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Neuregulin1-ErbB Signaling in Doxorubicin-Induced Cardiotoxicity

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1. Introduction

This chapter will review basic and clinical findings regarding the cardioprotective role of Neuregulin1-ErbB signaling against the cardiotoxicity of doxorubicin, a widely used chemotherapeutic agent. In 2001, *The New England Journal of Medicine* published the results from clinical trials in breast cancer patients using Trastuzumab, a monoclonal antibody that blocks the ErbB2 receptor. These studies showed that the incidence of New York Heart Association (NYHA) class III/IV heart failure was 16% in patients who were concurrently treated with doxorubicin and Trastuzumab compared to 3% and 2% respectively in patients who were treated with doxorubicin or Trastuzumab alone (Slamon et al., 2001). These results for the first time suggest that the ErbB signaling has cardioprotective effects against doxorubicin-induced cardiotoxicity. Since then, a significant amount of basic and clinical research has been conducted to investigate the mechanisms of the ErbB signaling pathway in protecting the heart from doxorubicin-induced toxicity. At the same time, studies have been performed searching for factors that stimulate ErbB signaling to protect the heart from doxorubicin. Although ErbB receptors have at least 13 ligands, Neuregulin1 proteins have become the focus of this search (Yarden and Sliwkowski, 2001a). Studies from cardiomyocyte culture, animal models and clinical trials have demonstrated that Neuregulin1 may be effective for preventing or treating doxorubicin-induced cardiotoxicity.

2. Doxorubicin-induced cardiotoxicity

2.1 Background

The anthracycline drug doxorubicin was discovered about 40 years ago and continues to be used as a first line antineoplastic drug (Outomuro et al., 2007a; Moretti et al., 2009). Doxorubicin is effective for the treatment of a wide variety of cancers in children and adults, such as leukemia, lymphoma, breast, lung and colon cancers (Outomuro et al., 2007a; Anderson and Sawyer, 2008). Doxorubicin has significantly improved survival of childhood cancer patients, in which the survival rate is now approaching 75% (Curry et al., 2006). There are an estimated 300,000 childhood cancer survivors in the United States (Jemal et al.,

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2006; Oeffinger et al., 2006). Nearly 60% of them have been treated with doxorubicin or its analogs (van Dalen et al., 2006; Bryant et al., 2007; LoPiccolo et al., 2008). In adult patients, such as breast cancer patients, it is estimated that 30% of them have been treated with doxorubicin (Paik et al., 2000; Pritchard et al., 2006). However, dose-related cardiotoxicity has limited the use of doxorubicin at all in a subset of cancer patients with pre-existing cardiovascular conditions and in cancer patients in general, due to significant risk of developing irreversible heart failure months and years after doxorubicin treatment (Barry et al., 2007a; Outomuro et al., 2007a; Anderson and Sawyer, 2008).

The incidence of doxorubicin-induced cardiotoxicity has been reported to be in a variable range of 0.4 - 41% (Outomuro et al., 2007a). The risk of doxorubicin-induced cardiotoxicity mainly depends on the cumulative dose of the drug administered. The incidence of doxorubicin-induced heart failure is about 3% at a cumulative dose of 400 mg/m^2 , 7.5% at a cumulative dose of 550 mg/m^2 and 18% at 700 mg/m^2 (Von Hoff et al., 1979; Swain et al., 2003; Outomuro et al., 2007a). Other factors, such as age (very young or elderly patients), pre-existing cardiovascular disease, and previous or concurrent use of other anti-cancer cytotoxic or targeted therapies, can increase the risk of doxorubicin-induced heart failure (Von Hoff et al., 1979; Safra, 2003; Outomuro et al., 2007b). Trastuzumab, a monoclonal antibody that blocks the HER2 receptor, is the first drug approved by the US Food and Drug Administration (FDA) for targeted cancer therapy. When Trastuzumab and doxorubicin were concurrently used in breast cancer patients, the incidence of New York Heart Association type III and IV heart failure rose from 2-3% to 16% over a period of 50 months of observation (Slamon et al., 2001).

Doxorubicin can cause acute, subacute and late cardiotoxicity. The acute doxorubicin cardiotoxicity starts within 24 hours of drug infusion, and may present as cardiac arrhythmia, myocarditis and pericarditis (Appelbaum et al., 1976). The long-term prognosis of acute doxorubicin cardiotoxicity is relatively good. Subacute doxorubicin cardiotoxicity occurs weeks and months after the treatment, while late cardiotoxicity can occur 4-20 years after the cessation of the treatment. The onset of chronic doxorubicin cardiotoxicity is insidious; the disease, however, progressively develops to severe and irreversible heart failure (Simsir et al., 2005). Therefore, doxorubicin cardiotoxicity is a Type I chemotherapy-related cardiac dysfunction (CRCD) (Ewer and Lippman, 2005). It is irreversible and presents a life-long threat for cancer survivors (Ewer and Lippman, 2005).

The pathological changes in chronic doxorubicin cardiotoxicity include cardiac hypertrophy, which may be followed with thinning of the ventricular wall (dilated cardiomyopathy), interstitial fibrosis, vascular and mitochondrial degeneration. Morphological changes within the cardiomyocyte include distention of the sarcotubular system (vacuolization), loss of myofibrils, as well as mitochondrial swelling and loss of cristae (Billingham et al., 1978; Mortensen et al., 1986; Rowan et al., 1988; Lipshultz et al., 1991; Mackay et al., 1994; Lipshultz et al., 2005; Barry et al., 2007b).

2.2 Potential mechanisms of doxorubicin-induced cardiotoxicity

There have been several proposed mechanisms for doxorubicin-induced cardiotoxicity:

(1) free radical generation and oxidative stress (Kang et al., 1996; Yen et al., 1996; Kang et al., 1997), (2) increased cardiomyocyte death by necrosis and apoptosis (Childs et al., 2002b; Green and Leeuwenburgh, 2002; Aries et al., 2004; Kalivendi et al., 2005a; Poizat et al., 2005),

(3) inhibition of cardiac specific muscle gene transcription and translation, in combination with an increase in myofibril protein degradation, leading to loss of myofibrils (Lewis and Gonzalez, 1987; Ito et al., 1990; Kurabayashi et al., 1994; Toyoda et al., 1998; d'Anglemont de Tassigny et al., 2004; Lim et al., 2004b), and (4) disturbance of intracellular calcium homeostasis (De Beer et al., 2001; Wallace, 2003). The mechanism of doxorubicin-induced free radical generation and oxidative stress has been reviewed in other chapters of this book as well as comprehensive reviews in the field (Singal et al., 2000; Berthiaume and Wallace, 2007; Simunek et al., 2009). In this chapter, we will focus on the mechanisms that connect the Neuregulin1-ErbB signaling to doxorubicin cardiotoxicity.

2.2.1 Doxorubicin-induced apoptosis in the cardiomyocyte

Studies have been conducted to investigate doxorubicin-induced cardiomyocyte apoptosis in H9c2 rat embryonic cardiomyocytes, neonatal and adult rat cardiomyocytes, as well as in hearts from rats and mice treated with doxorubicin (Childs et al., 2002a; Fukazawa et al., 2003; Liu et al., 2005; Youn et al., 2005; Fu and Arcasoy, 2007; Bian et al., 2009).

Doxorubicin induces apoptosis via both intrinsic and extrinsic pathways. Doxorubicin alters the ratio of pro-apoptotic and anti-apoptotic Bcl-2 family proteins, including Bcl-2, Bad, Bim, Bax, Bak and Bik (Aries et al., 2004; Rohrbach et al., 2005b; Kobayashi et al., 2006); it also causes DNA damage and p53 activation (Liu et al., 2004; L'Ecuyer et al., 2006). All these changes can cause loss of mitochondrial integrity, the leakage of cytochrome c and the activation of caspase 9 (Hengartner, 2000; Green and Leeuwenburgh, 2002). Mitochondrial dysfunction is an early indicator of doxorubicin-induced apoptosis in cardiomyocytes (Green and Leeuwenburgh, 2002). Within the extrinsic pathway, doxorubicin increases Fas and FasL, followed by the activation of caspase 8 (Nakamura et al., 2000; Kalivendi et al., 2005b). The activation of caspase 9 and/or caspase 8 eventually leads to the activation of caspase 3, cleavage of genomic DNA and apoptosis (Hengartner, 2000).

2.2.2 Doxorubicin-induced myofibril loss in cardiomyocytes

Doxorubicin selectively down-regulates cardiac specific muscle gene expressions. These may involve decreases in cardiac muscle gene transcription and translation, as well as increases in selective proteasome degradation of these proteins (Poizat et al., 2000).

Studies have shown that doxorubicin decreases the expression of α-sarcomeric actin, cardiac troponin I (cTnI), and myosin light chain 2 (MLC 2) (Ito et al., 1990). Doxorubicin treatment decreases cardiac troponins in left ventricular tissues of mice and in cultured rat neonatal cardiomyocytes (Bian et al., 2009). Down-regulations of cardiac troponins by doxorubicin are caused by decreased transcription and translation as well as increased caspase and proteasome degradation of these proteins (Bian et al., 2009). Caspase 3, 5, 6 or 10 directly cleaves cardiac troponins, while caspase 9 or 13 may indirectly cause degradation of these proteins (Bian et al., 2009).

Other reports also have shown that doxorubicin inhibits the expression of transcription factors or cofactors that are important for regulation of cardiac-specific gene transcription. These include GATA4, MEF2C, dHAND, Nkx2.5 and p300 (Poizat et al., 2000; Aries et al., 2004). In addition to cardiac genes, GATA4 may also regulate genes that are involved in the process of apoptosis. Overexpression of GATA4 in cardiomyocytes or mouse hearts

attenuates doxorubicin-induced apoptosis. On the other hand, GATA4 null mice are more susceptible to doxorubicin cardiotoxicity (Aries et al., 2004).

In addition to myofibril loss, doxorubicin also induces myofibril disarray in cardiomyocytes (Sawyer et al., 2002). Degradation of titin, a myofilament protein, may contribute to this effect of doxorubicin. Titin is a scaffold protein that assembles myofilament proteins into sarcomeres. It regulates cardiomyocyte contractile function via length-dependent activation in stretched sarcomeres during the transition from diastole to systole (Helmes et al., 2003). Doxorubicin activates calcium-dependent proteases calpains which in turn cause titin degradation (Lim et al., 2004a).

2.2.3 Doxorubicin disturbs calcium homeostasis in cardiomyocytes

The sarcoplasmic reticulum Ca^{2+} pump (SERCA2a) plays a pivotal role in intracellular calcium mobilization and thus myocardial contractility. The sarcoplasmic reticulum (SR) orchestrates the movement of calcium during both contraction and relaxation of the heart. Excitation leads to the opening of voltage gated L-type calcium channels, allowing the entry of calcium, which then stimulates the release of a much larger amount of calcium from SR and subsequent contraction. During relaxation, calcium is re-sequestered into SR by SERCA2a and extruded to the extracellular fluid by the sarcolemmal sodium-calcium exchanger (NCX) (del Monte et al., 1999; Wehrens and Marks, 2004). A decrease in SR Ca^{2+} ATPase activity and Ca^{2+} uptake is responsible for the abnormal Ca^{2+} homeostasis in human cardiomyocytes from failing hearts (Schmidt et al., 1998; Schmidt et al., 1999).

Studies have shown that doxorubicin can either increase or decrease cardiomyocyte contractility. The discrepancy of these findings may be caused by different animal and cell culture models, the dosage of doxorubicin, the duration of the treatment and especially the developmental stage of the disease. In the early stage of the disease, doxorubicin tends to induce Ca^{2+} release from SR and increase cardiomyocyte contractility (Brown et al., 1989b; Kim et al., 1989; Ondrias et al., 1990; Kapelko et al., 1996). In the late stage of the disease, doxorubicin inhibits Ca^{2+} regulatory proteins and reduces cardiomyocyte contractility (Ondrias et al., 1990; Dodd et al., 1993; Maeda et al., 1998; Boucek et al., 1999; Chugun et al., 2000; Gambliel et al., 2002; Timolati et al., 2006). In a subacute doxorubicin mouse model, doxorubicin induces an increase in cardiac contractile function as measured by dP/dtmax and dP/dtmin during the first few days after the doxorubicin injection; however, cardiac function declines later on (our unpublished data). These results are consistent with the findings in doxorubicin-treated patients (Brown et al., 1989b; Barry et al., 2007a).

Doxorubicin-induced reduction of cardiomyocyte contractility is often associated with decreased expression of SERCA2a (Dodd et al., 1993; Boucek et al., 1999; Gambliel et al., 2002), suggesting that impaired SERCA2a function may contribute to doxorubicin-induced cardiomyocyte contractile dysfunction.

Studies in mice with cardiomyocyte-specific overexpression of SERCA2a, however, showed that SERCA2a overexpression exacerbated doxorubicin-induced mortality and morphological damage to cardiac tissue (Burke et al., 2003). These results may be caused by constitutive activation of SERCA2a, especially during the early stage of doxorubicin cardiac injury. Increase of SERCA2a activities at the early stage of the disease may further aggravate

the adverse effects of doxorubicin on Ca^{2+} homeostasis, thereby exacerbating the disease. On the other hand, activation of SERCA2a at a later stage of the disease may be beneficial.

3. The Neuregulin1-ErbB signaling and its physiological functions in the heart

The Neuregulin1-ErbB signaling pathway is an evolutionally conserved signaling pathway. It is pivotal for the development of various organ systems including the heart. It is also important for maintaining normal physiological functions of these organs. During the past two decades, studies using genetically modified mouse models, and most recently systems biology approaches, have revealed that ErbB receptors and their ligands form a complex signaling network, which includes an input layer, signal-processing layers and an output layer. This signaling system regulates a wide range of functions of the cell. In the heart, a significant number of studies have been performed in this area.

3.1 The ErbB receptor tyrosine kinases

3.1.1 Members of the ErbB receptor family

The ErbB receptors, also known as HER receptors, are epidermal growth factor (EGF) receptor tyrosine kinases (RTKs). Worm *C. elegans* contains one ErbB receptor and one ligand (Aroian et al., 1990). In Drosophila, there are one ErbB receptor and four ligands (Freeman, 1998). In humans, there are four members of this RTK family, which include ErbB1 (also known as EGFR, HER1), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4) (Citri and Yarden, 2006). There are 13 polypeptide extracellular ErbB ligands, which include Neuregulin proteins, epidermal growth factor (EGF), epiregulin, betacellulin and others (Yarden and Sliwkowski, 2001b). All ErbB ligands contain a conserved epidermal growth factor (EGF)-like domain (Yarden and Sliwkowski, 2001b; Citri and Yarden, 2006). This multilayered and various combinations of ErbB receptors and ligands suggest an apparently more sophisticated and fine-tuned NRG1-ErbB axis-dependent regulation of signal transduction and biological responses in humans.

A unique feature of the ErbB family is that there is no known ligand for the ErbB2 receptor (Klapper et al., 1999), while ErbB3 lacks intrinsic kinase activity (Guy et al., 1994). However, ErbB2 and ErbB3 can form heterodimers to generate potent cellular signals (Citri et al., 2003). The ErbB2 receptor is a preferred heterodimeric partner of the other three ErbB receptors (Graus-Porta et al., 1997). Heterodimers which contain ErbB2 have higher affinity and broader specificity for ligands.

The ErbB receptors are expressed in various types of cells, including epithelial, mesenchymal, neuronal and cardiomyocytes. The ErbB receptors play important roles in organ development and maintaining the normal physiological function of adult tissues. In cancer cells, ErbB receptors are aberrantly expressed and constitutively activated. Therefore, they are major drug targets for cancer therapy (Alimandi et al., 1995; Moasser, 2007). In the developing heart, the ErbB2 and ErbB4 receptors are detected in the cardiac myocardium and endocardium (Erickson et al., 1997; Meyer et al., 1997; Zhao et al., 1998; Fuller et al., 2008; Pentassuglia and Sawyer, 2009; De Keulenaer et al., 2010), while the ErbB3 receptor is expressed in the cardiac endocardium and mesenchyme (Erickson et al., 1997; Camenisch et

al., 2002). ErbB2 and ErbB4 are expressed in adult cardiomyocytes (Zhao et al., 1998). Further more, recent studies have shown that adult cardiomyocytes also express the ErbB3 receptor (Camprecios et al., 2011).

3.1.2 The signaling network activated by the ErbB receptors

The ErbB receptors contain an extracellular ligand binding domain, a single transmembrane domain and intracellular kinase domain. Upon ligand binding, ErbB receptors form homo- or heterodimers, which trigger auto- or trans-phosphorylation on tyrosine residues of the receptors. These residues then serve as docking sites for recruiting signaling molecules from the cytosol to the cell membrane, which then activate various signaling pathways.

The dimerization of ErbB receptors induces activation of a wide range of signaling molecules. The PI3K and MAPK pathways are among the most studied. In addition, the ErbB receptors activate other pathways, such as STATs, PLCs and JNK. These molecules further regulate the activities of key transcriptional factors, such as Jun, fos and Myc, leading to the modulation of various aspects of cell function including survival, proliferation, and migration.

Systems biology studies have revealed that this signaling network is highly organized and precisely regulated by multiple layers of regulatory and control mechanisms, which include activation of specific pathways, positive and negative feedback loops and horizontal control by parallel signaling networks.

Phosphoproteomics studies have shown that each ErbB receptor has a specific and preferred binding pattern with signaling molecules (Schulze et al., 2005). The ErbB2 receptor has few binding partners, among which, Shc is the most common partner. The ErbB3 receptor has multiple binding sites for the PI3K subunit p85. ErbB1 and ErbB4 receptors show a diversity of interaction partners including STAT5 and Grb2.

Feedback loops in and outside of this network help maintain homeostasis. For example, ErbB-mediated activation of the MAPK pathway induces the transcription of TGFα and HB-EGF (Schulze et al., 2001), which in turn further activate the ErbB receptors. ErbB activation can also lead to transcription of proteins that inhibit further activation of a particular pathway. For example, ErbB1 receptor activation by EGF causes expression of the suppressor of cytokine signaling (SOCS), which in turn promotes ErbB1 degradation (Kario et al., 2005). In addition, the ErbB signaling can be regulated by G-protein coupled receptors (GPCRs). Studies have shown that thrombin and endothelin have positive effects on the ErbB signaling via activation of matrix metalloproteinases (MMPs), subsequent activation of ErbB ligands, or activation of Src or Pyk2 which phosphorylate ErbB receptors (Dikic et al., 1996; Yarden and Sliwkowski, 2001b; Negro et al., 2006).

3.2 Neuregulin1 proteins

Neuregulin1 proteins were discovered during 1992-1993 by four independent research groups. At the time, two of the groups were searching for a ligand for the oncogene ErbB2 (Holmes et al., 1992; Peles et al., 1992; Wen et al., 1992). One group was searching for a factor that stimulated the proliferation of Schwann cells (Marchionni et al., 1993), and the third was searching for a factor that stimulated the synthesis of muscle receptors for acetylcholine (Falls

et al., 1993). Subsequently, it was found that all these factors were encoded by the same gene, which is the Neuregulin1 gene (Burden and Yarden, 1997). Therefore, Neuregulin1 proteins were also called Neu differentiation factor (NDF), heregulins, glial growth factor (GGF), acetylcholine receptor inducing activity (ARIA). Subsequently, three other Neuregulin genes were discovered, which are Neuregulin2, Neuregulin3 and Neuregulin4 (Carraway et al., 1997; Zhang et al., 1997; Harari et al., 1999). However, limited information is available regarding the biological functions of Neuregulin2, 3 and 4 gene encoded proteins. Neureuglin1 proteins bind directly to the ErbB3 and the ErbB4 receptors. The ErbB2 receptor does not bind directly to Neureuglin1; rather it serves as a co-receptor and forms heterodimers with the ErbB3 or the ErbB4 receptor (Falls et al., 1993; Burden and Yarden, 1997).

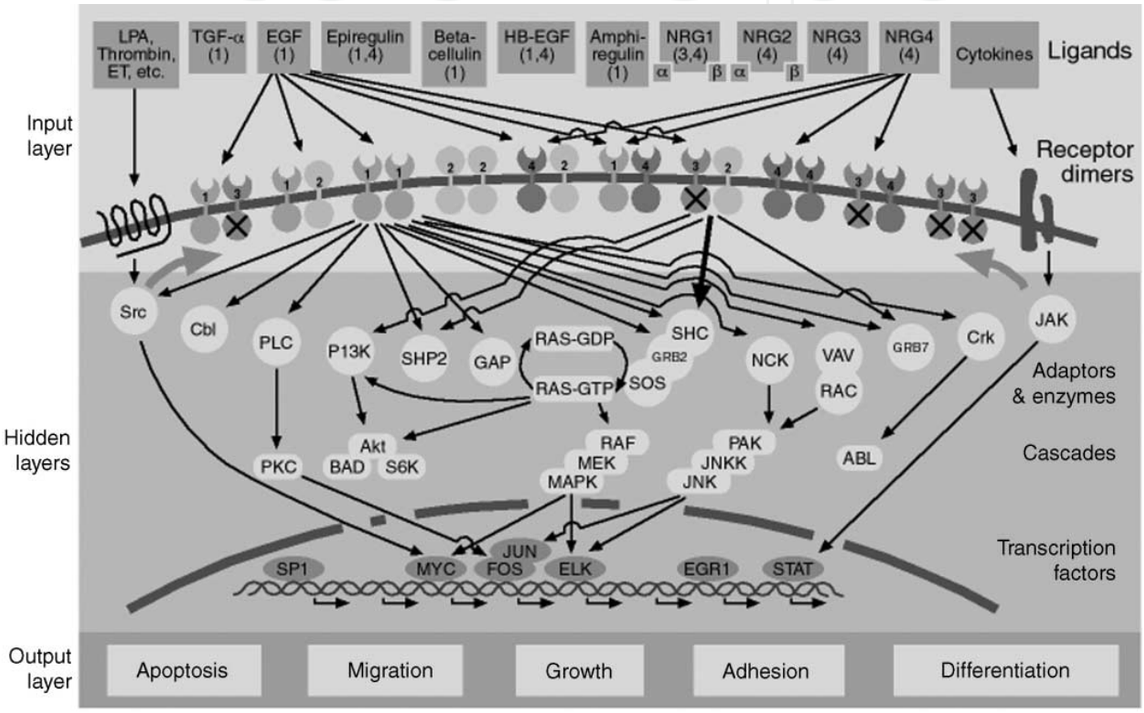

Fig. 1. The ErbB Signaling Network. ErbB receptors and their ligands form a complex signaling network which regulates a wide variety of cell functions. There are four ErbB receptors (ErbB1-ErbB4). The ErbB2 receptor does not bind to any ligand, but is a preferred partner of other ErbB receptors. The ErbB3 receptor is devoid of kinase activity. ErbB2 and ErbB3 can form functional and potent heterodimers. There are at least 13 known ligands for the ErbB receptors, including Neuregulin1 proteins. Ligand binding with the ErbB receptors activates various signaling molecules in the cell. The PI3K and MAPK pathways are among the most studied pathways. Multiple feedback loops exist within and outside of this signaling network. The activation and integration of these pathways lead to the regulation of cell survival, growth, proliferation, migration and differentiation. NRG, Neuregulin; EGF, epidermal growth factor; LPA, lysophosphatidic acid; ET, endothelin; GAP, GTPase activating protein; HB-EGF, heparin-binding EGF; Jak, Janus kinase; PKC, protein kinase C; PLC, phospholipase C; Shp2, Src homology domain-2-containing protein tyrosine phosphatase 2; Stat, signal transducer and activator of transcription. Reprinted by permission from *Nat Rev Mol Cell Biol; 2, 127-137, 2001*.

The Neuregulin1 gene is located on the short arm of the human chromosome 8 (Stefansson et al., 2002). At least 15 Neuregulin1 isoforms are produced by the Neuregulin1 gene as a result of alternative splicing and multiple promoters (Falls, 2003; Hayes and Gullick, 2008). All Neuregulin1 proteins contain the epidermal growth factor (EGF)-like domain, which binds to ligands and also is sufficient for activation of the ErbB receptors. According to the N-terminal structural differences, Neuregulin1 proteins are divided into three types (type I, II and III). Type I Neuregulin1 proteins contain an immunoglobulin (Ig)-like domain, an EGF-like domain, a proteolysis site, a hydrophobic transmembrane domain and a cytoplasmic tail. Like type I Neuregulin1, Type II Neuregulin1 isoforms contain an Ig-like domain and EGF-like domain. In addition, type II isoforms contain a signal peptide, a kringle-like sequence in their N-terminal. Type III isoforms do not have an Ig-like domain; instead, they contain a cysteine-rich domain in the N-terminal part of the protein. Therefore, Type I and II Neuregulin1 proteins are known as Ig-Neuregulin1s. The Type III Neuregulin1 proteins are known as CRD-Neuregulin1s. Other factors that differentiate

Neuregulin1 proteins are the type of EGF-like domain (α, β) and whether the isoform is initially synthesized as a transmembrane or non-membrane protein (Falls et al., 1993).

Type I and II Neuregulin1 proteins are synthesized as secreted proteins or single-pass transmembrane proteins. Proteolytic cleavage of the Ig-Neuregulin1s releases the N-terminal fragment that contains the EGF-like domain to the extracellular space, which may, in turn, bind to the ErbB receptors in a paracrine or autocrine manner. Studies have shown that metalloproteases, such as ADAM17 and ADAM19, are capable of mediating the shedding of Neuregulin1 proteins from cells (Kuramochi et al., 2004; Kalinowski et al., 2010). Type III Neuregulin1 proteins are synthesized as two-pass transmembrane proteins, with a transmembrane domain located at the C-terminal of the EGF domain and a transmembrane domain within the CRD-domain. Cleavage of the type III Neuregulin1 proteins exposes the EGF-like domain of the protein, which may interact with ErbB receptors in a juxtacrine mechanism (Falls et al., 1993).

3.3 Neuregulin-ErbB signaling in developing and adult hearts: Findings in transgenic mouse models

The physiological function of Neuregulin1 proteins and their ErbB receptors have been intensively studied during the past two decades, mainly in transgenic mouse models, as well as in mice treated with recombinant Neuregulin1s and in cell culture. Neuregulin1 proteins are localized in the cardiac endocardium and the endothelium of the cardiac microvasculature (Meyer and Birchmeier, 1995; Lemmens et al., 2006). The ErbB2 and ErbB4 receptors are expressed in cardiac myocardium (Gassmann et al., 1995; Lee et al., 1995) and ErbB3 proteins are expressed in endocardial cushion mesenchyme (Erickson et al., 1997). Neuregulin1s activate ErbB receptors in a paracrine manner (Marchionni, 1995). Studies have demonstrated that Neureuglin-ErbB signaling is essential for cardiac development in embryos and pivotal for protecting adult hearts from stress.

3.3.1 Transgenic mouse models with mutations of the Neuregulin1 gene

Several transgenic mouse models are generated with deletion/disruption of the EGF-like domain, Ig-like domain or the cytoplasmic tail of the Neureuglin1 proteins (Meyer and Birchmeier, 1995; Kramer et al., 1996b; Liu et al., 1998a).

A common phenotype of these transgenic mice is that all homozygous mice die around E10.5 during embryogenesis with the absence or underdevelopment of ventricular trabeculae. EGF-like domain disruption also causes cardiac endocardial cushion defects (Meyer and Birchmeier, 1995). Hearts around E10.5 display depressed contractility, dilatation of the common ventricle, decreased emptying of the ventricle and slow irregular heart rate (Kramer et al., 1996a; Liu et al., 1998b).

In mice with EGF-like domain disruption, the activity of all Neureuglin1s is abolished; while in mice with Ig-like domain deletion, Type III Neuregulin1s are still functional; in mice with cytoplasmic tail disruption, secretive Neuregulin1 proteins are not affected. The Ig-like domain interacts with the extracellular matrix (Kramer et al., 1996a), while the cytoplasmic tail regulates the proteolytic release of the Neuregulin1 extracellular domains (Liu et al., 1998b). Therefore, these studies suggest that Ig-like Neuregulin1s and the release of membrane anchored Neuregulin1s are necessary for cardiac trabeculae formation. They also suggest that Type III Neuregulin1s may not be pivotal for cardiac development. In mice with CRD-Neureuglin1 knockout, no cardiac defects were reported (Wolpowitz et al., 2000).

Recently, Hedhli and colleagues tested whether endothelial-derived Neuregulin1s are important for protecting the heart from ischemia-reperfusion injury (Hedhli et al., 2011). They generated mice with tamoxifen-inducible and endothelium-selective Neuregulin1 gene knockout by cross-breeding mice with VE-cadherin promoter driven Cre-ER (Monvoisin et al., 2006) and mice carrying homozygously floxed alleles of the Neuregulin1 gene (Yang et al., 2001). At the baseline, hearts from these knockout mice showed normal wall thickness, left ventricular chamber size, and systolic function. Cardiac morphology, including capillary density was not different from non-transgenic mice. However, after ischemia-reperfusion, the infarct area, the number of TUNEL positive cells, and the number of infiltrating leukocytes were significantly increased in knockout mice compared with controls. The activation of ErbB4 receptors was decreased in ischemia-reperfusion treated knockout mouse hearts. In addition, injection of a recombinant Neuregulin1 (EGF-like domain) reversed the adverse effects observed in the knockout mice. These results suggested that loss of endothelium-derived Neuregulin1 was the cause of worsening ischemia-reperfusion cardiac injury. This study has identified that Neuregulin1 is one of the protective factors derived from the cardiac endothelium.

3.3.2 Transgenic mouse models with mutations of the ErbB receptors

To understand the physiological roles of the ErbB receptors, transgenic mice with deletion of the ErbB2, ErbB3 or ErbB4 gene were generated (Gassmann et al., 1995; Lee et al., 1995; Erickson et al., 1997; Crone et al., 2002; Ozcelik et al., 2002). Deletion of the ErbB2 or the ErbB4 gene caused a similar cardiac phenotype as that observed in pan-Neuregulin1 knockout mice, which was the underdevelopment of cardiac ventricular trabeculae (Gassmann et al., 1995; Lee et al., 1995; Meyer and Birchmeier, 1995). ErbB2 or ErbB4 knockout mice died around E10.5 due to cardiac defects. Cardiac specific overexpression of the ErbB2 receptor in ErbB2 knockout mice restored normal ventricular trabeculation (Morris et al., 1999). These data suggest that the effects of Neuregulin1 proteins on cardiac development depend on both ErbB2 and ErbB4 receptors. They also suggest that ErbB2 and ErbB4 heterodimers are needed for propagating Neuregulin1 signals in the developing hearts. In mice with the ErbB3 gene knockout, however, the defect of ventricular trabeculation was not observed. Instead, cardiac cushions lacked mesenchyme and cardiac

valves were underdeveloped (Erickson et al., 1997). These findings are consistent with the expression pattern of the ErbB3 receptor in the heart.

The studies in mice with ErbB2 cardiac-specific conditional knockout provided further information on the ErbB2 receptor in the adult heart (Crone et al., 2002; Ozcelik et al., 2002). Two independent groups generated these mice by crossbreeding animals carrying the Cre-coding sequence (driven by myosin light chain 2v MLC2v or muscle creatine kinase promoter) and those with the loxP-flanked ErbB2 gene. Loss of the ErbB2 expression was observed perinatally in 50-60% of cardiomyocytes. Cardiac function was initially normal but progressively worsened. At the age of 3 months, these mice developed dilated cardiomyopathy with enlarged ventricular chambers, increase in the heart to body weight ratio and increase in atrial natriuretic factor and skeletal α-actin expression. Electron microscopy showed increased numbers of mitochondria and vacuoles in cardiomyocytes. Apoptosis as measured by TUNEL staining was increased in ErbB2 conditional knockout mice. Aortic stenosis caused more severe cardiac dysfunction in mutant mice. In addition, cardiomyocytes isolated from these mice were more susceptible to doxorubicin. Collectively, these data demonstrate that the ErbB2 receptor is essential for maintaining normal cardiac physiological function and morphology.

Mouse models		Cardiac phenotype	References
Neuregulin1 knockout (NRG1 KO)	EGF-like domain (pan NRG1 KO)	Homozygous die around E10.5 Absence of ventricular trabeculae; defect of endocardial cushion. Heart failure	(Meyer and Birchmeier, 1995)
	Ig-like domain (Type I and II NRG1 KO)	Homozygous die around E10.5 Lack ventricular trabeculae. Heart failure	(Kramer et al., 1996b)
	Cytoplasmic tail (Type I NRG1 KO)	Homozygous die around E10.5 Lack ventricular trabeculae. Heart failure	(Liu et al., 1998a)
	Endothelial specific conditional NRG1 KO	Normal cardiac function at baseline. Increased infarct area and apoptosis after ischemia-reperfusion	(Hedhli et al., 2011)
ErbB2 KO	Constitutive ErbB2 KO	Homozygous die around E10.5. Defect of ventricular trabeculae	(Lee et al., 1995)
	Cardiomyocyte-specific conditional KO	Survived to adulthood. Mice progressively developed dilated cardiomyopathy at the age of 3-6 months.	(Crone et al., 2002; Ozcelik et al., 2002)
ErbB4 KO		Homozygous die around E10.5. Defect of ventricular trabeculae	(Gassmann et al., 1995)
ErbB3 KO		Homozygous die around E13.5. Defect of cardiac cushions and valves.	(Erickson et al., 1997)

Table 1. Cardiac phenotypes in transgenic mouse models with disruption of the Neuregulin1 gene or the ErbB receptor genes

4. Neuregulin1-ErbB signaling protects the heart from doxorubicin cardiotoxicity

Studies in animal models show that Neuregulin1 injections alleviate doxorubicin-induced cardiac dysfunction in mice. Further, studies show that the Neuregulin1-ErbB signaling protects the heart from doxorubicin cardiotoxicity via several mechanisms which include inhibition of doxorubicin-induced apoptosis, loss of mitochondrial integrity, loss of myofibrils, and impaired calcium homeostasis.

4.1 Neuregulin1 protects the heart from doxorubicin-induced cardiac dysfunction: Studies in animal models

Studies from our laboratory show that doxorubicin induces a worsened cardiac dysfunction in Neuregulin1 knockout mice (Liu et al., 2005). We used heterozygous mice with Neuregulin1 EGF-like domain deletion (Meyer and Birchmeier, 1995). The RNA expression of Neuregulin1α and β isoforms was decreased in the hearts of knockout mice (unpublished data). These mice were fertile and no abnormalities were found in cardiac function and morphology at the baseline. When mice were injected with doxorubicin (20 mg/kg, i.p. once), however, survival was decreased in doxorubicin-treated knockout mice compared to wild type mice (two week survival: 13 vs. 33 %). Cardiac function as assessed by echocardiography and left ventricular catheterization showed worsened cardiac systolic function in doxorubicin-treated knockout mice. We further showed that the activation of ErbB2 and downstream signaling molecules Akt, mTOR and MAPK in the heart was more depressed in doxorubicin-treated knockout mice vs. wild type mice. These results suggest that Neuregulin1 is necessary for protecting the heart from doxorubicin.

Our laboratory and Liu et al. further demonstrated that injections of recombinant human Neuregulin1 proteins improve cardiac function in doxorubicin-treated animals (Liu et al., 2005; Liu et al., 2006; Bian et al., 2009). Comparing the two studies, we used recombinant human glial growth factor 2 (GGF2), and a subacute doxorubicin cardiotoxicity mouse model (doxorubicin, 20 mg/kg, i.p. once). GGF2 was injected subcutanously daily using the dosage of 0.75 mg/kg/day (Bian et al., 2009); while Liu et al. used a recombinant EGF-like domain (β_{2a} isoform) of Neuregulin1 proteins, and a chronic doxorubicin rat model (3.3 mg/kg/week, i.v. for 4 weeks). Recombinant Neuregulin1 was injected i.v. daily for the first 7 days using the dosage of 20μg/kg/day (Liu et al., 2006). Despite the different isoforms of Neuregulin1, the dosage, the route of drug administration and the animal models used, both studies showed that injections of recombinant Neuregulin1 significantly improved survival and cardiac systolic function in doxorubicin-injured mice and rats. These studies demonstrate that Neureuglin1 injections can protect the heart from doxorubicin-induced heart failure.

4.2 Neuregulin1-ErbB signaling inhibits doxorubicin-induced cardiomyocyte apoptosis

The anti-apoptotic effects of the Neureuglin1-ErbB signaling pathway activation were assessed in doxorubicin-treated neonatal rat cardiomyocytes and adult rat cardiomyocytes in culture, in Neuregulin1 EGF-like domain knockout mice and in ErbB2 conditional knockout mice.

In cultured neonatal rat cardiomyocytes, Fukazawa et al showed that daunorubicin (anthracycline drug) significantly increased apoptosis as assessed by three different methods which were TUNEL staining, flow cytometric quantification of subG1 cell fraction and caspase3 activation. Co-incubation of recombinant Neuregulin1 (GGF2) with daunorubicin significantly reduced apoptosis. This effect of Neuregulin1 was associated with the activation of Akt. Adenoviral infection of a dominant negative Akt abolished this effect of Neuregulin1, suggesting Akt is necessary for neuregulin1's anti-apoptotic effects. In addition, activation of Akt by neuregulin1 was abolished by the ErbB4, but not ErbB2, inhibitor. Together, these observations suggest that Neuregulin1 protects cardiomyocytes from daunorubicin-induced apoptosis via activations of ErbB4 and Akt (Fukazawa et al., 2003). Another study by Rohrbach et al. also showed that Neuregulin1 inhibited daunorubicin-induced cytochrome c release, and caspase3 activation. In addition, Neuregulin1 inhibited daunorubicin-induced increase in the ratio of pro-apoptotic protein Bcl-xS to anti-apoptotic protein Bcl-xL (Rohrbach et al., 2005a).

In doxorubicin-treated mice, we observed increases of caspase3, 6, 9 and caspase8 activations in the heart, suggesting both intrinsic and extrinsic apoptosis pathways were activated in these hearts. Concomitant Neureuglin1 injections significantly reduced the activations of these caspases (Bian et al., 2009). However, decreased activation of the Neuregulin-ErbB signaling does not further increase anthracycline drug doxorubicin or daunorubicin–induced apoptosis as measured by TUNEL staining. In mice with heterozygous knockout of the Neuregulin1 EGF-like domain, the number of TUNEL-positive cardiomyocytes was increased in doxorubicin-treated mouse hearts; however, there were no significant differences between doxorubicin-treated wild type and knockout mice (Liu et al., 2005). Similarly, in neonatal and adult rat cardiomyocytes, neither the anti-ErbB2 antibody nor the ErbB2 inhibitor further increased doxorubicin or daunorubicin-induced TUNEL staining (Fukazawa et al., 2003; Pentassuglia et al., 2009). These results, however, may be caused by the TUNEL staining method, which is relatively insensitive. In ErbB2 conditional knockout mouse hearts, very low levels of apoptotic cells was detected by TUNEL assay, while a more sensitive ligation-mediated PCR DNA fragmentation assay was able to detect the increase of apoptotic cells in ErbB2 knockout hearts (Crone et al., 2002).

Studies have also demonstrated that ErbB2 inhibition itself can induce mitochondrial dysfunction and increase apoptotic signals in cardiomyocytes as well as in adult hearts (Crone et al., 2002; Grazette et al., 2004). In cultured neonatal and adult rat cardiomyocytes, anti-ErbB2 antibody increased the ratio of Bcl-xS/Bcl-xL, translocation of BAX to mitochondria, cytochrome c release and caspase activation. These changes caused a loss of mitochondrial membrane potential and a decrease of ATP levels. Restoration of Bcl-xL levels prevented mitochondrial dysfunction (Grazette et al., 2004). In ErbB2 conditional knockout mice, apoptosis was increased in the heart. *In vivo* expression of the anti-apoptotic gene Bcl-xL partially reduced chamber dilation and restored contractility in the ErbB2 knockout mice. Cardiomyocytes isolated from the ErbB2 knockout mice were more sensitive to doxorubicin-induced cell death (Crone et al., 2002).

4.3 Neuregulin1-ErbB signaling prevents doxorubicin-induced cardiac myofibrillar disarray and loss

One of the pathological features of doxorubicin-induced cardiotoxicity is myofibril loss and disarray in cardiomyocytes. In adult rat cardiomyocytes, doxorubicin treatment resulted in

myofilament disarray (Sawyer et al., 2002; Pentassuglia et al., 2009). Simultaneous treatments with doxorubicin and an anti-ErbB2 antibody, or a dual inhibitor that targets both ErbB2 and ErbB1, caused an additive effect on myofibril damage. Conversely, recombinant Neuregulin1 reduced this effect of doxorubicin. Neuregulin1 activated both PI3K and MAPK pathways in cardiomyocytes. Inhibition of ErbB2 prevented activations of these pathways by Neuregulin1. In addition, inhibition of MAPK, but not PI3K, induced myofrilament damage in adult rat cardiomyocytes similar to that observed in ErbB2 antibody or inhibitor treated cardiomyocytes (Pentassuglia et al., 2009). These results suggest that Neuregulin1 may prevent myofibril damage by activating ErbB2 and MAPK.

In addition to preventing doxorubicin-induced myofibrillar disarray, the Neuregulin1-ErbB signaling also reduces doxorubicin-induced loss of cardiac troponin proteins (Bian et al., 2009). In doxorubicin-treated mouse hearts, cardiac troponin proteins (cTnI, cTnT and cTnC) were down-regulated. These were associated with a moderate increase in serum cTnI, but not cTnT, suggesting mechanisms other than releasing troponin proteins to serum may exist. Concurrent Neuregulin1 injections significantly prevented doxorubicin-induced loss of cardiac troponin proteins in the heart.

These phenotypes were again observed in cultured neonatal rat cardiomyocytes. Neuregulin1 co-treatment prevented doxorubicin-induced down-regulation of cTnI and cTnT proteins in cultured cardiomyocytes. These effects of Neuregulin1 were abolished by ErbB2, but not ErbB4, inhibition. These effects were also prevented by PI3K, Akt and mTOR inhibitors (Bian et al., 2009).

Further studies showed that doxorubicin reduced RNA expression of cTnI and cTnT in cardiomyocytes, while Neuregulin1 treatment reversed these effects of doxorubicin. The effect of Neuregulin1 on maintaining cTnI and cTnT proteins in doxorubicin-treated cardiomyocytes was reduced by cycloheximide (Bian et al., 2009). These results suggest that Neuregulin1 may maintain cTnI and cTnT levels by increasing transcription and translation of these proteins.

Neuregulin1 may also maintain cardiac troponins by decreasing doxorubicin-induced degradation of these proteins. Doxorubicin-activated caspases directly degraded cTnT. Doxorubicin also increased ubiquitination of cTnI. Neuregulin1 inhibited these effects of doxorubicin (Bian et al., 2009).

Results from Neuregulin1 treated normal cardiomyocytes provided clues on how it may protect cardiomyocytes from stresses, such as doxorubicin. In cultured adult rat cardiomyocytes, Neuregulin1 induced lamellipodia formation and elongation of cardiomyocytes, which restored the cell-to-cell contact of cultured cardiomyocytes. This was associated with Neuregulin1 activation of focal adhesion kinase (FAK) and formation of a complex which includes ErbB2, FAK, $p130^{CAS}$ and paxillin. FAK is pivotal for formation of the focal adhesion complex, cell spreading and motility. Neuregulin1-induced activation of FAK was inhibited by an anti-ErbB2 antibody.

In cultured adult rat cardiomyocytes, Neuregulin1 also increased the phosphorylation of GATA4, a transcriptional factor that regulates genes encoding cardiac sarcomeric proteins and important for cardiac development. Inhibition of the ErbB2 receptor or the MAPK pathway prevented phosphorylation of GATA4 by Neuregulin1. Further studies are needed to demonstrate whether these mechanisms are involved in the cardioprotective effects of Neuregulin1 in doxorubicin-injured cardiomyocytes or hearts.

4.4 Neuregulin1-ErbB signaling maintains calcium homeostasis in doxorubicin-treated cardiomyocytes

Doxorubicin disturbs calcium homeostasis and contractile function of the heart. In the acute phase, doxorubicin increases cardiac systolic and diastolic functions; while in the later stage, it causes heart failure in patients (Brown et al., 1989a).

Timolati et al. established a model in cultured cardiomyocytes which mimics this change (Timolati et al., 2006). When adult rat cardiomyocytes were treated with 1μM doxorubicin for 18 hours, cardiomyocytes calcium amplitude and fractional shortening were significantly increased. Intracellular diastolic calcium and time to 50% relaxation were modestly increased, suggesting increased cardiomyocyte systolic function in these cardiomyocytes. On the other hand, treatment of cardiomyocytes with 10μM doxorubicin for 18 hours caused significant decreases in calcium amplitude and fractional shortening as well as increases in intracellular diastolic calcium and time to 50% relaxation, suggesting systolic and diastolic function were decreased in these cardiomyocytes. In addition, doxorubicin (10μM) induced a down-regulation of SERCA proteins and SR calcium content.

Neuregulin1 treatment 3 hours prior to doxorubicin attenuated doxorubicin-induced alterations of contractility in cardiomyocytes (Timolati et al., 2006). Neuregulin1 decreased calcium amplitude and fractional shortening in 1μM doxorubicin-treated cardiomyocytes while increased these indices in 10μM doxorubicin-treated cells. Neuregulin1 also normalized the diastolic calcium and time to 50% relaxation in both 1 and 10μM doxorubicin-treated cardiomyocytes. In addition, Neuregulin1 increased SERCA proteins in 10μM doxorubicin-treated cardiomyocytes.

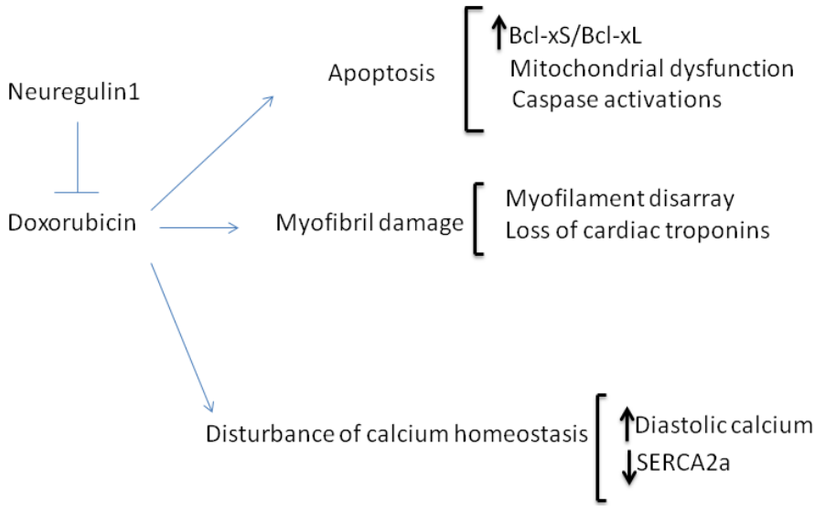

Fig. 2. The potential mechanisms of Neuregulin1-ErbB signaling in protecting the heart from doxorubicin-induced cardiotoxicity. The Neuregulin1-ErbB signaling protects cardiomyocytes from doxorubicin-induced apoptosis which include mitochondrial dysfunction, caspase activation and increase in the Bcl-xS/Bcl-xL ratio, myofibril damage which includes myofilament disarray and loss of cardiac troponins, and disturbance of calcium homeostasis which includes the increase of intracellular diastolic calcium and the decrease of SERCA2a.

In another study, Pentassuglia et al. showed that ErbB2 receptor inhibition aggravated doxorubicin-induced decrease in cardiomyocyte fractional shortening (Pentassuglia et al., 2009).

Taken together, these studies demonstrated that Neuregulin1-ErbB signaling is pivotal for inhibiting doxorubicin-induced mitochondrial dysfunction, apoptosis, myofibril disarray and loss, as well as disturbance of calcium homeostasis in the heart. They have provided mechanisms of Neuregulin1's protective effects in doxorubicin-injured hearts, as well as Trastuzumab-induced cardiotoxicity (Figure 2).

5. Conclusions

Doxorubicin-induced cardiotoxicity is a severe side effect of, and therefore, a major clinical obstacle to, cancer therapy by using this drug. The discovery of the cardiac protective role of the Neuregulin1-ErbB signaling pathway in doxorubicin-injured hearts has opened a new area for developing methods to prevent or cure doxorubicin-induced heart failure. The Neuregulin-ErbB signaling is an evolutionally conserved signaling pathway which plays critical roles in the development of multiple organ systems, including the heart. In addition to the cardiac protective effects in heart failure, this signaling pathway is one of the most mutated pathways in cancer. Understanding how this pathway protects the heart from doxorubicin injury is a first and necessary step towards using Neuregulin1 and potentially other ErbB ligands in cancer survivors.

During the past 10 years, the cardioprotective effects of the Neuregulin-ErbB signaling have been tested in other cardiovascular diseases. Studies have demonstrated that in addition to doxorubicin-induced heart failure, Neuregulin1 also improves cardiac function in ischemia-reperfusion, viral infection, and pacing induced heart failure (Liu et al., 2006; Bersell et al., 2009; Hedhli et al., 2011). In addition, Neuregulin1 promotes cell cycle reentry of differentiated adult cardiomyocytes, improves angiogenesis in the heart (Bersell et al., 2009; Hedhli et al., 2011), and promotes embryonic stem cell differentiation into the cardiac lineage (Sun et al., 2011). Clinical trials are ongoing using Neureuglin1 for the treatment of heart failure in patients (Gao et al., 2010). It is conceivable that Neuregulin1 may become a new drug for heart failure, including doxorubicin-induced cardiotoxicity.

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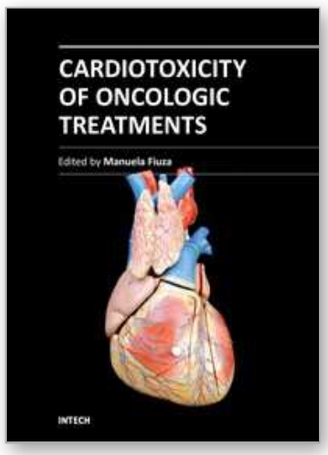

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The possibility of getting a cardiovascular disease or cancer increases with advancing age. At the same time, relevant improvements in cancer therapy have resulted in the improvement of quality of life and the increasement of the survival rate of such patients. As a result we have larger number of patients that experience the cardiac side effects of chemotherapy. The extent of cardiotoxicity is variable, depending on the type of drug used, combination with other drugs, prior mediastinal radiotherapy and the presence of cardiovascular risk factors or history of heart disease. Early detection of the patients proneness for developing cardiotoxicity is the key issue to decrease morbidity and mortality. It also facilitates more tailored therapeutic interventions. Therefore, the collaboration and interaction of cardiology and oncology may contribute to reducing the cardiovascular adverse effects and improving the results in the treatment of patients with cancer.

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