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Aging of signal transduction pathways, and pathology

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Abstract

The major cell signaling pathways, and their specific mechanisms of transduction, have been a subject of investigation for many years. As our understanding of these pathways advances, we find that they are evolutionarily well-conserved not only individually, but also at the level of their crosstalk and signal integration. Productive interactions within the key signal transduction networks determine success in embryonic organogenesis, and postnatal tissue repair throughout adulthood. However, aside from clues revealed through examining age-related degenerative diseases, much remains uncertain about imbalances within these pathways during normal aging. Further, little is known about the molecular mechanisms by which alterations in the major cell signal transduction networks cause age-related pathologies. The aim of this review is to describe the complex interplay between the Notch, TGF β , WNT, RTK-Ras and Hh signaling pathways, with a specific focus on the changes introduced within these networks by the aging process, and those typical of age-associated human pathologies.

Keywords

aging; cell signaling; Notch; WNT; Hedgehog; TGF- β ; RTK; cancer; tissue regeneration

Introduction

There is a growing consensus that cell signaling pathways are organized as intertwined communication networks, which process and interpret inputs using multimeric protein complexes as relay stations. Fine-tuned positive and negative regulation of signaling networks is a critical feature of normal, physiological signaling balance in any given cell. This is particularly evident with respect to Notch, TGF β , WNT, RTK-Ras and Hh signaling pathways, which possess numerous signaling attenuators and dampeners. In embryonic development, mutation within any one such signal transducing protein often results in deleterious consequences, e.g., failure in organ formation [1–5]. Further, despite the presence of cellular protective responses [6,7], pathologies (e.g. cancers) frequently develop in tissues due to somatic mutations within these key signal transduction networks [8–10]. Interestingly, during the aging process, changes in the signaling intensities of these networks have been noted throughout various tissues, many of which frequently manifest in age-related pathologies. Understanding these specific pathway defects, evident in current cancer and degenerative disorder models, provides a valuable perspective for further defining them within the context of aging itself.

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Notch, TGF β , WNT, RTK-Ras, Hh: role in the building and maintenance of healthy tissues

Notch

The Notch signaling network has been identified within a variety of metazoans [1,11], and plays a principle role in development and organogenesis - largely through coordinating cell-fate determination events in adjacent stem and progenitor cells (via inductive interactions and lateral-specification) [8,11]. The role of Notch in human embryonic development was recently approached in studies with human embryonic stem cells (hESCs). These studies revealed that hESC Notch1 activation and endogenous NICD up-regulation affects numerous target HES1 transcripts, many of which promote neural lineage and suppress mesodermal differentiation [1]. Notch also has a major impact on adult tissue maintenance and repair. For example, maintenance of skeletal muscle requires Notch signaling and greatly depends on Delta up-regulation for satellite cell activation [12]. In neural tissues, Notch suppresses the differentiation of neural stem cells, thereby promoting their continued self-renewal. Notch signaling also regulates differentiation and self-renewal of adult stem cells in skin, intestine and blood, reviewed in [8].

In mammals, there are 4 isoforms of the Notch receptor (Notch1-4) [1,13]. As a 300kDa protein, immature Notch undergoes S1 cleavage in the trans-golgi, prior to its presentation at the cell surface as a mature heterodimer (ectodomain with EGF-like repeats and calcium-dependent, non-covalently associated transmembranal domain) [1]. Direct interaction with DSL (Delta/Serrate/Lag-2) family transmembrane ligands (Delta-like 1, 3, 4 and Jagged 1, 2 in mammals) is responsible for the activation of Notch signaling [13,14] (depicted in Figure 1); although Delta-like3 is implicated in attenuating Notch activation. Ligand activation is further mediated by E3 ubiquitin ligases (e.g. Neuralized (Neur) and Mindbomb (Mib)) [1,14].

The EGF-like repeats within both Notch receptor and DSL ligands confer site-specific glycosylation events, which are important for Notch-ligand interactions and Notch-ligand binding affinities and consequently affect signal potentiation (e.g. Delta vs. Jagged) [1,15]. Following ligand binding, proteolytic cleavage ensues (S2 cleavage catalyzed by ADAM family metalloproteases), releasing the extracellular Notch-DSL portion (NECD). In the second cleavage (S3), a presenilin-1-dependent, γ -secretase complex releases the Notch intracellular domain (NICD). Nuclear localization motifs, present within NICD, confer its translocation to the nucleus where NICD binding displaces the CSL (CBF1 – humans, Suppressor of Hairless (Su(H)) – *D. melanogaster*, LAG-1 – *C. elegans*) transcriptional corepressor complex [16,17] (RBP-J in mice). As a transcriptional co-activator, NICD-CSL binding further releases transcriptional repression by recruiting a coactivation complex (Mastermind (Mam)/LAG-3/SEL-8 and the histone acetylase, p300), thereby driving Notch-target gene transcription [1]. Of note, NICD activity can be antagonized by the ubiquitylation ability of Numb. CSL-independent Notch signaling events are also described [18].

Primary transcriptional output of Notch signaling includes the upregulation of basic helix-loop-helix (bHLH) transcription factors of the [HES – *Hairy/E(spl)* and (HESR/HEY) – HES-related genes in vertebrates] class [19]. These proteins are known to regulate a diversity of genes, broadly associated with tissue-specific differentiation and cell cycle regulation.

TGF β

The Transforming-Growth-Factor Beta (TGF- β) superfamily proteins are ubiquitously secreted multifunctional cytokines, capable of signaling to virtually every cell type. These ligands influence many aspects of cell behavior including proliferation, differentiation, migration and apoptosis [20]. TGF β signaling affects numerous cellular functions and

processes, in both embryonic developmental stages (germ-layer specification and patterning) and in the adult. For example, as multifunctional regulators, TGF β s broadly influence the maturation and homeostasis of various cell lineages in the embryo, and also play a role in suppression of carcinogenesis in the adult [2]. TGF β is also involved with apoptosis in many cell types (SMAD induction or triggering of the death associated protein 6 (DAXX adapter protein association with Type II receptors) [21]

In mammals, TGF β ligands represent the following primary isoforms: 4 activin β -chains, nodal, 10 bone morphogenetic proteins (BMPs) and 11 growth and differentiation factors (GDFs) [22]. TGF β signaling is further controlled by signal attenuators, such as the BMP antagonists chordin and noggin, activin/BMP inhibitors (e.g. follistatin) and members of the Cerberus family and sclerostin [23,24]. Initially synthesized as intracellular dimeric pre-proteins, most TGF β ligands undergo pre-secretory processing by the subtilisin-like proprotein convertase (SPC) family [22]. Exceptions include GDF8/GDF11 and TGF β s, which are secreted as latent forms, requiring metalloprotease BMP1 activation [25]. Most ligands are present as cysteine knot-stabilized homodimers, although, heterodimers are also described (e.g. activin B α -B β , nodal-BMP4, nodal-BMP7) [22].

Binding of activated TGF β ligands to their receptors induces the formation of a heterotetrameric receptor complex (Type II-Type I receptor dimer bridging) [26], with subsequent Type II receptor kinase phosphorylation of Type I receptor Ser and Thr residues. Some ligands also require membrane-embedded co-receptors (Type III) [22]. Type I phosphorylation contributes to the recruitment of receptor-regulated SMADs (R-SMADs – SMAD1/SMAD2/SMAD3/SMAD5/SMAD8) and their phosphorylation (P-SMADs), allowing heteromeric rSMAD-SMAD4 and homomeric SMAD complexes to form [26,27]. rSMAD-SMAD4 complexes translocate and accumulate in the nucleus, thereby regulating target gene transcription – conferred by DNA-MH1 domain binding (except for SMAD2) and MH2 domain interactions between SMADs and various transcription factors, co-activators/co-repressors and chromatin remodeling complexes (SWI-SNF and histone-modifying enzymes, e.g. p300 and CBP). MH2 domains can also mediate SMAD-receptor and SMAD-SMAD interactions [26]. Of note, non-SMAD phosphorylation targets are also described for Type II and Type I receptors [28].

The TGF β signaling pathway (outlined in Figure 1) is traditionally represented as two branches that are downstream of Type I receptors: (1) ALK4 (activin receptor-like kinase), ALK5 and ALK7 phosphorylate SMAD2 and SMAD3, whereas (2) ALK1, ALK2, ALK3 and ALK6 phosphorylate SMAD1, SMAD5 and SMAD8. However, reports suggest that TGF β signaling is more promiscuous than previously conceived, implying shared cross-SMAD phosphorylation capabilities between the various ligands and receptors [26,29]. Conversely, Signal termination/attenuation is mediated by the inhibitory SMADs (I-SMADs), SMAD6 and SMAD7. I-SMADs are likely induced by TGF β s and BMPs, and have been functionally implicated in R-SMAD-Type I receptor binding competitive inhibition, receptor degradation (via the ubiquitin ligases SMURF1/SMURF2), receptor dephosphorylation, and transcriptional silencing [22].

WNT

WNT signaling encompasses a vast network of well-conserved proteins, which broadly influence changes in gene expression that govern embryogenesis and postnatal responses, such as cell proliferation, cell-fate determination and survival [3]. Canonical WNT signaling is associated with body axis specification and morphogenic signaling, whereas non-canonical WNT signaling is associated with planar cell polarity and axon guidance [30]. In the adult, WNT signaling promotes the differentiation, maintenance and proliferation of numerous stem

cell types (e.g. myogenic lineage development [31], intestinal stem cell self-renewal and hematopoietic stem cell expansion) [9].

Receptor-mediated WNT pathway activation occurs through the WNT proteins (19 reported in mammals), which comprise a major class of secreted cysteine-rich glycoproteins [32]. Conversely, WNT pathway activation can be inhibited or attenuated through the interaction of extracellular WNT ligands with secreted WNT antagonists (e.g. Frizzled-related protein (sFRP) and Dickkopf (DKK)) [9], or by antagonist binding to WNT receptor proteins (e.g. Sclerostin-LRP binding) [33].

Although WNTs may activate multiple signaling pathways [32], the most comprehensively studied and canonical pathway is the WNT/ β -catenin pathway (reviewed in [9,34], and depicted in Figure 1). In the absence, or repression, of WNT ligand-receptor stimulation, β -catenin is maintained at low cytoplasmic and nuclear levels through association with an adenomatous polyposis coli (APC)/AXIN complex, which confers β -catenin phosphorylation by casein kinase1a (CKIa) and glycogen synthase kinase 3 (GSK3). This non-cadherin associated β -catenin is targeted for destruction via β TRCP1 and β TRCP2 recognition, with subsequent ubiquitylation and proteasomal degradation. Nuclear repression of WNT target genes is further assured by DNA-binding of T-cell factor (TCF) and lymphoid enhancer-binding protein (LEF) transcription factor, and associated co-repressors.

During active WNT signaling, WNTs interact with members of the Frizzled (FZD) family (cell-surface seven-transmembrane-type receptors), and with LDL-receptor-related protein co-receptors (i.e. single-pass transmembrane proteins, such as LRP5, LRP6 that possess extracellular WNT-binding regions and cytoplasmic AXIN-binding motifs) [35]. WNT-FZD binding leads to Disheveled (DSH) family protein activation and recruitment of a secondary protein complex (mediated by protein kinase/phosphatase signaling intermediates [36], including GSK3 and APC-AXIN). Activated DSH contributes to GSK3 inhibition, while AXIN is degraded following LRP5/6 binding, thus diminishing β -catenin degradation. WNT-activated G-proteins likely participate in the disassembly of the GSK3, APC-AXIN complex [37]. With the " β -catenin destruction complex" inhibited, cytoplasmic unphosphorylated β -catenin levels rise and accumulate in the nucleus. Nuclear β -catenin then activates target gene expression through interactions with DNA-bound TCF/LEF family transcription factors (TCF1, LEF1, TCF3 and TCF4), as well as others [10].

Raf/MEK/ERK and PI3K/Akt

The Raf/MEK (mitogen-activated protein kinase, MAPK, or ERK kinase)/ERK (extracellular signal-regulated kinase) and PI3K (phosphatidylinositol 3-kinase)/Akt (or protein kinase B) signaling pathways are involved in diverse and seemingly contradictory cellular functions, such as: apoptosis, proliferation, survival, growth arrest, differentiation, motility, metabolism and senescence [38,39]. As summarized in Figure 1, nearly all cell-surface receptors (including G-protein-coupled receptors and receptor tyrosine kinases) [4], stimulate Ras GTPases, which in turn can activate both Raf/MEK/ERK and PI3K/Akt pathways [38,39]. Raf/MEK/ERK signaling alone targets more than 70 known substrates, distributed throughout the nucleus, cytosol, cytoskeleton and membranes [38]. There are currently five distinct categories of mitogen-activated protein kinases (MAPKs) identified in mammalian systems: ERKs 1 and 2 (ERK1/2), ERKs 3 and 4, ERK5, c-Jun amino-terminal kinases (JNKs) 1, 2, and 3, and p38 isoforms α , β , γ , and δ [4]. ERK1/2, JNKs and p38 kinases are the best characterized groups [4]. Activation of ERK1/2 is achieved by the Son of Sevenless (SOS)-mediated exchange of GDP to GTP by Ras, which allows ERK1/2 interaction with Raf isoforms, leading to phosphorylation of MEK 1 and 2 (MEK1/2), and eventually ERK1/2 [4,38]. The p38 module is activated by several MAPK kinase kinases (MAPKKKs), such as MEK kinases 1–4, which phosphorylate MEKs 3 and 6, resulting in activation of all p38 isoforms via MEK6, or p38a/

p38b by MEK3 [4]. The JNK module is also activated by the same MAPKKs that stimulate the p38 group, but MEKs 4 and 7 actually mediate phosphorylation of JNKs 1–3 [4].

Association of PI3K with phosphorylated growth factor receptors and other proteins is mediated by SH2, causing PI3K membrane localization, where it can interact with activators (e.g., Ras) [40]. Once active, PI3K catalyzes the formation of 3-phosphorylated phosphoinositides, such as phosphatidylinositol 3,4,5-triphosphate and phosphatidylinositol 3,4-bisphosphate [4]. These lipids can then stimulate numerous members of the protein kinase A, G and C family, including the p70 ribosomal protein, S6 kinase and Akt [41]. 3-phosphoinositide-dependent protein kinase-1 (PDK1) is responsible for phosphorylating Akt and many other AGC family protein kinases [41]. Additionally, the PI3K/Akt and Raf/MEK/ERK pathways can interact in several ways. For example, there is evidence that PDK1 is able to directly phosphorylate and activate MEK1/2 at the same sites used by Raf kinases [41]. Pathway crosstalk can also be inhibitory, since Akt may phosphorylate Raf and thus, block Raf/MEK/ERK cascade activation [39]. Also, the interaction of Ras with PI3K can activate Cdc42/Rac, which triggers Pak activation and consequently leads to Raf phosphorylation [42]. Crosstalk can occur even further upstream, since there is data supporting a role in basal PI3K activity for Ras activation at low levels of receptor stimulation [43].

Hedgehog

There are three hedgehog genes in vertebrates, namely Desert hedgehog (*Dhh*), Indian hedgehog (*Ihh*) and Sonic hedgehog (*Shh*), out of which *Shh* is the most well-studied [5,44]. Hedgehog (Hh) signaling plays vital roles in embryonic development, such as in fruit fly segmental patterning, left-right asymmetry in vertebrates and in adult tissue pattern maintenance [5,45,46]. Hedgehog gets its name from mutation of the single *Hh* gene in *Drosophila melanogaster*, which causes the appearance of continuous spikey cuticular processes (denticles) on the back of larvae [5,44]. One unconventional characteristic of Hh proteins is their unusual post-translational modifications, which include signal sequence removal and autocatalytic cleavage to yield an 18 kDa N-terminal (Hh-N) fragment retaining all signaling domains [5]. During this cleavage, a cholesterol moiety is linked to the C-terminal end of Hh-N, via a covalent bond, and then undergoes palmitoylation at the N-terminal site [5]. These modifications affect Hh protein activity in several ways. For example, the lipid moieties in Hh proteins are essential for their proper intracellular transport [5], and are also required for their secretion from cells (mediated by the transmembrane protein, Dispatched) [44]. In addition, the diffusion and spatial distribution of Hh proteins are modulated by Hh protein acylation [5,44]. Also, the N-terminal palmitoylation allows targeting of Hh proteins to lipid rafts, which in turn assists Hh-receptor binding [5].

The general mode of Hh signaling is presented in Figure 1. Hh proteins bind to the 12-transmembrane receptor protein, Patched (Ptc), aided by the interaction with two additional transmembrane proteins, Interference hedgehog (IHOG) and Brother of IHOG (BOI) [44]. Upon binding, Hh-Ptc becomes internalized and the inhibitory effect of Ptc on the serpentine protein, Smoothened (Smo), is alleviated [5,44]. In the absence of Hh, a microtubule-bound protein complex [composed of Fused (Fu), Costal2 (Cos2), Suppressor of Fused (SuFu), protein kinase A (PKA), glycogen synthase kinase 3 β (GSK3 β), and casein kinase 1 (CK1)] sequesters and cleaves the Glioma-associated oncogene homologue (Gli)/Cubitus interruptus (Ci) transcription factor [5,44]. The resulting N-terminal 75 kDa Gli/Ci fragment localizes to the nucleus and suppresses Hh target gene expression. In the case of Hh binding, however, activated Smo leads to phosphorylation of Fu and Cos2 and releases full-length Gli/Ci from the complex, which translocates into the nucleus and activates Hh gene targets [5,44]. In vertebrates, there are 3 distinct Gli proteins, namely Gli1, Gli2, and Gli3. Gli2 and Gli3 are

the effectors of the Hh pathway, while Gli1 is part of a positive feedback loop, as it is a Hh target itself and also enhances Gli2 activity [44].

Signal integration and signal transduction imbalance in aging and age-associated diseases

Cellular responses to internal and external stimuli are regulated by numerous positive and negative feedback loops, both within and between the herein reviewed signaling pathways. Accordingly, pathway cross-talk is a very complex, yet highly utilized feature required for the molecular regulation of cell homeostasis and adaptation, where modulation of one pathway can affect multiple regulatory circuitries. Considering that precise signaling strength is important for productive cell responses, it is not surprising that change in the major biochemical pathways [47], and in pathway crosstalk, manifests within many diseases that typically accompany human aging. In addition to outlining the individual signal networks, Figure 1 also summarizes the key components of pathway integration, and identifies deregulations that are associated with aging and age-related diseases.

The Notch pathway has been well-studied with respect to its interactions with other networks, such as RTK-Ras-MAPK, Jak-STAT, TGF β , WNT and Hh pathways [48–50]. For example, under conditions of normal development, astrocyte differentiation during embryogenesis is promoted by Notch-STAT3 interactions that drive JAK2-STAT3 complex formation [50]. Notch-WNT interactions are also implicated in several developmental stages, such as somitogenesis and hematopoiesis (WNT activates Notch signals for self-renewal in hematopoietic stem cells) [51], as well as adult intestinal and skin regeneration [51–53].

Additionally, one of the major components of WNT pathway, GSK3 β , is also a crosstalk integrator of multiple signal transduction networks [35]. For example, recently published work suggests that a balance between Notch and WNT signaling controls cellular homeostasis during the regeneration of adult skeletal muscle [54]. Namely, Notch promotes the proliferation of myogenic progenitor cells, and inhibits their precocious terminal differentiation by inhibiting WNT via GSK3 β activation. Conversely, during later stages of muscle repair and regeneration, WNT inactivation of GSK3 β thereby promotes myoblast and myotube differentiation [54]. Increased WNT is also reported to skew the differentiation of muscle stem cells (satellite cells) towards a fibroblast lineage, during muscle repair in an age-dependent manner [55]. It remains to be determined how these reciprocal effects of WNT (i.e. promoting myogenic differentiation in the young, and inhibiting myogenic differentiation in the old) are regulated. Lastly, the pro-aging role of elevated WNT signaling was further supported in studies using *klotho* (a WNT antagonist)-deficient mice [56], although other factors (e.g. insulin and FGF23) are also implicated in the aging phenotype of these animals [57,58].

With advancing age, many stem cell-regulating cues become adversely altered, thus precluding productive tissue repair. Moreover, in recent years, specific alterations within repair-specific signal transduction have been implicated with such changes in cell behavior. For example, Notch pathway is an essential and age-specific molecular determinant of adult skeletal muscle myogenesis [12], whose activation becomes lacking with age (due to failure of Delta upregulation), thus leading to diminished activation of endogenous satellite cell and defective repair of muscle injury [12,59,60]. Of note, the intrinsic repair potential of aged satellite cells remains intact, and can be restored to young levels by exposure to young environments [12, 61].

Aberrant Notch-TGF β interactions are also implicated in age-associated diseases, such as specific cancers [52,62,63], where TGF β -Notch-WNT crosstalk is altered (e.g., by alternative β -catenin control) [9]. Additionally, in neural stem cells, mutations in presenilin (part of the

Notch-associated γ -secretase complex), adversely affect progenitor function [64], thus implicating Notch alterations in the neurodegenerative aspects of Alzheimer's disease. Interestingly, aberrant WNT regulation (such as attenuated β -catenin signaling), is also implicated in Alzheimer's disease [9].

In summary, Notch's role in the age-related dysfunction of other tissues remains largely unresolved. Given its broad influence on various stages of lineage specification, however, it is probable that Notch signaling plays a key role. For example, disruption of the Notch-WNT-FGF and TGF β -Hh balance in various stem and progenitor cell subsets causes loss of cellular homeostasis, resulting in congenital diseases and cancers [65]. In addition, although not fully understood in the context of disease, there are several points of Notch-Ras communication identified. These include: Ras-mediated blocking of NICD transcriptional targets (e.g. attenuation of corepressor Groucho via phosphorylation), Notch stimulation of Ras pathway inhibitors (e.g. YAN in *Drosophila*) and non-antagonistic crosstalk, such as Ras promotion of DSL ligand expression (Delta derepression of nuclear Su(H) via Sno and Ebi in *Drosophila*) [48].

Signal integration of TGF β often occurs at the level of its SMAD effectors, which possess Pro-rich linker regions (commonly between MH1 and MH2 domains) that can be phosphorylated by MAPKs, GSK3 β and multiple CDKs [22,66,67]. Furthermore, some TGF β trap-ligands can inhibit WNT proteins (e.g. Cerberus [68]). Integration with other pathways is also facilitated by SMAD-interacting transcription factors, such as p53 integration between RTK and TGF β signaling [69]. TGF β signaling is well-known to play a role in cancer, scar formation/inflammation [70,71], senescence and diminished proliferation of many cell-types [26,29]. However, despite apparent crosstalk, specific alterations are only beginning to be understood. For example, the role of TGF β /Notch balance in aged-skeletal muscle repair deficiency stems from elevated levels of P-SMAD present on the promoter regions of specific CDK (cycling-dependent kinase) inhibitors [72]. Notch activation overrides these inducing effects of TGF β via P-SMAD removal, but Notch activation itself becomes lacking with age [12] for reasons unclear. In this manner, the necessity to impose cell-cycle check points becomes antagonistic to aged stem cell regenerative responses. This notion is further supported by recent work, implicating the activation of the INK4/p16 locus in the age-specific decline and productive maintenance of the hematopoietic system, pancreatic islet cells and adult neurogenesis [73–75]. Along these lines, TGF β signaling is also associated with cell cycle inhibition, via CDK inhibitor activation and cMyc inactivation [76–78].

Raf/MEK/ERK and PI3K/Akt signaling cascades interact with several pathways through various mechanisms. For example, the Raf/MEK/ERK pathway has been shown to crosstalk with TGF β , Notch and Wnt pathways, with the transcriptional corepressor Groucho (Gro)/Transducin-Like Enhancer of split (TLE) central to the crosstalk interactions among these pathways [79]. Active ERK/MAPK phosphorylates Gro/TLE, causing downregulation in its activity, which in turn relieves the inhibition of TGF β , Notch and WNT target genes by the repressors Brinker, HES and TCF/LEF, respectively [79]. Moreover, the JNK module of the cascade can have a positive effect on Smad2/3 by enhancing its interaction with Smad4 and thus increasing nuclear accumulation of transcription factors [80]. In contrast, ERK/MAPK might act as negative regulator of TGF β signaling by phosphorylating Smad1/5/8 and Smad2/3 in their linker regions, therefore preventing nuclear translocation and Smad-dependent transcriptional activation [80].

The Raf/MEK/ERK pathway is likely affected by senescence in a tissue-specific manner. For example, in the liver of aged mice, activated ERK1/2 levels decrease while phosphorylated JNK1/2 and p38 MAPK levels are enhanced [81]. Conversely, aging decreases the ability of rat aortas to respond to multiaxial stretch by phosphorylating JNK and p38 MAPKs, but does

not change the levels of activated ERK1/2, JNK and p38 MAPKs under uniaxial stretch [82]. As compared to young, old human skeletal muscle was found to have a higher content of phosphorylated ERK1/2, JNK and p38 MAPK under resting conditions. Upon exercise, ERK1/2, JNK and p38 MAPK phosphorylation was found to decrease in the old, while ERK1/2 activation increased in the young [83]. In addition, although the total amount of MAPK proteins was similar between age groups, there was a higher level of total MKP1 protein in the old [83]. With respect to bone marrow and blood lineages, aged bone marrow stromal cells (BMSCs)/osteoblasts upregulate IGF-I receptor expression. However, their ability to respond to IGF-I binding is impaired as the receptor fails to maintain its phosphorylated state in the presence of IGF-I, thus ERK1/2 phosphorylation is diminished [84]. Additionally, aged human T-cells fail to properly activate ERK and p38 MAPK upon anti-CD3 stimulation [85]. With respect to cells of the nervous system, injection of nerve growth factor (NGF) into the hippocampus of old rats failed to both upregulate its receptor and phosphorylate ERK in the basal forebrain, as typically seen in young animals [86]. Such results may help explain how Alzheimer's disease and normal aging affect memory [86]. Importantly, age-specific alterations in the RTK-Ras signaling pathway have been associated with enhanced tumorigenesis; as mutations that render Ras constitutively active are present in ~30% of human tumors, whereas the Raf gene is mutated largely in melanomas, thyroid, colon and ovarian cancers [38].

In addition to the many the age-specific changes in the RTK-Ras pathway, specific RTK-Ras-TGF β -Notch-WNT signal integration points may also be altered in multiple ways. For example, PI3K/Akt cascade interacts with BMP signaling through a BMP-mediated increase in PTEN activity (negative regulator of PI3K) [87]. This BMP-PI3K/Akt signaling link may play a role in senescence, as PTEN activity increases in aged, but not young, rat aortas stimulated by uniaxial stretch [82]. Moreover, inactivating PTEN mutations might enhance tumorigenicity by inducing Akt hyperactivation [88], which in turn may dysregulate both Hh and WNT pathways. This prediction is substantiated by the fact that almost 40% of pancreatic cancers, which are extremely fatal carry mutations in PTEN [89].

PI3K/Akt signaling also interacts with WNT pathway via the signaling molecule 14-3-3 ζ , which acts as a facilitator of β -catenin activation by Akt, thus stabilizing the β -catenin complex and promoting its nuclear translocation [87]. Akt can also negatively regulate GSK, thus attenuating its inhibitory effect on β -catenin and enhancing WNT signaling [87]. Consequently, this PI3K/Akt-WNT crosstalk, mediated by Akt, may become aberrant in aging as Akt phosphorylation is diminished in old rat aortas under uniaxial stretch, and in old BMSCs/osteoblasts under IGF-I treatment [84]. Similarly, the hormone-induced activation of both PI3K and Akt is impaired in rat enterocytes isolated from old animals [90]. However, phosphorylation of both PI3K and Akt is reported to increase during aging of the rat kidney [91].

Hh signaling is also capable of upstream and downstream interaction with the TGF β , Raf/MEK/ERK and WNT pathways. At the level of Hh proteins, experiments in zebrafish and chick embryos suggest that TGF β /Nodal signaling activates Shh expression in the ventral neural tube [92]. This signal integration point may be deregulated in age-related diseases, such as cardiovascular disease and diabetes. Injecting Shh into ischemic mice hind limbs, or Shh DNA into myocardial ischemia models, resulted in enhanced revascularization and organ salvage [93]. Thus, these studies suggest that endogenous Shh signaling is diminished by aging. Additionally, angiogenesis is dependent on Shh activity in an age-specific manner (89), and is crucial for wound healing (typically impaired in old and in diabetic tissues) [94]. The requirement for Hh signaling in normal tissue maintenance, and its deregulation in diabetic tissues, is further evidenced by data showing Ptc and Gli1 upregulation during wound repair

(via Shh stimulation), as well as accelerated wound healing (via topical Shh DNA administration) on a diabetic mouse model of cutaneous ulcers [94].

At the level of coordination between nervous and immune systems, age-specific changes in Hh signaling are also implicated in the pathophysiology of Multiple Sclerosis (MS) and Parkinson's disease (PD). Shh-N (N-terminal) levels are reduced in both grey and white matter from MS patients. However, the 45 kDa precursor Shh protein is still present, suggesting a defect in the autocatalytic cleavage reaction [95]. Intrastriatal injections of Shh-N (as protein or DNA packaged in adenovirus) resulted in partial protection of dopaminergic nigrostriatal neurons in a rat model of PD [96,97]. This protection likely occurs via normal Shh signaling, since transfection of Gli1 DNA in the rat striatum had a similar effect [96]. However, it remains to be determined why Hh declines with age, as signal attenuation in PD patients has not been linked to mutations in promoter/enhancer, or coding regions of *Shh* [98].

Considering the emerging evidence of age-related diminished Hh signaling, several age-specific changes in other key networks could be predicted from the herein reported signal integration. For example, Hh and Raf/MEK/ERK crosstalk can occur at the level of Gli through basal MEK1 activity (direct or via ERK/downstream proteins), which is required for Gli1/Gli2 activation and nuclear translocation [88]. This point of signal integration may thus be potentially involved in specific age-related changes of cell behavior. On the other hand, impaired Hh signaling with age could potentially decrease Hh-induced upregulation of RTKs (e.g., PDGFR α), therefore reducing Raf/MEK/ERK cascade activation and disrupting positive feedback loops [88]. Decreased Hh pathway activation may also lower Smo-induced PI3K activity upregulation, a process which seems pivotal in capillary morphogenesis of endothelial cells [99]. In this case, as PI3K-mediated subsequent activation of Akt would be reduced, such defect in Hh-PI3K/Akt crosstalk could interrupt the positive feedback on Hh signaling via GSK inhibition.

Another signal integration point likely affected by age-specific changes in the Hh pathway, and contributing to age-related pathologies, is the Wnt-Hh cross-talk. The Hh pathway interconnects with WNT signaling in two ways: (1) Gli1 and Gli2 were shown to positively regulate expression of secreted frizzled-related protein-1 (sFRP-1), which inhibits WNT ligands and/or their receptors [100,101] and (2) via downstream GSK3 β (a main component of complexes that inhibit Hh and WNT morphogenetic pathways) [5]. Both pathways have been implicated in a variety of cancers [102]. For instance, mutations causing loss of Ptc function, or constitutive activation of Smo/Gli1 overexpression, lead to Hh-independent pathway activation and are associated with basal cell carcinoma, medulloblastoma and other tumor types [102]. It is therefore possible that aberrant Hh pathway activation manifests cancer phenotypes by also directly altering normal WNT and PI3K/Akt signaling. Moreover, PI3K activity seems indispensable for Hh signaling, through the Akt-regulated control of Gli phosphorylation via PKA [88]. This is important in the context of cancer, since mitogens utilizing the PI3K/Akt cascade can make cells hypersensitive to Hh proteins. Additionally, many cancers (e.g., stomach, pancreas and prostate) are reliant upon high levels of Hh ligands [89]. Thus, it is plausible to propose treatments of Hh-dependent tumors using inhibitors of PI3K, Akt, MEK or ERK [89].

Conclusion

It remains a great challenge to integrate networks of biochemical pathways into a single framework of cellular communication that confers complex biological responses, such as tissue homeostasis, maintenance and repair. As interactions between the herein reviewed pathways are sensitive to even incremental changes in the cellular environment, there is a need for continued identification and characterization of signal integration features. It is becoming

increasingly clear that aging affects, not only multiple signal transduction pathways, but also their crosstalk. Such age-dependent changes are likely to be deleterious to cell homeostasis hence, inevitably contributing to tissue pathology. Therefore, a better understanding of the age-specific changes in signal transduction networks is critical to understanding aging in molecular terms and to development of novel therapies for age-specific disorders.

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Figure 1. Key signaling networks in normal cells and their age-related changes

The five major cell signaling pathways are illustrated and described in the text. Interactions within the same pathway are shown as black arrows and blue bars: (→, positive) (→, negative) Points of pathway crosstalk and convergence are highlighted with dashed arrows in black and dashed bars in blue: (→, positive interaction) (→, negative interaction) Question marks (?) indicate unknown beginnings or endpoints, in the crosstalk targets. For visual clarity, wavy lines are used to indicate pathway intersection (~). Critical defects in these pathways, attributable to both age-related diseases/degenerative disorders and to aging itself, are depicted as green, red, and yellow contours/bars/arrows in order to denote upregulation/activation. (○, →, →) downregulation/inactivation, (○, →, →) or both scenarios (○, →, →, →). Some key points on the aging of signaling networks are exemplified as follows: (a) presenilin mutations (of Notch-associated γ-secretase complex) in neural stem cells implicated in neurodegenerative disorders, such as Alzheimer's disease [64]. (b) attenuated β-catenin signaling is implicated in Alzheimer's disease [9]. (c) TGFβ/Notch balance associated with skeletal muscle repair deficiency, due to elevated levels of P-SMAD present on CDK inhibitor promoter regions [72]. (d) Altered MAPK signaling in mouse liver [81], rat aorta [82], human skeletal muscle [83], mouse bone marrow stromal cells (BMSCs)/osteoblasts [84], human T cells [85] and rat basal forebrain [86].

(e) aberrant Akt phosphorylation levels in rat aorta under uniaxial stretch [82], mouse BMSCs/osteoblasts under IGF-I treatment [84], rat enterocytes [90], and rat kidney [91].

(f) exogenous Shh administration promoted revascularization and improved wound repair in mouse myocardial ischemia [93] and mouse diabetic ulcer models [94], respectively, and also protected dopaminergic neurons in a rat model of Parkinson's Disease (PD) [96,97]; reduced Shh levels in grey and white matter of Multiple Sclerosis patients [95].

Notably, it is likely that many of these identified signaling aberrations deleteriously affect numerous targets. Deciphering these interactions, as well as uncovering other modes of pathway integration, will play a prominent role in the future development of targeted signaling manipulation for therapeutic purposes. This schematic diagram is by no means exhaustive. Many pathway crosstalk points were omitted for visual clarity, and due to manuscript length restrictions.

Abbreviations: NICD, notch intracellular domain; Su(H), suppressor of hairless; CBF1, *Candida glabrata* centromere binding factor 1; RBP-J, recombination signal sequence binding protein for Jk genes; Sno, strawberry notch; HES, hairy/enhancer of split; Ac-Sc, achaete-scute; Gro/TLE, groucho/ transducin-like enhancer of split; FZD, frizzled; Dsh, disheveled; APC, adenomatous polyposis coli; GSK, glycogen synthase kinase; β -CAT, beta-catenin; TCF/LEF, T-cell factor/lymphoid enhancer-binding protein; sFRP, secreted frizzled-related protein; RTK, receptor tyrosine kinase; FGF, fibroblast growth factor; MEK, MAPK or ERK kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun amino-terminal kinases; PNT, pointed; MKP, MAPK Phosphatase; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B; PDK, 3-phosphoinositide-dependent protein kinase; PTEN,; Shh, sonic hedgehog; Ptc, patched; Smo, smoothened; Fu, Fused serine threonine kinase; SuFu, suppressor of fused protein; Cos2, costal2; PKA, protein kinase A; CK1, casein kinase 1; Gli/Ci, Glioma-associated oncogene homologue/cubitus interruptus; IHOG/BOI, interference hedgehog/brother of IHOG; TGF β , transforming growth factor beta; BMP, bone morphogenetic protein; ACT, activin type receptor; CDK, cyclin-dependent kinase; P, phosphate group.