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Impact of Reactive Oxygen and Reactive Nitrogen Species for Stem Cell Mobilization, Function and Cardiovascular Differentiation

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1. Introduction

Reactive oxygen species (ROS), i.e. substances like hydrogen peroxide (H_2O_2), the superoxide anion (O_2^-) or the highly reactive hydroxyl ion (HO^-) as well as reactive nitrogen species (RNS) with nitric oxide (NO) as its most important member are ideally suited to serve as signaling molecules since they are locally generated, are highly and rapidly diffusible and can be neutralized by a bulk of anti-oxidative agents organized in the cellular anti-oxidative defense system. So far the signaling pathways regulating organ-specific differentiation of stem cells are largely unknown. Differentiation processes of stem cells embedded in tissues and organs are tightly regulated by the cellular microenvironment which is critically determined by the availability of nutrients and oxygen as well as by the balance of ROS and NO generation. Already during early embryogenesis NADPH oxidases and NO synthases are expressed in the growing embryo, suggesting that gradients of ROS and NO may exist in the developing organs and may be involved in proper functioning of commitment programs. During pathophysiological insults, e.g. during hypertension, atherosclerosis and cardiac infarction high levels of ROS and NO are generated, thus creating an inflammatory microenvironment which on one side contributes to cell damage, apoptosis and remodeling, however, on the other side may activate repair processes that involve recruitment and differentiation of stem cells of the cardiovascular cell lineage. During recent years emerging evidence suggests that ROS and RNS are involved in cardiovascular differentiation of embryonic (ES) and adult stem cells. Comparable effects may occur during differentiation processes of resident cardiac stem cells. A pivotal role for NADPH oxidases and NO synthases in cardiomyogenesis and vasculogenesis of ES cells has been recently outlined. In this chapter the current knowledge on activation, recruitment and differentiation of different cardiovascular stem cell populations by ROS and NO and the involved signal transduction cascade is reviewed. Furthermore the specific microenvironmental requirements for proper stem cell engraftment and maintenance are outlined.

2. Hypoxia and the anti-oxidative defense of stem cells

Life on earth requires oxygen for generating energy by metabolic processes. Paradoxically, life is on the same time threatened by oxygen, since highly reactive ROS may destruct cellular components such as DNA, proteins and lipids by oxidation. Organisms have acquired a complex system of antioxidant metabolites and antioxidative enzymes to prevent oxidative damage to cellular components. This system comprises of divergent agents such as ascorbic acid (Vitamin C), polyphenols, flavonoids, tocopherols, uric acid, glutathione and thioredoxins as well as antioxidant enzymes e.g. peroxiredoxins, superoxide dismutase and catalase. These antioxidant molecules may be supplemented by the antioxidative function of other enzymes that are not directly related to the anti-oxidative defense, like the sirtuins which are a phylogenetically conserved NAD+-dependent protein deacetylase/ADP-ribosyltransferase family implicated in diverse biological processes. Treatment of mouse preimplantation embryos with sirtuin inhibitors resulted in increased intracellular ROS levels and decreased blastocyst formation. These effects were recapitulated by siRNA-induced knockdown of Sirt3, which is involved in mitochondrial energy metabolism, and in Sirt3-/- embryos (Kawamura et al., 2010). Furthermore Prdm16, a transcription factor that regulates leukaemogenesis, palatogenesis and brown-fat development has been demonstrated to be involved in the maintenance of stem cell function by modulating the intracellular redox state. In stem cells of the haematopoietic and nervous systems, Prdm16 deficiency led to increase in ROS levels, depletion of stem cells, increased cell death and altered cell-cycle distribution. This could be rescued in the presence of the anti-oxidant, N-acetyl-cysteine (Chuikov et al., 2010). Previously it was assumed that antioxidant systems either prevent reactive species from being formed, or remove them before they can damage vital components of the cell. However, research of the last decade has unraveled a decisive role of ROS and RNS in cellular signaling where reactive species are involved in growth factor, hormone and cytokine function. From this time on it was speculated that the function of the antioxidative system is to keep ROS and RNS at an optimum homeostatic level that allows the regulation of specific signaling pathways. Apparently physiological levels of ROS are necessary to maintain genome stability in ES cells and cardiac stem cells. Interestingly antioxidants suppressed DNA damage at low concentrations, but potentiated such damage at higher concentrations. High-dose antioxidants decreased cellular levels of ATM (ataxia-telangiectasia mutated) and other DNA repair enzymes (Li & Marban, 2010). How the redox state inside cells is balanced is yet not known. Current methods of cellular analysis are apparently not sensitive enough to discriminate the time and spatial distribution of antioxidants within cells. It may be speculated that distinct combinations of antioxidant molecules are spatially distributed within cells and/or may be bound to specific cell structures thus allowing the generation of gradients of the anti-oxidative defense within cells. These gradients may be further diversified by the different anti-oxidative strength of members of the anti-oxidative capacity as well as different time durations of the chemical reactions that lead to the neutralization of the respective reactive species and termination of the respective signal transduction pathway.

During embryogenesis the early embryo is extremely sensitive towards oxidative stress. Extensive research on in vitro fertilization and embryo transfer has demonstrated that during embryo culture cell culture media have to be supplemented with distinct cocktails of anti-oxidants to avoid serious tissue damage (Agarwal et al., 2008). In the morula state the

early embryo passing through the ovary duct as well as the blastocyst prior to implantation in the uterus is subjected to severe hypoxia. Since hypoxia via hypoxia-activated transcription factors can regulate gene expression it has been speculated that the low oxygen pressure present in early embryo may direct the earliest possible differentiation steps (Simon & Keith, 2008). Also during early organogenesis, the embryo is in a state of relative hypoxia associated with a major decrease in terminal electron transport system activity and a marked increase in anaerobic glycolysis (Shepard et al., 2000). Hypoxia can be harmful to the embryo which requires the development of protection mechanisms. As recently described one of those may involve the adenosine A1 receptor (A1R) since $A1R^{-/-}$ embryos were more sensitive towards ischemic stress then normoxic controls and just the heart was identified as the site of A1R-mediated embryo protection (Wendler et al., 2007; Wendler et al., 2010). The susceptibility of the very early embryo towards oxidative stress may imply that also stem cells need either a low oxygen and/or high anti-oxidative status for stem cell maintenance. Indeed it has been demonstrated that human ES cells remained in an undifferentiated state when cultivated under low oxygen conditions (Ezashi et al., 2005). Furthermore it has been shown that bovine blastosysts produced under reduced O_2 (< 2% O_2) tensions displayed significantly more inner cell mass cells displaying pluripotency (Harvey et al., 2004). In the adult one of the major sources for stem cells is the bone marrow. The bone marrow microenvironment displays a lower oxygen concentration than other tissues, and stem cells are localized within the hypoxic regions thus suggesting that hypoxia may be crucial for stem cell maintenance. In hypoxic human ES cells hypoxia inducible factor-1α (HIF-1α), a principal mediator of hypoxic adaptations, modulates Wnt/β-catenin signalling by enhancing β-catenin activation and expression of the downstream effectors LEF-1 and TCF-1. O_2 availability, therefore, may have a direct role in stem cell regulation through HIF-1α modulation of Wnt/β-catenin signalling (Mazumdar et al., 2010). In a further study it has been recently shown that external addition of anti-oxidant and low oxygen tension results in reprogramming of human adipose stromal cells towards a more primitive stem cell type (Jee et al., 2010). HIF-2α but not HIF-1α is a direct upstream regulator of the stemness gene Oct-4 since HIF-2α was shown to be capable of binding hypoxic regulatory elements in the murine *Oct-4* promotor (Covello et al., 2006).

3. ROS generation during embryogenesis

During later stages of embryogenesis, when the utero-placental circulation is established the embryo is more capable to defend against oxidative stress which is due to a stronger anti-oxidative stress response and at least partially due to the metabolic switch from glycolysis to oxidative phosphorylation which occurs at times where the embryonic heart starts to contract, thus requesting more energy for heart performance. Despite the sensitivity of the early embryo towards oxidative stress few studies demonstrated that ROS at very low concentrations are already actively generated during the blastocyste state in rabbits and in postimplantation mouse embryos harvested on day 8 of pregnancy; here ROS generation is localized to the trophoblast cell layers (Gagioti et al., 1995). Moreover, placental NADPH oxidase-mediated ROS generation occurs in women during early pregnancy and may contribute to elevated ROS levels in embryos (Raijmakers et al., 2006). These data suggest that very low but physiologically relevant concentrations of ROS may already be involved in developmental processes during organogenesis and differentiation of stem cells from the inner cell mass. The meaning of ROS during later stages of organ maturation and

morphogenesis is not well defined but may at least be involved in neuronal, cardiac and vascular growth, where ROS have been shown in several studies to be involved in growth factor and cytokine-mediated signaling pathways such as the vascular endothelial growth factor/flk-1 (VEGF/flk-1) (Roy et al., 2008), platelet-derived growth factor BB (PDGF-BB) (Lange et al., 2009), cardiotrophin-1 (CT-1) (Sauer et al., 2004) and nerve growth factor (NGF)-mediated signaling pathways (Suzukawa et al., 2000). These pathways are associated to vasculogenesis, angiogenesis as well as the development of the central and peripheral nerve system, where ROS may be involved in the regulation of axon guidance through semaphorin 3A (Schwamborn et al., 2004). Furthermore, high levels of ROS have been implicated in site-specific cell death in inter-digital regions of the developing limb (Schnabel et al., 2006), where peroxidase activity and glutathione peroxidase-4 gene (Gpx4) expression were restricted to the non-apoptotic tissue (e.g., digits) of the developing autopod, thus suggesting that differential tissue growth may be regulated by redox gradients which are determined by distinct expression patterns of anti-oxidant molecules.

4. Oxidative stress during myocardial infarction – a potential stimulus for stem cell activation

During cardiovascular repair processes embryonic genes are activated, suggesting that comparable signaling pathways are involved in embryonic development of the cardiovascular system and in cardiac repair during adult live. During hypertension and hypertrophic cardiac growth (Akki et al., 2009; Anilkumar et al., 2009) but also in acute myocardial infarction (Hori & Nishida, 2009; Di Lisa et al., 2007; Webster et al., 2006), ROS are generated in the ischemic myocardium especially after reperfusion. ROS in high concentrations directly injure the cell membrane and cause cell death. However, ROS in low concentrations also stimulate signal transduction to elaborate inflammatory cytokines, e.g. tumour necrosis factor-α (TNF-α), interleukin (IL)-1β and -6, in the ischemic region and surrounding myocardium as a host reaction. These inflammatory cytokines regulate cell survival and cell death in the chain reaction with ROS (Frangogiannis, 2008). Other cytokines like transforming growth factor-β (TGF-β) are upregulated upon inflammation (Czarkowska et al., 2004), suggesting that TGF-β signaling may be crucial for repression of inflammatory gene synthesis in healing infarcts, presumably by mediating resolution of the inflammatory infiltrate. Furthermore TGF-β may play an important role in modulating fibroblast phenotype and gene expression, promoting extracellular matrix deposition in the infarct by upregulating collagen and fibronectin synthesis and by decreasing matrix degradation through induction of protease inhibitors (Frangogiannis, 2008). TGF-β is also a key mediator in the pathogenesis of hypertrophic and dilative ventricular remodeling by stimulating cardiomyocyte growth and by inducing interstitial fibrosis (Ellmers et al., 2008). Furthermore TGF-β has been demonstrated to enhance cardiomyogenesis of mouse ES cells, thus suggesting that stem cell differentiation requires a paracrine pathway within the heart (Behfar et al., 2002).

5. Stem cells within the heart and potential redox-regulated signaling pathway involved in stem cell proliferation and specification

Cardiac repair following myocardial injury is restricted due to the limited proliferative potential of adult cardiomyocytes. The ability of mammalian cardiomyocytes to proliferate

is lost shortly after birth as cardiomyocytes withdraw from the cell cycle and differentiate. However, recent research using integration of carbon-14, generated by nuclear bomb tests during the Cold War, into DNA to establish the age of cardiomyocytes in humans revealed that cardiomyocytes indeed renew, with a gradual decrease from 1% turning over annually at the age of 25 to 0.45% at the age of 75. Fewer than 50% of cardiomyocytes are exchanged during a normal life span (Bergmann et al., 2009). In contrast Hsieh et al did not find significant cardiac repopulation to occur during normal aging in mice, However, they found cardiomyocyte repopulation, albeit modest, by endogenous progenitors following injury, e.g. during cardiac infarction (Hsieh et al., 2007), thus suggesting that cardiac repair and renewal processes may occur through stem cell-mediated cell replacement.

The cellular basis for the exchange of cardiomyocytes during human life is not yet known but could be comparable to mice due to mobilization of bone marrow-derived stem cells (BMSC) and/or the activation of resident stem cells in the heart. Several studies on patients have shown that myocardial infarction results in mobilization of various populations of BMSCs which may be involved in cardiac repair processes (Leone et al., 2005; Leone et al., 2005; Leone et al., 2006; Wojakowski et al., 2009; Wojakowski et al., 2006) Besides BMSCs and circulating multipotent progenitor cells (Cesselli et al., 2009) several populations of resident cardiac stem cells have been described during recent years. In the early embryo, progenitor cells in pharyngeal mesoderm contribute to the rapid growth of the heart tube during looping morphogenesis. These progenitor cells constitute the second heart field and were first identified in 2001 (Rochais et al., 2009). Side population (SP) cells residing within the adult heart and comprising about 1 % of all cells were identified in 2002 by Hierlihy et al. who used the Hoechst 33342 dye exclusion procedure which was previously used to isolate stem cell populations expressing ATP-binding cassette (ABC) membrane transporters, e.g. P-glycoprotein, which confers multidrug resistance in cancer disease (Hierlihy et al., 2002). Upon coculture of SP cells from GFP^+ mice with adult cardiac cells from wild type mice this cell population gained positive α-actinin immunoreactivity, suggesting that a cardiac phenotype was attained (Martin et al., 2004). A subpopulation of SP cells comprising approximately 10% of the total SP cells expressing the stem cell marker Sca-1 was identified by Pfister et al. in 2005. This cell population was negative for the endothelial cell marker CD31, expressed Nkx2.5 and GATA-4, but not α-actinin or α-MHC. The cells could be differentiated into a more mature cardiac phenotype upon coculture with ventricular cardiomyocytes (Pfister et al., 2005). Upon cardiac infarction the CD31 negative cell population in the heart was depleted both within the infarct and non-infarct areas. SP pools were subsequently reconstituted to baseline levels within 7 days after myocardial infarction, through both proliferation of resident SP cells, as well as through homing of BMSCs to specific areas of myocardial injury and immunophenotypic conversion of BMCs to adopt a SP phenotype (Mouquet et al., 2005). Besides the SP cell population $Sca\text{-}1^+$ $c\text{-}Kit^-$ cells have been reported to be present in the mouse heart (Tallini et al., 2009), and so-called cardiospheres were isolated by mild enzymatic digestion of mouse and human heart tissues (Messina et al., 2004). A further resident stem cell population within the heart are $Isl1^+$ cells which express the islet-1 (Isl1) LIM homeodomain transcription factor (Laugwitz et al., 2005). $Isl1^+$ cells give rise to cardiomyocyte, endothelial, and smooth muscle lineages *in vitro* and may be involved in embryonic development of the coronary artery tree and for coronary artery growth. Previously it was shown that $Isl1^+$ cells with the transcriptional signature of $Isl1^+/Nkx2.5^+/flk1^+$ defines a multipotent cardiovascular progenitor which is not only capable to differentiate into cardiac cells but also to smooth muscle and endothelial

cells which may participate in coronary artery formation (Moretti et al., 2006). During embryonic development Isl1 is expressed by progenitor cells of the second heart field that gives rise to the formation of the outflow tract, the atria and the right ventricle and is required for proliferation, survival, and migration of these progenitors into the forming heart (Cai et al., 2003). Isl1 also marks cardiac progenitors found within postnatal hearts of rodents and humans (Laugwitz et al., 2005). Recently it has been shown that β-catenin directly regulates *Isl1* expression in cardiovascular progenitors and is required for multiple aspects of cardiogenesis (Lin et al., 2007). β-catenin is also required upstream of a number of genes required for pharyngeal arch, outflow tract, and/or atrial septal morphogenesis, including *Tbx2*, *Tbx3*, *Wnt11*, *Shh*, and *Pitx2* (Lin et al., 2007).

The signaling pathways that regulate differentiation of BMSCs and resident cardiac stem cells and/or stimulate proliferation of cardiac progenitor cells are just emerging. Potentially inflammation and elevation of ROS levels following cardiac infarction are involved in the initiation of signaling pathways that activate quiescent resident cardiac stem cells and BMSCs. A beneficial effect of pro-inflammatory signals during bone marrow stem cell therapy has been recently outlined (Sun et al., 2009). In the latter study transplanted BMSCs increased heart tissue inflammation, and elevated TNF-α, TGF-β and fibroblast growth factor-2 (FGF-2) levels which resulted in improved heart function and capillary density in the border zone of the myocardial infarct (Sun et al., 2009). Recently it has been shown that the redox effector protein-1 (Ref-1) which plays an essential role in DNA repair and redox regulation of several transcription factors is involved in the maintenance of cardiac stem cells, since Ref-1 inhibition in the presence of exogenous hydrogen peroxide resulted in the initiation of cardiac differentiation programs thus suggesting that Ref-1 plays an important role in maintaining the redox status of cardiac stem cells and protects them from oxidative injury-mediated cell death and differentiation (Gurusamy et al., 2009).

Many answers on signaling pathways involved in stem cell activation can be given from lessons in cardiac embryology where several signaling pathways that are involved in the development of the first heart field and the second heart field have been recently deciphered (Rochais et al., 2009). One of the main features of second heart field is the control of cardiac progenitor cell proliferation. The latter has been recently shown to be regulated by β-catenin, the intracellular mediator of the canonical Wnt pathway, which is likewise known to be involved in the regulation of several stem cell populations (Reya & Clevers, 2005). Wnt signaling displays positive as well as negative effects on early mesoderm commitment and cardiac specification depending on the developmental stage of the embryo (Rochais et al., 2009). In ES cells Wnt signals are required for early mesoderm differentiation (Lin et al., 2007) whereas during later stages of cardiomyogenesis Wnt signaling restricts cardiac differentiation to the lateral splanchnic mesoderm (Tzahor & Lassar, 2001; Nakamura et al., 2003). Recently it was shown that the Wnt/β-catenin pathway is essential for cardiac myogenesis to occur in ES cells, acting at a gastrulation-like stage, and mediating mesoderm formation and patterning. Among genes associated temporally with this step was Sox17, encoding an endodermal HMG-box transcription factor (Liu et al., 2007). β-catenin interacts with TCF/LEF-1 transcription factors to activate the expression of Wnt target genes. In the absence of Wnt signaling, β-catenin function is blocked by a destruction complex consisting of Axin, APC and the kinases GSK3ß and CK1α, which targets β-catenin for destruction by the proteasome. Binding of Wnt to its receptor Frizzled and LRP leads to inhibition of the destruction complex and allows β-catenin signaling. The cytoplasmic protein Dishevelled (Dvl) is involved in this process by binding to the redox-sensitive protein nucleoredoxin

(NRX) which belongs to the thioredoxin protein family known to be involved in the regulation of a variety of ROS mediated signaling pathways (Korswagen, 2006). ROS are presumably involved in a variety of signaling pathways that are crucial for heart development. Recently it was shown that ROS can modulate signaling by the Wnt/β-catenin pathway (Funato et al., 2006). Oxidative stress inhibits the interaction between NRX and Dvl thus stabilizing β-catenin and leading to an increase in the expression of endogenous Wnt target genes. Further studies have demonstrated that ROS can also inhibit Wnt/β-catenin signaling (Shin et al., 2004) which suggests that a specific time frame and concentration of ROS may be necessary for redox-mediated modulation of the Wnt/β-catenin signaling pathway. Another important pathway known to be crucial for cardiac mesoderm specification and differentiation is bone morphogenic protein (BMP) pathway. *BMP-4* overexpression promotes a cardiac cell lineage in the cranial mesoderm (Tirosh-Finkel et al., 2006). BMP-4 is known to be regulated by Wnt/β-catenin and FGF signaling and is involved in outflow tract septation which includes smooth muscle and endocardial cushion development (Liu et al., 2004). Furthermore BMP-2, another member of the BMP family is essential for cardiac cushion epithelial-mesenchymal transition and myocardial patterning (Ma et al., 2005). Proinflammatory cytokine TNF-α and H_2O_2 significantly increased endothelial expression of BMP-2 but not BMP-4 and induced a proinflammatory endothelial phenotype (Csiszar et al., 2006). In further studies the same group demonstrated that BMP-4 exerts prooxidant, prohypertensive, and proinflammatory effects but only in the systemic circulation, whereas pulmonary arteries are protected from these adverse effects of BMP-4 (Csiszar et al., 2008). BMP-4 by itself may increase ROS generation which has been shown in endothelial cells where oscillatory shear stress elevates BMP-4 and induces monocyte adhesion by stimulating ROS production from a Nox-1-based NADPH oxidase (Sorescu et al., 2004). In malformed embryos from diabetic rats which exert elevated levels of systemic ROS sonic hedgehog homolog (Shh) expression was decreased, and BMP-4 was increased, thus pointing to a redox sensitive regulation of the Shh/BMP-4 pathway. Recently it has been shown that Shh, which is secreted by stem cells in the amphibian intestine, induces BMP-4 in subepithelial fibroblasts suggesting that both Shh and BMP-4 are involved in the development of the cell-renewable epithelium (Ishizuya-Oka & Hasebe, 2008).

6. Impact of redox-regulated pro-angiogenic signals during cardiac infarction

During cardiac insults not only growth factors and cytokines which are involved in the proliferation and differentiation of resident cardiac stem cells towards cardiac cells are upregulated. The healing of infarction is also grossly dependent on proper revascularization. Healing may be dependent on redox-mediated expression/release of pro-angiogenic growth factors like FGF-2 (Detillieux et al., 2003), VEGF (Wojakowski et al., 2004) and PDGF (Zymek et al., 2006) which has been demonstrated to occur after cardiac infarction. Pro-angiogenic factors are also released by monocytes and neutrophils (Lambert et al., 2008) which are migrating to the area of infarction where they induce formation of granulation tissue, containing myofibroblasts and neovessels (Nahrendorf et al., 2007). Increasing angiogenic growth factors in the infarcted hearts has therefore been recently used for cardioprotection and/or to improve cardiac healing (Lahteenvuo et al., 2009; Zhang et al., 2009; Harada et al., 1994; Hsieh et al., 2006b; Hsieh et al., 2006a; House et al., 2003). Conversely, inhibition of pro-angiogenic signaling, e.g. PDGF-signalling in infarcted hearts of mice resulted in impaired maturation of the infarct vasculature, enhanced capillary

density, and formation of dilated uncoated vessels. Defective vascular maturation in antibody-treated mice was associated with increased and prolonged extravasation of red blood cells and monocyte/macrophages (Zymek et al., 2006). VEGF is critical for stem cell-mediated cardioprotection which was shown in experiments where VEGF was downregulated in mesenchymal stem cells by si-RNA approaches. When these cells were infused in the coronary circulation the increase in postischemic myocardial recovery after ischemia reperfusion injury was significantly impaired (Markel et al., 2008). Recently suicide genes under the control of endothelium (endothelial nitric oxide synthase)-, smooth muscle (SM22α)-, and cardiomyocyte (α-MHC)-specific promoters, were used to selectively deplete the individual cell lineage acquired by transplanted undifferentiated bone marrow-derived cells into an acute myocardial infarction model. It was demonstrated that elimination of transplanted endothelium-committed or SM22α-expressing cells, but not cardiac-committed cells, induced a significant deterioration of ejection fraction. Moreover, elimination of endothelial NO synthase-expressing cells 2 weeks after injection reduced capillary and arteriole density (Yoon et al., 2010). The angiogenic factors VEGF, PDGF-BB and FGF-2 are all upregulated by exogenous ROS (Sen et al., 2002; Eyries et al., 2004) and exert cardioprotective effects under conditions of ischemia-reperfusion injury (Hsieh et al., 2006c; Iwai-Kanai et al., 2002). Bone marrow mesenchymal stem cells by themselves release VEGF as a potentially beneficial paracrine response which is enhanced by TGF-α and TNF-α (Wang et al., 2008). Furthermore VEGF upregulation has been observed under tissue stress conditions associated with ROS generation, e.g. physical exercise (Roy et al., 2008) and cardiac infarction, where not only the *VEGF* gene but also the VEGF receptors flt-1 and flk-1 were upregulated (Li et al., 1996). Exogenous FGF-2 increased endogenous FGF-2 promoter activity and protein levels in ovine pulmonary arterial smooth muscle cells (PASMC). These increases in FGF-2 expression were mediated by elevations in superoxide levels via NADPH oxidase activation. In addition, FGF-2-mediated increases in FGF-2 expression and pulmonary arterial smooth muscle cell (PASMC) proliferation were attenuated by inhibition of phosphatidylinositol 3-kinase, Akt, and NADPH oxidase (Black et al., 2008). Comparably exogenous ROS increased VEGF and VEGFR expression (Gonzalez-Pacheco et al., 2006; Chua et al., 1998), stimulated endothelial cell proliferation and migration (Luczak et al., 2004) as well as cytoskeletal reorganization (Vepa et al., 1999) and tubular morphogenesis (Shono et al., 1996) which all utilize ROS within their signal transduction pathways. Addition of PDGF-BB, FGF-2 and VEGF to non-phagocytic cells has been shown to rapidly increase ROS generation (Thannickal et al., 2000) which may likewise occur in stem cells thus stimulating cardiovascular differentiation.

7. Redox-regulated pathways involved in mobilization of stem cells from the bone marrow

Stem cells and progenitor cells are mobilized from the bone marrow in response to inflammation, tissue injury and cytokines (Aicher et al., 2005). A cytokine playing a prominent role in stem cell mobilization, endothelial cell differentiation and vascular repair is stromal cell–derived factor-1α (SDF-1α), a CXC chemokine known to play a critical role in the trafficking of hematopoietic, lymphopoietic cells as well as stem cell progenitors, and in maintaining hematopoietic stem cell niches in bone marrow (Kucia et al., 2004). The high SDF-1α content in the bone marrow creates a concentration gradient, which retains hematopoietic stem cells within the stem cell niche. Disruption of this SDF-1α gradient

results in mobilization of stem cells into the circulation. This degradation occurs after upregulation of G-CSF levels during systemic stress or injury. Under these conditions elastase is secreted from neutrophils which cleaves membrane-bound SDF-1/CXCR4 complexes on the surface of bone marrow stem cells in the marrow (Heissig et al., 2002; Jin et al., 2008). SDF-1 is released by stromal cells and binds to its CXCR4 receptor on stem and progenitor cells. The signaling cascade following interaction between SDF-1 and CXCR4 may involve the generation of ROS. This has been recently evidenced in studies on B-lymphocytes where ROS were involved in CXCR4-induced Akt activation (Lee et al., 2007). If high concentration gradients of circulating SDF-1 exist, CXCR4-positive cells are leaving the bone marrow to be directed to sites of tissue injury. During tissue damage, ischemia and inflammation plasma and tissue levels of SDF-1α are upregulated (Schober, 2008). Consequently SDF-1α expression is significantly upregulated in experimental rat and mouse models of infarction (Pillarisetti & Gupta, 2001), and in plasma and cardiac tissue of patients with myocardial infarction (Yamani et al., 2005). Furthermore, SDF-1α expression has been shown to increase under hypoxic conditions (Ceradini et al., 2004), and thus may serve to attract stem cells to sites of tissue injury and ischemia. Recently it has been shown that expression of SDF-1α on circulating platelets is increased in patients with acute coronary syndrome and correlates with the number of $CD34^+$ progenitor cells (Stellos et al., 2009). Expression of SDF-1α appears to be correlated to the expression of eNOS in the heart since eNOS $^{-/-}$ mice displayed reduced SDF-1α levels in isolated cardiomyocytes. eNOS in the host myocardium promoted mesenchymal stem cell migration to the ischemic myocardium and improved cardiac function through cGMP-dependent increases in SDF-1α expression (Li et al., 2009). The local inflammatory response implying adhesion molecule expression and eNOS-dependent signaling was required for SDF-1α-induced adhesion of c-kit^+ cells to the vascular endothelium (Kaminski et al., 2008). Furthermore, oxidative stress from lactate metabolism by circulating stem/progenitor cells accelerated further stem cell recruitment and differentiation through thioredoxin-1 (Trx1)-mediated elevations in HIF-1 levels and the subsequent synthesis of HIF-1-dependent growth factors including VEGF and SDF-1α (Milovanova et al., 2008). Taken together these data suggest a model whereby, in response to tissue injury and inflammation, stem cells within the bone marrow are expanded and primed through G-CSF which then results in mobilization of stem cells via degradation of SDF-1α in the marrow and recruitment of the stem cells to sites of elevated SDF-1α levels within the injured, inflamed or ischemic tissues. Mobilization is then terminated when the increased SDF-1α gradient in the marrow is re-established and retains newly formed or non-mobilized stem cells as a reserve for future emergency signals (Mays et al., 2007). Interestingly G-CSF stimulation induced ROS generation in bone marrow neutrophils correlating with activation of Lyn, PI3-kinase and Akt, whereas the anti-oxidant N-acetyl cysteine diminished G-CSF-induced ROS production and cell proliferation (Zhu et al., 2006). Further research on the priming function of G-CSF on ROS generation by neutrophils revealed that mitogen-activated protein kinase (MAPK) pathways are involved in phosphorylation of Ser345 of p47phox, a cytosolic component of NADPH oxidase in human neutrophils (Dang et al., 2006). Previously it was shown that several hematopoietic growth factors including G-CSF signal through the formation of ROS (Sattler et al., 1999) which has been associated to a stimulation of cell proliferation of hematopoetic stem cells upon treatment with G-CSF (Pyatt et al., 1996). Furthermore the blood oxidative status was found to be significantly increased in healthy hematopoetic stem cell donors receiving G-CSF,

which indicated that during stem cell mobilization a transient inflammatory status is generated (Cella et al., 2006) which may facilitate further stem cell mobilization. ROS mediated stem cell mobilization and recruitment may be used in therapeutic angiogenesis approaches. In this respect hyperbaric oxygen has been shown to stimulate recruitment and differentiation of circulating stem/progenitor cells in subcutaneous Matrigel which was inhibited by antagonists of NADPH oxidase and free radical scavengers (Milovanova et al., 2009).

Mostly ROS elicited upon growth factor and cytokine signaling are only acting within a narrow time window. Recently the interesting concept of the redox window of coronary collateral growth was formulated. This concept suggests that the redox window constitutes a range in the redox state of cells, which not only is permissive for the actions of growth factors but amplifies their actions. Initial changes in cellular redox are arising from different events, e.g. from the oxidative burst during reperfusion following ischemia, to recruitment of various types of inflammatory cells capable of producing ROS. Any event that upsets the normal redox equilibrium is capable of amplifying growth. However, extremes of the redox window, oxidative and reductive stresses, are associated with diminished growth factor signaling and reduced activation of redox-dependent kinases (Yun et al., 2009). Previously the same group had demonstrated that ROS are involved in human coronary artery endothelial cell (HCAEC) tube formation, coronary collateral growth in vivo, and signaling (p38 MAP kinase) by which ROS may stimulate vascular growth (Yun et al., 2009).

8. ROS and NO generation in bone marrow-derived stem cells

Besides the role of ROS and NO generated during states of tissue inflammation, ischemia and injury, stem cells per se are generating ROS as well as NO which may be involved in proliferation and differentiation processes. ROS and NO generation in stem cells could occur in response to transient changes in systemic redox balance and could initiate a feedforward cycle of ROS/NO generation and elaboration of a balanced anti-oxidative response system that may be the basis of stem cell proliferation, migration and differentiation. An increasing number of studies reported on the crucial role of ROS/NO on mesenchymal stem cell differentiation. It was shown that neuronal differentiation of mesenchymal stem cells involved upregulation of NADPH oxidase and increased ROS generation (Wang et al., 2007). Furthermore physical shockwave treatment was shown to increase osteogenic activity of human umbilical cord blood (HUCB) mesenchymal progenitor cells through superoxide-mediated TGF-β1 induction (Wang et al., 2004). ROS generation through the activity of the Nox-2 and Nox-4 isoform of NADPH oxidase has been demonstrated in human $CD34^+$ cells which may contribute to the activation of intracellular signaling pathways leading to mitochondriogenesis, cell survival, and differentiation in hematopoietic stem cells (Piccoli et al., 2007a). In the latter study the authors suggested that the coordinated activity of the Nox isoforms in hematopoietic stem cells functions as environmental oxygen sensor and generates low level of ROS, which likely serve as second messengers. The pro-oxidant setting, entering into play when hematopoietic stem and progenitor cells leave the hypoxic bone marrow niche, would enable them to be more responsive to proliferative/differentiative stimuli. Moreover it is suggested that enhanced ROS elicit mitochondrial "differentiation" in a pre-commitment phase needed to match the bioenergetic request in the oncoming proliferation/differentiation process (Piccoli et al., 2007b). Mesenchymal stem cells from the bone marrow have been shown to express

iNOS (Sato et al., 2007) as well as eNOS (Klinz et al., 2005). Recently it was shown that hematopoietic stem cell development is dependent on blood flow and and is closely associated to NO generation since intrauterine NOS inhibition or embryonic eNOS deficiency resulted in a reduction of hematopoietic clusters and transplantable murine hematopoietic stem cells (North et al., 2009). Generation of NO by eNOS has been reported in mouse endothelial progenitor cells (EPCs) and was utilized to identify the EPC population (Loomans et al., 2006). Administration of Angiotensin II (Ang II) significantly promoted NO release, inhibited EPC apoptosis and enhanced EPC adhesion potential (Yin et al., 2008). In a recent study it was shown that two NO agents (SNAP and DEA/NO), able to activate both cGMP-dependent and -independent pathways, were increasing the cardiomyogenic potential of bone marrow-derived mesenchymal stem cells and adipose tissue-derived stem cells (ADSCs) (Rebelatto et al., 2009). Furthermore it was recently shown that the nitric oxide-soluble guanylyl cyclase pathway is involved in the oxytocin-mediated differentiation of porcine bone marrow stem cells into cardiomyocytes (Ybarra et al., 2010).

9. ROS and NO in cardiovascular differentiation of ES cells

Most evidence about the role of NO and ROS in cardiovascular differentiation has been obtained in mouse ES cells. It was shown that undifferentiated self-renewing stem cells are devoid of endogenous ROS generation and expression of NADPH oxidase. Undifferentiated ES cells were demonstrated to be equipped with highly efficient mechanisms to defend themselves against various stresses and to prevent or repair DNA damage. One of these mechanisms is high activity of a verapamil-sensitive multidrug efflux pump. During the differentiation process anti-oxidative genes are downregulated which should result in increased ROS generation (Saretzki et al., 2004). Consequently, during the differentiation process the gp91-phox homologues Nox-1, Nox-2 and Nox-4 are upregulated in a distinct time frame, starting with Nox-1 and followed by Nox-4 (Buggisch et al., 2007), whereas Nox-2 is closely correlated to the differentiation of phagocytic cells from ES cells which occurs subsequent to cardiovascular differentiation (Hannig et al., 2010). During the early stages of ES cell differentiation, i.e. between day 4 and day 10 of cell culture ROS generation is elevated and downregulated during later stages. The stages of active ROS generation are just those where cardiovascular differentiation occurs, i.e. between day 4 and day 9 of cell culture. Any approaches to increase intracellular ROS, e.g. by addition of nanomolar concentrations of H_2O_2 to differentiating embryoid bodies (Buggisch et al., 2007; Sauer et al., 1999; Sauer et al., 2000), treatment with direct current electrical fields (Sauer et al., 2005; Sauer et al., 1999; Serena et al., 2009), application of mechanical strain (Schmelter et al., 2006), treatment with cardiotrophin-1 (CT-1) (Sauer et al., 2004), PDGF-BB (Lange et al., 2009) or peroxisome proliferator-activated receptor α (PPARα) (Sharifpanah et al., 2008) resulted in prominent stimulation of cardiovascular differentiation of ES cells. Interestingly elevation of intracellular ROS by exogenous stimulators resulted in upregulation of Nox-1 and Nox-4 thus initiating a feed-forward stimulation of prolonged ROS generation (Schmelter et al., 2006; Buggisch et al., 2007). Consequently, si-RNA inactivation of Nox-4 resulted in complete inhibition of embryonic stem cell-derived cardiomyogenesis (Li et al., 2006). Stimulation of ROS generation by different means resulted in activation of the MAPK pathways ERK1,2, p38 and JNK. Furthermore stimulation of embryoid bodies by ROS resulted in activation of the cardiogenic transcription factors BMP-10, MEF2C, GATA-4, DTEF-1 and Nkx-2.5 (Buggisch et al., 2007). Interestingly vasculogenesis required activation

of ERK1,2 and JNK whereas p38 activation was dispensable. Cardiomyogenesis, however, required the activation of all three pathways since pharmacological inhibition of either pathway abolished cardiac cell differentiation (Schmelter et al., 2006). Recently it was demonstrated that also smooth muscle differentiation from ES cells is under the control of Nox-4. Overexpression of Nox-4 specifically resulted in increased smooth muscle cell marker production, whereas knockdown of Nox-4 induced a decrease. Furthermore, smooth muscle cell-specific transcription factors, including serum response factor (SRF) and myocardin were activated by Nox4 gene expression (Xiao et al., 2009). When cardiomyogenesis was stimulated with CT-1, additionally activation of NF-κB and the JAK/STAT signaling pathway in a redox-sensitive manner was observed (Sauer et al., 2004). CT-1 has been previously shown to exert cardioprotective effects which may be related to the activation of anti-apoptotic signaling pathways (Calabro et al., 2009). CT-1 is expressed in post-myocardial infarct heart, and may play an important role in infarct scar formation and ongoing remodeling of the scar (Freed et al., 2003). Furthermore, CT-1 is a cytokine that induces hypertrophy and has been shown to be increased in hypertensive patients (Lopez et al., 2009). An additional role of CT-1 may involve the activation and differentiation of resident cardiac stem cells. In this respect is has been recently shown that CT-1 signaling through glycoprotein 130 (gp130) regulates the endothelial differentiation of cardiac stem cells (Mohri et al., 2009). Recently CT-1 in combination of 5-azacytidine which is an inhibitor of DNA methylation was shown to induce cardiac gene expression in mesenchymal stem cells (Xinyun et al., 2009). In human ES cells telomere maintenance, oxidative stress generation, and genes involved in antioxidant defense and DNA repair were investigated during spontaneous differentiation of two human embryonic stem cell lines. Telomerase activity was quickly downregulated during differentiation, probably due to deacetylation of histones H3 and H4 at the hTERT promoter and deacetylation of histone H3 at hTR promoter. Telomere length decreased accordingly. Mitochondrial superoxide production and cellular levels of ROS increased as result of stimulated mitochondrial biogenesis. The expression of major antioxidant genes was downregulated despite this increased oxidative stress. DNA damage levels increased during differentiation, whereas expression of genes involved in different types of DNA repair decreased (Saretzki et al., 2008). Besides the evident role of ROS for cardiovascular differentiation a prominent involvement of NO in cardiomyogenesis of ES cells has been evidenced. In murine undifferentiated ES cells, NOS-1, NOS-3, and sGCβ(1) were detected while NOS-2, sGCα(1), and PKG were very low or undetectable. When ES cells were subjected to differentiation, NOS-1 abruptly decreased within one day, NOS-2 mRNA became detectable after several days, and NOS-3 increased after 7-10 days (Mujoo et al., 2006). Components of NO signaling were likewise expressed in human ES cells (Mujoo et al., 2006). Nkx2.5 and myosin light chain (MLC2) mRNA expression was increased on exposure of mouse and human ES cells to NO donors and a decrease in mRNA expression of both cardiac-specific genes was observed with nonspecific NOS inhibitor (Mujoo et al., 2008). Recently it was shown that NO acts as a repressor of the stemness gene *Nanog* in mouse and human ES cells. The suppressive action of NO on *Nanog* gene depended on the activation of p53 repressor protein by covalent modifications, such as pSer15, pSer315, pSer392 and acetyl Lys 379. NO-induced repression of *Nanog* was also associated with binding of trimethylated histone H3 and pSer315 p53 to its promoter region (Mora-Castilla et al., 2010). In several studies it was reported that NO is acting as signaling molecule during cardiomyogenesis of ES cells (Mujoo et al., 2008; Kanno et al., 2004; Bloch

et al., 1999). Furthermore NO donors stimulated vasculogenesis of ES cells (Milosevic et al., 2010). Studies on NO generating agents revealed that sGC activators alone exhibited an increase in mRNA expression of cardiac genes (MLC2 and Nkx2.5). Robust inductions of mRNA and protein expression of marker genes were observed when NO donors and sGC activators were combined. Measurement of NO metabolites demonstrated an increase in the nitrite levels in the conditioned media and cell lysates on exposure of cells to the different concentrations of NO donors. cGMP analysis in undifferentiated stem cells revealed a lack of stimulation with NO donors. Differentiated cells however, acquired the ability to be stimulated by NO donors (Mujoo et al., 2008). Generation of NO is apparently also the mediator of cardiomyogenesis of mouse ES cells achieved with the hormone oxytocin (Muller et al., 2008) and arginine vasopressin (Gassanov et al., 2007). Hence these data suggest that the interplay of ROS and NO is required to direct undifferentiated ES cells into the cardiovascular cell lineage.

10. Conclusions

Emerging evidence of recent years outlines a decisive role of ROS and RNS in the initiation of differentiation programs of stem cells. How this can be achieved is currently difficult to investigate due to technical limitations of in vivo detection of short lived radical species and subcellular analysis of the distribution of diffusible intracellular anti-oxidants. More than 20 years ago the free radical theory of development was established by Allen and Balin (Allen & Balin, 1989). According to this theory metabolic gradients exist in developing organisms and are believed to influence development. Metabolically generated oxidants that are counteracted by the antioxidative defense may be one decisive factor that directs the initiation of certain developmental events (Allen & Balin, 1989). The literature reviewed in the current article outlines that ROS as well as RNS are involved in stem cell mobilization, function and differentiation in a very complex way. To understand how this can function in a tissue where many different cell types exist in close proximity remains to be unraveled. Barriers of antioxidants have to be postulated that hinder the diffusibility of oxidants in one and pave their way in other cellular locations thus achieving distinct redox microenvironments and differentiation patterns. The prooxidant role of NO is often attributed to RNS intermediates rather than NO itself. NO may react with superoxide which – in contrast to H_2O_2 – is not diffusible across cell membranes. The resulting peroxynitrite ($ONOO^-$) can diffuse freely within and out of the cell, and react with lipids, proteins and DNA. The balance between NO and superoxide may help to define the prooxidant action of NO versus ROS in biological tissues. This interplay may discriminate between pathways resulting in oxidative stress and induction of apoptotic pathway versus signaling cascades resulting in stem cell maintenance and/or stem cell differentiation.

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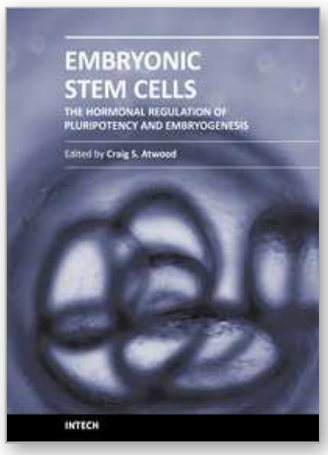

Embryonic Stem Cells: The Hormonal Regulation of Pluripotency and Embryogenesis

Edited by Prof. Craig Atwood

ISBN 978-953-307-196-1
Hard cover, 672 pages
Publisher InTech
Published online 26, April, 2011
Published in print edition April, 2011

Pluripotency is a prerequisite for the subsequent coordinated differentiation of embryonic stem cells into all tissues of the body. This book describes recent advances in our understanding of pluripotency and the hormonal regulation of embryonic stem cell differentiation into tissue types derived from the ectoderm, mesoderm and endoderm.

How to reference

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Heinrich Sauer and Maria Wartenberg (2011). Impact of Reactive Oxygen and Reactive Nitrogen Species for Stem Cell Mobilization, Function and Cardiovascular Differentiation, Embryonic Stem Cells: The Hormonal Regulation of Pluripotency and Embryogenesis, Prof. Craig Atwood (Ed.), ISBN: 978-953-307-196-1, InTech, Available from: http://www.intechopen.com/books/embryonic-stem-cells-the-hormonal-regulation-of-pluripotency-and-embryogenesis/impact-of-reactive-oxygen-and-reactive-nitrogen-species-for-stem-cell-mobilization-function-and-card

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