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REVIEW

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Notch-ing up knowledge on molecular mechanisms of skin fibrosis: focus on the multifaceted Notch signalling pathway

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Abstract

Fibrosis can be defined as an excessive and deregulated deposition of extracellular matrix proteins, causing loss of physiological architecture and dysfunction of different tissues and organs. In the skin, fibrosis represents the hallmark of several acquired (e.g. systemic sclerosis and hypertrophic scars) and inherited (i.e. dystrophic epidermolysis bullosa) diseases. A complex series of interactions among a variety of cellular types and a wide range of molecular players drive the fibrogenic process, often in a context-dependent manner. However, the pathogenetic mechanisms leading to skin fibrosis are not completely elucidated. In this scenario, an increasing body of evidence has recently disclosed the involvement of Notch signalling cascade in fibrosis of the skin and other organs. Despite its apparent simplicity, Notch represents one of the most multifaceted, strictly regulated and intricate pathways with still unknown features both in health and disease conditions. Starting from the most recent advances in Notch activation and regulation, this review focuses on the pro-fibrotic function of Notch pathway in fibroproliferative skin disorders describing molecular networks, interplay with other pro-fibrotic molecules and pathways, including the transforming growth factor-β1, and therapeutic strategies under development.

Keywords: Dermal fibroblasts, Hypertrophic scar, Keloid, Systemic sclerosis, Epidermolysis bullosa, Transforming growth factor-β1, Extracellular matrix, JAG1, NOTCH1, Gamma-secretase inhibitor

Background

Tissue homeostasis and function ground on the equilibrium between extracellular matrix (ECM) synthesis and degradation. However, in specific biological contexts the remodelling cycle of the ECM transiently shifts towards one of its two phases, a phenomenon that occurs for example during the physiological wound healing process. A wide range of genetic, immunological, metabolic and environmental cues can perturb the homeostatic turnover of ECM, leading to fibrosis: the disproportionate and

disorganized accumulation of ECM components, mainly collagens, in various organs [1, 2]. Dermal fibrosis underlies a large and heterogeneous spectrum of pathological conditions which can affect exclusively the skin or where the skin represents one of the targeted organs within the context of a multisystem disorder. Fibrotic diseases involving the skin include disorders due to an aberrant cutaneous wound healing process (e.g. hypertrophic scars and keloids), systemic sclerosis, dystrophic epidermolysis bullosa, chronic graft-versus-host disease, eosinophilic fasciitis and nephrogenic systemic fibrosis [3–5].

Progresses in deciphering the cellular and molecular bases of fibrosis have revealed that a multitude of mechanisms are able to trigger or sustain fibrotic events in a context-dependent fashion. Transforming growth factor

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(TGF)-β1 represents the best-characterized player in fibrosis onset and maintenance [6, 7]. However, other factors, ranging from an inflammatory *milieu* to additional pro-fibrotic pathways, can overlap or support the TGF-β1 activity shaping the fibrotic processes and determining a wide range of disease manifestations [8–10].

Notch signalling is a highly conserved, ubiquitous, cell–cell communication pathway involved in cell fate, proliferation and tissue homeostasis both in embryonic development and adult life [11]. Notch activation requires binding between the Notch receptor exposed on the surface of a "signal-receiving cell" and the Notch ligand on a juxtaposed "signal-sending cell". Receptor-ligand interactions commit Notch receptor to a two-step proteolytic cascade generating a transcriptionally active intracellular fragment. Given its pleiotropic actions, the correct timing and magnitude of Notch activation are crucial to avoid detrimental consequences. In particular, gene mutations in the Notch core pathway members and network components are causative of neoplastic disorders [12–14] and of a number of rare, mainly developmental, diseases affecting almost all body districts [11, 15, 16]. In addition, Notch signalling is dysregulated during the onset and course of a variety of diseases, including fibrosis. Notch can exert a pro-fibrotic role in lung, kidney, liver and skin by regulating myofibroblast activation and epithelial-to-mesenchymal transition (EMT), or by dialoguing with other potent fibrogenic pathways, in particular the TGF-β1 signalling [17].

This review focuses on the pro-fibrotic role of Notch pathway in acquired and inherited fibroproliferative disorders affecting the skin. After briefly introducing the general mechanisms of Notch activation and regulation, we will summarize Notch involvement in tissue fibrosis with a specific emphasis on the Notch crosstalk with ECM components and the TGF-β signalling pathway. Thereafter we will discuss Notch role in skin physiopathology, focusing on the most recent findings on the fibrogenic activity of Notch in systemic sclerosis, hypertrophic scars, keloids and dystrophic epidermolysis bullosa. Finally, the current molecular approaches to counteract Notch activation will be reviewed.

Notch activation, processing and regulation
Notch signalling cascade: from receptor-ligand binding to transcriptional outcomes

In mammals, the Notch family is made of four Notch receptors (Notch 1–4) and five ligands (Jagged 1 and 2, Delta-like 1, 3 and 4) differently expressed depending on the specific cell type and its biological context. Notch activation usually requires the binding between a Notch receptor and a Notch ligand exposed on two different, neighboring cells. Ligand engagement triggers two subsequent proteolytic cleavages (S2 and S3 cleavages) of Notch receptor to release a biologically active Notch intracellular domain (NICD), which translocates into the nucleus where it activates the transcription of its downstream targets, thereby mediating a multitude of biological effects (Fig. 1) [11]. A "pulling force" exerted by the Notch ligand toward its tied receptor drives Notch activation by unmasking an ADAM (short for A Disintegrin And Metalloproteinase) metalloprotease cleavage site at the level of the Notch regulatory region (NRR), close to Notch receptor transmembrane segment [18, 19]. The NRR site is targeted by ADAM proteins, which operate the first ligand-induced proteolytic cleavage (S2 cleavage) and produce the Notch extracellular truncation (NEXT) fragment. In the second activation step, the NEXT fragment is cleaved by the γ-secretase complex (S3 cleavage) to generate the NICD protein product (Fig. 1) [20, 21].

Although the mature Notch receptor usually interacts with its Notch ligand by direct cell–cell contact (transactivation), several evidences indicate that Notch signalling can also take place over long distances through dynamic filopodia [25, 26], basal protrusions in epithelial cells [27] or exosomes [28, 29].

In the nucleus, NICD interacts with the DNA-binding protein RBPJ (recombination signal binding protein for immunoglobulin kappa J region) and the co-activator Mastermind-like protein 1 (MAML1) creating a ternary complex able to trigger target gene transcription. A growing body of evidence indicates that RBPJ acts as transcription repressor in the absence of NICD, whilst the recruitment of NICD determines the transition from an "OFF" to a transcriptionally active state by complex mechanisms including (i) the displacement of a set of RBPJ-bound co-repressors, (ii) modifications in RBPJ affinity/specificity for selected DNA loci or (iii) chromatin rearrangements favouring accessibility and function of distal enhancer elements [30–32]. In mammals, the prototypical bona fide Notch target genes are represented by the basic helix-loop-helix (bHLH) transcriptional factors of the hairy and enhancer of split (HES) family, including *HES1* and *HES5*, as well as by the *HEY* gene family (HES related with YRPW motif) such as *HEY1* [30]. HES and HEY family members act as transcriptional repressors of gene expression by direct and indirect mechanisms including the recruitment of histone deacetylases or the heterodimeric binding of specific bHLH activators, respectively [33]. HES and HEY factors critically drive differentiation of various cell types, and, for these reasons, are tightly regulated and subjected to rapid cycles of activation and repression [33]. Beyond *HES* and *HEY* genes, multiple factors including (i) the context-dependent accession by Notch transcriptional complex to specific DNA response elements, (ii) the NICD cooperation
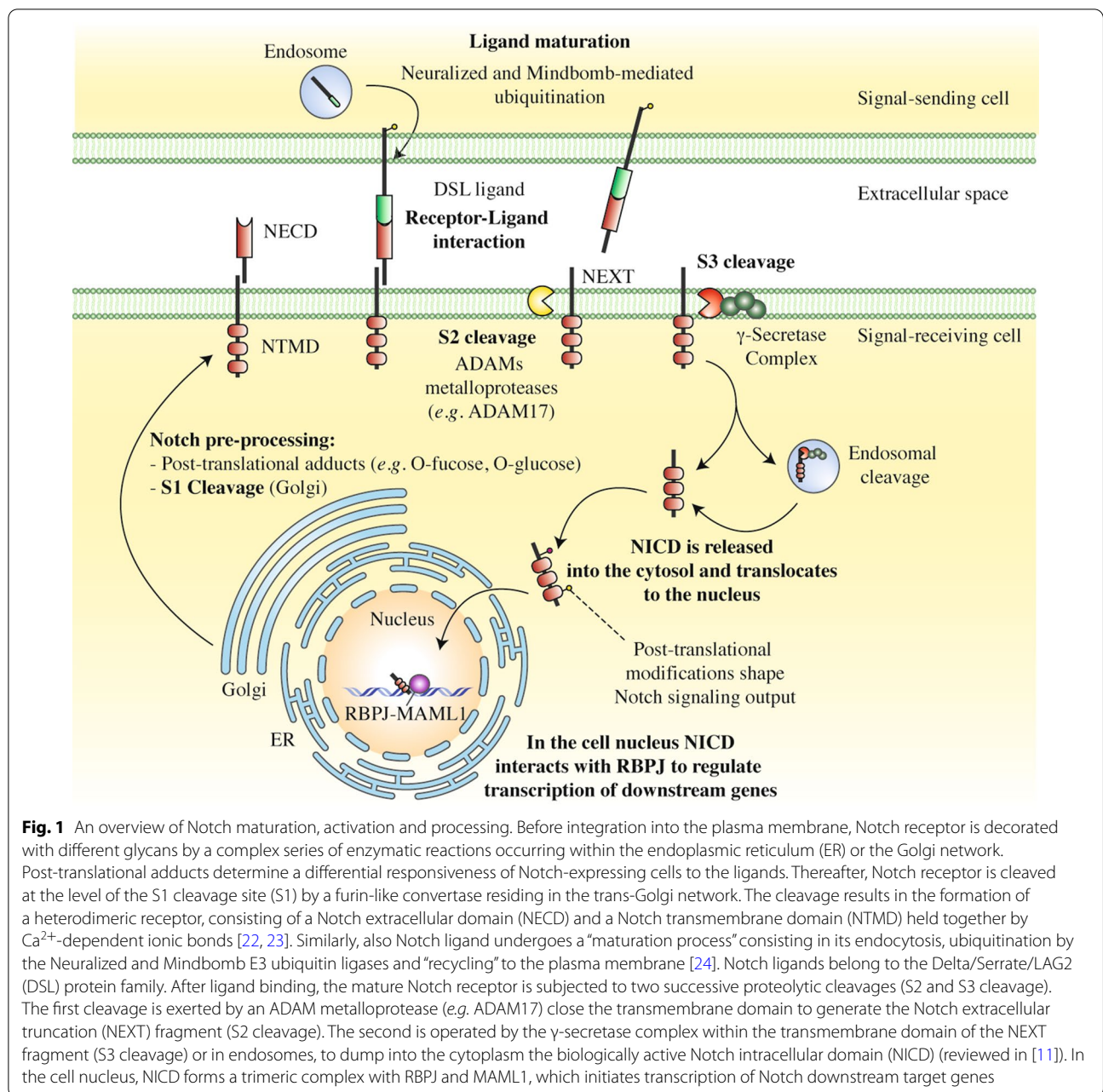
Fig. 1 An overview of Notch maturation, activation and processing. Before integration into the plasma membrane, Notch receptor is decorated with different glycans by a complex series of enzymatic reactions occurring within the endoplasmic reticulum (ER) or the Golgi network. Post-translational adducts determine a differential responsiveness of Notch-expressing cells to the ligands. Thereafter, Notch receptor is cleaved at the level of the S1 cleavage site (S1) by a furin-like convertase residing in the trans-Golgi network. The cleavage results in the formation of a heterodimeric receptor, consisting of a Notch extracellular domain (NECD) and a Notch transmembrane domain (NTMD) held together by Ca^{2+}-dependent ionic bonds [22, 23]. Similarly, also Notch ligand undergoes a "maturation process" consisting in its endocytosis, ubiquitination by the Neuralized and Mindbomb E3 ubiquitin ligases and "recycling" to the plasma membrane [24]. Notch ligands belong to the Delta/Serrate/LAG2 (DSL) protein family. After ligand binding, the mature Notch receptor is subjected to two successive proteolytic cleavages (S2 and S3 cleavage). The first cleavage is exerted by an ADAM metalloprotease (*e.g.* ADAM17) close the transmembrane domain to generate the Notch extracellular truncation (NEXT) fragment (S2 cleavage). The second is operated by the γ-secretase complex within the transmembrane domain of the NEXT fragment (S3 cleavage) or in endosomes, to dump into the cytoplasm the biologically active Notch intracellular domain (NICD) (reviewed in [11]). In the cell nucleus, NICD forms a trimeric complex with RBPJ and MAML1, which initiates transcription of Notch downstream target genes

with different co-activators, and (iii) the effects of post-translational modifications (PTMs) and epigenetic modulators (see below) underlie the qualitatively divergent Notch-derived transcriptomes and determine the exceptionally wide range of Notch-based biological responses [11, 34].

Regulation of Notch pathway by post-translational modifications

Notch signalling is under the control of multiple mechanisms in each phase of its complex intracellular routes

[35]. A variety of regulatory processes modulate Notch receptor and ligand expression, maturation, localization, trafficking and stability prior, during and after the ligand-receptor interaction at the plasma membrane. An important role in controlling Notch pathway is played by post-translational modifications (PTMs) that occur at different stages of Notch member molecular life course. Indeed, both Notch receptors and ligands can undergo glycosylation, methylation, hydroxylation, acetylation, ubiquitination, and, not least, phosphorylation [34]. Notch PTMs cooperate to finely tune the pathway

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functioning or to terminate the signal [36]. For a recent review of PTM-mediated regulation of Notch pathway see [34]. Among PTMs, ubiquitination of NICD by the ubiquitin E3 ligase FBXW7 (also known as SEL10) has been validated as a key mechanism to switch-off Notch effects [37]. Of note, FBXW7-dependent ubiquitination requires the concomitant phosphorylation of specific amino acid residues within the Pro-Glu-Ser-Thr (PEST) domain of NICD, by different kinases, including the Down-syndrome-associated kinases DYRK1A and DYRK1B [38] and the cyclin-dependent kinase 8 (CDK8) [39]. Thus, PTMs and their interplay represent an additional wall to break down in understanding the complexity of Notch cascade, and potential druggable targets to modulate Notch activity in disease conditions [34].

Epigenetic regulation of Notch signalling

microRNAs (miRNAs or miRs) are a class of non-coding RNAs (nc-RNAs) that regulate gene expression at the posttranscriptional level. Each miRNA binds to specific messenger RNAs (mRNAs), thus indicated as miRNA targets, leading to their degradation or translational inhibition. miRNAs are involved in almost all physiological and pathological events, including the fine regulation of the Notch pathway. Indeed, an ever-growing number of miRNAs have been reported as modulators of the Notch pathway [40–43]. A list of miRNAs experimentally-validated by gene reporter assay as negative regulators of Notch receptors and ligands is available as Supplementary Information [see Additional file 1: Table S1].

The impaired regulation of miRNAs targeting Notch pathway members contributes to many disease conditions, including fibrosis of various organs [43–47]. Notably, the Notch signalling pathway can also up- or down-regulate the expression of specific miRNAs. For instance, Notch activation in vascular smooth muscle cells (VSMCs) results in a direct, RBPJ-dependent, up-regulation of miR-143/145 cluster which in turn cooperates with Jagged 1 (JAG1)/Notch signalling to promote VSMC contractile phenotype [48]. On the other hand, the Notch-RBPJ-MAML1 complex can repress miRNA expression, as described in CD4 T cells for miR-29 family members [49].

Long nc-RNAs (lncRNAs) are a broad and heterogeneous class of RNA molecules (> 200 nucleotides in length) including intergenic transcripts and sense/antisense RNAs overlapping protein-coding genes. Functional studies revealed that lncRNAs can act both as enhancers and repressors of gene expression through in cis and in trans mechanisms. However, the roles and biological relevance of the vast majority of them remain elusive [50]. As for the interplay between Notch signalling and lncRNA activity, it has been shown that the lncRNA

NEAT1 (Nuclear-Enriched Abundant Transcript 1) controls miR-129-5p levels and indirectly regulates the abundance of NOTCH1, a miR-129-5p target, in rat astrocytes [51]. In neural progenitors, the lncRNA LncND (Neuro Development) controls miR-143-3p activity, and in turn the expression levels of NOTCH1 and NOTCH2, two miR-143-3p targets [52]. On the other hand, the Notch pathway drives the expression of the pro-tumorigenic lncRNA LUNAR1 (Leukemia-Associated Non-coding IGF1R Activator RNA 1) in T-ALL [53] and colorectal cancer [54]. Review articles focused on the crosstalk between Notch signalling pathway and lncRNAs have been recently published [55, 56].

Besides miRNA- or lncRNA-based regulatory mechanisms, other processes such as histone [57–60] and mRNA modifications, the latter known as epitranscriptome, have been demonstrated to regulate Notch activity in a cell-specific manner [61, 62]. In particular, the reversible methylation of the N6 position of specific adenosine bases (m^6A) within target mRNAs and nc-RNAs in eukaryotic cells has recently emerged as a pervasive modulator of gene expression, with roles in health and disease conditions. Briefly, m^6A influences the mRNA structure and the binding of specific regulatory proteins, with implications in splicing, nuclear retention, mRNA stability and translation efficiency [62–66]. As for the interplay between Notch and m^6A, a recent study revealed that METTL3 (methyltransferase like 3), a subunit of the N6-methyltransferase complex, is able to methylate several Notch transcripts and potentiate Notch activity in glioma stem-like cells [62].

Notch pathway in tissue fibrosis

Fibrosis is a pathological condition marked by excessive deposition of fibrous connective tissue in an injured or inflamed tissue. It can affect all organs resulting in disruption of the physiological tissue architecture and function [67]. A wound healing (WH) response ensues any tissue injury to rapidly restore homeostasis. The WH process is classically resumed in four successive but overlapping phases: haemostasis, inflammation, new tissue formation (or proliferative phase) and remodelling. In the WH context, fibrosis is due to several interlinked mechanisms that affect the proper magnitude and spatiotemporal sequence of the WH phases, leading to a chronic WH response [68, 69], with continued tissue damage, repair and regeneration. Different acute or chronic stimuli including infections, autoimmune and inflammatory reactions and mechanical injury contribute to fibrosis onset, often in a disease-dependent manner [67].

The phenoconversion of fibroblasts (FBs) and other mesenchymal precursor cells into a highly specialized cell type called myofibroblast is a crucial process that triggers

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and sustains fibrogenesis in all fibrotic diseases. Myofibroblasts are characterized by contractile and secretory abilities given by the production of specific contractile proteins (*e.g.* α-SMA, α-smooth muscle actin) and secretion of ECM matricellular proteins [70, 71]. Of note, many of the classical fibrogenic signalling pathways such as TGF-β, platelet-derived growth factor (PDGF), WNT and hedgehog (Hh) are strictly connected and cooperate to induce myofibroblast differentiation and persistence, driving disease progression and maintaining it over time [2, 7, 8].

Notch and fibrosis
Notch signalling is emerging as a potent inducer of fibrosis in liver, lung, kidney and skin [17, 72–74]. The Notch cascade is involved in FB proliferation [75], myofibroblast differentiation [74, 76], contractile phenotype induction and acts synergistically with other pro-fibrotic pathways, primarily the TGF-β signalling. These pro-fibrotic features are particularly evident in VSMCs, where: (i) the JAG1-NOTCH1-RBPJ axis and the myocardin (MYOCD) signalling synergistically activate expression of smooth muscle cell (SMC) marker genes such as myosin, heavy polypeptide 11, smooth muscle (MYH11) and transgelin (TAGLN) [77] and (ii) JAG1/NOTCH3 interactions lead to the induction of contractile marker proteins (α-SMA and calponin—CNN1) and, in turn, of the contractile phenotype in in vitro SMCs-endothelial cells 3D coculture models [78] as well as in VSMC disorders driven by inactivating mutations in the NOTCH3/TGF-β regulator gene *HtrA* serine peptidase 1 (HTRA1) [79]. Moreover, Noseda and coll. demonstrated that the human *ACTA2* gene, encoding α-SMA, contains a RBPJ consensus binding site, whose activation is necessary and sufficient to obtain a Notch-mediated α-SMA transcription in endothelial cells and primary FBs [80–82]. In addition to its direct effects in regulating myofibroblast differentiation as well as the synthetic/proliferative and contractile features of activated FBs, Notch signalling possesses an important role in inflammation and EMT [83–85], two processes involved in fibrosis outcome. However, studies in this field, in relation to fibrotic mechanisms, are still limited.

Notch cross-talk with the extracellular matrix and its pro-fibrotic components
The extracellular matrix (ECM) is the non-cellular constituent of all tissues and organs. It contains a hydrated multifaceted mixture of macromolecules (*e.g.* fibrous glycoproteins, glycosaminoglycans, proteoglycans) which is synthetized by all resident cells, especially by FBs [86–88], in a tissue- and context-dependent manner [89, 90]. ECM works as a structural scaffold but also importantly

as a biologically active system, able to modulate the behaviour of the surrounding cells and orchestrate a plethora of processes including cell proliferation, motility, differentiation, polarity and WH. Given its pleiotropic roles, ECM homeostasis is crucial for health maintenance [91–93]. Indeed, ECM architecture and composition are altered in multiple pathological conditions, including fibrosis [94–96]. Specific to fibrosis, it is well established that ECM composition and mechanical properties strongly impact on the bio-availability and activity of key anti- and pro-fibrotic factors involved in fibrosis, first and foremost the fibrogenic growth factor TGF-β1 [94, 95, 97–99].

At the cellular level, surface receptors and proteins decorating the membranes act as effective "mechanosensors" able to intercept biochemical and biophysical ECM modifications (*e.g.* composition, force and rigidity—the so-called ECM stiffness) and to convert them into molecular and functional inputs through the induction of intracellular signalling cascades [100].

The pro-fibrotic Notch pathway is also emerging as a "sensing system" able to intracellularly transmit a variety of microenvironmental cues, including chemical and physical ECM modifications [101]. On the other hand, ECM modulates Notch signalling activation through direct and indirect mechanisms involving both core ECM components and ECM-related pathways. A pertinent example is represented by MAGP-2 (Microfibril Associated Glycoprotein-2)—a microfibril/elastin network structural component also involved in fibrosis [102]—which directly interacts with the tandem EGF-like repeats in DSL (Delta/Serrate/LAG2) ligands and NOTCH1 receptor. MAGP-2-NOTCH1 interactions promote the receptor heterodimer dissociation and its activation through an ADAM-independent mechanism [103, 104]. On the contrary, in endothelial cells MAGP-2 co-operates with the integrin αVβ3 to recruit the c-SRC kinase which phosphorylates N1ICD at specific tyrosine residues, leading to a reduction of its half-life and transcriptional ability [105, 106]. Additional ECM proteins able to modulate Notch activation via direct or indirect routes include periostin (POSTN), an ECM matricellular protein which binds NOTCH1 and preserves its expression in stress conditions [107], type I and type IV collagens [108] and laminin-111 [109].

Notch and TGF-β1: the fibrogenic dialogue
The TGF-β pathway is a paradigm of signal transduction mechanisms initiated by the activation of a serine/threonine kinase receptor at the plasma membrane. Briefly, TGF-β cascade is triggered by the binding between a member of the TGF-β and BMP (Bone Morphogenetic Protein) ligand subfamilies, and a ligand-specific

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heteromeric receptor complex [110]. Then, the signal is propagated by the receptor-mediated recruitment and phosphorylation activation of the cytoplasmic SMAD proteins (SMADs) (TGF-β canonical pathway) or by alternative molecular cascades, in particular the MAPK (Mitogen Activated Protein Kinase) signalling (TGF-β non-canonical pathway) [110–112]. Phosphorylated SMAD2 and SMAD3 proteins form a complex with SMAD4 to convey signals from TGF-β receptors into the nucleus, where SMADs act as transcriptional factors. The intricate interplay of ligand and receptor types, SMADs and cofactors as well as of signal-driven transcription factors (SDTFs) and lineage-determining transcription factors (LDTFs) underlies the various TGF-β-dependent transcriptional outcomes in relation to specific cell contexts and biological conditions [110].

Notably, TGF-β1 signalling plays an outstanding role in fibrosis of several tissues and organs, due to the potent induction of ECM protein synthesis and myofibroblast differentiation [113, 114]. Importantly, TGF-β-mediated modifications of the ECM composition and properties (e.g. ECM stiffness) contribute to perpetuate, in a vicious-cycle, myofibroblast activity and TGF-β production [97, 114].

Several reports highlighted a cooperation between different Notch and TGF-β pathway members in a variety of cellular contexts and biological processes [83, 115–119], including fibrosis.

The Notch pathway members JAG1 and HES1, are early transcribed in response to TGF-β stimulation in HaCaT keratinocytes [115]. Similarly, the TGF-β/Notch axis synergistically acts to promote the transcription of Hes1 in chicken embryos, neural stem cells, myoblasts and epithelial cells through SMAD3 and SMAD4 activation [83, 120]. Interestingly, SMAD3 is able to directly interact with NICD and can be recruited to RBPJ-binding sites on DNA in the presence of RBPJ and NICD in C2C12 myoblast cells [120]. In the context of fibrosis, the Notch/TGF-β axis cooperates to induce fibrosis in several tissue and organs, and its pharmacological targeting represents a powerful strategy to counteract the fibrogenic process [121–124]. The dialogue between Notch and TGF-β induces the expression of contractile and pro-fibrotic markers (e.g. α-SMA, CNN1 and TAGLN) in different cell types including SMCs [125], mesenchymal stem cells (MSCs) [126], lung FBs [122] and RLE-6TN rat alveolar epithelial cells [127]. In RLE-6TN cells, the cis-elements CArG box ($CC(A/T)_6GG$ box) and TCE (TGF-β control element) lying in the promoter of *ACTA2* gene are critical for the transcriptional induction of α-SMA in response to both TGF-β and NICD [127]. The well-established role of Notch in contractility-associated gene expression, coupled with its mechanosensitive properties [27] and its

TGF-β-mediated induction in response to mechanical stress [128], suggest that the fibrosis-driven activation of Notch signalling could prime or enforce, in a self-powered loop, the fibrotic behaviour of myofibroblasts.
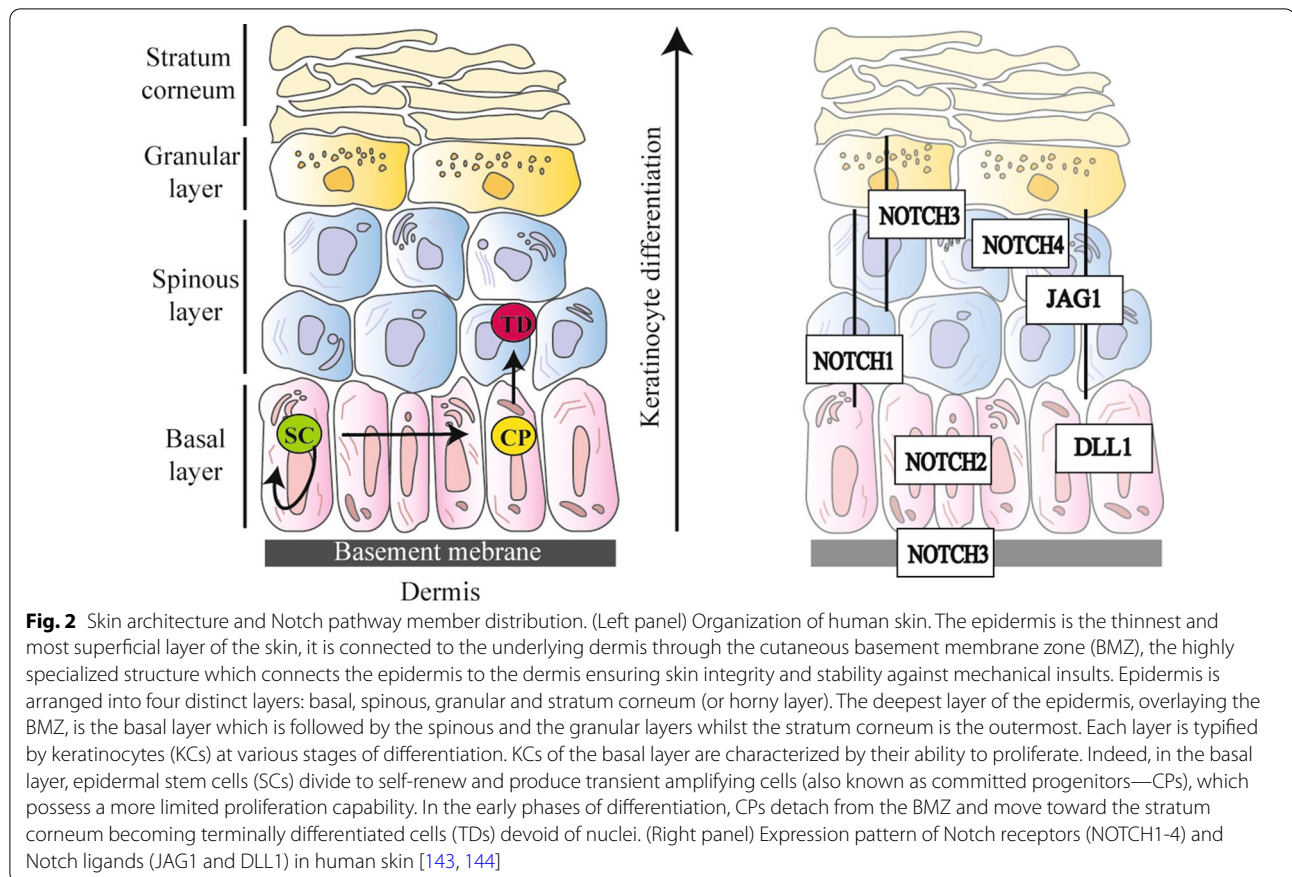
The Hippo member Yes-associated protein 1 (YAP1) is emerging as an important mechanosensitive system, able to convert external ECM-dependent inputs into pro-fibrotic outputs at the intracellular level. In TGF-β1–treated FBs as well as in primary myofibroblasts from patients with Dupuytren disease, a fibroproliferative disorder of the hands and fingers, YAP1 activation leads to induction of a pro-fibrotic phenotype with increased ECM production and cell contractility [129]. Interestingly, the two highly related transcriptional cofactors YAP and TAZ can also interact with the Notch cascade by different modalities in relation to the different cellular contexts. In general, YAP/TAZ can (i) mediate transcriptional regulation of Notch family members; and (ii) cooperate with Notch to transcribe common, downstream target genes [130].

Beyond the TGF-β, a cross-talk between Notch and the fibrogenic Wnt pathway [131] has been shown to occur at multiple levels ranging from a reciprocal regulation to an opposite or synergistic activity [119, 132–137]. Despite the well-established relationship between Notch and Wnt signalling, mechanistic studies exploring their interplay in fibrotic disease models are still missing [138].

Notch expression and role in skin physiology and pathology

Notch in keratinocyte differentiation and proliferation
From a simplified perspective, human skin is composed of a stratified squamous epithelium, the epidermis, standing above a connective tissue, the dermis (Fig. 2, left panel). Epidermal tissue is continually self-renewing, and keratinocytes (KCs), the major cell type of the epidermis, represent its foremost "shaping force". Epidermis is organized into four distinct layers typified by KCs at various stages of differentiation (Fig. 2, left panel). In brief, moving upward from the basal layer to the top of the epidermis (i.e. the horny layer) KCs undergo a complex series of molecular events that progressively modify their features, such as morphology and keratin type expression and organization, and commit the cell to terminal differentiation (TD) (Fig. 2, left panel). Epidermal homeostasis rests on a perfect equilibrium between KC proliferation and differentiation programs. The Notch signalling pathway exerts a crucial role in regulating and maintaining skin homeostasis, orchestrating KCs differentiation at the level of inter-follicular epidermis (IFE) and hair follicles (HFs), and finally working in epithelial barrier formation [139–142]. The analysis of Notch signalling components in skin revealed that Notch receptors and ligands exhibit

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Fig. 2 Skin architecture and Notch pathway member distribution. (Left panel) Organization of human skin. The epidermis is the thinnest and most superficial layer of the skin, it is connected to the underlying dermis through the cutaneous basement membrane zone (BMZ), the highly specialized structure which connects the epidermis to the dermis ensuring skin integrity and stability against mechanical insults. Epidermis is arranged into four distinct layers: basal, spinous, granular and stratum corneum (or horny layer). The deepest layer of the epidermis, overlaying the BMZ, is the basal layer which is followed by the spinous and the granular layers whilst the stratum corneum is the outermost. Each layer is typified by keratinocytes (KCs) at various stages of differentiation. KCs of the basal layer are characterized by their ability to proliferate. Indeed, in the basal layer, epidermal stem cells (SCs) divide to self-renew and produce transient amplifying cells (also known as committed progenitors—CPs), which possess a more limited proliferation capability. In the early phases of differentiation, CPs detach from the BMZ and move toward the stratum corneum becoming terminally differentiated cells (TDs) devoid of nuclei. (Right panel) Expression pattern of Notch receptors (NOTCH1-4) and Notch ligands (JAG1 and DLL1) in human skin [143, 144]

stringent qualitative and quantitative expression patterns within the different areas of IFE and HFs [140, 143, 144] (Fig. 2, right panel). As for the specific effects of Notch receptors and ligands in human KCs, functional investigations revealed that NOTCH1 reduces the proliferative rate of stem cells (SCs) compartment, while NOTCH2 and NOTCH3 cooperate to induce TD [145]. The antiproliferative role of NOTCH1 has been also documented in mouse epidermis [146] and is in line with its tumour suppressor function in squamous cell carcinoma (SCC) [147]. For these reasons, dysregulation of Notch signalling can contribute to the pathomechanisms of various skin disorders and syndromes marked by an altered KC proliferation/differentiation rate including Adams-Oliver syndrome—a congenital disorder characterized by terminal transverse limb malformations, skin and skull bone defects—as well as psoriasis and atopic dermatitis [148].

Notch in fibroproliferative skin diseases

Multiple lines of evidence revealed that Notch activation is involved in the pathogenesis of skin fibroproliferative diseases, both acquired (e.g. dermatofibromas, hypertrophic scars, keloids and systemic sclerosis) and inherited (i.e. dystrophic epidermolysis bullosa, DEB).

A comparative immunohistochemical analysis of skin biopsies from patients affected with different fibroproliferative diseases revealed that NICD staining, suggestive of Notch pathway activation, varies in a cell-dependent manner according to the disease types (Fig. 3) [149]. In particular, abundant expression of NICD was present in FBs from keloids, hypertrophic scars and dermatofibromas, while it was barely detectable in FBs from normal control skin. The following paragraphs describe the most recent advances in Notch-mediated mechanisms of fibrosis in skin diseases.

Systemic sclerosis

Systemic sclerosis (SSc) is an autoimmune connective tissue disorder of unknown etiology affecting the skin, muscles and multiple internal organs, characterized by a highly heterogeneous evolution and outcomes. In SSc patients, the hyperactivated immune response against a multitude of autoantigens leads to vascular dysfunction [150], inflammation and fibrosis of the skin and visceral organs [151]. The molecular mechanisms underlying SSc onset are intricate, and many pathways contribute to its pathogenesis, including the signalling cascade initiated by TGF-β [152] and the Notch pathway [153].
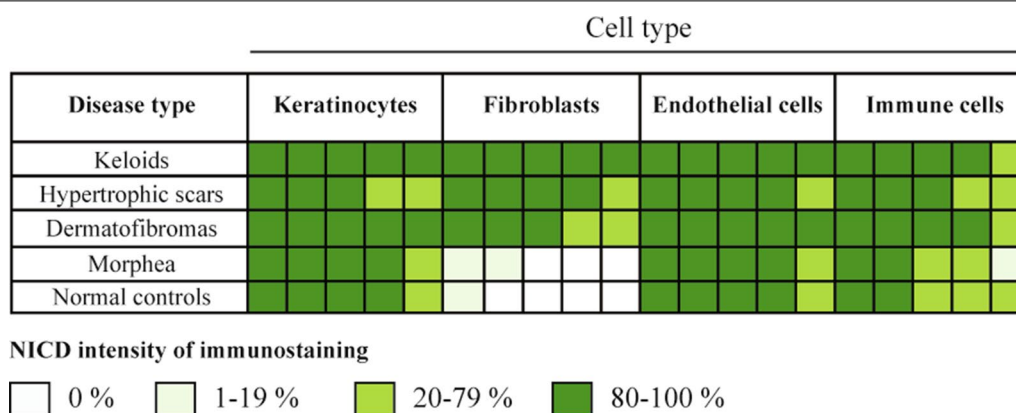
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Fig. 3 NOTCH1 activation in fibroproliferative skin diseases. Immunohistochemical staining pattern showing the expression levels of the cleaved, biologically active NOTCH1 protein (NICD, Notch intracellular domain) in skin biopsies from patients affected with four different fibroproliferative diseases and normal controls. In general, NICD is constitutively expressed in keratinocytes, endothelial cells and immune cells of patients affected with the various fibroproliferative disorders and healthy biopsies, with no significant variation among them. As for NICD expression in fibroblasts, it was generally low in morphea and barely detected in healthy controls. On the other hand, Notch pathway is activated in fibroblasts from keloids, hypertrophic scars and dermatofibromas. Figure adapted from [149]

In vivo and in vitro evidences showed that Notch signalling is activated in SSc and drives fibrosis [76, 154]. High levels of NICD and JAG1 have been found in lesional skin biopsies as well as in cultured skin FBs from SSc patients. Interestingly, in SSc fibrotic skin the expression of JAG1 ligand is particularly prominent in inflammatory infiltrates, suggesting that T-cells might contribute to activate Notch cascade in resident FBs [76]. Pharmacological and siRNA-mediated inhibition of Notch signalling in primary SSc FBs reduces *COL1A1* and *COL1A2* expression and α-SMA abundance. On the other hand, healthy FBs treated with the recombinant JAG1 protein (Jag1-Fc) show an increased release of ECM and fibroblast-to-myofibroblast differentiation [76]. Inflammation- and ROS-depended hyperactivation of ADAM17 could represent a mechanism underlying the aberrant levels of NICD in SSc skin [154–156].

As for epigenetic involvement in SSc fibrosis, Wasson and coll. recently described the up-regulation of the lncRNA HOX transcript antisense RNA (HOTAIR) in primary SSc FBs, and validated its involvement in fibrogenesis via the EZH2-mediated activation of Notch signalling [47]. In addition, exosomes (EXOs)—small membrane-bound vesicles of endocytic origin involved in cell–cell communication [157, 158]—derived from serum [159] and neutrophils of SSc patients [160] display a specific set of differentially expressed miRNAs and lncRNAs involved in fibrosis and regulation of profibrotic pathways, including Notch [159, 160]. Imatinib is a tyrosine kinase (TK) inhibitor, with an anti-inflammatory and anti-fibrotic role in preclinical and clinical models of SSc and other disorders [161, 162]. Notably,

recent pharmacokinetic analyses revealed that the Notch pathway also controls imatinib uptake in SSc FBs through the reduction of expression levels of key organic molecule transporters, such as the multidrug and toxin extrusion transporter MATE1 [163]. Finally, specific *NOTCH3* polymorphisms correlate with an increased susceptibility to develop various forms of SSc, suggesting that they can prime disease pathomechanisms or modulate clinical manifestations of fibrosis [164].

Hypertrophic scar

Hypertrophic scar (HS), clinically appearing as a raised scar confined to the site of injury, is a common WH complication, deriving from aberrant abnormal proliferative and remodelling phases on a background of genetic susceptibility [9, 165]. HS is typified by dermal alterations, in particular FB hyperproliferation, overproduction of ECM and by the persistence of α-SMA-positive myofibroblasts [166]. In addition, the epidermis is thickened and its major constituents, the KCs, are activated and undergo accelerated differentiation [166, 167]. Finally, the KC-FB cross-talk plays an important role in HS development [168, 169]. A study by Li and coll. revealed that Notch signalling is activated in the epidermis of HS patients, regulates production of fibrotic factors in KCs, both in vitro and in vivo models, and significantly contributes to scar hyperplasia [168]. Inflammatory burden is one the most important factors in pathological scarring and macrophages are well-known determinants in the pathological drift of the healing process in several skin diseases, including the HS [170]. A recent study showed that blocking Notch activity in macrophages alleviates

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scar formation by lessening inflammatory response and collagen accumulation [171]. At the same time, in vivo experiments revealed that RBPJ knock-out (KO) mice are characterized by a reduced expression of type I and type III collagens and of several fibrotic markers in the healed skin [171].

Adipose-derived mesenchymal stem cells (AMSCs) are considered promising tools to counteract fibroproliferative disorders such as HS and keloids in accordance to their ability to reduce the production of pro-fibrotic factors and lessen myofibroblast features. Recently, Han and coll. demonstrated that conditioned medium from AMSCs alleviates the fibrotic phenotype (*i.e.* fibrosis-associated ECM synthesis, proliferation, migration) of HS and KD FBs by reducing the activity of TGF-β1 and NOTCH1 cascade [172].

Keloid disease

Keloid disease (KD), which appears clinically as a raised scar that grows beyond the injury site and has no tendency to regress, is a benign although disabling fibro-proliferative skin disorder derived by an excessive and abnormal WH process [173]. The dermis of KD is characterized by abundant thick hyalinized collagen bundles, also known as "keloidal collagen", and by the persistence of myofibroblasts, and is surmounted by a thickened epidermis which undergoes accelerated differentiation [166]. Though Notch signalling has been less investigated in KD than in other fibrotic skin diseases, available experimental evidences support its involvement in KD pathomechanisms [174, 175]. A study by Syed and Bayat revealed that (i) NOTCH1, NOTCH2 and JAG1 mRNA and protein levels are significantly up-regulated in KD skin biopsies and primary FBs from KD patients (KD FBs) with respect to healthy tissues and FBs, respectively; (ii) Notch pathway stimulates cell proliferation, migration, invasion and angiogenetic properties of cultured KD FBs; and (iii) Notch activation positively correlates with inflammatory degree in KD tissues, suggesting that immune cells might turn on Notch cascade in vivo [174]. Furthermore, a recent study reported that KD FBs from subjects affected with active KD (*i.e.* patients with a recent keloid and complaining keloid pruritus and pain) exhibit a more prominent NOTCH1 activation as compared to KD patients with stable lesions [175]. In addition, KD FBs were hallmarked by: (i) a reduced autophagic flux, which has been associated with a reduced autophagy-mediated degradation of Notch, and (ii) a NOTCH1-mediated induction of α-SMA, TGF-β3 and NLRP3 (NACHT, LRR and PYD domains-containing protein 3) inflammasome, which primes the inflammatory cascade in KD. Finally, treatment of KD FBs with rapamycin—an inducer of autophagy—determined NOTCH1, and in turn NLRP3,

down-regulation in KD FBs [175]. Deregulation of the Notch pathway in KD has been recently described by an integrative analysis of mRNA and miRNA expression levels at the wound site of KD-prone individuals [176].

Dystrophic epidermolysis bullosa

Epidermolysis bullosa (EB) embraces a heterogeneous group of inherited skin fragility disorders typified by skin blistering and superficial wounds [177]. The underlying genetic defects are the major determinants of disease extent and clinical phenotype. However, genetic, epigenetic and environmental factors can deviate the expected genotype–phenotype correlations, contributing to disease severity variability in EB individuals carrying the same mutations [178]. The recessive dystrophic EB (RDEB) subtype is caused by biallelic mutations in the *COL7A1* gene, encoding type VII collagen (COL7), the major component of anchoring fibrils which ensure adhesion of the cutaneous basement membrane zone to the dermis. In RDEB patients, loss of the structural function of COL7 disrupts skin resilience to mechanical stress and impairs the WH process. In RDEB, wound sites are enriched in immune cells, bacteria and myofibroblasts that fuel, in a self-renewing loop, the inflammatory burden and the development of inflammation- and injury-driven soft tissue fibrosis [5]. Fibrosis is a regular and devastating disease complication in RDEB patients that leads to joint contractures, hand and foot digit fusion and mitten deformities and favours the onset of aggressive and metastasizing squamous cell carcinomas (SCCs) [179, 180]. Understanding the molecular mechanisms regulating fibrosis in RDEB represents a critical step towards the development of novel therapeutic strategies to counteract disease progression and improve patients' quality of life. Despite the pro-fibrotic role of Notch signalling in a wide range of fibrotic disorders and the paradigm of RDEB as a powerful model to investigate common mechanisms of fibrosis [5], the involvement of this pathway in EB-associated fibrosis remains almost unexplored.

In a recent study, our group showed for the first time that JAG1 protein levels and the cleaved/activated form of NOTCH1 are increased in RDEB FBs as compared to primary skin FBs from healthy subjects and positively correlate with the abundance of the pro-fibrotic miR-145-5p [45]. In accordance with these findings, our previous genome-wide expression analysis performed on RDEB FBs from a monozygotic twin pair with markedly different phenotypic disease manifestations revealed the up-regulation of JAG1 and Notch family members in the more severely affected twin [178]. We are currently exploring the Notch role in RDEB-associated fibrosis, including its interplay with the TGF-β pathway.

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In addition to fibrosis, Notch could be an important factor in regulating RDEB-associated inflammation due its well-established involvement in immune cells development and function [84]. Finally, inactivating mutations in NOTCH1, NOTCH2 and NOTCH4 are recognized genetic determinants in RDEB-associated SCCs [180, 181]. Although the pro-tumorigenic properties of Notch pathway, alone or in cooperation with other signalling mechanisms, are well investigated in human cutaneous malignant melanoma and SCC [146, 182–184], mechanistic studies in RDEB-SCC models are missing.

Therapeutic routes

The fibrosis-limiting effects of Notch signalling inhibition have been extensively and successfully described in numerous preclinical models. However, safety and therapeutic potential of Notch inhibitors remain to be fully elucidated in the clinical practice, in particular in the context of long-term treatments for chronic diseases such as fibrosis [17, 185]. Of note, the Notch cascade represents a relevant therapeutic target in cancer, where over 70 clinical trials have been registered [186–188]. Interestingly Nirogacestat (PF-03084014), a Notch GSI (γ-secretase inhibitor), entered a phase III clinical study (ClinicalTrials.gov Identifier: NCT03785964) for the treatment of desmoid tumour/aggressive fibromatosis, rare, slow-growing malignancies arising from FBs and characterized by a heterogeneous outcome.

GSIs are a wide family of molecules able to halt γ-secretase enzymes activity [189]. Initially, they were developed to block presenilin 1 and 2, the enzymes responsible for amyloid precursor protein formation in Alzheimer's disease, but nowadays GSIs represent the prototypical drugs to counteract Notch pathway activation in blood malignancies and solid cancers [188, 190–192]. Unfortunately, GSIs are nonspecific Notch inhibitors and the definition of their therapeutic window is critical to avoid toxic side effects [193]. Indeed, low-dose, combinatorial therapies against the main morphogenic pathways (i.e. Hedgehog, Wnt and Notch) have been shown effective in murine models of SSc, and could represent a safer approach to counteract skin fibrosis in human patients [194].

In addition to the GSIs, several molecules able to inhibit the initial steps of Notch trafficking and processing, uncouple receptor-ligand interactions or selectively prevent the interaction between NICDs and their nuclear co-activators have been developed [195–200] (Fig. 4 and Additional file 1: Table S2) and clinical testing

is underway for some of them. Non-GSIs Notch inhibitors range from monoclonal antibodies (mAbs) to Notch ectodomain-based molecular decoys. In the clinical practice, antibody- and ectodomain-based inhibition strategies could halt the Notch signalling cascade in a selective manner, avoiding the potential harmful effects given by pan-Notch inhibitors. Moreover, both repurposing drugs such as artesunate, an anti-malarial agent, as well as plant-derived natural products, such as astragaloside, have been reported to down-regulate TGF-β and Notch signalling cascades and lessen pulmonary fibrosis in vitro and in rat models [122, 201].

In conclusion, the intricate mechanisms underlying Notch maturation, processing, regulation and activity represent as many steps of complexity in pathway understanding, but at the same time they make Notch a powerful druggable target in multiple phases of its molecular life, by an ever-growing range of potential therapeutic tools (Fig. 4 and Additional file 1: Table S2).

Conclusions

Despite Notch signalling represents a timeless research topic in a multitude of physiological and pathological conditions, its mechanism of action remains intricate. Notch-based biological outputs are complex, subjected to a tight regulation and extremely diversified, often in a cell- and context-dependent fashion. This intrinsic complexity makes Notch investigation challenging but fascinating. In the skin, Notch is a well-established regulator of keratinocyte differentiation and, in turn, loss-of-function mutations in genes encoding Notch members are important players in the onset of SCC. On the contrary, though several in vivo and in vitro studies established a role for Notch cascade activation in different fibroproliferative diseases, also in combination with several pro-fibrotic pathways, in particular the TGF-β1, its involvement in FB behaviour appears as yet incompletely investigated. Similarly, interactions between Notch and ECM members in fibroproliferative skin disorders have been described but not fully elucidated. The findings summarized in this review show that the Notch pathway pervasively regulates different aspects of skin homeostasis and its dysregulation can underlie the pathomechanisms of fibrosis. Thus, targeting the Notch cascade could represent a relevant tool for future therapeutic approaches in fibrotic skin disorders.
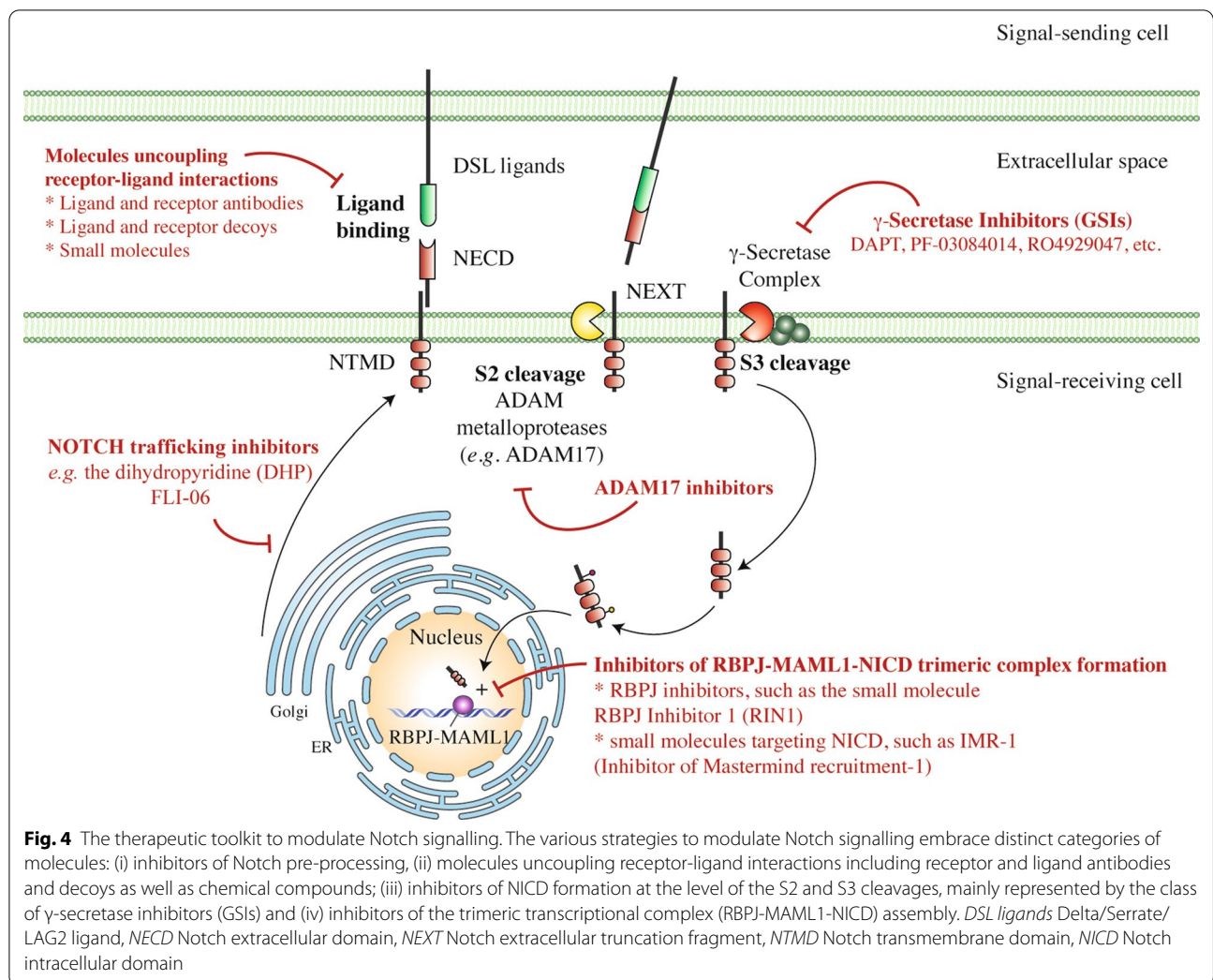
Fig. 4 The therapeutic toolkit to modulate Notch signalling. The various strategies to modulate Notch signalling embrace distinct categories of molecules: (i) inhibitors of Notch pre-processing, (ii) molecules uncoupling receptor-ligand interactions including receptor and ligand antibodies and decoys as well as chemical compounds; (iii) inhibitors of NICD formation at the level of the S2 and S3 cleavages, mainly represented by the class of γ-secretase inhibitors (GSIs) and (iv) inhibitors of the trimeric transcriptional complex (RBPJ-MAML1-NICD) assembly. *DSL ligands* Delta/Serrate/LAG2 ligand, *NECD* Notch extracellular domain, *NEXT* Notch extracellular truncation fragment, *NTMD* Notch transmembrane domain, *NICD* Notch intracellular domain

Abbreviations

α-SMA: Alpha-smooth muscle actin; BMZ: Basement membrane zone; CNN1: Calponin; DAPT: N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; EB: Epidermolysis bullosa; ECM: Extracellular matrix; FBs: Fibroblasts; HS: Hypertrophic scar; KD: Keloid disease; KCs: Keratinocytes; GSI: γ-Secretase inhibitor; lncRNAs: Long non-coding RNAs; MAPKs: Mitogen-activated protein kinases; miRNA (or miR): MicroRNA; NECD: Notch extracellular domain; NEXT: Notch extracellular truncation fragment; NICD: Notch intracellular domain; nc-RNAs: Non-coding RNAs; PTMs: Post-translational modifications; RBPJ: Recombination signal binding protein for immunoglobulin kappa J region (also known as CSL or CBF1); RDEB: Recessive dystrophic epidermolysis bullosa; SCC: Squamous cell carcinoma; SSc: Systemic sclerosis; TAGLN: Transgelin; TD: Terminal differentiation; TGF-β1: Transforming growth factor-β1; VSMCs: Vascular smooth muscle cells; WH: Wound healing.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12929-021-00732-8.

Additional file 1: Table S1. Luciferase reporter assay-validated miRNAs regulating Notch receptors and ligands. **Table S2.** Drugs and compounds targeting Notch pathway.

Declarations

Ethics approval and consent to participate
Not applicable.

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