Journal of Dermatological Science 75 (2014) 100–108

Contents lists available at ScienceDirect

Journal of Dermatological Science

journal homepage: www.jdsjournal.com

The roles of Frizzled-3 and Wnt3a on melanocyte development: In vitro studies on neural crest cells and melanocyte precursor cell lines

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ARTICLE INFO

Article history:
Received 10 October 2013
Received in revised form 13 April 2014
Accepted 21 April 2014

Keywords:
Wnt3a
Frizzled-3
Neural crest
Melanocyte precursor
Melanoblast

ABSTRACT

Background: Wnt3a and Frizzled-3 are both expressed in the dorsal neural tube that gives rise to the neural crest in *Xenopus*, zebrafish and mice. Melanocytes originate from the neural crest (NC) and postnatally, melanocyte stem cells reside in the hair follicle bulge and in the dermis. However, the roles of Wnt3a and Frizzled-3 in melanocyte development have not been clarified.
Objective: The aim of this study was to delineate the expression of Frizzled-3 in murine melanocyte lineage and human melanocytes, and to study the effects of Wnt3a on melanocyte development at various stages.
Methods: Murine NC explant cultures and three NC-derived melanocyte lineage cell lines, including NCCmelb4M5 (Kit^- melanocyte precursors), NCCmelb4 (Kit^+ melanoblasts) and NCCmelan5 (differentiated melanocytes), and human epidermal melanocytes were treated with pure recombinant Wnt3a protein and their cell behaviors were analyzed including their proliferation, Kit expression, tyrosinase (Tyr) activity, melanin production, dendrite formation and migration.
Results: Frizzled-3 was expressed in Tyr-related protein (TRP)-1^+ cells in NC explant cultures, in all 3 melanocyte precursor cell lines and in human melanocytes. Wnt3a increased the population of TRP-1^+ cells, the number of L-3,4-dihydroxyphenylalanine (DOPA)$^+$ cells and dendrite formation in NC explant cultures. Wnt3a stimulated the proliferation of all 3 melanocyte precursor cell lines in a dose-dependent manner and also stimulated human melanocyte proliferation. Moreover, Wnt3a increased Tyr activity and melanin content of differentiated melanocytes, but did not activate Tyr activity in melanoblasts. Wnt3a stimulated dendrite formation in differentiated melanocytes, but not in melanoblasts. Wnt3a did not affect melanoblast or melanocyte migration. Wnt3a did not induce c-Kit expression in Kit^- NCCmelb4M5 cells and did not affect c-Kit expression in any cell line tested.
Conclusions: Frizzled-3 is constitutively expressed in murine melanocyte precursors, melanocytes and human melanocytes. Wnt3a and Frizzled-3 signalings play important roles in regulating the proliferation and differentiation of murine NCCs and various developmental stages of melanocyte precursors. The effect of Wnt3a on human melanocytes is similar to its effects on murine melanocytes. Therefore Wnt3a/Frizzled-3 signaling is a promising target for human melanocyte regeneration.

1. Introduction

Melanocytes are generated either directly from the neural crest (NC) (in the mouse at approximately E9) or indirectly from nerve cells (around at E11). Transcription factors, such as Sox10, Pax3, FoxD3 and Mitf, participate in a genetic network regulating melanocyte formation from the neural crest. The activity of these

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http://dx.doi.org/10.1016/j.jdermsci.2014.04.012
0923-1811/

intrinsic factors is controlled and modulated by extracellular signals including canonical Wnt, Edn, Kitl, and other signals that remain to be identified [1]. In the trunk region of the mice, founder melanoblasts are determined around E8.5–E9.5. Precursor melanoblasts arising from founder melanoblasts can be visualized from E10.0. The melanoblasts begin to migrate from the migration staging area (MSA) through the dermis along a dorsolateral pathway underneath the ectoderm from E10.5, the first wave melanocyte in skin. From E11, the second wave dermal melanoblasts appear which are generated from the neural precursors (with neural crest stem cells) migrating along the ventral pathway. On E13.5, melanoblasts migrate from the dermis to the epidermis where they continue to proliferate and migrate actively [2]. Melanoblasts that enter developing hair follicles around E15.5 and E16.5 appear to survive without a Kit signal until they are reactivated upon initiation of the first wave of the hair cycle after birth [3,4]. Using the Dct-LacZ transgenic mice for melanocyte lineage tracing, melanocyte stem cells responsible for melanocyte regeneration during hair cycle are identified in the bulge area [5], which are a c-Kit-negative population [6]. Using double transgenic Wnt1-Cre/R26R mice, neural crest cells (NCCs) can be traced in adult animals by detecting β-galactosidase expression. β-galactosidase-positive cells can be identified in basal layers of the outer root sheath from the hair bulge to the matrix at the base of the hair follicle, which are possible melanocyte precursors or more primitive NC progenitors. Hair bulge explant cultures give rise to pluripotent NCCs, which can differentiate into neurons, smooth muscle cells, Schwann cells, chondrocytes and melanocytes [7]. Stem cells with NC characteristics can be derived from the bulge area of cultured human hair follicles and dermis-derived spheres, and can give rise to myogenic, melanocytic and neuronal cell lineages [8,9]. Thus, the bulge area of adult hair follicles and dermis are reservoirs of pluripotent NC stem cells. Factors guiding the development of the melanocyte lineage in the NC may play important roles in the development of melanocyte stem cells that reside in the bulge area and dermis.

Most mammalian genomes, including the human genome, harbor 19 Wnt genes, which fall into 12 conserved Wnt subfamilies. Wnt proteins are ~40 kDa in size and contain many conserved cysteine residues. When interacting with target cells, Wnt proteins bind a heterodimeric receptor complex, which consists of a Frizzled and an LRP5/6 protein. The 10 mammalian Frizzled proteins are seven-transmembrane receptors and have large extracellular N-terminal cysteine-rich domains that provide a primary platform for Wnt binding. The Wnt–Frizzled interaction is promiscuous: a single Wnt can bind multiple Frizzled proteins and vice-versa [10]. Wnt genes play crucial roles in the early steps of NC formation including the induction, maintenance of presumptive NC fate and proliferation of NC progenitors, and later steps of specification, proliferation and migration of differentiated NCC types [11–13].

Melanoblast specification from NC precursors is governed primarily by Wnt and BMP signaling molecules; BMP can suppress both Wnt-induced sensory neurogenesis in mouse [14] and Wnt-induced melanocyte generation in quail neural crest cells [15]. Wnt/β-catenin plays a dual stage-dependent role in neural crest stem cell lineage decisions: at an early stage (presumably in the premigratory neural crest), canonical Wnt controls sensory neurogenesis; at a somewhat later stage, it might regulate melanocyte formation, although this remains to be confirmed [2]. Wnt signals that influence NC formation and melanocyte lineage specification act through the stabilization of β-catenin and its regulation of transcription by binding to Tcf/Lef transcription factors [13]. Wnt1 and Wnt3a are expressed in the dorsal neural tube at the specific time and site of NC formation [16]. In Wnt1- and Wnt3a-deficient mouse embryos, melanocyte precursors are markedly deficient [11]. Wnt3a-conditioned medium dramatically increases the number of melanocytes in quail NC cultures, but decreases the number of neurons and glia cells [15]. Over-expression of Wnt signaling genes, including β-catenin, Wnt1 and Wnt3a, in murine neural tube explant cultures induces the expansion of melanocyte numbers, but Wnt1 and Wnt3a act through distinct modes. Wnt1 acts on melanocyte precursors to expand the melanocyte lineage, while Wnt3a expands melanocyte numbers by biasing the fate of NCCs [17]. Ablation of β-catenin in mutant animals causes the loss of melanocytes [18]. Frizzled-3 is expressed at the gastrula stage early enough to mediate Wnt-dependent NC induction. Frizzled-3 is then expressed restrictively in the neural plate and in the dorsal neural tube after neural tube closure in *Xenopus*, mice, chickens and zebrafish [13]. Frizzled-3 and Wnt3a are highly localized to dorsal neural tissues that give rise to the NC [11,19,20]. However, the expression of Frizzled-3 in the melanocyte lineage has not yet been clarified.

The Wnt/Frizzled/β-catenin signaling pathway has the potential to be a therapeutic target for melanocyte regeneration. However, the expression of the Frizzled receptor on melanocytes is not well understood and the effects of Wnts on the hierarchy of melanocyte development have not been comprehensively delineated. These circumstances prompted us to investigate in detail the effect of Wnt3a/Frizzled-3 on melanocyte development. In this study, we used culture systems of NC explants and three cell lines derived from NCCs as well as human melanocytes to investigate the effect of Wnt3a on melanocyte development [21]. Namely, murine NCCs at E9.5 serve as an excellent experimental model for studying the development of melanocyte precursors in normal embryonic skin and specific gene-mutated skins [21–23]. The three cell lines derived from murine NCCs, NCCmelb4M5, NCCmelb4 and NCCmelan5, represent specific developmental stages of melanocyte precursors. NCCmelb4M5 cells do not express Kit and are immortal and stable in the absence of Kit ligand. They are positive for TRP-1 and TRP-2 and contain stage I melanosomes [24]. Glial fibrillary acidic protein (GFAP), which is a marker for glial cells, is also expressed by NCCmelb4M5 cells, while NCCmelb4 cells are negative for this protein [24], indicating that NCCmelb4M5 cells are c-Kit-negative bipotent glia/melanocyte precursors. NCCmelb4 cells are melanoblasts that are Kit^+, Tyr^-, $TRP\text{-}1^+$, $TRP\text{-}2^+$ and L-3,4-dihydroxyphenylalanine $(DOPA)^-$. Electron microscopic observations revealed that these cells contain only stage I melanosomes [25]. In contrast, NCCmelan5 cells have the characteristics of differentiated melanocytes and are Tyr^+, Kit^+, $TRP\text{-}1^+$, $TRP\text{-}2^+$ and $DOPA^+$ [26]. In this study, using NCCs derived from NC explant cultures and NCCmelb4M5, NCCmelb4 and NCCmelan5 cell lines as well as human melanocytes, the localization of Frizzled-3 and the effects of Wnt3a on the proliferation, migration and differentiation of melanocytes were investigated in detail.

2. Materials and methods

2.1. Mice

C57BL/6 (B6) mice obtained from the National Laboratory Animal Center of Taiwan (Tainan, Taiwan) were used at E9.5 for NC explant cultures.

2.2. Wnt3a protein

Recombinant mouse Wnt-3A derived from Wnt-over-expressing Chinese hamster ovary cells was purchased from R&D Systems, Minneapolis, MN, USA (Catalog No. 1324-WN). Mouse Wnt3a shares 96% amino acid identity with human WNT3a. Wnt3a was resuspended in Minimal Essential Medium (GIBCO, USA) to a concentration of 10 ng/ml for cell culture or explant culture.