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ANTI-CANCER TYROSINE KINASE INHIBITORS INCREASE OXIDATIVE STRESS IN PRIMARY CARDIAC FIBROBLASTS

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Anti-cancer tyrosine kinase inhibitors (TKI) are known to exert cardiotoxicity that can result in heart failure. Here we have used primary adult rat cardiac fibroblasts (CFs) to examine whether imatinib and sunitinib (both TKIs) increase oxidative stress and cause increased oxidation and activation of calcium/calmodulin dependent protein kinase II (CaMKII). Activation of CaMKII is known to be important for cellular function but excessive activation is linked to cardiac pathology.

CFs, isolated from adult Wistar rat hearts, were maintained in culture and treated over 24 hour with the TKIs sunitinib and imatinib (0.1–20 μ M) in serum-free medium (DMEM). Cell phenotype and growth was monitored using a Nikon Eclipse (TE300) inverted microscope and ROS assays performed using a specially designed assay kit (Abcam, UK) which uses DCFDA to measure hydroxyl, peroxy and other ROS activity. Quantitative immunoblotting was used to assess ox-CaMKII protein levels.

Treatment with both TKIs caused a dose-dependent decrease in cell viability. Sunitinib exerted more toxic effects at lower concentrations than imatinib. Decreased cell viability was evident at concentrations as low as 1 μ M for sunitinib with dramatic cell loss (>50%) evident at 10–20 μ M. Significantly increased ROS levels (using the DCFDA-based assay) were observed in the presence of both imatinib (4956 $\pm$ 102 vs 1984 $\pm$ 358 (imatinib vs vehicle (a.u.), n=3, p<0.05) and sunitinib (15186 $\pm$ 3856 vs 1984 $\pm$ 358 (sunitinib vs vehicle (a.u.), n=3, p<0.01). Quantitative immunoblotting suggested that ox-CaMKII levels were increased following treatment with either 1 μ M imatinib (1.4-fold (ox-CaMKII/GAPDH)) or 1 μ M sunitinib (1.2-fold (ox-CaMKII/GAPDH)) however at higher concentrations (particularly for sunitinib) increased cell loss and resultant loss of total protein was observed.

In conclusion, both imatinib and sunitinib cause increased oxidative stress in adult primary CFs with resultant activation of CaMKII. These effects are evident at lower concentrations of sunitinib than for imatinib and reflect the increased cardiotoxicity of this TKI reported in the patient.

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STEM-CELL DERIVED MYOGENIC PROGENY ENRICH FOR VASCULAR SMOOTH MUSCLE CELL EPIGENETIC MARKS AT THE MYOSIN HEAVY CHAIN 11 PROMOTER *IN VITRO*

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Epigenetics plays a critical role in SMCs differentiation where histone proteins associated with the myosin heavy chain 11 (*Myh11*) promoter are post-translationally modified while promoter sequences are de-methylated by the DNA-modifying

enzyme ten-eleven translocation-2 (TET2). Di-methylation of lysine 4 on histone 3 (H3K4me2) is generally associated with euchromatin and active gene expression whereas, tri-methylation of lysine 27 on histone 3 (H3K27me3) is generally associated with formation of heterochromatin and gene suppression. Our aim was to determine the level of H3K4me2 and H3K27me3 enrichment on *Myh11* promoter of differentiated SMCs and compare that signature to undifferentiated resident vascular stem cells and their stem cell-derived myogenic progeny. Murine Sca1⁺ stem cells (APCs), rat medial Sox10⁺ multipotent vascular stem cells (MVSCs) and CD44⁺ bone-marrow derived mesenchymal stem cells (MSCs) were all examined for H3K4me2 and H3K27me3 enrichment on *Myh11* promoter, before and after myogenic differentiation and compared to freshly isolated aortic differentiated SMCs and sub-cultured 'de-differentiated SMCs' *in vitro* by Chromatin Immunoprecipitation (ChIP) assay. Differentiated rat and murine SMCs were highly enriched for H3K4me2 mark at the *Myh11* promoter compared to the H3K27me3 mark, concomitant with enhanced TET-2 binding. Phenotypical 'de-differentiated' sub-cultured rat and murine SMC were also enriched for H3K4me2 mark when compared to H3K27me3, but to a much lesser extent. In contrast, all three stem cell populations were highly enriched for H3K27me3. The levels of enrichment of H3K27me3 significantly reduced following myogenic differentiation with TGF- β 1 concomitant with a significant enrichment of H3K4me2 mark and TET-2 binding to levels that mimicked the level of enrichment in de-differentiated SMCs at the same locus. We conclude that stem-cell derived myogenic progeny enrich for the H3K4me2 mark at the *Myh11* promoter and thus may be responsible for the presence of this mark within arteriosclerotic cells following injury.

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GENERATION OF χ -SECRETASE INHIBITOR-LOADED PLGA-Fe₃O₄ MAGNETIC NANOPARTICLES

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Stem cell derived myogenic progeny play important roles in the pathophysiological processes of various vascular disorders, such as arteriosclerosis, atherosclerosis, and in-stent restenosis. Targeting these cells is an attractive therapeutic strategy for treating vascular remodelling. However, while polymer-coated DES have significantly reduced the incident of in-stent restenosis, current DESs lack the fundamental capacity for (i) adjustment of the drug dose and release kinetics and the (ii) ability to replenish the stent with a new drug on depletion. This limitation can be overcome by a strategy combining magnetic targeting via a uniform field-induced magnetization effect and a biocompatible magnetic nanoparticle (MNP) formulation designed for efficient entrapment and delivery of specific drugs that target the resident vascular stem cell source of the SMC. Magnetic nanoparticles (MNP's) containing magnetite (Fe₃O₄) were fabricated, polymer coated with poly (DL-lactide-co-glycolide) polyvinyl alcohol [PLGA-PVA] and loaded with a χ -secretase inhibitor (GSI) of Notch signalling, DAPT using an oil in water emulsification technique. The free GSI's and GSI-loaded MNP's were assessed for drug release, the efficacy at

controlling mesenchymal stem cell (MSC) growth (proliferation and apoptosis) and inhibiting myogenic differentiation under magnetic and non-magnetic conditions. The DAPT-loaded MNPs had an average hydrodynamic diameter of 351 d.nm. Up to 40% of drug was released from MNPs within 48 hours rising to 65% after 1 week under magnetic conditions. The Notch ligand, Jagged1 increased Hey1 mRNA levels and promoted myogenic differentiation of MSCs *in vitro* by increasing SMC differentiation markers, myosin heavy chain 11 (Myh11) and calponin1 (CNN1) expression, respectively. This effect was significantly attenuated following treatment of cells with MNP's loaded with DAPT when compared to unloaded MNP's. Notch GSI -loaded magnetic nanoparticles are functional at targeting vascular stem cells *in vitro*.

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CHARACTERISATION OF RESIDENT MULTIPOTENT VASCULAR STEM CELLS (MVSCs) FROM SUSCEPTIBLE AND NON-SUSCEPTIBLE ARTERIOSCLEROTIC REGIONS OF THE MOUSE AORTA

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Vascular remodelling leading to arterial obstruction is a hallmark of arteriosclerosis and in-stent restenosis and is due in part to the accumulation of vascular smooth muscle (SMC)-like cells within the vessel wall. The source of these vascular cells has been controversial with many studies providing

compelling evidence for a putative role for stem cell-derived myogenic progeny. It is known that neuroectoderm-derived (NE) vascular regions (ascending aorta, aortic arch, carotid artery) are more susceptible to arteriosclerotic lesion formation in comparison with paraxial mesoderm-derived (PM) regions (descending and abdominal aorta, femoral artery). Our aim was to isolate and characterise stem cells from arteriosclerotic-susceptible and non-susceptible regions and determine their differential responsiveness to discrete myogenic inductive stimuli. A population of myosin heavy chain (Myh11⁻) negative, Sca1/S100 β /Nestin⁺ multipotent vascular stem cells (MVSCs) was first shown to accumulate within the intima of murine carotid arteries following injury using e-GFP Sca1/S100 β transgenic mice. Resident MVSCs were isolated from mouse aorta arch (susceptible) and descending aorta (non-susceptible) and grown in B27 supplemented maintenance media. Cells were characterised by fluorescent immunocytochemistry using stem (Sca1/S100 β /Nestin) and vascular SMC differentiation cell markers (Cnn1 and Myh11). MVSCs from both aortic arch and descending aorta were positive for stem cell markers but negative for Myh11 and Cnn1. Treatment of both cell populations with TGF- β 1 or the Notch ligand, Jagged-1 for 7 and 14 days promoted myogenic differentiation by increasing the number of cells expressing SMC markers concomitant with increased Myh11 and Cnn1 mRNA levels, respectively. Moreover, MVSCs from the NE susceptible regions significantly increased their expression of Myh11 in response to Jagged1, when compared to PM derived cells. We conclude that the aortic arch and descending aortic regions both house a neuroectoderm-derived Sca1/S100 β /Nestin⁺ stem cell population that responds to myogenic inductive stimulation *in vitro*.