The Role of the Neurotrophin Network in Skin Squamous Cell Cancer and the Novel Use of the Zebrafish System

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Cutaneous squamous cell carcinoma (cSCC) is the second most prevalent form of skin cancer. An increasing number of cSCCs are associated with dysregulation of key molecules that control skin homeostasis. These observations have increased interest in the role of neurotrophins and their receptors in the pathogenesis of cSCC. They have been demonstrated to have a considerable impact on the aggressiveness potential of skin cancer by both in vitro and in vivo models. In this context, mouse models are classically used to dissect proliferation versus differentiation balance, but they have some limitations in terms of time, space, and costs. Recently, zebrafish models have been implemented as a new tool to obtain information regarding the invasive capacity and metastasis of neoplastic cells. By xenotransplantation technique, cSCC cells from a patient's biopsy or cell line can be successfully characterized, with or without the presence of genetic manipulation or treatments. In addition, the evaluation of the immune microenvironment contributes to potentially identifying connections and homologies with humans. In this review, we retrace the role of the neurotrophin network in healthy and pathological skin, particularly in cSCC. We review how zebrafish models can be important tools for studying cSCC development, growth, and potential treatments.

Keywords: CD271, cSCC, Neurotrophins, TRK receptors, Zebrafish models

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Abbreviations: 3D, 3-dimensional; AA, alopecia areata; AD, atopic dermatitis; BCC, basal cell carcinoma; BDNF, brain-derived neurotrophic factor; COL7, collagen VII; cSCC, cutaneous squamous cell carcinoma; DF, dermal fibroblast; EMT, epithelial-to-mesenchymal transitions; GBM, patient-derived glioblastoma; HNSCC, head and neck squamous cell cancer; KC, keratinocyte; NGF, nerve GF; NMSC, nonmelanoma skin cancer; NT, neurotrophin; PDX, patient-derived xenotransplantation; PNI, perineural invasion; SCC, squamous cell carcinoma; TRK, tropomyosin receptor kinase

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INTRODUCTION

Neurotrophins (NTs) are a family of GFs initially identified for their important roles in neuronal survival and development. Indeed, they are implicated in several functions, including axonal growth, synapse formation, and maintenance (Chao, 2003; Teng and Hempstead, 2004). NT family members comprise nerve GF (NGF), brain-derived neurotrophic factor (BDNF), NT3, and NT4, which interact with and exert their functions through 2 types of transmembrane receptors: the high-affinity receptor tropomyosin receptor kinase (TRK) and the common NT receptor p75NTR (also known as CD271). CD271 is a transmembrane glycoprotein belonging to the TNF receptor superfamily. Unlike TRK receptors, CD271 binds all NTs, although with varying binding kinetics that allow it to discriminate between different NTs. On the other hand, TRKs preferentially interact with a specific NT and with CD271 and, together, act as coreceptors to improve TRK-mediated functions (Skaper, 2012). Moreover, CD271 can signal alone and exerts a proapoptotic role because its cytoplasmic tail contains several potential motifs for interactions with downstream signaling molecules and comprises the so-called death domain (Gentry et al, 2004). Since their initial discovery, several works have addressed the expression and function of NTs and their receptor in non-neuronal tissues, such as the skin. Moreover, an increasing number of evidence suggests that an interplay may exist between skin cells and the inflammatory system (Manti et al, 2017; Minnone et al, 2017; Vidal Yucha et al, 2019), which becomes relevant in the context of dermatological diseases, such as skin cancer, namely, cutaneous squamous cell carcinoma (cSCC).

cSCC is the second most frequent type of nonmelanoma skin cancer (NMSC), after basal cell carcinoma (BCC) (Karia et al, 2013; Karia and Schmults, 2014; Leiter et al, 2020; Que et al, 2018). In the last decade, the incidence of cSCC has grown enormously worldwide, reaching up to 200% (Muzic et al, 2017). According to the latest epidemiological studies, an estimated 2 million cases are identified each year in the United States, which means that about 200 cSCCs are diagnosed every hour (Lukowiak et al, 2020). Moreover, given that the major onset of the tumor is in the second half of life, the increase in average lifetime promotes cSCC therapies within the primary needs of public health (Lukowiak et al, 2020). A range of treatment options is available for the cure of low-risk cSCC, mostly surgery based (Peris et al, 2023). Conversely, in the case of advanced cSCC, where surgery cannot be used, radio or systemic chemotherapy and, more recently, immunotherapy with checkpoint inhibitors become the first choice (Lupu et al, 2017; Stratigos et al, 2020).

In the last years, several efforts have been made to achieve novel tools to understand the pathogenesis of cSCC (Quadri et al, 2022b). Moreover, given the high heterogeneity of cancer, precision medicine has become increasingly popular. Indeed, it allows the recognition of patient-specific clinical, genetic, and molecular features and seeks to identify the most effective therapy for an individual patient (Millner and Strotman, 2016; Schmidt et al, 2016). In this contest, in vivo and in vitro models have become increasingly sophisticated, by including patient-derived cells.

With this ongoing recognition of the genetic molecular complexity of the pathogenesis of cSCC, the zebrafish is emerging as an alternative approach to the study of cSCC. This model offers several advantages compared with mouse models, such as lower costs, the possibility of high-throughput drug screening, and live cell invasion imaging, and provides unique advantages in advancing the application of precision medicine for the treatment of cSCC (Al-Hamaly et al, 2023; Quadri et al, 2022b).

In this review, we defined the impact of the NT network on skin pathology and, specifically, on its role in cSCC. We focus on the most recent updates in the field reviewing the importance of NTs and their receptors in carcinoma and healthy and neoplastic skin. Given its increasing importance for cancer research, we illustrate the novel use of the zebrafish model in the study of the cSCC by focusing on the effect of the NT network on skin cancer features and treatment.

NT NETWORK IN EPITHELIAL CARCINOMA

NTs and their receptor expression and function have been evaluated in different types of cancer, with possible implications in epithelial carcinoma pathophysiology (Foerster et al, 2019; Khwaja et al, 2008, 2004; Verbeke et al, 2010).

TRK receptor signaling alteration, including gene fusion, protein overexpression, and single nucleotide modification, has been extensively reported as being implicated in the pathogenesis of many types of tumors (Khotskaya et al, 2017). In fact, a novel class of compounds blocking the TRK fusion protein molecular pathways are currently under early clinical investigation for several cancer types, including carcinomas (Doebele et al, 2015). On the other hand, many conflicting data have been reported on the localization and function of CD271 in epithelial cancer, especially in cSCC.

Head and neck squamous cell cancers (HNSCCs) are members of a group of neoplastic diseases affecting the oral and neck region and represent one of the most common types of cancer with aggressive, invasive behavior. Oral, pharyngeal, and laryngeal squamous cell carcinoma (SCC) represent almost 90% of the cases (Chi et al, 2015; Leiter et al, 2020). HNSCC is characterized by high tissue heterogeneity, and to date, an accurate target for prevention and therapy is still missing (Porcheri and Mitsiadis, 2021). Several reports describe the heterogeneous expression of the NT network in HNSCC (Huang et al, 2009; Khwaja et al, 2008; Kiyosue et al, 2013; Verbeke et al, 2010). Studies have reported a higher expression of TRK receptors associated with cancer cell proliferation and demonstrated that the use of TRK inhibitors decreases the activation of TRK-dependent mitogenic signaling (Doebele et al, 2015; Khotskaya et al, 2017).

In Palazzo et al (2019), the analysis of the Oncomine public datasets (www.oncomine.org) reveals a highly heterogeneous expression of *NTRK1*, *NTRK2* and *NTRK3* mRNA (encoding for TRKA, TRKB and TRKC, respectively) in HNSCC. *NGFR* mRNA levels (encoding for CD271) were found statistically downregulated in mucosal SCC compared with that in normal tissue and correlated with the modulation of other tumor-associated markers (Palazzo et al, 2019). A role for the BDNF/TRKB system has also been observed in cisplatin resistance development in head and neck tumors, through protein kinase B–dependent signaling pathways (Lee et al, 2012). Moreover, BDNF, together with TGF-β1, has been shown to improve tumor cell survival and contribute to worsening patient prognosis (Rössing et al, 2011).

Several works confer to CD271 the role of cancer stem cell marker in HNSCC (Elkashty et al, 2020; Huang et al, 2009; Khwaja et al, 2008; Kiyosue et al, 2013; Verbeke et al, 2010). CD271 immunohistochemical analysis localizes it in the basal layers in oral epithelial dysplasia and oral SCC (Teixeira Buck et al, 2018). Moreover, CD271-positive cells display self-renewal ability and chemotherapy resistance in esophageal SCCs (Huang et al, 2009; Li et al, 2015). Recently, it has been found that CD44+/CD271+ oral SCC cells exhibited higher cell proliferation, sphere/colony formation, chemo and radioresistance, upregulation of cancer stem cell–related genes, and in vivo tumorigenicity than the CD44+/CD271− cells (Elkashty et al, 2020). In murine HNSCC models, the treatment of the cancer cells with NGF results in upregulation of the epithelial-to-mesenchymal transition (EMT) markers SNAI2 and SLUG, which in turn give rise to a more invasive phenotype and an enhanced capacity for metastasis to regional lymph nodes. This behavior seems to be sustained by CD271 expression (Chung et al, 2018).

BCC is the most frequent nonmelanoma skin cancer derived from keratinocytes (KCs) within the basal layer of the skin (Cameron et al, 2019). Most of the lesions are commonly easy to treat and undergo complete surgery; in contrast, the advanced type of BCC, with a high risk of recurrence, requires a multidisciplinary approach, including radio and immunotherapy among the therapeutic options (Peris et al, 2023). Analysis of the NT network by examining CD271 expression was initially used to distinguish between BCC and desmoplastic trichoepithelioma because the histological discrimination between these skin diseases is challenging when only morphological criteria are applied. However, the authors reported some limitations due to CD271 diffuse immunoreactivity (Jedrych and McNiff, 2013). The expression of CD271 has been found in most tumor cells for desmoplastic trichoepitheliomas, infiltrative BCC, and microcystic adnexal carcinoma. Therefore, there are no statistically significant differences providing the efficacy in the use of CD271 as a diagnostic marker for sclerosing neoplasms of the skin.

More recently, studies conducted on BCC indicate that patients with BCC and cell lines possess lower levels of CD271 and pro-BDNF. In fact, their overexpression can promote tumor cell death, including inflammatory-silent apoptosis and lytic inflammatory–activated necroptosis (Lu et al, 2021). Using a syngenic mouse model of BCC, the authors demonstrated that CD271/pro-BDNF activities

induce tumor-associated macrophage (M1) and T-cell recruitment within the tumor area, thus determining the BCC carcinoma immune microenvironment (Lu et al, 2021).

Regarding other epithelial squamous carcinomas, such as the cervical SCC, the expression of NTs and their receptors still needs major clarification. However, NGF and TRKA overexpression has been found in cervical SCC, also indicating them as targets in this subtype of cervical tumor (Faulkner et al, 2020). On the other hand, the role of CD271 in cancer appears to differ among the different cancers. Induction of CD271 in prostate and bladder cancer stimulates apoptosis (Khwaja et al, 2008; Ratliff, 2005). In breast cancers, however, CD271 upregulation promotes cell survival by NF-kB pathway (Bashir et al, 2022), and its overexpression increases the survival of breast cancer cells through p21 (WAF1) (Verbeke et al, 2010).

NT NETWORK IN HEALTHY AND PATHOLOGICAL SKIN

In healthy skin, NTs and their receptors create a complex network made of different cell types (specifically KCs, dermal fibroblast [DFs], and melanocytes), releasing NTs and expressing CD271 or TRK receptors according to their differentiation stage or localization (Pincelli, 2017).

NTs influence KC proliferation, differentiation, and globally, the maintenance of skin homeostasis (Botchkarev et al, 2006). KCs express the high-affinity receptors TRKA and TRKC and a truncated TRKB isoform (Marconi et al, 2003). NGF is mainly expressed in KC stem cells and stimulates cell proliferation and survival through the TRKA receptor (Marconi et al, 2003; Pincelli and Marconi, 2010), thus contributing to the maintenance of stemness. On the other hand, BDNF and NT4 induce KC apoptosis through CD271 (Truzzi et al, 2011). Recently, it has been demonstrated that CD271 characterizes a population of early differentiated transit-amplifying KC and is implicated in the switch between stem cells and their progeny (Lotti et al, 2022; Truzzi et al, 2015).

Concerning the role of NTs in human DFs, the expression of NT receptors as well as the release of all NTs have been provided (Palazzo et al, 2012). The NT networks can induce the differentiation of DFs into myofibroblasts by promoting the expression of α-smooth muscle actin protein as well as sustain their proliferation and contribute to tissue remodeling and wound healing.

Human melanocytes produce all NTs in different amounts, whereas they only release NT4 (Marconi et al, 2006; Yaar et al, 1994). Both UVR and treatment with phorbol 12-myristate 13-acetate or 12-O-tetradecanoylphorbol 13-acetate in culture modulate the expression of TRKA and TRKB and NT release. NTs fail to stimulate melanocyte proliferation, whereas they stimulate the synthesis of tyrosinase and TRP1 and increase melanin production. Taken together, these results demonstrate an interaction between melanocytes and the nervous system, and NTs might also be a target of therapy for skin pigmentation disorders.

All these works strongly suggest the involvement of the NT network in the maintenance of skin features, and their dysregulation is critically involved in different types of dermatological diseases. It has been shown that NT-dependent signaling has been implicated in different inflammatory skin diseases, such as psoriasis, alopecia, or atopic dermatitis (AD), where NTs can contribute to cell loss or hyperproliferation (Figure 1 and Table 1).

After wound healing, a higher expression of CD271 has been demonstrated in injured skin (Shi et al, 2021), and its overexpression seems likely to sustain the hypertrophic scar formation while reducing the expression of those proteins correlated with autophagy. Similarly, the role of CD271 in promoting wound healing and decreasing autophagy in diabetic wounds has been reported (Wu et al, 2024). In the context of burn wound healing, NGF has been demonstrated to accelerate the healing process through TRKA and CD271, thus improving the structural organization and resolution of the scar (G El Baassiri et al, 2023). Indeed, NGF is released by skin cells as well as nerve cells, endothelial cells, mast cells, macrophages, and neutrophils, and molecules such as TGF-β1, IL-1β, and TNF-α can increase NGF expression (Kritas et al, 2014).

The NT network is also involved in the development of hair structure and hair cycling (Adly et al, 2006; Botchkarev et al, 2004, 2000; Peters et al, 2006). Among the hair disorders, alopecia areata (AA) is an autoimmune disease of actively growing hair follicles (Price, 1991; Shapiro and Price, 1998), characterized by immune cell infiltration (Gilhar et al, 1998; Hoffmann, 1999) with a higher level of proinflammatory cytokines (McElwee et al, 2002). In mouse models affected by AA, higher levels of NGF and BDNF were found in the outer and inner root sheath, respectively, and all TRK receptors were downregulated in the outer root sheath. CD271 was upregulated in the outer root sheath and was ectopically expressed in the dermal papilla (Palkina et al, 2005). The same authors reported the release of NGF by macrophages within the affected area, thus indicating a role for NTs in CD8-dependent apoptosis in the context of AA (Palkina et al, 2005).

In psoriasis, NGF influences KC proliferation, angiogenesis, and T-cell activation (Raychaudhuri et al, 2008). Indeed, NGF upregulation was found not only in psoriatic plaque but either after trauma and in nonlesional skin, indicating a major release of NGF in the context of lymphocyte recruitment. Furthermore, given the higher expression of TRK and NGF (Pincelli, 2017) in psoriasis, topical treatment with the alkaloid K252, which acts by inhibiting TRK receptor phosphorylation, improves psoriasis in the immunodeficient mouse–human skin model (Raychaudhuri et al, 2004). A decrease in CD271 levels, particularly in lesional psoriatic skin, is correlated with apoptosis resistance and disrupted skin maintenance. The overexpression of the proapoptotic CD271 receptor restores susceptibility to cell death in normally resistant psoriatic KC (Truzzi et al, 2011).

Finally, NGF-positive cells were observed in AD epidermis, mostly in the dermal papilla, whereas in the papillary dermis, a larger number of cells demonstrated strong TRKA expression. High NGF serum levels in AD serum have also been reported (Papoiu et al, 2011). In affected AD skin, CD271-positive nerve fiber profiles were increased in terms of number and size and terminated at the level of CD271-positive basal cells (Papoiu et al, 2011)). As reported for other conditions, such as lichen simplex chronicus (Altunay et al, 2021), the higher NGF levels might be responsible for

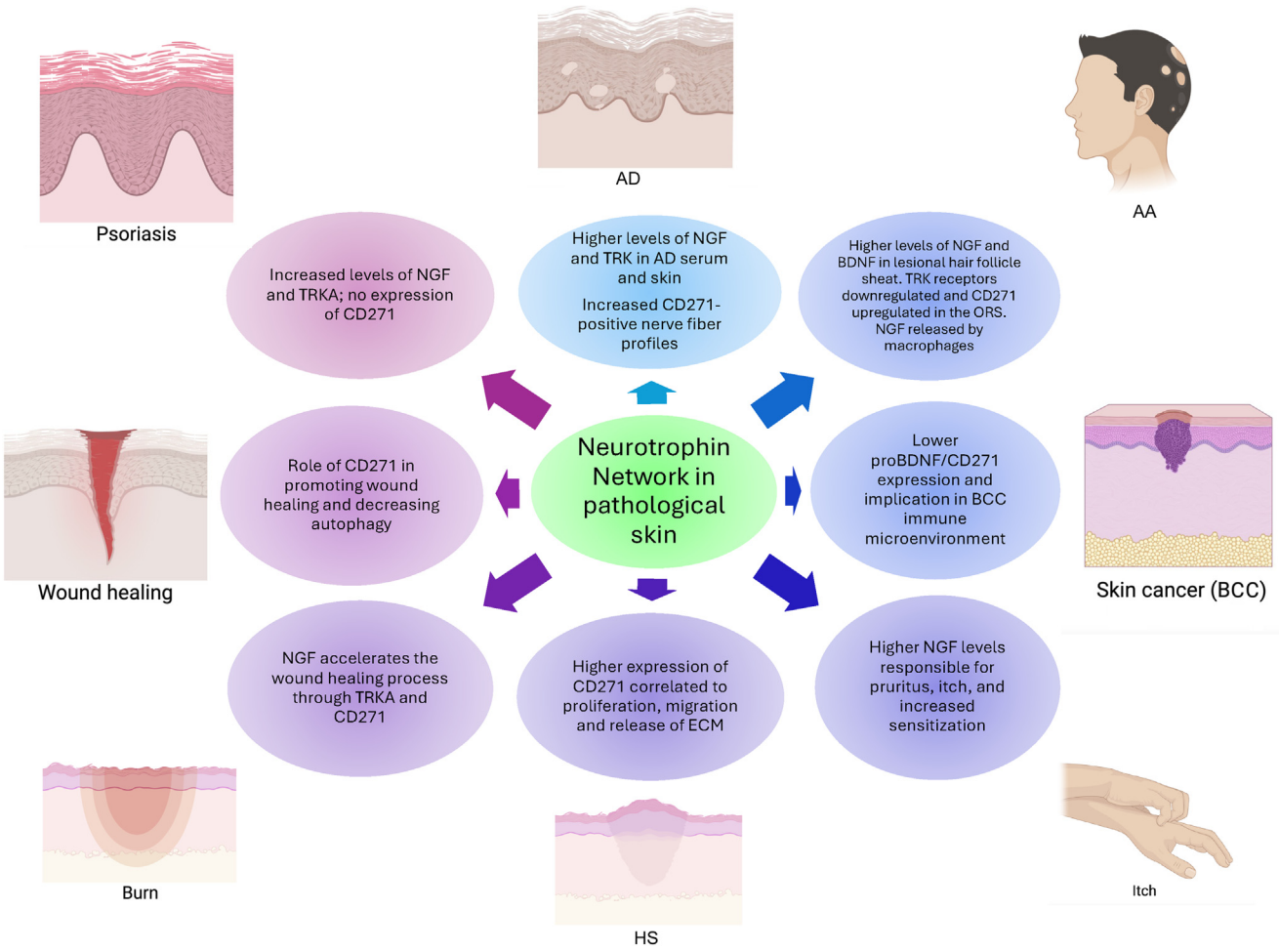

Figure 1. Role of NTs and their receptors in pathological skin. The dysregulation of the NT network is implicated in skin alteration that leads to pathological conditions. In detail, CD271 expression correlates with promoting wound healing. The HSs are a result of an altered wound-healing process and dysregulated autophagy. Higher expression of CD271 has been associated with altered proliferation, migration, and release of ECM components as well as increased autophagy, which is responsible for HS. Similarly, after burning, NGF accelerates wound healing through TRKA and CD271. Higher levels of NGF are responsible for pruritus, itch, and increased sensitization. Psoriasis showed an increased expression level of NGF and TRKA but no expression of CD271. Higher levels of NGF and TRK were found in patients with AD as well as an increase in CD271-positive nerve fiber profiles. Higher levels of NGF and BDNF were found in the outer and inner root sheath of AA, respectively, and all TRK receptors were downregulated in the outer root sheath. However, CD271 was upregulated in the outer root sheath and was ectopically expressed in the dermal papilla. In BCC, the expression of pro-BDNF/CD271 is low, and their signaling is implicated in the formation of the BCC immune microenvironment. Figure was created with BioRender.com. Refer to Table 1 for specific references reporting NT and NTR expression and function findings in skin pathological conditions. AA, alopecia areata; AD, atopic dermatitis; BCC, basal cell carcinoma; BDNF, brain-derived neurotrophic factor; ECM, extracellular matrix; HS, hypertrophic scar; NGF, nerve GF; NT, neurotrophin; NTR, neurotrophin receptor; TRK, tropomyosin receptor kinase.

pruritus and itch in lesional skin as well as for increased sensitization (Andersen et al, 2018). In urticaria, the serum level of NGF has increased (Basak et al, 2014), whereas CD271, together with the stem cell factor, is selectively and systemically downregulated in the skin of patients with urticaria and may represent negative feedback to increased mast cell reactivity and proliferation (Hermes et al, 2003). Similarly, BDNF is increased in serum and diseased skin of patients with chronic spontaneous urticaria (Rössing et al, 2011), suggesting a role for NTs in the pathophysiology of this chronic inflammatory skin disease.

In summary, the role of the NT network in the skin is quite complex and related to the multiple different skin cell types and their variable interactions in both health and diseases. Therefore, further studies are needed to fully understand the expression and function of the NT network within skin physiology and pathology.

NT NETWORK IN cSCC

Recently, a stage-specific localization and an initial functional evaluation of NT receptors in cSCC have been elucidated (Dallaglio et al, 2014; Palazzo et al, 2019; Quadri et al, 2023). A summary of the most important work reporting the expression and function of NT and their receptors in cSCC is displayed in Table 2.

CD271 is scarcely detectable in cSCC tumor biopsies, and cSCC cells expressing survivin, which is associated with cancer stem cells, do not express keratin 10, which is a marker of early KC differentiation, and CD271 (Dallaglio et al, 2014). Similarly, CD271 expression was found

Table 1. Expression and Function of Neurotrophins and Neurotrophin Receptors in Skin Pathologies

Skin Pathology	References
Wound healing	Shi et al, Can J Physiol Pharmacol, 2021 Wu et al, Biochim Biophys Acta Mol Basis Dis, 2024
Burn	G El Baassiri et al, Burns, 2023 Kritas et al, J Biol Regul Homeost Agents, 2014
Hypertrophic scar	Shi et al, Can J Physiol Pharmacol, 2021
Itch	Andersem et al, Exp Dermatol, 2018 Basak et al, Indian J Dermatol Venerol Leprol, 2014 Hermes et al, Br J Dermatol, 2003 Rossing et al, Clin Exp Allergy, 2011
Psoriasis	Raychaudhuri et al, J Invest Dermatol, 2004 Truzzi et al, Cell Death Differ, 2011
Atopic dermatitis	Papoiu et al, Neuropeptides, 2011
Alopecia areata	Palkina et al, J Invest Dermatol Symp Proc, 2005
Basal cell carcinoma	Krahl and Sellheyer, Br J Dermatol, 2010 Jedrych and McNifff, Am J Dermatopathol, 2013 Lu et al, J Immunol Res, 2021

significantly lower in cSCC cells in vitro by both monolayer and 3-dimensional (3D) culture (Palazzo et al, 2019). Given that its expression correlated with spheroid compactness and the expression of prodifferentiating factors, such as DLX3, CD271 downregulation and the interplay between DLX3 and CD271 have been indicated as an essential path to prevent those alterations of epidermal homeostasis, which leads to cSCC development.

A critical analysis of the expression of the NT receptors during the transition from actinic keratosis to cSCC development and progression has been given by Quadri et al (2023). Their findings proved that CD271 is upregulated in well-differentiated cSCC compared with that in the more aggressive moderately/poorly differentiated cSCC, whereas the expression of TRKA directly correlates with the level of malignancy. CD271 activities were found to reduce proliferation and malignancy marker expression in patient-derived cSCC spheroids at each tumor grade by increasing neoplastic cell differentiation. Both CD271 overexpression and activation decrease cSCC cell invasiveness in 3D cSCC tumor spheroids. Treatment with K252a reduces the size of CD271-overexpressing spheroids to a higher degree than with the controls, by synergistically acting with CD271 activities. This was confirmed by the significant increase of apoptosis when CD271 is overexpressed and TRK signaling is inhibited by K252a. However, the exact molecular interactions between K252a and CD271 signaling have not been clarified. Similarly, TRKA/Fc, a human recombinant TRKA Fc (crystallizable fragment region) chimera protein derived from mouse myeloma cell line, treatment by reducing the availability of NGF and NT3 can significantly reduce cSCC spheroid growth, and its effect synergizes with β-amyloid treatment, which in turn activates CD271. These data reveal that CD271 activation prevents low- to high-risk progression of cSCC and that TRK and CD271 receptor modulation might result in an effective strategy for inhibiting cSCC cell viability.

Table 2. Expression and Function of Neurotrophins and Neurotrophin Receptors in cSCC

Protein/Gene	Main Findings with References
TRKA *NTRK1*	TRKA expression associated with the PNI area (Chen-Tsai et al, Dermatol Surg, 2004; Frydenlund and Mahalingam et al, Vitam Horm, 2017) No significant TRKA expression was observed in tumoral cSCC cells near the PNI area (Brugière et al, J Invest Dermatol, 2018) TRKA and NTRK1 expressions directly correlate with the cSCC aggressiveness (Quadri et al, J Exp Clin Cancer Res 2023)
TRKB *NTRK2*	TRKB expression associated with the PNI area (Chen-Tsai et al, Dermatol Surg, 2004) TRKB expression was found in perineural tumor cSCC cells (Brugière et al, J Invest Dermatol, 2018) TRKB/BDNF associated with EMT (Deborde et al, J Clin Invest, 2026; Jia et al, Oral Oncol 2015, Shan et al, Oncol Rep, 2016) NTRK2 markedly expressed in MD/PD patient–derived spheroids (Quadri et al, J Exp Clin Cancer Res 2023)
TRKC *NTRK3*	TRKC expression associated with the PNI area (Chen-Tsai et al, Dermatol Surg, 2004) No significant TRKC expression was observed in tumoral cSCC cells near the PNI area (Brugière et al, J Invest Dermatol, 2018)
CD271 *NGFR*	CD271 expression was mainly associated with the PNI area (Chen-Tsai et al, Dermatol Surg, 2004) CD271 was strongly expressed in perineural tumor cells (Brugière et al, J Invest Dermatol, 2018) Scarcely detectable in aggressive cSCC (Dallaglio et al, Br J Cancer, 2014) CD271 expression correlates with spheroids compactness in vitro and the expression of prodifferentiated factors (Palazzo et al, Int J Mol Sci, 2019) CD271 and NGFR are upregulated in WD cSCC compared with those in healthy skin and premalignant lesions, whereas the expression decreases in the more aggressive MD/PD tumors (Quadri et al, J Exp Clin Cancer Res, 2023). CD271 reduced the proliferation and malignancy of patient-derived cSCC spheroids by increasing neoplastic cell differentiation and improved therapy outcome in SCC13 spheroids (Quadri et al, J Exp Clin Cancer Res 2023).
NGF *NGF*	NGF expression was mainly associated with the PNI area (Chen-Tsai et al, Dermatol Surg, 2004) No significant NGF expression was observed in tumoral cSCC cells near the PNI area (Brugière et al, J Invest Dermatol, 2018) High NGF mRNA expression in MD/PD patient–derived spheroids (Quadri et al, J Exp Clin Cancer Res 2023)
BDNF *BDNF*	BDNF expression associated with PNI potential (Brugière et al, J Invest Dermatol., 2018) TRKB/BDNF associated with EMT (Deborde et al, J Clin Invest, 2026, Jia et al, 2015, Shan et al, Oncol Rep, 2016) High BDNF mRNA expression in MD/PD patient–derived spheroids (Quadri et al, J Exp Clin Cancer Res 2023)
NT3 *NTF3*	No significant NT3 expression was observed in tumoral cSCC cells near the PNI area (Brugière et al, J Invest Dermatol, 2018)
NT4 *NTF4*	No significant NT4 expression was observed in tumoral cSCC cells near the PNI area (Brugière et al, J Invest Dermatol, 2018)

Abbreviations: BDNF, brain-derived neurotrophic factor; cSCC, cutaneous squamous cell carcinoma; EMT, epithelial-to-mesenchymal transition; MD, moderately differentiated; NGF, nerve GF; NGFR, nerve GF receptor; NT3, neurotrophin 3; NT4, neurotrophin 4; PD, poorly differentiated; PNI, perineural invasion; WD, well-differentiated.

According to the American Joint Committee on Cancer, cSCC staging criteria include perineural invasion (PNI) as a high-risk tumor feature (Farasat et al, 2011), and this seems to be a specific characteristic of the cSCC from the head and neck. In detail, large-caliber nerve invasion, together with other nerve invasion–dependent risk factors, has been associated with a poor outcome in patients with cSCC, with an elevated risk of metastasis and death (Carter et al, 2013; Ross et al, 2009). The analysis of the expression of the NT receptors and, particularly TRKA, has been initially associated with PNI in the context of not-skin tumors. Subsequent evaluations refer to a major expression of TRKA in cSCC from the head and neck, but its expression was not directly correlated with PNI, being more heterogeneous concerning the anatomical site (Frydenlund and Mahalingam, 2017). Further studies that include the analysis of neural adhesion factors (ie, neural cell adhesion molecule); NGF; TRKA, B, and C; and CD271 indicate a major association of the expression of NT molecules on cSCC tumor cells in the areas of PNI. In detail, elevated levels of TRK receptors may predict PNI, and higher CD271 expression in SCC cells within PNI areas could be used for PNI detection. This work supports the important role of the NT network in the identification of cSCC tumors with greater aggressiveness and PNI potential (Chen-Tsai et al, 2004).

With a deep focus on tumor cells distant from the PNI area versus perineural tumor cells, Brugière et al (2018) found that in perineural tumor cells, there is a strong expression of BDNF, TRKB, and CD271, contrasting with weak expression of these markers in tumor cells distant from the PNI areas, whereas no significant expression was observed for TRKA, TRKC, NGF, NT3, or NT4. A decrease in E-cadherin and an increase in SNAIL1 and NCAM1 expressions were shown in perineural tumor cells compared with those in tumor cells distant from PNI areas, associating BDNF/TRKB paths with EMT, as reported in several other studies concerning epithelial cancers (Deborde et al, 2016; Jia et al, 2015; Shan et al, 2016). This study demonstrates that NTs are linked to EMT in human cSCC and involve NCAM1, suggesting direct interactions between perineural tumor cells and the nerve.

IMPACT OF THE NT NETWORK ON cSCC

Managing nonmelanoma skin cancers is one of the elements constantly on the clinical agenda, both in terms of the number of patients and type of treatments, which must correspond to the patient's characteristics in terms of age or, eventually, concomitant therapies for other health problems. Clinicians often assist patients who, owing to surgery, receive a very invasive treatment, and of course, they need to be further assisted from wound treatment to healing, which could be delayed or altered by the current pharmacological treatment of the patient. During advanced cSCC conditions, it will also be necessary to consider the side effects of chemotherapeutics or immunomodulatory drugs administered systemically (Aboul-Fettouh et al, 2021; Baggi et al, 2021). Another point to consider in the management of cSCC is that other than being caused by gene alterations induced by UVR exposure, several factors might contribute to its pathogenesis (Winge et al, 2023). In addition to skin aging, various genetic conditions, including dystrophic bullous epidermolysis, can cause lesions and comorbidities (Winge et al, 2023).

What does this have to do with the analysis of the NT network? From what has been studied so far, not only in the context of cSCC but also in the regulation of epidermal homeostasis and hyperproliferative skin conditions, it is clear that we have several factors, from the evaluation of the NT expression to the activation or inhibitor of their receptors, to be considered as an effective tool and targets for future pharmacological development. In fact, the NT network contributes to the maintenance of skin features by exerting regulatory functions in all skin cells (Botchkarev et al, 2006; Lotti et al, 2022). Specifically, their activity in healthy KCs modulates stemness, proliferation, and differentiation, thus contributing to epidermal homeostasis.

NT network modulation, by mean of CD271 receptor activity, would seem to be able to greatly sustain both the effectiveness of therapies, potentially favoring a better patient outcome and/or determining, in the first instance for localized cSCC lesions, a rapid prodifferentiation effect that could limit the progression and aggressiveness of the neoplasms. Therefore, there is a strong need to investigate further the mechanisms through which the NT network can interfere with the proliferation and eventually differentiation of cutaneous cancer cells.

Going into precision oncology, we have to talk about individual responses to treatment, and zebrafish avatars make it possible to investigate a priori how and if a cancer treatment can be effective on the individual patient' cells (Fazio et al, 2020; The Lancet, 2021). Using the NT system with cSCC cell xenografting in zebrafish, as will be described further, we could introduce a novel tool that might help in the diagnosis and treatment of the advanced disease. Thanks to all these instruments, we could envision a future improvement in both the quality of care, with a strong reduction of invasive methods, and, not less importantly, their cost to public health.

ZEBRAFISH XENOGRAFTING MODELS AND AVATARS

Zebrafish (*Danio rerio*), a vertebrate species traditionally used in developmental biology and vertebrate genetics studies, has rapidly emerged as a promising animal model for human diseases, including cancer. Despite its appearance, zebrafish shows several similarities to *Homo sapiens*, around 70% of genome homology, which increases to 82% in the case of human disease–related genes (Delvecchio et al, 2011; Howe et al, 2013; Lieschke and Currie, 2007; White et al, 2013). Interestingly, zebrafish skin shows several similarities to human skin, which renders this vertebrate particularly suitable for the study of several cutaneous disorders, including neoplasms (Russo et al, 2022).

Other than being easy and inexpensive to maintain compared with other mammalian models, zebrafish is highly useful for chemical and genetic screens, transgenesis, and large-scale studies as high-throughput in vivo assays. In addition, its small size, external development, and the optical transparency of the embryos and larvae facilitate in vivo fluorescent imaging, without using invasive procedures (Zhao et al, 2015). Another advantage of using zebrafish at a developmental stage consists of the ease of producing and

injecting many embryos in a short amount of time (Zhang et al, 2015).

Importantly, there is a temporal separation in the maturation of the innate and adaptive immunity in zebrafish. The innate immune system is functional 2 days after fertilization, whereas the adaptive immune system is not active until 28 days after fertilization. This allows for xenotransplantation of human cancer cells and monitoring of their growth, invasion, and metastasis as well as the evaluation of drug responses (Zhao et al, 2015; Zon and Peterson, 2010; Quadri et al, 2022b). However, transplantation in adults has also been reported, where immune rejection was avoided by inducing immunosuppression with sublethal γ-irradiation or dexamethasone or using the immunocompromised zebrafish strain, such as *Rag2* or *Il2rga* transgenic animals (Tang et al, 2014; Yan et al, 2019).

The creation of transparent albino zebrafish strains, harboring *Roy* and *Nacre* allelic variants, allowed for live visualization of cancer cell progression also in adult animals (Lawson and Weinstein, 2002; White et al, 2008). Therefore, zebrafish xenotransplantation models offer the possibility to study many features of cancer, such as growth, invasion and dissemination, and drug responses also at a later stage. However, cancers can also be induced by a transient knockdown of the target genes achieved by microinjection of morpholino antisense oligonucleotides or the synthetic TALENs (Huang et al, 2016; Nasevicius and Ekker, 2000). These techniques have been gradually replaced by the CRISPR/Cas9 system, which allows for both stable knock-in and knockdown of different types of genes (Dovey et al, 2009; Hwang et al, 2013). Altogether, these models clarified the gene alterations that are involved in the step-by-step progression from healthy to neoplastic cells and can be used for drug screening (Dovey et al, 2009; Quadri et al, 2022b; Travnickova et al, 2019).

Zebrafish response to the injected cancer cells in terms of the innate immune response or angiogenesis can be reached by transgenic zebrafish strains' where immune or endothelial cells are labeled (Beis et al, 2005; Cascallar et al, 2022; Jacob et al, 2016; Lawson and Weinstein, 2002). In 1997, Long et al (1997) first established a transgenic zebrafish line by microinjecting the promoter sequence of GATA-1 (the erythroid-specific transcription factor) fused to GFP into 1-cell-stage zebrafish embryos. Given the transparency of zebrafish, this approach permitted the real-time and in vivo visualization of fluorescent circulating blood cells. Moreover, the germline from the parental transgenic fish continued to express GFP in the F1 and F2 generations. This work was the starting point for the generation of numerous transgenic zebrafish lines expressing several types of fluorescent proteins (ie, GFP, DsRed2, mCherry, etc) in molecules, intracellular organelles, cells, or tissue of interest, allowing the visualization of them in vivo and in real time (Choe et al, 2021). For example, the *Tg(mpx:GFP)*, *Tg(lysC:DsRed2)*, and *Tg(mpeg1:EGFP)* zebrafish fluorescently label neutrophils and macrophages (Ellett et al, 2011; Hall et al, 2007; Renshaw and Trede, 2012), whereas the *Tg(fli1:EGFP)* and the *Tg(kdrl:EGFP)* label the vascular endothelial cells. Furthermore, the expression of human oncogenes in zebrafish can be tracked and be particularly useful in understanding their role in tumor development (Michailidou et al, 2009; Park et al, 2008; Patton et al, 2005). These models could be potentially applied in the studies of several types of cancer, including cSCC.

In recent years, patient-derived xenotransplantation (PDX) models (or zebrafish avatar) have been established (Fazio et al, 2020). In this case, zebrafish are amenable to modeling the metastatic potential, growth, immune response, angiogenesis, and drug response of cancer cells derived from patient specimens. Marques et al (2009) showed the transplantation of direct explants from gastrointestinal human tumors into zebrafish embryos and larvae, both into the yolk sac and in the liver. In this study, the zebrafish model permitted a rapid analysis of human tumor metastatic capacity (Marques et al, 2009). Moreover, Finotto et al (2023) showed the real-time in vivo monitoring of patient-derived glioblastoma (GBM) stem cells and GBM-associated macrophage interaction in an orthotopic zebrafish xenograft model. They identify *Lgals1* as a primary regulator of immunosuppression in zebrafish in vivo models (Finotto et al, 2023).

Given this evidence, the PDX zebrafish model will allow the characterization of the primary tumor cell behavior in vivo and real time, reflecting the clinical course of the patient's medical history, providing noteworthy benefits for evaluating the patient's prognoses, and identifying the most appropriate individualized therapy (Howe et al, 2013; White et al, 2013).

ZEBRAFISH MODELS IN THE STUDY OF cSCC AND THE NT NETWORK

Thanks to all its features, zebrafish represents an outstanding model in cancer research given its high versatility in different aspects of tumor development, progression, migration, and drug responses. Moreover, the PDX models, which used patient-derived cells, proved to be very useful in the precision medicine era (Fazio et al, 2020). Although several transgenic models have been developed, a zebrafish transgenic model of cSCC does not exist, maybe because of the extreme difficulty in fish's KC transformation. Chen et al (2014) tried to establish a novel NMSC model in zebrafish by inducing the overexpression of CDC6 and c-MYC in the epidermis under the keratin-14 promoter. They found that only the co-overexpression of CDC6 and c-MYC in zebrafish skin triggered a tumor-like transformation in the embryonic stage (Chen et al, 2014).

However, given the difficulty found in generating neoplastic transformation in zebrafish KCs, most of the cSCC research takes advantage of the xenografting procedure, which allows to obtain information on cell growth and invasive capacity within a week (Martins et al, 2016; Paulson et al, 1990; Quadri et al, 2023, 2022b). Moreover, the use of the fluorescent transgenic reporter zebrafish strains significantly increases the power of the information obtainable related to the interaction between cancer cells and host, such as angiogenesis and immune response, which remain the major hallmarks of aggressive tumors (Codolo et al, 2022; Pan et al, 2022; Quadri et al, 2022a; Roth et al, 2021; Wang et al, 2024; Zhao et al, 2024). For example, Martins et al (2016) reported the use of the zebrafish model to evaluate the role of collagen VII (COL7) in cSCC. It is well-known that

individuals with dystrophic epidermolysis bullosa, an inherited blistering disorder caused by sequence variations in the *COL7A1* gene, develop a very aggressive form of cSCC. To elucidate the role of COL7 in cSCC, *COL7*-null EB3K cells were injected into transgenic *Tg(fli1:EGFP)* zebrafish yolk 1 day after fertilization, with or without the human recombinant COL7. After 3 days, EB3K cells stimulated angiogenesis, as demonstrated by intratumoral vascularization. Conversely, the presence of the human recombinant COL7 reversed the angiogenic process (Martins et al, 2016). The xenotransplantation model was also used to evaluate the function of the Y kinase receptor AXL in cSCC. Cichoń et al (2014) showed that the injection of AXL-knockdown cells in zebrafish promoted a loss in tumor mass formation, confirming the role of AXL in promoting cSCC aggressiveness.

Recently, Quadri et al (2023) reported for the first time the use of zebrafish to elucidate the role of the common NT receptor CD271 in cSCC. Their work revealed the role of CD271 in inhibiting the aggressiveness of cSCC in vivo. In addition, they demonstrated that CD271 expression and activation significantly improve the outcome of chemotherapy in vivo. Moreover, using the *Tg(mpeg1:mCherry)g123* zebrafish transgenic strains or *mpeg1.1* in situ hybridization, they showed an increase in the recruitment of leucocytes by CD271-overexpressing cells. Interestingly, the same experiment was performed in zebrafish, which was subjected to a depletion of the immune cells. Mock or CD271-overexpressing cSCC cells were injected in wild-type or *Tg(lysC:DsRed2)nz50* transgenic zebrafish strain with or without the simultaneous treatment with a toxic compound for macrophage, NK cells, cytotoxic T lymphocytes, and skin mast cells. Stimulatingly, the immune cell ablation induced a significant increase in zebrafish metastasis, suggesting that the zebrafish immune system is important in preventing cSCC cell invasion. The block of cell spreading observed in zebrafish injected with CD271-overexpressing cells was partially reversed after leucocyte ablation. In their work, Quadri et al (2023) elucidated the role of CD271 in promoting the recruitment of the immune cells, therefore suggesting a role of the NT network in regulating the cSCC tumor microenvironment.

CONCLUSION

The use of zebrafish models results in a novel and feasible tool for the study of neoplastic KC behavior in vivo and allows for multiple applications, including personalized medicine, in the field of cSCC research. A significant amount of work reported the invaluable importance of the NT network in regulating skin homeostasis and inflammation, becoming seriously involved in skin diseases once a profound dysregulation of the homeostatic balance occurs. Recently, a demonstration of the NT network function in cSCC has been given by in vitro and zebrafish models (Figure 2). In this study, CD271 could be seen as a protective or suppressing factor in

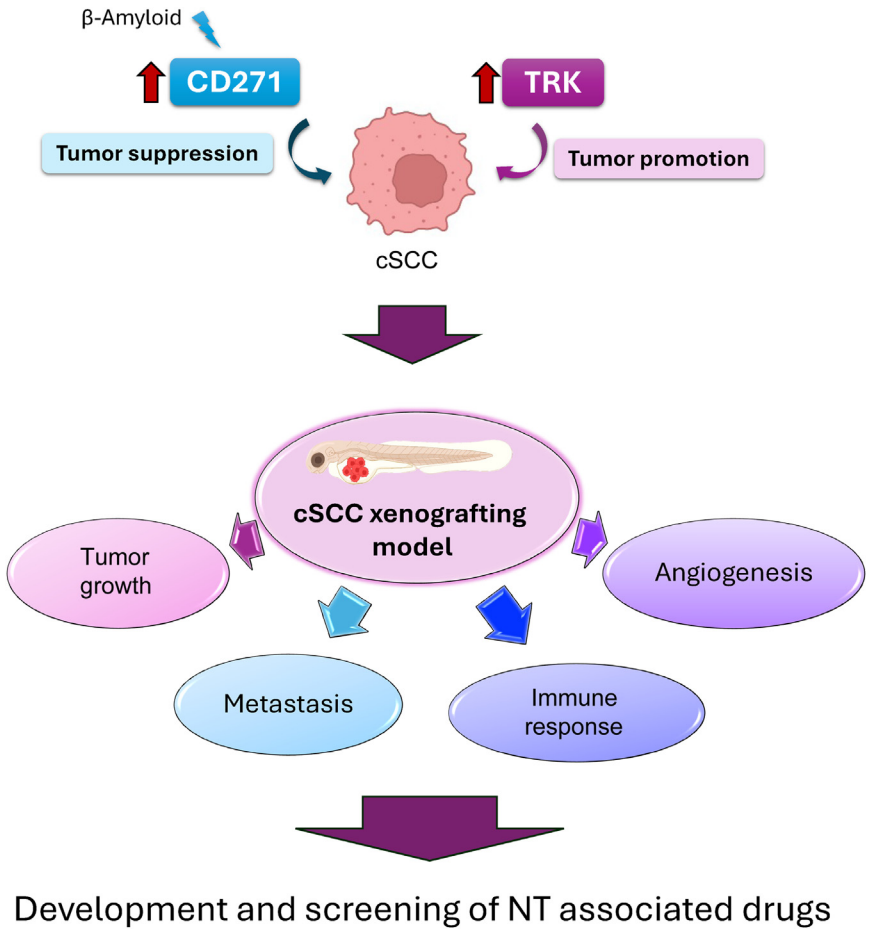

Figure 2. NT receptor functions in cSCC and the use of the cSCC xenografting zebrafish model. The cSCC xenografting zebrafish model is an invaluable tool for the study of the role of the NT network and for evaluating new therapeutic pathways. cSCC cells (either cell lines, such as SCC13 or primary cSCC cells isolated from tumor biopsies) can be directly injected, w/wo genetic modification or transient transfection/transduction, to study specific aspects of the NT pathway in vivo. Alteration in CD271 expression or activation by factors such as b-amyloid or modulation of TRK expression or signaling can be performed in vitro or followed by xenografting to better characterize the role of the NT network in cSCC and for testing new therapies. The zebrafish models allow the evaluation of tumor growth, metastasis, immune response, and angiogenesis, thus providing a fast and significant indication of the effectiveness of drugs. Figure was created with BioRender.com. cSCC, cutaneous squamous cell carcinoma; NT, neurotrophin; TRK, tropomyosin receptor kinase; w/wo, with/without.

cSCC, and thanks to the use of transgenic zebrafish strains, its importance in stimulating the recruitment of the immune cells as well as in determining tumor killing after its activation has been demonstrated. Therefore, the integration of the zebrafish model within the oncological research instruments will surely provide significant advances in skin cancer research and will offer an enormous platform for the study of cell biology and drug discovery.

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CONFLICT OF INTEREST
The authors state no conflict of interest.

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