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# Non-classical Signalling Mechanisms in Stem Cells

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## 1. Introduction

There are many gaps in our understanding of stem cell biology that need to be explored in order to realize the full potential of stem cells for therapies. Understanding how stem cells communicate within their microenvironment is important to be able to manipulate their functions. Indeed, the stem cell microenvironment is critical for their maintenance, and communication between neighbouring cells plays an important part in determining cell fate. Most studies of the stem cell niche focus on paracrine or juxtacrine cell interactions, particularly the influence of cytokines and various G-protein coupled receptor ligands, influences which have been reviewed elsewhere (Kobayashi et al 2010). Other signalling mechanisms that have received less attention include oxidant signalling through various protein kinase pathways, and intercellular communication through gap junctions. Not surprisingly gap junctional intercellular communication (GJIC) has been implicated in regulating crucial biological events in many stem cells, including proliferation, differentiation and apoptosis. Understanding and modulating GJIC in stem cells could potentially lead to the development of novel methods for expanding stem cells *in vitro* and directing their differentiation into functional mature cells. In this review we have summarized current knowledge on the identified roles of gap junctions and of reactive oxygen species (ROS) in stem cells, and speculate on how these may be exploited to develop the therapeutic potential of stem cells.

## 2. Gap junctions and gap junction intercellular communication

The classical background on gap junctions and GJIC has been covered extensively in a number of excellent recent reviews and therefore will only be briefly discussed here (Martin and Evans, 2004). Gap junctions are intercellular junctions found to be either in an opened or a closed conformation. They are the only intercellular junctions that allow direct transfer

of signalling molecules and metabolites to adjacent cells (Alexander and Goldberg, 2003; Kumar and Gilula, 1996). Gap junctions are hydrophilic channels consisting of two connexons, hemichannels localized in the membrane of adjacent cells, each of them consisting of six connexins (Cx) (Kumar and Gilula, 1996; Sosinsky and Nicholson, 2005) which can be assembled from either a single type or multiple types of connexins. Importantly, the connexin constitution of the gap junctions will define the pore size of the junction, hence allowing different permeability for the transfer of molecules (Saez et al., 2003). It is however generally accepted that only molecules less than 1-1.5kDa diffuse through gap junctions (De Maio et al., 2002; Evans et al., 2006). It is now also suggested that unpaired connexon hemichannels can mediate intercellular communication without forming gap junctions (Ebihara 2003; Goodenough and Paul 2003; Evans, De Vuyst et al. 2006).

## 3. Gap junctions in undifferentiated stem cells

### 3.1 Mouse embryonic stem cells

Mouse embryonic stem cells (mESC) display functional GJIC, express mRNA transcripts of various connexins: Cx26, Cx30.3, Cx31, Cx32 and Cx37, Cx43 and Cx45 but only Cx31, Cx43 and Cx45 proteins, suggesting a translational regulation of connexins in mESC (Nishi et al., 1991; Oyamada et al., 1996; Worsdorfer et al., 2008). Studies so far seem to indicate that Cx45 does not play a fundamental role in mESC regulation of pluripotency and differentiation and suggest a role of Cx43, although still unclear, in these processes. Indeed, Cx45-null mESC are able to differentiate into cells of the three germ layers following embryoid body formation (Egashira et al., 2004). In contrast, some studies showed that Cx43-knock down mESC display decreased cell proliferation, down-regulation of several stem cell markers and up-regulation of differentiation markers and inability to form embryoid bodies (Todorova et al., 2008; Worsdorfer et al., 2008). However, other data suggest the opposite where the down-regulation of Cx43 resulted in Src phosphorylation and increase in cell proliferation (Kim et al., 2010). In the same study, the adenosine analogue 5′-N-ethylcarboxamide (NECA) was shown to stimulate Cx43 phosphorylation and to inhibit GJIC in mESC, through the activation of PI3K/Akt, PKC, MAPK and NFκB signalling pathways. The closure of GJIC would subsequently lead to Src-induced cell proliferation and migration (Kim et al., 2010). Lastly, Cx43-null mESC show not only a defective differentiation to oligodendrocytes but also an increase in differentiation to astrocytes (Parekkadan et al., 2008). Hence there are conflicting findings on the role of GJIC in mESC, with some data suggesting that Cx43 and open gap junctions are necessary for mESC proliferation, for survival and for maintaining them in the pluripotent state, while other data suggest that the closure of gap junctions is in fact a signal for proliferation and migration. Clearly further work is required to properly define the roles GJIC in mESC under different conditions.

### 3.2 Human embryonic stem cells

Human embryonic stem cells (hESC) are also coupled through functional gap junctions (Bhattacharya et al., 2004; Carpenter et al., 2004; Wolvetang et al., 2007; Wong et al., 2006; Wong et al., 2004) (Figure 1) and express almost all transcripts of human connexin isoforms, except Cx40.1 and Cx50 (Huettner et al., 2006). Functional GJIC seems to be a common characteristic of hESC maintained in different culture conditions (Wong et al., 2006; Wong et al., 2004). So far, only a few factors have been shown to inhibit GJIC in hESC such as bone morphogenetic protein (BMP)-4 (Wong et al., 2006), endothelin (ET) 1 and ET-2 (Wong et al.,

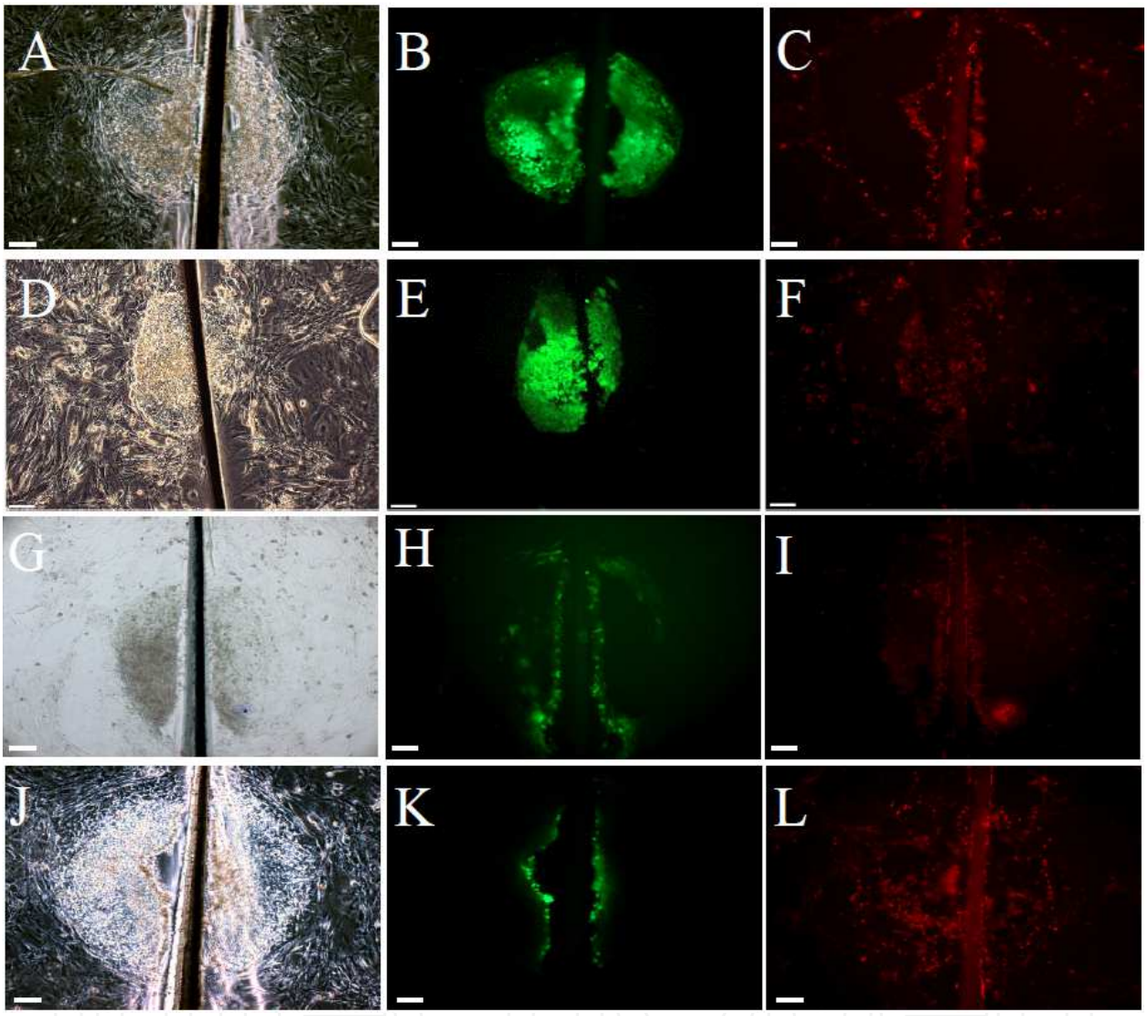


Fig. 1. GJIC in hESC. **(A, D, G, J):** Light and fluorescence micrographs with Lucifer yellow **(B, E, H, K)** and rhodamine-dextran **(C, F, I, L)** in HES-3 cells. Rhodamine-dextran was used as a negative control, showing no dye transfer across to the neighboring cell. Cells were incubated in the presence **(A–C)** or absence **(D–F)** of Ca2+Mg2+ or in the presence of phorbol 12-myristate 13-acetate **(G–I)** or U0126 **(J–L)**. Scale bars = 100 μm. Reproduced, with permission, from (Wong et al., 2004).

2009). Similar to mESC, studies suggest that gap junctions play an important role in the regulation of hESC pluripotency and differentiation. Indeed, long-term chemical inhibition of GJIC in hESC can increase apoptosis (Wong et al., 2006) and in pro-differentiation conditions (such as the absence of a feeder cell layer, and non-conditioned medium), hESC do not communicate through gap junctions (Wong et al., 2006). Furthermore, the pro-differentiation factor BMP-4 (Pera et al., 2004; Xu et al., 2002) inhibits GJIC in hESC, an effect that can be prevented by the addition of the BMP antagonist noggin (Wong et al., 2006). The inhibitory effect of ET-1 is short-lasting and is not associated with changes in colony size, morphology or stem cell marker expression (Wong et al., 2009). These studies suggest that open gap junctions are required for the maintenance of hESC pluripotency and that closure of gap junctions is associated with differentiation or cell death. Further work is however needed to understand the precise implications of GJIC for maintaining hESC pluripotency.

### 3.3 Somatic stem cells

The inhibition of GJIC in somatic stem cells prevents regeneration of the planarian flatworm, suggesting a conserved role of gap junctions in regulating stem cell fate (Oviedo and Levin, 2007). However, not all somatic stem cells communicate through functional gap junctions. Some somatic stem and putative progenitor/stem somatic cells lack connexin expression and/or functional GJIC : keratinocyte stem cells (Matic et al., 2002), corneal epithelial stem cells (Matic et al., 1997), pancreatic ductal epithelial stem cells (Tai et al., 2003), neural-glial stem cells (Dowling-Warriner and Trosko, 2000), bovine mammary gland progenitor cells (Holland et al., 2003), human breast epithelial stem cells (Kao et al., 1995) and human kidney epithelial stem cells (Chang et al., 1987). Other somatic stem cells, in particular hematopoietic stem cells (HSC), mesenchymal stem cells (MSC) and neural stem/progenitor cells (NS/PC) express connexins and/or possess functional gap junctions that seem to play a role in regulating their homeostasis, pluripotency and/or differentiation. Current knowledge is however sparse. It has been proposed that Cx32, Cx43 and GJIC play a role in HSC maintenance and differentiation (Hirabayashi et al., 2007b; Ploemacher et al., 2000; Rosendaal et al., 1997). Indeed, Cx32 knockout mice exhibit more undifferentiated HSC and fewer progenitor cells, suggesting a role of Cx32 in maturation of HSC to progenitor cells (Hirabayashi et al., 2007a; Hirabayashi et al., 2007b). Moreover, Cx43-deficient mice demonstrate defects in blood cell formation (Montecino-Rodriguez et al., 2000). Cx43 mRNA is not expressed in undifferentiated and quiescent HSC (Montecino-Rodriguez et al., 2000) but is up-regulated in adult mouse bone marrow upon stem cell division (Rosendaal et al., 1994). These data thus suggest an important role of Cx43 in hematopoiesis, but the precise molecular mechanisms remain to be elucidated. Human MSC can communicate through GJIC and express Cx40, Cx43 and Cx45 (Lin et al., 2007; Valiunas et al., 2004). Moreover, human MSC have been demonstrated to form Cx43-mediated GJIC with umbilical vein endothelial cells, which is of importance for their osteogenic differentiation (Villars et al., 2002). Lastly, rat brain derived NS/PC express Cx43 and Cx45 and communicate through GJIC, which is essential for their survival and proliferation (Cai et al., 2004). In these cells, Cx32 and Cx43 are upregulated during differentiation (Yang et al., 2005). Similar data were observed in mouse fetal NS/PC, where the closure of gap junctions decreases cell proliferation and reduces cell survival (Cheng et al., 2004; Duval et al., 2002). Interestingly the overexpression of Cx43 stimulates proliferation of these cells (Cheng et al., 2004), findings somewhat different to the observations in rat-derived NS/PC. Furthermore, in

mouse embryonic NS/PC, Cx43 and GJIC allow interkinetic nuclear migration, an apical basal movement of the nucleus observed during cell cyle and necessary for corticogenesis (Liu et al., 2010). In addition, NS/PC from other species have also been demonstrated to express Cx43 and communicate via GJIC (Wen et al., 2008) (Russo et al., 2008), suggesting a conserved and critical role of GJIC in NS/PC self-renewal and differentiation. Finally, it is notable that gap junctions appear to be important for establishing functional interactions between grafted NS/PC and host (Jaderstad et al., 2010).

## 4. What goes through gap junctions and how can this modify stem cell fate?

GJIC refers to the passive diffusion of intracellular molecules through gap junctions to a neighboring cell (Kumar and Gilula, 1996). Numerous cytoplasmic molecules can diffuse through gap junction channels, including small ions ($Na^+$, $K^+$, $Ca^{2+}$, $H^+$, $Cl^-$), second messengers (cyclic nucleotides, inositol triphosphate), amino acids (glycine, glutamate), cellular metabolites (glucose, glutathione, adenosine, AMP, ADP, ATP), short interfering RNA (siRNA) and peptides involved in cross-presentation of major histocompatibility complex class I molecules (Alexander and Goldberg, 2003; Krysko et al., 2005; Neijssen et al., 2005; Valiunas et al., 1997). In adult cells and some tissue systems, GJIC has long been known as crucial for certain cellular functions, such as electrical synchronization, intercellular buffering of cytoplasmic ions, cell metabolism, control of cell migration and cell fate including carcinogenesis (De Maio et al., 2002; Krysko et al., 2005; Mesnil et al., 2005; Parekkadan et al., 2008; Todorova et al., 2008; Vine and Bertram, 2002). However, few studies have addressed the importance of gap junctions for promulgating intercellular $Ca^{2+}$-waves in stem or progenitor cells. Early studies suggested that $IP_3$ can induce intracellular $Ca^{2+}$ release from endoplasmic-reticulum, and both $Ca^{2+}$ and $IP_3$ can permeate gap junction channels to the neighbouring cells (Boitano et al., 1992; Saez et al., 1989). Such diffusion of $IP_3$ and $Ca^{2+}$ through gap junctions effectively forms a positive feedback loop allowing intercellular communication between distant cells. Alternatively, other studies have demonstrated that such intercellular $Ca^{2+}$ waves can also be maintained by the paracrine messenger ATP (Guthrie et al., 1999). It was demonstrated that $IP_3$ can trigger ATP release to the extracellular space through connexon hemichannels, and act on G-protein coupled receptors on neighbouring cells, leading to phospholipase C activation, $IP_3$ production and subsequent $Ca^{2+}$ release in the neighbouring cells (Braet et al., 2003; Ebihara et al., 2003; Guthrie et al., 1999). Gap junction-mediated transmission of $Ca^{2+}$ waves has been inferred in some progenitor cells. In NS/PC cells, transmission via $Ca^{2+}$ waves appears to control their proliferation in the ventricular zone (Weissman et al., 2004) as well as in retinal neural progenitor cells (Pearson et al., 2005). Moreover, transient increases in intracellular $Ca^{2+}$ can also stimulate differentiation and neurite outgrowth of different NS/PC cells, but whether such $Ca^{2+}$ signalling occurs through gap junction-mediated waves remains to be determined (Carey and Matsumoto, 1999; Gomez and Spitzer, 1999; Gu and Spitzer, 1995). Recent transplantantion studies by Jaderstad et al. (2010) showed that establishment of GJIC that allows synchronized $Ca^{2+}$ waves is important for grafted NS/PC to integrate functionally to the host neural circuitry. Such GJIC between grafted NS/PC and host cells thus provided a neuroprotective effect in mouse models of neurodegeneration (Jaderstad et al., 2010). Interestingly, diffusion of $Ca^{2+}$ via gap junctions has also been suggested to modulate differentiation of other somatic stem cells. Previous studies by Muller-Borer et al. (2004) suggested that synchronized $Ca^{2+}$ signalling via GJIC co-cultured with neonatal

cardiomyocytes induces rat liver stem cells to express cardiac transcription factors and acquire a phenotype resembling cardiomyocytes (Anderson et al., 2007; Muller-Borer et al., 2004). Similarly, we have recently shown gap junctions established between human adipose-derived MSC and neonatal rat cardiomyocytes in co-culture, which induces the expression of cardiac genes and spontaneous cardiomyocyte-like contractions in the human cells (Choi et al., 2010a) (Figure 2). All this points to a growing appreciation of the role of gap-junction-mediated $Ca^{2+}$ waves in modulating gene expression and promoting differentiation of stem cells. Other metabolites that can diffuse through gap junction include cyclicAMP and glutamate, which were previously shown to act as signals for cell death (Amsterdam et al., 1996; Ozog et al., 2002). Although it remains to be proven, it is possible that cyclicAMP and glutamate might play a role in propagation of death signals in stem cells that possess GJIC, such as hESC and NS/PC. Finally, interesting candidates amongst many that might yet be exposed to diffuse through gap junctions, are small RNAs. Previous studies provided evidence that exogenous siRNA can diffuse through gap junction to the neighbouring cells

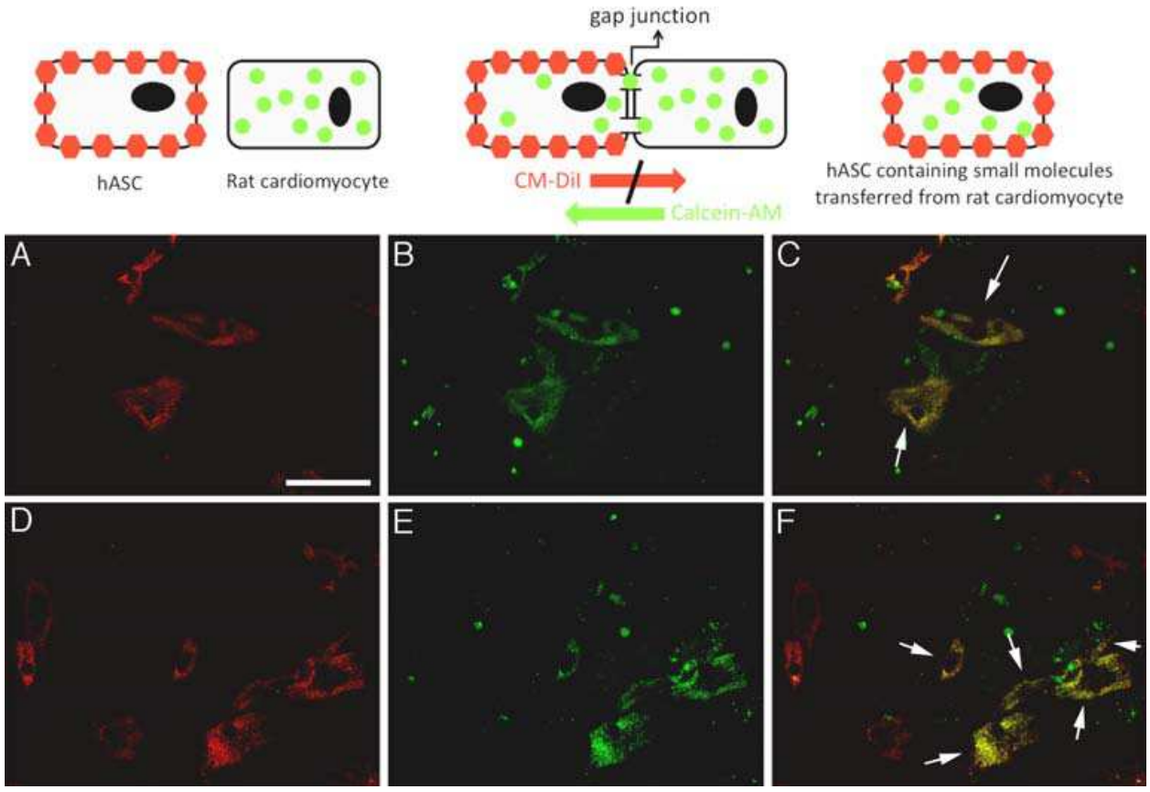


Fig. 2. Functional gap junctions formed between human adipose-derived MSC and rat neonatal cardiomyocytes co-cultured for 24 hours. The human stem cells are labeled red with DiI, which is unable to transfer between cells, and the rat cardiomyocytes were stained by calcein-AM which can transfer from the cytosol of one cell to the adjacent ones through gap junctions. After co-culture, double labeled cells (human cells – cardiomyocyte-like-differentiated from MSC) can be seen as indicated by the arrows (Choi, Dusting, Dilley et al – unpublished, similar to Choi et al 2010a)

(Valiunas et al., 2005; Wolvetang et al., 2007) to silence genes therein. Thus, it is possible that this mechanism may also apply to endogenous microRNAs that provide post-transcriptional regulation of a diverse array of genes. Given the emerging role of microRNA in regulating various physiological processes in hESC and other somatic stem cells (Liu and Zhao, 2009; Mallanna and Rizzino, 2010; Navarro and Lieberman, 2010), future work is likely to explore gap junction-mediated transfer of microRNA in modulating the fate of adjacent stem cells.

## 5. Oxidant signalling and NADPH oxidase in proliferation and survival of vascular cells

Over-production of reactive oxygen species (ROS) and diminished antioxidant systems (e.g superoxide dismutase, catalase, glutathione peroxidase and glutathione) may lead to oxidative stress, and this is known to contribute to the pathogenesis of several diseases. These include ischemic-reperfusion injury (e.g. heart attack and stroke), atherosclerosis, hypertension, ischemic heart disease, cancers and neurodegeneration. However, given that all types of cells generate low but detectable amounts of ROS under different circumstances, it is likely that ROS serve as important mediators under physiological conditions. In fact, the production of ROS is tightly regulated by antioxidant systems, which maintain redox homeostasis within the cellular environment. As a consequence, ROS have distinct functional effects, which are dependent on a number of factors such as the type of cell within which ROS are generated, and the type and ultimate concentration of ROS at sub-cellular sites where they may modulate enzyme activity and influence gene expression. One of the most important ROS in the vasculature is superoxide anion, formed enzymatically and non-enzymatically, by the univalent reduction of oxygen. The best characterized source of superoxide is the mitochondrial electron transport chain, but many other intracellular enzymes such as xanthine oxidase (XO), cyclooxygenase (COX), nitric oxide synthase (NOS), cytochrome $P_{450}$ oxidase and NADPH oxidase are capable of producing this radical. All these enzymes, save NADPH oxidase, have important cellular functions apart from superoxide generation, whereas the NADPH oxidase enzyme complex is the only known enzyme dedicated to production of ROS, using intracellular NADPH as the "substrate" and electron donor. Since the mid 1990's it has become evident that many cells produce superoxide constitutively by an enzyme with all the characteristics of the NADPH oxidase previously shown to be present in dedicated phagocytic or inflammatory cells. Although constitutively active, NADPH oxidases in blood vessels can be further activated by stimuli such as angiotensin-II (Ang-II), tumour necrosis fector-alpha (TNFα), TGFβ, thrombin, platelet-derived growth factor (PDGF) and by specific ROS themselves (Barry-Lane et al., 2001; Lassegue et al., 2001; Li and Shah, 2003; Moe et al., 2006; Patterson et al., 1999; Suh et al., 1999). The NADPH oxidase is comprised of a membrane-bound heterodimeric unit called flavocytochrome *b558,* composed of small subunit p22phox and gp91phox (aka Nox2). The catalytic moiety of gp91phox (Nox2) contains flavin-adenine dinucleotide (FAD) binding site, two heme components and one NADPH binding site. In the presence of stimuli such as phorbol ester, bacterial lipopolysaccharides or formyl-methionyl-leucyl-phenylalanine (fMLP), protein kinase C (PKC) causes phosphorylation of p47phox and initiates the translocation of p47phox, and its associated proteins p67phox, p40phox and small G-protein Rac1 to the membrane to bind to the cytochrome *b558* complex. The fully assembled complex allows NADPH to bind to gp91phox on the cytoplasmic side of the membrane to initiate a series of electron transfers starting from NADPH to FAD then to

heme and finally to oxygen to produce two molecules of superoxide anion radical. To date five isoforms of the catalytic subunit Nox have been identified (Nox1 to Nox5). In addition two homologs of the associated intracellular proteins p47phox and p67phox known as NoxA1 (NADPH oxidase activator1) and NoxO1 (NADPH oxidase organizer), respectively, have been identified (Babior et al., 1973; Lassegue and Clempus, 2003; Li and Shah, 2004). We and others have shown that NADPH oxidase-derived ROS have important functions in survival and proliferation of vascular cells (Ago et al., 2004; Chen et al., 2008; Peshavariya et al., 2009; Petry et al., 2006). Petry et al. showed that suppression of either Nox2 or Nox4 reduce endothelial cell proliferation in vitro, whereas over-expression of these isoforms increased cell proliferation (Petry et al., 2006). Similarly, we have shown that suppression of Nox4 only reduces proliferation, whereas suppression of Nox2 increases apoptosis and therefore also effectively promotes proliferation of endothelial cells (Peshavariya et al., 2009). Vascular smooth muscle cells (VSMC) from different sources express highly both Nox4 and Nox1, but do not express Nox2 (Chan et al., 2009). It is well documented that several growth factors increase Nox1 expression or activity or both, and they also enhance proliferation of VSMC (Lassegue et al., 2001; Suh et al., 1999). However the role of Nox4 in VSMC proliferation is complicated. For instance, it has been shown that transforming growth factor-beta (TGFβ) - induced Nox4 is important in pulmonary smooth muscle cell proliferation (Sturrock et al., 2006). In contrast, the expression of Nox4 increased under quiescent (serum-deprived) conditions and this leads to aortic VSMC differentiation rather than proliferation under these conditions (Clempus et al., 2007). Despite such inconsistencies in the literature regarding the role of Nox4-derived ROS in proliferation versus differentiation, almost all studies indicate that Nox4 is involved in migration of VSMC and endothelial cells (Datla et al., 2007; Lyle et al., 2009; Sturrock et al., 2006). Taken together these findings suggest that NADPH oxidase and its isoforms have distinct roles in vascular cell survival and proliferation, and the disparity of functions may be due to the different ligand receptor couplings and sub-cellular localization of the NADPH oxidase complexes involved.

## 6. Intracellular kinase pathways induced by oxidant signalling promote cell survival or proliferation.

There is compelling evidence that low levels of oxidants activate several cell signalling pathways and regulate cell survival and proliferation, whereas high levels of oxidants stimulate stress-activated signalling pathways leading to cell death. For example, nanomolar to sub-micromolar concentrations of hydrogen peroxide ($H_2O_2$) stimulate the proliferation of several cell types, but higher concentrations of $H_2O_2$ (>100μM) leads to cell death (Giorgio et al., 2007; Kim et al., 2009; Stone and Yang, 2006). However, the effect of $H_2O_2$ is cell type dependent: $H_2O_2$ (100μM) increased VSMC proliferation whereas the same concentration inhibits the proliferation of endothelial and fibroblast cells (Rao and Berk, 1992). It is important to note, however, that provision of exogenous $H_2O_2$ may produce very different effects from stimulation of endogenous $H_2O_2$ release at particular subcellular sites (Forman, 2007). Ligand-receptor interactions also result in the generation of $H_2O_2$ by mammalian cells, but these may have different effects on their downstream signalling pathways and thus exert distinct effects on cell survival, proliferation and differentiation (Chen et al., 2008; Datla et al., 2007; Suh et al., 1999; Wang et al., 2000). ROS have several targets such as transcription factors, phosphatases, and enzymes of the receptor kinase family. It has

become evident that ROS regulate activity of MAP kinases (MAPKs), a family of serine/threonine kinases, including extracellular signal–regulated kinases 1 and 2 (ERK1/2), c-Jun *N*-terminal kinases (JNKs, also termed stress activated protein kinase; SAPKs) and p38MAPK (Figure 3) Several growth factors (Vascular endothelial growth factor, VEGF; epidermal growth factor, EGF; PDGF and thrombin) and cytokines (Ang-II and TNF-α) induce proliferation of endothelial and VSMC via ROS-mediated activation of MAPK family members (Chen et al., 2008; Datla et al., 2007; Lassegue et al., 2001; Li et al., 2005; Li and Shah, 2003; Park et al., 2009; Patterson et al., 1999; Suh et al., 1999; Ushio-Fukai et al., 1998; Ushio-Fukai et al., 1999; Ushio-Fukai et al., 2002). For example, VEGF, EGF, and TNF-α stimulate proliferation or angiogenesis of endothelial cells which is dependent upon ROS-induced phosphorylation of ERK (Chen et al., 2004; Chen et al., 2008; Datla et al., 2007; Li et al., 2005; Ushio-Fukai et al., 2002). The effect of TNF-α on endothelial cell proliferation is concentration dependent: lower concentrations of TNF-α induce angiogenesis whereas higher concentrations induce apoptosis of endothelial cells (Chen et al., 2004; Deshpande et al., 2000). Similarly, Ang-II and PDGF induced the phosphorylation of p38MAPK, JNK and ERK, but only the phosphorlyation of p38MAPk and JNK are ROS sensitive and lead to VSMC growth (Lassegue et al., 2001). Furthermore, in human aortic VSMC 7-ketocholesterol induces ROS via Nox4 at the endoplasmic reticulum and activates pJNK, but this leads to cell death (Pedruzzi et al., 2004). In contrast to the MAPK, Akt/protein kinase B has been identified as an important component of a pro-survival signalling pathway. Addition of either exogenous $H_2O_2$ or receptor-mediated intracellular $H_2O_2$ leads to the phosphoryaltion of Akt (Esposito et al., 2003; Ushio-Fukai et al., 1999). Recently, it has been demonstrated that ROS-mediated activation of the PI3kinase/Akt pathway increased the production of

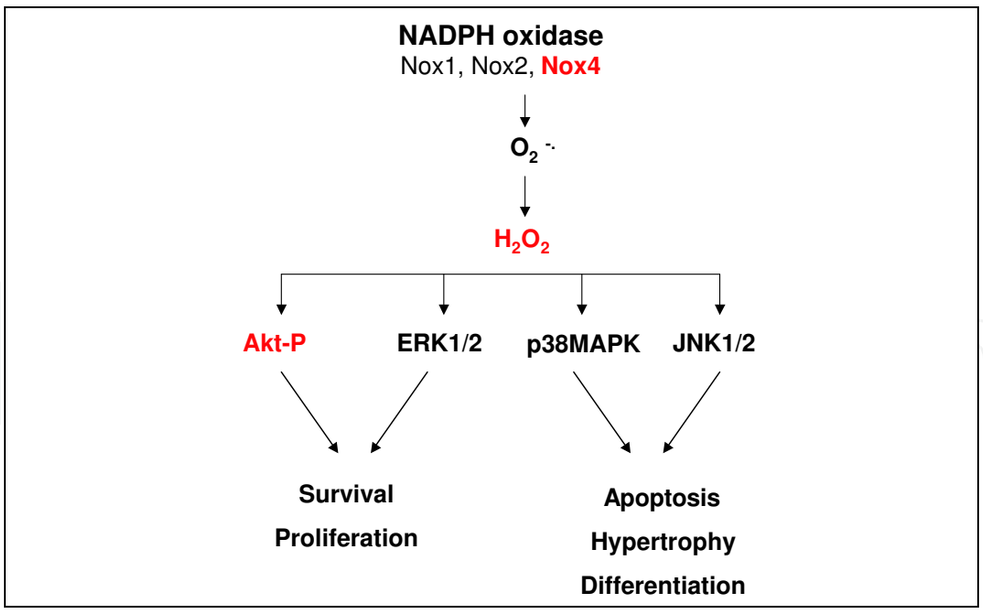


Fig. 3. NAPDH oxidase and ROS signalling. Extracellular ligand-activated membrane receptors (including GPCRs) linked to NADPH oxidase produce ROS intracellularly (superoxide anion or H2O2 in the case of Nox4), which in turn, lead to the phosphorylation and activation of the MAP kinases indicated. The different kinases have different effects in

adult vascular smooth muscle and endothelial cells, as indicated, some of which are shared in stem cells (see text).

nitric oxide and again promoted survival of endothelial cells (Bodiga et al.; Dhanasekaran et al., 2009). Previously, we showed that the proliferative state of endothelial cells exhibits higher ROS production and phosphorylation of Akt compared to quiescent cells, and inhibition of either ROS production or the PI3kinase/Akt pathway reduces endothelial cell proliferation (Peshavariya et al., 2009). ROS-mediated activation of Phospho-Akt (pAkt) promoting cell survival has also been reported in other cell types, such as HeLa, NIH3T3 cells (Wang et al., 2000) and hepatocytes (Kim et al., 2008) . Thus it emerges that activation of ROS-dependent signalling pathways are influenced by several factors such as ligand-receptor interaction, the type of cell, sub-cellular localisation of ROS producing enzymes and the antioxidant status of cells. Therefore, ROS mediated signalling pathways are fine-tuned to differential functions such as proliferation, survival or apoptosis in different cell types under different conditions.

## 7. Intracellular kinase pathways promoting survival of stem cells and oxidant signalling inducing differentiation

Given that ROS signalling clearly has important roles in the proliferation, survival and differentiation of several cell types, it should be considered whether or not this applies to stem cells. In hESC, the PI3k/Akt and ERK1/2 pathways are constitutively active in most culture media used for the maintenance of hESC (Lin et al., 2007). Moreover, the pro-maintenance factors bFGF, neutrophins, S1P and PDGF activate these kinase signalling pathways, suggesting an essential role of these pathways in hESC maintenance and pluripotency. Furthermore, inhibition of either pathway results in differentiation of hESC or cell death (Armstrong et al., 2006; McLean et al., 2007; Wong et al., 2007). Recently the link to ROS activation of these pathways has been demonstrated in hESC, where ROS stimulation leads to differentiation to mesodermal cells (Ji et al., 2010). Recently we have explored intracellular pathways promoting cell survival after hypoxic pre-conditioning of adipose-derived mesenchymal stem cells (MSC). In these cells it was clear that VEGFA was the major cytoprotective cytokine released during hypoxia, and again VEGF acted via Akt1 phosphorylation to protect these MSC from apoptosis during subsequent severe ischaemia, for the protective effect of the preconditioning was blocked by a VEGF antibody and the PI3 kinase inhibitor LY294002 (Stubbs et al., 2010). Interestingly this paracrine protective effect could be imparted to endothelial cells subjected to hypoxia, revealing an interesting way that adipose-derived MSC that we have utilised in tissue engineering of cardiac tissue, could promote the growth of any complex tissue that requires a vasculature (Chan et al., 2009; Choi et al., 2010b). Several other studies have suggested that ROS signalling can determine the fate of stem cells (Lee et al., 2009; Li et al., 2006; Li and Marban, 2010). For example an early study showed that mESC generate intracellular ROS, and addition of exogenous $H_2O_2$ promotes their differentiation to cardiomyocytes. This study suggested that PI3 kinase/Akt pathway is upstream of ROS, for inhibition of PI3 kinase reduced ROS formation and cardiac cell differentiation (Sauer et al., 2000; Sauer and Wartenberg, 2005). The enzymatic source of ROS and their downstream signalling pathways have been further explored in differentiation of ESC down the cardiac lineage. Interestingly, Nox4 emerged as the main source of ROS involved in cardiac differentiation, and it appears to regulate

phosphorylation of p38MAPK and the cardiac differentiation markers Nkx2.5 and myocyte enhancer factor 2C (MEF2C; (Li et al., 2006)). Mechanical strain induces cardiac differentiation of mESC and both ROS and their downstream signalling pathways were shown to be involved. Antioxidants N-(2-mercapto-propionyl-glycine (NMPG) and vitamin E suppress mechanical strain-induced ROS and reduces the critical downstream signalling pathways p38MAPK, ERK and JNK and also compromises cardiac differentiation and vasculogenesis. Thus it seems that mechanical strain activates these three members of the MAPK family via ROS and there is no specificity at this level (Schmelter et al., 2006). Intracellular ROS derived from mESC not only enhanced the differentiation to cardiomyocytes but also increased their proliferation, underlining the importance of ROS in cardiomyogenesis (Buggisch et al., 2007). Finally, ESC-derived ROS signalling is not limited to cardiac differentiation but is also involved in differentiation to VSMC and vascular stabilization. Xiao et al (2009) demonstrated that Nox4 over-expressing ESC showed enhanced differentiation to VSMC, involving transcription factors including serum response factor (SRF) and myocardin.

## 8. Conclusion

We are just beginning to understand the signalling that occurs between stem cells, both adult mesenchymal and embryonic, and soon studies will be focused on these mechanisms in induced pluripotent stem cells. There is emerging evidence on the importance of intercellular and intracellular signalling mechanisms that use gap junctions and oxidant signalling to regulate maintenance of stemness and proliferation, or alternatively trigger differentiation or apoptosis to grow new tissues. Defining these mechanisms will lead to greater efficiency in developing stem cell therapies for the clinic, and we amongst others are using this to build larger, more robust constructs from stem cells through tissue engineering (eg Chan et al 2009). The fruits of this signalling research will enhance approaches to regenerative medicine in many fields, and hopefully allow the great promise of stem cells to realise its full potential.

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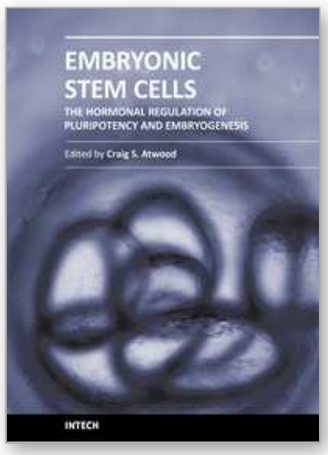


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Pluripotency is a prerequisite for the subsequent coordinated differentiation of embryonic stem cells into all tissues of the body. This book describes recent advances in our understanding of pluripotency and the hormonal regulation of embryonic stem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

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