Neuregulin-1, in a Conducive Milieu with Wnt/BMP/Retinoic Acid, Prolongs the Epicardial-Mediated Cardiac Regeneration Capacity of Neonatal Heart Explants

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Abstract

Rationale: Cardiac sympathetic nerves are required for endogenous repair of the mammalian neonatal heart *in vivo*, but the underlying mechanism is unclear.

Objective: We tested the hypothesis that a combination of cardiac developmental growth factors Wnt3a, BMP4 and Neuregulin (NRG-1), compensate for denervation and support cardiac regeneration in explanted neonatal mammalian hearts.

Methods and Results: Hearts from 2-day old neonatal mice were harvested, lesioned at the apex and grown *ex vivo* for 21 days under defined conditions. Hearts grown in canonical cardiomyocyte culture media underwent complete coagulative necrosis, a process resembling ischemic cell death, by day 14. However, the addition of Wnt3a, BMP-4 and NRG-1, maintained cellular integrity and restored the endogenous regenerative program. None of these factors alone, or in any paired combination, were sufficient to induce regeneration in culture. rNRG-1 alone significantly reduced the accumulation of double strand DNA damage at Day 3; (–NRG-1: 60±12%; +NRG-1: 8±3%; P<0.01) and prevented coagulative necrosis at Day 14. Short-term addition of rWnt3a and rBMP-4 (day 0-3, NRG-1+) increased WT1 expression (a marker of epicardial cells) 7-fold, epicardial proliferation (78±17 cells vs. 21±9 cells; P<0.05), migration and recellularization (80±22 vs. zero cells; P<0.01; n=6) at the injury site on day 14.

Conclusions: A novel explant culture system maintains three-dimensional neonatal mouse hearts and the mammalian neonatal cardiac regenerative program *ex vivo*. We identified that rNRG-1, plus short-term activation of Wnt- and BMP-signaling, promotes cardiac repair *via* epicardial cell activation, their proliferation and migration to the injury site, followed by putative cardiomyocyte recruitment. This novel technique will facilitate future studies of mammalian cardiac regeneration and may be useful in cardiac-specific drug testing.

Keywords: Cardiac regeneration; Tissue regeneration; Epicardial stem cells; Drug model; Heart repair; Neonatal mouse; Cardiomyocytes; Nerves; Regenerative medicine

Introduction

Cardiovascular disease accounts for more deaths than any other disease^[1], in large part due to an inability of the adult mammalian heart to sufficiently regenerate lost tissue following injury. Lower vertebrates, e.g., teleost fish species and urodele amphibians, such as the newt, retain a capacity to regenerate lost cardiac tissue through a process that involves activation of the epicardium and cardiomyocyte proliferation^[2-5]. Until recently, it was thought that the mammalian heart was incapable of such a response to acute injury. However, recent discoveries challenge this view; neonatal mouse and pig hearts undergo complete repair following resection^[6] and infarction^[7, 8] of the left ventricle, respectively. Furthermore, 0.45-1% of human cardiomyocytes are replaced per year throughout life^[9]. These findings have propelled a major drive toward determining the mechanisms of endogenous mammalian cardiac regeneration.

It is clear that the absence of cardiac autonomic nerves compromises repair of the neonatal mouse heart^[10-12]. Ablation of either the sympathetic^[12] or parasympathetic^[11] cardiac nerves abrogates the innate regenerative potential of the neonatal heart, suggesting that non-redundant contributions from both branches of autonomic nerves are required to support full cardiac regeneration. Neuronal input may also be required for cardiac homeostasis, as heart failure following cardiac transplantation,

where the transplanted heart is denervated, remains a common outcome^[13].

Following experimental parasympathectomy, local administration of Neuregulin (NRG-1), a cardiomyocyte growth factor, rescued regeneration following apical injury to murine neonatal hearts through a mechanism of cardiomyocyte proliferation^[14]. In these *in vivo* experiments, sympathetic nerves are presumably intact, making it difficult to tease apart the relationship and relative contributions of each branch of the autonomic nervous system.

In the heart, NRG-1 is produced primarily by cardiac endothelial cells in response to β -adrenergic signals from sympathetic cardiac nerves. The tyrosine kinase receptors for NRG-1 (ErbB2 and ErbB4) are located on the surface of neighboring cardiomyocytes^[14], where signals are transduced into the cell following receptor heterodimerization. During the first seven postnatal days, ErbB2 expression is progressively downregulated on the membrane surface of cardiomyocytes^[15], coinciding temporally with a loss in regenerative potential^[6].

To address the role of NRG-1 in the denervated heart, we developed a novel explant model of mammalian cardiac regeneration. With this technique, whole neonatal hearts can be maintained in culture under defined conditions. Explanted neonatal hearts are totally

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denervated and exhibit no regenerative activity. Here, we report that continuous administration of NRG-1 and RA plus brief exposure to Wnt3a and BMP-4 was sufficient to induce epicardial activation and homing of epicardial cells to the site of experimental injury, activated embryonic transcription factors, Nkx2-5 and GATA416 and subsequent recellularization of the ventricular myocardium in explanted, injured neonatal hearts. Together, these data suggest that the regenerative contribution from cardiac nerves can be compensated for, and the neonatal regenerative window can be extended, with appropriate and timely exposure to Wnt/ β -catenin and BMP/RA signaling together with NRG-1 in mammalian cardiac explants.

Methods

Culture Media

Maintenance media (Base+P+N) consisted of DMEM/F12 (Invitrogen, cat. no. 15090), 2% probumin (Millipore, cat. no. 82-100-5), 50 U/mL Penicillin-streptomycin (Gibco, #15140-148), 2 mM l-Alanyl-l-glutamine (Cellgro, cat. no. 25-015-CI), 1% MEM non-essential amino acids (Cellgro, cat. no. 25-025-CI), 0.1 mM 2-Mercaptoethanol (Invitrogen, 21985-CI), 50 μ g/mL (+)-Sodium l-ascorbate (Sigma, #A4034), 10 μ g/mL bovine transferrin (Invitrogen, #11107-018), 8 ng/mL rhFGF2 (Invitrogen, #PHG0023), 10 ng/mL rhActivin-A (R&D, #338-AC), 200 ng/mL LONGR3 IGF-I human (Sigma, #85580C) and 10 ng/mL rhNRG-1 (R&D, #396-HB). Activation media (AM) consisted of maintenance media (Base+P+N) with the addition of 25 μ g/L rmWnt3a (R&D #1324-WNP), 100 μ g/L rmBMP4 (R&D #5020-BP) and 4 μ M retinoic acid (Sigma, #R2625). Regenerative media (Base+NWB) consists of maintenance media (Base+P+N) with the addition of 4 μ M retinoic acid.

Ex vivo Maintenance of Neonatal Mouse Heart

Two-day-old mice were decapitated and partially exsanguinated by blotting with absorbent paper. Cadavers were submerged in cold PBS on a 10 cm petri dish and opened using needle nose tweezers (Roboz, Gaithersburg, MD, USA). Hearts were removed by pinching the outflow tract at the base of the heart and placed into a clean petri dish containing ice cold PBS (Cellgro, #21-031). Hearts were exsanguinated by extensive massaging and palpating with the flat portion of the tweezers. During collection, hearts were temporarily stored in clean ice-cold PBS, prior to culture. Hearts were placed into 60 mm tissue culture plates (BD, #35004) and maintained (4-5 per plate) in 4 mL maintenance media in a 37°C, 5% CO₂ humidified cell culture incubator. Every third day of culture, 80% of the media was gently aspirated and replaced with fresh, pre-warmed media.

Induction of Endogenous Cardiac Regeneration

To induce innate cardiac regeneration, hearts were mechanically injured and cultured in regeneration media for up to 21 days. Hearts were obtained and washed as described above. While submerged in ice cold PBS each heart was visualized with a stereo dissecting microscope and injured by cutting the apex (at left ventricular free wall) with the tip of a 30G hypodermic needle (BD, #305106) through the application of downward force. Hearts were immediately placed in 4 mL activation media at a density of 4-5/60 mm plate and maintained at 37°C in a 5% CO₂ humidified cell culture incubator. After 72 hours, 80% of media was carefully aspirated and replaced with an equal volume of regeneration media (Base+NWB). Hearts were incubated for 72 hours. Again 80% of media was aspirated and replaced with regeneration media. Hearts were maintained in regeneration media up to day 21, exchanging 80% of the media every 72 hours.

Histologic Analysis

Hearts were harvested at various time points for analysis by immunohistochemistry and immunofluorescence. The tissue was washed in PBS prior to fixation in 4% PFA (Thermo Scientific Rockford, IL, #28906) at room temperature for 15 minutes, prepared

for cryo-embedding by placing into 30% sucrose solution until reaching equilibrium and subsequently embedded in clear frozen section compound (VWR, #95057-838). Tissue sections were cut at a thickness of 10 μ M using a Leica CM1850 cryostat and mounted on positively charged microscope slides (Fisher, #12-550-17), air dried and stored at negative 80°C prior to histology. Gross morphology was evaluated with hematoxylin and eosin (Sigma, #HMS16, #318906), immunohistochemistry rabbit anti-mouse Wt1 (abcam, #ab96792), mouse anti- β -MyHC (DSHB, #A4.951-c) and immunofluorescence rabbit Nkx2.5 (Pierce, #PA5-21686) rabbit Isl1 (millipore, #AB4326), goat anti-mouse TBX18 (Santa Cruz, #sc-17869), rabbit anti-mouse GATA4 (Pierce, #PA1-102), anti-BrdU-FITC (Millipore, #MAB3262F), DAPI (Life Technologies, #R37606). Hearts were incubated with EdU (details of dosage and length of exposure are specified in Figure legends) and harvested as described. After being allowed to dry at room temperature, sections were stained using a Click-iT EdU Alexa Fluor 594 Imaging Kit (Life Technologies, 1753456) and counterstained with DAPI (Life Technologies, R37606). Positive cells were imaged using an Olympus IX81 fluorescent microscope and quantified using NIH Image J software.

RNA Preparation and Quantitative Real-Time PCR

Total RNA was extracted from cells using the TRIzol method (Thermo Fisher Scientific, #15596026), and then reverse transcribed to complementary DNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, USA) according to the manufacturer's protocol. Quantitative RT-PCR for indicated genes was performed using TaqMan™ probes ERBB2, WT1, NKX2.5, GATA4, TBX5, and c-KIT and TaqMan™ Universal PCR Master Mix (Applied Biosystems, USA) and TaqMan™ Gene Expression Assays according to manufacturer's protocol. Samples were analyzed using the BIORAD sequence detection system (Bio-Rad, Hercules, CA, USA). All PCR reactions were performed in triplicate, and the specificity of the reaction was determined by melting curve analysis at the dissociation stage. The relative quantitative method was used for quantitative analysis. The calibrator was the averaged Δ Ct from untreated tissue. The endogenous control was glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Western Blots

Cells were harvested and lysed in RIPA buffer and Protease Inhibitor Cocktail (Sigma, St. Louis, MO). Protein extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to polyvinylidene fluoride membranes (Bio-Rad). Membranes were incubated with phospho-GSK3B, GSK3B (Santa Cruz Biotechnology Inc., Santa Cruz, CA), ATK, phospho-AKT (Abcam, Cambridge, UK), phospho-p38 (Cell Signaling Technology, Danvers, MA, USA), antibodies in Tris-buffered saline, Tween 20 buffer with 5% Bovine Serum Albumin in Tris-buffered saline with 0.1% Tween-20, followed by incubation with horseradish-peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc.). Immunoreactive bands were visualized using the Chemiluminescent Substrate Kit (Thermo Fisher Scientific).

Results

Long-term, Whole Heart Explant

Canonical cardiomyocyte culture media used in the maintenance of cardiomyocyte monolayers contains several growth factors together with fetal bovine serum (Base+FBS). However, under these conditions, explanted neonatal hearts rapidly accumulated double strand DNA damage and underwent coagulative necrosis and cell death due to insufficient access to nutrients and growth factors, as early as 3 days after the initiation of culture (Figure 1A). Replacing FBS with probumin (2% albumin), as a source of protein, plus the addition of 50 ng/mL rNRG-1 (Base+P+N) was sufficient to maintain DNA integrity, and cardiomyocyte function and structure

Figure 1. A novel ex vivo culture method containing rhNRG-1 can maintain structural, cellular and signalling properties in neonatal mouse heart explants. Explanted neonatal hearts rapidly accumulate double stranded DNA damage and undergo coagulative necrosis soon after plating in canonical cardiomyocyte culture media (Base + FBS) (A). Replacing FBS with probumin (2% albumin), together with 10 ng/mL rNRG-1 (Base+P+N) maintains DNA integrity, cardiomyocyte function and structure for greater than 21 days in culture (A). Phosphorylation of AKT and the serine/threonine protein kinase GSK-3 was increased by NRG-1. In contrast, phosphorylation of p38 MAP kinase signalling, was not altered by the addition of rNRG-1 at protein level (B) quantified in (C). H2AX immunofluorescence showed a significant reduction in DNA damage in rNRG-1 treated explants compared to controls (D, E). Immunostaining (F, G) and qRT-PCR (H) showed that, in the presence of rNRG-1, the expression of ErbB2 on cardiomyocytes was maintained at high levels during long-term culture (12-14 days).

for >21 days in culture (Figure 1A). Histologic evaluation of cardiomyocytes showed intact nuclei and sarcomeres, indicative of healthy, viable cells. Indeed, >75% of hearts cultured in the presence of rNRG-1 retained a (non-coordinated) beating phenotype for up to 14-days following harvest (Supplemental video 1).

We next evaluated the mechanisms contributing to tissue survival in the cardiac cultures. rNRG-1 promoted anti-apoptotic and anti-necrotic signaling at early time-points. Figure 1B and 1C show that by day 3, enhanced PI3K/AKT signaling was evident, which was sensitive to the PI3K inhibitor, wortmannin. Furthermore, phosphorylation (deactivation) of the serine/threonine protein kinase GSK-3 was increased by rNRG-1, an effect that was not blocked by wortmannin, as expected. In contrast, phosphorylation of p38 MAP kinase signaling, which can inhibit cardiomyocyte proliferation, was not affected by the addition of rNRG-1 (Figure 1B and 1C). We observed a significant reduction in double strand DNA damage in rNRG-1 treated explants compared to controls ($-NRG1$ 60 ± 12 cells per panel vs. $+NRG1$ 8 ± 3 , $p < 0.01$) based on H2AX immunofluorescence (Figure 1D and 1E). Interestingly, the expression of ErbB2, the tyrosine kinase receptor for NRG-1, declines *in vivo* within the first 7 postnatal days^[15] and this decay coincides with a loss of innate regenerative ability^[6]. In the salamander (axolotl), NRG-1 protein is abundant in the regenerative blastema, but both NRG-1 and ErbB2 expression is reduced upon denervation, suggesting that peripheral nerves support a positive-

feedback loop that sustains NRG-1 and ErbB2 expression^[17, 18]. We tested if exposure of explant neonatal hearts to rNRG-1 would similarly maintain expression of ErbB2 on cardiomyocytes. We observed that in the presence of rNRG-1, ErbB2 was maintained at high levels during long-term (14 days) *in vitro* culture compared to *in vivo* counterparts (Figure 1F-H). This result suggests that supplementing explant media with rNRG-1 is necessary and sufficient to maintain robust ErbB2 expression in the absence of cardiac sympathetic nerves, a situation that would facilitate cardiomyocyte responsiveness to the pro-survival and anti-apoptotic effects of NRG-1^[19, 20].

Activation of Ex Vivo Injury Response

Recent reports in zebrafish and mice suggest that NRG-1 treatment *in vivo* acts as a potent mitogen, triggering cardiomyocyte proliferation and cardiac repair^[11, 21]. In neonatal heart explant cultures treated with rNRG-1, we did not observe analogous cardiomyocyte proliferation, despite robust activation of pro-survival and anti-apoptotic signaling (Figure 1B-E). In the presence of rNRG-1 hearts remain viable for >21 days; however, these injuries fail to initiate robust mitosis within the myocardium. Indeed, cellular proliferation, as determined by 5-ethynyl-2'-deoxyuridine (EdU)^[22] incorporation, is minimal at day 3 post-injury (21 ± 11 EdU+ cells; $n=4$ hearts, 3 tissue sections from each) and is exclusively limited to the epicardial layer surrounding the heart (Figure 2A).

Figure 2. Epicardial cell activation and proliferation can be initiated by the addition of Wnt/BMP signalling in whole, cultured murine heart explants. 5-ethynyl-2'-deoxyuridine (EdU) staining shows limited epicardial cell proliferation when explanted hearts are cultured in rNRG-1 alone (Base+P+N) for 3 days (A). In the presence of Wnt3a, BMP-4 and RA (Base+NWB), explants showed robust epicardial activation and proliferation at day 3 as indicated by EdU staining (B,C) and immunohistochemistry against Wt1 (D) compared to a 2-day old freshly isolated neonatal heart (E). qRT-PCR identified epicardial Wt1 expression peaked at day 10 in culture (Base+NWB) before gradually declining towards baseline (F,G).

Ex Vivo Epicardial Mobilization

The epicardium is substantively involved in zebrafish cardiac regeneration and supports cardiomyocyte proliferation early in development. Therefore, we sought to assess if enhanced epicardial activation in murine neonatal cardiac cultures promotes myocyte proliferation^[23, 24]. Epicardial cell fate determination, differentiation and migration from the proepicardial organ during embryogenesis is coordinated through the regulated expression of growth factors that are under tight temporal control^[25, 26]. Wnt/ β -catenin signaling plays an important role during vertebrate heart development, and is re-activated in response to cardiac injury^[27]. Following acute ischemic injury, the epicardium is activated, organ-wide, in a Wnt-dependent manner. Cells subsequently proliferate and undergo epithelial-mesenchymal transition^[28, 29]. Both epicardial maturation and cardiomyocyte specification require BMP-4^[30-32], which together with the morphogenetic factor Retinoic acid (RA), contributes to normal cardiac development during embryogenesis. Together Wnt3a, BMP-4 and RA constitute a potent group of growth factors that orchestrate epicardial and myocardial development and response to injury^[33]. We therefore, supplemented our MM with Wnt3a (25 ng/mL), BMP-4 (100 ng/mL) and RA (4 μ M) (NWB). By day 3 this treatment produced robust epicardial activation and proliferation (–NWB: 21 ± 9 cells vs. +NWB: 78 ± 17 cells; $P < 0.05$), Figure 2A-E). Epicardial *Wt1* expression peaked at day 10 in culture before gradually declining (Figure 2F and 2G).

Recellularization of Myocardial Injury

While removing the heart from the neonatal mouse might constitute “injury” we reasoned that a direct physical injury to the ventricular myocardium might be necessary to assess cardiac repair and myocardial proliferation in response to rNRG-1 in explants. Therefore, we mechanically injured the free ventricular wall by introducing a small cut into the myocardium. The cut opens to form a wedge-shaped injury (Figure 3A and 3B). When the ventricular free wall of explants is mechanically injured and the epicardium activated, we observed a robust recellularization of the injury site. Within the first three days the injury is filled with extracellular matrix (C) and an influx of proliferative migratory epicardial cells (Figure 3C-E). By day 21 the injury site has become completely recellularized (Figure 3F and 3G), with little or no evidence of fibrosis (Figure 3H), resembling the *in vivo* regenerative process in neonates^[6].

Although cardiac injury proceeds in the presence of cardiomyocyte proliferation *in vivo*^[6, 11, 21, 23, 34] we did not observe cardiomyocyte mitosis within the myocardium in this *ex vivo* system. Rather, our data suggest that proliferation is restricted exclusively to the epicardium and cells located within the injury site itself (Figure 3I). However, this observation does not preclude the possibility that cardiomyocytes migrate to the injury in response to epicardial tropic signaling^[35].

To confirm the epicardial origin of cells responsible for early recellularization of the injury, we utilized a transgenic reporter system to exclusively label *Wt1*⁺ epicardial cells. We crossed B6.Cg-*Gt(ROSA)26Sor^{tm14(CAG-eYFP)}/Hze/J* with *Wt1^{tm2(Cre/ERT2)Wtp/J}* (*Wt1^{CreERT2/+}; Rosa26^{mTmG/+}*) mouse strains. These mice harbor a targeted mutation of the *Gt(ROSA)26Sor* locus with a *loxP*-flanked “stop” cassette preventing transcription of a CAG promoter-driven red fluorescent protein variant (tdTomato), which is expressed following *Wt1*Cre-mediated recombination^[36] (Figure 3J). Breeding these transgenic mouse lines together, generated progeny where all tissues are tdTomato positive unless *WT1* is expressed, at which point recombination results in permanent GFP expression. Uninjured, freshly isolated neonatal hearts are dtTomato⁺ except for the single layer of *WT1*⁺ epicardial cells surrounding the entire ventricular myocardium (Figure 3K). When cultured in regeneration media (Base + NRG-1, Wnt3a, BMP4, RA), *WT1*⁺/GFP⁺ cells are

Figure 3. Mechanical injury to cardiac explants leads to epicardial cell proliferation and migration into the site of injury. To investigate the regenerative potential of neonatal explanted hearts a small cut was introduced into the left ventricular free wall at the apex (A,B). Within three days post injury, while in culture (Base+NWB), the injury site fills with extracellular matrix (C) and an influx of proliferative cells as indicated by immunohistochemistry (D) and EdU staining (E). Complete recellularization of the site was observed by day 21 (F,G) with limited evidence of fibrosis (arrowhead) as indicated by Masson's Trichrome immunohistochemistry (H). Immunostaining revealed that cellular proliferation is exclusive to epicardial cells surrounding the heart (EdU) and to cells localized within injury site (BrdU) (I). To confirm the epicardial origin of cells responsible recellularization of the injury we utilized a transgenic reporter system to exclusively label *Wt1*⁺ epicardial cells (J). In this model all cells are red with the exception of those of epicardial origin, which recombine the “stop” cassette and express GFP (K). Upon injury and *ex vivo* culture (Base+NWB) GFP⁺ cells can be seen populating the site of injury by day 3 (K), confirming an epicardial lineage.

found within the site by day 3 post-injury (Figure 3K), confirming their epicardial origin. These cells also re-express the embryonic transcription factor TBX18 suggestive of immature cardiomyoblast formation (Supplemental Figure 1). Despite a robust initial recellularization by WT1⁺ epicardial cells, GFP signal is progressively lost within the injury between day 12-21 (data not shown), suggesting that the primary cellular influx is replaced by an, as-of-yet unidentified, non-epicardial cellular source. Our data support the consensus within the field that epicardial cells do not differentiate directly into cardiomyocytes, but rather play an instructive role, potentially establishing a suitable cellular environment for cardiomyoblasts to repair. Indeed, our data promote a hypothesis where cardiomyocytes disassemble their sarcomeres⁶, becoming circular rather than elongated, and migrate into and populate the injury from the surrounding myocardium (Supplemental Figure 2).

Cellular Source of Injury Responsive Cells.

Following an initial influx of epicardial cells, WT1 surface expression is gradually lost as the repair matures past day 12 (Figure 2G and 3G). This loss in WT1 antigen expression coincides with a loss of WT1;GFP expression, suggesting the elimination of

epicardial-derived cells from the site of injury. At this time, immunofluorescence analysis indicates that expression of the transcription factors GATA4 and Nkx2-5 is reactivated within the proliferating population, (Figure 4A-D) and qPCR (Figure 4E and 4F), indicative of cardiomyocyte differentiation, but no increase in the late cardiomyocyte differentiation factor Tbx5 or the cardiac stem cell marker c-Kit was observed (Figure 4G and 4H). This result suggests that putative cardiac stem cells do not contribute to a *de-novo* population of cardiomyocytes within the lesion.

Our preliminary data suggest a potential clue as to the source of cardiomyocytes. The FUCCI system marks specific cell cycle stages within live cells^[37]. A cell in the G1 phase of cell cycle expresses a red nuclear reporter, which can be visualized by epifluorescence histology (Supplemental Figure 3). In our experiments, by day 21 following injury, the site is resolved and closely resembles the surrounding myocardium (Supplemental Figure 3, DAPI). However, by using FUCCI mice we see that the entire subepicardium is populated with cardiomyocytes in a pre-mitotic state in G1 (Supplemental Figure 3, G1). These cells are restricted to the subepicardium and they surround the site of injury, yet are not represented in the myocardium adjacent to the endocardium, in the epicardium or within the injury itself (Supplemental Figure 3, Merge).

Figure 4. Cells expressing embryonic transcription factors Nkx2-5 and GATA4 evolve within the site of recellularization in an ex vivo model of mammalian cardiac regeneration. After 12 days in culture (Base+NW) WT1 expression is lost from the injury site. Within the proliferating population (A,B) transcription factors NKX2-5 (C) and GATA4 (D) are reactivated as indicated by immunostaining. The gene expression profile of NKX2-5 (E) and GATA4 (F) demonstrates the opposite temporal profile compared to WT1 expression, demonstrating an increase from day 10 to day 12, which is indicative of cardiomyocyte differentiation. TBX5 (G) and c-kit (H) RNA levels do not follow the same trajectory, but rather are immediately reduced suggesting putative cardiac stem cells are not involved with this regenerative response.

Discussion

We tested the ability of recombinant NRG-1 to rescue regeneration following injury to the apex of neonatal hearts, *ex vivo*. Unlike when parasympathetic denervation in reversed *in vivo* with exogenous administration of rNRG-1^[11], rNRG-1 was not sufficient to induce regeneration in cardiac explants, *ex vivo*. However, epicardial cell activation *via* Wnt and BMP signaling, in combination with NRG-1, was necessary and sufficient to restore the endogenous regenerative program lost due to the denervation of explanted hearts. Sympathetic nerve-derived Wnt mediates repair programs in several animal models^[27, 38, 39], plays an important role during vertebrate heart development, and is re-activated in response to cardiac injury^[27]. Following acute ischemic injury, the epicardium is activated, organ-wide, in a Wnt-dependent manner. Cells subsequently proliferate and undergo epithelial-mesenchymal transition^[28,29]. Both epicardial maturation and cardiomyocyte specification require BMP-4^[30-32], which together with the morphogenetic factor Retinoic acid (RA), contributes to normal cardiac development during embryogenesis. RA has both early patterning roles and subsequent mitogenic roles^[40, 41]. Together Wnt3a, BMP-4 and RA constitute a potent group of growth factors that orchestrate epicardial and myocardial development and response to injury^[33].

The essential role that peripheral nerves play in tissue regeneration is well-established across a wide array of species and tissues^[18], but only recently has the role of cardiac nerves in mammalian cardiac tissue regeneration been explored^[11,12,42-49]. Having now established a link between cardiac nerves, heart regeneration and disease progression, understanding the mechanism of nerve-mediated cardiac responses to injury is needed. Again, we looked to established models for clues into the signaling pathways and cellular contribution during an injury response. NRG-1 plays a critical role in a broad range of tissue homeostasis and regeneration models^[9,14,17,19-21,50,51], it is essential for nerve-dependent axolotl limb regeneration^[14] and is an injury-induced cardiomyocyte mitogen for the endogenous heart regeneration in zebrafish^[18]. Understanding the role of NRG-1, and other putative nerve-dependent growth factors, in mammalian cardiac regeneration is confounded by *in vivo* complexity. To make it easier to address the mechanisms underlying cardiac repair, we developed an *ex vivo* culture system where explanted neonatal hearts maintain their three-dimensional structure and perinatal regenerative ability^[6]. Despite the absence of the cardiac nerves that are required for homeostasis and regeneration^[8,9,48], we maintained neonatal hearts for more than 21 days under defined *ex vivo* conditions by the addition of rNRG-1. While rNRG-1 alone does not enable cardiac repair of the injured neonatal explant, as previously described in related models^[11, 20], NRG-1 increased Pi3K/AKT and decreased GSK-3 activation, reduced double strand DNA damage, enhanced cardiomyocyte survival in long-term culture and maintained high levels of expression of ERBB2 on the surface of cardiomyocytes, which has been previously shown to trigger mammalian heart regeneration by promoting cardiomyocyte dedifferentiation and proliferation^[12].

Wnt3a, BMP-4 and RA were needed, in addition to rNRG-1, to induce cardiac regeneration *ex vivo*. Sympathetic nerve-derived Wnt mediates repair programs in several animal models^[27, 38, 39], although the mechanism of repair is incompletely understood. Interestingly, treatment of human iPSCs with Wnt3a and BMP-4 followed by Wnt inhibition causes an increase in biologically functional cardiomyocytes, suggesting that BMP-4 and Wnt/ β -catenin signaling pathways play a major role in the production new cardiomyocytes following cardiac injury^[30]. Both factors are critical during embryogenesis and in established models of tissue regeneration^[52], and provide insight into the mechanisms of mammalian cardiac regeneration that would have been difficult to identify using an *in vivo* model. Wnt/ β -catenin signaling plays a key role in regeneration of the heart^[27,28,53], and certain other tissues^[38,39,54]. In the heart, this reparative effect of Wnt/ β -catenin signaling may function through the activation of epicardial cells^[28, 41].

The epicardium, a single-cell, thick mesothelial layer surrounding the heart, has a key regulatory role in the regenerative process in competent species such as zebrafish and the neonatal mouse^[2, 4, 5, 55]. In combination with Wnt/ β -catenin signaling, retinoic acid represents a robust co-activator and mitogen of both epicardium and myocardial cells^[40, 41, 55, 56]. Following activation and epicardial endothelial to mesenchymal transition, the injury response in cardiac explants can be further enhanced with the removal of Wnt3a and BMP4^[25, 30, 32, 57]. Several reports suggest that cardiac regeneration is mediated through the proliferation of cardiomyocytes^[58, 59], however, our data do not support that model in the *ex vivo* heart, at least outward of the injury site. Our data agree with published reports suggesting that epicardial cells do not transdifferentiate to generate *de-novo* cardiomyocytes in the post-embryonic heart. Rather these data suggest that resident cardiomyocytes disassemble their sarcomeres and cytoplasmic structure and migrate to populate the site of injury (Supplemental Figure 2)^[15]. Interestingly, we found no evidence of cardiomyocyte proliferation within the injury site by either EdU or Phospho-histone-H3 immunofluorescence. This finding underscores the complexity of the regenerative process and supports further studies focusing not only on the biochemical mechanisms, but also the temporal cellular dynamics within the injury and in the surrounding tissue.

Our preliminary data suggest a potential clue as to the source of cardiomyocytes. The FUCCI system marks specific cell cycle stages within live cells where a cell in the G1 phase of cell cycle expresses a red nuclear reporter^[37]. In our experiments, by day 21 following injury, the site is resolved and closely resembles the surrounding myocardium. However, by using FUCCI mice we see that the entire subepicardium is populated with cardiomyocytes in a pre-mitotic state in G1. These cells are restricted to the subepicardium and they surround the site of injury, yet are not represented in the myocardium adjacent to the endocardium, in the epicardium or within the injury itself.

Wnt3a and Bmp4 signaling activate specific, non-overlapping genes in cardiac progenitors with Nkx2-5 expression controlled by Wnt/ β -catenin, and Gata4 controlled by BMP signaling^[57]. In murine models, genetic lineage-tracing studies indicate that the proepicardium originates from Nkx2-5- and ISL1-expressing lateral plate mesoderm progenitors^[60]. ISL1, a marker of the secondary heart field, remained negative throughout the *ex vivo* regenerative process (data not shown). Several studies have reported that the epicardium is derived from Nkx2-5⁺ progenitors^[61] and the fate of epicardial and myocardial cells bifurcate at this stage. As a consequence, epicardial cells do not contribute to neo-myogenesis in the post-embryonic mammalian heart. Our data support this argument as no cardiomyocytes within injury sites expressed GFP or EdU. Rather, at approximately day 10 of culture, the fully recellularized injury began to lose the founding population of cells (Supplemental Figure 4a). These cells appear to be replaced by resident cardiomyocytes that migrate from the neighboring myocardium (Supplemental Figure 2). Once the injury resolves it was difficult to identify the site by histological methods. However, a vestige of the injury can be resolved by identifying non-specific auto-fluorescence (Supplemental Figure 4B). This feature together with a subtle concave indentation in the ventricular wall represents a signature for the identification of former injury sites. These sites are populated with cardiac troponin⁺ (cTnT⁺) cells. However, rather than having the well-defined sarcomeric structure characteristic of adjacent cardiomyocytes, these cells demonstrate a rounded phenotype with disassembled sarcomeres as previously described in the regenerating apex of neonatal mouse hearts *in vivo*^[6, 62] (Supplemental Figure 4C). The mechanisms driving this migration and maturation process are currently unresolved, however, a similar, distinct bilayer of cardiomyocytes demonstrating differing regenerative potential was recently seen in zebrafish^[63]. We speculate that this subepicardial layer represents a pool of injury-responsive cells within the myocardium, although further work will be required to advance this hypothesis.

The signaling pathways activated in response to injury and the mechanisms by which these signals activate the epicardium and drive regeneration are currently unknown. This report provides a novel and much-needed platform from which to address these important questions. Understanding the cell-intrinsic and extrinsic mechanisms governing regeneration of the neonatal murine heart can illuminate the fixed and flexible components of a cellular and molecular response to cardiac injury^[64]. Such knowledge is critical to understanding the current limitations and future potential for repair in the injured adult human heart.

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Abbreviations

NRG-1	: Neuregulin 1
rNRG-1	: recombinant Neuregulin 1
Wnt3a	: Wingless-related integration site 3a
BMP4	: Bone morphogenetic factor 4
WT1	: Wilms tumor 1
ErbB2	: Receptor tyrosine-protein kinase erbB-2
ErbB4	: Receptor tyrosine-protein kinase erbB-4
RA	: Retinoic Acid
Nkx2-5	: NK2 homeobox 5
GATA4	: GATA Binding Protein 4
EdU	: 5-ethynyl-2'-deoxyuridine
GAPDH	: glyceraldehyde 3-phosphate dehydrogenase
H2AX	: H2A histone family member X
Pi3K/AKT	: phosphoinositide-3-kinase/ Protein kinase B
MM	: Maintenance Media
NWB	: Neuregulin, Wnt3a, BMP4
GFP	: Green Fluorescent Protein
FUCCI	: Fluorescent Ubiquitination-based Cell Cycle Indicator
cTnT	: Cardiac Troponin T

Potential Conflicts of Interests

1. Author Joshua Hare is Consultant/Shareholder/Board Member of Longeveron; Consultant/Shareholder/Board Member of Vestion; Consultant/Shareholder/Board Member of Heart Genomics and has obtained grants from NHLBI.
2. Author Ian White is Founder and CSO NeoBiosis, LLC.

Additional Information

Supplementary Information accompanies this article. Supplementary information, Video and figures are linked to the online version of the article.

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