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Rescue of Developmental Defects by Blastocyst Stem Cell Injection: Towards Elucidation of Neomorphic Corrective Pathways

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Abstract

Stem cell-based therapy is an exciting area of high potential for regenerative medicine. To study disease prevention, we inject mouse embryonic stem cells (ESCs) into a variety of mouse blastocysts, most of which harbor mutations. Mice derived from these mutant blastocysts develop human-like diseases, either at developmental stages or in the adult, but blastocyst injection of ESCs prevents disease from occurring. Rather than entirely repopulating the affected organs, with just 20% of chimerism, the ESCs replenish protein levels that are absent in mutant mice, and induce novel or “neomorphic” signals that help circumvent the requirements for the mutations. We also show data indicating that the “neomorphic” mechanisms arise as a result of blastocyst injection of ESCs, regardless of the nature of the host blastocyst (mutant or wild-type). Thus, blastocyst injection of ESCs not only allows the study of disease prevention, but also unveils novel pathways whose activation may aid in the correction of congenital or acquired disease.

Keywords

Embryonic Stem Cells; Blastocyst; Congenital Heart Disease; Muscular Dystrophy; Myocardial Infarction; Mouse Model of Human Disease; Neomorphic Effects

Introduction

Embryonic stem cells (ESCs) possess the unique capacity to differentiate into a diverse range of cell types that compose the human body. For this reason, ESC research is an area of study that holds significant promise for the development of regenerative treatments for a wide array of diseases [12,16,18,26]. Through the injection of wild-type (WT) ESCs into early mutant mouse blastocysts—preimplantation embryos which would otherwise be prone to disease development—our lab studies mechanisms for the correction of congenital diseases.

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The mice generated by this technique are termed “chimeras” as they truly are a composition of both innately and externally derived cells of both mutant and wild-type genotypes. These ESCs undergo mutual recognition and synergistically contribute to murine development. Blastocyst injection is a classic protocol normally used to generate mutant mouse lines via the injection of mutant ESCs into WT donors. Our protocol reverses this concept mechanistically to determine if we can use blastocyst injection as a model to both prevent disease from occurring and to then elucidate the underlying mechanisms governing ESC-mediated corrections.

Through the use of protein and gene expression assays, we are able to examine molecular differences between WT, mutant, and chimeric mice. These assays allow us to pinpoint future candidate molecules for continued study. These candidates will be eventually tested for their ability to replace the therapeutic capabilities of ESCs as a potential strategy for disease treatment. In some situations, the corrections are achieved via supply of proteins from the ESCs that are absent in the mutant mouse due to the mutation, thus restoring normal protein levels. The microarray analysis also reveals that “neomorphic” events occur; molecular changes or novel findings induced by the injected WT ESCs in a chimeric environment. These neomorphic proteins that emerge as a result of mixing two populations of cells (chimerism) have the ability to bypass the requirement of the mutation. In this review, we summarize data indicating that WT ESCs incorporated into mutant blastocysts are capable of preventing disease from occurring in mouse models of human disease. We describe the induction of neomorphic events implicated in the rescue of congenital heart disease and muscular dystrophy. We also show that WT ESCs incorporated into WT blastocysts also induce neomorphic corrective processes, in this case, leading to prevention of heart failure when a heart is ischemically stressed.

Therefore, developmental injection of WT ESCs prevents congenital or acquired disease from occurring.

Prevention of Congenital Heart Defect

The “thin myocardial syndrome” is an example of a congenital heart defect, and represented by the inhibitor of DNA binding (Id) knockout (KO) mice [3]. The Id proteins (Id1 to Id4) are dominant negative antagonists of basic helix-loop-helix (bHLH) transcription factors and regulate differentiation in multiple lineages [27]. The Id genes are expressed in embryonic tissues during development in partially overlapping patterns, but their expression declines in the adult [9,10]. Within the developing heart, Id genes are expressed in nonmyocardial layers (epicardium, endocardium, endothelium, and endocardial cushion) [6]. However, the primary affected target of the Id proteins is the myocardium, which does not express Id proteins. This pattern suggests some type of layer-to-layer communication. Loss of one Id gene (Id1, Id2, or Id3) does not result in any developmental defect, but double KO embryos (Id1/Id3, Id1/Id2, or Id2/Id3) in any combination display a prominent cardiac phenotype that leads to midgestational demise. This is consistent with the redundant role and overlapping pattern of expression of Id genes. In these double KO embryos (Id1/Id3, Id1/Id2, and Id2/Id3), the primary defect is in myocyte proliferation, resulting in a thin myocardial wall. Additionally, the trabeculae have disorganized sheets of myocytes surrounded by an impaired endocardial cell lining, the endocardial cushion is hypoplastic, and the muscular portion of the ventricular septum is incomplete [5,6].

With the intent to extend the viability of the Id1/Id3 double KO embryos for 1–2 days and unveil novel roles for these factors during development, we injected ROSA26 LacZ-marked WT ESCs into mutant (Id1/Id3 double KO) blastocysts. Rather than surviving two more days, the resultant embryos survived to birth. In the WT/Id chimeras, all the cardiac structures appeared normal histopathologically and functionally. Intriguingly, only a small fraction of the chimeric hearts originated from the WT ESCs, just 20–30%. In other words, 70–80% of the chimeric hearts were composed of Id1/Id3 double KO mutant cells. To study variations in

gene expression profiles in the chimeric heart, we performed a microarray analysis. Most of the genes dysregulated in the Id KO hearts were restored to WT levels, even in the mutant compartment of the heart. Thus, the corrections were achieved globally, suggesting the presence of non-cell autonomous corrective factors [5,6].

To identify potential secreted factors that could be bridging corrective information between the WT ESC-derived and the mutant components, we performed a microarray analysis with ribonucleic acid from epicardial cultures (one source of Id proteins) of WT, KO, and chimeric (WT/Id) hearts. One of the factors we became interested in was insulin-like growth factor 1 (IGF-1), because IGF-1 was lower in the Id KO cultures (versus WT) and normalized in the chimeric cultures (versus WT). IGF-1 is known to promote cardiac myocyte proliferation and is released into the bloodstream. In addition, IGF-1 and Id gene expression patterns overlap, and the loss of IGF-1 or Id genes has remarkable similarities [13,23,24]. To test if IGF-1 could replace the rescue effect of the ESCs, we intraperitoneally injected mouse recombinant IGF-1 into the mother harboring Id1/Id3 double KO embryos. Notably, the Id1/Id3 double KO embryos, which were predisposed to die in utero, developed to term. Although the proliferation defects were corrected by IGF-1 administration, the rescue was partial, as some of the structural defects failed to reverse [6]. We then hypothesized that in addition to IGF-1, the ESCs could be supplying paracrine factors, which could be required to accomplish a full rescue of the cardiac phenotype. We, therefore, turned our attention to Wnt5a. Unlike IGF-1, which is dependent on the presence of Id proteins, Wnt5a was overproduced in the chimeric epicardial cultures (versus WT). The fact that the chimeric heart had more Wnt5a than the WT heart suggested a neomorphic effect of the ES cells. The ES derived cells, surrounded by a mutant environment, in this case, Id KO cells, would generate disproportionate levels of Wnt5a protein to compensate for a reduction in Id dose. Wnt5a is a lipid-modified glycoprotein. Wnt signaling is required for cardiac development and its loss leads to ventricular septal defects [2,19,28]. Because Wnt5a cannot be injected maternally, as it cannot travel long-distances to reach the embryo, we designed an ex vivo experiment to test its potential corrective role. In a control experiment, WT and KO heart explants were cocultured with murine embryonic fibroblasts (MEFs), and subsequently arrayed. In this control condition, over 600 genes were dysregulated in the Id KO explants (relative to WT explants). Next, we cocultured WT and KO heart explants with MEFs that overexpress Wnt5a to mimic a chimeric environment and observed that 85% of the dysregulated genes were normalized (relative to WT explants). Therefore, over-expression of Wnt5a corrects gene expression profiles [6].

We propose that congenital heart defects initiated by the absence of Id proteins can be rescued by blastocyst injection of WT ES cells. We also propose that the ESCs are responsible for the secretion of two proliferative factors: IGF-1, whose presence is dependent on the presence of Id proteins (non-neomorphic), and Wnt5a, whose presence is independent of the presence of Id, but emerges as a result of mixed chimerism (neomorphic). Therefore, neomorphic and non-neomorphic events account for the correction of the thin myocardial syndrome [3,5,6].

Prevention of Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is an X-linked congenital neuromuscular degenerative disease that results from a mutation in the protein dystrophin. Mdx mice represent a mouse model of DMD [30]. Dystrophin is the key protein in the dystrophin-glycoprotein complex [8]. The dystrophin-glycoprotein complex (DGC) is a trans-sarcolemmal protein complex in skeletal, cardiac, and smooth muscle that behaves as a link between the actin cytoskeleton and the extracellular matrix [4]. Examples of DGC components are the dystroglycans, sarcoglycans, dystrobrevins, syntrophins, and neuronal nitric oxide synthase (nNOS). Without dystrophin, the DGC is unstable, causing muscular tissue to be extra vulnerable to stress-induced damage from normal day-to-day contractions. The resulting muscular degeneration

forces patients into a wheelchair as early as 10 years old. Death commonly occurs in the late teens or early 20s due to cardiopulmonary defects associated with the dystrophic heart and diaphragm.

Our lab hypothesized that the injection of ROSA26 LacZ-marked WT ESCs into mutant mdx blastocysts would restore dystrophin to levels high enough to rescue the resultant WT/mdx chimeras. These WT/mdx chimeras varied in their degrees of skeletal muscle rescue due to their levels of chimerism. Chimerism values were determined by Xgal staining of 3 week tail biopsies and on various tissues harvested at the time of animal sacrifice at 4 months of age. Our results showed that WT/mdx chimeras with >10% ESC incorporation produced morphological and functional recoveries similar to wild-type mice. However, in the WT/mdx chimeras with <10% ESC incorporation, there were no observable signs of corrections, thereby indicating a threshold of ESCs necessary for suitable levels of dystrophin to be distributed throughout the skeletal muscle syncytium [31].

In the >10% WT/mdx chimeras, we observed a functional and morphological recovery similar to wild-type levels. Also, histological analysis of the >10% WT/mdx chimeras showed little, if any, of the necrosis and mononuclear invasion that is paramount in mdx skeletal muscle. In addition to this, these chimeras were also resistant to the skeletal muscle hypertrophy commonly associated with DMD. For example, the thickness of the >10% WT/mdx chimera diaphragms were at least twice as thin as mdx diaphragms and almost identical to the control WT diaphragms. Overall, the >10% WT/mdx chimera diaphragms displayed organized structure, a smaller amount of fibers across the width and a more uniform cross-sectional area of such fibers [31]. Conversely, subtle signs of hypertrophy, associated with increased levels of AKT (a mediator of hypertrophy) protein were apparent.

The regenerative capacity of the satellite cells cannot remedy the massive structural damage associated with DMD and this ultimately results in the loss of muscle fibers and an increase in fibrosis over time. The most common indicator of skeletal muscle regeneration is central nucleation of the fiber. Our >10% WT/mdx chimera showed little of this central nucleation indicating a significant slowdown in the demand for skeletal fiber regeneration. In addition, we performed immunofluorescence for embryonic myosin heavy chain (eMHC), which is a marker for regeneration, and found that eMHC was greatly reduced in the skeletal muscle of the >10% WT/mdx chimeras [31].

In heterozygote DMD hearts, only half of the cardiac cells contain dystrophin due to X-chromosome inactivation, but the skeletal muscle contains normal levels of dystrophin. This is because the skeletal muscle fiber is a histological syncytium, and the dystrophin protein finds no physical boundaries to spread across the fiber. Similar to heterozygotes, western blot analysis showed that our >10% WT/mdx chimeras had dystrophin protein levels of 70–100% of WT levels, but as expected, this observation was not observed in the heart, where only a functional syncytium forms. In addition, other DGC members, such as dystrobrevin, syntrophin, and nNOS, all had increased protein levels. Immunofluorescence for dystrophin and these DGC proteins supported this evidence and also confirmed their collective localization to their sarcolemmal niche. It was concluded that, dystrophin levels are elevated in skeletal relative to heart muscle because WT ESC-derived myonuclei have the ability to overproduce dystrophin to make up for the ability of the other mdx myonuclei in the same fiber, to produce this key structural protein, and/or reduce its degradation [31].

Not only were skeletal fibers corrected from the WT ESC injections, but the >10% WT/mdx chimeras showed nearly fourfold improvement in abdominal fat mass almost equal to WT. This is a significant gain being that mdx mice are normally very lean and is attributed to an increase in adipocyte population rather than adipocyte size. Intriguingly, the fat recovery

depends on the presence of dystrophin, as ES cells derived from mdx mice and, therefore, without dystrophin cannot rescue defects in either the skeletal muscle or fat. However, the fat does not produce dystrophin. Thus, we speculate that the presence of dystrophin, supplied from the ESCs from non-fat tissues (i.e., skeletal muscle) triggers the activation of downstream pathways and produces rescue molecules that act at a distance to rescue the fat. In turn, the fat, which is a rich source of bioactive factors, could be contributing to corrections in the skeletal muscle by secreting hypertrophic factors. What would be these potential factors, and how can we identify them? As in the case of the congenital heart disease rescue, we performed microarray analysis with WT, mdx, and WT/mdx fat. Microarray analysis indicated a 27-fold increase in follistatin like-1 (*Fstl1*) in the WT/mdx compared to the WT fat. This finding was validated via semi-quantitative polymerase chain reaction. *Fstl1* has previously been coined as a factor responsible for both hypertrophy and revascularization of skeletal muscle via the Akt pathway [20,21]. We have seen signs of both hypertrophy and increased levels of AKT in the rescued chimeric skeletal muscle. This disproportionate neomorphic induction of *Fstl1* in the chimeric adipose tissue is a result of mixed chimerism between two types of cells, in this case WT cells (derived from the ESCs) and mdx cells (derived from the mdx blastocyst). This suggests a positive feedback loop of tissue communication between the fat and skeletal muscle that is likely regulated by the secretion of circulating factors, in some cases neomorphic, that are only present due to the unique WT/mdx chimera phenotypes. We conclude that the recovery of muscular dystrophy via blastocyst injection of ES cells is primarily dependent on the replenishment of dystrophin throughout the skeletal muscle. This dystrophin supply not only targets the skeletal muscle but also triggers modifications in other tissues that do not produce dystrophin, like the fat. In turn, the fat produces neomorphic corrective molecules that may aid in the prevention of muscular dystrophy (Table 1).

Prevention of Myocardial Infarction

The rescue processes of congenital heart disease and muscular dystrophy summarized in the above sections have in common that the WT ES cells are injected into blastocysts harboring a mutation. Here, we ask if WT ES cells injected into WT blastocysts can trigger novel beneficial outcomes in a stressed adult heart. The central question is: can pre-natal ESC transplantation yield resistance to post-natal myocardial infarction? It has recently been established that heart muscle continuously rejuvenates during lifespan through renewal of cardiomyocytes [1], yet in the context of large-scale destruction—as it occurs following ischemic insult—the regenerative potential is insufficient to ensure organ salvage. Indeed, acquired heart disease is a leading cause of morbidity and mortality despite advances in our understanding of cardioprotective processes [33]. A preemptive intervention capable to engineer tolerance would offer prevention to tissue at risk avoiding the anticipated progression towards debilitating heart failure.

To test whether pre-natal incorporation of ESCs could augment stress tolerance and ensure lifelong protection, WT ROSA26 (LacZ-marked) ES cells were injected into WT blastocysts to generate stochastic integration throughout differentiating lineages [34]. Surgical transfer of derived blastocysts into the uterus of pseudopregnant females yielded full-term chimera. Within offspring, tissues derived from ROSA26 ESCs were traced by the LacZ transgene (Xgal staining) demonstrating that transplantation of ESCs into pre-implantation blastocysts generated a mosaic embryo with sustained engraftment of stem cell-derived tissue into adulthood. Noticeably, chimera displayed central obesity, associated with dominant depots of subcutaneous and visceral fat, but were otherwise devoid of obesity-related morbidity. This observation suggests that WT/WT chimeric mice differ from WT mice, and that some novel (neomorphic) molecular pathways that result in body changes take place. Throughout the 1 year follow-up, non-chimera and chimera exhibited similar cardiac performance. Importantly, however, upon permanent ligation of the left anterior coronary artery and despite comparable

ischemic insult, middle-age non-chimeric hearts rapidly deteriorated with irreversible decline in contractile function, in contrast to chimera that displayed progressive recovery of heart performance. Contrary to the vulnerable non-chimera, 12–14 month-old age-matched chimera were remarkably resistant to imposed myocardial infarction and regained within 1 month contractile performance at pre-occlusion levels. In fact, preemptive transplantation of ESCs at the embryonic stage halted development of cardiomyopathy traits in the adult chimera, averting progression of disease, which manifested in failing non-chimera as electrical remodeling and ventricular enlargement with fibrosis. In contrast, activation of the tissue repair process in the chimeric cohort was characterized by an increased stem cell load in adipose tissue and upregulated markers of biogenesis Ki67, c-Kit, and Sca-1 in the myocardium. Favorable outcome in infarcted chimera translated into an overall benefit in workload capacity and survival. In this way, stem cell transplant into pre-implantation embryo yielded a myocardial infarction-tolerant adult phenotype mitigating the clinically relevant endpoints of the heart failure syndrome [34].

The beneficial effect of chimeric tissue in the adult heart under imposed stress provides the initial evidence of preventive regenerative medicine in the setting of myocardial infarction implemented through prophylactic intervention. Delivery of exogenous embryonic stem cells into the blastocoel of an early embryo joined native to non-native progeny to yield composite, chimeric blastocysts with a finite blastomere number. In the context of the developing embryo, microsurgical transfers allow effective and sustained integration of independent sources of pluripotent progenitors that are in principle competent to differentiate into all lineages. In view of the physical restrictions imposed by the inner cell mass, the dual population of coexisting native and implanted progenitor cells creates the opportunity for competitive selection and titration of overall chimerism to meet developmental requirements [17]. Predetermined chimerism through embryo manipulation allowed assessment of the therapeutic impact of pre-natal enrichment with non-native progenitors upon cardiac stress challenge in the adulthood. The pattern of tissue formation optimized to accommodate the high-demand of initial cardiogenesis reinforced a failure-safe blueprint that was maintained in offspring throughout lifespan. Although the spectrum of contributing cell types and their origin remains to be defined, the favorable outcome in infarcted chimera corresponded to reparative features of cardiac biogenesis characterized by increased mitotic activity and tissue-specific progenitor cell load associated with reduced fibrosis. Moreover, the surplus of adipose tissue that developed in chimera contained elevated levels of non-hematopoietic mesenchymal progenitor pools, in line with the therapeutic potential of adult stem cells in the setting of myocardial infarction. Thus, the neomorphic augmentation of the cardiac and adipose stem cell pools could be responsible for the beneficial outcomes observed in the chimeric ligated heart [17]. Preemptive stem cell-based intervention in utero, thus, provides a strategy to engineer tolerance, and prevent incidence of life-threatening organ failure in the adult. In this way, pre-natal transplantation of embryonic stem cells expands the scope of traditional retrospective therapy to the previously unexplored prospective protection.

Neomorphic Commonalities in Blastocyst Rescue

Using blastocyst injection, we have shown that there are multiple methods by which corrections to a disease state phenotype can be achieved in a variety of mouse models. In the mouse model of thin myocardial syndrome, corrections are achieved predominantly by the secretion of healing factors, such as Wnt5a, whose production does not depend on the presence of the mutations observed in the Id KO mice. Despite being Id-independent, Wnt5a induction circumvents the Id mutation. The factors responsible for the neomorphic effects are neomorphic factors, and these events would not occur in the absence of WT ESCs, as the neomorphic induction is solely observed in the chimeric tissue. Models such as the mdx mouse require not only the replenishment of missing proteins, such as dystrophin but also the

upregulation of factors thought to contribute to corrections. For example, the neomorphic upregulation of follistatin-like proteins from neighboring adipose tissue appears to enhance hypertrophy of chimeric skeletal muscle. Recently, other labs have rescued central nervous system demyelination disease in “shiverer” mice by injecting ESCs at blastocyst stage [14]. It would be intriguing to ascertain if this rescue mechanism also involves neomorphic mechanisms. The neomorphic induction requires a mixed population of cells, but not necessarily distinct. For example, we have also seen neomorphic corrective processes in the hearts of mice composed of WT ES cells and WT blastocysts. The difference between these two WT components is that in one case, the cells (ES cells) are externally supplied at preimplantation embryo stage. We speculate that the driving force of the neomorphic induction is the process of ES cell/inner cell mass mutual recognition and selection that occurs in the blastocyst. Eventually, if the corrections can be achieved solely via neomorphic mechanisms, the mutation that produces the phenotype does not need to be genetically reversed or “fixed”. In this regard, the ESCs incorporated at blastocyst stage will be acting as “delivery vehicles” of the neomorphic factors, and thus, the therapeutic value of the cells could ultimately be replaced by the administration of the defined, neomorphic factors. This finding will have enormous therapeutic capabilities. Towards this, we are currently testing whether transgenic overexpression of Wnt5a can overcome the defects in the Id1/Id3 double KO embryos/mice.

There is high enthusiasm for the concept of cell-based therapy. Our approach, blastocyst injection, aims at exploiting the neomorphic abilities by which ESCs prevent a disease phenotype and provide researchers with candidate corrective molecules for continued research. The identification of corrective mechanisms may have tremendous impact on the treatment of human congenital and acquired disease. Researchers now possess the ability to generate pluripotent cells from adult somatic cells by the addition of four defined transcription factors (induced pluripotent stem cells or iPSCs). iPSCs are able to differentiate into a wide range of cell types, resembling ESCs in their differentiation capabilities as well as their ability to contribute to chimeric generation [7,15,22,25,29,32,35]. A fundamental difference between human ESCs and iPSCs is that the latter can be obtained from adult tissue of a patient carrying a disease, circumventing possibilities of immune system rejection. However, the iPSCs need to be perfected, as they carry (retro-/adeno-) viral insertions that may have undesired oncogenic activity. The scientific community is currently working towards the production of a new generation of iPSCs without viral/oncogenic insertions [11,29]. In the meantime, it would be intriguing to follow up our ESC studies using iPSCs to study their ability to correct disease by triggering conventional and neomorphic processes.

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Table 1

Neomorphic corrections observed in rescued chimeras via blastocyst injection of WT ESCs

Mutation	Neomorphic		
	Protein	Source	Target
Thin myocardial syndrome			
Id1,3	Wnt5a	Smooth muscle, epicardium	Developing heart
Muscular dystrophy			
Dystrophin	Follistatin-like 1	Abdominal fat	Skeletal muscle
Myocardial infarctium			
None	Unknown	Stem cells from myocardium and fat	Myocardium