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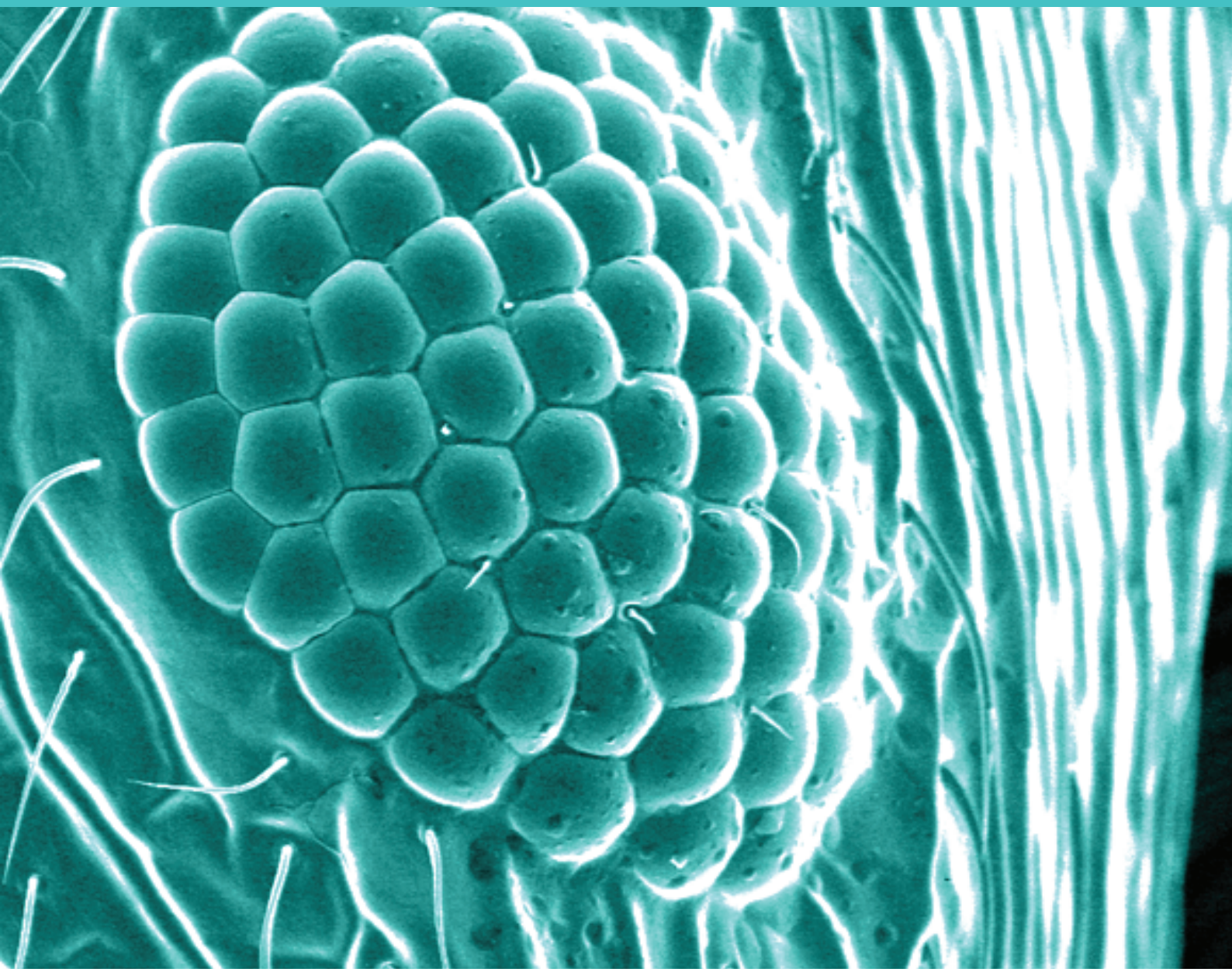

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THE ROLE OF THE NEUREGULIN-1/ERBB SIGNALING PATHWAY IN CARDIAC MORPHOGENESIS AND REMODELING

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Summary

Neuregulin-1 (NRG1) signaling through tyrosine kinase receptors erbB2 and erbB4 was revealed essential for cardiac development as mouse mutated in the *Nrg1* or either cognate receptors *Erbb2* or *Erbb4* genes lack the formation of trabeculae at the ventricular wall. Indeed, the injection of the NRG1 active peptide in developing embryos induced trabeculation of the ventricular free wall. The components of the NRG1 pathway have been identified and the cardiac activities are being progressively characterized. An acquired form of dilated cardiomyopathy was evidenced in a subpopulation of breast tumor patients undergoing a combined treatment with antibodies against erbB2 and chemotherapy. In this regard, the cardiomyocyte-specific gene deletion of either *erbb2* or *erbb4* leads to ventricular dilation in adult mice, providing an experimental model system to examine the NRG1-mediated activities. We reviewed the evidence on the growing field of research for NRG1 signaling in both cardiac morphogenesis and in the postnatal myocardial remodeling.

Introduction

1. The Neuregulin pathway

The neuregulins are transmembrane proteins of four isotypes (NRG1-4), which active domain is related to the epidermal growth factor (EGF). The NRG1 is classified into three subgroups (I–III), which includes about 30 isoforms as the result of the expression from different promoters and of splicing variants encoded in over 1.2 Mb, one of the largest genes in the mammalian genome [1]. The active amino terminal domain of the type I and II is released by metalloproteinase (ADAM 17, 19), modulated by PKC-delta and cleaved by α-secretase activity [2, 3]. The extracellular active domain of type III NRG1 remains attached to the transmembrane domain, which is therefore suggested to signal in the immediate environment **(Fig 1A).** The NRGs activate the tyrosine kinase receptors, erbB2, erbB3 and erbB4 that belong to the EGF receptor family (erbB1) [4]. The neuregulin and erbB2 were first identified in unrelated research

topics of the neuromuscular junction and cancer cells respectively, and it had been ten years later, in 1993, that these molecules were characterized as the ligand and co-receptor of the same signaling pathway.

2. The NRG1-erbB signaling activities in cardiac development

In the heart, the type I and II of NRG1 are expressed in endocardial cells of the endocardium which are subjected to proteolytic cleavage **(Fig 1A).** The released active peptides of the NRG1β isoform bind and activate erbB2/erbB4 heterodimers, expressed in cardiomyocytes **(Fig 1B).**

In the late 1995, the phenotype of genetically recombined *Nrg1* or either *Erbb2* and *Erbb4* genes in mouse revealed the critical activities of this pathway for embryonic development, which embryos died *in utero* at E10.5 days post coitum (dpc) [5-7]. The developing heart of these 3 lines of gene knockout mice had impaired or nil formation of the trabeculae at the ventricular free wall. Trabeculae are a network of cardiomyocytes growing into the chamber lumen that contribute to the thickening of the myocardium and to the formation of the ventricular conduction system. The first evidence for the role of NRG1 in the developing heart prompted the study of the pathway activities in experimental model systems. In an ex-vivo developing embryo culture system, the cardiac injection of NRG1β and α active peptides (70 aminoacids) was sufficient to induce trabeculation of the ventricular wall **(Fig. 1C)**, which stronger activity was displayed by the β relative to the α isoform [8].

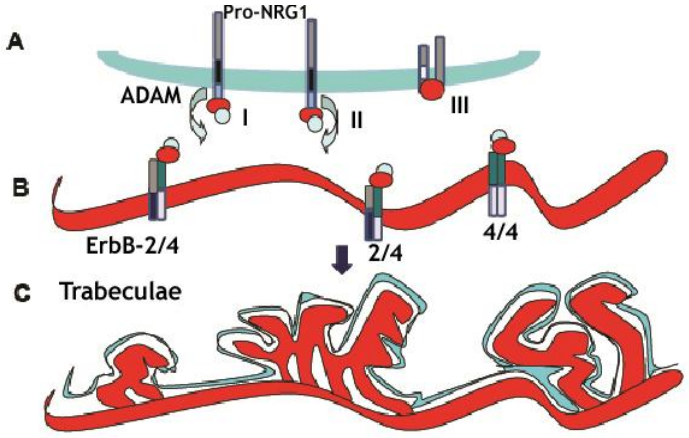

Figure 1. A) Schematic representation of type I, II, III NRG1. Although not expressed in the heart, type III was included for graphic purpose. B) NRG1 binding and activation of erbB receptors. C) Formation of Trabeculae by NRG1 through erbB2/erbB4 activation.

In addition to the NRG1-mediated differentiation and specification of cardiomyocytes into trabeculae, NRG1 was found to induce cardiomyocytes to proliferate at the compact zone of the ventricular wall when co-injected with IGF-I peptides. These results suggest that growth factor activities may interact by modulating the dominant individual effects and indicate that NRG1/IGF-I induce differentiation in proliferating cardiomyocytes, forming trabeculae [8]. Experiments carried out in zebrafish indicate that the NRG1-induced trabeculation is mediated by proliferation followed by the directional migration of differentiating cardiomyocytes, as opposed to a directional budding of proliferative cardiomyocytes into the chamber lumen [9].

Recent experiments employing homozygous mice for hypomorphic *Nrg1* alleles, which lack the transmembrane domain, demonstrated that the central outermost part of the left ventricle was the most affected region as monitored by the early inactivation of gene expression at E8.5 dpc. This result indicates a role for NRG1 beyond the formation of trabeculae, in cardiomyocytes of the compact zone, potentially linking biochemical feedback to molecular pathways for growth and differentiation, acting on the central part of the ventricular wall that sustains contraction [10]. In this scenario, the NRG1 pathway displays morphogenetic activities on diverse cardiac tissues. The erbB receptors are involved in cushion morphogenesis, which is initiated by epithelial-mesenchyme transformation giving rise to the prospective valves. Indeed, a murine model of defective valve formation by hyaluronan gene inactivation was rescued by the administration of NRG1 with the concomitant phosphorylation of erbB2 and erbB3. These results associate the NRG1 pathway to valve formation through mesenchyme-extracellular matrix interactions [11].

The septation and maturation of the outflow tract depends on erbB4-positive cardiomyocytes and erbB3-positive neural crest cells that migrate to the lateral mesenchyme of the dorsal aorta in mice. A congenital form of left ventricular outflow tract defects was positively associated with a specific haplotype in the *ERBB4* gene [12]. Recently, certain polymorphisms in *ERBB2* [13] and in the *NRG1* [14] genes have been suggested to be biologically significant in the pathogenesis of nonneoplastic disease, such as schizophrenia or cardiovascular disorders. Thus far, further experimentation is required to directly address the specific functions of ErbB and NRG1 isoforms in disease.

Altogether, it is suggested that NRG1 orchestrates gene expression in a feedback modulation with wall stress, contractility and blood flow parameters, acting on the coordinated growth of the cardiac chambers during morphogenesis.

3. The NRG1 intracellular signaling cascade. The erbB-dependent intracellular cascades have been extensively studied because of the important role in cancer cells, thereby, providing the basis for the analyses of signaling mechanisms in other cell types. The NRG activation of erbB receptors mediates auto- and trans-phosphorylation of tyrosine residues at the receptor intracellular domain. A subgroup of phosphotyrosine residues bind specific adaptor molecules (e.g. Grb, Shc, Src, SH3 domain) [13], ultimately inducing intracellular pathways, e.g. MAP kinase, and PI 3´-kinase cascades, PLC γ, the regulation of the Ca^{2+}-dependent Protein Kinase C and NFAT activity **(Fig. 2)** [4]. A link between NRG1 and focal adhesion kinase (FAK) has been found in proliferative and migrating cells.

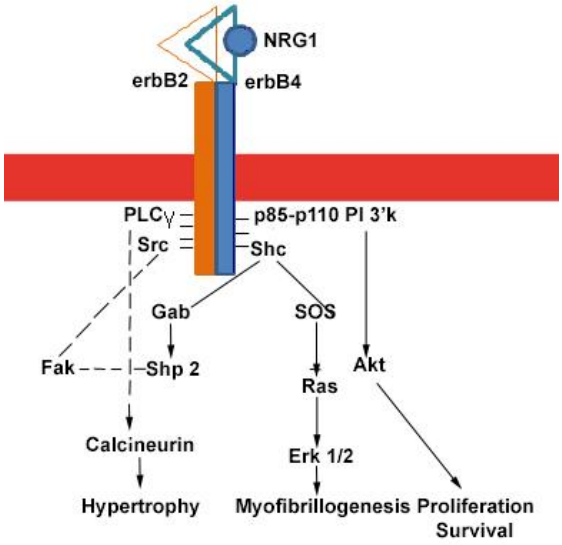

Figure 2. Schematic representation of NRG1-erbB2/erbB4 intracellular signaling

In cultured cardiomyocytes, the Ras/MAPK/erk1/2 pathway was required for the NRG1-driven myofibrillogenesis in cultured cardiomyocytes. This activity was mimicked by a constitutively active form of Ras and inhibited by its dominant negative form and by the MEK1 inhibitor PD98059 [8]. The induced ability of cardiomyocytes to proliferate was dependent on NRG1/IGF-I through the activation of the PI 3´-kinase/Akt pathway, which was verified by the wortmannin inhibition of cardiomyocyte DNA synthesis in embryonic development and in cultured neonatal cardiomyocytes. The cellular transfection with adenovirus harboring a

constitutively active Akt mimicked the proliferative and protective activities, which were inhibited by a dominant negative form of Akt in the presence of NRG1/IGF-I [8].

ErbB non-phosphorylated interactions. Intracellular signaling also depends on the binding ability of specific non-phosphorylated residues at the C-terminal domain of erbB receptors to PDZ domain containing proteins. This protein domain was named by homology to the interacting region of the Post synaptic protein 95, Disc large and Zona occludens ZO-1 proteins with four highly conserved residues GLGF. The interaction to PDZ domain proteins is relevant for the specific location of erbB proteins into particular membrane compartments and for the modulation of the receptor stability and activity [14]. Despite the significance of PDZ domain proteins in the heart (e.g. MAGUK, actinin binding proteins), there is not yet evidence for the specific PDZ-erbB-interacting proteins in cardiomyocytes. The erbB4 receptors, which are endocytosis-impaired, are also regulated through proteolysis mediated by the proteasome system and by the alternative transcriptional activity of the cleavable juxtamembrane isoform JMa. These mechanisms either drive the erbB4 protein degradation or induce the nuclear translocation of the JMa intracellular domain. As it occurs for the release of the NRG1 active peptides, the release of the erbB4 JMa C-terminal domain is modulated by the activation of PKC and mediated via the activity of tumor necrosis factor-alpha converting enzyme and further cleavage by γ-secretase at the plasma membrane. In the heart, the identified erbB4 protein in cardiomyocytes is the JMb non-cleavable splice variant which may be proteolyticaly modulated by the proteasome system [15]. Three PPXY motifs couple erbB4 with WW domain proteins, such as Wwox and ubiquitin ligases, modulating the activity of the c-terminal domain[16].

3. The requirement of erbB in the adulthood

The clinical impact of the NRG1 signaling in cardiology was the increased incidence of dilated cardiomyopathies in a subpopulation of breast tumor patients undergoing the combined administration of anthracycline chemotherapy and a humanized antibody against erbB2 protein (trastuzumab, pertuzumab) [19]. The cardiac effect of these humanized antibodies, which are species specific and do not cross-react with the mouse protein, was experimentally assessed through the ventricular cardiomyocyte-specific-deletion of either *Erbb2* or *Erbb4* in mouse. The tissue-specific mutation in any of these genes in mouse led to dilated cardiomyopathy in the

adulthood **(Fig. 3)** [20, 21]. These murine models were useful to demonstrate the cardiomyocyte-autonomous and essential requirement of the erbB2/erbB4 during the postnatal remodeling of the myocardium.

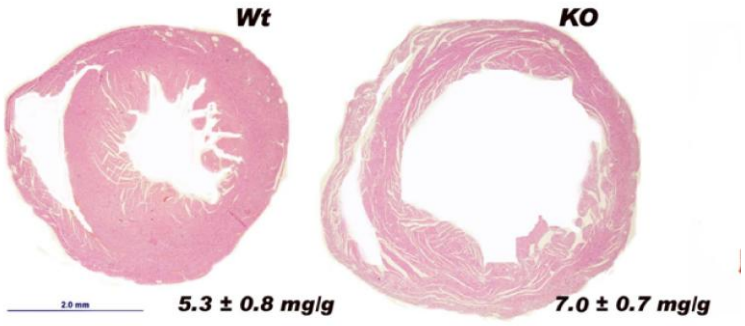

Figure 3. Ventricular specific erbB4-KO leads to adult dilated cardiomyopathy.

Subcellular localization of erbB2/erbB4. Clues about a local cardiomyocyte effect of NRG1 for the maintenance of myocardial architecture arise from the subcellular localization of the receptors. The erbB2/erbB4 proteins were found accumulated in the T-tubule membrane system of cardiomyocytes and in the intercalated discs (ID) [21] **(Fig. 4)**

The relevance of the receptor localization is that the T-tubules place membrane molecules in close apposition of the myofibril Z-disc, providing a specific context for the functional interactions among molecules at the plasma membrane, sarcoplasmic reticulum (SR) and the myofibril Z-disc. The ID are highly specialized Z-disc structures at the cell-cell contacts that provide the anchorage of the sarcomeres to the membrane and placed the connexin-43 gap junctions, assuring electric transmission and the rhythmic contraction of cardiomyocytes of the ventricular chamber.

The erbB subcellular context suggests that NRG1 may act on intersecting cues to cytoskeletal pathways required for myocardial remodeling. A connection of NRG1 signaling with the cytoskeleton has been also suggested during cardiac morphogenesis in a feedback modulation with changes in wall stress and contractile parameters.

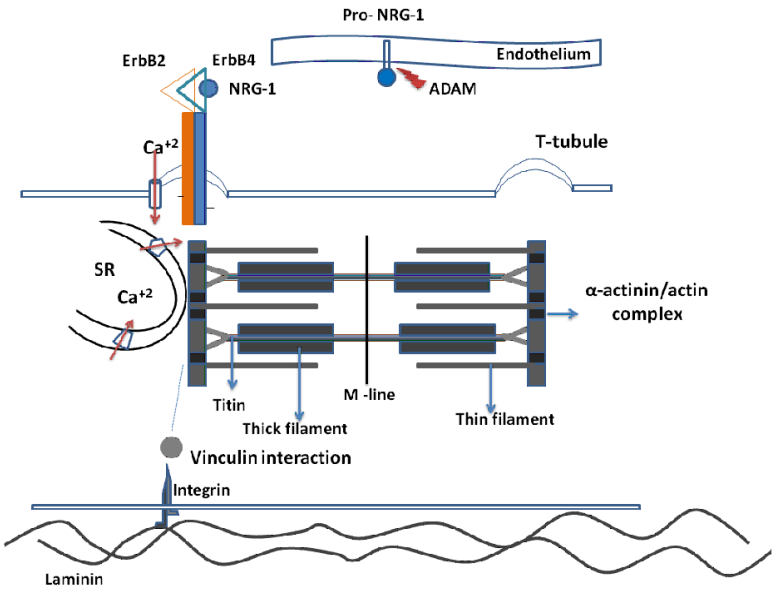

Figure 4. Endothelium-Cardiac muscle interactions through paracrine NRG1 signaling. Functional interaction of molecules placed at the T-tubules, SR, Z-band, ID and costameres

4. Cardioprotective and regenerative activities

In agreement with the requirement of the NRG1-erbB2/erbB4 pathway for the prevention of ventricular dilation during remodeling, NRG-1/erbB2 signaling has been shown to be critical for the physiologic cardiac hypertrophy manifested during pregnancy in rodents. Accordingly, the lapatinib-mediated inhibition of erbB2 phosphorylation resulted in a pathologic pregnancy-related dilation of the ventricular chambers that occurs without apparent apoptotic cell death [22]. Downregulation of erbB2 and erbB4 expression was evidenced during the pathologic remodeling of the myocardium in rodents under pressure overload and in humans with a failing myocardium [23].

The *in vivo* administration of NRG1 in different conditions of mouse cardiac pathology contributed to the amelioration of contraction (reduced left ventricular end systolic dimensions, increased ejection fraction). In these experiments, the NRG1-mediated functional performance of the ventricular chambers was correlated with the increased phosphorylation of the myosin regulatory light chain (RLC) [24]. However, the administration of NRG1 in knockout mouse for the myosin light chain kinase, *Mlck*, gene improves cardiac performance without an increase in the phosphorylation level of RLC [25], implicating additional mechanisms for the contractile improvement. In cultured cardiomyocytes, NRG1 appears to exert a negative inotropic effect through either NOS activity [26] or via the activation of the muscarinic response [27]. Both NO

production and active muscarinic receptors modulate the inotropic response to beta-adrenergic stimulation, which may result in an improved fractional shortening. Indeed, there is a general lack of evidence of a direct inotropic effect, or induced changes in calcium handling or in myofilament calcium sensitivity that may explain the NRG1-mediated enhancement in the cardiac systolic function.

Additional repair activities have been suggested in a murine model of cardiac infarction. In this setting, the exogenous administration of NRG1 was shown to induce mononucleated cardiomyocytes to proliferate contributing to cardiac repair mechanisms, without affecting the level of apoptotic cell death [28].

In cultured cardiomyocytes, NRG1 appeared to protect doxorubicin-mediated myofibril disarray [29], and the toxic degradation of the troponins [30]. These results are in line with the beneficial cardiac effect of NRG1 in one week old myocardial infarction [28].

The cardioprotective activity of NRG1 was also inferred by the doxorubicin-aggravated contractile dysfunction in heterozygous NRG1 mutant mice [31] and the exacerbated ventricular dilation in erbB4-KO [32]. The administration of doxorubicin to erbB4-KO mice led to the downregulation of components of the IGF-I pathway accompanied by deregulation of the ubiquitin-proteasome system [32]. The mechanisms leading to cardiomyocyte death by autophagic vacuolization and necrosis were synergistically induced in the doxorubicin-treated erbB4-KO heart. The relevance of protein-ubiquination was documented for autophagic vacuolization as a useful predictor of myocardial deterioration [33]. Moreover, the monitoring of necrotic cardiomyocytes by the determination of cTnI serum level is a highly sensitive cardiotoxic marker for breast tumor patients following trastuzumab and doxorubicin therapy [34].

Conclusions

The relevance of the NRG1-erbB2/erbB4 pathway in the heart was characterized by the induced formation of trabeculae at the ventricular free wall during embryonic development, promoting differentiation in proliferative cardiomyocytes. The embryonic activities of NRG1 signaling appeared to be also required beyond the formation of trabeculae, acting on the regulation of gene expression in cardiomyocytes of the central part of the ventricular wall, which is critical for pressure development. The NRG1-erbB2/erbB4 pathway is critical for the maintenance of the

myocardial structure in the adult heart, and moreover, an impaired NRG1 signaling exacerbates the anthracycline-mediated cardiotoxicity. The accumulated data indicates a panel of NRG1-dependent protective and repair activities displayed in the heart during the lifespan of the individuals, which molecular events remain unclear. There are central questions about the NRG1/erbB biology to be addressed as to how NRG1 affects cytoskeletal pathways during cardiac remodeling.

The continuous research in this area will provide the critical molecules and targets which may help the design of diagnostic tools and therapeutic strategies, particularly relevant for patients with erbB2-positive breast tumors undergoing the combined treatment with anthracyclines and trastuzumab.

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