Title: Novel regulatory mechanisms controlling inducible nitric oxide synthase (iNOS) expression and function

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Abstract

Introduction:

Nitric oxide (NO) is a bioactive gas produced by a family of enzymes known as the nitric oxide synthases (NOS). When produced by the inducible form of NOS (iNOS) through inflammatory cytokine stimulation, NO plays a role as an immune effector molecule. Importantly, dysregulation of iNOS and the resultant production of NO is implicated in the pathogenesis of many immune-mediated diseases where it affects tissue injury and regeneration. Therefore, elucidating novel regulatory mechanisms controlling iNOS expression is critical to understanding its role in these diseases. Here, we identified novel protein interactors with iNOS that may have potential roles in regulating iNOS protein expression and function at the post-translational level.

Approach and Results:

iNOS fused to a protein tag (Halotag) was transfected into A549 human lung epithelial cells in order to overexpress iNOS protein. Utilizing the Halotag, iNOS was affinity purified under normal conditions or treated with 1400W (a chemical inhibitor of iNOS protein activity that prevents the production of NO), then the samples were analyzed using a Tandem Mass Tag mass spectrometry workflow. It was observed that Annexin-A1 (ANXA1) protein levels were reduced in samples where iNOS activity was inhibited, suggesting that NO regulates iNOS interaction with ANXA1. The BAG family molecular chaperone regulator 2 (BAG2) protein that is known to regulate protein ubiquitination was also found to significantly interact with iNOS, suggesting that it may play a role in regulating iNOS protein degradation. ANXA1 and BAG2 are currently being pursued in validation experiments.

Conclusions:

Novel iNOS protein interactors have been identified and are currently being studied. The findings made here will elucidate novel regulatory mechanisms by which iNOS protein levels are controlled. These results may be significant for understanding and treating diseases where iNOS expression is dysregulated.

Colony stimulating factor 1 dependent tissue-resident macrophages play crucial role in muscle regeneration and are promising targets to treat muscular dystrophies

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Macrophages are key regulators of muscle regeneration and fibrosis. During muscle damage, two pools of morphologically indistinguishable phagocytic cells accrue from proliferating resident macrophages and recruitment of blood-borne monocytes. The absence of a specific marker has made it challenging to precisely determine the kinetics and roles of these two different populations. Herein, using parabiosis, RNA sequencing and fate-mapping approaches we identified *timd4* as a transcript heavily expressed by muscle resident macrophages that is completely absent from muscle infiltrating macrophages. We characterized the heterogeneity, turn over and origin of myeloid populations present in skeletal muscle at steady state, which in contrast to many other tissues, has not been well-characterized. We found three major subpopulations of myeloid cells: a population of $F4/80^{+}TIM4^{+}$ macrophages that are long term and minimally got replaced by blood monocytes; and two other populations ($F4/80^{+}TIM4^{-}$ and $F4/80^{low}$ $CD11C^{+}MHCII^{+}$) that were in continuous exchange with blood cells. We investigated the function of these subsets and showed that $TIM4^{+}$ macrophages played an indispensable role in clearance of damaged-induced apoptotic cells as in their absence, secondary necrosis of non-engulfed apoptotic cells resulted in unique accumulation of widespread patchy necrotic fibers in later stages of the regeneration process. Our results reveal a distinct role for muscle long-term resident macrophages during the regeneration process and dispute the notion that phagocytic infiltrating macrophages could compensate for their absence. Finally, we found that modulation of colony stimulating factor-1 (CSF-1) dependent resident macrophages in mdx mice improves muscular dystrophy as shown by enhanced muscle resistance to damage from eccentric contraction mainly due to fiber type switching from fast-twitch fibers (type IIB) to type IIX and type IIA fibers. This suggests that CSF-1 receptor inhibitors that are already being tested in clinical trials to treat rare cancers may have therapeutic potential for human Duchenne muscular dystrophies.

Engineering a drug-inducible caspase-9 system in human embryonic stem cells to improve the safety of cell therapies

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Human pluripotent cell lines and their derivatives have great potential to provide cell-based therapies for the treatment of multiple diseases, including type 1 diabetes. To add an additional layer of safety, we have introduced a dual inducible caspase-9/luciferase transgene at the AAVS1 locus of H1 human embryonic stem cells. In the event of possible unfavourable outcomes following cell administration to a patient, the inducible caspase-9 can be used to initiate implant-specific apoptosis by exposure to the AP20187 pro-drug. The luciferase provides a means to monitor cell survival post-implant into live animals and to visualize the efficiency of induced cell death. Preliminary *in vitro* studies have been performed to functionally test this system in two successfully targeted hemizygous clones. The presence of luciferase was confirmed by addition of D-luciferin and an AP20187 time course experiment detected cell death within the first hour of treatment, and over 99% of the cells were eliminated within 24 hours. Notably, we observed several rare genetic events in a small subpopulation of cells that prevented them from responding to the pro-drug treatment. The safety-switch also functioned in cells differentiated to pancreatic progenitors and then exposed to AP20187. Implantation studies in mice will test the functionality of the safety-switch *in vivo,* both in teratomas and in a cell therapy model for diabetes. Further iterations of this transgene design are currently under investigation to target both AAVS1 alleles for functional redundancy and to target a region further upstream within the locus that we predict will be less amenable to potential silencing.

Investigating atrial- and ventricular-specific effects of titin truncating variants in induced pluripotent stem cell-derived cardiomyocytes

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Titin truncating variants (TTNtv) have been associated with structural heart diseases like dilated cardiomyopathy where they have shown an increased propensity to early and life-threatening ventricular arrhythmias. Multiple genetic association studies have also linked TTNtv to early-onset atrial fibrillation in the absence of structural heart disease. The mechanism behind TTN-related arrhythmias that occur without structural disease is unknown.
Existing studies have utilized human induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CMs) to investigate patient-specific TTNtv. However, most studies were conducted in the context of dilated cardiomyopathy and employed cardiac differentiation protocols that generate a mixed population of cardiomyocytes with a predominantly ventricular phenotype. This makes it difficult to identify any atrial-specific effects of TTNtv. By altering retinoid signaling during cardiomyocyte differentiation, we can generate atrial-like hiPSC-CMs to compare potential cell-type specific effects of TTNtv.
Our study focuses on a family carrying a TTNtv, c.55695_55698delCAGC p.(Ser18566Trpfs*25), that was identified through whole exome sequencing of patients with unexplained atrial fibrillation. Two out of five family members are genotype positive and have experienced arrhythmias in the absence of structural disease. One individual has early-onset atrial fibrillation and the other was resuscitated from ventricular fibrillation. To study the specific effects of this TTNtv, we will generate hiPSCs from all family members and use CRISPR-Cas9 to generate isogenic controls. We will study atrial and ventricular hiPSC-CMs using microelectrode arrays and optical mapping of voltage and calcium transients to characterize the cell-type specific electrophysiological profiles of this variant. The findings from this study may further our current understanding of Titin and the role of TTNtv in arrhythmia.

A Simple, Reproducible Method to Generate Red Blood Cells from Human Pluripotent Stem Cells

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Methods to generate erythroid cells from human pluripotent stem cells (hPSCs) are important for disease modeling and therapeutic development, specifically for hemoglobinopathies and other red blood cell (RBC) disorders. PSC-derived erythroid cells also offer a potentially unlimited supply of RBCs for transfusion, especially for rare blood types. Existing protocols for generating RBCs from hPSCs are typically complex, require co-culture with feeder cells, and exhibit large variability between hPSC lines.

We have developed a simple, serum- and feeder-free method for generating erythroid cells from multiple human embryonic stem (H1, H7, H9) and induced pluripotent stem cell lines (WLS-1C, STiPS-F016, STiPS-B004). This protocol involves two phases: specification of hPSCs to hematopoietic progenitor cells (HPCs), followed by their differentiation into erythroid cells. In phase 1, aggregates of hPSCs generated from maintenance cultures are plated at 4-10 aggregates per cm^2 (~400 – 1,000 cells per cm^2 assuming 100 cells per aggregate) onto matrigel-coated wells. The cells undergo mesoderm specification (day 0-3) with subsequent differentiation into HPCs (day 3-10). On day 10, an average of $2.2x10^5$ HPCs were generated per cm^2 (range: $1.3x10^4$ – $7.7x10^5$; 2-11 experiments; 6 cell lines), with 65-99% and 24-88% of cells expressing CD43 and CD34 respectively. In phase 2, the erythroid differentiation phase (day 10-24), HPCs expanded 230-fold (range: 48 - 1036), with 76% (range: 62 - 91%) of cells expressing the erythroid marker Glycophorin A (GlyA). Overall, the cultures yielded $4.0x10^7$ $GlyA^+$ cells per cm^2 (range: $1.8x10^6$ – $3.1x10^8$) after 24 days. Further maturation for 7 days with EPO and human serum resulted in >90% $GlyA^+$ cells. Notably, there was no cell loss in the maturation phase, resulting in a final yield of $9.3x10^7$ (range: $1.5x10^7$ – $4.13x10^8$) $GlyA^+$ cells per cm^2 on day 31. Differentiated cells exhibited orthochromatic normoblast morphology and low CD71 expression, consistent with erythroid maturation. Erythroid cells expressed a mix of 'primitive' and 'definitive' hemoglobin types, with higher levels of adult and fetal than embryonic hemoglobin.

In summary, we have developed a two-step, serum- and feeder-free method to generate large numbers of erythroid cells from multiple hPSC lines, providing a standardized and reproducible platform for basic and translational research.

Title: Characterization and application of a novel microsphere-laden bioink for generating stem cell-derived neural tissues

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3D bioprinting can fabricate precisely controlled 3D tissue constructs with tissue-like complexity. The development of novel bioinks requires that they mimic the extracellular environment by supporting cell adhesion, proliferation, and differentiation after printing. The mechanical properties of bioinks such as the gel stiffness and porosity should match the properties of the target tissue *in vivo*. Here we used the RX1 bioprinter from Aspect Biosystems to print dome shaped neural tissue constructs using our fibrin-based bioink containing drug releasing microspheres and neural progenitors derived from human induced pluripotent stem cells (hiPSCs). The constructs contained three different types of microspheres that released guggulsterone, retinoic acid, and purmorphamine as these small molecules promote differentiation into mature neural tissues similar to that found in the brain. We evaluated how different concentrations and combinations of microspheres affected the composition of the bioprinted tissues. We also printed acellular constructs that were used for rheological testing. However, the rheological properties of soft materials such as elastic moduli are difficult to measure precisely using pre-existing protocols such as atomic force microscopy or shear rheology. This procedure involves spherical indenters that are positioned on top of the bioink samples, generating an indentation depth that is then correlated with elastic modulus. Here, we measured the elastic moduli of our fibrin-based bioink with or without microspheres with a direct method using a modified Hertz model for thin films. Overall, we demonstrate the versatility of our ink and how incorporation of drug releasing microspheres can alter the composition of our bioprinted tissues.

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A feedforward controller for decoupling resource-limited genetic modules in mammalian cells

Synthetic biology has the potential to bring forth advanced genetic devices for applications in regenerative medicine. However, accurately predicting the behavior of engineered genetic devices, especially across diverse cell types, remains difficult due to lack of modularity, wherein a device's output does not depend uniquely on its intended inputs but also on its context. One contributor to lack of modularity is competition among genes for shared cellular resources, such as those required for transcription and translation, which can induce `coupling' among otherwise independently-regulated genes. Here we develop an endoribonuclease (endoRNase)-based incoherent feedforward loop (iFFL) to make gene expression levels robust to changes in resource availability. We demonstrate that this iFFL can accurately control gene expression levels in the presence of significant resource sequestration by transcriptional activators (*i.e.* 'squelching'). Across various mammalian cell lines, the iFFL output level is robust to resource sequestration, indicating that the iFFL broadly mitigates the effects of cellular context on gene expression. In addition, the iFFL improves the precision of gene expression levels across cell lines, reducing another important source of context-dependence that may result from differences in resource availability in different cell types. Finally, the iFFL output is also robust to cell-to-cell variation in DNA copy number, substantially improving upon previously-described miRNA-based iFFLs. Ultimately, endoRNase-based iFFLs will enable predictable, robust, and context-independent control of gene expression in mammalian cells, opening the door for precision control of cell fate decisions and of the behavior of cell therapies.