ANTIOXIDANTS & REDOX SIGNALING
Volume 7, Numbers 11 & 12, 2005

Forum Review

Role of Rac-GTPase and Reactive Oxygen Species in Cardiac Differentiation of Stem Cells

MICHEL PUCEAT

ABSTRACT

In the life of a cell, there is a constant balance between generation of reactive oxygen species (ROS) and activity of antioxidant defense mechanisms. Besides the damaging effects of ROS on many biomolecules, ROS also play a significant role in signal transduction pathways of growth factors suggesting a role of oxidative species in cell differentiation. ROS have recently been involved in the process of cardiac differentiation of stem cells. Several molecular mechanisms, including ones mediated by the GTPase Rac that underlie the regulatory role of ROS in the process of stem cell differentiation toward a cardiac lineage, are reviewed. *Antioxid. Redox Signal.* 7, 1435–1439.

INTRODUCTION

Intracellular reactive oxygen species (ROS) are generated by any organism living in an aerobic environment. Furthermore, biological systems are continuously exposed to exogenous oxidants. ROS include both free radicals, such as superoxide ($O_2^{\cdot -}$) and hydroxyl radical (OH·), and nonradical components, such as hydrogen peroxide (H_2O_2). Several sources of ROS have been identified in the cell, the main one being the mitochondria respiratory chain. In the cytosol, other intracellular sources of ROS include xanthine oxidase, cytochrome P450, as well as the membrane NADPH oxidase and nitric oxide synthase.

ROS have both deleterious (at high concentration) and beneficial (at low concentrations) effects on the function of a cell. Indeed ROS can damage a variety of biomolecules, including proteins and nucleic acids. On the other hand, ROS activate many transcription factors, such as nuclear transcription factor-κB (NFκB) and activator protein-1 (AP-1) (6), required for cell proliferation and differentiation. ROS are thus involved in both cell proliferation and differentiation, as well as in cell survival and death, depending on their intracellular concentration and possibly their subcellular source. The cells have developed defense mechanisms (superoxide dismutase, catalase, gluthatione reductase and peroxidase, etc.) to maintain a redox state compatible with their life. However, once again in biology the frontier between normality and pathology remains hazy, and a thorough characterization of ROS signaling in the cells has to be pursued to understand better the physiological role of these oxidants.

This review will focus on the role of ROS in the process of early cardiogenesis. I will review data from the literature and will propose some new lines of research for the near future.

ROLE OF ROS IN CARDIAC CELL DIFFERENTIATION AND CARDIOGENESIS

There are no data available as to the direct role of ROS in formation of the heart in embryos. Some studies investigating the effect of hyperbaric oxygen enhancing the production of ROS suggested a teratogenic effect of oxygen in rat and in mouse (12, 17, 20, 35).

To investigate the molecular mechanisms of cardiac cell differentiation, most authors use an *in vitro* system, namely, the embryonic stem (ES) cells. Indeed, pluripotent ES cells derived from the inner cell mass of the blastocyst are capable of giving rise to different progeny representative of the three embryonic layers, namely, the endoderm, the mesoderm, and the ectoderm. ES cells have the capability to self-renew and to differentiate (31). ES cells are differentiated within embry-

CRBM, CNRS FRE 2593, Montpellier, France.

oid bodies (EBs), a three-dimensional structure that recapitulates the early steps of embryogenesis and specifically cardiogenesis (16). ES cells feature a high level of antioxidant defense and can even grow under 40% hyperoxia. These cells express a high content of thioredoxin, glutathione reductase, glutathione *S*-transferase, and manganese superoxide dismutase. This capability to withstand high oxidative stress decreases upon cell differentiation. It is thus tempting to propose that a decrease in that capability to maintain thiol–disulfide equilibrium with glutathione/thioredoxin redox couple may allow the cell to adjust its level of ROS compatible with a role of these molecular species as second messenger in cell differentiation (27).

Sauer *et al.* (28) were the first to suggest a participation of ROS in cardiac cell differentiation. The authors treated ES cell-derived EBs at an early stage of differentiation, with 10 n*M* H_2O_2 or 20 μ*M* menadione, a compound known to generate H_2O_2. They observed an increase in the percentage of beating EBs, indicating the presence of cardiac cells in the EBs. Although inhibitors of phosphatidylinositol trisphosphate decreased beating activity of EBs, this effect was counteracted by addition of prooxidants H_2O_2 or menadione, suggesting that endogenous phosphatidylinositol 3-kinase (PI3K)-mediated ROS formation is required for cardiac differentiation of ES cells. The same authors further documented the ROS-dependent signaling pathway and reported that cardiotrophin induced proliferation of ES cell-derived cardiomyocytes through ROS generation. This effect is mediated by the JAK/STAT and mitogen-activated protein kinase (MAPK) pathways (29). However, the previously described effects of ROS have to be attributed to a low concentration of oxygen species. Indeed, forced expression of constitutive active Rac1, which generates ROS in ES cell-derived cardiomyocytes, or addition of 100 n*M* H_2O_2 dramatically impaired the process of cardiac cell differentiation (24). Similarly, the anticonvulsant valproic acid, which induces generation of ROS, delayed cardiac differentiation of ES cells (19). Na *et al.* pointed out that valproic acid-induced generation of ROS overwhelmed the antioxidative defense of the EBs. Despite all these reports, little is known as to the molecular mechanisms that underlie the effect of ROS on cardiac cell differentiation

SIGNALING PATHWAYS UNDERLYING ROS GENERATION

A Rac-dependent signaling pathway generates intracellular ROS

Rac is a monomeric GTPase. Inactive Rac is bound to GDP and constitutively inhibited by guanine dissociation inhibitors (GDIs) to prevent the exchange of GDP for GTP. Once an agonist (platelet-derived growth factor, epidermal growth factor, insulin growth factor, angiotensin, tumor necrosis factor-α) induces Rac activation, guanine nucleotide exchange factors (GEFs) catalyze the exchange of GDP for GTP and membrane localization. GTPase-activating proteins then regulate the hydrolysis of GTP back to inactive GDP-bound Rac. The membrane NADPH oxidase is a major Rac1/Rac2 effector enzyme (1, 2). The NADPH oxidase comprised of five components ($p40^{phox}$, $p47^{phox}$, $p22^{phox}$, $p67^{phox}$, and $gp91^{phox}$ or Nox2) generates ROS.

The major evidence for the regulation of NADPH oxidase by Rac was first uncovered in phagocytes. Indeed, in neutrophils stimulated with formylmethionyl-leucylphenylalanine, concanavalin A, or phorbol 12-myristate 13-acetate, both Rac 1 and Rac 2 are translocated from the cytosol to the membranes, and this phenomenon requires $p67^{phox}$, a subunit of the NADPH complex (7). In nonphagocytic cells, agonist stimulation was also shown to activate Rac and generates ROS (32). Rac-induced ROS activation mediates many cell functions, including gene expression, cell proliferation, and cell–cell or cell–matrix adhesion (34), pointing to a central role of the GTPase-mediated ROS generation in the cell life.

Several models have been proposed for NADPH oxidase regulation by Rac. Upon stimulation of cells with agonists, Rac dissociates from its cytosolic complex with GDI. GDP is then exchanged for GTP, a reaction catalyzed by a GEF. Rac further binds $p67^{phox}$ (21) through its switch 1 domain (3), allowing for activation of the NADPH oxidase. A Rac GEF (pix) was recently found to be associated with Nox1, another member of NADPH Nox family. Sequential activation of phosphatidylinositol 3-kinase, β Pix, Rac1, and Nox1 leads to ROS generation (22).

We showed that expression of a constitutively active mutant of Rac (RacV12) dramatically impaired cardiac differentiation of ES cells. $Gp91^{phox}$, $p22^{phox}$, and $p67^{phox}$, a Rac target, are highly expressed in embryonic tissues, including the heart, and in early differentiation stages of ES cell-derived EBs (5, 24, 28). ROS repress expression of numerous cardiac and muscle genes (18). We found that expression of RacV12 under the transcriptional control of the cytomegalovirus (CMV) promoter, which generates ROS at high concentration (23), decreased expression of Mef2C, a cardiac transcription factor required for expression of genes encoding cardiac constitutive proteins. Expression of a constitutively activated mutant of Rac (RacL61D38), which lost the ability to bind $p67^{phox}$ and in turn to activate the NADPH oxidase (21), did not prevent ES cells from differentiating into functional beating cardiac cells. The ROS scavenger, catalase, added to the culture medium of EBs rescued Mef2C expression, myofibrillogenesis, and in turn beating activity of RacV12 EBs. These data demonstrated the crucial deleterious role of Rac-induced ROS generation in early stages of cardiac cell differentiation. The molecular mechanisms of ROS regulation of Mef2c gene expression remains to be established.

On the contrary, at a later stage of differentiation, expression of RacV12 under the transcriptional control of the ventricular myosin light chain (MLC) favors cardiac differentiation of ES cells mainly by facilitating the myofibrillogenesis. It is possible that moderate production of ROS, because of the mild activity of the MLC promoter compared with the strong activity of a CMV promoter, rather induces proliferation of cardioblasts (29) and may facilitate the process of differentiation through a PI3K-dependent pathway (28). Alternatively, expression of Rac under the transcriptional control of the MLC promoter and in turn production of intracellular ROS occur at a later stage of differentiation, at which cells develop

more specific defense mechanisms against ROS, including high expression of myoglobin, and feature a better control of their redox state. ROS at low concentration may facilitate proliferation of cardiac progenitors while improving their differentiation toward mature cardiomyocytes, both cell processes not being mutually exclusive in cardiac cells.

NFκB and chromatin remodeling

NFκB has been proposed together with AP-1 and hypoxia-inducible factor-1 as a major target of ROS although this has been recently challenged (10). NFκB induces hypertrophy of cardiac cells (14, 33) following ROS generation (11). NFκB is thus, in principle, capable of triggering an embryonic genetic program at the adult stage. Despite the lack of available information, a role of NFκB can be predicted in the process of cardiac cell differentiation.

More generally, ROS have been suggested to influence chromatin remodeling. As ES cells undergo differentiation, they spontaneously execute developmentally regulated programs of gene expression and silencing that are both associated with alterations in chromatin structure (26). ROS increase histone acetylation by regulating histone acetyltransferase and deacetylase activity and phosphorylation (25). This enhances transcription factor activity with recruitment of coactivators, such as CBP and p300. p300 is required for Mef2c and GATA4 transcriptional activity (9, 36). Thus, ROS may act directly on the activity of two key transcription factors in cardiogenesis.

Chromatin remodeling is essential for normal cardiogenesis (8, 15). ROS impact on the high order chromatin may thus dramatically affect the process of cardiac cell differentiation both *in vitro* (26) and *in vivo*.

Ca^{2+}-dependent and kinase-dependent signaling pathways

ROS and Ca^{2+} interact to regulate mitochondrial metabolism (34). Ca^{2+} is known to regulate many processes of gene transcription and cell differentiation. More specifically, the early steps of cardiogenesis are dependent on Ca^{2+} signaling (13). Indeed, the activity of several proteins involved in the process of cardiac gene transcription or of formation of the contractile apparatus (myofibrillogenesis) is dependent on intracellular Ca^{2+}-dependent kinase or phospholipase activities, generating second messengers mobilizing intracellular Ca^{2+} (inositol trisphosphate). The effects of ROS on the activity of numerous kinases and phospholipases (tyrosine kinases, protein kinase Cs, MAPKs, phospholipase Cγ, growth factor kinases) strongly suggest that ROS mediate some effects of growth factors on cardiac cell differentiation.

ROS also affect $p21^{Cip}$ and cyclin D1, and several phases of the cell cycle (4). Up-regulation of $p21^{Cip}$ and cyclin D1 by transforming growth factor β participates in cardiac commitment of ES cells (unpublished data). It is tempting to speculate that the switch between a high proliferative capability of ES cells (with prolonged S phase, absence of G1/S checkpoint) (30) and a MAPK-regulated somatic cell cycle upon cell differentiation can be tightly regulated by intracellular ROS levels.

CONCLUSIONS

The effects of ROS on the process of cardiac cell differentiation are just emerging (Fig. 1). There are specific questions that need to be answered to make progress in this issue. One

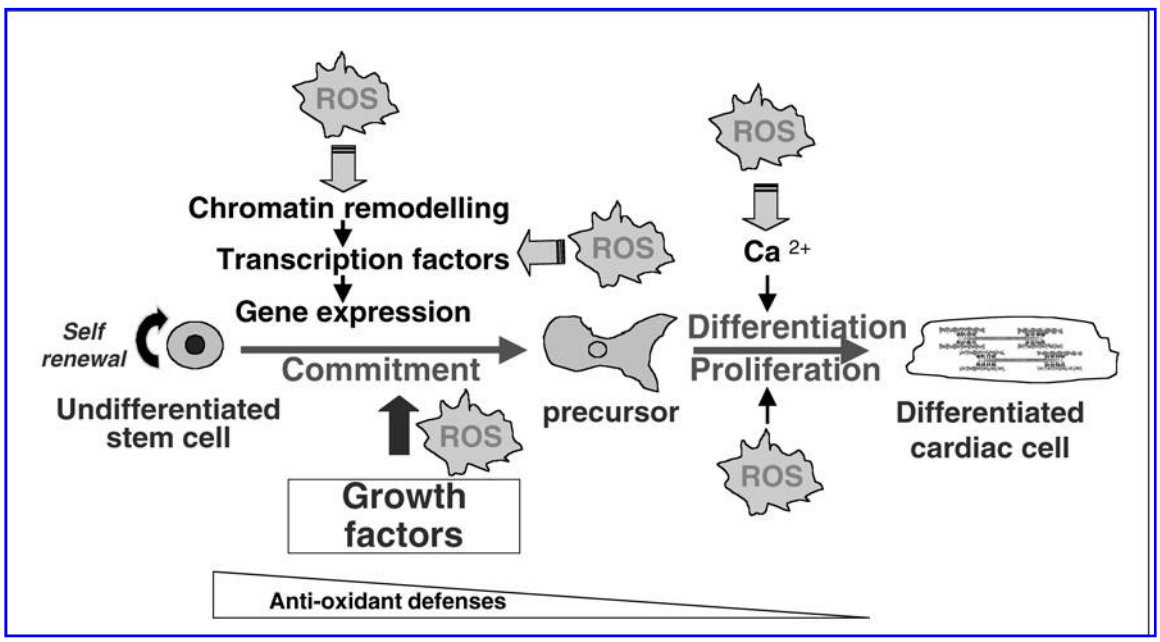

FIG. 1. Targets of ROS in processes of cardiac commitment and differentiation of ES cells.

needs to better investigate spatially localized ROS generation in cardiac progenitors and to quantify the phenomenon. Indeed any response of the cell at the molecular level (*e.g.,* gene transcription, protein synthesis) will depend on the level of ROS in a local environment. How ROS specifically target expression of muscle transcription factors (Mef2c, MyoD) has to be elucidated.

Stem cell differentiation represents the early step of embryogenesis and is essentially an attempt to achieve tissue-specific patterns of gene expression *in vitro*. Studies of the effects of ROS on chromatin in differentiating ES cells will greatly help in understanding the regulatory role of ROS in cardiac cell differentiation.

ABBREVIATIONS

AP-1, activator protein-1; CMV, cytomegalovirus; EB, embryoid body; ES, embryonic stem; GDI, guanine dissociation inhibitors; GEF, guanine nucleotide exchange factors; H_2O_2, hydrogen peroxide; MAPK, mitogen-activated protein kinase; MLC, myosin light chain; NFκB, nuclear factor-κB; PI3K, phosphatidylinositol 3-kinase; ROS, reactive oxygen species.

REFERENCES

1. Archer H and Bar-Sagi D. Ras and Rac as activators of reactive oxygen species (ROS). *Methods Mol Biol* 189: 67–73, 2002.
2. Babior BM. NADPH oxidase: an update. *Blood* 93: 1464–1476, 1999.
3. Bokoch GM and Diebold BA. Current molecular models for NADPH oxidase regulation by Rac GTPase. *Blood* 100: 2692–2696, 2002.
4. Boonstra J and Post JA. Molecular events associated with reactive oxygen species and cell cycle progression in mammalian cells. *Gene* 337: 1–13, 2004.
5. Cheng G, Cao Z, Xu X, van Meir EG, and Lambeth JD. Homologs of gp91phox: cloning and tissue expression of Nox3, Nox4, and Nox5. *Gene* 269: 131–140, 2001.
6. Dalton TP, Shertzer HG, and Puga A. Regulation of gene expression by reactive oxygen. *Annu Rev Pharmacol Toxicol* 39: 67–101, 1999.
7. Dusi S, Donini M, and Rossi F. Mechanisms of NADPH oxidase activation in human neutrophils: p67phox is required for the translocation of rac 1 but not of rac 2 from cytosol to the membranes. *Biochem J* 308 (Pt 3): 991–994, 1995.
8. Gottlieb PD, Pierce SA, Sims RJ, Yamagishi H, Weihe EK, Harriss JV, Maika SD, Kuziel WA, King HL, Olson EN, Nakagawa O, and Srivastava D. Bop encodes a muscle-restricted protein containing MYND and SET domains and is essential for cardiac differentiation and morphogenesis. *Nat Genet* 31: 25–32, 2002.
9. Hasegawa K, Meyers MB, and Kitsis RN. Transcriptional coactivator p300 stimulates cell type-specific gene expression in cardiac myocytes. *J Biol Chem* 272: 20049–20054, 1997.
10. Hayakawa M, Miyashita H, Sakamoto I, Kitagawa M, Tanaka H, Yasuda H, Karin M, and Kikugawa K. Evidence that reactive oxygen species do not mediate NF-kappaB activation. *EMBO J* 22: 3356–3366, 2003.
11. Higuchi Y, Otsu K, Nishida K, Hirotani S, Nakayama H, Yamaguchi O, Matsumura Y, Ueno H, Tada M, and Hori M. Involvement of reactive oxygen species-mediated NF-kappa B activation in TNF-alpha-induced cardiomyocyte hypertrophy. *J Mol Cell Cardiol* 34: 233–240, 2002.
12. Jaffee DC. Hemodynamics and cardiogenesis: the effects of physiologic factors on cardiac development. *Birth Defects Orig Artic Ser* 14: 393–404, 1978.
13. Li J, Puceat M, Perez-Terzic C, Mery A, Nakamura K, Michalak M, Krause KH, and Jaconi ME. Calreticulin reveals a critical Ca^{2+} checkpoint in cardiac myofibrillogenesis. *J Cell Biol* 158: 103–113, 2002.
14. Li Y, Ha T, Gao X, Kelley J, Williams DL, Browder IW, Kao RL, and Li C. NF-kappaB activation is required for the development of cardiac hypertrophy in vivo. *Am J Physiol Heart Circ Physiol* 287: H1712–H1720, 2004.
15. Lickert H, Takeuchi JK, Von Both I, Walls JR, McAuliffe F, Adamson SL, Henkelman RM, Wrana JL, Rossant J, and Bruneau BG. Baf60c is essential for function of BAF chromatin remodelling complexes in heart development. *Nature* 432: 107–112, 2004.
16. Maltsev VA, Wobus AM, Rohwedel J, Bader M, and Hescheler J. Cardiomyocytes differentiated in vitro from embryonic stem cells developmentally express cardiac-specific genes and ionic currents. *Circ Res* 75: 233–244, 1994.
17. Miller PD, Telford IR, and Haas GR. Effect of hyperbaric oxygen on cardiogenesis in the rat. *Biol Neonate* 17: 44–52, 1971.
18. Morel Y and Barouki R. Repression of gene expression by oxidative stress. *Biochem J* 342 Pt 3: 481–496, 1999.
19. Na L, Wartenberg M, Nau H, Hescheler J, and Sauer H. Anticonvulsant valproic acid inhibits cardiomyocyte differentiation of embryonic stem cells by increasing intracellular levels of reactive oxygen species. *Birth Defects Res A Clin Mol Teratol* 67: 174–180, 2003.
20. New DA and Coppola PT. Effects of different oxygen concentrations on the development of rat embryos in culture. *J Reprod Fertil* 21: 109–118, 1970.
21. Nisimoto Y, Freeman JL, Motalebi SA, Hirshberg M, and Lambeth JD. Rac binding to p67(phox). Structural basis for interactions of the Rac1 effector region and insert region with components of the respiratory burst oxidase. *J Biol Chem* 272: 18834–18841, 1997.
22. Park HS, Lee SH, Park D, Lee JS, Ryu SH, Lee WJ, Rhee SG, and Bae YS. Sequential activation of phosphatidylinositol 3-kinase, beta Pix, Rac1, and Nox1 in growth factor-induced production of H_2O_2. *Mol Cell Biol* 24: 4384–4394, 2004.
23. Price MO, Atkinson SJ, Knaus UG, and Dinauer MC. Rac activation induces NADPH oxidase activity in transgenic COSphox cells, and the level of superoxide production is exchange factor-dependent. *J Biol Chem* 277: 19220–19228, 2002.
24. Puceat M, Travo P, Quinn MT, and Fort P. A dual role of the GTPase Rac in cardiac differentiation of stem cells. *Mol Biol Cell* 14: 2781–2792, 2003.

25. Rahman I, Marwick J, and Kirkham P. Redox modulation of chromatin remodeling: impact on histone acetylation and deacetylation, NF-kappaB and pro-inflammatory gene expression. *Biochem Pharmacol* 68: 1255–1267, 2004.
26. Rasmussen TP. Embryonic stem cell differentiation: a chromatin perspective. *Reprod Biol Endocrinol* 1: 100, 2003.
27. Saretzki G, Armstrong L, Leake A, Lako M, and von Zglinicki T. Stress defense in murine embryonic stem cells is superior to that of various differentiated murine cells. *Stem Cells* 22: 962–971, 2004.
28. Sauer H, Rahimi G, Hescheler J, and Wartenberg M. Role of reactive oxygen species and phosphatidylinositol 3-kinase in cardiomyocyte differentiation of embryonic stem cells. *FEBS Lett* 476: 218–223, 2000.
29. Sauer H, Neukirchen W, Rahimi G, Grunheck F, Hescheler J, and Wartenberg M. Involvement of reactive oxygen species in cardiotrophin-1-induced proliferation of cardiomyocytes differentiated from murine embryonic stem cells. *Exp Cell Res* 294: 313–324, 2004.
30. Savatier P, Lapillonne H, van Grunsven LA, Rudkin BB, and Samarut J. Withdrawal of differentiation inhibitory activity/leukemia inhibitory factor up-regulates D-type cyclins and cyclin-dependent kinase inhibitors in mouse embryonic stem cells. *Oncogene* 12: 309–322, 1996.
31. Smith AG. Embryo-derived stem cells: of mice and men. *Annu Rev Cell Dev Biol* 17: 435–462, 2001.
32. Sundaresan M, Yu ZX, Ferrans VJ, Sulciner DJ, Gutkind JS, Irani K, Goldschmidt-Clermont PJ, and Finkel T. Regulation of reactive-oxygen-species generation in fibroblasts by Rac1. *Biochem J* 318 (Pt 2): 379–382, 1996.
33. van Empel VP and De Windt LJ. Myocyte hypertrophy and apoptosis: a balancing act. *Cardiovasc Res* 63: 487–499, 2004.
34. Werner E. GTPases and reactive oxygen species: switches for killing and signaling. *J Cell Sci* 117: 143–153, 2004.
35. Wolff-Quenot MJ and Vidal S. [Teratogenic effect of hyperbaric oxygen in rat and mouse (author's translation)]. *Arch Anat Histol Embryol* 62: 3–11, 1979.
36. Yanazume T, Morimoto T, Wada H, Kawamura T, and Hasegawa K. Biological role of p300 in cardiac myocytes. *Mol Cell Biochem* 248: 115–119, 2003.

Address reprint requests to:
M. Pucéat, Ph.D.
CRBM, CNRS FRE 2593
1919, route de Mende
34293, Montpellier, France

E-mail: michel.puceat@crbm.cnrs.fr

Received after final revision June 20, 2005; accepted June 30, 2005.

This article has been cited by:

1. Clifford D.L. Folmes, Timothy J. Nelson, Petras P. Dzeja, Andre Terzic. 2012. Energy metabolism plasticity enables stemness programs. *Annals of the New York Academy of Sciences* **1254**:1, 82-89. [CrossRef]
2. Jörg-Detlef Drenckhahn. 2011. Heart Development: Mitochondria in Command of Cardiomyocyte Differentiation. *Developmental Cell* **21**:3, 392-393. [CrossRef]
3. Lee Anna Cunningham, Kate Candelario, Lu Li. 2011. Roles for HIF-1# in neural stem cell function and the regenerative response to stroke. *Behavioural Brain Research* . [CrossRef]
4. Elena Serena, Elisa Figallo, Nina Tandon, Christopher Cannizzaro, Sharon Gerecht, Nicola Elvassore, Gordana Vunjak-Novakovic. 2009. Electrical stimulation of human embryonic stem cells: Cardiac differentiation and the generation of reactive oxygen species. *Experimental Cell Research* **315**:20, 3611-3619. [CrossRef]
5. S. A. Eremeyev, V. I. Kargin, K. A. Motovilov, V. N. Tashlitsky, V. Yu. Markov, G. A. Korshunova, N. V. Sumbatyan, M. Yu. Vyssokikh, L. S. Yaguzhinsky. 2009. Molecular mechanisms of transformation of SkQ mitotropic quinones and the search for new approaches to creation of selective free radical traps. *Biochemistry (Moscow)* **74**:10, 1114-1124. [CrossRef]
6. Elisa Bregant, Giovanni Renzone, Renata Lonigro, Nadia Passon, Carla Loreto, Maura Pandolfi, Andrea Scaloni, Gianluca Tell, Giuseppe Damante. 2009. Down-regulation of SM22/transgelin gene expression during H9c2 cells differentiation. *Molecular and Cellular Biochemistry* **327**:1-2, 145-152. [CrossRef]
7. Nan Wang, Chunhui Sun, Siwei Huo, Yun Zhang, Jing Zhao, Shangli Zhang, Junying Miao. 2008. Cooperation of phosphatidylcholine-specific phospholipase C and basic fibroblast growth factor in the neural differentiation of mesenchymal stem cells in vitro. *The International Journal of Biochemistry & Cell Biology* **40**:2, 294-306. [CrossRef]
8. Nicanor I. Moldovan . 2005. Emerging Roles of Reactive Oxygen and Nitrogen Species in Stem/Progenitor Cells. *Antioxidants & Redox Signaling* **7**:11-12, 1409-1412. [Citation] [Full Text PDF] [Full Text PDF with Links]