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Review Article

Neuregulins: protective and reparative growth factors in multiple forms of cardiovascular disease

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Neuregulins (NRGs) are protein ligands that act through ErbB receptor tyrosine kinases to regulate tissue morphogenesis, plasticity, and adaptive responses to physiologic needs in multiple tissues, including the heart and circulatory system. The role of NRG/ErbB signaling in cardiovascular biology, and how it responds to physiologic and pathologic stresses is a rapidly evolving field. While initial concepts focused on the role that NRG may play in regulating cardiac myocyte responses, including cell survival, growth, adaptation to stress, and proliferation, emerging data support a broader role for NRGs in the regulation of metabolism, inflammation, and fibrosis in response to injury. The constellation of effects modulated by NRGs may account for the findings that two distinct forms of recombinant NRG-1 have beneficial effects on cardiac function in humans with systolic heart failure. NRG-4 has recently emerged as an adipokine with similar potential to regulate cardiovascular responses to inflammation and injury. Beyond systolic heart failure, NRGs appear to have beneficial effects in diastolic heart failure, prevention of atherosclerosis, preventing adverse effects on diabetes on the heart and vasculature, including atherosclerosis, as well as the cardiac dysfunction associated with sepsis. Collectively, this literature supports the further examination of how this developmentally critical signaling system functions and how it might be leveraged to treat cardiovascular disease.

Introduction

Neuregulins (NRG) are polypeptide growth factors belonging to the epidermal growth factor family that signal through receptor tyrosine kinases encoded by the erythroblastic leukemia viral oncogene homolog receptor (ErbB) family [1]. ErbB receptor dimerization by NRG activates tyrosine kinases that induce vital pathways in embryogenesis, including cardiac development, neural development, and myogenesis [2–6]. Four NRG genes have been identified, each producing isoforms with specific post-translation modifications that have been demonstrated to regulate tissue morphogenesis and repair via context-specific cellular processes including proliferation, differentiation, survival, apoptosis, and migration.

The neuregulin/ErbB pathway has been a source of interest in cardiovascular biology since the disrupted expression of neuregulin 1 (NRG-1), ErbB2, and ErbB4 all produce an identical lethal phenotype from myocardial trabeculation failure at 10.5 days gestation [2–4]. The clinical cardiovascular relevance of this biology was awakened when Herceptin (Trastuzumab), an ErbB2 (HER-2, c-neu) monoclonal antibody used in breast cancer, was found to be associated with an unacceptably high incidence of left ventricular dysfunction when used with anthracycline therapy [7]. Since those initial reports, NRG-1 has been found to have cardioprotective effects, leading to work examining recombinant NRG-1 as a heart failure therapy with regenerative capabilities. This review aims to discuss the most recent discoveries regarding NRG/ErbB cardiovascular biology, clinical applications in cardiovascular repair, and areas of proposed future investigation.

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Neuregulins

NRG-1 is the most extensively studied NRG and is one of four separate genes (*Nrg1–Nrg4*), each producing unique proteins able to signal through ErbB receptors. The *Nrg1 gene* can produce multiple different isoforms via alternative splicing [8–10]. The three structural components that differentiate these isoforms include either an α- or β-splice variant of the EGF-like domain, the N-terminal sequence, and a transmembrane domain. The EGF-like domain is the peptide sequence that provides binding specificity to ErbB3 and ErbB4 [11]. The immense diversity of isoforms has been predominantly studied in neuronal development and neuronal plasticity [12,13]. With regards to the adult cardiovascular system, NRG-1 type 1 isoforms are expressed in the microvascular endothelium of the heart, with NRG-1α expressed at higher levels than NRG-1β [14]. The NRG-1 isoforms 1 and 2 with β EGF domains are of critical importance as loss of function of these subunits results in a lethal phenotype from myocardial trabecular failure at 10.5 days of life [4].

NRG-1 subtypes expressed in the heart are pro-NRG transmembrane proteins that require protease processing for activation [14]. Early studies implicated the ADAM family of matrix metalloproteinases (MMPs), specifically ADAM17 and ADAM19, in the release of endothelial membrane pro-NRG-1 [15–18]. Further studies had shown ADAM17 to be the primary protease involved in the release of pro-NRG-1, while ADAM19 has no role [19]. There are likely other proteases that are able to activate pro-NRG-1, as ADAM17 knockout mice do not resemble NRG-1 deficient mice with respect to embryonic heart defects [19]. There remains much that is unknown about what regulates the activity of ADAM17 in relation to NRG-1 shedding from the cardiac endothelium and how these are altered in pathologic states such as heart failure. For example, in a canine tachycardia-induced heart failure model, NRG-1 expression increased without a change in the mRNA expression of ADAM17 [20]. More studies are required to fully decipher how NRG-1 is released from the endothelium during physiologic conditions and cardiovascular stress.

As will be discussed in more detail later, NRGs can be found in the circulation, although whether circulating NRGs are active has been questioned. Endocardial and endothelial cells of the cardiovascular system are the source of NRG-1, which are responsible for cardiac development and cardiac protection from stress, respectively [21]. Of the remaining NRGs, NRG-2, and NRG-4 are believed to have cardiovascular functions, while NRG-3 appears to be expressed predominantly in the central nervous system.

The *Nrg2* gene produces the NRG-2 protein that has homology to NRG-1 but has a different activity and different selectivity for ErbB receptors. NRG-2 is expressed in the endocardial lining of the heart with higher expression in the atrium and lower expression in the ventricular outflow tracts [22]. Carraway et al. [22] demonstrated that NRG-1 favored heterodimerization of ErbB3 or ErbB4 with ErbB2 while NRG-2 favored ErbB3 heterodimerization with ErbB1 resulting in different cellular responses. In neuronal cell lines, ADAM10 and BACE2 are the predominant proteases required for proteolytic membrane release [23]. Further investigation is required to assess the role of NRG-2 in the heart.

The *Nrg4* gene produces the NRG-4 protein, which was initially thought to have little influence on the cardiovascular system. However, recently NRG-4 serum levels have been associated with insulin resistance [24], metabolic syndrome [25], nonalcoholic fatty liver disease [26], and the severity of atherosclerosis [27]. The *Nrg4* gene is capable of producing five splice variants with a wide tissue distribution [28]. The A1 and A2 variants of NRG-4 contain a transmembrane domain and are likely activated by proteolysis while the three B-isoforms lack a transmembrane domain and are likely secreted [29]. NRG-4 selectively binds ErbB4 producing homodimerization or heterodimerization with ErbB1 and ErbB2. NRG-4 was found to have approximately 8-fold lower binding affinity then NRG-1 [30].

ErbB receptors

The four ErbB receptor tyrosine kinases are ErbB1, ErbB2, ErbB3, and ErbB4. Of the four receptors, NRG-1 is known to directly interact with ErbB3 and ErbB4, causing a conformational change allowing homodimerization or heterodimerization with other ErbB receptors. Dimerization allows transphosphorylation catalyzed by the intracellular kinase domains of the receptor complex. ErbB2 has no natural ligand but gains kinase activity through heterodimerization. ErbB3 can bind NRG-1 but has low kinase activity; thus, homodimerization leads to minimal downstream signaling. When NRG binds ErbB4, a conformational change allows homodimerization with another ErbB4 or heterodimerization with ErbB1, ErbB2, or ErbB3 [31–33]. Expression levels of ErbB receptors vary in space (subcellular location and tissue distribution) and in time (e.g., developmental stage, physiologic, and pathologic stress). In embryonic mouse cardiac myocytes, the expression levels of ErbB2 and ErB4 are highest, with a progressive decline in myocyte expression starting early after birth [34]. ErbB3 is expressed in the myocardium at stable levels throughout postnatal development [35,36].

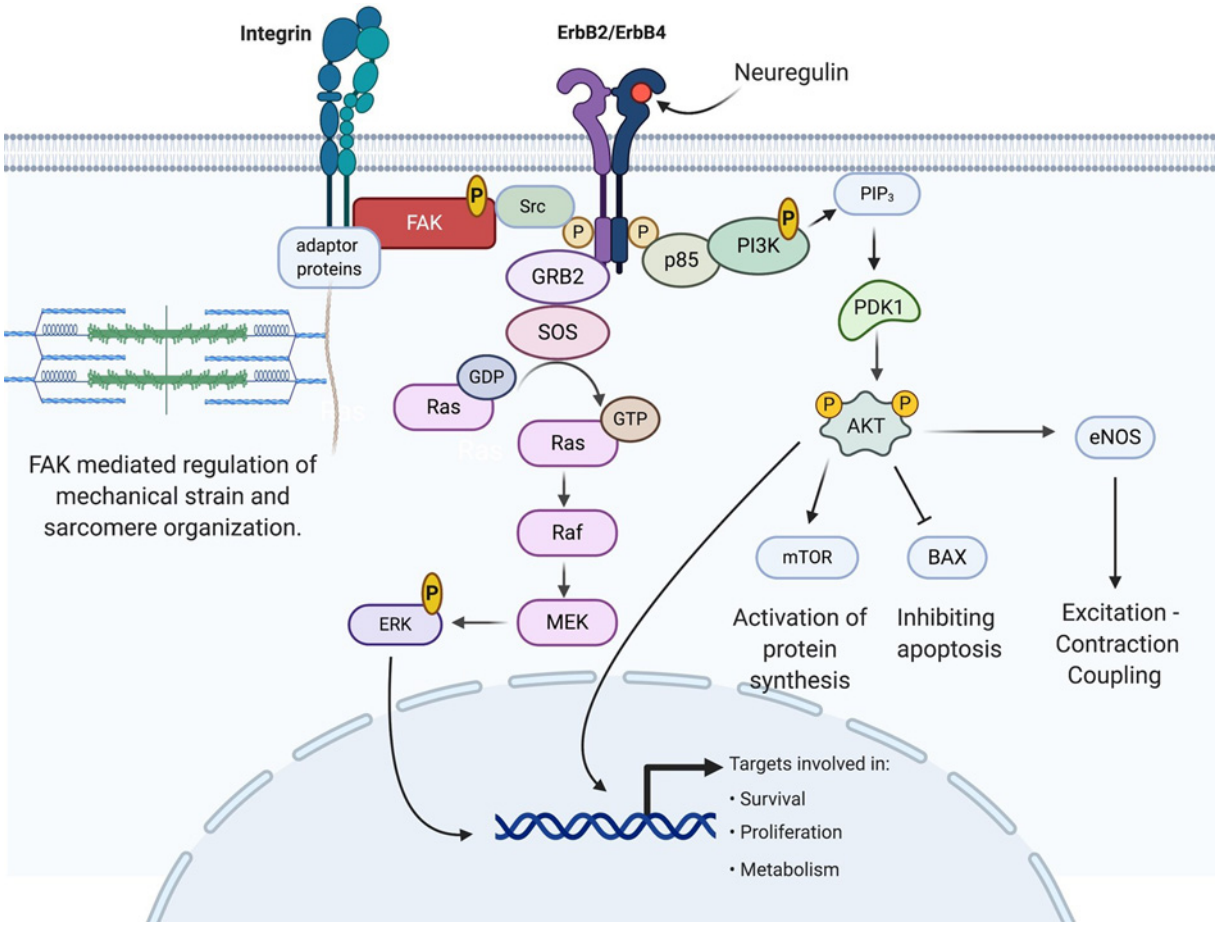

Figure 1. The NRG-1/ErbB signaling pathway in cardiac myocyte

NRG-1 binds ErbB4 and induces dimerization with ErbB2, allowing receptor tyrosine kinase transphosphorylation. Activated ErbB receptors interact with and activate several pathways including RAS/ERK, PI3K/Akt, and Src/FAK [171–174]. Abbreviations: AKT, serine/threonine-specific protein kinase B; BAX, bcl-2-associated x protein; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; GDP, guanosine diphosphate; GTP, guanosine triphosphate; GRB2, growth factor receptor-bound protein 2; MEK, mitogen-activated ERK kinase; mTOR, mammalian target of rapamycin; PDK1, phosphoinositide-dependent kinase 1; PIP3, phosphotidyl inositol (4,5,6)-triphosphate; PI3K, phosphatidyl inositol-3 kinase; p85, regulatory subunit of PI3K; Raf, proto-oncogene serine/threonine-protein kinase; Ras, Ras proteins, members of a large superfamily of small GTPases; SOS, son of sevenless; Src, proto-oncogene tyrosine-protein kinase.

The NRG/ErbB signaling system regulates diverse cellular responses that are context specific. The molecular regulators of this specificity are not fully understood but include several factors such as specific isoforms of NRG-1, ErbB receptor expression, subcellular localization, and coupling to intracellular signaling cascades (ERK 1/2, PI3K/Akt, STAT, and FAK) that ultimately regulate cellular responses based on the cell type (Figures 1 and 2). ErbB2 and ErbB4 in adult mouse ventricular cardiomyocytes are enriched in a specific membrane compartment, the T-tubule system [37]. The T-tubules are invaginations of the sarcolemma that are exposed to the extracellular space and function to link depolarization to intracellular calcium release and subsequent myocyte contraction. ErbB2 and ErbB4 are present at intercalated discs and have a T-tubule like striated pattern [38]. In that context, ErbB2 interacts with FAK, which is also located at the intercalated disc, and NRG activation induces focal adhesion complex formation in adult rat cardiac myocytes [39]. The localization in both sarcolemma T-tubules and intercalated discs suggests that ErbB2 and ErbB4 support the hypothesis that NRG/ErbB signaling in the adult heart regulates myocyte-to-myocyte connectivity. Further investigation is needed to fully understand this and other functions of the NRG/ErbB system in the adult heart.

Clinical Science (2020) **134** 2623–2643
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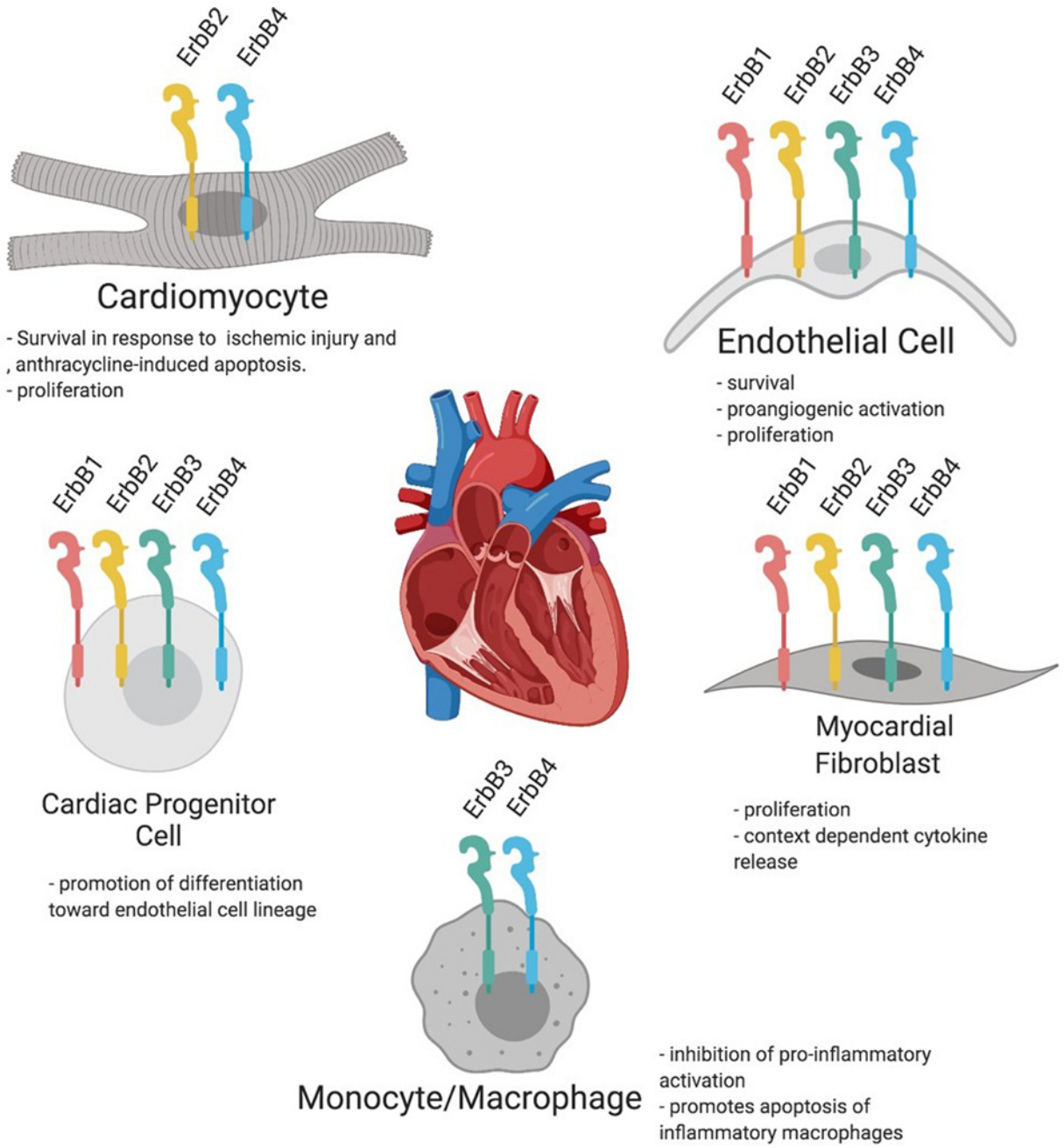

Figure 2. ErbB receptor expression in different subpopulations of cardiac cells

Adult cardiomyocytes express ErbB2 and ErbB4. Expression of all ErbB receptors, including ErbB1, ErbB2, ErbB3, and ErbB4, has been demonstrated on cardiac endothelial cells, fibroblasts, and highly proliferative cells. ErbB3 and ErbB4 are expressed on monocytes and cardiac macrophages. See the text for references.

Myocardial NRG/ErbB levels in relation to physiologic and pathologic cardiac stress

NRG-1, ErbB2, and ErbB4 are essential for cardiac development as discussed above, as well as the preservation of heart function and adaptation to physiologic and pathologic stresses (Figure 3 and Table 1). NRG-1 appears to be constitutively active at some level, as it can be found in the coronary effluent of isolated hearts [40]. In pregnancy, the heart adapts to physiologic stress induced by an increase in effective circulating volume, leading to left ventricular (LV) growth and eccentric remodeling. Left ventricular tissue samples from pregnant rats have both elevated NRG-1

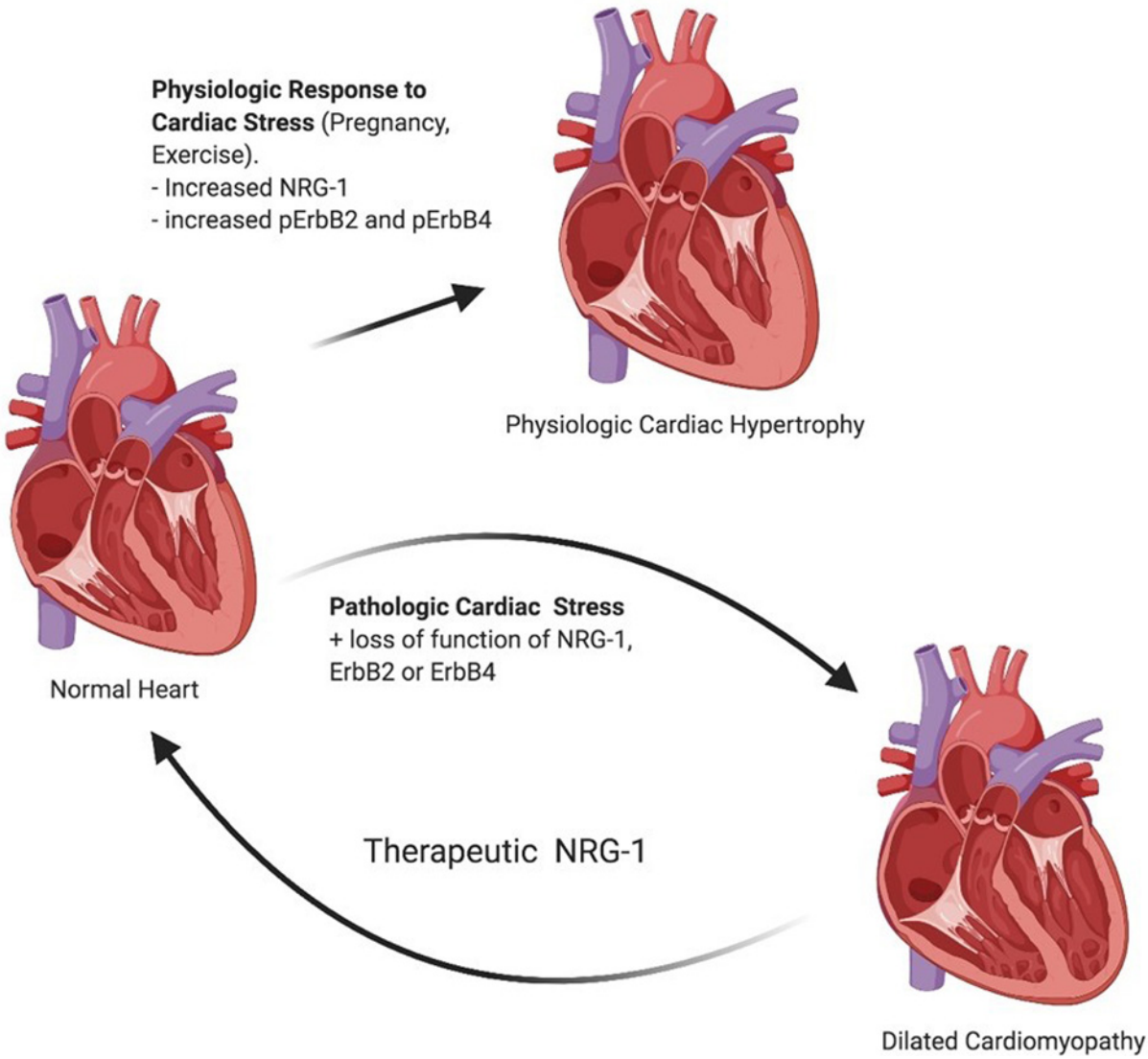

Figure 3. NRG-1 signaling through ErbB2 and ErbB4 regulate growth and survival pathways leading to physiological hypertrophy

The loss of NRG-1, ErbB2, or ErbB4 results in dilated cardiomyopathy under cardiac stress. *In vivo* studies have suggested that NRG-1 can improve left ventricular ejection fraction in subjects with heart failure with a reduced ejection fraction. *pErbB2 and pErbB4 represent phosphorylated (activated) forms of ErbB receptors.

Table 1 NRG-1 and myocardial ErbB receptors expression in different types of physiologic and pathologic stress

Cardiac stress	NRG expression	Myocardial ERBB expression and activity
Physiologic:		
Exercise	↑ NRG1 [42–44,175]	↑ p-ErbB2 and p- ErB4 [44]
Pregnancy	↑ NRG1 [41]	↑ p-ErbB2
Early aortic stenosis	No change	No change in ErbB2 and ErbB4
Early pacing induced cardiomyopathy	↑ NRG1	↑ p-ErbB2 and p- ErbB4 with increased PI3K/pAkt and p ERK 1/2.
Pathologic:		
Late aortic stenosis	No change [47]	↓ ErbB2 and ErbB4 [47]
Late pacing induced cardiomyopathy	↑ NRG1 [20]	↑ p-ErbB2 and ↑ p- ErbB4 but decreased PI3K, pAkt, and p- ERK1/2 [20]
Decompensate systolic heart failure	↑ NRG1 [50]	↓ ErbB2, p-ErbB2 and ↓ ErbB4,p-ErbB4 [50]
Post LVAD implantation	↓ NRG1 [50]	↑ErbB2 and ErbB4 [50]

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and activated ErbB2/ErbB4, implicating NRG/ErbB signaling in cardiac adaptation to physiologic stress [41]. Exercise, another physiologic cardiovascular stressor, activates NRG-1, in skeletal muscle [42], and circulating NRG-1 is positively correlated with cardio- respiratory fitness [43]. In a post-myocardial infarction rat model, exercise increases left ventricular tissue levels of NRG-1 as well as ErbB2/ErbB4 activation [44]. These studies all suggest that in adulthood, endogenous NRG/ErbB acts to preserve myocardial function in the setting of physiologic stress. Interestingly, sustained high level of NRG-1 expression in the cerebella throughout life is associated with an increased life span when compared across seven rodent species [45]. In correlation, diminished myocardial NRG-1/ErbB expression has been proposed to be related to age-associated myocardial senescence and loss of cardiomyocytes through apoptosis [46,47] suggesting NRG-1 has a role in the prevention of cardiovascular aging.

The activation of ErbB receptors by NRG *in vivo* has consistently been shown to be protective against pathological stress. Trastuzumab, a monoclonal antibody that disrupts ErbB2 function, is associated with systolic dysfunction and heart failure in patients treated for Her2/Neu positive breast cancer. Cardiomyopathy seen with Trastuzumab treatment was most prevalent when given in combination with anthracyclines [48], suggesting NRG/ErbB regulates the myocardial response to pathologic stress of anthracycline-induced injury. Signaling through the NRG/ErbB pathway diminishes in advanced LV dysfunction, potentially contributing to disease acceleration. Diminished NRG/ErbB signaling occurs in rats with aortic banding. Initially, aortic banding increased expression of NRG, but after persistent stress, LV expression of ErbB2 and ErbB4 decreases as decompensated heart failure develops [47,49]. The correlation between diminished ErbB2/ErbB4 expression and decompensated heart failure was also found when comparing myocardial samples before left ventricular assist device placement (LVAD) with normal unused donor hearts. Patients undergoing LVAD had lower expression of activated ErbB2/ErbB4 despite increased NRG-1 [50]. There is a clear correlation between diminished ErbB2/ErbB4 function and progression to decompensated heart failure, but it is unclear what is affecting ErbB expression. It has been postulated by others that down-regulation of NRG-1/ErbB signaling associated with pump failure is related to increased levels of angiotensin II, and epinephrine [51] as *in vitro* both angiotensin II and epinephrine have been found to suppress NRG-1 mRNA synthesis in cardiac endothelium [49].

Others have postulated that an inhibitory cross-talk between ErbB receptor tyrosine kinases and G-protein–coupled receptors (GPCR) is responsible for shifting the balance between pathologic and physiologic cardiac hypertrophy. This inhibitory cross-talk in the myocardium has been described by Chung et al. [52] after they found that activation of endothelin type A receptors (ET_A) inhibited ErbB2/ErbB4 at multiple levels, including NRG-1 induced ErbB2/ErbB4 autophosphorylation and downstream phosphorylation of Akt. However, studies there are also reports of positive interactions between GPCR and ErbB receptors. GPCR receptors can transactivate ErbB receptors via at least two mechanisms: phosphorylation of ErbB receptors via other tyrosine kinases such as Src [53] and through activation of the metalloproteinases leading to NRG activation [54–57]. Further studies are necessary to understand the role of cross-talk between GPCR and ErbB receptors in the progression of heart failure.

ErbB receptor expression is also regulated by micro-RNAs (miR) in many tissues, and this may also play a role in cardiac pathology. Cardiotoxic anthracycline treatment induces expression of miR-146a in mouse cardiac myocytes, which suppresses ErbB4 expression thereby increasing myocyte damage [58]. Pressure overload increases expression of miR-199a [59], which is suppressed by STAT3 (signal transducer and activator of transcription 3) [60]. Cardiac myocyte STAT3 deficienty in mice leads to cardiomyopathy [61]. Cardiac myocytes from STAT3-deficient mice have reduced ErbB4 as a result of increased miR-199a, resulting in impaired energy utilization and cardiac myocyte death [62]. Further studies are required to assess miRNA regulation of other ErbBs and NRGs.

Circulating neuregulin levels

The levels of circulating NRG-1 and NRG-4 in steady-state conditions have been examined in several studies (Table 2). We and others have found that the level of NRG-1 is characterized by large inter-individual variability with the range of absolute values covering two orders of magnitude, from hundreds to tens of thousands of picograms per milliliter of blood in healthy donors. The analysis of variability revealed the presence of high values of quartile coefficient of dispersion ranging between 0.8 and 0.9 in the blood plasma or serum [43,63]. This large inter-individual variability can explain the significant differences between levels of circulating NRG-1 found in different studies [43,63,64]. In contrast with large inter-individual variability, the individual level of circulating NRG-1 is stable, not changing even after exersice [43] in healthy volunteers. Similar to NRG-1, the levels of circulating NRG-4 also vary significantly with values of quartile coefficient of dispersion between 0.5 and 1.0 [24,65].

Several clinical studies have demonstrated that the level of circulating NRG-1 and NRG-4 changes during the development and progression of cardiovascular disease. For example, the level of circulating NRG-1 is increased in patients with paroxysmal atrial fibrillation [66]. While the underlying mechanisms remain to be investigated, the

Table 2 The levels of circulating NRG-1 and NRG-4 in human cohorts

Neuregulins	Mean ± SD	Median [IQR]	Plasma or serum	*N*	Reference	Major finding
NRG-1, ng/ml	217 ± 170		Serum	*n*=9	[43]	NRG-1 correlates with exercise capacity
	0.77 ± 0.37		Serum	*n*=36	[64]	NRG-1 correlates with proangiogenic factors, VEGF and Angiopoietin-1
	5.3 ± 8.8	0.9 [0.6, 7.0]	Platelet-free plasma	*n*=10	[63]	Circulating NRG-1 is functionally inactive
	9.0 ± 11.4	0.4	Plasma	*n*=62	[176]	NRG levels are reduced after exposure to cardiotoxic chemotherapy
		2.6 [0.2, 19.1]/4.1 [2.0, 12.9]	Serum/plasma	*n*=21	[70]	NRG-1 inversely correlates with CAD severity
		1.4 [0.2, 14.2]	Serum	*n*=319	[177]	No changes during acute coronary syndrome
		4.4 [2.8, 8.7]	Serum	*n*=899	[71]	Circulating NRG-1 is associated with heart failure severity and risk of death or cardiac transplantation
NRG-4, ng/ml		2.3 [1.1, 3.7]	Serum	*n*=129	[65]	The level of circulating NRG-4 is inversely associated with the risk of Type 2 diabetes mellitus
	1.1 ± 0.9		Serum	*n*=57	[78]	Circulating NRG-4 is inversely associated with the risk of acute coronary syndrome
		0.08 [0, 0.55]	Serum	*n*=83	[24]	The circulating NRG-4 is an independent risk factor associated with diabetes
	4.1 ± 2.0		Plasma	*n*=41	[178]	Circulating level of NRG-4 is reduced in diabetic peripheral neuropathy
	1.4 ± 0.2		Plasma	*n*=32	[27]	NRG-4 inversely correlates with the severity of CAD
	1.4 ± 0.1		Serum	*n*=24	[179]	NRG-4 is increased in patients with Type 2 diabetes mellitus

increased level of NRG-1 may be associated with the induction of compensatory mechanisms in response to cardiac stress associated with increased inflammation, pressure overload, and generation of reactive oxygen species [67–69]. Increased level of NRG-1 has also been found in patients with stress-induced ischemia [70], as well as severe heart failure, where it associates with the risk of death or cardiac transplantation [71]. A recent study demonstrated the positive correlation between circulating NRG-1 and left ventricular ejection fraction [72], supporting the idea that NRG-1 is contributing to compensatory cardiac responses.

The levels of both NRG-1 and NRG-4 correlate inversely with the severity of coronary artery disease (CAD) [27,70] and coronary collateral circulation [73]. The potential explanation may include the reduction in the synthesis or secretion of NRG-1 and NRG-4 by endothelial cells [74,75] associated with endothelial dysfunction in CAD [76,77]. In addition, an association between higher NRG-4 level and lower risk of acute coronary syndrome has been described in patients with coronary artery disease [78].

These and other changes in the levels of circulating NRG-1 and NRG-4 indicate regulation of activation and/or expression, but not necessarily changes in circulating activity. Nitration of NRG-1's EGF-like domain, found in circulating NRG-1, results in the inactivation of the ligand [79]. This may explain our recent finding that circulating

Clinical Science (2020) **134** 2623–2643
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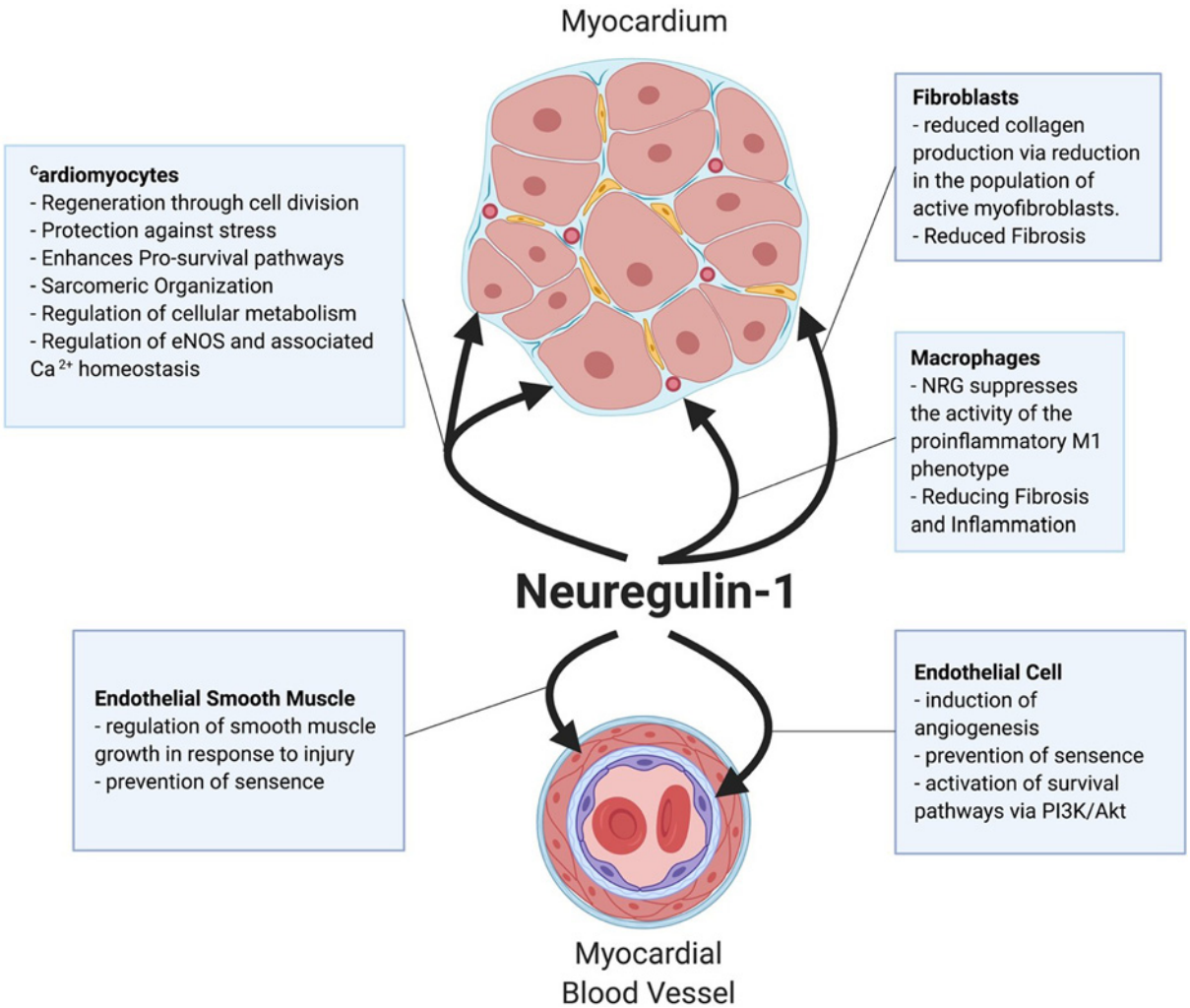

Figure 4. NRG-1 regulates functional responses in different cell types within the myocardium

Heart injury is associated with the proteolytic activation and release of NRG-1 from the surface of cardiac endothelial cells. Active NRG-1 protects cardiomyocytes, reduces pro-fibrotic activation of fibroblasts, promotes survival of endothelial cells, and angiogenesis, and prevents pro-inflammatory activation of immune cells.

NRG-1 is functionally not active in blood obtained from healthy donors [63]. In addition to NRG-1 inactivation, there are circulating NRG-binding proteins such as the endogenous soluble form of the human ErbB3 receptor that may negatively regulate circulating NRG activity [80]. The inactivation of NRGs and the presence of soluble binding partners, which limit the functional activity of NRGs, are adding to the complexity in interpreting the study results and emphasize the importance of functional testing of NRGs in healthy donors and patients with cardiovascular disease, using cell-based assays [63].

Neuregulin in repair of systolic heart failure

The clinical experience with Trastuzumab-associated cardiac dysfunction led to studies designed to understand the role of this signaling system in the adult myocardium [7]. Mice were created with inducible suppression of ErbB2 in cardiac myocytes using Cre-recombinase technology resulting in a dilated cardiomyopathy phenotype exacerbated by anthracyclines [37,81]. An identical dilated cardiomyopathy phenotype was also seen in a Cre-recombinase myocardial specific ErbB4 mouse model [82]. Recombinant NRG-1 was thus examined as a potential therapy for systolic heart failure [83]. The mechanisms of NRG-1's beneficial effects in cardiac repair in these studies are still being investigated, as the biology of this system in the heart and other tissues is complex and incompletely understood (Figure 4).

Table 3 Neuregulins studied as therapies for systolic heart failure

Recombinant forms of NRG	Commercial name	Domains	Examples of *in vivo* studies	Human studies	Results of human studies
Human recombinant Neuregulin 1 (rhNRG1β)	Neucardin	β-Isoform of the growth factor domain (EGF-domain) of NRG-1	Liu et al. [83], Gu et al. [180], Guo et al. [181], Fang et al. [182]	Jabbour et al. [116], Gao et al. [117]	Both phase 1 and 2 trials have been completed finding sustained improvement in LVEF in systolic heart failure patients at 90-day follow-up. A phase 3 trial is currently on going.
Human recombinant Neuregulin 1 (rdNRG1β)	None	β-Isoform of the growth factor domain (EGF-domain) of NRG-1 with Ig domain	Bersell et al. [89]	None.	None
Human recombinant glial growth factor 2 isoform of NRG1β (GGF2)	Cimaglermin Alfa	β-isoform of the growth factor domain (EGF-domain), type 2 isoform of NRG-1 gene, containing IgG and kringle domains	Bian et al [183], Hill et al. [184], Galindo et al. [95]	Lenihan et al. [118]	A phase 1 trial was completed finding sustained improvement in LVEF in systolic heart failure patients. There was a dose limiting side effect at the highest planned dose. See the text for details.
Engineered bivalent NRG1β (bivNRG)	None	2 β-Isoform of the growth factor domain (EGF-domain) of NRG-1 connected by a linking peptide	Jay et al. [145]	None	None

Cardiac myocyte growth, survival, and proliferation

The first evidence of direct effects of NRG-1 in cardiac myocytes was obtained from *in vitro* experiments using cells isolated from rat hearts [84]. The recombinant human glial growth factor 2 (GGF2), a recombinant protein with the active EGF-binding domain of NRG-1 (see Table 3 for further details), can improve embryonic cardiac myocytes survival in conditions of serum deprivation. Further studies demonstrated pro-survival effects of NRG-1, including protection from norepinephrine cytotoxicity [85]. NRG-1 also protects cardiac myocytes from anthracycline-induced apoptosis via ErbB4-dependent activation of the PI3K/Akt signaling pathway [86]. In addition, the mitogen-activated protein kinase signaling pathway is involved in NRG-1 mediated reprogramming toward enhanced cardiac myocyte growth and survival [87]. Endothelial cell-derived NRG-1 protects cardiomyocytes against hypoxia and reperfusion injury, both *in vitro* and *in vivo* [88]. The ability of NRG-1 to promote the survival of cardiomyocytes in response to a wide variety of cardiovascular stresses appears to be a critical role for NRG-1 in the postnatal heart.

Neuregulins, via activation of ErbB receptors, can also stimulate the proliferation of cardiac myocytes in the adult heart. Bersell et al. [89] demonstrated that NRG-1, signaling through ErbB2/ErbB4, can induce mononuclear cardiomyocytes to disassemble their myofibrils, enter the cell cycle, divide and regenerate injured myocardial tissue. Less than 10% of cardiomyocytes are mononuclear, and *in vivo*, only 0.3% of mononuclear cardiomyocytes under the influence of exogenous NRG-1 underwent division [89] suggesting that pharmacologic NRG-1 produces limited regeneration. D'Uva et al. [36] demonstrated that the manipulation of ErbB2 signaling augments cardiac regenerative properties. Mice with constitutively active cardiomyocyte ErbB2 show significant cardiac hypertrophy, with the cellular division of both mononuclear and binuclear myocardial cells. Mice with constitutively active ErbB2 that underwent myocardial ischemia showed a robust dedifferentiated population of cardiac myocytes in the infarcted tissue associated with improved cardiac function. Polizzotti et al. [90] demonstrated, in mice subjected to cryoinjury, NRG induces cardiac myocyte regeneration in newborn mice, but this response was diminished 5 days post-birth. Similarly, they showed that cardiac myocytes obtained from human infant myocardium respond to NRG in an age-dependent manner, with cell division only seen in cells obtained from younger subjects (<6 months) [90]. Based upon a series of experiments in zebrafish and mice, it appears that NRG-1-dependent cardiac regeneration in early postnatal life is dependent on cardiac innervation [91].

Anti-fibrotic and anti-inflammatory effects of neuregulins

NRG/ErbB signaling appears to regulate inflammation and fibrotic responses to injury in multiple tissues, including the heart. NRG-1's anti-inflammatory effects were first identified in CNS microglial cells after ischemic insult, where

Clinical Science (2020) **134** 2623–2643
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NRG-1 treatment reduced NF-kB mediated expression of TNFα and IL-6 [92]. NRGs appear to regulate the inflammatory response of the heart to injury, though the mechanisms are complex and incompletely understood. NRG-1 has direct anti- inflammatory effects in nonclassical monocytes in humans, acting via ErbB3, reducing TNFα production [93]. NRG-1 regulates macrophage phenotype, increasing the CD206 positive 'regenerative phenotype' when administered into a rat heart after myocardial infarction. This may relate to observations in other inflammatory diseases, including inflammatory bowel disease, where colonic macrophages exposed to inflammatory stimuli increased ErbB4 expression. NRG-4 administration reduced the number of colonic macrophages via induction of macrophage apoptosis and attenuated inflammation [94].

The effects of NRG-1 on the inflammatory response to injury are likely linked to the reduced fibrosis observed in several experimental systems. In left ventricular samples of post-MI swine treated with GGF2, there was more organized myofibril structure, reduced fibrosis, attenuated expression of pro-fibrotic genes, and a reduced number of myofibroblasts [95]. Similar anti-fibrotic effects were reported in a mouse model where treatment with recombinant human NRG1β (rhNRG-1β) attenuated the adverse effects of angiotensin II on cardiac hypertrophy by inhibiting fibrosis and activation of myocardial fibroblasts [96]. In mice with ErbB4-deficient macrophages, angiotensin exposure resulted in worse myocardial fibrosis, suggesting that active NRG/ErbB4 attenuates the fibrosis effects of fibroblasts [96]. An additional study found NRG-1 to have anti-fibrostic effects by suppressing macrophage activation and preventing bleomycin-induced fibrosis in the heart, lung, and skin tissue *in vivo* [96].

All of these data together provide strong support for the role of NRG-1 signaling through ErbB4 and ErbB3 in the control of myeloid cell activation, reduction of the number of pro-inflammatory macrophages via targeted apoptosis and suppressing the release of pro-inflammatory cytokines in multiple tissues. Because myocardial fibrosis and remodeling are leading molecular mechanisms in the progression of systolic heart failure, NRGs ability to prevent inflammation and pro-fibrotic transformation has substantial translational implications, providing a rationale for the use of NRGs as a therapy in systolic heart failure.

Regulation of remodeling via neuregulin's activation of focal adhesion kinase

Focal adhesion kinase (FAK) is a nonreceptor tyrosine kinase that is critical for the transmission of mechanical strain into biochemical signals that regulate cardiac myocyte growth and survival [97,98]. GGF2 treatment of adult rat ventricular myocytes induced formation of a multiprotein complex containing ErbB2, FAK p130^{CAS}, and paxillin, which induces lamellipodia formation and myocyte elongation that ultimately restored myocyte cell-to-cell contact and synchronous beating [39]. Increased mechanical load on the heart results in increased tension and stretch on cardiomyocytes, in association with activation of FAK [99] and NRG/ErbB [20]. Complete knockout of the FAK protein results in embryonic lethality, and myocardial specific Cre-recombinase deletion results in a dilated cardiomyopathy after exposure to angiotensin II [100]. An important part of normal physiologic cardiac remodeling is maintaining optimal sarcomere length despite cardiac myocyte growth and lengthening [97,101]. The physiologic growth of the heart during pre- and postnatal maturation [34] or during pregnancy is regulated by NRG/ErbB signaling [41]. An attractive hypothesis emerges from this work is that load-dependent NRG/ErbB regulation of FAK orchestrates the normalization of sarcomere load via cardiac myocyte growth, and perhaps cell division, as discussed previously. Similarly, the reparative effects of NRGs in systolic heart failure may in part be mediated by ErbB2 activation of FAK. These results support the hypothesis that NRG/ErbB signaling through FAK regulates mechanical strain-induced pathologic eccentric hypertrophy [102].

NRG/ErbB regulation of angiogenesis

Angiogenesis plays a critical role in myocardial development, growth, and recovery from injury. ErbB1, ErbB2, ErbB3, and ErbB4 are expressed on endothelial cells (Figure 2), and NRG-1 can regulate their proliferation, function, and participation in angiogenesis. NRG-1 induces the growth of vascular endothelial cells *in vitro* in a manner independent of VEGF [103]. NRG-1 treatment reverses diabetic vascular injury by increasing myocardial capillary density and increasing myocardial blood flow in mice [104]. In ischemic injury, NRG-1 treatment increases capillary density within the peri-infarct region [83]. These and other studies highlight the ability of NRG-1/ErbB signaling to augment myocardial recovery by increasing angiogenetic potential.

The downstream signaling mechanisms by which NRG-1 induces angiogenesis are not completely clear. Recent literature demonstrates that ErbB2 heterodimerizes with neuropilin (Nrp1) on cardiac endothelium to form a functional receptor for a vascular guidance molecule semaphorin 3d, leading to the development of coronary veins [105]. Nrp1 is a transmembrane protein that interacts with co-receptors and has been shown to regulate angiogenesis and

endothelial sprouting [106]. While Nrp1 is required for the embryonic vascular morphogenesis, it also has a role in the regulation of angiogenesis in adults [107]. The recent discovery of Nrp1's dimerization of ErbB2 did not test NRGs ability to induce angiogenesis but further supports ongoing investigation into the ErbB-dependent regulation of angiogenesis. NRG-1 induces increased production and release of VEGF by endothelial cells *in vivo* [104]. However, in an *in vivo* model of corneal angiogenesis, NRG-1 induces angiogenesis independent of VEGF, suggesting that NRG is functionally angiogenic. Hedhli et al. [21] developed an endothelial-specific inducible NRG-1 knockout mouse. Mice lacking endothelial NRG-1 had decreased blood flow in response to femoral artery ischemic injury due to reduced angiogenesis that recovered with exogenous NRG-1. NRG-1 has therapeutic potential in ischemic cardiomyopathy as it can stimulate angiogenesis in post-infarction myocardium and could be studied as a therapeutic in stable coronary artery disease to induce collateralization [21,103,108].

NRG-1 can also regulate angiogenesis via the prevention of apoptosis of endothelial progenitor cells [109]. We have recently demonstrated that human cardiac highly proliferative cells are characterized by the expression of all four ErbB receptors [110]. These cells are characterized by high proliferative activity *in vitro*, and have been shown to possess progenitor properties. NRG-1-dependent stimulation of ErbB receptors of these cells induces their differentiation toward endothelial cell lineage, providing an additional mechanism by which NRG-1 regulates cardiac microvasculature formation.

Regulation of cardiac myocyte metabolism

NRG-1 regulates glucose uptake into skeletal muscle via GLUT4 [111] and cardiac myocytes in a manner independent of insulin [112]. NRG-1β induces glucose uptake in cardiac myoctyes [14] via activation of the PI3K/Akt pathway [113]. In animal models of heart failure, recombinant forms of NRG-1 induce glucose uptake by induction of glycolytic enzyme expression and suppression of oxidative phosphorylation in rhesus monkeys [114], mice [115], and in swine; this leads to symptomatic hypoglycemia [95]. In human phase I and II trials, NRG-1 did not show hypoglycemic effects [116–118]. The effects of NRG-1 on glucose uptake, as well as hepatocyte gluconeogenesis, have led to the concept that NRG-1 may have therapeutic potential in the treatment of Type 2 diabetes mellitus [119–121].

NRG's effects on cardiac myocyte metabolism appear to be essential for its regenerative properties. Honkoop et al. [122] used a zebrafish model of cardiac regeneration to demonstrate that proliferating border zone cardiac myocytes have a very distinct transcriptome compared with nonproliferative cardiac myocytes in remote areas, with a definite shift toward anaerobic respiration. NRG-1/ErbB2 signaling induced this metabolic reprogramming, and the change toward anaerobic respiration was essential for myocardial regeneration [122].

The ability of the myocardium to utilize both fats and carbohydrates is well established, with oxidative phosphorylation being dominant in the adult myocardium [123–125]. The progression to decompensated heart failure is associated with a shift to anaerobic respiration, which is thought to be pathogenic [126–130]. Thus, the NRG-1 associated shift to anaerobic metabolism in the zebrafish model would seem to be maladaptive. This may be context or species-specific, as NRG-1 stimulation of adult rat cardiomyocytes increases the expression of many genes involved in mitochondrial β-oxidation [131]. Similarly, NRG-1 induces oxidative phosphorylation in cardiomyocytes generated from human embryonic stem cells [132]. Treatment of post-MI rats reverses the pathologic changes in metabolism transcriptome [115]. In post-MI swine, similar effects were seen along with normalization of mitochondrial structure as seen by transmission electron microscopy [95]. Although the exact mechanisms of improved mitochondrial function in cardiomyocytes requires further investigation, NRG-1 acts on complex 2-mediated mitochondrial respiration in skeletal muscles [133]. Incorporating noninvasive metabolic imaging [134] into future clinical trials with recombinant NRG-1 in heart failure may help to sort out to what extent changes in myocardial metabolism are mechanistic in humans.

Clinical trials of recombinant neuregulins in heart failure

Two forms of recombinant NRG-1β have been studied in clinical trials to examine the potential for a therapeutic effect in systolic heart failure (Table 3). Jabbour et al. [116] administered the EGF domain-only form of rhNRG-1β (Neucardin) to patients with chronic systolic heart failure and observed a sustained improvement in ejection fraction 84 days after therapy was completed. A nonsustained acute increase in cardiac output with rhNRG-1β infusion [135] was observed, likely due to a vasodilatory response, as rhNRG-1β has negative inotropic effects on isolated myocytes [136–138]. A phase II trial was completed by Gao et al. [117] where chronic systolic heart failure (EF<40%) patients were administered increasing doses of rhNRG-1β again producing sustained improvement in ejection fraction at the 90 days follow up. A phase 3 study with the same form of NRG-1 has been presented in abstract form reporting a

Clinical Science (2020) **134** 2623–2643
https://doi.org/10.1042/CS20200230

mortality benefit in systolic heart failure patients [139]. An ongoing phase 3 trial will provide further insight into the potential mortality benefit with NRG-1 in systolic heart failure (Clinical Trials ID#: NCT03388593).

The larger rhNRG-1 GGF2 (Cimaglermin Alfa) has been studied in a phase 1 trial, where a single dose was associated with a sustained improvement in LVEF after 90 days in patients with chronic systolic heart failure. The most common side effect with both forms of rhNRG-1β was nausea. The GGF2 study reached its safety endpoint after one patient at the highest planned dose of GGF2 developed transient hyperbilirubinemia and elevated aminotransferases [118]. The mechanism for nausea, as well as the transient transaminitis most likely is due to on-target effects of the recombinant NRG, given the wide tissue distribution of ErbB receptors. Mosedale et al. [140] examined the impacts of GGF2 on the liver and found no evidence of hepatocyte necrosis but did find ´marked' hyperplasia and hypertrophy of bile duct epithelium. Studies in cultured hepatocytes showed that GGF2 treatment changes the expression of genes regulating bilirubin metabolism and transport. While a major concern in the development of rhNRG-1β as a cardiac therapy has been the promotion of tumor growth given the well-established role of ErbB2 in oncogenesis [141], such an effect has not been reported in any trials to date.

Motivated by the potential oncogenic effects, a recombinant form of NRG that circumvents ErbB2 has been developed and studied in preclinical models. A bivalent neuregulin (bivNRG) containing two identical amino acid sequences, corresponding to the ErbB receptor binding domain, was created by linking them through a hydrophilic, protease-resistant spacer [142]. BivNRG promotes the dimerization (homo- and hetero-) of ErbB3 and ErbB4 receptors, inducing their transphosphorylation and activation. In cancer tissues, ErbB3 receptors often interact with ErbB2 [143] to form the most biologically active heterodimer activating the PI-3K/Akt signaling pathway [144]. Through the induction of minimally active ErbB3 homodimer formation at the expense of the more potently active ErbB2/ErbB3, bivNRG has a cytostatic effect in cancers. Through the induction of highly active ErbB4 homodimers, bivNRG retains its cardioprotective effects [145]. This unique property makes bivNRG a promising candidate for the treatment of anthracycline-induced cardiomyopathy. BivNRG and NRG-1 have similar cardioprotective effects during cardiac myocyte exposure to doxorubicin, while bivNRG, but not NRG-1, showed a growth-suppressive effect in a neoplastic cell line expressing ErbB2 and ErbB3 receptors [145]. Whether bivNRG will induce cardiac repair in other circumstances, where ErbB3/ErbB2 activation may be necessary for antifibrotic, angiogenic, and anti-inflammatory effects discussed previously will require further investigation.

Neuregulins in atherosclerosis

There is growing evidence that NRG maintains normal vascular function via signaling in endothelial cells, smooth muscle cells, and macrophages, and disruptions in this signaling system may be involved in atherosclerosis. Circumstantial evidence comes from human studies, with cell and animal studies providing more mechanistic information. Immunohistochemistry analysis of human coronary atherosclerotic lesions revealed an increased expression of NRG-1 in macrophages [146]. In a cohort of subjects with angiographic evidence of coronary disease, reduced levels of circulating NRG-1 was associated with the severity of disease [70]. Xu et al. [147] reported that NRG-1 reduced cholesterol ester accumulation in monocyte-derived macrophages in a dose-dependent manner *in vitro*, and administration of rhNRG-1 in ApoE-/- mice produced significantly reduced aortic atheromatous plaque surface area. In addition to the lipid regulation, NRG has anti-inflammatory effects mediated by ErbB4 on macrophages. Macrophages exposed to inflammatory cytokines increase their expression of ErbB4 (but not ErbB1, ErbB2 or ErbB3) and subsequent exposure to NRG-4 induces apoptosis [94]. NRG-1 suppresses macrophage cytokine release and subsequent myocardial fibrosis induced by angiotensin II [96]. NRG-1 has protective effects against oxidative stress-induced senescence in aortic endothelial and smooth muscle cells [148]. This could be of particular importance as the underlying pathophysiology of acute coronary syndrome is related to the thinning and rupture of the endothelium-smooth muscle layer over fatty atheromatous plaques [149]. All these studies taken together provide strong evidence that NRG has anti-inflammatory properties attributed to modulated macrophage function, and it could be a potential therapy to prevent or treat atherosclerosis.

Neuregulin 4 and its potential role in linking metabolic syndrome, inflammation, and coronary artery disease

Recently, NRG-4 has become an area of interest within endocrinology because of its association with obesity and the progression to diabetes. NRG-4 was first identified in 1999 by Harari et al. [30] where they found it to be highly expressed in the pancreas, adipose tissue, and skeletal muscle. Interestingly, the EGF domain of NRG-4 is highly selective for ErbB4 as opposed to NRG-1, which interacts with both ErbB3 and ErbB4 [30]. NRG-4 is expressed in brown adipose tissue and acts in an endocrine-like function on hepatocyte regulation of gluconeogenesis and lipogenesis [150].

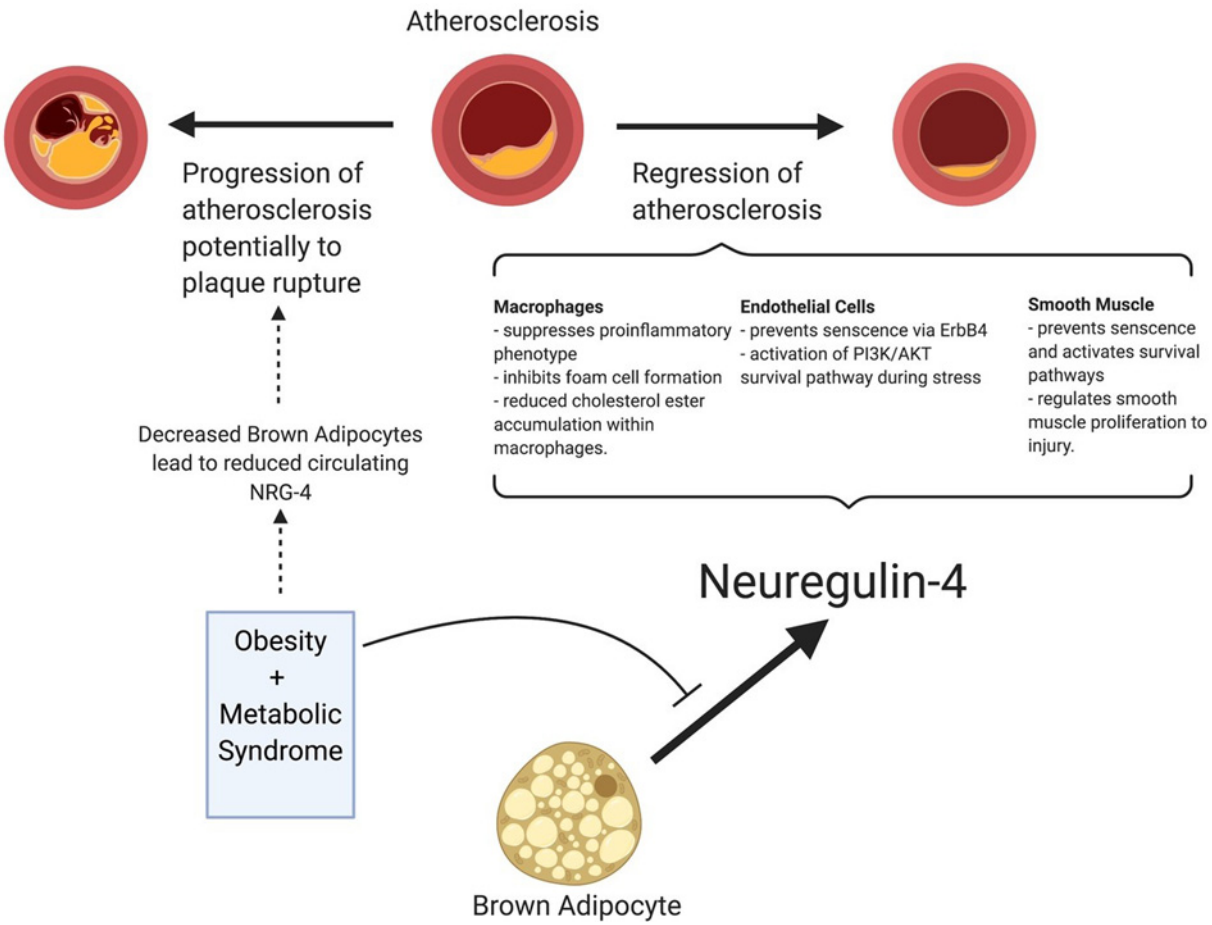

Figure 5. NRG-4 protects against atherosclerosis progression

NRG-4 is released from brown adipose tissue and reduces pro-inflammatory activation of tissue-resident macrophages and their differentiation into foam cells. NRG-4 promotes endothelial cell survival and prevents smooth muscle cell senescence, both of which suppress atherogenesis.

Several studies have found an inverse relationship between circulating NRG-4 and metabolic syndrome [25,26,151]. In addition, it has been shown that low levels of NRG-4 are an early marker of insulin resistance [24,152]. Reduced levels of NRG-4 have been associated with increased carotid intimal thickness [153], higher high-sensitivity c-reactive protein [154], increased angiographic severity of coronary artery disease [27], and acute coronary syndrome [155], suggesting that NRG-4 is an adipokine that mediates the link between metabolic syndrome and atherosclerosis (Figure 5).

As discussed previously, there are many known risk factors of atherosclerosis but few known associations between the progression of atherosclerosis to plaque rupture events. NRG-4, the ErbB4 selective ligand, has been highlighted throughout this review to have beneficial effects on cardiac myocytes and the endothelium while having anti-inflammatory effects on macrophages leading to decreased tissue fibrosis and decreased atherosclerotic plaque burden. Further prospective studies are needed to investigate the role of NRG-4 in the progression of CAD.

Neuregulin in diastolic heart failure

There are very few clinical strategies that effectively treat diastolic heart failure despite numerous trials [156,157]. NRG-1 has been postulated to be a therapy for a subtype of diastolic heart failure—diabetic cardiomyopathy [158]. The Framingham Study established that men and women with diabetes are at higher risk for developing heart failure after adjusting for comorbid conditions [159]. Diabetic cardiomyopathy is often accompanied by comorbidities, including coronary artery disease, hypertension, autonomic neuropathy, and microvascular dysfunction. However, diabetes is independently associated with progressive left ventricular remodeling and dysfunction characteristic of

Clinical Science (2020) **134** 2623–2643
https://doi.org/10.1042/CS20200230

diastolic heart failure [160]. There are a multitude of neurohormonal factors that influence the pathophysiology of diabetic cardiomyopathy, but decreased myocardial compliance is a hallmark [160]. NRG-1 has consistently been shown to increase myocardial compliance [96,161]. It has been suggested that mechanisms of NRG-1 mediated changes in compliance are attributed to calcium homeostasis [138], reduced inflammatory response and resulting fibrosis [96], and direct effects on sarcomeric tension [162].

Rats with diabetes, induced by streptozotocin, have impaired left ventricular function by echocardiography accompanied by significant fibrosis [163]. The level of NRG-1 in the hearts of diabetic rats is decreased along with reduced phosphorylation of ErbB2 and ErbB4 [163], suggesting that diminished levels of NRG-1 could be associated with the development of diastolic heart failure in diabetic cardiomyopathy. Animal models have suggested diabetes mellitus Type 2 (DM2) is associated with altered phosphorylation of titin, leading to reduced compliance [164,165]. The phosphorylation balance of a specific region of titin determines the compliance of the cardiac myocyte sarcomere. Hopf et al. [162] demonstrated rhNRG-1 modified the phosphorylation of titin in diabetic mice and found that rhNRG-1 could rapidly decrease the end-diastolic pressure–volume relationship (indicating improved diastolic relaxation) in Zucker diabetic obese rats [158]. The significance of this study is the suggestion that NRG-1 can revert the metabolic effects of insulin resistance on the sarcomere and supports the role of NRG-1 in the treatment of diabetic cardiomyopathy.

Activation of the pro-inflammatory immune cells, including macrophages, has been implicated in the pathogenesis of diabetic cardiomyopathy [160]. As previously mentioned in this review, NRG-1 and NRG-4, acting through ErbB3 and ErbB4 have been shown to induce apoptosis of the pro-inflammatory macrophages and reduce pro-inflammatory activation of myeloid cells. NRGs ability to modulate chronic inflammation in diabetic cardiomyopathy may also be an additional therapeutic mechanism.

Neuregulin in prevention of septic cardiomyopathy

Sepsis is a condition with life-threatening organ dysfunction caused by a dysregulated host response to infection [166]. Sepsis is characterized early on by an increase in cardiac output, but in some cases of sepsis, there is transient cardiomyopathy with reduced ejection fraction. [167]. Based on the observation of reduced NRG-1 in neuromuscular junctions during sepsis, Zhou et al. [168] suggested that exogenous NRG-1 may be beneficial during sepsis. They found that treatment of rats with rhNRG-1 improved survival. Hemodynamic measurements revealed improved mean arterial pressure, isovolumetric relaxation, and decreased left ventricular end-diastolic pressure accompanied by reduced levels of troponin, TNF-, IL-1β, and IL-6 in the NRG-1 treatment group [168]. The reduction of inflammatory cytokines and improved mortality suggest that NRG-1 has protective effects in septic cardiomyopathy via down-regulation of immune response-related cardiac injury. Kang et al. [169] further investigated the effects of NRG-1 in sepsis by examining the impact in endothelial cells where they demonstrated an inhibitory effect of NRG-1 on ICAM-1, VEGF, and nitric oxide, factors which increase in sepsis. The investigators also found that NRG-1 inhibited RhoA/ROCK signaling, which has previously been shown to promote endothelial cell shedding and increase vascular permeability [169]. Along with improvements in cardiac dysfunction in sepsis, NRG-1 appears to protect endothelial cell injury and the resulting loss of barrier function that contributes to multi-organ failure [170].

Conclusions

NRGs have therapeutic effects in multiple forms of cardiac disease through direct actions on cardiac myocytes, endothelial cells, inflammatory cells, and fibroblasts. Recent literature has expanded our knowledge of NRGs regulation of metabolism in multiple tissues, implicating NRGs as potential links between metabolic syndrome, diabetes, and coronary artery disease. The mechanisms by which NRG/ErbB signaling is cardioprotective have been elucidated, but the mechanisms by which these cardioprotective pathways are regulated *in vivo* remain unanswered. Promising clinical trial results with two distinct forms of recombinant NRG-1 in systolic heart failure support further investigation of this biologic therapy. Future NRG research is warranted that focuses on NRG-4/ErbB4 due to new literature suggesting the connection between metabolic syndrome, diabetes, and coronary artery disease. Acute coronary syndrome remains a plague on the cardiology community as we have excellent risk stratification tools for the development of CAD but no means to predict who is at risk for plaque rupture events and the deadly consequences. NRG-4 could be a biomarker for the risk of ACS as well as a potential therapy. NRG is protective of cardiovascular systolic dysfunction, and the literature supports a protective role of NRG in CAD, diastolic heart failure, diabetes, and inflammatory conditions affecting the heart. There remain many questions regarding the differential response between different NRGs and ErbB combinations across different tissue types and understanding these pathways in greater detail has the potential to greatly expand the clinical utility of NRG (Figure 6).

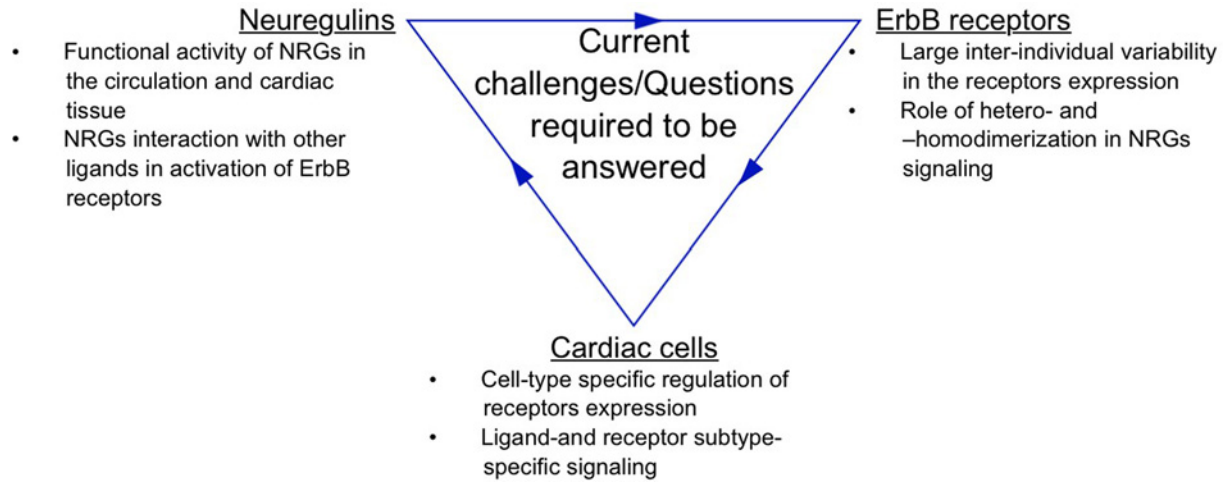

Figure 6. Current challenges and questions

Large inter-individual variability in both NRG's and ErbB receptors expression, the presence of active and inactive forms of NRG's, and their interaction with different ErbB receptors expressed on a variety of cardiac cells form a complex NRG/Erbb signaling network. A better understanding of cell-type- specific effects mediated by NRG's in the heart is necessary for the development of cardioprotective therapies.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Abbreviations

CAD, coronary artery disease; ET_A, endothelin type A receptors; FAK, focal adhesion kinase; NRG, neuregulin; Nrp1, neuropilin; STAT3, signal transducer and activator of transcription 3.

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