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Existing and Developing Preclinical Models for Neurofibromatosis Type 1–Related Cutaneous Neurofibromas

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CONFLICT OF INTEREST

DL holds equity in, is a member of the Board of Directors, and serves as a Senior Scientific Advisor to Recombinetics, a genome-editing company, and Makana, a xenotransplantation company focused on pig kidney transplantations into patients. DL collaborated with Recombinetics to produce and characterize a porcine model of the neurofibromatosis type 1 syndrome. This model is described in this manuscript. VS, PT, LQL, RLC, MRS, JOB, SDR, IL, CGR, SYL, and ES receive support from the Neurofibromatosis Therapeutic Acceleration Progam (NTAP) at Johns Hopkins University. VS, LQL, MRS, JOB, and IL receive funding from the Department of Defense. IL and JOB are consultants for SpringWorks Therapeutics. The remaining authors state no conflict of interest.

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Abstract

Neurofibromatosis type 1 (NF1) is caused by a nonfunctional copy of the *NF1* tumor suppressor gene that predisposes patients to the development of cutaneous neurofibromas (cNFs), the skin tumor that is the hallmark of this condition. Innumerable benign cNFs, each appearing by an independent somatic inactivation of the remaining functional *NF1* allele, form in nearly all patients with NF1. One of the limitations in developing a treatment for cNFs is an incomplete understanding of the underlying pathophysiology and limitations in experimental modeling. Recent advances in preclinical in vitro and in vivo modeling have substantially enhanced our understanding of cNF biology and created unprecedented opportunities for therapeutic discovery. We discuss the current state of cNF preclinical in vitro and in vivo model systems, including two- and three-dimensional cell cultures, organoids, genetically engineered mice, patient-derived xenografts, and porcine models. We highlight the models' relationship to human cNFs and how they can be used to gain insight into cNF development and therapeutic discovery.

INTRODUCTION

Neurofibromatosis type I (NF1) is one of the most common human genetic disorders, affecting one in 3,000 individuals worldwide (Evans et al., 2010). NF1 is caused by pathogenic variants in the *NF1* gene, which encodes neurofibromin, a RAS-GTPase activating protein that inhibits RAS signaling. Inactivating mutations in *NF1* lead to increased RAS signaling and complex multisystem manifestations (Gutmann et al., 2017). A hallmark of NF1 is the development of multiple peripheral nerve sheath tumors, called neurofibromas. The two major types are plexiform neurofibromas (pNFs), which involve multiple deep nerves or a nerve plexus, and cutaneous neurofibromas (cNFs), which are limited to the skin. cNFs present as a soft mass, which can have a wide range of appearances ranging from a barely visible lesion with subtle discoloration of the overlying epidermis (nascent, latent cNFs) to lesions that are raised, including flat, sessile, globular, and pedunculated forms (Ly et al., 2022; Ortonne et al., 2018).

Histologically, a cNF is a nonencapsulated lesion composed of a cellular component and an extracellular matrix. It is a hypocellular lesion with small, comma-shaped cells dispersed

in an extracellular matrix. The cellular component is polymorphous with a variety of cell types, including Schwann cells (SCs), fibroblasts (FBs), perineurial/perineurial-like cells, and immune cells, as well as residual axons embedded in the matrix and variable amounts of collagen bundles in the myxoid (Brosseau et al., 2021, 2018). SCs harboring biallelic inactivation of *NF1* are considered the tumorigenic cells within cNFs. Notably, in a single individual, there are multiple unique somatic *NF1* variations across cNFs (Maertens et al., 2006; Serra et al., 2001; Thomas et al., 2012), and it is unknown how the various somatic and germline *NF1* variations influence the neurofibromagenesis.

Almost all adults with NF1 will form cNFs that can number in the thousands for individual patients and involve the entire body (Ortonne et al., 2018; Williams et al., 2009). In addition to physical symptoms such as pain and itching, cNFs are a major source of emotional distress and may dramatically impact QOL (Ortonne et al., 2018; Williams et al., 2009). Surgical removal is the main treatment to address singular, symptomatic cNFs, with no curative or preventative drug treatment yet available (Ortonne et al., 2018). Until recently, a major impediment to cNF therapeutic discovery has been a lack of well-characterized and reliable preclinical cNF models to address the complex variables in the biology of these skin tumors. In this review, we will discuss in vitro and in vivo model systems, highlight recent progress, and comment on prospective directions for developing cNF preclinical platforms to advance the understanding of NF1 and cNFs biology and provide relevant models for drug discovery and testing.

IN VITRO CULTURE SYSTEMS FOR cNFs

A range of in vitro models have been established using both human and animal cells for cNFs. These include two-dimensional (2D) or three-dimensional (3D) formats; single-cell cultures or cocultures; and the application of cNF-derived primary cells, semi-immortalized cells, or pluripotent-derived cells depending on the application (Table 1).

2D cNF cell culture models

Models using primary cells.—The initial cNF models used primary cells directly isolated from human cNFs. Different methodologies were set up more than two decades ago to establish pure SC cultures isolated from the multicellular cNFs (Rosenbaum et al., 2000; Rutkowski et al., 2000; Serra et al., 2000; Wallace et al., 2000), taking advantage of the identification of ß-heregulins as potent SC mitogens (Levi et al., 1995) and using them for SC culture (Rutkowski et al., 1995). In fact, $NF1^{-/-}$ and $NF1^{+/-}$ SCs could be isolated from cNFs using distinct culture conditions (Serra et al., 2000). These SC cultures have been used to investigate the type of second *NF1* hits present in cNFs (Maertens et al., 2006; Serra et al., 2001), facilitate the diagnosis of people with mosaic forms of NF1 (Maertens et al., 2007), and test the effects of hormones (Pennanen et al., 2018) or drugs (Mazuelas et al.[1]) on cellular behavior. FBs, the second most abundant cell component in cNFs after SCs, can be easily expanded as single cultures in conventional culture conditions. Using single primary cultures, SC–FB cocultures have also been established to investigate the

[1]Mazuelas H, Magallón-Lorenz M, Uriarte-Arrázola I, Negro A, Rosas I, Blanco I, et al. Unbalancing cAMP and Ras/MAPK pathways as a therapeutic strategy for cutaneous neurofibromas. 2022. pre-pint in bioRxiv; DOI: 10.1101/2022.12.23.521754.

cross-talk between these cells (Mazuelas et al.[1]). In addition, primary cultures from animal cNFs have been established to study the biology and evaluate the efficacy of drugs before in vivo studies. For example, cultures of wild type (WT) $NF1^{+/+}$ SCs from porcine sciatic nerves and primary cNF-derived SCs and FBs have been established from the $NF1^{R1947*/+}$ Ossabaw minipigs (Isakson et al., 2018), which have been used for preclinical drug testing.

Models using semi-immortalized cells.—Primary cells from cNF can be expanded for only a limited number of passages, limiting applications such as drug screens. The Wallace laboratory developed 14 new semi-immortalized cNF-derived SC lines (from cNFs donated by adults with NF1) by transduction with expression constructs for both *hTERT* and *mCdk4* genes (Wallace et al., unpublished data), in a similar approach used for pNF-derived SCs (Li et al., 2016). Before being used for preclinical testing, these cell lines are undergoing genomic and transcriptomic characterization, as was done with pNF-derived immortalized SCs (Ferrer et al., 2018). The immortalized cNF cells will offer several advantages, including cost-effectiveness, convenience (no longer requiring complex factors for propagation), data consistency, availability, and bypassing the ethical concerns associated with the use of animal and human tissue. However, a known limitation is that semi-immortalized cNF cells will not have the exact same physiological status as nonmodified primary cells and that genetic drift as well as adaptations to the nonphysiological conditions may occur in long-term passaged cells, leading to reduced reproducibility of the results (Ben-David et al., 2018; Ferrer et al., 2018; Hirsch and Schildknecht, 2019; Li et al., 2016).

Models using pluripotent cells.—Given the limitations reviewed earlier and the multicellular nature of cNFs, researchers are devising various systems to investigate each cell type and its interactions more reliably. As such, human induced pluripotent stem cells (iPSCs) harboring *NF1* gene pathogenic variations that have been differentiated into Schwannian lineage cells can serve as a cNF model to study the impact of *NF1* alterations on SC lineage differentiation and to generate humanized neurofibromas in mice for therapeutic testing (Mo et al., 2021). Other methodologies for generating iPSC-derived $NF1^{-/-}$ neural crest (NCs) cells and differentiating SCs (Carrió et al., 2019) have been used as a base for generating neurofibromaspheres (see the section below) (Mazuelas et al., 2022). Finally, using a human SC specification system (GF-free and chemical compound—based protocol that differentiates toward SC fate in 3 weeks) and temporally tunable and reversible control of NF1 protein production/degradation, the Lee laboratory developed a humanized cNF model with human embryonic stem cells (Lee et al., unpublished data). This model is useful for studying the effect of different levels of *NF1* loss on SC behavior.

The 3D cNF cell culture models

The 2D cNF models cannot recapitulate the in vivo microenvironment architecture, and drugs that have a measurable effect on the viability of cells grown in 2D culture conditions may not have the same efficacy in more complex, realistic 3D in vitro and in vivo models (Chaicharoenaudomrung et al., 2019). Therefore, the use of 3D cell culture approaches has grown to serve as an intermediary between 2D and in vivo studies. The 3D cNF models exist in spheroid structures, matrix-embedded systems, or multicellular organoids/tumoroids. These models mimic the core physical and biochemical features of cNFs and

allow the spatial effects of multicellular interactions (Kapałczyńska et al., 2018). However, 3D models also have several limitations, such as difficulty in constructing and maintaining 3D cultures, which affects reproducibility, and costs and challenges in completing and interpreting readouts to measure drug response (Chaicharoenaudomrung et al., 2019; Jensen and Teng, 2020).

The 3D suspension culture systems.—Using a methodology similar to that used to create 3D pNF models (Kraniak et al., 2018), the Mattingly group is developing 3D culture techniques using semi-immortalized cNF cells (cNF93.1a) (Muir et al., 2001), either in a single culture or in cocultures combined with related primary FBs (Fb93.1) to create a quantifiable platform for preclinical drug testing (Mattingly et al., unpublished data). Furthermore, 3D coculture models for cNFs using iPSC-derived SCs together with cNF-derived primary FBs have been generated and successfully used for drug testing (Mazuelas et al.[1]), similar to the earlier described neurofibromaspheres (Mazuelas et al., 2022).

In addition to human cell—based systems, a porcine 3D cNF in vitro system that forms spheres in culture was generated from the $NF1^{R1947*/+}$ Ossabaw minipigs. Furthermore, the Topilko laboratory created a murine 3D in vitro model from $Nf1^{-/-}$ boundary cap (BC)—derived stem-like cells from the cNF of $Prss56^{Cre}$, $NF1^{fl/fl}$, $Rosa26^{Tom}$, and *Nf1*-knockout (KO) mice (Coulpier et al., unpublished data). This neurosphere cell line has been successfully used for 3D in vitro drug studies.

Organoids/tumoroids.—cNF organoids from primary cNFs of patients with NF1 were recently generated using a specific geometry to elucidate disease biology and for high-throughput drug screening (Nguyen et al., 2022[2]). Using this technology, several effective candidates against cNF cells were identified after testing 43 kinase inhibitors on cNF organoids established from two patients (Nguyen et al., 2022[2]). Finally, cNF tumoroids were generated using a modified 3D murine skin graft culture system (Liao et al., 2016). These skin grafts were composed of $Nf1^{-/-}$ skin neurosphere cells cocultured with $Nf1^{+/-}$ dorsal root ganglia (DRGs), sciatic nerves, and WT human foreskin FBs on a layer of collagen type I. The grafts could be cultured for up to 5 months, and the skin neurosphere cells were neurotropic, were infiltrative to the nerve tissue, and could differentiate into SCs. Furthermore, this skin graft system can be used to define the interactions and spatial relations between different cell types in cNFs and test the effects of various extracellular components. It is also amenable to preclinical drug testing.

IN VIVO MODELS FOR cNFs

Several in vivo models have been developed to study cNF tumor biology, host—tumor interaction, the role of the neural environment and microenvironment, as well as therapeutic discovery for both prevention of cNF development and treatment of emergent cNFs. In general, pharmacologic animal models of human disease are imperfect proxies to predict therapeutic efficacy in humans. This is particularly true for NF1-associated cNFs for

[2]Nguyen HTL, Kohl E, Bade J, Eng SE, Tosevska A, Al Shihabi A, et al. A rapid platform for 3D patient-derived cutaneous neurofibroma organoid establishment and screening, 2022. Preprint in bioRxiv; DOI: 10.1101/2022.11.07.515469.

several reasons (Table 2). First, it has to be acknowledged that because of the large diversity of *NF1* gene alterations in humans (Bergoug et al., 2020) and poor genotype—phenotype correlation, any experimentally induced inactivating germline pathogenic variant in animals will not recapitulate the entire human disease but rather model a limited spectrum of symptoms. Second, animal skin differs anatomically from human skin. Third, the pathophysiology of cNFs is complex (i.e., multicellular tumors with multiple different *NF1* variants) and only partially understood (Brosseau et al., 2021). Fourth, human cNFs vary widely in presentation, number, and growth rate. Finally, there are no drugs that have been proven successful in human cNF by which animal models could be validated. Thus, currently available models will provide only partial insights into human cNF and therapeutic response, and accordingly, a broad range of models, each contributing to our understanding of cNFs and potential treatments, is needed.

Nonmammalian models

Drosophila has many of the key organs impacted by NF1, including the brain, peripheral nerves, and heart muscle. Although there are key structural differences between the organs of the fly and those of humans, expressing human *NF1* in *Nf1*-mutant flies can rescue multiple NF1-dependent phenotypes (Hannan et al., 2006). Studies in *Drosophila* have emphasized the complexity of *Nf1* downstream signaling, including the function of neurofibromin in both the RAS and cAMP pathways, later confirmed in a murine model (Brown et al., 2012; Dasgupta et al., 2003; Guo et al., 2000, 1997; Tong et al., 2002). The structure of *Drosophila* peripheral nerves is similar to that of human: motor and sensory nerve processes are enwrapped by peripheral and perineurial glia for peripheral nerves, analogous to our Schwann and perineurial cells, respectively. Activating the *NF1* target RAS1 ($RAS1^{G12V}$) in the peripheral glia mirrored aspects of cNF in patients (Lavery et al., 2007) and promoted perineurium enlargement (Johannessen et al., 2005).

Mammalian models

Murine models.—Genetically engineered mouse models (GEMMs) that enable the spontaneous formation of cNFs have allowed for key insights into cNF genesis (Table 3). GEMMs for cNFs rely on the Cre-recombinase/LoxP system, which conditionally knocks out the *Nf1* gene in specific cell stages of the NS—SC lineage, whereas contributory environmental factors, for example, ultraviolet radiation, have not yet been explored. Although there are documented differences between the structure and physiology of rodent and human skin, these rodent models can provide valuable insight into mechanistic studies, including characterizing the cell of origin, genomics, transcriptomics, immunologic contributions to cNF progression, and preclinical drug testing in specific biologic settings.

HoxB7-Cre; $Nf1^{fl/fl}$ and Sox10-CreERT2; $Nf1^{fl/fl}$ mouse models.—Conditional deletion of *Nf1* in the HOXB7 (homeobox 7) lineage in mice recapitulated the development of both human cNFs and pNFs (Chen et al., 2019). HOXB7 is a transcription factor expressed in migrating NC cells, the cell population that generates SCs (López et al., 1995). Lineage tracing using *Hoxb7* in *Rosa26-lacZ* mice showed that *Hoxb7*-lineage cells give rise to DRG cells, peripheral nerves, and nerve endings in the dermis of the skin. Furthermore, *HOXB7* expression is present in SCs in human cNFs. To assess whether HOXB7 is

expressed in the cells that give rise to cNFs, isolated skin neurosphere cells from *Hoxb7-Cre; Nf1*$^{fl/fl}$ and *R26-lacZ* mice were implanted into the sciatic nerve of naked mice. These allograft mice developed neurofibromas that were positive for *lacZ* expression, showing that *Hoxb7*-lineage cells indeed give rise to neurofibromas. By comparison, analysis of the skin in *Hoxb7-Cre, Nf1*$^{fl/fl,}$ and *R26-lacZ* mice revealed that by age 1 year, a majority of the mice had developed skin lesions with fully pigmented, thickened skin indicative of diffuse infiltrating neurofibroma (Chen et al., 2019), a pattern seen in some patients with NF1. Similar to those in humans, these lesions developed predominantly in the head, neck, and upper limb areas. In addition, at around age 5 months, some of the mice developed pNF in the spinal cord. Thus, *Hoxb7-Cre*–driven deletion of *Nf1* results in the development of diffuse infiltrating cNF and pNF but not the discrete cNFs seen most commonly in patients with NF1.

To model the more common, discrete cNFs, Mo et al. (2021) circumvented early *Nf1* deletion in the *Hoxb7-Cre* mice (which led to diffuse cNFs) and instead used a tamoxifen-inducible *Sox10-CreERT2* driver to delete *Nf1* later in life, after the cells of origin migrated to the dermis. These mice developed discrete papules on their backs and necks that were restricted to the dermis, recapitulating human cNFs. Inducing the mice with tamoxifen when they were aged >1 month resulted in the development of sessile cNFs that protruded from the skin. Some mice also developed pNFs. Importantly, treating cNFs of *Sox10-CreERT2; Nf1*$^{fl/fl}$ mice with the MAPK/extracellular signal—regulated kinase (ERK) kinase (MEK) inhibitor PD0325901 reduced phosphorylated ERK expression and the number of *Sox10*-positive tumor cells (Mo et al., 2021). Thus, this GEMM recapitulates both human discrete cNFs and pNFs and was shown to be a robust preclinical model for drug testing in a proof-of-principle experiment.

Prss56Cre, Nf1-KO mouse model.—The Prss56Cre, *Nf1*-KO mouse model, in which biallelic loss of *Nf1* and expression of the fluorescent reporter Tomato were simultaneously targeted in PRSS56-expressing cells, including the BC cell population, successfully recapitulates the development of cNFs and pNFs and the spontaneous progression of pNFs to malignant peripheral nerve sheath tumor, a rare complication in NF1 (Radomska et al., 2019). BC cells are present during embryonic development, between embryonic day 10.5 and embryonic day 13.5, and were found at the dorsal root (sensory) entry zone and ventral (motor) exit points of all spinal and cranial nerves (Radomska and Topilko, 2017). Using *Prss56*^{Cre} in combination with the Cre-inducible reporter line *Rosa26*^{Tom}, BC cells were shown to give rise to derivatives that migrate along the nerves to the periphery (Radomska and Topilko, 2017). The dorsal BC cell derivatives migrate into the dorsal (sensory) roots and give birth to most if not all myelinating and nonmyelinating SCs. The ventral root BCs expressing PRSS56 migrate along the spinal nerves to reach the dorsal and ventral skin, where they will differentiate into melanocytes and myelinating and nonmyelinating SCs in the hypodermis and dermis, suggesting that skin BC derivatives might be one of the sources for the origin of cNFs (Radomska et al., 2019).

Prss56^{Cre} mice were crossed with *Nf1*$^{fl/fl}$, *Rosa26*^{Tom,} or *Nf1*$^{fl/—,}$ *Rosa26*^{Tom} lines to generate *Nf1-KO* mutants on WT and heterozygous (Nf1) backgrounds, respectively. Both mutants shared the biallelic loss of *Nf1* in the *Prss56*-expressing cells, and both developed

cNFs, suggesting that in this mouse model, the heterozygous background does not play a decisive role in the pathophysiology of neurofibromas (Radomska et al., 2019). cNFs emerged spontaneously at ages 10–12 months on the back and belly skin, and their cellular composition was similar to that of patient cNFs (Radomska et al., 2019). However, in contrast to common patient cNFs, which are prominent and polypoid, mouse cNFs were flat and punctate. Notably, skin trauma (small incisions or bites due to the natural aggression of house-grouped males) accelerated the development of cNFs and probably pNFs, suggesting that inflammation, nerve damage, wound healing, or a combination of all are possible triggers (Radomska et al., 2019). Using a so-called inducible model (*Nf1-KOi),* in which trauma to the skin is induced, more than 90% of $Prss56^{Cre/+}$, $Nf1^{fl/fl}$, and $Rosa26^{Tom/+}$ males developed cNFs by age 4 months (Coulpier et al., unpublished data), thereby enabling the generation of large, uniform cohorts of mice with numerous cNFs necessary for preclinical drug testing.

Porcine models.—Despite their high cost and maintenance, swine cNF models may have several advantages over rodents and are believed to be a better avatar for predicting drug efficacy and toxicology in humans given the similarities in drug metabolism and skin architecture between both species (Swindle et al., 2012). Three publications describe the creation of porcine minipig models of NF1 (Isakson et al., 2018; Rubinstein et al., 2021; White et al., 2018) (Table 3). In two of them, the mutations were produced by TAL effector nuclease—targeted gene editing and homologous recombination (Isakson et al., 2018) or recombinant adeno-associated virus homologous recombination (White et al., 2018) in fetal FBs, followed by somatic cell nuclear transfer to generate fully $NF1^{+/-}$ heterozygous founders that were then propagated. The resultant NF1-mutant minipigs, the $NF1^{R1947*/+}$ nonsense allele in Ossabaw minipigs (Isakson et al., 2018), and the exon 42 deletion mutant $NF1^{+/ex42del}$ in Yucatan minipigs (Uthoff et al., 2020; White et al., 2018), were described in detail. A third paper describes the generation of *NF1* loss-of-function (LOF) mutant alleles (in some cases along with TP53 LOF-mutant alleles) in pigs but were produced by single-cell embryo injection of CRISPR/Cas9 reagents (Rubinstein et al., 2021). This approach was complicated by mosaic offspring, cryptic alleles that were difficult to validate, and off-target editing. Germline transmission of three *NF1*-mutant alleles was achieved by this method, but so far, no detailed analyses of the resultant phenotypes have been described (Rubinstein et al., 2021).

Both of the published porcine models of NF1 that include detailed phenotyping describe the development of spontaneous cNFs (Isakson et al., 2018; Uthoff et al., 2020; White et al., 2018). The cNFs developed primarily (Isakson et al., 2018) or only (White et al., 2018) in uncastrated males, suggesting a strong androgen effect on cNF formation in minipigs. The penetrance of cNF formation was ~40% by age 4 months for the $NF1^{R1947*/+}$ nonsense allele in Ossabaw minipigs (Isakson et al., 2018), and this model has been successfully used for pharmacokinetic and pharmacodynamic studies (Osum et al., 2021). The penetrance of cNF formation was ~55% by age 20 months for the $NF1^{+/ex42del}$ in Yucatan minipigs (White et al., 2018). Both the Ossabaw and Yucatan minipig NF1 models show a similar diffuse cNF pathology, in which the cNF appears as a smooth, raised lesion often covering a large area of skin and increasing in size with time (not multiple discrete lesions). Histologically,

the porcine cNFs contained low cellularity, neoplastic clusters of S100+ SCs, endothelial cells, and eosinophils and mast cells.

FUTURE AREAS OF FOCUS

Despite the high degree of amino acid sequence homology among mammalian species, including humans, mice, rats, pigs, chimpanzees, and dogs (Kaufmann De, 2008), only a few germlines' heterozygous *NF1*-mutant models develop cNFs. Haploinsufficient minipigs bearing germlines *Nf1* mutations are known to spontaneously develop cNFs in the skin (Isakson et al., 2018; Uthoff et al., 2020; White et al., 2018), whereas many germline mouse and rat models do not (Dischinger et al., 2018; Jacks et al., 1994). Only when total *Nf1* inactivation is induced within the neuroectodermal lineage or dermal cells in mice is the cNF phenotype penetrant (Chen et al., 2019; Le et al., 2009; Mo et al., 2021; Radomska et al., 2019; Wu et al., 2008).

To date and to the best of our knowledge, no comparative genomics studies have been performed across species to quantify the effects of genetic background on the cNF phenotype or therapeutic response. It is also unclear how the biology of human cNFs differs from that of mouse and pig cNFs. Given the number of available genetically engineered models and recent efforts to bank human cNF tissue specimens, there is now an unprecedented opportunity to use cross-species experimental approaches to better interrogate cNF biology and therapeutics and, ultimately, to reduce the number of animal subjects required to determine drug effectiveness and improve clinical translatability. Each model system presents specific opportunities and limitations that may be selected for specific applications. As drug therapies continue to be developed for pNFs, these agents may also be assessed in the preclinical models of cNFs. Comparing the similarities and differences between cNFs in humans and animal models or organoids regarding their physiology, appearance (phenotype), and molecular and phenotypic responses to therapy will greatly enhance the ability to determine which model best serves which translational question. In particular, species cross-validation will increase confidence in the translation of drug discovery efforts from preclinical models to clinical trials.

Comparing single-cell analytics between human cNF and the various preclinical models can shed further light on the contributions of the constituent cell types to tumor formation and propagation. This is a tremendous opportunity to evaluate core aspects of cNF pathophysiology and gain insight into the tumor-intrinsic and -extrinsic mechanisms that contribute to pathogenesis as well as how the models are similar and dissimilar to human cNFs and cNF subtypes.

Ultimately, the rapid proliferation of in vitro and in vivo cNF models combined with ongoing therapeutic developments in the field of NF1 and rasopathies provides a ripe opportunity to assess the utility of various cNF models for each stage of therapeutic discovery: from pathophysiologic evaluation to target identification and efficacy assessment.

CONCLUSION

cNFs are tumors of the skin that have a tremendous negative impact on people living with NF1. Although they continue to represent a great unmet medical need, recent scientific developments are accelerating the discovery and likelihood of meaningful therapeutic progress in the near term. The development, characterization, and validation of preclinical models of cNFs are a major advance in the mission to develop efficacious therapies to prevent or treat cNFs. Existing cNF models are highly sophisticated and can meet a range of research needs. All models incorporate at least one type of *NF1* alteration (because loss of *NF1* underlies all cNF development), and some have accounted for somatic alterations. Work is ongoing to characterize how each model can contribute to our understanding of human cNF pathophysiology, which in turn may lead to the identification and validation of therapeutic targets, the establishment of optimal metrics for therapeutic response, and the prediction of clinical success.

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Abbreviations:

2D	two-dimensional
3D	three-dimensional
BC	boundary cap
cNF	cutaneous neurofibroma
DRG	dorsal root ganglion
ERK	extra-cellular signal—regulated kinase
FB	fibroblast
GEMM	genetically engineered mouse model
iPSC	induced pluripotent stem cell
KO	knockout
LOF	loss of function
NC	neural crest
NF1	neurofibromatosis type 1
pNF	plexiform neurofibroma
SC	Schwann cell

WT wild type

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Table 1.

Characteristics of cNF in vitro models

2D Versus. 3D	Species	Cell Type: Primary	Cell Type: Semi-Immortalized	Cell Type: iPSC or ESC derived	Source	Description	Comment	Reference
2D	Human	X			cNF, pNF	Methodology for expanding cNF SCs cultures by differential attachment		Wallace et al., 2000
							Used for testing in vivo tumorigenic properties of cNF-derived SCs	Muir et al., 2001
2D	Human	X			cNF	Methodology for expanding *NF1*$^{+/-}$ cNF SCs		Rosenbaum et al., 2000
2D	Human	X			cNF	Methodology for expanding *NF1*$^{-/-}$ cNF SCs		Serra et al., 2000
							Used for identifying somatic *NF1* pathogenic variants	Maertens et al., 2006; Serra et al., 2001
							Used for genetic diagnosis of mosaic NF1 cases	Maertens et al., 2007
							Used for testing of hormones	Pennanen et al., 2018
							Drug screening	Serra laboratory Mazuelas et al.[1]
2D	Human	X			cNF	Cocultures of 70% of *NF1*$^{-/-}$ SC and 30% of *NF1*$^{+/-}$ FB	Drug screening	Serra laboratory Mazuelas et al.[1]
2D, 3D	pig	X			cNF	cNF-derived SCs and FBs from *NF1*$^{R1947*/+}$ Ossabaw minipigs	Drug testing	Largaespada laboratory (unpublished data); Isakson et al., 2018
2D	Human		X		cNF	cNF-derived semi-immortalized SC lines	*NF1*$^{+/-}$;*NF1*$^{-/-}$ immortalized SCs from cNF, similar to the pNF-immortalized SCs (Li et al., 2016)	Wallace laboratory; Wallace et al. (unpublished data)
2D	Human			X	iPSC derived	Differentiation of *NF1*$^{+/+}$,*NF1*$^{+/-}$ and *NF1*$^{-/-}$ hiPSCs into SLCs	*NF1*$^{+/+}$ and *NF1*$^{+/-}$ hiPSCs SLCs did not form tumors upon engraftment, whereas NF1$^{-/-}$ hiPSCs SLCs did	Mo et al., 2021
2D	Human			X	iPSC derived	*NF1*$^{-/-}$ edited/reprogrammed iPSCs differentiated into NCs and further to SCs	iPSC-derived *NF1*$^{-/-}$ differentiating SCs show a high proliferation and poor differentiation capacity in 2D	Carrió et al., 2019
2D	Human			X	ESC derived	SOX10:GFP reporter hESC line; *NF1*$^{-}$/SMASh	In development Testing of SC behavior in response to different doses of *NF1*. Not suitable for drug testing.	Lee et al. (unpublished data)
2D	Human			X	ESC derived		In development Useful to study cNF pathogenesis and the effect of different levels of NF1 loss on SC development.	Lee et al. (unpublished data)

2D Versus. 3D	Species	Cell Type: Primary	Cell Type: Semi-Immortalized	Cell Type: iPSC or ESC derived	Source	Description	Comment	Reference
3D/organoid	Human	X			cNF	*NF1*$^{+/-}$;*NF1*$^{-/-}$ cells seeded in Matrigel in miniring format	Setting up conditions Drug testing of 43 kinase inhibitors	Nguyen et al., 2022[2]
3D	Human		X		cNF	cNF-derived semi-immortalized SC lines (Wallace et al., unpublished data)	MEKi tested had activity in 3D but not in 2D	Mattingly laboratory (unpublished data)
3D	Human			X	iPSC derived	*NF1*$^{+/-}$;*NF1*$^{-/-}$ hiPSCs into SCs, alone or cocultured with cNF-derived primary fibroblasts	Drug testing	Serra laboratory Mazuelas et al.[1]
3D	Mouse	X			cNF	Spherogenic culture o *Nf1*$^{-/-}$ BC-derived stem-like cells	Drug screening (compared with MEKi) Mechanisms governing spontaneous LOH	Topilko laboratory Coulpier et al. (unpublished data)
3D/ organoid skin raft	Mouse	X			cNF	*Nf1*$^{-/-}$ skin neurosphere cells cocultured with *Nf1*$^{+/-}$ DRGs, sciatic nerves, and FBs on a layer of collagen type I	Interrogate interactions and spatial relations between different cell types; preclinical drug testing	Liao et al., 2016

Abbreviations: 2D, two-dimensional; 3D, three-dimensional; BC, boundary cap; cNF, cutaneous neurofibroma; DRG, dorsal root ganglion; ESC, embryonic stem cell; FB, fibroblast; hESC, human embryonic stem cell; hiPSC, human induced pluripotent stem cell; iPSC, induced pluripotent stem cell; LOH, loss of heterozygosity; MEKi, MAPK/extracellular signal—regulated kinase kinase inhibitor; NC, neural crest; NF1, neurofibromatosis type 1; pNF, plexiform neurofibroma; SLC, Schwannian lineage cell.

Table 2.

Ideal Animal Model and Challenges in cnf

Desired Feature	Challenges
Same genetic basis	Large multifunctional gene with >3,000 gene alterations resulting in disease phenotypes (Bergoug et al., 2020)
Similar anatomy and physiology	Porcine model most closely approximates human skin and drug metabolism but is a more challenging and expensive model for genetic manipulation
Similar pathology and causative mechanism	The pathophysiology of cNF is poorly understood, with at least six different cell types and a complex ECM (Brosseau et al., 2021)
Similar phenotype as endpoint	Human neurofibromas have large variability in presentation
Responsive to known efficacious drugs	There are no proven drugs to treat human cNF
Predictive of prior efficacious drugs	There are no proven drugs to treat human cNF

Abbreviations: cNF, cutaneous neurofibroma; ECM, extracellular matrix. The table was adapted from McGonigle and Ruggeri (2014).

Table 3.

Characteristics of cNF in Vivo Models

Model	Species	Description	Tumor Types	Comments	Reference
Prss56; *Nf1*-KO and Prss56; *Nf1*-Koi	Mouse	cNF cell of origin: boundary cap cell Spontaneous cNF arises in the skin as flat lesions >1 year Induced skin trauma (Prss56; *Nf1*-Koi) at 2 months promotes synchronous emergence of multiple cNF after three steps stages: initiation (2–3 months), progression (3–4 months), and stabilization (>4 months)	cNF pNF pNF–MPNST progression	Suitable for drug prevention and efficacy testing (responsive to a MEK inhibitor) Suitable for studying triggers, for example, inflammation, wound healing Unknown effects of skin injury on cNF	Coulpier et al. (unpublished data); Radomska et al., 2019
HoxB7-Cre; Nf1$^{fl/fl}$ and *Sox10-CreERT2; Nf1*$^{fl/fl}$ mouse models (with tamoxifen induction)	Mouse	cNF cell of origin: Schwann cell precursor Spontaneous cNF arises in the skin as diffuse or sessile lesions Able to induce cNF with tamoxifen	cNF pNF pNF–MPNST progression	Ideal to study cNF biology Suitable for drug testing, responsive to MEK inhibitor PD0325901 Modulation of the Hippo pathway acted as a modifier for neurofibroma tumorigenesis MPNST might limit therapeutic value	Chen et al., 2019; Mo etal., 2021
NF1$^{R1947*/+}$ Ossabaw minipigs	Pig	Spontaneous cNF formation at ~40% by age 4 months primarily in uncastrated males cNF pathology: collagen rich, diffuse, infiltrating	cNF OPG	No drug response data yet Suitable for agents targeting collagen or fibroblast activity Difficult to engineer Costly, difficult husbandry	Isakson et al., 2018
NF1$^{+/ex42del}$ Yucatan minipigs	Pig	Spontaneous cNF formation at ~55% by age 20 months, only in uncastrated males cNF pathology: myxoid, diffuse	cNF	As stated earlier	White etal., 2018; Uthoff etal., 2020.
CRISPR/Cas9-engineered *NF1* and *TP53*-mutant model	Pig	No detailed analyses of the phenotype	unknown	Mosaic offspring	Rubinstein et al., 2021

Abbreviations: cNF, cutaneous neurofibroma; KO, knockout; MEK, MAPK/extracellular signal–regulated kinase kinase; MPNST, malignant peripheral nerve sheath tumor; NF1, neurofibromatosis type 1; OPG, optic pathway glioma; pNF, plexiform neurofibroma.