subsequently, NIC translocates to the nucleus and functions as a transcription factor to influence gene expression including HES, Hey and other targets.

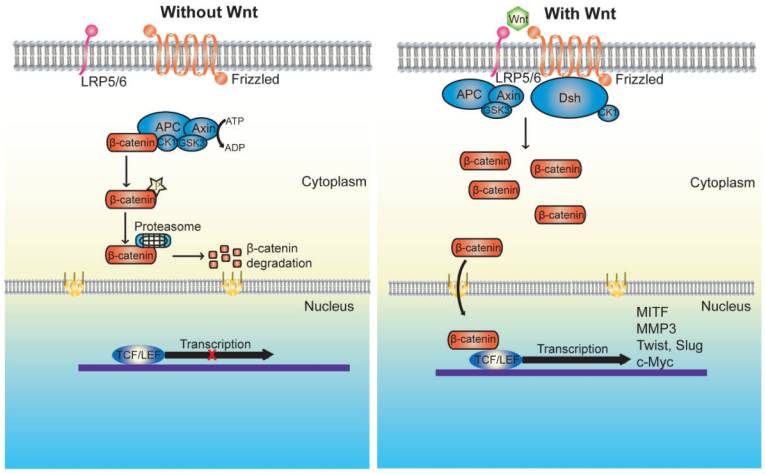

Figure 2. Canonical Wnt signaling

Wnt signaling is activated upon ligation of Wnt proteins to their respective dimeric cell surface receptors composed of the seven transmembrane Frizzled proteins and the LRP5/6. In the absence of Wnt ligand, β-catenin is not accumulating in the cytoplasm, as it is degraded by a destruction complex including Axin, adenomatosis polyposis coli (APC), protein phosphatase 2A (PP2A), glycogen synthase kinase 3 (GSK3) and casein kinase 1α (CK1α). In the presence of Wnt ligand, the cytoplasmic protein disheveled (Dvl) is recruited to the plasma membrane, phosphorylated and activated there. Activation of Dvl causes the dissociation of GSK-3β from Axin and leads to the inhibition of GSK-3β. Subsequently, the destruction complex is disrupted, and the phosphorylation and degradation of β-catenin is inhibited. Stabilized β-catenin then translocates into the nucleus leading to changes in different target gene expression.

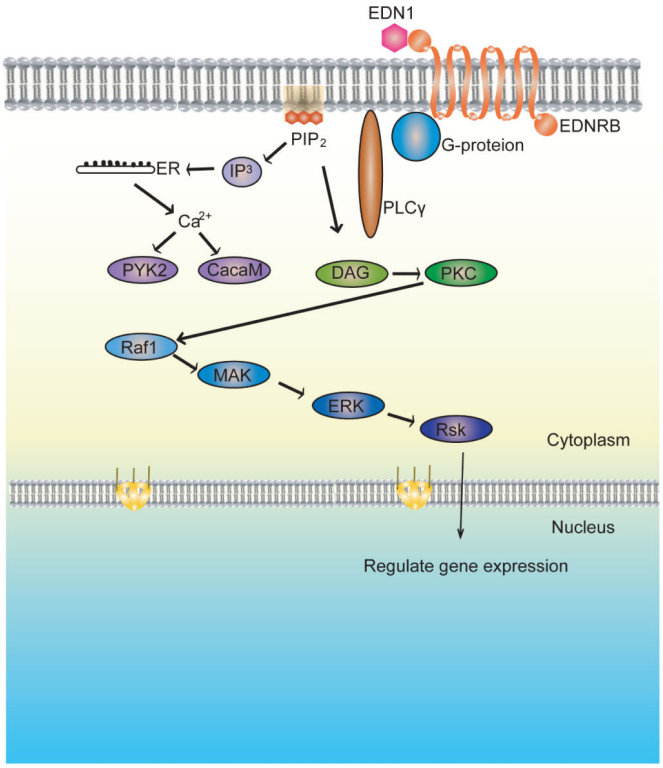

Figure 3. Endothelin signaling pathway

Binding of endothelins to their receptors initiates a series of intracellular signal transduction events via heterotrimetic G-proteins. Activation of Ednr leads to the activation of phospholipase Cβ, inhibition of adenyl cyclase, activation of plasma membrane Ca2+ channels, and activation of non-receptor tyrosine kinases. In melanocytes, binding of Edn1 or Edn3 to Ednrb causes the activation of the pathways that include protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and Raf-1.

Table 1

A

	Melanocyte Fate					Expression in human MC	Expression with melanoma prpgression			
	RBP-J KO mice	GSI / DAPT	GSI/YO01027	KO mice			MC	NV	PM	MM
Notch1	Loss of pigmentation (no MC)	Loss of pigmentation (no MC)	Loss of pigmentation (no MC)	Hair Gray (low MC)	Hair white in Notch1 −/−Notch2−/− double KO mice (No MC)	low	Increasing			
Notch2				Hair Gray (low MC)		–	Increasing			
Notch3				NA		–	?			
Notch4				NA		low	Increasing			

B

	MC fate in KO mouse model	Expression in human MC	Expression with melanoma prpgression			
			MC	NV	PM	MM
Wnt1	no Mbs in Wnt1−/− and Wnt3a−/− double KO mice	+	Decreasing			
Wnt3a		+	Decreasing			
Ednrb	Loss of pigmentation (no mc)	+	Increasing			
Edn1	NA	NA	NA			
Edn3	Loss of pigmentation (no mc)	NA	Increasing			
SOX9	complete loss of trunk NCCs	+	Decreasing			
SOX10	complete absence of NCCs	−	Increasing			

RBP-J: A transcription factor that is associated with Notch to generate the transactivation complex, which initiates transcription of target genes.

DAPT: N-[N-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester, a gamma-secretase inhibitor (GSI).

YO01027: a gamma-secretase inhibitor (GSI).

MC: melanocyte; KO:knockout; NV: nevi; PM: primary melanoma; MM: metastatis melanoma; Mbs: melanoblasts; NCCs: neural crest cells; NA: not available.