4

IDENTIFY NOVEL THERAPIES FOR VASCULAR DISEASE AND REGENERATIVE MEDICINE BASED ON THE LANDMARK DISCOVERY OF INDUCED PLURIPOTENT STEM CELLS

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Cardiovascular disease (CVD) is the leading cause of death globally, in 2013 it was responsible for 30% of all deaths worldwide. It has been shown that endothelial dysfunction is a major pathological mechanism that precedes CVD, yet these damaged endothelial cells (ECs) display poor regenerative capabilities. Current therapy is inadequate or unsuitable for a significant number of patients, however it is thought that the ability to replace damaged ECs could serve as a valuable therapeutic option for the future. Recent capability to generate induced pluripotent stem cells (iPSCs) from adult cells and differentiate these towards ECs holds great promise in the area of regenerative medicine. Nevertheless, the differentiation process involved is complex and not fully understood. An appreciation of a variety of molecular mechanisms is required in order to enhance protocols that could be replicated for novel therapies. This project has derived ECs from iPSCs using a reliable protocol. Results here confirmed successful differentiation with an up-regulation in important endothelial markers at the mRNA level alongside positive immunofluorescent staining. These differentiated cells were characteristically similar to mature ECs. In order to expose underlying mechanisms of EC differentiation, the novel gene SETSIP was shown to be upregulated during this process. Further investigation to stimulate differentiating cells with a SETSIP peptide noted an induction in endothelial markers. Finally, experiments involving epigenetic drugs induced changes in both endothelial marker and SETSIP expression. This project has elucidated the role of SETSIP and data shown here may be important in advancing therapeutic potential through the development of more robust protocols concerning EC differentiation.

5

RESIDENT S100 β /SCA1⁺ MULTIPOTENT VASCULAR STEM CELLS UNDERGO MYOGENIC AND VASCULOGENIC DIFFERENTIATION *IN VITRO*

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Arteriosclerosis is an important age-dependent disease that encompasses atherosclerosis, in-stent restenosis, autologous bypass grafting and transplant arteriosclerosis. Vascular smooth muscle (vSMC)-like accumulation is a key event leading to intimal-medial thickening (IMT), vessel remodelling and an important marker of subclinical disease. Vascular stem cell progeny in addition to de-differentiated SMC and/or SMC derived from endothelial-mesenchymal transition (EndoMT) are all reported to contribute to IMT as they become activated/dysfunctional, differentiate down vascular and myeloid lineages and subsequently dictate, in-part, vessel remodelling. In this study we examined the multipotent potential of a

specialised population of rat adult resident multipotent vascular stem cells (rMVSC) located within the vessel wall and their capability to differentiate down both myogenic (muscle) and vasculogenic (endothelial) lineages when given the appropriate stimulus. Using Sca1-eGFP transgenic mice *in vivo*, there was a significant increase in the number of Sca1⁺ cells within the intima of the left carotid artery (LCA) following partial carotid artery ligation-induced injury after 3 days that co-localised with endothelial nitric oxide synthase (eNOS) and CD31⁺ positive cells, when compared to the sham-operated control vessels and the contralateral right carotid artery (RCA). The number of Sca1-eGFP⁺ cells significantly increased over time within the adventitial, medial and neointimal layers following ligation-induced injury after 7 and 14 days, respectively. *In vitro*, S100 β /Sca1⁺ rat MVSCs cultured in vasculogenic inductive media for 7 days underwent differentiation to an endothelial cell phenotype characterised by the appearance of a cobblestone morphology and increased eNOS expression. In contrast, MVSC exposed to media supplemented with TGF- β 1 for 7 days underwent myogenic differentiation to SMC-like cell. These data suggest that resident S100 β /Sca1⁺ MVSCs are capable of both myogenic and vasculogenic differentiation depending on the inductive stimulus and may contribute in part to intimal medial thickening (IMT) and endothelial regeneration following injury *in vivo*.

6

PARALLEL ASSESSMENT OF CELL VIABILITY IN CARDIAC AND CANCER CELLS FOLLOWING TREATMENT WITH SUNITINIB

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Various cardiopathological effects have been observed following chemotherapy treatment in cancer patients, due to anti-cancer drug-induced cardiotoxicity (CTX). A retrospective study of cancer survivors reported a 50% and 10% incidence of hypertension and heart failure respectively following treatment with the tyrosine kinase inhibitor sunitinib, licensed to treat pancreatic neuroendocrine tumours. The cellular mechanisms underlying CTX are not known. Here, for the first time, we compare the potency of sunitinib in both cardiac cells (primary cardiac fibroblasts (CFs)) and cancer cells (a pancreatic adenocarcinoma cell line (PANC-1)).

Adult rat CFs were isolated by bulk collagenase digestion, maintained in culture and used between passages 1–2. PANC-1 cells, from previously-frozen stocks, were used between passages 41–49. Cells were treated with sunitinib (0–10 μ M in CFs; 0–100 μ M in PANC-1) for 24 hour prior to epifluorescent imaging for phenotypic assessment. Cell viability was examined by alamar blue assays following 24 hour sunitinib treatment (0–100 μ M).

Overall, results indicated increased sensitivity of CFs to sunitinib compared with PANC-1 cells. Phenotypic changes indicative of cell death, including appearance of intracellular vacuoles, were evident in CFs following 1 μ M sunitinib treatment whereas similar effects were not induced until 10 μ M treatment in PANC-1 cells. Alamar blue assays demonstrated a dramatic increase in CF death compared to PANC-1 death following treatment with 10 μ M sunitinib (11.6 $\pm$ 0.02 vs 56.5

± 1.5 (% viability) CF vs PANC-1, $n=3$). A lower IC_{50} value for sunitinib was required to exert the same effects on CF ($IC_{50} 5.2 \mu M$) vs PANC-1 ($IC_{50} 13.5 \mu M$) cell viability.

These results suggest sunitinib can cause lethal effects in cardiac cells at lower doses than those required to induce pancreatic cancer cell death. Future work will aim to identify cellular mechanisms responsible for these toxic effects. Parallel studies in cardiac and cancer cells will be beneficial in distinguishing how focused anti-cancer drug delivery could be improved to avoid CTX.

7 NOX4 NADPH OXIDASE IS A KEY REGULATOR OF ENDOTHELIAL CELL FUNCTION IN EXPERIMENTAL DIABETES

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Introduction The characteristic hyperglycaemia of diabetes drives reactive oxygen species (ROS) production in endothelial cells (ECs), leading to microvascular dysfunction and cardiovascular complications. NADPH oxidases are enzymes whose primary function is to generate ROS, and which contribute to development and progression of cardiovascular disease. This study aimed to investigate the role of the NOX4 isoform, which is highly expressed in ECs, under hyperglycaemic conditions, in EC signalling and paracrine communication with fibroblasts, as a major determinant of diabetic cardiovascular remodelling.

Methods Human aortic endothelial cells (HAoECs) were treated with normal (NG, 5.5 mM) or high (HG, 25 mM) glucose for up to 5 days with or without NOX4 siRNA knockdown (KD) prior to assessment of mRNA expression (real-time RT-PCR; relative to β -actin) and superoxide generation (DHE fluorescence). NIH 3 T3 fibroblasts were treated with conditioned media from NOX4-modified HAoECs for 24 hour to interrogate effects on paracrine signalling.

Results HG treatment of HAoECs for 5 days increased mRNA expression of NOX4 (NG 1.01 ± 0.06 , HG 1.27 ± 0.06 ; $n=9$, $p<0.05$) and associated antioxidant and proinflammatory genes (e.g. NRF2: 0.91 ± 0.04 vs 1.22 ± 0.04 ; IL-6: 1.03 ± 0.13 vs 2.59 ± 0.58 ; $n=6$, $p<0.05$). This was associated with increased superoxide production (262 ± 12 vs 338 ± 11 arbitrary units; $n=4$, $p<0.05$) at 2 but not 5 days. Interestingly, NOX4 KD under HG conditions increased mRNA expression of endogenous antioxidant enzymes after 2 days (e.g. NRF2: 1.90 ± 0.06 vs 2.21 ± 0.06 ; $n=3$, $p<0.05$) whilst normalising increased superoxide production. Furthermore, increased TGF β -induced differentiation of NIH 3 T3 fibroblasts observed in the presence of conditioned media from HG-treated HAoECs was ablated by NOX4 KD (e.g. α -SMA: scrambled control 1.31 ± 0.04 , NOX4 KD 0.94 ± 0.07 ; $n=3$, $p<0.05$).

Conclusions HG-induced NOX4 signalling regulates ROS production and endogenous antioxidant expression in ECs, driving paracrine stimulation of fibroblast differentiation. It therefore seems likely that EC NOX4 NADPH oxidase signalling contributes significantly to adverse diabetic cardiovascular remodelling.

8 HEDGEHOG RESPONSIVE STEM CELL ANTIGEN-1/S100 β RESIDENT VASCULAR STEM CELLS CONTRIBUTE TO NEOINTIMAL FORMATION

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Intimal medial thickening (IMT) and vascular remodelling are hallmarks of arteriosclerotic disease. The origin of neointimal cells and the signalling molecules that direct their fate and function is controversial. Here, we demonstrate that Hedgehog (Hh) responsive S100 β ⁺/Sca1⁺ perivascular stem cells substantially contribute to IMT within carotid arteries of transgenic mice following ligation-induced injury *in vivo*. Genetic lineage tracing analysis using S100 β -eGFP/Cre/ERT2 transgenic mice to mark resident vascular stem cells before injury demonstrated that Hh responsive S100 β ⁺/Sca1⁺ cells substantially contribute to IMT, an effect significantly attenuated following treatment with the Hh smoothened inhibitor, cyclopamine. *In vitro*, recombinant SHh (rSHh) treatment of multipotent S100 β ⁺/Sca1⁺ resident stem cells increased target gene Gli expression, decreased telomerase activity, and promoted myogenic differentiation and cell growth; effects significantly attenuated following Hh inhibition. These findings suggest that perivascular S100 β ⁺/Sca1⁺ stem cells are a major source of neointimal cells contributing to IMT and suggest that this cohort may be a relevant therapeutic target to prevent arteriosclerosis.

9 ENDOTHELIAL NOX4 NADPH OXIDASE PROTECTS AGAINST ADVERSE CARDIAC REMODELLING ASSOCIATED WITH EXPERIMENTAL DIABETES

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Background Chronic heart failure (CHF) is a major cause of mortality in diabetes due to characteristic alterations in cardiac structure and function. The diabetic heart is typified by increased fibrosis, inflammation and microvascular remodelling together with hyperglycaemia-induced endothelial dysfunction and reactive oxygen species (ROS) generation, which may predispose to cardiovascular stress. The aim of this study was to investigate the specific contribution of endothelial Nox4 NADPH oxidase, a major source of cardiovascular ROS, to cardiac remodelling in experimental diabetes.

Methods Diabetes was induced in endothelial-specific Nox4 transgenic (Tg) mice and WT littermate controls (10–12 weeks of age; $n=8$ –12/group) by streptozotocin (STZ) injection. After 6 months, echocardiography was performed and blood and cardiac tissue collected for metabolic and gene expression analyses, respectively.

Results Endothelial Nox4 overexpression did not affect blood glucose or HbA1c levels in control or diabetic animals.