A specified therapeutic window for neuregulin-1 to regenerate neonatal heart muscle

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The most prominent cause of impaired cardiac function in adults is ischemic heart disease such as myocardial infarction, whereas decreased heart function in children is generally the consequence of congenital heart disease (CHD), structural malformations of the heart that are present at birth. Heart defects are the most common birth defect in humans—and the most deadly too, boasting the infamous title of leading cause of birth defect-related morbidity and mortality (1). One in 100 newborns display signs of minor CHD, but in one out of 1,000 cases the malformations are severe enough to necessitate surgical intervention. The therapeutic conundrum is that surgical repair is often life-saving, but may not be enough by itself to guarantee the child a carefree existence later on. The human heart has virtually no inherent regenerative capability and despite long-standing efforts, no valid cardiac regenerative therapy proven to be clinically effective exists to date. Since scarred and dysfunctional regions of the heart cannot be replaced, children with CHD are at greater risk of developing chronic heart failure—even after surgical correction of the malformation (2,3). Unfortunately, currently available pharmacological therapies for heart failure were developed with the adult patient in mind and proved ineffective in pediatric trials, highlighting the need for child-specific heart failure therapies (4).

Adult mammalian heart muscle cells, or cardiomyocytes, display extremely limited proliferation potential (5,6). After an intense proliferative phase, which in mice occurs during the first week of postnatal life, cardiomyocytes exit the cell cycle and thereafter undergo only hypertrophic growth—an increase in cell size that is not accompanied by DNA replication or cell division (7)—with the exception of a brief, additional proliferative burst that occurs during preadolescence (8). Perhaps not surprisingly, the developmental window of cardiomyocyte proliferation coincides with the ability of the heart to regenerate after injury: until day 7, neonatal mice are capable of scarless regeneration, after which fibrotic scar formation predominates over tissue replacement, with preadolescent mice displaying only partial regeneration after myocardial infarction (8-10). Administering factors that will boost cardiomyocyte proliferation after injury is one of the strategies being pursued in the quest for the ultimate cardiac regenerative therapy (11). To identify such factors, scientists have turned to cardiogenesis and pinpointed the signaling networks driving normal heart development. A plethora of endogenous cardiac growth regulators have since been thrown into the spotlight as potential heart regeneration tools, one of them being neuregulin-1 (NRG-1).

In a study published a few years ago, the Kühn lab showed that exogenous administration of recombinant NRG-1 could promote cardiac regeneration in adult mice by stimulating cardiomyocyte proliferation (12). This year, they tested whether NRG-1 could do the same in neonatal animals, which bear more proliferation-competent cardiomyocytes than adult animals and should theoretically be more responsive to proliferative stimuli. Their results, published in a recent issue of Science Translational Medicine (13), indicate that NRG-1 can promote cardiomyocyte proliferation and thus heart regeneration in neonatal animals after injury, but only within a certain time window. Importantly, they also tested NRG-1 treatment on diseased human heart biopsies that were taken from newborns with CHD and cultured in vitro, and found that NRG-1 is effective in driving cardiomyocyte proliferation if applied before 6 months of age. Consequently, the authors concluded that...
if NRG-1 were to be administered to CHD patients during the first few months of life, as co-adjuvant therapy together with surgery, it might be helpful to regenerate the missing cardiac muscle at an early age and prevent heart failure from developing later on.

**NRG-1: a promising growth factor for heart regeneration**

The neuregulin family of growth factors was originally discovered in the early 1990s in the context of cancer and neural research, but it was not until the first systematic studies of NRG signaling disruption were published that its crucial role in heart development became clear (14-16). Fifteen NRG isoforms have been identified so far, of which NRG-1, a trans-membranous protein that requires proteolytic cleavage to be activated and secreted, is the one that is expressed at highest levels in the heart (17). Secreted NRG-1 signals through the ErbB receptor tyrosine kinases of the family of epidermal growth factor receptors (18). In cardiomyocytes, NRG-1 binds to the extracellular ligand-binding domain of ErbB4, triggering a conformational change in the receptor, which then dimerizes with either ErbB2, the preferred heterodimerization partner for all ErbB receptors, or another activated ErbB4 receptor (19).

Several lines of evidence point to a critical role of NRG-1/ErbB signaling in cardiac development, homeostasis and response to injury. Germline knock-out of the NRG-1, ErbB2 or ErbB4 gene in mice causes significant impairment of myocardium development and death at midgestation, indicating that each of these genes is independently required for cardiomyocyte generation (14-16). NRG-1 was later shown to act synergistically with insulin-like growth factor 1 (IGF-1) to drive cardiac chamber morphogenesis in the mouse embryo (20). Although its expression levels in the myocardium decrease after development of the heart, NRG-1 also plays an important function in postnatal cardiac homeostasis, as shown by mice with cardiac-restricted ablation of the ErbB2 or ErbB4 receptor, which although normal at birth, develop lethal dilated cardiomyopathy later in life (21,22). In the clinical setting, interest in NRG-1 has been sparked by the observation that chemotherapeutic drugs targeting this signaling pathway produce adverse cardiac side effects such as cardiomyopathy, which can be seen when women with breast cancer are treated with the inhibitory ErbB2 antibody Herceptin/Trastuzumab, especially in combination therapy with anti-neoplastic agents such as anthracyclines (23,24). Moreover, NRG-1 and ErbB4 genetic variants have recently been associated with sudden cardiac death and congenital left ventricular outflow tract defects, respectively (25,26).

Cumulative experimental evidence suggests a crucial role of the NRG-1/ErbB pathway in cardiomyocyte growth and proliferation. Treating neonatal rat ventricular cardiomyocytes in vitro with NRG-1 resulted in hypertrophy (27) and when applied to mouse or human embryonic stem cells differentiating along the cardiac lineage, NRG-1 increased the production of ‘working-type’ cardiomyocytes (17). In zebrafish, NRG-1 could trigger heart muscle cell dedifferentiation and proliferation in both uninjured and injured conditions, resulting in cardiomegaly or heart regeneration respectively (28). In rats, both NRG-1 and active, phosphorylated ErbB4 levels were shown to increase after myocardial ischemia/reperfusion injury, and treatment with NRG-1 could reduce infarct size and fibrotic scars, improve overall cardiac function and increase cardiomyocyte proliferation after injury (29-31).

**Can NRG-1 regenerate the infant heart?**

In the recent study by Polizzotti et al., the effects of NRG-1 treatment were tested in the neonatal mouse heart after cryoinjury, which causes scarring and reduced cardiac function (13). Cryoinjury was performed on the first day of life, and mice were then given daily NRG-1 injections for 34 days, starting at birth (early administration) or 5 days after birth (late administration). Animals receiving NRG-1 at birth fared best, displaying a sustained increase in heart function that persisted for 30 days after suspension of therapy. Late NRG-1 administration on the contrary did not cause significant improvement in cardiac function compared to control mice 30 days after the last injection. Similarly, a decrease in scar size was observed after early but not late administration, indicating that the timing of NRG-1 therapy is important for long-term results. The authors then went on to test whether NRG-1 therapy could benefit human pediatric patients affected by CHD. To this end, they focused on children affected by Tetralogy of Fallot, a common form of CHD that usually involves four heart malformations and requires surgical repair. Myocardial samples collected at the time of surgery were cultured as 3D organotypic systems in the presence or absence of NRG-1. Normal cardiomyocytes from healthy myocardium displayed a certain degree of cell cycle activity, which naturally declined by 6 months of age. In basal conditions, cardiomyocytes from diseased myocardium stopped...
proliferating much earlier than those from healthy donors and NRG-1 treatment could boost their cycling activity, but only within 6 months of age. Collectively, their experiments in mice and human led the authors to conclude that NRG-1 can trigger heart regeneration and improve overall cardiac function after injury in newborn mice and that it can also stimulate cardiomyocyte proliferation in human CHD patients. In both species however, cardiomyocytes can only take advantage of the effects of NRG-1 during a defined time window, after which they become insensitive to NRG-1 therapy.

What defines the NRG-1 therapeutic window?

The data reported by Polizzotti et al. are in line with an earlier study, which showed that although NRG-1 treatment could increase hypertrophy in both neonatal and adult rat ventricular myocytes, it could only stimulate proliferation in neonatal ones, implying a temporal context-dependence of NRG-1 signaling (32). Cardiomyocyte proliferation levels normally peak during embryonic development and decrease drastically to negligible levels in adulthood (8). Interestingly, another recent report suggested that the window of NRG-1 activity is defined by ErbB2 receptor levels (33). The authors of this study confirmed that in vivo administration of NRG-1 at birth induces a dose-dependent increase in cardiomyocyte proliferation and that the effect is profoundly reduced if the growth factor is delivered a week after birth. They then observed that whereas NRG-1 and ErbB4 levels remain unchanged over the first week of life, ErbB2 expression is down-regulated at the same time as cardiomyocyte proliferation comes to a halt, suggesting that declining levels of the ErbB2 coreceptor may limit the postnatal window of cardiomyocyte proliferation and regeneration. In fact, they proved that NRG-1 cannot induce neonatal cardiomyocyte proliferation in the absence of ErbB2, and then pushed their observation further by testing whether reactivating ErbB2 in juvenile and adult animals could prolong the cardiac regenerative window. Transient ErbB2 induction after myocardial infarction induced cardiomyocytes to reenter the cell cycle and improved overall cardiac function, suggesting that augmentation of ErbB2 signaling (in the absence of exogenous NRG-1) is sufficient to prolong the regenerative capacity of the heart past its normal window.

Together, the data by D’Uva et al. (33) and Polizzotti et al. (13) point to the fact that NRG-1 mediated stimulation of cardiomyocyte proliferation and heart regeneration after injury declines quickly after birth. However, an earlier study by the Kühn lab indicates that NRG-1 can promote cardiomyocyte division in young adult mice after myocardial infarction (12). So how do we reconcile two seemingly contradictory observations? One possibility is that the NRG-1 mediated response differs depending on the type of cardiac injury model employed—cryoinjury in the current study or LAD ligation previously (12,13). In the neonatal mouse heart, cryoinjury inhibits endogenous cardiomyocyte proliferation, and heart regeneration does not occur to the same degree as reported in hearts after LAD ligation or myocardial resection (9,10,34)—although it should be noted that whether the latter injury models truly lead to scarless repair in neonatal mice is controversial (35). Nevertheless, Polizzotti et al. argue they opted for cryoinjury, rather than the previously employed LAD ligation method, because it recapitulates the scar formation process and the decrease in cardiomyocyte proliferation that are often seen in young patients with heart disease, thus likely providing a better model for human pediatric CHD (34). Whether the observed proliferation-boosting effects of NRG-1 on neonatal mouse and human cardiomyocytes would still be confined to early postnatal stages in the context of a different injury model is unknown so far.

How does NRG-1 regenerate the heart?

Whether the ability of NRG-1 to promote cardiomyocyte renewal declines shortly after birth or persists into adulthood is a controversial issue, as is the exact mechanism through which NRG-1 benefits cardiac function. In a study published last year, NRG-1 was reported to promote cardiac repair in an adult rat model of myocardial infarction via a combination of cardiomyogenic stem cell activation and cardiomyocyte cell cycle induction (31). However, issues regarding the fidelity of the assay used to detect cardiomyocyte proliferation in that study had previously been raised (36). Similarly, concerns have recently been voiced around a number of technical issues in the study by Bersell et al. (12), including the poor fidelity of the reporters used to mark cardiomyocyte nuclei and the inadequate sensitivity of the assays employed to detect DNA synthesis (37). Although adult mice were subjected to myocardial infarction followed by recombinant NRG-1 injections using the same experimental setup as described by Bersell et al. (12), no significant increase in the percentage of cardiomyocyte nuclei undergoing DNA synthesis, as measured by both bromodeoxyuridine (BrdU) and
3H-thymidine (3H-Thy) incorporation, was detected in NRG-1-treated mice compared to control animals (37). This would suggest that any beneficial impact of NRG-1 treatment on adult injured hearts is likely not attributable to true myocardial proliferation and definitive evidence of this phenomenon is yet to be presented. Even in neonatal hearts, where according to Polizzotti et al. (13) NRG-1 acts mostly by stimulating cardiomyocyte cell cycle in an ErbB4-dependent manner, cardiac regeneration and improvement of heart function by NRG-1 treatment after injury might not be derived from one mechanism, such as proliferation of cardiomyocytes, alone. After early NRG-1 administration in neonatal mice, the authors calculated that more than 60% of cardiomyocytes arose through proliferation of pre-existing heart muscle cells, as measured by the number of phosphorylated histone H3- and Aurora B kinase-positive cells. The remaining quota was ascribed to cardiomyocyte protection, with NRG-1 possibly stimulating the growth of new blood vessels and/or preventing cardiomyocyte apoptosis through its paracrine effects (13). NRG-1 may also exert its beneficial effects by down-regulating genes involved in fibrosis, such as matricellular proteins, collagens and basil lamina components (38). Thus, a combination of all the above mechanisms may account for the observed beneficial impact of NRG-1 on the neonatal mouse heart after cryoinjury.

Polizzotti et al. attempted to shed some light on NRG-1 effectors and mediators by analyzing transcriptomic changes in treated versus untreated control samples. Their RNA-Seq experiment identified over 600 genes whose expression is affected by early NRG-1 treatment, including cytokines, transcription factors and known regulators of cell division. Generic gene ontology analyses led the authors to conclude that the cardiac structural and functional changes they observed after NRG-1 treatment were paralleled by broad changes in gene regulation, although deeper analyses will need to be conducted to determine which genes truly explain NRG-1 beneficial effects on cardiac injury. It should also be noted that on this occasion the authors limited their RNA-Seq study to the early NRG-1-treated sample alone, but a comparison with the late treated sample, where no significant regeneration occurs, could help pinpoint genes with a crucial role in the regenerative response window. In conclusion, NRG-1 likely acts through a variety of pathways to protect and help restore heart function during the remodeling process that follows ischemic injury and further investigation will need to be conducted in the future to elucidate authentic key players.

Looking ahead: how far are we from a NRG-1 therapy for CHD patients?

The endogenous supply of heart cells that can regenerate and repair damage decreases dramatically with age, but the study by Polizzotti et al. suggests that NRG-1 can enhance proliferation in cells that have not lost that capacity yet. In CHD patients, delivering NRG-1 early in life to encourage the production of new cardiomyocytes could thereby aid cardiac function, support the surgical approach adjunctively and reduce the risk of developing heart failure later on. So far, clinical trials for NRG-1 therapy in heart disease have only been performed on adult patients, reporting conflicting results. In one study, 44 chronic heart failure patients were enrolled and given intravenous injections of recombinant NRG-1 for 10 days. After 90 days, left ventricular ejection fraction improved compared to patients treated with placebo, indicating that short-term administration can exert long-term effects on cardiac function (39). In a second trial enrolling 15 chronic heart failure patients, however, the long-term effects of NRG-1 treatment were not as pronounced (40). At least five more trials of NRG-1 administration in adult chronic heart failure are currently underway (17).

Before we will be able to utilize the full potential of NRG-1 based therapy and tailor it to pediatric CHD patients, we must untangle the mechanisms through which NRG-1 causes the observed effects on homeostatic and cardiac functions. It is also imperative that we consider potential pitfalls, risks and limitations of NRG-1 therapy. The first obvious concern relates to the tumorigenic potential of the NRG-1/ErbB signaling pathway. ErbB2 was originally identified as an oncogene and expression of a mutated, constitutively active receptor is a poor prognosis indicator for breast cancer (41). To minimize oncogenic risk, we should aim to design cell-specific therapies to target NRG-1 to cardiomyocytes alone, or alternatively limit treatment duration to avoid unnecessary and unspecific over-stimulation of the pathway. Fine-tuning the dosage and/or duration of NRG-1 administration will also ensure that the desired outcome is achieved with as little adverse effects on cardiac function as possible. The results by Polizzotti et al. show that the ability of NRG-1 to stimulate cardiomyocyte proliferation in human CHD samples is greatly reduced by 6 months of age, suggesting there may be a narrow ‘window of opportunity’ in which NRG-1 treatment can improve the success of reconstructive surgery. So ideally, NRG-1 therapy for pediatric CHD patients...
should start as early as possible, but how long should it last for and what dosage should be used? In both zebrafish and mouse, unrestrained cardiomyocyte proliferation induced by activation of the NRG-1/ErbB2 pathway leads to enlargement and eventually failure of the heart (28,33). This indicates that although it may be beneficial to induce cardiomyocyte proliferation with NRG-1, it is imperative to achieve the correct level of signaling and to turn off the pathway so that the appropriate number of cells is maintained in the heart. Finally, the overall safety and any possible side effects of NRG-1 administration in pediatric patients will have to be assessed through carefully designed and controlled pilot clinical studies before adopting it as novel regenerative therapeutics for human CHD.

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References