Role of Agrin in tissue repair and regeneration: From mechanisms to therapeutic opportunities (Review)

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Abstract. Tissue regeneration is a complex process that involves the recruitment of various types of cells for healing after injury; it is mediated by numerous precise interactions. However, the identification of effective targets for improving tissue regeneration remains a challenge. As an extracellular matrix protein, Agrin plays a critical role in neuromuscular junction formation. Furthermore, recent studies have revealed the role of Agrin in regulating tissue proliferation and regeneration, which contributes to the repair process of injured tissues. An in-depth understanding of the role of Agrin will therefore be of value. Given that repair and regeneration processes occur in various parts of the human body, the present systematic review focuses on the role of Agrin in typical tissue and highlights the potential signaling pathways that are involved in Agrin-induced repair and regeneration. This review offers important insight into novel strategies for the future clinical applications of Agrin-based therapies, which may represent a feasible treatment option for patients who require organ replacement or repair.

Contents

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Abbreviations: LRP4, low density lipoprotein receptor-related protein 4; MuSK, muscle-specific kinase; NMJ, neuromuscular junction; AChR, acetylcholine receptor; NSPCs, neural stem/progenitor cells; MG, myasthenia gravis; DNMG, double-seronegative myasthenia gravis; YAP, yes-associated protein; VEGFR2, vascular endothelial growth factor receptor 2; FAK, focal adhesion kinase; ERK, extracellular regulated protein kinases; CREB, cAMP responsive element binding protein; BMP, bone morphogenetic protein; MMP12, matrix metalloproteinase-12

Key words: Agrin, repair, regeneration, mechanism

1. Introduction

Organ damage and loss are generally caused by congenital abnormalities or acquired disorders. The clinical application of regenerative medicine includes the use of biological products, stem cell therapy, tissue engineering, cellular reprogramming and gene therapy (1). Recently, a regenerative approach, in which the local milieu of the diseased tissue or organ is modulated into a regenerative environment to aid in the healing process, has attracted attention and provided new insights into 'translational medicine'. It is speculated that this approach will replace traditional transplantology in the near future. Thus, this new regenerative approach needs to be explored.

Agrin is an extracellular matrix protein that has heparan sulfate proteoglycan as the core protein. It is encoded by the *AGRN* gene and has a relative molecular weight of ~220 kDa, including nine protein kinase inhibitor domains, four epidermal growth factor-like domains and one adhesion molecule G homologous domain (2). The *AGRN* transcript can be spliced to produce different isoforms: Either a type II transmembrane

protein or a secreted protein (3) (Fig. 1). This splicing determines both the localization and function of Agrin.

Agrin was originally isolated from tissue with electrical activity. It binds to low-density lipoprotein receptor-related protein 4 (LRP4) and activates muscle-specific kinase (MuSK), thus forming a multiprotein complex at the neuromuscular junction (NMJ) (4). The function of Agrin in the formation and maintenance of the NMJ is relatively well known; however, it also has roles in proliferation, motility, cell adhesion and even the regulation of progression in certain types of cancer (5-7). In a previous study, the role of Agrin in regulating tissue proliferation and regeneration was investigated, revealing that Agrin exerts a pleiotropic therapeutic response, including intrinsic and extrinsic arms, which contributes to the repair process in injured animals (8). Agrin therapy has also been reported to promote both cardiac regeneration after myocardial infarction and the proliferation of human induced pluripotent stem cell-derived cardiomyocytes (9). However, the role of Agrin during tissue repair and regeneration remains largely unknown and investigations are needed to reveal whether Agrin employs a similar strategy to stimulate the proliferation of tissue and organs. In the subsequent sections of the present review, the roles and mechanisms of Agrin in regulating the repair of different tissues and organs were discussed, which may offer important insights into novel strategies for regenerative therapies. The search sequence (PRISMA flow diagram) is provided in Fig. S1.

2. Regulation of Agrin in neurogenesis

Possible mechanisms of Agrin in neurogenesis. Over the last several decades, Agrin has been identified to play a central role in the formation of skeletal neuromuscular synapses between presynaptic motor neurons and postsynaptic muscle fibers (10). During this process, Agrin is released by motor neurons and interacts with the transmembrane LRP4, which leads to acetylcholine receptor (AChR) aggregation via the activation of MuSK; this is important for the formation of a functional NMJ (4).

A previous study demonstrated that local Agrin stimulation induces the clustering of mitochondria and synaptic vesicles, two well-known presynaptic markers, and regulates vesicular trafficking (11). Furthermore, Gautam *et al* (12) reported that postsynaptic AChR aggregates are significantly reduced in number, size and density in the muscles of Agrin-deficient mutant mice, suggesting that mice lacking Agrin are unable to make synapses. This defect may be caused by the absence of a neuron-specific isoform, with an exon encoding only eight amino acids, which is a critical nerve-derived inducer of postsynaptic differentiation (13). However, two reports have indicated that synapses can form in the absence of Agrin (14,15). Cyclin-dependent kinase 5 is activated by ACh agonists, and is required for the ACh agonist-induced dispersion of AChR clusters that have not been stabilized by Agrin (14). Similarly, a study by Misgeld *et al* (15) demonstrated that the action of Agrin *in vivo* is critically dependent on cholinergic neurotransmission. Using double-mutant mice, these authors demonstrated that synapses can form in the absence of Agrin, provided that ACh is also absent. These results suggest that ACh causes instability in newly generated postsynaptic sites and indicate that a major physiological role of Agrin is to counteract this 'anti-synaptogenic' influence (15).

Of note, ectopic MuSK expression promotes ectopic synapse formation, and ectopic MuSK expression stimulates synapse formation in the absence of Agrin and rescues the lethality of *AGRN*-mutant mice. Furthermore, Agrin ablation is accompanied by a second gene transformation, leading to increased postsynaptic MuSK concentrations. These results further indicate that the postsynaptic cell, and particularly MuSK, has a crucial role in the regulation of synapse formation (16). Furthermore, studies suggest that Agrin may stabilize existing AChR aggregates in the presence of neurally derived dispersants (17,18). Although MuSK and the synapse-specific cytoplasmic protein rapsyn are required in the initial steps of postsynaptic differentiation and the formation of an end-plate band, Agrin is not essential. However, nerve-derived Agrin is required in the subsequent stages of synaptic growth and maintenance (17).

Neurogenesis occurs at every stage of life, including in adults. It has been reported that neurogenesis is associated with learning, memory and mood regulation, and its attenuation contributes to emotional and cognitive deficits during aging and neurological diseases such as Alzheimer's disease (19). In the adult hippocampus, neural stem/progenitor cells of the subgranular zone reorganize and proliferate to generate newborn neurons, which then integrate into the granule cell layer of the dentate gyrus (20). During this dynamic process, the activation and proliferation of quiescent stem cells, neuronal fate specification, cell migration and synaptic integration are all involved (21). In addition, Agrin mRNA levels are reportedly increased in the mouse hippocampus following exposure to an enriched environment, suggesting that Agrin expression is dependent on activity (22). Rather than as a key synaptic organizer, Agrin has been designated as a stabilizer that can induce postsynaptic differentiation in the absence of nerves (17,23). Another study reported that all three members of the Agrin-MuSK-LRP4 complex are involved in the induction of presynaptic differentiation (24), and particularly strong evidence supports the role of LRP4 (25).

During Agrin-induced neurogenesis, multiple types of cells and signaling pathways are involved (26). Transforming growth factor-β1 has been reported to enhance synaptogenesis via the upregulation of neuronal Agrin expression in Schwann cells, which is both sufficient and necessary for mediating synapse-promoting effects in the developing NMJ (27). In addition, Zhang *et al* (28) revealed that the combined treatment of Agrin and laminin in a co-culture system is able to enhance functional NMJ formation through a primarily neural mechanism, which has potential clinical importance for treating denervation injuries and creating functional neuromuscular constructs for muscle tissue. Zhang *et al* (22) have also reported that Agrin activates the receptor tyrosine kinase orphan receptor 2 (ROR2) through LRP4 in a mouse model, identifying a role for Agrin-LRP4-ROR2 signaling in adult neurogenesis. Similarly, Ma *et al* (29) determined the role of Agrin in botulinum neurotoxin type A-induced nerve sprouting in a rat model by regulating downstream MuSK and upstream microRNA (miR)-144, and confirmed that Agrin can regulate nerve sprouting via the miR-144-Agrin-MuSK

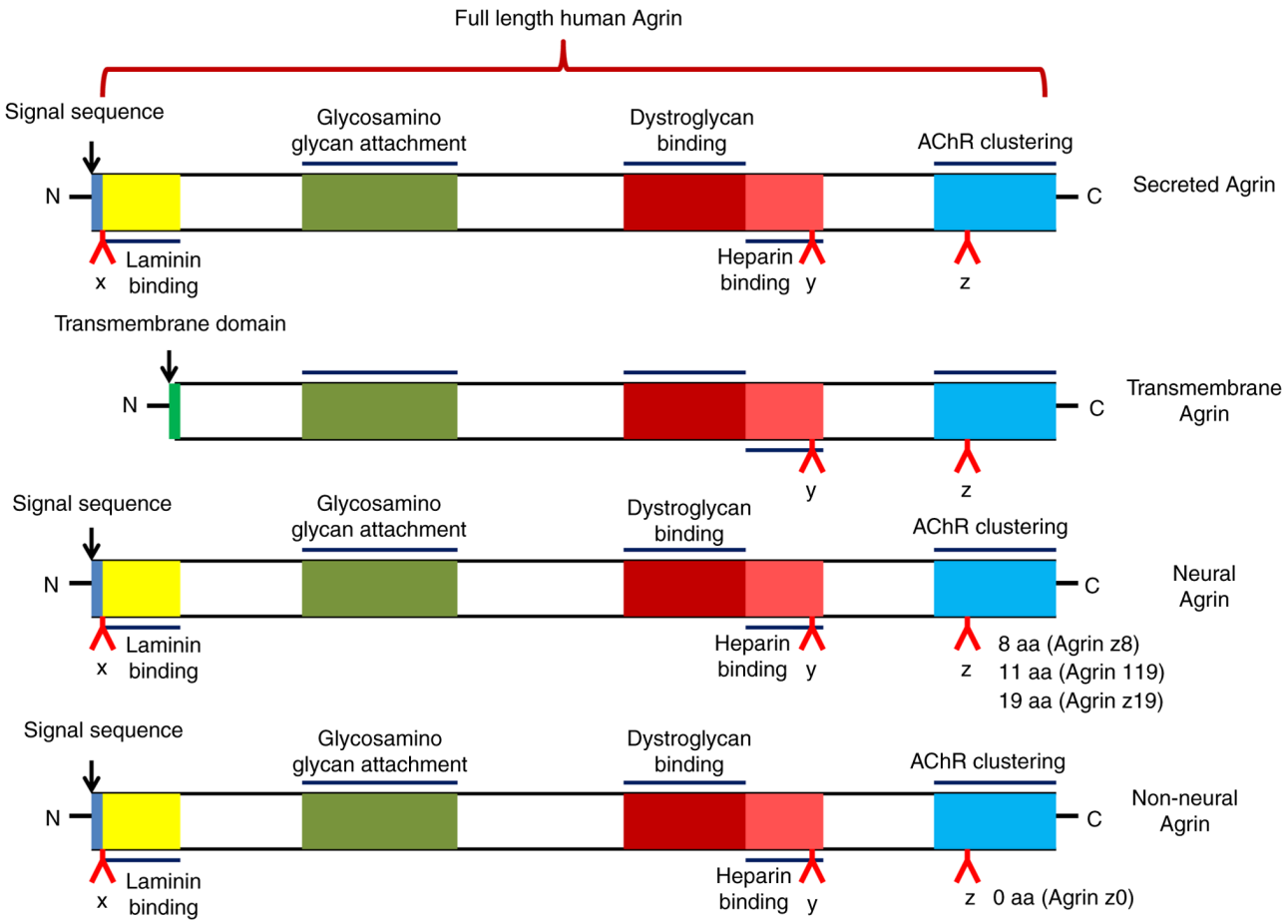

Figure 1. Schematic showing different forms of Agrin. Referenced by Chakraborty *et al* (3) and recreated with BioRender.com. AChR, acetylcholine receptor.

signaling pathway. Yang *et al* (23) reported that the expression of the active Agrin isoforms B11 and B19 is upregulated in Schwann cells during nerve regeneration in adults. As well as reporting that neurons express active Agrin, they also noted that glial cells express active Agrin and play a role in inducing AChR clusters beneath perisynaptic Schwann cell sprouts (23), suggesting that Agrin may play an indispensable role in the repair of central nervous system injury.

Possible role of Agrin in nerve disease. Myasthenia gravis (MG) is an autoimmune disease in which antibodies against AChR, MuSK or other AChR-related proteins in the NMJ cause localized or general muscle weakness (30). In patients with MG, autoantibodies bind to the components of postsynaptic muscle endplates and destroy the structure and function of NMJ, thus leading to impaired neuromuscular transmission (31). Recent studies have revealed that antibodies against Agrin and its receptor LRP4 are both critical for NMJ formation and maintenance in patients with MG (32). Furthermore, Yu *et al* (33) revealed that anti-LRP4/Agrin antibodies in patients with MG are pathogenic; they impair the NMJ by interrupting Agrin-dependent LRP4-MuSK interactions. A multicenter study revealed that LRP4/Agrin antibody-positive patients with double-seronegative MG have more severe clinical disease than antibody-negative patients (34). These results suggest that Agrin-LRP4-MuSK signaling may be a potential therapeutic target for MG and other neuromuscular disorders (35). NT-1654 is a C-terminal fragment of mouse neural Agrin; it possesses the same mechanism of action as Agrin, by binding to LRP4 to activate MuSK protein Docking protein 7 signaling at the NMJ. It has also been demonstrated to induce AChR aggregation and alleviate a sarcopenia-like phenotype (36). Li *et al* (37) similarly reported that NT-1654 attenuates the clinical severity of this phenotype, effectively promotes AChR aggregation at the NMJ and attenuates the repair of NMJ transmission and the reduction of MuSK in rats with experimental autoimmune MG.

Agrin may also play an important role in other nerve-related diseases. Adult neurogenesis in the hippocampus may represent the plasticity of brain functions, including emotions, learning and memory. A decline in hippocampal neurogenesis is thought to cause emotional and cognitive deficits in aging and Alzheimer's disease. A recent study reported that neurogenesis in the brains of healthy individuals may be more conservative (38,39). Furthermore, Zhang *et al* (22) revealed that Agrin is upregulated in the hippocampus of mice stimulated by an enriched environment. The genetic deletion of *AGRN* in excitatory neurons decreases the proliferation of neural stem/progenitor cells and increases depressive-like behavior (22). A further analysis led to a working model in which Agrin activates ROR2 via LRP4 to promote adult neurogenesis.

Sepsis is another infection-induced neuromuscular dysfunction; it can induce denervation-like alterations in the

NMJ, which may cause muscle weakness (40). Lv *et al* (41) reported that decreased Agrin expression may induce skeletal muscle dysfunction in sepsis, whereas exogenous Agrin alleviates neuromuscular dysfunction and downregulates γ- and α7-nicotinic AChR expression.

3. Role of Agrin in the tumor microenvironment

In the process of tumor progression, tumors make new capillaries by angiogenesis, and the targeting of angiogenesis contributes to tumor treatment (42). Localized benign tumors are surrounded by well-developed basement membranes, which limit tumors from migrating from the surrounding tissue into blood vessels. However, in malignant tumors, the tumor triggers an 'angiogenesis switch', thus tipping the balance between pro- and anti-angiogenic factors toward vascularization (43). Angiogenesis is a hallmark of cancer, and although multiple naturally occurring compounds have anti-angiogenic effects, these effects are not absolute (44,45). A recent study demonstrated that different protein modules within proteoglycans can enhance tumor cell plasticity and metastasis (46). However, the molecular mechanisms underlying their recruitment of blood vessels within the tumor niche remain largely elusive.

As a surface proteoglycan, the role of Agrin in promoting cancer angiogenesis has been demonstrated recently (7,47). He *et al* (47) reported a high expression of Agrin in cholangiocarcinoma tissue compared with that in adjacent non-tumor tissues; further analysis revealed that Agrin expression is associated with poorer prognosis, such as portal vein tumor thrombus and intrahepatic metastasis. Furthermore, forced Agrin expression in cholangiocarcinoma cells appears to promote tumor growth-related processes such as proliferation, colony formation, migration and invasion. In rectal cancer, Agrin expression has also been revealed to be markedly increased, and its upregulation is associated with poor prognosis. However, Agrin inhibition suppresses cell growth in rectal cancer, whereas Agrin overexpression prompts these behaviors *in vitro* (7). Mechanistically, multiple signaling pathways may participate in Agrin-regulated cancer progression. Agrin reportedly activates the Hippo signaling pathway and induces the translocation of yes-associated protein (YAP) to the nucleus in cholangiocarcinoma (47). However, Agrin also elevates Wnt pathway activity by increasing cyclin D1, c-Myc, phosphorylated glycogen synthase kinase-3β and phosphorylated β-catenin levels (7). These results indicate that Agrin may function as an oncogenic indicator of cancer progression through its activation of various pathways, which may be helpful for developing optimized therapies for cancer.

In the healthy liver, Agrin expression in hepatocytes is minimal and its expression is limited to certain regions surrounding blood vessels. However, during the transformation of liver cirrhosis and hepatocellular carcinoma, Agrin levels in hepatocytes are significantly increased (22). The role of Agrin in extracellular matrix sensing and mechanical conduction has been reported to integrate integrin and focal adhesion, as well as the activation of YAP/tafazzin (TAZ) to promote liver tumor growth (48,49). Furthermore, the depletion of Agrin in cancer cells reduces blood vessel infiltration and suppresses tumor growth, as well as the metastasis of hepatocellular carcinoma cells to mouse lungs. Strikingly, metastatic lesions that can colonize the lungs by Agrin deficiency lack the capacity to attract peripheral pulmonary vessels within these lesions (50).

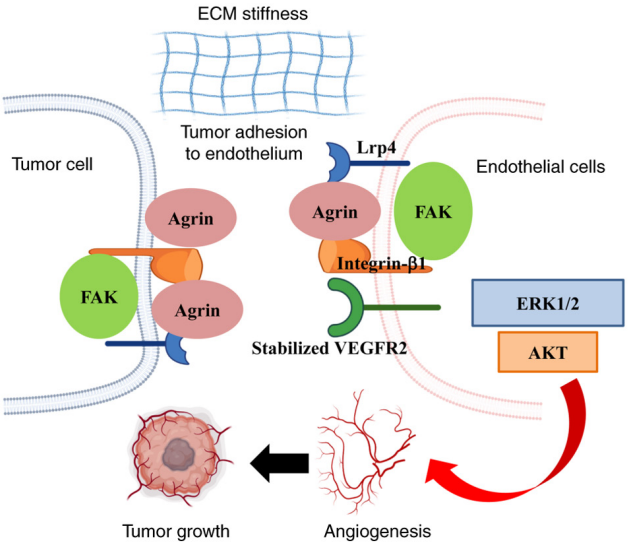

Figure 2. Agrin promotes adherence of endothelial cells to tumor cells and enhances tumor angiogenesis. The primary mechanism is extracellular matrix stiffness and Agrin stabilizes VEGFR2 by enhancing interactions with LRP4-Integrin-b1-FAK. Referenced by Njah *et al* (50) and recreated with BioRender.com. LRP4, low-density lipoprotein receptor-related protein 4, FAK, focal adhesion kinase; ECM, extracellular matrix.

Endothelial cell recruitment is critical for tumor vascularization. Njah *et al* (50) demonstrated that Agrin promotes the adherence of endothelial cells to tumor cells by recruiting blood vessels, and then facilitates tumor angiogenesis. Further analysis revealed that Agrin stabilizes vascular endothelial growth factor receptor 2 (VEGFR2) by enhancing its interactions with LRP4-integrin-b1-focal adhesion kinase (FAK) (Fig. 2). This suggests that tumor angiogenesis may be inhibited by targeting Agrin-induced VEGFR2 reductions. Furthermore, by inactivating the Hippo pathway, Agrin can promote extracellular matrix remodeling and stiffness by enhancing YAP/TAZ/transcriptional enhancer associated domain-dependent transcription, which then promotes tumorigenesis (49,51,52). Notably, consistent with other proteoglycans that affect endothelial cell migration, it has been reported that Agrin-either secreted by cancer cells or exogenously supplied - is essential for angiogenesis (53).

In the context of oral squamous cell carcinoma progression, Agrin is upregulated in oral squamous cell carcinoma and promotes cell migration and adhesion, suggesting that Agrin also plays an oncogenic role in oral cancer (54). Agrin can be cleaved through protein hydrolysis to produce bioactive fragments (55). One of the cleavage products is the C-terminal fragment, which reportedly has a role in multiple pathological processes, including sarcopenia (56), renal dysfunction (57) and cancer (58). Rivera *et al* (59) reported that invasive oral carcinomas have higher Agrin expression than benign tissue. These results suggest that the presence of Agrin may promote cancer progression in the tumor microenvironment. In oral squamous cell carcinoma cells, Agrin can activate FAK by binding to integrins or dystroglycan complexes, and can then induce cell invasion and metastasis through growth factor receptor-bound protein 2, proto-oncogene tyrosine-protein

kinase Src, extracellular regulated protein kinases and cyclin D (60,61). Nonetheless, the role of C-terminal fragment Agrin in angiogenesis needs to be further investigated in oral cancer, with a focus on tumor progression.

Other studies have reported that high Agrin expression is also associated with tumor progression and poor prognosis in hepatocellular carcinoma and lung adenocarcinoma (62,63). Agrin-positive staining can be used to identify patients with an increased risk of metastasis after surgery for lung adenocarcinoma, and may therefore be a valuable prognostic marker (62). Furthermore, the recurrence-free survival rate of Agrin-positive patients with hepatocellular carcinoma was reported to be significantly lower than that of Agrin-negative patients (63). The potential mechanism may be the result of Agrin-mediated tumor-related angiogenesis in the tumor microenvironment (53). Furthermore, Agrin knockdown in multiple human endothelial cell lines, including human umbilical vascular, human retinal, human dermal microvascular and human aortic endothelial cells, was observed to be associated with significantly reduced *in vitro* angiogenesis after treatment with soluble Agrin (50). However, in endothelial cell-specific Agrin knockout mice, normal endothelial and hematopoietic cell development was observed during embryogenesis (64). Of note, the growth of localized or metastatic cancer cells is not affected after their implantation into mice with Agrin-depleted endothelial cells. This finding suggests that Agrin may not play an important role in endothelial development during physiological and tumor-related angiogenesis; targeting endothelial-derived Agrin may therefore not be effective in inhibiting tumor angiogenesis.

Any discrepancies among these studies may be attributed to the availability of Agrin *in vitro* vs. *in vivo*, which may rescue the loss of Agrin in endothelial cells in *AGRN*-knockout mice during tumor angiogenesis. This suggests that in the early stages of tumor growth, endothelial cell recruitment does not require endothelial Agrin, as tumor-derived Agrin can compensate for the endothelial loss of Agrin. Thus, the profiles and mechanisms of Agrin in tumorigenesis need to be further studied.

4. Agrin-mediated cardiac regeneration

At present, heart disease is the leading cause of death worldwide, and repairing the damaged heart remains one of the most critical challenges in cardiovascular disease. In mammals, post-mitotic adult cardiomyocytes lose their proliferative capacity for replenishing damaged tissue (65). Upon injury, cardiomyocytes are replaced by fibrotic tissue, which usually has deleterious consequences. Hypertrophy therefore becomes responsible for most remaining heart growth. Notably, cardiomyocyte proliferation is sufficient for the repair of cardiac injury in neonatal mice; however, this ability is greatly diminished by 1 week after birth (66). In the past decade, however, researchers have questioned whether mature hearts truly lack the ability to produce new myocardium after injury; indeed, their findings suggest the possibility of a substantial endogenous regenerative capacity.

Studies have demonstrated that the proteoglycan Agrin can promote cardiomyocyte proliferation as an extracellular matrix component and is involved in neonatal heart repair (9,67). Of note, Agrin is reportedly enriched in the matrix on postnatal day 1 but decreased by postnatal day 7. Crucially, the administration of recombinant Agrin promotes cardiac regeneration in adult mice after myocardial infarction *in vivo* through the reactivation of cardiomyocyte proliferation. A porcine model of acute myocardial infarction, which is closely related to human cardiac physiology, has been used to demonstrate that recombinant human Agrin delivered to the infarcted heart can target the affected regions in an efficient and clinically relevant manner. These results indicate that recombinant Agrin may be a novel therapy for acute myocardial infarction and may prevent the onset of chronic heart failure (68). The data obtained from these mouse and porcine models highlight that Agrin treatment may strengthen pleiotropic effects and the broad cardiac repair process.

Agrin has been reported to bind and signal through α-dystroglycan (DAG1) (69) with C-terminus laminin G-like domains (LG1 and LG2) (60,70). In the heart, DAG1 serves as an Agrin receptor that is expressed by cardiomyocytes and mediates mild dedifferentiation and proliferation (9). The aforementioned reports emphasize the potential therapeutic role of Agrin in cardiac repair; this therapy is simple, safe and clinically relevant, and may be used in patients. However, further research is needed regarding the molecular mechanisms, effective dose ranges, toxicity and pharmacokinetics of Agrin.

The results from mouse (9) and porcine (68) myocardial infarction models that received local injections of recombinant Agrin into the damaged heart indicate that Agrin may have an overall cardioprotective effect. This was also accompanied by anti-inflammatory effects and blood vessel production, which adds to cardiomyocyte protection and induces proliferation. These findings suggest that Agrin has the potential to regulate cardiomyocyte cell proliferation. However, epicardial cells with multiple differentiation potential are also of great importance for cardiac regeneration (71). Normal development of the embryonic heart is reportedly regulated by epicardial cells through differentiation and proliferation (72,73). However, it remains unclear whether Agrin can promote the proliferation of embryonic epicardial cells. Jing *et al* (74) revealed that epicardial cell proliferation is promoted by Agrin through its regulation of YAP activity. Furthermore, YAP is key for the regulation of Hippo signaling (75), and Agrin has been reported to inhibit the Hippo signaling pathway and promote YAP activity (76). Although cardiac dysplasia can be induced by specific exfoliation of the embryonic epicardium (77), the abnormal epithelial-mesenchymal transition, migration and differentiation of epicardial cells can also be induced by proliferation disorders (78,79). The migration and epithelial-mesenchymal transition of epicardial cells are controlled by interactions between epicardial cells and the extracellular matrix (80,81). Thus, Agrin may play an important role in cardiac regeneration through its regulation of epicardial cells.

Considering the various risks and technical issues of current cardiac regenerative strategies, Agrin therapy has potential as a relatively safe and effective drug for repairing damaged hearts. However, caution is needed when performing this cardiac regenerative strategy because of the role of Agrin in tumorigenesis and angiogenesis. Furthermore, to evaluate the potential benefits of therapeutic Agrin, it should

be considered whether Agrin can stimulate human cardiac fibroblast hyperproliferation. It would also be worthwhile to investigate whether Agrin has a similar mechanism of action for stimulating cardiomyocyte and cancer cell proliferation.

5. Agrin mediates cartilage formation

Cartilage loss leads to osteoarthritis; cartilage has a low turnover and often fails to repair after injury because it is devoid of blood vessels (82). Cartilage regeneration is therefore a priority in medicine. A study by Erickson *et al* (83) indicated that *AGRN* was initially upregulated throughout fracture healing, suggesting that Agrin may have a novel function in non-neural tissue, including cartilage. Consistent with a role for Agrin in skeletal development, a study by Hausser *et al* (84) demonstrated that Agrin is involved in postnatal skeletal development and endochondral bone formation in transgenic mice. Furthermore, chondrocytes have high Agrin expression in the growth plate, suggesting that the expression of Agrin in cartilage may have a critical role in normal skeletal growth (84).

Agrin is composed of a large N-terminal portion, which binds to components of the basal membrane, and a biologically active C-terminal portion, which contains three globular domains separated by epidermal growth factor-like repeats (85). Eldridge *et al* (86) reported that Agrin is expressed in a splice isoform without y and z motifs; they also identified Agrin as having strong therapeutic potential in cartilage regeneration. Agrin is expressed in normal cartilage but is progressively lost in osteoarthritis, and Agrin knockdown induces downregulation of the cartilage transcription factor SOX9, as well as other cartilage-specific extracellular matrix molecules. Conversely, cartilage differentiation *in vitro* and ectopic cartilage formation *in vivo* are supported by exogenous Agrin. These results suggest that Agrin plays an important role in chondrogenesis and the repair of osteochondral defects (87).

Reduced growth and impaired skeletal development have been observed in Agrin-null mice, thus indicating an important role for Agrin in chondrocyte biology (84). A study by Eldridge *et al* (87) also revealed that Agrin is upregulated in injured cartilage and induces chondrogenic differentiation in synovial membrane mesenchymal stem cells. A single intra-articular administration of Agrin induces the long-lasting regeneration of critical-size osteochondral defects by attracting joint-resident progenitor cells to the injury site. Furthermore, the simultaneous activation of cAMP responsive element-binding protein and the suppression of canonical Wnt signaling downstream of b-catenin are critical for inducing stable articular chondrocyte differentiation and the formation of stable articular cartilage. In addition, considering the close relationship between cartilage and bone, Agrin may be involved in the replacement of cartilage by bone; this process involves multiple steps and several components of the extracellular matrix (84,86,88,89).

Agrin plays an important role in chondrocyte biology and participates in multiple signal transduction pathways. The Wnt pathway has been well characterized, and is thus an attractive therapeutic target for bone repair and skeletal homeostasis (90). As described in the previous paragraph, β-catenin has various roles at different stages of bone repair and regulates the ratio of osteoblasts and chondrocytes in the callus, which arises from pluripotent mesenchymal stem cells in the early phases after injury (91). Later in the bone healing process, β-catenin induces osteoblast differentiation and osteoblastic matrix production (92). As the main receptor of Agrin, LRP4 is also involved in the modulation of Wnt signal transduction, which is an important pathway in osteogenic differentiation and bone formation (93-95). Souza *et al* (96) reported that Agrin and its receptors (LRP4 and DAG1) are expressed during the differentiation of osteoblasts from three different sources. Furthermore, Agrin disruption impairs the expression of its receptors, as well as osteoblast differentiation, and treatment with recombinant Agrin slightly improves this process. Agrin knockdown also downregulates the expression of genes related to Wnt and bone morphogenetic protein (BMP) signaling pathways. These results highlight the contribution of the Agrin-Wnt-BMP pathway to osteoblast differentiation and suggest that Agrin is a candidate target in the development of new therapeutic strategies for bone-related diseases and injuries (96).

Together, the aforementioned results indicate that Agrin may be an orchestrator of repair morphogenesis at the joint surface through its modulation of multiple signaling pathways. We therefore anticipate that it represents a unique therapeutic opportunity in the field of osteoarthritis, which opens new avenues of investigation for cartilage-regenerative medicine. However, in the clinic, cartilage defects are often associated with meniscal/ligament injury and are at times accompanied by osteoarthritis. Given that joint instability can be compromised in the presence of inflammation, it remains to be explored whether Agrin can induce cartilage regeneration under these conditions.

6. Role of Agrin in aging and disease

During aging, AChR clusters at the NMJ become fragmented and denervated. As the site of information exchange and storage between motor neurons and muscles, deleterious morphological, functional and molecular features are acquired in the NMJ with advancing age, and the NMJ ultimately degenerates (97). NMJ activity requires communication (through molecular mechanisms) among all three cellular components: The presynapse, postsynapse and postsynaptic currents. These three cellular components collaborate through synapse-associated molecules to regenerate adult NMJs following injuries that cause severed motor axons, postsynaptic current loss and muscle fiber atrophy (98). A study suggested that age-related NMJ decline is induced by compromised Agrin-LRP4-MuSK signaling (99). This highlights the interdependence between all three cellular components of the NMJ, as well as the roles of synapse-associated molecules, such as Agrin, LRP4 and MuSK receptors, in the maintenance and repair of adult NMJs. In this regard, a study has shown that Agrin deletion in a subset of adult motor neurons causes postsynaptic disintegration and motor axon degeneration, suggesting that synaptic molecules-including Agrin-remain essential in adult NMJs (100). This indicates that synaptic molecules that are essential for NMJ formation continue to play important functions in adulthood. Together, these findings suggest that

Table I. Signaling network during Agrin-induced regeneration.

Author(s), year	Cell type	Function	Targets	Mechanism	(Refs.)
Bassat *et al*, 2017	Cardiomyocyte	Promotes division	DGC/YAP	Disassembly of the DGC, and Yap- and ERK-mediated signalling	(9)
Zhang *et al*, 2019	NSPCs	Neurogenesis	LRP4/Ror2	Promotes adult neurogenesis through proliferation and maturation of NSPCs	(22)
Jing *et al*, 2021	Epicardial Cells	Promotes proliferation	YAP	Increases the expression of Ki67, pH3 and Aurora B through YAP	(74)
Hou *et al*, 2020	Limbal stem cells	Promotes proliferation	YAP/Cyclin D1	Facilitates the dephosphorylation of Yap1 and activates transcription of Cyclin D1	(76)
Eldridge *et al*, 2016	Chondrocyte	Differentiation	LRP4/DAG/ DKK1	Inhibition of WNT pathway and promotion of SOX9 expression	(86)
Eldridge *et al*, 2020	Chondrocyte	Differentiation	LRP4/DAG/ CaMKII	Inhibition of WNT signaling in a CREB-dependent manner	(87)
Chakraborty *et al*, 2021	Keratinocytes	Enhances mechanically competent	MMP-12	Overhauls cytoskeletal architecture via enhancing actomyosin cables	(122)
Chakraborty *et al*, 2018; Calvo *et al*, 2013	Cancer cell	Carcinogenesis	MuSK	Inhibition of the Hippo pathway by promoting the FAK-ILK-PAK1 axis	(3,124)
Sun *et al*, 2021	Epicardial cells	Regulator of EMT	β-catenin/ pFAK	Enhances EMT by decreasing β-catenin and promoting pFAK localization and the aggregation of dystroglycan	(125)

LRP4, low-density lipoprotein receptor-related protein 4, DKK1, dickkopf WNT signaling pathway inhibitor 1; DAG, dystroglycan; FAK-ILKPAK1, focal adhesion kinase-integrin linked kinase-p21 activated kinase; CaMKII, calcium/calmodulin-dependent protein kinase II; DGC, dystrophin-glycoprotein complex; Musk, muscle-associated receptor tyrosine kinase; NSPCs, neural stem/progenitor cells; YAP, yes-associated protein; EMT, epithelial-mesenchymal transition.

age-related changes in NMJs may be mitigated by targeting these molecules. Studies that have assessed therapeutic potential following injury and in disease have demonstrated that Agrin and other integral components of the NMJ can repair age-related damage. Furthermore, following sciatic nerve crush surgery in young animals, administration of biologically active Agrin fragments into skeletal muscles can accelerate the rate of NMJ remodeling (36).

Sarcopenia is characterized by the loss of skeletal muscle mass, strength and function, and is a strong predictor of multiple adverse health outcomes, such as physical disability, hospitalization and mortality (101,102). During the neuromuscular remodeling process, the neuronal protease neurotrypsin proteolytically cleaves and inactivates *AGRN*, thus dissociating a 22-kDa C-terminal Agrin fragment (103). Recently, the C-terminal Agrin fragment concentrations in the circulation have emerged as a potential biomarker of skeletal muscle deterioration (104), which may signal the onset of sarcopenia.

Intriguingly, a study has demonstrated that appendicular lean mass, age and sex are significant explanatory factors for C-terminal Agrin fragment concentrations. In male individuals especially, there is a strong correlation between serum C-terminal Agrin concentrations and appendicular lean mass. Furthermore, vitamin D supplementation and physical exercise are significantly associated with lower C-terminal Agrin concentrations (105). This finding indicates that C-terminal Agrin fragments may be a potential marker for identifying sarcopenia in a subgroup of affected individuals in the future. Furthermore, the viability of the C-terminal Agrin fragment as a biomarker for sarcopenia has been confirmed in a variety of subpopulations (106). These results have also revealed that z-Agrin degradation during aging may contribute to NMJ pathology. Pratt *et al* (107) reported that plasma C-terminal Agrin fragment concentrations are significantly higher in sarcopenic individuals than in nonsarcopenic individuals, suggesting the potential relevance of C-terminal Agrin fragments as an accessible biomarker for skeletal muscle health. Together, these findings indicate that C-terminal Agrin fragments may be used for the diagnosis of sarcopenia, and may also have potential as an early indicator of denervation.

7. Functions of Agrin in other tissue and organs

In tissues such as the kidneys and lungs, Agrin plays a role in mechanotransduction by linking the cell cytoskeleton to other basement membrane components, including DAG1 and laminin-γ1 (108,109), through either direct binding or

indirectly via integrins (60). Raats *et al* (110) concluded that the presence of Agrin in the glomerular capillary wall and its ultrastructural localization are involved in linking the podocyte cytoskeleton to the glomerular basement membrane. However, other studies have directly demonstrated the contribution of Agrin to glomerular basement membrane functionality in podocytes and have reported that the glomerular basement membrane shows normal renal function with no changes in glomerular architecture, suggesting that Agrin is not required for the establishment or maintenance of glomerular basement membrane architecture (111,112). Vestentoft *et al* (113) reported that the levels of extracellular matrix constituents, including Agrin, are significantly upregulated after rat liver injury, mainly in the second tier of defense. These findings suggest that Agrin may play an important role in the hepatic progenitor cell response process for tissue repair and indicate a potentially important biological role for Agrin in other tissues and organs; however, the detailed roles and mechanisms remain unclear.

Limbal stem cell deficiency is an ocular surface disorder that is caused by the decreased population of corneal epithelial stem or progenitor cells and their dysfunction, which leads to vision loss and corneal blindness (114-116). Corneal epithelium regeneration depends on limbal stem cells, which may contribute to maintaining corneal epithelium homeostasis (117). Hou *et al* (76) reported that Agrin promotes limbal stem cell proliferation *in vitro* and noted that Agrin accelerates the wound-healing rate of corneal epithelium by activating limbal stem-cell proliferation *in vivo*. Further analysis of the underlying mechanism revealed that Agrin can facilitate the nuclear translocation of YAP1 and the expression of cyclin D1 induced by YAP1 dephosphorylation, which subsequently promotes limbal stem cell proliferation (76).

Hematopoiesis is a dynamic process that refers to the production of all hematopoietic-lineage cells generated by multipotent hematopoietic stem cells (118). Among the extracellular matrix components, heparan sulfate proteoglycans reportedly play a crucial role in controlling the structural and functional organization of the bone marrow hematopoietic stem cell niche (119). Agrin is expressed by multipotent nonhematopoietic mesenchymal stem cells as well as by differentiated osteoblasts from the surface of the bone endometrium. Furthermore, Mazzon *et al* (69) reported that Agrin is a critical niche-derived signal that controls the survival and proliferation of hematopoietic stem cells, and demonstrated that, although Agrin-deficient mice display impaired hematopoiesis, this can be reverted by Agrin-sufficient stroma. These findings suggest that Agrin may play a crucial role in the hematopoietic niche and in the cross-talk between stromal and hematopoietic stem cells.

Wound healing represents a complex biological program for restoring damaged tissue architecture to a normal state (120). During this process, a major factor for effective wound healing following injury is the rate of deposition of new extracellular matrix and its components that favor the healing process, which may subsequently support keratinocyte proliferation, migration and angiogenesis (121). A clinical trial study by Chakraborty *et al* (122) revealed that the recombinant Agrin fragment has great potential to accelerate wound healing as a bio additive material, and enrichment of the proteoglycan Agrin occurs early in the wound microenvironment, which indicates that it is essential for healing. Importantly, Agrin enhances the actomyosin cables by sensing geometric stress and force after injury, and then completely alters the cytoskeletal structure. Furthermore, matrix metalloproteinase-12 (MMP12) has been identified as a downstream effector of Agrin (123), and the Agrin-MMP12 pathway integrates a broad range of mechanical stimuli to promote optimal mechanical biology for wound healing and the generation of pro-angiogenic parameters. Therefore, injury-triggered Agrin enrichment has been proposed to integrate a broad range of mechanical stimulation and enhanced mechanical sensation through MMP12 activity in keratinocytes, which ultimately favors wound healing.

8. Conclusions and perspectives

Although Agrin was initially identified as a factor that is critical for NMJ function, its function in other tissues-including cardiac regeneration, tumor growth and cartilage formation-implies a widespread role for this protein. Existing evidence suggests that Agrin function is associated with the regulation of tissue proliferation and regeneration. During this process, multiple fundamental signaling pathways that are modulated by Agrin are involved (3,9,22,74,76,86,87,122,124,125) (Table I), and different molecular mechanisms appear to play functional roles in different cell types. For instance, Agrin promotes epithelial-mesenchymal transition by decreasing β-catenin and promoting phosphorylated FAK localization at focal adhesions in human embryonic stem cell-derived epicardial-like cells, and enhances dystroglycan aggregation in the Golgi apparatus. The absence of Agrin leads to the dispersal of dystroglycan *in vivo*, thus disrupting basement membrane integrity and impairing epithelial-mesenchymal transition. Injury-triggered Agrin enrichment induces a broad range of signaling pathways in different tissues, which ultimately favors the development of an injury microenvironment. In summary, Agrin represents a unique therapeutic opportunity in regenerative medicine, and a new and exciting research avenue involves understanding how to achieve a high specificity of biological effects by regulating otherwise-pleiotropic signaling pathways. However, despite initial findings, the precise function of Agrin in repair and regeneration-at both the molecular and whole-organism levels-remains to be elucidated.

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Availability of data and materials

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Authors' contributions

YYM conceived the study. XL reviewed the literature and wrote the draft. YX interpreted the information and checked the manuscript. JXS and FG collected part of the information and produced the figures. YYM supervised the preparation of the study and revised the manuscript. All authors read and approved the final manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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