Differential effects of overexpression of two forms of ephrin-A5 on neonatal rat cardiomyocytes

YUN YOU LI, ZHIBAO MI, YIQIN FENG, CHARLES F. McTIERNAN, RENPING ZHOU, PAUL D. ROBBINS, SIMON C. WATKINS, and ARTHUR M. FELDMAN

Cardiovascular Institute and Department of Molecular Genetics and Center for Biological Imaging, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213, and Laboratory for Cancer Research, College of Pharmacy, Rutgers University, Piscataway, New Jersey 08854

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The Eph family of receptors constitutes the largest subfamily of receptor tyrosine kinases (31). Eph receptors have recently been classified into two subfamilies, EphA and EphB, according to their preference for either glycosylphophatidylinositol-anchored ephrin-A ligands or transmembrane ephrin-B ligands (6, 36). While the function of the Eph receptor tyrosine kinases is not fully defined, some may play a role in signal transduction during differentiation, development, neuronal axon guidance, bundle formation, and control of cytoskeletal architecture (14, 15, 33). Ephrin-A1 (B61) was first identified as a proinflammatory cytokine-inducible endothelial cell product (16, 25, 27, 28) that plays a role in angiogenesis. Similarly, the activation of EphB receptors also directs vascular network differentiation and assembly (1, 30, 32), which suggests additional roles for Eph receptors and their ligands in both inflammation and cardiovascular development and diseases.

Recently, we (22) cloned and examined the regulation of EphA3 in neonatal rat cardiomyocytes. EphA3 is downregulated by proinflammatory cytokines in neonatal rat cardiomyocytes. We hypothesized that the downregulation of the EphA3 receptor may be mediated by its cognate ligands. Of the ephrin ligands, EphA3 binds ephrin-A5 with the highest affinity and vice versa (affinity for EphA3 > EphA8 > EphA4 = EphA5) (7, 18, 19). Ephrin-A5, initially affinity purified using an EphA5-IgG fusion protein column and named AL-1, is expressed as multiple transcripts at high levels in the human brain, heart, and kidney (17, 18, 33). It was reported that ephrin-A5 plays a role in the fasciculation and guidance of axons during development of the nervous system (9, 10). However, the downstream events regulated by the activation of EphA3 receptor, the functional significance of their activation, and the role of ephrin-A5 in the cardiovascular system have not been defined. In addition, the nature of the multiple transcripts is not clear. Direct activation of the receptor using its cognate ligands may provide invaluable information on the function of Eph receptors in cardiac myocytes.

To elucidate the role of Eph receptors and ephrin ligands in the cardiovascular system, we studied the expression of ephrin-A5 and isolated two forms of ephrin-A5 from cardiomyocytes. The effect of ephrin-A5 overexpression on EphA3 expression and the role of ephrin-A5 in neonatal cardiomyocyte biology and development were further explored.
MATERIALS AND METHODS

Cell culture and cytokine treatment. Cardiac myocytes were isolated from the ventricles of 1-day-old Sprague-Dawley rats as previously described (24). After being plated on pronectin (Promega; Madison, WI)-coated tissue culture plates, myocytes were grown at 37°C in 5% CO2 for 24 h. The medium was then changed, and the cells were allowed to grow for an additional 48 h. Medium containing 100 U/ml tumor necrosis factor-α (TNF-α; Biosource International; Camarillo, CA) was then added to the cultures. The cardiac myocyte preparations routinely contained >95% sarcomeric myosin-positive cells. All cell preparation and culture procedures were carried out under sterile conditions, and experiments conformed with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, Revised 1996).

Northern blot analysis. Total RNA was isolated from cultured cardiomyocytes via acid phenol extraction (4). RNA samples were enriched for polyadenylated species by oligo(dT)/magnetic bead capture with a PolyATtract mRNA isolation system (Promega). Poly A+ mRNA enriched from 150 μg of total RNA was resolved in formaldehyde-agarose gels, transferred to nitrocellulose membrane (Schleicher and Schuell; Keene, NH), and fixed by ultraviolet cross-linking. There was no known sequence for rodent ephrin-A5, when this cloning experiment was performed. Human AL-1, later named ephrin-A5, was used to select a pair of degenerate primers for the isolation of rat ephrin-A5. The amplified cDNA was sequenced and used as a probe for Northern blot analysis. The rat ephrin-A5 probe was prepared by PCR amplification of rat cardiomyocyte cDNA with rTth DNA polymerase and a pair of degenerate primers (sense: 5'-GTA CGC GGT A-3', antisense: 5'-GGG GGA TCC CTA TAA TGT CAA G-3'), corresponding to 1–25 and 666–687 of the cloned full-length sequence, respectively. The PCR products were cloned into a TA vector (Invitrogen; Carlsbad, CA). Clones homologous to human ephrin-A5 were isolated and sequenced. The full-length ephrin-A5 cDNA clone was used as a probe in Northern blot analysis. Prehybridization, hybridization, and quantification of the signals were performed as previously reported (22). After hybridization, the membranes were stripped with 2 mM Tris·HCl (pH 8.0), 0.2 mM EDTA, and 0.1% SDS at 75°C for 1 h and rehybridized sequentially with radiolabeled glyceraldehyde-3-phosphate dehydrogenase probe.

For the analysis of brain natriuretic peptide (BNP) expression, 6 μg of total RNA was resolved in formaldehyde-agarose gels and transferred to nitrocellulose membrane, and Northern hybridization was performed as described above using a BNP cDNA probe.

Cloning of rat heart ephrin-A5 cDNAs and construction of recombinant adenovirus containing ephrin-A5α and ephrin-A5β. A pair of specific primers that flanked the ephrin-A5 coding sequence with a XbaI or a BamHI site, respectively (sense: 5'-GGA TCT CGA ATG TTG CAC GTG GAG ATG TGT GAC GTC-3', antisense: 5'-GGG GGA TCC CTA TAA TGT CAA GGA CAT CGC C-3'), corresponding to 1–25 and 666–687 of the cloned full-length sequence, respectively, were used in the PCR amplification of ephrin-A5 in an attempt to isolate different forms of ephrin-A5. An ephrin-A5 with an 81-bp deletion (hereafter named as ephrin-A5β) was isolated in addition to the full-length ephrin-A5 (named as ephrin-A5α). The amplified cDNA products were sequenced and cut with 275 to 747, respectively. The specific primers used for cloning of the shorter ephrin-A5 cDNA were located at 1–25 and 666–687, respectively. A: RT-PCR amplification of ephrin-A5 cDNAs. Lane 1, cardiomyocytes; lane 2, heart; lane 3, brain; M, DNA marker. B: cDNAs were cloned and sequenced, and the nucleotide and amino acid sequences of the full-length and deleted forms of ephrin-A5 are shown. The 81-bp missing exon region of ephrin-A5β is underlined.

Fig. 2. Cloning of 2 forms of ephrin-A5 from the rat heart and cardiomyocyte. The degenerative primers used for the isolation of the full-length ephrin-A5 cDNA were located at 48 to 27 and 725 to 747 of the cloned full-length sequence, respectively. The specific primers used for cloning of the shorter ephrin-A5 cDNA were located at 1–25 and 666–687, respectively. A: RT-PCR amplification of ephrin-A5 cDNAs. Lane 1, cardiomyocytes; lane 2, heart; lane 3, brain; M, DNA marker. B: cDNAs were cloned and sequenced, and the nucleotide and amino acid sequences of the full-length and deleted forms of ephrin-A5 are shown. The 81-bp missing exon region of ephrin-A5β is underlined.

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Fig. 1. Expression of ephrin-A5 in cardiomyocytes. A: Northern blot analysis showing multiple ephrin-A5 transcripts that were upregulated by tumor necrosis factor (TNF-α). B: Western blot result of ephrin-A5 proteins. Results are representative of 3 independent experiments. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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XbaI and BamHI, and the cDNAs were inserted into a pAdlox shuttle vector (13). The recombinant adenoviral vector originates from replication-deficient type 5 adenovirus (lacking E1 and E3 loci) and was constructed through Cre-lox recombination (13, 34). The ephrin cDNAs and LacZ gene were inserted in place of the E1 region, and expression is driven by the cytomegalovirus promoter. High-titer suspensions of recombinant adenoviruses were prepared by established methods (13). Briefly, a confluent 10-cm dish with $1.6 \times 10^7$ Cre8 cells was split into five 6-cm dishes and incubated at 37°C for 4 h. Transfection of these cells with pAdlox recombinant plasmids was performed by the calcium phosphate precipitation method with $3 \mu g$ of the construct digested with SfiI and $3 \mu g$ of helper virus DNA. The media was changed 16 h posttransfection. The transfected Cre8 cells were carefully fed daily until large scales of plaques appeared. The cells were then suspended in their media and placed into a sterile tube. The viral lysate was obtained by freezing/thawing three times. The recombinant virus was purified and amplified by infecting two 10-cm dishes of Cre8 cells using 0.1 ml of the lysate. One dish was used for a virus stock, and the other dish was used to make DNA to confirm the identity of the virus. The viruses were propagated in Cre8 cells, purified by cesium chloride density centrifugation, and stored in aliquots at $-80°C$ as previously described (34). The viral titer was equal to optical density at 260 nm wavelength (OD 260) times dilution and divided by $9.09 \times 10^{13}$ particles/ml. A hundred particles were assumed to be one plaque-forming unit.

Adenoviral vector-mediated ephrin-A5α and ephrin-A5β gene transfer. After being plated, the cells were incubated for 24 h, the media was then changed, and cardiac myocytes were transduced with Ad.LacZ, Ad.ephrin-A5α, or Ad.ephrin-A5β vectors ($2 \times 10^9$ particles in 2 ml media for $1 \times 10^6$ cells), respectively, and further incubated for 8–40 h before analysis.

In situ staining of ephrin-A5α and ephrin-A5β after gene transfer. After transduction with Ad.ephrin-A5α and Ad.ephrin-A5β for 24 h, cardiac myocytes were stained with the EphA5 receptor fused with alkaline phosphatase (EphA5-AP) as described (5). Cells were incubated with media containing EphA5-AP for 2 h at room temperature and rinsed with Hank’s balanced salt solution containing 0.5 mg/ml bovine serum albumin and 20 mM HEPES. The cells were fixed with 60% acetone and 3% formaldehyde in 20 mM HEPES (pH 7.5), and heated at 65°C for 15 min. Positive staining was visualized with 0.17 mg/ml sodium 5-bromo-4-chloro-3-indolyl phosphate, 0.33 mg/ml nitro blue tetrazolium, and 2.24 mg/ml L-homoarginine in color development buffer [100 mM Tris-HCl, 100 mM NaCl, and 5 mM MgCl₂ (pH 9.5)] at 37°C.

cDNA array analysis of ephrin-A5α- and ephrin-A5β-responsive genes in cardiomyocytes. Total RNA was isolated from neonatal rat cardiomyocytes transduced with Ad.LacZ, Ad.ephrin-A5α, or Ad.ephrin-A5β, respectively, for 40 h and treated with RQ1 RNase-free DNase I (Promega). Poly A⁺

**Fig. 3.** Gene transfer and expression of ephrin-A5α and ephrin-A5β in cardiomyocytes. EphA5-AP staining shows the expression of both ephrin-A5α and ephrin-A5β in cardiomyocytes. A–C: cardiomyocytes transduced with adenoviral LacZ (A), ephrin-A5α (B), or ephrin-A5β (C). D: downregulation of the EphA3 receptor by both ephrin-A5α and ephrin-A5β. Extracellular signal-regulated kinase (ERK)1 was not changed and was used as control of protein loading. E: quantitative results of EphA3 protein levels; $n = 4$ each. *$P < 0.05$ compared with Ad.LacZ; †$P < 0.05$ compared with Ad.ephrin-A5α.
mRNA was then isolated as described above. Total cDNA probes were labeled directly by reverse transcription of the poly A+ mRNA with specific oligo primers for the 588 genes in the cDNA expression array (Clontech; Palo Alto, CA) in the presence of [α-32P]dATP (3,000 Ci/mmol, NEN Life Science; Boston, MA). The probes were purified with a Sephadex-G50 spin column. Prehybridization was done in the ExpressHyb solution (Clontech) for 1 h. The solution was then replaced with fresh ExpressHyb solution with the addition of radiolabeled cDNA probes. After overnight hybridization at 68°C, the membranes were washed four times with 2× saline-sodium citrate (SSC) and 1% SDS at 68°C and 0.1× SSC and 0.5% SDS and then exposed in a PhosphorImager cassette overnight, and the results were scanned with a PhosphorImager (Molecular Dynamics; Sunnyvale, CA). After each hybridization, the filters were stripped and rehybridized with different probes. Hybridization results of probes from Ad.LacZ-, Ad.ephrin-A5a-, and Ad.ephrin-A5β-transduced cells were compared.

**Western blot analysis.** The total protein of cultured neonatal cardiomyocytes was collected through lysis of the cells into RIPA buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail, Sigma; St. Louis, MO) after 8–40 h of transduction with the adenoviral vectors. Protein concentration was determined using Bradford protein assay with bovine IgG as a standard (Bio-Rad Laboratories; Hercules, CA). Equal amounts of total protein were separated in a standard SDS-polyacrylamide gel and electroblotted onto nitrocellulose membranes (Micron Separations; Westborough, MA). The membrane was blocked with 3% fat-free dry milk in PBS and 0.05% Tween 20 for 1 h.

EphA3, ephrin-A5, cyclin D2, cyclin-dependent kinase (CDK)4, and extracellular signal-regulated kinase (ERK)1 proteins were detected with specific antibodies (all from Santa Cruz Biotechnologies; Santa Cruz, CA) at a concentration of 0.3–0.5 μg/ml for 1 h at room temperature. The membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibodies. The reactions were developed with enhanced chemiluminescence reagents (Pierce; Rockford, IL), and the images were obtained by exposure to X-ray films.

**Quantification of neonatal cardiomyocyte DNA synthesis.** For the quantification of bromodeoxyuridine (BrdU) incorporation, the cardiomyocytes were incubated for 24 h, transduced with Ad.ephrin-A5α or Ad.ephrin-A5β for 12 h, and then pulsed with 20 μM BrdU for 24 h. The incorporated BrdU was stained using a FITC-conjugated monoclonal anti-BrdU antibody at a dilution of 1:20 (33284X, PharMingen; San Diego, CA). Myosin heavy chain staining with MF20 at a dilution of 1:4 (developed by D. A. Fischman, obtained from the Developmental Studies Hybridoma Bank maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA) was used as a marker of cardiomyocytes. The cells were cultured in the absence of a high concentration of BrdU (100 μM, used for inhibition of fibroblast growth in other routine studies). Because of the absence of high concentrations of BrdU in the initial 24 h of culture, fibroblasts presented at significantly higher proportions in these cell preparations for the DNA synthesis studies. However, because cardiomyocytes were counted separately under the microscope and were clearly identified, the higher proportion of fibroblasts in the DNA synthesis studies was acceptable.

**Fig. 4.** Gene expression in cardiomyocytes over-expressing ephrin-A5s. cDNA expression array analysis shows the regulation of cyclin D2 mRNA by ephrin-A5α and ephrin-A5β. Hybridization results of cDNA probes radiolabeled by reverse transcription of mRNA were isolated from Ad.LacZ-transduced cardiomyocytes (A), Ad.ephrin-A5β-transduced cardiomyocytes (B), and Ad.ephrin-A5α-transduced cardiomyocytes (C). Each paired dot on the blot represents a specific gene. The intensity of the hybridization signal corresponds to the abundance of transcript levels of the individual gene. D and E: equal amounts of protein extracts were analyzed using Western blots (top) for the expression of cyclin D2 (D) and cyclin-dependent kinase (CDK)4 protein expression (E). Shown are the results after 40 h of transduction. Bottom: summary of the quantitative results of cyclin D2 and CDK4 protein expression. n = 4 each. *P < 0.05 compared with Ad.LacZ.
synthesis studies should not interfere with the result interpretation for cardiomyocytes.

Flow cytometry. After infection of the cultured cardiomyocytes with Ad.LacZ, Ad.ephrin-A5α, or Ad.ephrin-A5β for 24–40 h, the cells were suspended using trypsin and stained with propidium iodide (50 μg/ml). Flow cytometry was then performed by a specialist at the flow cytometry facility of the University of Pittsburgh using a FACSscan flow cytometer (Becton Dickinson; San Jose, CA). The percentages of cells at sub-G0/G1, G0/G1, S, and G2/M phases were recorded and compared.

Statistical analysis. One-way analysis of variance was applied to compare EphA3, cyclin D2, and CDK4 expression levels and cell counts in different experimental groups. Comparison among the means was performed with the post hoc Student-Newman-Keuls test using SPSS statistical analysis software (11). Results are presented as means ± SE. Statistical significance was considered at P < 0.05.

RESULTS

Expression of ephrin-A5 transcripts by cardiac myocytes. At the time of these experiments, there was no known sequence of rodent ephrin-A5. The full-length ephrin-A5 was amplified using a pair of degenerate primers selected from human AL-1, sequenced, and used as a probe for Northern blot analysis. Northern blot analysis revealed multiple transcripts of ephrin-A5 at various levels of expression in rat cardiomyocytes. Up to seven ephrin-A5 transcripts ranging in size from 1.7 to 7.4 kb in the heart and cardiomyocytes were found. Consistent with a previous report (17), the expression of these transcripts was regulated by TNF-α (Fig. 1). The significance of multiple ephrin-A5 transcripts is not clear; therefore, we assessed whether these transcripts might represent different gene products. A new pair of primers were then selected from the full-length sequence and used to amplify shorter forms of ephrin-A5. RT-PCR of cardiomyocyte and brain cDNA with rTth DNA polymerase identified two ephrin-A5 cDNA isoforms with different lengths. The nucleotide and deduced amino acid sequences of both forms of ephrin-A5 are shown in Fig. 2. The full-length ephrin-A5 sequence was deposited into the GenBank in 1997 (Accession No. RNU69279). The full-length ephrin-A5α and ephrin-A5β reduced bromodeoxyuridine (BrdU) incorporation into cardiomyocytes. Nuclei were stained blue with 4′,6-diamidino-2-phenylindole (DAPI), nuclei with DNA synthesis and the incorporation of BrdU were stained green with anti-BrdU antibody, and cardiomyocytes were stained red with MF-20 anti-myosin heavy chain antibody. A–C: Ad.LacZ-transduced cells; D–F: Ad.ephrin-A5β-transduced cells; G–I: Ad.ephrin-A5α-transduced cells. C, F, and I: overlay of DAPI, BrdU, and MF-20 staining. Arrow, cardiomyocytes with active DNA synthesis. J: there was a reduced number of BrdU-positive cardiomyocytes after Ad.ephrin-A5β or Ad.ephrin-A5α transduction. *P < 0.05, compared with Ad.LacZ.
Ephrin-A5 cDNA (designated as ephrin-A5α) coding sequence encompassed 687 bp in length, whereas the shorter cDNA (designated as ephrin-A5β) had a 81-bp deletion in the region coding for the spacer domain of ephrin-A5. Ephrin-A5β was the only one found using this strategy. Two cDNA isoforms with the same sequences were reported recently (21). Therefore, of the multiple transcripts, at least one splices in the coding region and generates a form of ephrin-A5 with a deletion (ephrin-A5β). Other transcripts of different sizes may be due to differential polyadenylation of the mRNA, which is very common among eukaryotic genes (12, 29).

Overexpression of ephrin-A5α and ephrin-A5β induced downregulation of EphA3. To assess the physiological role of ephrin-A5α and the function of ephrin-A5β, cardiomyocytes were transduced with recombinant adenoviruses carrying each of the two forms of ephrin-A5 cDNAs. Overexpression of ephrin-A5α and ephrin-A5β was highly efficient. Both forms of ephrin-A5 expressed in cardiomyocytes specifically bound the EphA5 receptor, as demonstrated by staining with EphA5-AP. More than 95% of the cells were positively stained (Fig. 3), whereas none of those transduced with Ad.LacZ was stained. Consistent with ligand-receptor interactions in other ligand-receptor systems, extended (40 h) high-level expression of ephrin-A5 by Ad.ephrin-A5 in cardiomyocytes induced downregulation of the EphA3 receptor (P < 0.05 compared with Ad.LacZ; Fig. 3). In addition, Ad.ephrin-A5β overexpression in cardiomyocytes appeared to be more effective in downregulating EphA3 than Ad.ephrin-A5α despite the fact that it harbored a deletion (P < 0.05). Because the expression of ERK1 protein was not changed by ephrin-A5 overexpression, it was used as a control for protein loading in Western blot analysis of EphA3.

Both ephrin-A5α and ephrin-A5β downregulated the expression of cyclin D2 and CDK4 and reduced neonatal cardiomyocyte DNA synthesis and growth. To further assess the molecular effects of ephrin ligand overexpression on myocyte, we performed cDNA expression array analysis of cardiomyocytes transduced with either ephrin-A5α, ephrin-A5β, or the control vector Ad.LacZ. The expression of cyclin D2 was reduced in ephrin-A5α- and ephrin-A5β-transduced cells (Fig. 4). To further explore whether the changes in the mRNA expression of cyclin D2 were associated with changes at the protein levels and changes in the levels of functionally associated CDK4, equal amounts of extracts from cardiomyocytes transduced with Ad.ephrin-A5α or Ad.ephrin-A5β were examined using Western blot analysis. The results showed that overexpression of either ephrin-A5α or ephrin-A5β reduced the expression of cyclin D2 and CDK4 proteins at 24 h postinfection. The representative results of cyclin-D2 and CDK4 protein expression at 40 h after transduction are shown in Fig. 4.

Because cyclin D2 and CDK4 play an active role in DNA synthesis and cell proliferation, we examined whether ephrin-A5α- and ephrin-A5β-induced cyclin D2 and CDK4 downregulation would have an effect on cardiomyocyte DNA synthesis and growth. Indeed, the...
number of neonatal cardiomyocytes with BrdU incorporation was significantly reduced after overexpression of ephrin-A5α or ephrin-A5β (P < 0.05), suggesting decreased DNA synthesis and growth (Fig. 5). In addition, FACScan analysis of the cardiomyocytes transduced with either ephrin-A5α or ephrin-A5β showed an increase in cell numbers in the sub-G0/G1 phase and a significant decrease in the number of cells in the G2/M phase (P < 0.05; Fig. 6).

Differential regulation of cardiomyocyte gene expression by ephrin-A5α and ephrin-A5β. To examine whether the two forms of ephrin-A5 have different effects on cardiomyocytes, the expression of 588 genes was examined using a cDNA expression array after transduction of the cells with Ad.LacZ, Ad.ephrin-A5α, or Ad.ephrin-A5β. Genes with their expression altered more than twofold by Ad.ephrin-A5α or Ad.ephrin-A5β relative to Ad.LacZ control are listed in Table 1. Whereas both Ad.ephrin-A5α and Ad.ephrin-A5β downregulated the expression of cyclin D2 mRNA and upregulated 27-kDa heat shock protein and macrophage migration inhibitory factor, the expression of other genes in Ad.ephrin-A5β-transduced cardiomyocytes differed from that in Ad.ephrin-A5α-transduced cells. Among the genes that showed significant alterations in their expression, platelet-derived growth factor (PDGF)-associated protein, carboxypeptidase E, Ral A GTP-binding protein, ras-related protein RAB2, eukaryotic translation initiation factor 2 (subunit 1), and insulin-like growth factor-binding protein may be involved in the gene regulation and growth modulating effects of ephrin-A5 (Table 1). Shown in Fig. 7 are the representative results of the cDNA expression array analysis and Northern blot analysis of BNP expression.

**DISCUSSION**

The prototypic member of the family of ligands for Eph receptors, ephrin-A1 (B61), was first identified as a protein whose expression was upregulated in response to proinflammatory cytokines and may function as a stimulator of angiogenesis (2, 16, 25, 27, 28). The role of ephrin ligands in cardiovascular development is seen mainly in vascular development (1, 23, 32). In the present study, we demonstrated that two alternatively spliced forms of ephrin-A5 (full-length ephrin-A5α and the 27-amino acid-deleted form ephrin-A5β) were expressed in the rat heart. This finding is consistent with earlier studies (8, 21) in mouse and rat nervous tissues that demonstrated alternatively spliced transcripts of ephrin-A5; however, it was not clear about their specific function and whether they are expressed in the heart.

To elucidate the role of the different splice forms of ephrin-A5 in cardiomyocytes, we overexpressed the two forms of ephrin-A5 in neonatal cardiomyocytes. The overexpression of both forms of ephrin-A5 resulted in downregulation of cyclin D2 and CDK4, which was associated with reduced DNA synthesis in neonatal cardiomyocytes. Thus ephrin-A ligands may have a unique function to withdraw those cells from the cell cycle by regulating the expression of cyclin D2 and CDK4, important regulators of cell cycling. These findings in cardiac cells are consistent with an earlier study (3) demonstrating that activation of EphA5 receptor using an ephrin-A1 recombinant fusion protein results in a marginal decrease in U-118 MG glioblastoma cell proliferation. Similarly, spinal cord cells cocultured with ephrin-A5-expressing cells reduced both neurite length and neuron cell density. Although the mechanisms responsible for neuronal cell loss are not fully defined, the effect may be apoptosis, attributable to ephrin-A5 expressed on the cell surface (35). Flow cytometry study of ephrin-A5α- or ephrin-A5β-transduced cardiomyocytes showed increased sub-G0/G1 cell populations, which suggest increased apoptosis in those cells (Fig. 6). Taken together, these results suggest a role of ephrin ligands in cardiac cell growth and survival. However, mice with ephrin-A5 gene knockout demonstrated defects in axon guidance in the midbrain and dorsal midline structure, with anencephaly or

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open crania with cleft nose and palate, but no obvious cardiac defects (9, 26). The inability of ephrin-A5 knockout to induce cardiac effects might suggest a large degree of redundancy in the ephrin-mediated pathways, although cardiomyocyte changes have not been assiduously evaluated in that model.

In the nervous system, ephrin-A5 is required for the proper guidance and mapping of retinal axons in the mammalian midbrain by a two-step mechanism of Eph receptor activation with distinct ligand binding and ligand-independent receptor-receptor oligomerization events (9, 20). Those various functions of ephrin-A5 in different systems may be achieved by different levels of its expression and differential regulation of genes (17).

Indeed, whereas ephrin-A5α and ephrin-A5β share common effects such as downregulation of cyclin D2 and upregulation of 27-kDa heat shock protein and macrophage migration inhibitory factor, ephrin-A5β markedly regulated other growth-regulating genes such as eukaryotic translation initiation factor 2 (subunit 1) and insulin-like growth factor-binding protein. Ephrin-A5 may also be involved in the regulation of gene expression through differential regulation of genes such as PDGF-associated protein, carboxypeptidase E, Ral A GTP-binding protein, and ras-related protein RAB2.

In conclusion, multiple ephrin-A5 transcripts were found to be highly responsive to TNF-α in neonatal rat cardiomyocytes. Two isoforms of ephrin-A5, ephrin-A5α and ephrin-A5β, were identified in rat cardiomyocytes. Overexpression of these two forms of ephrin-A5 reduces BrdU incorporation in cardiomyocytes. In addition, ephrin-A5α and ephrin-A5β regulated different sets of gene expression. These differential effects of ephrin-A5α and ephrin-A5β on cardiomyocyte gene expression suggest that these ligands may have additional different functions in cardiomyocytes.
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REFERENCES


