Published in final edited form as:

J Mol Cell Cardiol. 2006 August ; 41(2): 228–235.

Neuregulin activates erbB2-dependent src/FAK signaling and cytoskeletal remodeling in isolated adult rat cardiac myocytes

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Abstract

Cardiac myocyte erbB2 expression is required for maintenance of normal cardiac structure and function, though its role in cardiac cellular physiology is incompletely understood. We tested the hypothesis that erbB2 signaling modulates focal adhesion formation via activation of a src/FAK pathway using adult rat ventricular myocytes in primary culture. The erbB ligand neuregulin-1 β (NRG-1 β) induced phosphorylation of Src at Y416 and Y215, and FAK at Y861. Using antibody and pharmacological inhibitor strategies, we found that FAK activation was erbB2- and Src-dependent, but independent of PI3-kinase/Akt pathway. Furthermore, NRG-1 β stimulated the formation of a multiprotein complex between erbB2, FAK, p130^{CAS} and paxillin within 30 min, and induced lamellipodia with longitudinal elongation of the myocytes within days. The extension of lamellipodia resulted in restoration of cell-to-cell contact between isolated myocytes, allowing for synchronous beating. These effects of NRG-1 β were prevented by a src inhibitor as well as an antibody to erbB2. These results suggest the potential role of NRG-1 β /erbB2/Src/FAK signaling in the maintenance and repair of electrical and mechanical coupling in cardiomyocytes.

Keywords

Neuregulin; erbB2; Focal adhesion complex; Cardiac myocyte; src; p130CAS; FAK

1. Introduction

Neuregulins (NRGs) and the erbB family of receptor tyrosine kinases are required for tissue morphogenesis during cardiac development[1–3]. The expression of NRG-1, erbB2 and erbB4 persist in the postnatal heart [4] and disruption of this system results in impaired cardiac function [5–9]. The mechanisms by which this signaling system acts to maintain cardiac structure and function remain incompletely understood. In vitro, NRG-1 β induces growth and survival in cardiac myocytes via activation of MEK/Erk and PI3-kinase/Akt pathways [10, 11]. However, there are also effects of NRG-1 β on myocyte structure both at baseline [10] and in response to injury [7] that are not fully accounted for by these signaling pathways.

Focal adhesion kinase (FAK) is a nonreceptor tyrosine kinase critical for the formation of focal adhesion complexes (FAC) and cell spreading, motility and survival [12]. FAK is a substrate of Src, and both FAK and Src are activated in cancer cells overexpressing erbB2 or stimulated with erbB ligand [13,14]. Furthermore, p130^{CAS} is a FAK-interacting protein, and is frequently hyper-phosphorylated in erbB2 overexpressing or Src transformed cells [15,16]. In ventricular myocytes, FAK and p130^{CAS} are known to be essential for the maintenance of sarcomeric organization [17–19] and for the regulation of cell survival [20]. FAK localization to the

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intercalated disks in freshly isolated ventricular myocytes [21] suggests that it may be critical for myocyte–myocyte interactions. Cardiac-specific inactivation of FAK results in cardiac failure, with marked histopathology of cardiac myocytes including myofibrillar disarray [22]. Collectively, these findings led us to hypothesize an erbB2/Src/FAK signaling pathway in ventricular myocytes stimulated by NRG-1 β . Our results demonstrate that NRG-1 β activates focal adhesion complex formation that leads to directional spreading initiating from the axial ends of myocytes. We propose that NRG-1 β -induced cardiomyocyte “remodeling” may represent a mechanism by which myocytes maintain electrical and mechanical coupling in the heart, and restore such coupling after tissue injury.

2. Experimental procedures

2.1. Chemicals

The recombinant NRG-1 β (glial growth factor 2) was kindly provided by Mark Marchionni (Cambridge Neuroscience, Inc, Arlington, MA). All other chemicals were purchased from Sigma and Calbiochem.

2.2. Cell preparation and culture

Adult rat ventricular myocytes (ARVM) were isolated as previously reported [4]. ARVM were plated at densities of 80–150 myocytes/mm² on P60 plates or 40 $\times$ 22 mm glass coverslips precoated with laminin (Becton-Dickinson) and were maintained in serum-free ACCT (albumin, L-carnitine, creatine, taurine) medium with 100 μ mol/L bromodeoxyuridine to inhibit nonmyocyte proliferation.

2.3. Cell treatment

NRG-1 β was used at 10 ng/mL. Preincubation with PI3-kinase inhibitor LY294002 (10 μ mol/L, Calbiochem) or Src-inhibitor PP2 (1 and 10 μ mol/L, Calbiochem) was for 30 min prior to NRG-1 β treatment. For signaling analysis, ARVM were cultured overnight at 37 °C before treatment.

2.4. Western blotting and immunoprecipitation

Anti-actin was obtained from Sigma. Antibodies against phospho-Src (pY416, pY527), phospho-FAK (pY925), phospho-Akt, Akt and phospho-Erk1/2 were from Cell Signaling. Anti-Src, FAK, erbB2 and Erk2 were from Santa Cruz Biotechnology. Monoclonal Src antibody was also purchased from Upstate Biotechnology. Phospho-Src (pY215), phospho-FAK (pY397, 407, 576, 577, 861) and paxillin antibodies were from Biosource. Anti-p130^{CAS} was from BD Biosciences. ARVM were lysed with modified RIPA buffer (1% NP-40, 50 mmol/L Tris–HCl, 1 mmol/L EDTA, 0.25% DOC, 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 2 μ g/mL leupeptin, 1 μ g/mL pepstatin, 1 μ g/mL aprotinin, 1 mmol/L sodium orthovanadate). Aliquots representing 30–60 μ g of protein were used for western blot, and 200–350 μ g for immunoprecipitation. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane (Bio-Rad). After membrane development with ECL-reagent (PIERCE), quantification was performed by densitometry (Molecular Analyst, Bio-Rad).

2.5. Immunocytochemistry

ARVM were washed twice with PBS, fixed with 4% paraformaldehyde for 10 min and permeabilized in 0.2% Triton X-100 before immunostaining. We blocked nonspecific binding with 4% Bovine serum albumin in PBS for 1 h at room temperature, and incubated cells overnight with anti-phospho-FAK (Y861) or anti- α -actinin diluted in the blocking buffer. After washing with PBS, secondary antibody conjugated with FITC antibody was added for 30 min,

followed by co-staining with TXRD-conjugated phalloidin (Molecular Probe). Cells were again washed in PBS and mounted using Vectashield with nuclear staining by DAPI (Vector laboratories). We viewed cells with a Nikon Eclipse E400 fluorescent microscope. The percentage of lamellipodia positive cells/rod-shaped ARVM was determined by randomly counting 300 rod-shaped ARVM in each coverslip. Both myocytes with directional and random shape of lamellipodia were counted as lamellipodia positive cells.

2.6. Statistical analysis

Results are expressed as mean $\pm$ SD of at least 3 different experiments. One-way ANOVA was used for multiple comparisons, with Bonferroni post-test analysis. Two-way ANOVA was used for examining interaction between parameters as indicated. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. NRG-1 β induces phosphorylation of Src at Y416 and Y215, and FAK at Y861

We tested the effect of NRG-1 β on Src and FAK signaling using phosphorylation site-specific antibodies. NRG-1 β induced rapid Src phosphorylation at both Y416 and Y215, known kinase activating sites for Src [23,24], and FAK at Y861 (Fig. 1). In contrast, we could not detect changes in FAK phosphorylation at other tyrosine-phosphorylation sites, although there was baseline phosphorylation at Y397, Y407 and Y925. We performed immunocytochemistry of myocytes to determine the localization of NRG-1 β -induced FAK phosphorylation (Fig. 2). Interestingly, while there was some baseline immunostaining of anti-FAK (pY861) along the length of the cell (Fig. 2A and C), NRG-1 β treatment induced an increase in staining that was most prominent at the axial ends of the cell (Fig. 2B and D).

3.2. FAK activation at Y861 is Src-dependent, but independent of PI3-kinase

Using a pharmacologic strategy, we examined whether there were any interactions between Src and other signaling pathways activated by NRG-1 β . We pretreated ARVM with Src inhibitor PP2 (1 or 10 μ mol/L) or PI3-kinase inhibitor LY294002 (10 μ mol/L), followed by NRG-1 β treatment for 15 min. Pre-incubation of myocytes at 2 different PP2 concentrations clearly dissected NRG-1 β -induced downstream pathways (Fig. 3A), while both DMSO and PP3, a negative control to PP2, had no influence on NRG-1 β induced signaling (data for PP3 not shown). LY294002 did not affect Src (Fig. 3B) or FAK (data not shown) phosphorylation. Thus, NRG-1 β -induced-FAK activation is Src-dependent, but independent of the PI3-kinase pathway.

3.3. NRG-1 β -stimulated Src/FAK pathway is erbB2-dependent

ARVM express both erbB2 and erbB4 receptors [4], and NRG-1 β binds to erbB4 and activates down-stream signaling through erbB4 homodimerization or hetero-dimerization of erbB4 with erbB2 [25]. To examine the role of erbB2 in NRG-1 β /Src/FAK signaling, we preincubated myocytes with normal mouse IgG (1 μ g/mL, Santa Cruz Biotechnology) or an antibody to erbB2 (1 μ g/mL, NeoMarkers clone B10 without sodium azide) that causes erbB2 homodimerization and downregulates erbB2 signaling [7,26]. Overnight incubation of ARVM with anti-erbB2 or normal mouse IgG did not change baseline expression of Akt, FAK or erbB2 (Fig. 4). Interestingly, anti-erbB2 pretreatment completely blocked NRG-1 β activation of Src and FAK, while there was no effect of control mouse IgG on NRG-1 β signaling. NRG-1 β treatment suppressed Src phosphorylation at Y527, a negative regulatory site of Src [27], and this was also prevented by anti-erbB2. In contrast, anti-erbB2 caused only partial inhibition of NRG-1 β -induced phosphorylation of Akt, with no effect on Erk1/2 phosphorylation. The

blocking effects of erbB2 antibody were very similar to that of PP2 (Fig. 3A). These results suggest that erbB2 is required for NRG-1 β activation of the Src/FAK signaling pathway.

3.4. NRG-1 β induces focal adhesion complex formation

We examined the effect of NRG-1 β activation of Src/FAK on formation of FAC using a co-immunoprecipitation strategy in ARVM before and after NRG-1 β treatment (Fig. 5A). At baseline, some interaction between FAK and p130^{CAS} was detectable. After 15 min of treatment, FAK/p130^{CAS} interaction increased. This complex included erbB2. While paxillin was only weakly detectable in this complex at 15 min, there was strong association by 30 min with increase in paxillin phosphorylation (data not shown). Thus, NRG-1 β stimulation over 30 min induces a multiprotein complex that includes the erbB2 receptor. Furthermore, FAK/p130^{CAS} interaction was completely suppressed by anti-erbB2 pretreatment, indicating that this NRG-1 β -induced multiprotein complex formation is also erbB2-dependent (Fig. 5B). Interestingly, PP2 (1 μ M) did not suppress NRG-1 β -induced p130^{CAS}–FAK interactions.

3.5. NRG-1 β leads to ARVM directional spreading and cell-to-cell contact

We assessed the effect of NRG-1 β -stimulated Src/FAK signaling on ARVM lamellipodia formation. For this study, we treated myocytes for 4–6 days and examined the formation of lamellipodia. Upon isolation from the intact heart, a fraction of myocytes (usually 10–20%) will round-up due to cellular injury. As ARVM structure would be disrupted in these cells, they were excluded from the analysis. By the 4th day of treatment, NRG-1 β -treated cells showed lamellipodia formation originating from the ends of the cells (Fig. 6A). Although lamellipodia were also observed in the untreated myocytes, these were in general smaller and occurred in a lower fraction of cells. Staining for pFAK (pY861) was obvious in the NRG-1 β -treated cells, though it was not necessarily localized to the ends of myocyte as was seen acutely after NRG-1 β treatment (data not shown). The effect of NRG-1 β was more prominent by the 6th day of treatment, where extension of lamellipodia resulted in restoration of cell–cell contact. Under phase contrast microscopy of living cells at this time point, myocytes treated with NRG-1 β were beating in synchrony, while asynchronous or no beating was observed in the untreated myocytes.

Baseline lamellipodia formation was suppressed by pre-incubation of myocytes with [PP2]_{1 μ mol/L}, but NRG-1 β -induced lamellipodia was not suppressed (Fig. 6B). Cell death occurred with long-term treatment of myocytes with [PP2]_{10 μ mol/L} (data not shown). In contrast, treatment with anti-erbB2 inhibited NRG-1 β -induced formation of lamellipodia (Fig. 6B). Collectively, these results suggest that activation of Src/FAK pathway by NRG-1 β induces cardiomyocyte coupling through lamellipodia formation.

4. Discussion

These results add a new dimension to our understanding of the NRG-1 β /erbB signaling system in the heart. To date, the explanation for the absolute requirement of NRG-1, and the erbB2/erbB4 receptors in cardiac development [1–3] focused on the ability of this system to regulate myocyte growth and survival [4,28]. Our findings that NRG-1 β /erbB2 signaling regulates FAK activation, FAC formation and directional spreading of myocytes demonstrate a novel function of NRG-1 β /erbB signaling and suggest that NRG-1 β regulates myocyte–myocyte and/or myocyte–matrix interactions.

NRG-1 β binding and activation of erbB4/erbB2 receptor tyrosine kinases in myocytes induce activation of a MEK/Erk pathway, as well as a PI3-kinase/Akt pathway, leading to increased protein synthesis [10] and cell survival in the presence of cytotoxic stress [11,28], respectively. The NRG-1 β /erbB2-dependent activation of Src/FAK observed here appears to be distinct

from those pathways, as it was selectively suppressed by low concentrations of Src inhibitor or chronic treatment with an anti-erbB2 antibody. However, since higher concentrations of PP2 and anti-erbB2 partially inhibited NRG-1 β -dependent phosphorylation of Akt (but not Erk), we cannot rule out the possibility that Src or FAK modulates PI3-kinase/Akt pathway in myocyte, similar to that reported in breast cancer cells and 293 cells [29,30]. The persistent activation of Akt and Erk in the presence of anti-erbB2 suggests that these pathways can be activated by erbB4 homodimerization alone.

NRG/erbB-dependent Src/FAK signaling has been observed in several contexts. In MCF-7 cells, erbB2-dependent phosphorylation of Src at Y215 and FAK Y861 has been observed and implicated in tumor metastasis and invasiveness [14]. In Schwann cells, NRG-1 β rapidly induced a direct association of erbB2 and erbB3 receptors with FAK [31], similar to what we observed in myocytes (although at later time points). The ‘growth’ we observed in myocytes stimulated with NRG-1 β is conceptually similar to glioblastoma cell growth where actin microfilament organization induced by FAK or p130^{CAS} is also directional. FAK/erbB signaling induced by NRG-1 β in Schwann cells similarly occurs at the leading edge of cell extension [31]. The activation of FAK via NRG-1b/erbB2 signaling is specific for the Y861, a phosphorylation site previously been shown to be a critical for coupling of FAK to p130Cas during transformation of NIH3T3 fibroblasts [32]. We observed high levels of baseline phosphorylation of FAK at multiple sites, likely due to integrin interaction and activation by the laminin matrix substrate that we use to plate myocytes upon. It is possible that these other sites, which are known to regulate FAK interactions with critical regulators of cell structure and motility (for recent review see [33]), are required for erbB2/FAK signaling. Collectively, it appears that NRG-1 β /erbB/Src/FAK-Y861 signaling may function in many tissues to create, and perhaps repair, tissue architecture by orchestrating cell–cell interaction. There are obvious limitations as to what can be learned in cell culture, and a critical step in developing these ideas will be to find a way to study this system in the intact heart, in the presence of functional cell–cell connections.

While we have focused in this study on the role of NRG-1 β -induced Src/FAK activation in lamellipodia formation, it is interesting that FAK and p130^{CAS} both appear to be critical for the regulation of cardiac sarcomere organization. Mice lacking p130^{CAS} die in utero with a failure of cardiac development [34], and overexpression of mutant p130^{CAS} or C-terminal focal adhesion-targeting domain of FAK in neonatal cardiac myocytes disrupts sarcomere structure [17,18]. Thus, it is tempting to speculate that FAK and p130^{CAS} interaction induced by NRG-1 β /erbB2 signaling plays a role in the maintenance of sarcomere structure. Cardiac-specific deletion of FAK results in a loss of sarcomere structure over time, and a heart failure phenotype [22]. Further in vitro work, perhaps over longer time frames, will be needed to address whether this is a primary defect, or if the disruption of sarcomere structure is secondary to some other stress induced by disruption of FAK.

The effect of anti-erbB2 on NRG-1 β activation of Src/FAK signaling suggests that conditions that suppress myocardial erbB2 expression or function will cause selective impairment of this signaling pathway. Mice with cardiac-specific erbB2 mutation can survive until birth with decreasing heart function and increasing susceptibility to stress [5,6]. Detailed histological examination of the hearts from these mice, in particular focusing on myocyte–myocyte and myocyte–matrix coupling, was not reported. Our present findings predict that these mice will show specific impairment in NRG-1 β -dependent activation of Src/FAK signaling and FAC formation, particularly under stressed conditions. In preliminary experiments, we have found that the potent activation of NRG-1 β /erbB signaling by myocardial ischemia–reperfusion injury [35] is accompanied by phosphorylation of FAK at Y861, and formation of a FAK/erbB2 complex (Kuramochi, Lim and Sawyer, unpublished observation). A morphological analysis

of the erbB2 knockout mice during such a stress will help to elucidate the functional significance of the erbB2/Src/FAK pathway.

In patients treated with the erbB2-targeted cancer therapy trastuzumab, there is an increased risk of clinical heart failure, particularly in those patients receiving anthracyclines [8]. The mechanism for this toxicity remains incompletely understood, but occurs primarily in the setting of concurrent or prior administration of cardiotoxic anthracyclines. Based upon our present findings, we speculate that toxicity of trastuzumab occurs due to the disruption of myocyte erbB2 signaling along the src/FAK pathway, leading to an impaired ability of the heart to manage repair of cell–cell and/or cell–matrix coupling in the setting of a cardiotoxic stress (i.e. anthracyclines). The suppression of Src and FAK phosphorylation, FAC formation, ARVM lamellipodia and cell–cell contact by treatment with anti-erbB2, support this thesis. Further study that more definitively assesses the role of NRG-1 β /erbB2/Src/FAK signaling in the maintenance and repair of myocyte coupling will help to explore this possibility.

Acknowledgements

We thank Mark Marchionni (Cambridge Neurosciences) for recombinant NRG-1 β , and Chee Lim, Narine Sarvazyan and Adam Lerner for helpful discussions. This study is funded by HL68144 and N01-HV-28178, a grant from the Juvenile Diabetes Research Association, an investigator initiated grant from Roche Ltd. to DBS.

References

1. Gassmann M, Casagrande F, Orioli D, Simon H, Lai C, Klein R, et al. Aberrant neural and cardiac development in mice lacking the erbB4 neuregulin receptor. *Nature* 1995;378:390–4. [PubMed: 7477376]
2. Lee KF, Simon H, Chen H, Bates B, Hung MC, Hauser C. Requirement for neuregulin receptor erbB2 in neural and cardiac development. *Nature* 1995;378:394–8. [PubMed: 7477377]
3. Meyer D, Birchmeier C. Multiple essential functions of neuregulin in development [published erratum appears in *nature* 1995 Dec 14;378 (6558):753]. *Nature* 1995;378:386–90. [PubMed: 7477375]
4. Zhao YY, Sawyer DR, Baliga RR, Opel DJ, Han X, Marchionni MA, et al. Neuregulins promote survival and growth of cardiac myocytes. Persistence of erbB2 and erbB4 expression in neonatal and adult ventricular myocytes. *J Biol Chem* 1998;273:10261–9. [PubMed: 9553078]
5. Crone SA, Zhao YY, Fan L, Gu Y, Minamisawa S, Liu Y, et al. ErbB2 is essential in the prevention of dilated cardiomyopathy. *Nat Med* 2002;8:459–65. [PubMed: 11984589]
6. Ozcelik C, Erdmann B, Pilz B, Wettschurek N, Britsch S, Hubner N, et al. Conditional mutation of the erbB2 (her2) receptor in cardiomyocytes leads to dilated cardiomyopathy. *Proc Natl Acad Sci U S A* 2002;99:8880–5. [PubMed: 12072561]
7. Sawyer DB, Zuppinger C, Miller TA, Eppenberger HM, Suter TM. Modulation of anthracycline-induced myofibrillar disarray in rat ventricular myocytes by neuregulin-1beta and anti-erbB2: potential mechanism for trastuzumab-induced cardiotoxicity. *Circulation* 2002;105:1551–4. [PubMed: 11927521]
8. Slamon DJ, Leyland-Jones B, Shak S, Fuchs H, Paton V, Bajamonde A, Fleming T, Eiermann W, Wolter J, Pegram M, Baselga J, Norton L. Use of chemotherapy plus a monoclonal antibody against her2 for metastatic breast cancer that overexpresses her2. *N Engl J Med* 2001;344:783–92. [PubMed: 11248153]
9. Garcia-Rivello H, Taranda J, Said M, Cabeza-Meckert P, Vila-Petroff M, Scaglione J, et al. Dilated cardiomyopathy in erb-b4-deficient ventricular muscle. *Am J Physiol, Heart Circ Physiol* 2005;289:H1153–60. [PubMed: 15863464]
10. Baliga RR, Pimental DR, Zhao YY, Simmons WW, Marchionni MA, Sawyer DB, Kelly RA. Nrg-1-induced cardiomyocyte hypertrophy. Role of pi-3-kinase, p70(s6k), and mek-mapk-rsk. *Am J Physiol* 1999;277:H2026–37. [PubMed: 10564160]
11. Fukazawa R, Miller TA, Kuramochi Y, Frantz S, Kim YD, Marchionni MA, et al. Neuregulin-1 protects ventricular myocytes from anthracycline-induced apoptosis via erbB4-dependent activation of pi3-kinase/akt. *J Mol Cell Cardiol* 2003;35:1473–9. [PubMed: 14654373]

12. Gu J, Tamura M, Pankov R, Danen EH, Takino T, Matsumoto K, et al. Shc and fak differentially regulate cell motility and directionality modulated by pten. *J Cell Biol* 1999;146:389–403. [PubMed: 10427092]
13. Muthuswamy SK, Li D, Lelievre S, Bissell MJ, Brugge JS. ErbB2, but not erbB1, reinitiates proliferation and induces luminal repopulation in epithelial acini. *Nat Cell Biol* 2001;3:785–92. [PubMed: 11533657]
14. Vadlamudi RK, Sahin AA, Adam L, Wang RA, Kumar R. Heregulin and her2 signaling selectively activates c-src phosphorylation at tyrosine 215. *FEBS Lett* 2003;543:76–80. [PubMed: 12753909]
15. Spencer KS, Graus-Porta D, Leng J, Hynes NE, Klemke RL. ErbB2 is necessary for induction of carcinoma cell invasion by erbB family receptor tyrosine kinases. *J Cell Biol* 2000;148:385–97. [PubMed: 10648571]
16. Kanner SB, Reynolds AB, Vines RR, Parsons JT. Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. *Proc Natl Acad Sci U S A* 1990;87:3328–32. [PubMed: 2110361]
17. Kovacic-Milivojevic B, Roediger F, Almeida EA, Damsky CH, Gardner DG, Ilic D. Focal adhesion kinase and p130cas mediate both sarcomeric organization and activation of genes associated with cardiac myocyte hypertrophy. *Mol Biol Cell* 2001;12:2290–307. [PubMed: 11514617]
18. Kovacic-Milivojevic B, Damsky CC, Gardner DG, Ilic D. Requirements for the localization of p130cas to z-lines in cardiac myocytes. *Cell Mol Biol Lett* 2002;7:323–9. [PubMed: 12097978]
19. Mansour H, de Tombe PP, Samarel AM, Russell B. Restoration of resting sarcomere length after uniaxial static strain is regulated by protein kinase cepsilon and focal adhesion kinase. *Circ Res* 2004;94:642–9. [PubMed: 14963000]
20. Heidkamp MC, Bayer AL, Kalina JA, Eble DM, Samarel AM. Gfp-frnk disrupts focal adhesions and induces anoikis in neonatal rat ventricular myocytes. *Circ Res* 2002;90:1282–9. [PubMed: 12089066]
21. Willey CD, Balasubramanian S, Rodriguez Rosas MC, Ross RS, Kuppuswamy D. Focal complex formation in adult cardiomyocytes is accompanied by the activation of beta3 integrin and c-src. *J Mol Cell Cardiol* 2003;35:671–83. [PubMed: 12788385]
22. Peng X, Kraus MS, Wei H, Shen TL, Pariaut R, Alcaraz A, et al. Inactivation of focal adhesion kinase in cardiomyocytes promotes eccentric cardiac hypertrophy and fibrosis in mice. *J Clin Invest* 2006;116:217–27. [PubMed: 16374517]
23. Pu M, Akhand AA, Kato M, Hamaguchi M, Koike T, Iwata H, et al. Evidence of a novel redox-linked activation mechanism for the src kinase which is independent of tyrosine 527-mediated regulation. *Oncogene* 1996;13:2615–22. [PubMed: 9000135]
24. Yang Z, Bagheri-Yarmand R, Wang RA, Adam L, Papadimitrakopoulou VV, Clayman GL, et al. The epidermal growth factor receptor tyrosine kinase inhibitor zD1839 (Iressa) suppresses c-src and pak1 pathways and invasiveness of human cancer cells. *Clin Cancer Res* 2004;10:658–667. [PubMed: 14760089]
25. Alroy I, Yarden Y. The erbB signaling network in embryogenesis and oncogenesis: signal diversification through combinatorial ligand–receptor interactions. *FEBS Lett* 1997;410:83–6. [PubMed: 9247128]
26. Yarden Y. Agonistic antibodies stimulate the kinase encoded by the neu protooncogene in living cells but the oncogenic mutant is constitutively active. *Proc Natl Acad Sci U S A* 1990;87:2569–73. [PubMed: 1969636]
27. Martin GS. The hunting of the src. *Nat Rev, Mol Cell Biol* 2001;2:467–75. [PubMed: 11389470]
28. Kuramochi Y, Lim CC, Guo X, Colucci WS, Liao R, Sawyer DB. Myocyte contractile activity modulates norepinephrine cytotoxicity and survival effects of neuregulin-1beta. *Am J Physiol, Cell Physiol* 2004;286:C222–9. [PubMed: 14522821]
29. Lu Y, Yu Q, Liu JH, Zhang J, Wang H, Koul D, et al. Src family protein-tyrosine kinases alter the function of pten to regulate phosphatidylinositol 3-kinase/akt cascades. *J Biol Chem* 2003;278:40057–66. [PubMed: 12869565]
30. Jiang T, Qiu Y. Interaction between src and a c-terminal proline-rich motif of akt is required for akt activation. *J Biol Chem* 2003;278:15789–93. [PubMed: 12600984]

31. Vartanian T, Goodearl A, Lefebvre S, Park SK, Fischbach G. Neuregulin induces the rapid association of focal adhesion kinase with the erbb2–erbb3 receptor complex in schwann cells. *Biochem Biophys Res Commun* 2000;271:414–7. [PubMed: 10799311]
32. Lim Y, Han I, Jeon J, Park H, Bahk YY, Oh ES. Phosphorylation of focal adhesion kinase at tyrosine 861 is crucial for ras transformation of fibroblasts. *J Biol Chem* 2004;279:29060–5. [PubMed: 15123632]
33. Mitra SK, Hanson DA, Schlaepfer DD. Focal adhesion kinase: in command and control of cell motility. *Nat Rev, Mol Cell Biol* 2005;6:56–68. [PubMed: 15688067]
34. Honda H, Oda H, Nakamoto T, Honda Z, Sakai R, Suzuki T, et al. Cardiovascular anomaly, impaired actin bundling and resistance to src-induced transformation in mice lacking p130cas. *Nat Genet* 1998;19:361–5. [PubMed: 9697697]
35. Kuramochi Y, Cote GM, Guo X, LeBrasseur NK, Cui L, Liao R, et al. Cardiac endothelial cells regulate ros-induced cardiomyocyte apoptosis through neuregulin-1beta/erbb4 signaling. *J Biol Chem* 2004;279 (49):51141–7. [PubMed: 15385548]

Fig. 1.

NRG-1 β activation of Src and FAK phosphorylation in myocytes. ARVM were stimulated with 10 ng/mL of NRG-1 β for indicated time periods. Equal amounts (50 μ g) of protein from myocyte lysates were blotted for (A) Src and (B) FAK phosphorylation. (A) NRG-1 β induced increases in phosphorylation of Src at Y215 and Y416, with a decrease in phosphorylation at Y527. Apparent molecular weight of bands shown is 60 kDa. (B) Treatment with NRG-1 β induced site-specific phosphorylation of FAK at Y861, but did not change degree of phosphorylation at other sites. Apparent molecular weight of bands shown is 125 kDa. (C) Densitometry of blots demonstrates activation of FAK at Y861 occurs within 2 min (* P <0.05 vs. 0 min, n =4). Blots are representative of 4 independent experiments.

Fig. 2.

NRG-1 β activation of FAK phosphorylation is localized in myocytes. ARVM cultures untreated (A and C: CTL) or treated with NRG-1 β (B and D, 10 ng/mL for 20 min) were fixed and stained with phospho-FAK (pY861: pFAK: Green). Cells were counterstained for filamentous actin (phalloidin, red) or DNA (DAPI, blue) (see Experimental procedures). Panels A and C show phospho-FAK staining alone, whereas panels B and D show overlay with actin and nuclear staining to demonstrate localization of phospho-FAK. Accumulation of phospho-FAK along the ends of myocyte is shown by arrowhead (scale bar=100 μ m).

Fig. 3.

NRG-1 β induced FAK (Y861) is Src-dependent, but independent of PI3-kinase. (A) ARVM were preincubated with or without PP2 (1 and 10 μ mol/L) for 30 min, followed by 15 min of NRG-1 β treatment (10 ng/mL). Cell lysates were used for western blotting with phospho-Akt (pAkt), Akt, phospho-Erk1/2 (p-Erk), Erk2, phospho-FAK (pY861: pFAK) and FAK antibodies. Results were quantified by densitometry (* P <0.05 vs. NRG group, † P <0.05 vs. NRG, PP2 (1 μ mol/L)+NRG groups, n =4). (B) ARVM were preincubated with LY294002 (10 μ mol/L) for 30 min, followed by treatment with NRG-1 β (10 ng/mL) for indicated times. Western blots were performed using antibodies to phospho-Src (pY416, pY215: p-Src; 60 kDa), phospho-FAK (pY861: 125 kDa), phospho-Akt (pAkt; 60 kDa), phospho-Erk1/2 (pErk:

44, 42 kDa) and Akt (60 kDa). Phospho-FAK (Y861) results were quantified by densitometry (* P <0.05 vs. 0 min, n =4).

Fig. 4.

NRG-1 β -stimulated Src/FAK phosphorylation is erbB2-dependent. (A) ARVM were preincubated with normal mouse IgG or anti-erbB2 overnight, and were treated with NRG-1 β (10 ng/mL) for 15 min. Immunoblot analysis was performed for phospho-Akt (pAkt), phospho-Erk1/2 (pErk), phospho-Src (pY416 and pY527: p-Src), phospho-FAK (pY861: pFAK), Akt (60 kDa), FAK and erbB2 (185 kDa), respectively. Blots are representative of 3 different experiments. (B) The results of immunoblots were quantified with densitometer (* P <0.05 vs. others, # P <0.05 vs. NRG-1 β treatment alone, n =3).

Fig. 5.

NRG-1 β induces focal adhesion complex formation in ARVM. (A) ARVM were stimulated with 10 ng/mL of NRG-1 β and lysed after the indicated time periods. Cell lysates (350 μ g) were immunoprecipitated (IP) with antibodies to FAK, p130^{CAS}, paxillin or erbB2, and immunoblotted (IB) after SDS-PAGE with antibodies to FAK (125 kDa), p130^{CAS} (130 kDa), erbB2 (185 kDa) or paxillin (68 kDa) as indicated. Blots are representative of at least 3 independent experiments. (B) After the preincubation with PP2 (1 or 10 μ mol/L) for 30 min, or with anti-erbB2 overnight, ARVM were treated with NRG-1 β at 10 ng/mL for 15 min. Cell lysates were immunoprecipitated with p130^{CAS} antibody, and immunoblotted with FAK (125 kDa), or p130^{CAS} (130 kDa) antibodies. Blots are representative of 2 independent experiments.

Fig. 6. NRG-1 β accelerates myocyte directional spreading and cell-to-cell contact through erbB2/Src signaling. (A) ARVM were cultured with or without NRG-1 β treatment for 4–6 days. Culture medium was changed everyday, with new NRG-1 β added into the medium after each change. Paraformaldehyde-fixed ARVM were stained with phalloidin (Red) and DAPI (Blue). NRG-1 β -treated myocytes demonstrate lamellipodia formation that was evident at 4 days, and became more prominent by 6 days (scale bar=100 μ m). (B) After PP2 or anti-erbB2 preincubation, ARVM were cultured for 4 days with or without NRG-1 β . Inhibitors were refreshed every 2 days, while NRG-1 β was added into medium everyday. Cells were fixed and stained, and counted under epifluorescence microscopy. The % lamellipodia-positive rod-

shaped ARVM/total rod-shaped ARVM was calculated as described in Experimental procedures. NRG treatment induced a significant increase in lamellipodia-positive myocytes (* $P < 0.05$ vs. others), that was significantly inhibited by treatment with anti-erbB2 (# $P < 0.05$ for NRG+anti-erbB2 vs. NRG alone). PP2 caused a significant inhibition in lamellipodia formation that was independent of NRG-induced lamellipodia ($P < 0.05$ for PP2 alone vs. control, PP2+NRG vs. NRG alone; $P = \text{n.s.}$ for interaction between NRG and PP2 by two-way ANOVA; $n = 4$).