

POST-REPERFUSION HEALING OF THE HEART:

FOCUS ON OXYGEN-SENSITIVE GENES AND DNA MICROARRAY AS A TOOL

CHANDAN K. SEN^{1†}, JOSEPH S. VERDUCCI³, VINCENT F. MELFI⁴,

SAVITA KHANNA¹, CATALIN BARBACIORU² AND SASHWATI ROY¹

¹Laboratory of Molecular Medicine and DNA Microarray Facility, Davis Heart & Lung Research Institute, Department of Surgery, and ²Department of Biomedical Informatics, The Ohio State University Medical Center; ³Department of Statistics, College of Mathematical and Physical Sciences; and ⁴Mathematical Biosciences Institute, The Ohio State University, Columbus, Ohio 43210

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Correspondence:

Professor Chandan K. Sen
512 Davis Heart & Lung Research Institute
The Ohio State University Medical Center
473 W. 12th Avenue, Columbus, OH 43210
Tel. 614 247 7658; Fax 614 247 7818
sen-1@medctr.osu.edu

Footnote to page 1. At least two different units are used in the literature for the expression of oxygenation, %O₂ and pO₂. Each may be appropriate for specific conditions, and often they are interconvertible. For example, one atmosphere (760 mm Hg) of air (20.95%O₂ / 78.08% N₂) is equivalent to pO₂ = 159 mm Hg or Torr. At 37°C, one atmosphere of humidified air corrected for the partial pressure of H₂O (47 mm Hg) is equivalent to 760-47 = 713 mmHg or Torr. Therefore, for humidified air in the body (say tracheal air) at 37°C, 1% and 7.13 mm Hg or Torr are equivalent. Because this article discusses *in vitro* studies involving cell culture incubators set at %O₂, this unit has been used.

ABSTRACT

Focal coronary artery blockage followed by further reperfusion injury is commonly involved in myocardial infarction. The injured heart has some inherent reparative responses. Although such natural healing mechanisms seem to be inefficient, a clear understanding of the underlying principles of myocardial healing holds the key to successful therapy. Under normoxic conditions, pO_2 ranges from 90 to <3 Torr in mammalian organs with the heart at ~ 35 Torr (5%) and arterial blood at ~ 100 Torr. Thus, “normoxia” for cells is an adjustable variable. In response to chronic moderate hypoxia, cells lower their normoxia set-point such that reoxygenation-dependent relative elevation of pO_2 ($+\Delta pO_2$) results in perceived hyperoxia. Perceived hyperoxia induces differentiation of cardiac fibroblasts to myofibroblasts in the peri-infarct region and represents one factor supporting myocardial healing. This review article discusses the significance of oxygen-sensitive genes in the cardiac fibroblasts in post-reoxygenation myocardial healing. In addition to being indispensable as a tool for identifying O_2 -sensitive genes in the cardiac fibroblast and categorizing them into functional groups, the DNA microarray plays a wider role in helping to identify pathways of development and repair, as well as potential targets for therapy. Because appropriate statistical analyses and development of pathway networks are critical for extracting useful information from microarrays, pointers to recent developments in DNA microarray as an analytical tool are presented.

INTRODUCTION

Focal coronary artery blockage followed by further reperfusion injury is a key sequence of events that are associated with myocardial infarction. The typical clinical case of reperfusion injury occurs in acute myocardial infarction in which an occlusion of a major epicardial coronary artery is followed by re-establishment of the vascular canal of the artery. Reperfusion therapy is often used in an attempt to salvage the ischemic tissues. Other conditions involving reperfusion include cardiac surgery when the heart is arrested with cardioplegia to facilitate surgical intervention and is subsequently reoxygenated after removal of the aortic cross-clamp. Acute hypoxia, followed by abrupt reoxygenation using say cardiopulmonary bypass, results in an unintended injury mediated by oxygen free radicals (2). While reperfusion has been mostly studied in the context of “oxygen wastage” (129) and related oxidative injury (2), it is important to underscore that reperfusion is necessary for revival of physiological functioning and survival of ischemic tissues. Recent studies clearly establish that the challenged heart does have some inherent reparative responses ranging from recruitment of progenitor cells from the bone marrow to the injury site (110), mitosis of cardiomyocytes (8) to differentiating fibroblasts at the injury site to myofibroblast (125). Although such naturally occurring myocardial reparative process seems to be relatively inefficient in preventing adverse functional outcomes, a clear understanding of the underlying principles of myocardial healing hold the key to successful therapy. Thus, substantial efforts have been directed towards the understanding of natural principles that underlie healing of the reperfused heart muscle. Such knowledge would serve as an indispensable tool to vitalize the natural healing responses towards improved functional outcomes. The objective of this article is to discuss the significance of oxygen-sensitive genes in the cardiac fibroblasts in post-reoxygenation myocardial healing followed by the analysis of DNA microarray as a tool in experimental heart research.

ISCHEMIA-REPERFUSION AND OXYGEN TENSION

Under conditions of systemic normoxia, the heart cells receive a limited supply of O₂ not to exceed a maximum of 10% (57, 163, 165). This is consistent with the recent observation that myocardial pO₂ in the working murine heart *in vivo* is 5% (123). The myocardium may be exposed to hypoxia under a number of conditions such as transplantation, myocardial ischemia after occlusion of a major coronary artery, high altitude, and anemia. Conditions of ischemia in the heart, such as caused by the occlusion of a distal arterial vessel, result in a hypoxic area containing a central focus of near-zero O₂ pressure bordered by tissue with diminished but nonzero pO₂ (Fig. 1). These border zones extend for several millimeters from the hypoxic core, with the O₂ pressures progressively increasing from the focus to the normoxic region (126). Moderate hypoxia is associated with a 30-60% decrease (~1-3% O₂) in pO₂ (141). Since prior studies showed that O₂ consumption becomes O₂-limited only below 0.1% O₂ in isolated rat cardiac myocytes and below 0.3% in isolated rat hearts, physiological or metabolic changes observed in cells at 1-3% O₂ would be unlikely to result from limited oxygenation of mitochondrial cytochromes (77, 166). In response to mild or moderate compromise in pO₂, adaptive processes in surviving cells allow for physiological functioning of the tissue. These adjustments are evident for example in the hibernating myocardium where the organ maintains vital functions in the face of prolonged moderate hypoxia (18). Although adjustments in metabolism and contractile function have been demonstrated to allow myocardial survival in the face of reduced O₂ supply, the cellular basis of such adaptation and the signaling pathways involved in the process remain to be defined (142).

OXYGEN AND OXYGEN SENSING

Oxygen got its name from "*Principe Oxygene*", which means acidifying principle. "Oxy" is

from Greek, and means sharp or acid; "gen" is also from Greek, and means the origin of. Taken together, oxygen means "the origin of acid". Joseph Priestly's (1774) "dephlogisticated air" (116) and Carl Scheele's (1771) "fire air" were soon characterized by Antoine Lavoisier as pure respirable air (83). Within decades of the first realization that oxygen is the element of life, Brizé-Fradin (14) noted in 1808 that "vital air" or pure oxygen would soon wear life out instead of maintaining it. That oxygen may be harmful to human health was first postulated in the late 19th century with Paul Bert's work (1878) on oxygen sickness. That observation was extended through Michaeli's theoretical considerations, Gerschman's experimental verification and finally caught the interests of biomedical scientists when in 1969 McCord & Fridovich demonstrated that a metalloenzyme produced hydrogen peroxide (H_2O_2) by combining a toxic metabolite of oxygen known as superoxide (O_2^-) with hydrogen (96, 97). Claude Bernard's claim that the "*fixity of the milieu interieur*" was essential to the life of higher organisms in the early 19th century was followed by Walter Cannon's concept of "homoeostasis" who refined and extended the concept of self-regulating mechanisms in living systems. As with numerous biological systems, the concept of homeostasis applies to the tissue biology of oxygen as well.

Cellular O_2 homeostasis is tightly maintained within a narrow range (perceived as "normoxia") due to the risk of oxidative damage from excess O_2 (hyperoxia), and of metabolic demise from insufficient O_2 (hypoxia) (137). pO_2 ranges from 90 to below 3 Torr in mammalian organs under normoxic conditions with arterial pO_2 of about 100 Torr or ~14% O_2 (112). Thus, "normoxia" for cells is a variable that is dependent on the specific localization of the cell in organs and functional status of the specific tissue. O_2 sensing is required to adjust to physiological or pathophysiological variations in pO_2 . Whereas acute responses often entail changes in the activity of pre-existing proteins, chronic responses invariably involve O_2 -

sensitive changes in signal transduction and gene expression (41). Several current articles have highlighted the key significance of understanding the fundamentals of O₂ sensing (15, 40, 42, 67, 68, 76, 80, 91, 104, 139, 173). The elegantly elaborated principles of hypoxia sensing in the bacteria and yeast (19, 56, 109) have still to find their matching counterparts in higher organisms. Even transient localized O₂ deficits can produce irreversible cellular damage. The lack of O₂ is critical in the pathogenesis of major causes of mortality such as stroke, myocardial infarction and chronic lung diseases. This provides for a compelling rationale to decipher the hypoxic response pathway. This pathway is implicated in adaptation to hypoxia-induced physiological stress by regulating changes in gene expression, and is also critical for the execution of many physiological events including formation of blood vessels during embryogenesis, and pathophysiological processes such as tumorigenesis (16). A family of hypoxia-inducible transcription factors (HIFs) lies at the heart of this adaptive pathway (137, 138). HIF-1 is a heterodimer composed of hypoxia-regulated HIF-1 α (or its paralogs HIF-2 α and HIF-3 α) and oxygen-insensitive HIF-1 β subunits which are basic-loop-helix-PAS domain proteins (136). The α -subunit of the heterodimeric transcription factors HIF-1, 2 and 3 is unstable under normoxia but is rapidly stabilized upon exposure to hypoxic conditions. Following heterodimerization with the constitutively expressed beta subunit, HIFs activate the transcription of an increasing number of genes involved in maintaining oxygen homeostasis at the cellular, local and systemic levels. A major advance in our comprehension of how hypoxia is sensed by mammalian cells came forth with the discovery of a family of oxygen-dependent enzymes responsible for the modulation of HIF-1 stability (15, 42, 68). More recently, a second oxygen-dependent post-translational modification of HIF that regulates the ability of HIF to recruit stimulatory transcriptional cofactors to its target genes has been identified (80). Degradation of HIF-1 α under normoxic conditions is triggered by

post-translational hydroxylation of conserved proline residues within a polypeptide segment known as the oxygen-dependent degradation domain (ODD). Hydroxylation of at least two prolyl residues in ODD of HIF-1 α regulates its interaction with the von Hippel-Lindau protein (VHL) that targets HIF-1 α for ubiquitination and proteosomal degradation (136). Induction of HIF-1 α in response to hypoxia is instantaneous arguing against a complex signal transduction pathway (69). Reactive oxygen species (ROS), present at higher concentrations under normoxia compared to hypoxia, have been proposed to be a key player in oxygen sensing (112). ROS is thought to oxidatively degrade HIF-1 α under normoxic conditions (112). Arguing against a role of ROS in HIF-1 α induction, Jaakkola et al. have demonstrated that in an *in vitro* system lacking cellular enzymes O₂⁻ or H₂O₂ fails to induce HIF-1 α degradation response (68). In this context it should be noted that while it is true that specific prolyl hydroxylase enzymes are necessary for oxygen sensing, dioxygen is a required cofactor (68) and that inside the cell H₂O₂ is known to serve as a precursor of dioxygen when acted upon by catalase (122). Several laboratories have reported that ROS negate hypoxia inducible cellular responses. The addition of exogenous H₂O₂ inhibited hypoxia-induced erythropoietin (Epo) production in HepG2 cells. Likewise, elevation of endogenous H₂O₂ levels by the addition of menadione or the catalase inhibitor, aminotriazole, dose-dependently lowered Epo production (44). Sequestration of H₂O₂ mimics hypoxia (20). Antioxidants have been able to mimic hypoxia by inducing HIF-1 α under normoxic conditions (28, 128). In striking contrast to these findings, Schumacker and associates reported that cardiomyocytes increase ROS production following exposure to hypoxia (37, 157) and that mitochondrial cytochrome c oxidase serves as the oxygen sensor that enhances ROS production (18, 21). While the dispute regarding the conditions under which ROS induces or suppress cellular responses to hypoxia requires additional definitive data, it may be summarized that there is a general

consensus and clear rationale supporting that ROS play a key role in oxygen sensing (161).

Current work in the field of oxygen sensing is almost exclusively focused on the study of hypoxia. Reoxygenation, on the other hand, has been mostly investigated in the context of oxidative injury and there is a clear paucity of data describing the O₂-sensitive signal transduction pathways under conditions of oxygenation that mildly or moderately exceed perceived normoxia. During hypoxia in the heart, cells adjust their normoxic set-point such that the return to normoxic pO₂ after hypoxia is perceived as “relative hyperoxia” (41, 103). Understanding the molecular responses to such hyperoxic challenge is important because they are triggered by tissue reoxygenation *in vivo*. “O₂ wastage” and oxidative injury (129) represent important aspects of ischemia-reoxygenation biology, however, it is important to underscore that reperfusion/reoxygenation is necessary for the revival of physiological functioning and long-term survival of ischemic tissues.

PERCEIVED HYPEROXIA : P21 AS A KEY EFFECTOR

Although cells are cultured in the laboratory at an ambient O₂ concentration of 20%, which corresponds to a pO₂ of approximately 140 mmHg at sea level, cells in the human body are exposed to much lower O₂ concentrations ranging from ~14% (100 mm Hg) in the pulmonary alveoli to 5% (35 mm Hg) in the heart. Culturing cells at room air is generally considered to be a “normoxic” condition. Studies related to cellular effects of hyperoxia have focused on concentrations of O₂ much higher than 20% (144). A quarter of a century ago it was published in *Nature* that human diploid fibroblasts grown at 10% O₂ have a longer life than cells grown at the routine 20.6% O₂ (106). Consistently, development of pre-implantation embryos clearly favors 7% O₂ over 20% O₂ ambience (7). Ambient O₂ is readily dissolved in the cell culture medium (93). Thus, isolating a primary heart cell from a 5% O₂ environment

and maintaining it at 20% O₂ room-air condition may be expected to subject the cells to “O₂ stress” that would be sensed by mechanisms responding to supraphysiological levels O₂. To address this issue, recent works in our laboratory have tested the hypothesis that that O₂, even in marginal relative excess of the pO₂ to which cells are adjusted, results in activation of specific signaling pathways that alter the phenotype and function of cells (123-125). We proposed that during mild hypoxia, myocardial cells adjust their normoxia set-point such that reoxygenation-dependent relative elevation of pO₂ results in "perceived hyperoxia" (Fig. 1).

The first line of evidence supporting the concept of perceived hyperoxia originated from *in vitro* studies. Adult ventricular cardiac fibroblasts, grown under conditions of 20% or 10%O₂ since isolation from 5%O₂ *in vivo*, proliferated significantly slower than cells grown at near-physiological 3%O₂. Growth arrest at G2/M phase was evident. On day 6 of culture, the number of cells at 3%O₂ was double compared to cardiac fibroblasts count at 20%O₂. The O₂-sensitive growth inhibition was reversible ruling out senescence as the primary route of growth arrest. Previous studies with non-cardiac fibroblasts suggest the differentiation of fibroblasts is subject to reversal (92, 159). Cardiac fibroblasts isolated from 5%O₂ *in vivo* and grown at a four-fold O₂-rich ambience underwent a clear change of phenotype indicative of differentiation. Such changes were clearly minimized in cells grown under 5%O₂ culture conditions. Markers of O₂-induced differentiation of cardiac fibroblasts to myofibroblasts included substantial increase in cell size, appearance of stress fibers and parallel reorganization of smooth muscle actin with the stress fibers. Furthermore, exposure of cardiac fibroblasts to supraphysiological concentration of O₂ enhanced vimentin and smooth muscle actin expression as well as increased contractility of cells in a collagen matrix. These observations confirmed that exposure of cardiac fibroblasts to elevated O₂ triggers differentiation of the cells to myofibroblasts. Here, elevated O₂ refers to an ambience

containing higher concentration of O_2 than the levels of O_2 to which the cells are adjusted *in vivo* (Fig. 2).

TGF β 1 is a known inducer of cardiac fibroblast differentiation (36). The morphological/cytoskeletal characteristics of cardiac fibroblasts observed in response to elevated O_2 matched those of cardiac fibroblast cultured at 3% O_2 but treated with TGF β 1. Strikingly, inhibition of p38MAPK significantly released both TGF β 1-induced as well as elevated O_2 -induced growth inhibition. These observations suggested a parallel between TGF β 1- and perceived hyperoxia induced changes in cellular responses. Application of the high-density DNA microarray approach coupled with bioinformatics tools (see latter section on *Microarray as Tool* for a treatise on analytical considerations) identified O_2 -sensitive genes in cardiac fibroblasts and categorized them into functional groups (125). Results from unbiased screening for O_2 -sensitive genes confirmed the p21-p53 axis as being a key target of cardiac fibroblast exposure to elevated O_2 . This finding is consistent with the current notion that many of the signaling pathways that control cellular decisions related to tissue remodeling are regulated by nuclear interactions of cell-cycle proteins (102). Microscopic visualization of cardiac fibroblasts revealed that the nucleus of cells exposed to 20% O_2 clearly stained more prominently for the presence of p21 protein compared to fibroblasts at 3% O_2 . Exposure of cardiac fibroblasts to 20% O_2 resulted in a significant increase in p21 promoter-driven luciferase reporter activity. To test the significance of elevated O_2 -induced p21 expression on growth arrest observed under conditions of room air, experiments were conducted using cardiac fibroblasts isolated from the heart of p21 knock-out mice. Strikingly, p21 deficient cells completely escaped from elevated O_2 -induced growth arrest. Thus, p21 has been identified as a key effector of perceived hyperoxia.

Quantitative analysis of gene expression revealed that in addition to p21, exposure of cardiac fibroblasts to elevated O_2 resulted in the marked induction of cyclin D1, D2, G1 and Fra-2. Elevated expression of these candidates are not only associated with growth inhibition but also with differentiation (5, 43, 47, 75, 95, 98, 105, 108). The D-type cyclins consist of cyclins D1, D2 and D3. Cyclin D1 synthesis is induced by p21 (25). Cyclin D2 expression is known to be induced in multiple states of growth arrest (98). Cyclin G1 is involved in G_2/M arrest (75). This is consistent with the observation that cardiac fibroblasts exposed to elevated O_2 contain higher levels of cyclin G1 mRNA and are in G_2/M arrest. Fos-related antigen 2 (Fra-2) is a member of the Fos family of immediate-early genes, most of which are rapidly induced by second messengers. All members of this family act by binding to AP-1 sites as heterodimeric complexes with other proteins. However, each appears to have a distinct role. Although the role and biology of Fra-2 are less understood than those of its relatives c-Fos, Fra-1, and FosB, it is evident that elevated Fra-2 is associated with cellular differentiation (5, 95, 105).

Because cellular phenotype induced by perceived hyperoxia compared well with $TGF\beta_1$ -induced morphological changes, the hypothesis that $TGF\beta_1$ is involved in conferring O_2 -sensitive phenotype to cardiac fibroblasts was proposed. Both total and active $TGF\beta_1$ were substantially higher in cardiac fibroblasts exposed to 20% O_2 . Experiments with conditioned cell culture media supported that the media of cardiac fibroblasts grown at 20% O_2 contain significantly higher amounts of active $TGF\beta_1$ compared to media from cells at 3% O_2 . In support of the hypothesis that ROS can trigger the displacement of latency-associated peptide from $TGF\beta_1$, it has been observed that exposure to $TGF\beta_1$ containing conditioned culture media to oxidant challenge (UVC) significantly increased the levels of active $TGF\beta_1$. It

is plausible that ROS generated by cells at elevated O_2 may activate TGF β 1 (123). p38MAPK represents a major down-stream mediator of TGF β signaling (29, 30, 54, 59, 63, 78, 133, 160, 162, 168). Using in-gel kinase assay it has been observed that the activation of p38MAPK in isolated primary cardiac fibroblasts is sensitive to ambient O_2 . Furthermore, the growth inhibition caused by exposure to elevated O_2 can be abrogated in the presence of a p38MAPK inhibitor. These observations lead to the hypothesis that exposure of cardiac fibroblasts to perceived hyperoxia induces the activation of p38MAPK which in turn plays a significant role in executing O_2 -induced growth inhibition via inducible p21 expression (Fig. 2).

CARDIAC FIBROBLASTS: KEY PLAYERS IN MYOCARDIAL REPAIR

In excess of 90% of the myocardium's interstitial cells are fibroblasts (39), which actively cross-talk with myocytes (107) to determine the quantity and quality of extracellular matrix (39, 74, 81). Compared to myocytes, cardiac fibroblasts are relatively more resistant to oxidant insult (170). Under certain pathological conditions such as aortic regurgitation, cardiac fibroblasts produce abnormal proportions of non-collagen extracellular matrix, specifically fibronectin, with relatively little change in collagen synthesis (13). A characteristic feature of cardiac fibroblasts is their ability to differentiate forming myofibroblasts. Specific factors that facilitate this differentiation process have been recently identified (46). Cardiac fibroblasts electrically couple with cardiac myocytes to contribute to the tissue's electrophysiological properties responsible for maintaining cardiac rhythm (45). The CMG cardiomyogenic cell line, which serves as precursor of a mixture of fibroblasts and spontaneously beating cardiomyocyte-like cells, generates myocytes with sustained functionality for transplantation purposes (50). In addition to the well-recognized co-operation with myocytes, cardiac fibroblasts are known to interact with endothelial cells to support angiogenesis in the heart

(171). Fibroblasts actively regulate interstitial fluid pressure in loose connective tissue by maintaining tension on the collagen network (10, 121). Taken together, cardiac fibroblasts represent an integral component of the key mechanisms that are required to maintain normal cardiac functioning, defend the heart against insults and orchestrate remodeling of injured tissue (149). It has been classically acknowledged that the mammalian heart has a very limited regenerative capacity and, hence, heals by scar formation (101), a process directly regulated by fibroblasts and their derivative matrix products. Culture of isolated myocardial cells, both myocytes and interstitial cells, have proven to be very useful over the last decade for studying molecular and cellular cardiac physiology and pathophysiology (11, 27, 35, 52, 58, 60, 73, 79, 81, 127, 150, 153, 158, 164). Cardiac fibroblasts are mainly responsible for the synthesis of major extracellular matrix (ECM) in the myocardium including fibrillar collagen types I and III and fibronectin. The cardiac ECM forms a stress-tolerant network that facilitates the distribution of forces generated in the heart and provides for proper alignment of cardiac myocytes. Effective reorganization of cells to regenerate an injured tissue requires the efficient laying out of a proper extracellular matrix bed. Studies with scaffolds have underscored the key significance of acellular extracellular matrix in facilitating tissue regeneration (4).

During the last two decades, the pursuit for unraveling the science of myocardial healing has developed asymmetrically with a vast majority of the studies focusing on cardiomyocytes with little or no recognition of the significance of the populous fibroblasts (89). Addressing this oversight may hold the key to more conclusive clinical outcomes than generated from current efforts (94, 135). Indeed, in cellular therapeutics aiming at myocardial repair the delivery of mesenchymal stem cells, progenitors of all connective tissue cells including cardiac fibroblasts, to the injury site in the heart has generated favorable outcomes. In adults of

multiple vertebrate species, mesenchymal stem cells have been isolated from bone marrow and other tissues, expanded in culture, and differentiated into several tissue-forming cells such as bone, cartilage, fat, muscle, tendon, liver, kidney, heart, and even brain cells. Adult mesenchymal stem cells isolated from fatty tissue can be chemically transformed into cardiomyocytes serving as a source of autologous cardiac fibroblasts and myocytes for myocardial repair (119). More recently it has been demonstrated that human mesenchymal stem cells can be differentiated *in vitro* into a mixture of fibroblast and cardiomyocyte-like cells which are potentially valuable for repairing the injured myocardium (167). Genetically modified human mesenchymal stem cells can express functional HCN2 channels *in vitro* and *in vivo*, mimicking over-expression of HCN2 genes in cardiac myocytes, and represent a novel delivery system for pacemaker genes into the heart or other electrical syncytia (113). Systemic delivery of bone marrow-derived mesenchymal stem cells has been tested for myocardial repair. Such delivery of mesenchymal stem cells to rats after myocardial infarction, although feasible, was limited by entrapment of the donor cells in the lungs. Direct left ventricular cavity infusion enhanced migration and colonization of the cells preferentially to the ischemic myocardium (6). The efficacy of human mesenchymal stem cells in cellular repair is dependent on its ability to communicate with target cells such as cardiac myocytes *via* gap junctions. Recently it has been demonstrated that human mesenchymal stem cells make cardiac connexins and form functional gap junctions in the myocardium (154).

One of the key determinants of the response of the cardiac fibroblast in the clinical context of myocardial damage is its transformation from a quiescent cell primarily responsible for extracellular matrix homeostasis, to an activated or differentiated cell which plays a central role in wound healing (89). Stimulation of fibroblast proliferation may contribute to fibrosis of the heart (120). However, cell-cycle arrest followed by differentiation to myofibroblast may

support wound contraction. The first evidence for perceived hyperoxia *in vivo* came from a survival surgery model involving ischemia-reoxygenation of the rat heart (125). Examination of the post-reoxygenation tissue revealed induction of p21 accompanied by differentiation of cardiac fibroblasts to myofibroblasts in the peri-infarct region (regions ii & iii, Fig. 1). Reoxygenation represents only one of numerous factors that are associated with reperfusion. Direct evidence supporting that inducible p21 expression in the heart *in vivo* is indeed sensitive to $+\Delta pO_2$ came from studies demonstrating that the gene can be induced in the heart *in vivo* simply by transiently exposing mice to an oxygen-rich ambience (125). p21 supports remodeling of tissues injured by oxygenation (147). Other responses to perceived hyperoxia include TGF β activation and differentiation of fibroblasts to myofibroblasts (123). Both of these processes have been identified in the myocardial tissue recovering from reoxygenation injury (48, 64). Current studies identify TGF β as an important mediator of post-reperfusion healing (23), consistent with the proposed role of perceived hyperoxia in facilitating tissue remodeling.

That low oxygen ambience serves as a cue to trigger angiogenesis is a well-accepted notion. Studies related to perceived hyperoxia establish that the sensing of oxygen environment is not limited to hypoxia. It demonstrates that in addition to being a trigger for injury as is widely recognized, reoxygenation insult has an inbuilt component of tissue remodeling in the peri-infarct region induced by perceived hyperoxia. Like other reparative responses, this mechanism is also clearly inadequate to heal the heart in the case of injuries with clinical manifestations. Understanding of the underlying mechanisms of this and other myocardial healing responses should, however, prove to be instrumental in developing productive therapeutic approaches.

DNA MICROARRAY AS A TOOL: AN UPDATE ON CONSIDERATIONS AND APPROACHES

In addition to being indispensable as a tool for identifying O₂-sensitive genes and categorizing them into functional groups (125), the DNA microarray plays a wider role in helping to identify pathways of development and repair, as well as potential targets for therapy. Here we describe recent use of the microarray in heart studies. Because motivated statistical analyses are critical for extracting useful information from microarrays, we present an overview of current statistical practice, with pointers to recent methodological research.

RESEARCH DESIGNS

Most microarray experiments in heart research have utilized one of two research designs, differential expression or longitudinal pattern. The goal of the first type is to identify which genes operate differently when a particular condition is changed. The goal of the second is to describe the time pattern genetic expression concurrent with developmental changes in the heart.

Differential expression

Typifying this design, several rodent studies have identified the genomic basis of cardiac hypertrophy (87, 114, 152). Other studies have identified over-expression of thrombospondin-2 and galectin-3 as predictors of heart failure (90, 134, 140). Prentice and Webster (115) provide a review of other recent heart studies for differential expression, and statistical methods for identifying differential expression are detailed in another section of this review. Once differentially expressed genes have been identified using these methods, current practice calls for confirming the results by running independent expression tests, such as quantitative PCR, ribonuclease protection assay or Northern blot, for the selected genes.

Differentially expressed genes play a critically important role in deducing pathways and thereby developing new therapeutic strategies. In a rat model of ischemia, gene expression profiling experiments resulted in the discovery of two different genetic programs in the heart, namely, a protective program activated upon brief episodes of transient ischemia and an injury-related one activated in response to irreversible ischemic injury (143). In addition to confirming suspected pathways, microarray analysis may help to generate new hypotheses. In another rat model (33) for differences in mRNA expression of hyperthyroid and normal heart, differentially expressed genes encoded proteins related to specific functional categories. Reduction of insulin-induced glucose uptake in hyperthyroid cardiomyocytes indicated impaired glucose transport in cardiac cells. Moreover, a few genes such as GLUT4, cytochrome P450 isoforms, superoxide dismutase (SOD), collagens, matrix metalloproteinases (MMP), and tissue inhibitors of matrix metalloproteinases had not been reported earlier. These findings suggest additional pathways that may be altered in hyperthyroid heart.

Longitudinal pattern

Generally speaking, longitudinal studies involve comparison of gene expressions at various intervals over a course of time. These designs fall into two categories: repeated measures, in which measurements are made on the same subject at different times, and cross sectional studies, in which samples are harvested from a diminishing pool of subjects at each time interval. An example of the former is a study where blood samples were taken from stenosis patients prior to stent placement, and afterwards at 2 weeks and 6 months. Peripheral blood mononuclear cells were used with Affymetrix U133A microarrays to identify longitudinal patterns of genes that were associated with in-stent restenosis (ISR). Identification of such genes aids in either screening patients or developing treatments such as coating of stent (51).

In this example, the goal again is to differentiate outcome using gene expression data, but here the expressions come as triples (pre-placement, 2 weeks, and 6 months) for each gene. Although there are highly developed statistical methods for analyzing the time course of a single gene using a sample of subjects (34), the problem with doing this for many genes is an area of current statistical research. The problem is a bit easier when only two time points are used. To study the effect of chronic ventricular unloading on myocardial gene expression, microarray profiling gene expression was compared before and after LVAD (26). Upregulated genes included a large proportion of transcription factors, genes related to cell growth/apoptosis/DNA repair, cell structure proteins, metabolism, and cell signaling/communication; downregulated genes were limited to cytokines. Identification of genes with significant changes in expression, in this instance, becomes similar to the procedures used for finding differential expression except that paired t-statistics are used in place of two-sample t-statistics (148).

Although it may seem that repeated measurement experiments are relatively rare in heart research, such is not the case if the repeated measurements on the same subject involve different tissues harvested at the same time. Examples include monitoring gene expression patterns in cerebellum, heart and white adipose tissue in a mouse model of mutation to thyroid hormone receptor β (TR β) (99), which helped uncover complex multiple signaling pathways that mediate the molecular actions of TR β mutants *in vivo*. Another example is where the researchers explored mRNA expression from all four chambers of eight explanted failing [idiopathic dilated cardiomyopathy (DCM), n=5; ischemic cardiomyopathy (ICM), n=3], and five non-failing hearts using high-density oligonucleotide arrays (Affymetrix U95Av2) (70). The goal is to recognize common gene expression patterns in heart failure. Although existing

statistical methods for paired comparisons can lead toward this goal, there remains a need for more effective statistical methods in this area. In contrast, cross sectional studies are easier to analyze statistically. The drawback is that they carry less information per microarray about the course of development than do repeated-measure designs. Nevertheless, such designs are plentiful and informative. In studying postnatal development of the mouse heart, several genes involving the cell cycle, growth factors, transcriptional regulation, cytoskeleton, and detoxification enzymes were identified by microarray analysis (24). In particular, *in vivo* treatment with one of these, pleiotrophin (PTN), increased bromodeoxyuridine incorporation and proliferating cell nuclear antigen expression during day 7 to day 14, indicating that PTN induces cell proliferation in mouse heart. Another study incorporated a similar design to identify genes with different longitudinal profiles of expression in control and transverse aortic constricted mice, implicating the involvement of several pathways regulating both cardiac structure and function (172). Confirmation of the expression levels with quantitative PCR was in greatest accordance with GeneChip data for those genes with the largest up- or down-regulation profiles.

STATISTICAL METHODS

Microarrays are imaged using an optical scanner. These images must then be background corrected to adjust for nonspecific binding, fluorescence from other chemicals on the slide, and the like. The next steps in analysis in most studies are *normalization*, to adjust for differences that are not biological in nature, but are due to the technology (e.g. dye effects); and *comparison testing*, to determine which genes are differentially expressed. These basic steps are often followed by *cluster analysis*, to search for groups of genes (or subjects) with similar gene expression patterns; and *functional assessment*, in which relational databases are used to identify pathway components compatible with the observed patterns of

expression. We illustrate these procedures using the data from our laboratory describing the oxygen-sensitive genes in adult primary cardiac fibroblasts (125).

Background adjustment and normalization

DNA microarrays contain multiple probes for each gene. The probes are typically scattered over the surface of the microarray chip. Variations in intensity from probe to probe or chip to chip for the same sample need to be resolved into a reliable level of expression. Observed intensities are sometimes modified based on comparison with nearby, *background*, probes whose expression is theoretically known. For cDNA microarrays, background adjustment is controversial, since although it can reduce bias, it can also increase variance. See Scharpf et al. (132) for discussion of the bias-variance tradeoff and Smyth et al. (146) for a description of some of the commonly used background adjustment methods. For Affymetrix arrays, each gene probe has a single nucleotide mismatch probe-mate. The MAS 5.0 method of Affymetrix, which use paired probes for adjustment, and the RMA method (12), which uses quantile adjustment, are both in common practice.

Microarray data can be quite noisy. Much of the variation in intensity levels can arise from technical rather than biological causes. Non-biological sources of variation can be introduced during sample preparation (e.g. dye effects), array manufacture (e.g. probe concentration), hybridization (e.g. amount of sample), and in the measurement process (e.g. scanner inaccuracies) (61). For a more complete discussion of possible sources of *obscuring variation* see Hartemink et al (61). Some biological sources of bias, such as comparison of *in vivo* with *in vitro* samples, may call for special adjustment. Hence it is important to normalize data to, as much as possible, remove the technical variation while still retaining the informative biological variation. Normalization is performed both within each array, and between arrays, to make comparisons more meaningful. As an example, boxplots of unnormalized and normalized intensity data from a microarray study with six Affymetrix arrays is given in

FIGURE LEGENDS

Figure 1. Cellular responses to reoxygenation following chronic hypoxia. Chronic hypoxia results in cellular adjustments such that reoxygenation causes hyperoxic insult as evident in part in the form of oxidative damage in numerous studies. *ischemic*, diagram of

ischemic tissue, with focus of insult represented by the blue center (region **i**). Focal ischemia is known to be associated with graded oxygenation from near-zero status at focus to levels increasing with distance from focus. The concentric circles represents regions (i-iv) of the tissue with increasing graded distance from the focus of insult (blue); **reperfused**, diagram of the reperfused tissue with corrected pO_2 state represented by change of color from a shade of blue (hypoxic) to red. Important elements triggered by oxygen, during the course of reoxygenation-associated remodeling include: in region **i** cell death or fatal oxidative injury at the focal point of insult, making room for regenerating tissues; in region **ii** non-fatal cellular stress, triggering reparative responses; in region **iii** survival of phenotypically altered cells that favor remodeling (physiological or pathological / fibrogenic). Fibrosis denies room to regenerating healthy cells; and in region **iv** correction of pO_2 of mildly hypoxic cells localized beyond a critical distance from the focus of insult, favoring regeneration and restoration of physiological functioning of the organ. Δ , represents ΔpO_2 in response to reoxygenation. ΔpO_2 : **i>ii>iii>iv**. While a large ΔpO_2 causes ROS mediate injury in reoxygenated region **i**, perceived hyperoxia supports remodeling in regions **ii** and **iii**.

Figure 2. Elevated oxygen sensitive signaling in adult primary cardiac fibroblasts. ROS generation is enhanced followed by growth-inhibition and differentiation. TGF 1 and downstream p38MAPK is activated. Cell cycle check-points known to be associated with differentiation eg p21, cyclins and Fra-2 are up-regulated. Broken lines represent effects where intermediary steps are expected. Grey solid lines represent association with differentiation phenotype.

Figure 3. Although normalization is somewhat ad-hoc, there are two basic ideas that are relevant to all microarray normalization. First, the normalization method must be tailored to

the microarray platform (12, 117, 130, 131, 169). Normalization of cDNA arrays is quite different from normalization of Affymetrix arrays, both in the sources of variation to be removed and the algorithms. Second, linear normalization methods often miss obscuring variation that can be removed, so nonlinear methods should be used (12, 86, 169).

Differential expression, multiple comparisons and false discovery rate

Identifying genes that are differentially expressed under two or more treatment conditions is a primary goal of most microarray studies. The two main issues in assessing differential expression are determining a method for assessing the extent of differential expression (*e.g.*, fold change, t-test, ANOVA), and adjusting the method for the effects of multiple comparisons, since typically there are thousands of genes being studied. The first issue is relatively straightforward, in the sense that it falls under the auspices of “classical” statistical methods that have been studied and taught for many years. One important point is the weakness of relying on fold change as the sole criterion, since fold change does not take into account the variability in the data. This can lead to two problems. First, genes with low expression levels and high variability may be identified as differentially expressed. Second, genes that display small but reproducible (*i.e.*, low variability) changes in gene expression may be missed. There have been some efforts to incorporate variability in methods that rely on fold change (86), but these still suffer from the latter of the two problems noted above.

The issue of multiple comparisons is more complex. Ideally the probability of a false positive (a gene incorrectly identified as differentially expressed) should be small, and the probability of correctly identifying genes that are differentially expressed should be large. Standard statistical methods are set up to balance these goals in the context of only one comparison, *i.e.*, if the microarray contained only one gene. Without adjustment, standard statistical

methods give incorrect results in the context of microarray data. For example, consider a microarray study with m genes, and suppose none are differentially expressed. For various values of m , the probability that a standard statistical tool set to reject the null hypothesis if a p-value is less than 0.05 will yield at least one false positive are given in Table 1. Since most microarrays contain thousands of genes, standard statistical methods are clearly unacceptable.

The Bonferroni method is a simple method to correct for multiple testing that is still widely used in microarray data analysis. This method just divides the p-value cutoff by the number of genes m . For example, if the probability of at least one false positive is to be limited to 0.01, and there are $m=5000$ genes on the array, the Bonferroni method would identify a gene as differentially expressed if its p-value were less than $0.01/5000 = 0.000002$. Although this method is quite generally applicable, it is usually not a good choice for microarray studies, because it has very low power, *i.e.*, the probability of correctly identifying differentially expressed genes is very small, so many potentially interesting genes may be missed. See

	Multiple Testing Adjustment		
	None	Bonferroni	FDR
Number of significant genes	601	23	75
p-value of Cappa2 gene	0.000014	0.171	0.002

Table 1 for an illustration of the weakness of the Bonferroni method of adjustment. For this and other reasons, different criteria than the probability of at least one false positive have been advocated. The most promising of these is the False Discovery Rate (FDR) (9). FDR is the expected proportion of false positives among all rejected hypotheses. Instead of trying to

avoid any false positives, the FDR controls the proportion of positive calls that are false positives. Designing procedures to control the FDR is challenging. The original technique of Benjamini and Hochberg, to control the FDR at level α , works as follows. First, compute p-values for each of the m genes, and order the p-values from smallest to largest. Second, plot the ordered p-values versus their rank along with the line with slope α/m and intercept zero. Next, find the last p-value, say p^* , that lies below the line, and reject the hypotheses corresponding to all p-values less than or equal to p^* . In Figure 4, the hypotheses corresponding to the three smallest p-values would be rejected. The Benjamini-Hochberg procedure has been shown to control the FDR under certain assumptions on the dependence structure of the genes' expression levels (8, 9). The procedure is in wide use, and is recommended by many journals (31).

	Multiple Testing Adjustment		
	None	Bonferroni	FDR
Number of significant genes	601	23	75
p-value of Cappa2 gene	0.000014	0.171	0.002

Table 1 contains some comparisons of the unadjusted p-values, Bonferroni-adjusted p-values, and Benjamini-Hochberg adjusted p-values for data from six Affymetrix mouse arrays. Unfortunately there are many microarray studies not covered by the assumptions underlying the Benjamini-Hochberg algorithm, so there is much work in the statistical community aimed at developing a method of controlling the FDR that is more generally applicable than the original Benjamini-Hochberg method. A promising method, which relies on the bootstrap technique, has been recently analyzed (111, 155, 156). However, this method achieves the FDR asymptotically, so is not suitable for studies involving small numbers (e.g. 5) of arrays.

Determining sample size needed to control FDR

In planning an experiment there are two major decisions to make about microarrays: the total number that should be used and the proportion that will be used for biological versus technical replication. The first decision is typically based on budget and the second on the reliability of the microarrays being used. The real question is whether a planned experiment has a realistic chance of detecting and identifying important biological processes. Recently a decision theoretic procedure was introduced (100), where a typical loss function is a weighted sum of the FDR and its counterpart false negative rate (FNR). The idea is to plot the expected loss versus sample size, and judge if a desired value can be achieved with a realistic sample size. The expected loss is estimated through simulating expression data and recording the behavior of the Benjamini-Hochberg method.

Clustering genes and/or subjects

The common way of presenting gene expression data is *via* a heat map. This is an array where, typically, genes index the rows, and chips index the columns. Chips may represent either different subjects or the same subject under different conditions. The array itself is color coded to display the, usually normalized, level of expression. The variation of colors along any row is called the expression pattern of the associated gene. If the genes in the array are arbitrarily ordered, it is difficult to perceive patterns in the heat map. For the subset of 67 genes most likely to be differentially expressed under 10% and 21% oxygen, Figure 5a illustrates an unclustered heat map that gives little visual information. The simplest remedy is to sort the genes in such a way that two genes with similar expression patterns are close together. Hierarchical procedures begin by putting each individual into its own group, combining the two closest, the next two closest, and so on. This requires a measure of

closeness between groups of individuals. Such measures are constructed by specifying a metric between individuals and a procedure of using this metric to induce a distance between two groups. For example, the Manhattan distance between two genes is the sum of absolute values of the difference in expression on each microarray. The complete linkage method of induction defines the distance between two groups as the largest metric distance between two individuals, one from each group. Different metrics and procedures may produce different blocks of patterns. For the 67 likely differentially expressed genes, the choice of Manhattan distance and complete linkage produces Figure 5b. Many authors (17, 22, 55, 88, 151) have reviewed the effectiveness of standard clustering algorithms in identifying clear patterns. A recently proposed method, called HOPACH (Hierarchical Ordered Partitioning And Collapsing Hybrid) alternates the “top down” method of partitioning with the “bottom up” method of agglomeration to produce clusters that are reliably reproduced when subjects are re-sampled or experiments are replicated. The ultimate criterion for clustering is whether or not clustered genes tend to act in conjunction with each other. For example, it is difficult to interpret some of the groupings in Figure 5b. As an alternative to searching through dozens of possible clustering combinations, a new approach is to append additional information to the expression matrix before attempting to cluster genes. The additional information may be an important aspect of the expression matrix, for example the difference in mean expression between low and high oxygenation experiments, or external information such as gene functioning categories. This method of appending onto expression matrices may afford a stronger aid to identifying relevant gene families and/or pathways, or may just simplify the heatmap. Figure 5c gives the simplified pattern when the difference in mean expression under high oxygenation is appended to the expression matrix before clustering. Although appending genes with functional categories may yield functionally interpretable groupings, it does not help all that much in identifying different pathways since, for example, all active

transcription factors tend to be clustered together even if they are operating in disjoint networks. Moreover, a single gene may be a functional part of more than one network or pathway, but traditional clustering methods allow a gene to be included in only one cluster. Plaid models (84) are an attempt to handle multiple group membership of genes. Regularized coloring patterns are imposed in layers, which ideally represent independent networks. Each layer is color coded with intensity of that color being proportional to the expression level attributable to activity in that layer. When the layers are overlaid, the result is a plaid pattern. To be successful, the method requires a precision and uniformity of variance in the microarray data that has not yet been achieved, but the method may be valuable as another tool in inferring pathways. A promising area of research is to guide the choice of layers using partial information about pathways.

Functional characterization of genes and their organization into pathway networks

Detecting and clustering differentially expressed genes, as described above, are just the first steps toward learning about gene function and genetic networks. There are many situations when limited gene expression data is available, but existing gene networks or functional classes of genes are known. In this case, one can try to relate newly acquired gene expression level data to known functional and partial pathway information. Important sources of information are the Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. The GO project (3) is a collaborative effort to address the need for consistent descriptions of gene products in different databases. GO produces a controlled vocabulary that can be applied to all organisms even as knowledge of gene and protein roles in cells is accumulating and changing. GO provides three structured networks of defined terms to describe gene product attributes. The three organizing principles of GO are *molecular function*, *biological process* and *cellular component*. A gene product has one or

more molecular functions and is used in one or more biological processes; it might be associated with one or more cellular components. KEGG (71, 72) is a suite of databases and associated software, integrating current knowledge on molecular interaction networks in biological processes (the PATHWAY database), on the universe of genes and proteins (the GENES/SSDB/KO databases), and on the universe of chemical compounds and reactions (the COMPOUND/GLYCAN/REACTION databases). Several methodologies have been proposed for constructing gene networks based on gene expression data, such as the Boolean networks (1) or differential equation models (32). Another way of addressing this question is the Bayesian networks framework (49, 62), where the expression level of each gene is treated as a random variable and each regulatory interaction as a probabilistic dependency between such variables. Bayesian networks are graph-based models of joint multivariate probability distributions that capture properties of conditional independence between variables. Such models are attractive for their ability to describe complex stochastic processes and provide a tool for learning from noisy observations. In addition, bootstrap methods can be used for estimating confidence in the learned structures (38). The main idea is to sample with replacement, observations from the given data set and learn for them. In this way many networks are generated, all of which are reasonable models, reflecting the effect of small perturbations in data, on the learning process (49). However, because of limited expression data typically available for any particular system in a given state, network reconstruction processes typically result in the identification of multiple networks that explain data equally well. In most cases causal relationships cannot be reliably inferred from gene expression data alone, since for any particular network changing the direction of the edge between two nodes has little effect on the model fit. To reliably infer causal relationships, additional information is required. Biological knowledge (66), including protein-protein and protein-DNA interactions (65), binding site sequences and transcription factors (85) is needed.

More recently, pathway reconstruction associated with complex disease traits was obtained by integrating genotype, transcription and clinical trait data (174). In this approach, gene expression data was treated as quantitative trait loci (QTL). Patterns of co-localization between disease trait QTL and gene expression QTL are indicative for causal inference.

There are several network/pathway reconstruction and analysis software packages. One example is GNA (Genetic Network Analyzer) which is a computer tool for the modeling and simulation of genetic regulatory networks. The aim of GNA is to assist biologists and bioinformaticians in constructing a model of a regulatory network using knowledge about regulatory interactions in combination with gene expression data. Another software tool is GenMAPP (Gene MicroArray Pathway Profiler), a free computer application designed to visualize gene expression data on maps representing biological pathways and groups of genes. Another useful software tool is Cytoscape, an open source software project for integrating biomolecular interaction networks with high-throughput expression data and other molecular states into a unified conceptual framework. Although applicable to any system of molecular components and interactions, Cytoscape is most powerful when used in conjunction with large databases of protein-protein, protein-DNA, and genetic interactions that are increasingly available for humans and model organisms.

As an illustration of some of the above ideas, the following approach is used below: First identify genes that are differentially expressed and then use either Fisher's exact test (82) or z-scores (65) to estimate significance for known gene networks or functional classes of genes defined by KEGG pathways or GO terms. This procedure was applied on the cardiac fibroblast microarray data from our laboratory investigating oxygen-sensitive genes (125). The 12,488 probes were normalized as in MAS5 and the 5389 genes present in all samples

in either 3% or 20% O₂ group were selected for further analysis. The t-statistic was used as a measure of differentiation between groups and p-values from the t-distribution are computed. After controlling the FDR at a 15% level, 129 genes showed significant change between the 3% and 20% O₂ groups. These genes were separated into groups based on known pathways (KEGG) or functional classes of GO terms (for “biological process”, “cellular component” and “molecular function”). The significant KEGG pathways or GO terms detected by this method are presented in Tables 3-5. An alternative goal is expanding an existing pathway related to growth inhibition and fibroblast differentiation shown to be induced by perceived hyperoxia as performed by Roy et al. (125) (Fig. 6). The expression of candidate genes shown in red ellipse with blue outline have been independently verified using either real-time PCR or ribonuclease protection assay (125). For three of them, CDKN1A, Tob1 and Wig1, TP53 is a hypothetical activator. We sought to identify other candidate activator/inhibitor genes capable of explaining, better than TP53 does, the variance in expression levels of these genes. Linear regression models for each of these genes are fit to generate hypothesis on candidate activators/inhibitors of these three genes. For any given gene G, ANOVA may be used to compare the linear models (1) $G \sim a + b \cdot TP53$ with (2) $G \sim a + b \cdot TP53 + c \cdot C$, where C is a gene differentially expressed under the two treatment conditions. Genes showing a significant improvement in model (2) when compared with (1) represent candidates for activation/inhibition of gene G. Table 4 lists these candidates for CDKN1A, Tob1 and Wig1 genes.

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References

1. **Akutsu T, Miyano S, and Kuhara S.** Identification of genetic networks from a small number of gene expression patterns under the Boolean network model. *Pac Symp Biocomput*: 17-28, 1999.
2. **Ambrosio G and Tritto I.** Reperfusion injury: experimental evidence and clinical implications. *Am Heart J* 138: S69-75, 1999.
3. **Ashburner M, Ball, CA, Blake, JA, Botstein, D, Butler, H, Cherry, JM, Davis, AP, K. Dolinski, Dwight, SS, Eppig, JT, Harris, MA, Hill, DP, Issel-Tarver, L, Kasarskis, Lewis, S, Matese, JC, Richardson, JE, Ringwald, M, Rubin, GM, Sherlock, G.** Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* 25: 25-29., 2000.
4. **Badylak S, Obermiller J, Geddes L, and Matheny R.** Extracellular matrix for myocardial repair. *Heart Surgery Forum* 6: E20-26, 2003.
5. **Banerjee C, Stein JL, Van Wijnen AJ, Frenkel B, Lian JB, and Stein GS.** Transforming growth factor-beta 1 responsiveness of the rat osteocalcin gene is mediated by an activator protein-1 binding site. *Endocrinology* 137: 1991-2000, 1996.
6. **Barbash IM, Chouraqui P, Baron J, Feinberg MS, Etzion S, Tessone A, Miller L, Guetta E, Zipori D, Kedes LH, Kloner RA, and Leor J.** Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium: feasibility, cell migration, and body distribution. *Circulation* 108: 863-868, 2003.
7. **Batt PA, Gardner DK, and Cameron AW.** Oxygen concentration and protein source affect the development of preimplantation goat embryos in vitro. *Reproduction, Fertility, & Development* 3: 601-607, 1991.
8. **Beltrami AP, Urbanek K, Kajstura J, Yan SM, Finato N, Bussani R, Nadal-Ginard B, Silvestri F, Leri A, Beltrami CA, and Anversa P.** Evidence that human cardiac myocytes

divide after myocardial infarction.[see comment]. *New England Journal of Medicine* 344: 1750-1757, 2001.

9. **Benjamini Y and Hochberg Y.** Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J Roy Statist Soc Ser B* 57: 289--300, 1995.

10. **Berg A, Ekwall AK, Rubin K, Stjernschantz J, and Reed RK.** Effect of PGE1, PGI2, and PGF2 alpha analogs on collagen gel compaction in vitro and interstitial pressure in vivo. *American Journal of Physiology* 274: H663-671, 1998.

11. **Bjercke RJ, Erichsen DA, Rege A, Lindner V, and Smith JD.** Soluble transforming growth factor-beta type II receptor inhibits negative remodeling, fibroblast transdifferentiation, and intimal lesion formation but not endothelial growth. *Circulation Research* 84: 323-328, 1999.

12. **Bolstad BM, Irizarry RA, Astrand M, and Speed TP.** A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 19: 185-193, 2003.

13. **Borer JS, Truter S, Herrold EM, Falcone DJ, Pena M, Carter JN, Dumlao TF, Lee JA, and Supino PG.** Myocardial fibrosis in chronic aortic regurgitation: molecular and cellular responses to volume overload. *Circulation* 105: 1837-1842., 2002.

14. **Brize-Fradin.** *La chimie pneumatique appliquee aux travaux sous l'eau.* Paris, 1808.

15. **Bruick RK and McKnight SL.** A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* 294: 1337-1340, 2001.

16. **Bruick RK and McKnight SL.** Oxygen sensing gets a second wind. *Science* 295: 807-808, 2002.

17. **Bryan J, Pollard KS, and van der Laan MJ.** Paired and unpaired comparison and clustering with gene expression data. *Statist Sinica* 12: 87--110, 2002.

18. **Budinger GR, Duranteau J, Chandel NS, and Schumacker PT.** Hibernation during hypoxia in cardiomyocytes. Role of mitochondria as the O₂ sensor. *Journal of Biological Chemistry* 273: 3320-3326, 1998.
19. **Bunn HF and Poyton RO.** Oxygen sensing and molecular adaptation to hypoxia. *Physiological Reviews* 76: 839-885, 1996.
20. **Canbolat O, Fandrey J, and Jelkmann W.** Effects of modulators of the production and degradation of hydrogen peroxide on erythropoietin synthesis. *Respiration Physiology* 114: 175-183, 1998.
21. **Chandel NS, Budinger GR, Choe SH, and Schumacker PT.** Cellular respiration during hypoxia. Role of cytochrome oxidase as the oxygen sensor in hepatocytes. *Journal of Biological Chemistry* 272: 18808-18816, 1997.
22. **Chen G, Jaradat SA, Banerjee N, Tanaka TS, Ko MSH, and Zhang MQ.** Evaluation and comparison of clustering algorithms in analyzing ES cell gene expression data. *Statist Sinica* 12: 241--262, 2002.
23. **Chen H, Li D, Saldeen T, and Mehta JL.** TGF-beta 1 attenuates myocardial ischemia-reperfusion injury via inhibition of upregulation of MMP-1. *American Journal of Physiology - Heart & Circulatory Physiology* 284: H1612-1617, 2003.
24. **Chen HW, Yu SL, Chen WJ, Yang PC, Chien CT, Chou HY, Li HN, Peck K, Huang CH, Lin FY, Chen JJ, and Lee YT.** Dynamic changes of gene expression profiles during postnatal development of the heart in mice. *Heart* 90: 927-934, 2004.
25. **Chen X, Bargonetti J, and Prives C.** p53, through p21 (WAF1/CIP1), induces cyclin D1 synthesis. *Cancer Res* 55: 4257-4263., 1995.
26. **Chen Y, Park S, Li Y, Missov E, Hou M, Han X, Hall JL, Miller LW, Bache RJ, Afrakhte M, and Schultheiss TM.** Alterations of gene expression in failing myocardium following left ventricular assist device support. *Physiol Genomics* 14: 251-260, 2003.

27. **Chiavegato A, Faggini E, Franch R, Puato M, Ausoni S, Pauletto P, and Tsuruda T.** Cardiotrophin-1 stimulation of cardiac fibroblast growth: roles for glycoprotein 130/leukemia inhibitory factor receptor and the endothelin type A receptor. *Circulation Research* 89: 1111-1121, 2001.
28. **Chilov D, Camenisch G, Kvietikova I, Ziegler U, Gassmann M, and Wenger RH.** Induction and nuclear translocation of hypoxia-inducible factor-1 (HIF-1): heterodimerization with ARNT is not necessary for nuclear accumulation of HIF-1alpha. *Journal of Cell Science* 112: 1203-1212, 1999.
29. **Chin BY, Mohsenin A, Li SX, Choi AM, and Choi ME.** Stimulation of pro-alpha(1)(I) collagen by TGF-beta(1) in mesangial cells: role of the p38 MAPK pathway. *American Journal of Physiology - Renal Fluid & Electrolyte Physiology* 280: F495-504, 2001.
30. **Chiu C, Maddock DA, Zhang Q, Souza KP, Townsend AR, and Wan Y.** TGF-beta-induced p38 activation is mediated by Rac1-regulated generation of reactive oxygen species in cultured human keratinocytes. *International Journal of Molecular Medicine* 8: 251-255, 2001.
31. **Curran-Everett D and Benos DJ.** Guidelines for reporting statistics in journals published by the American Physiological Society. *Physiol Genomics* 18: 249-251, 2004.
32. **de Hoon M, Imoto, S, Kobayashi, K, Ogasawara, N, Miyano, S.** Inferring gene regulatory networks from time-ordered gene expression data of *Bacillus subtilis* using differential equations. *Pac Symp Biocomput*: 17-28, 2003.
33. **De K, Ghosh G, Datta M, Konar A, Bandyopadhyay J, Bandyopadhyay D, Bhattacharya S, Bandyopadhyay A, Chen Y, Park S, Li Y, Missov E, Hou M, Han X, Hall JL, Miller LW, Bache RJ, Afrakhte M, and Schultheiss TM.** Analysis of differentially expressed genes in hyperthyroid-induced hypertrophied heart by cDNA microarray. *J Endocrinol* 182: 303-314, 2004.

34. **Diggle PJ, Heagerty PJ, Liang K-Y, and Zeger SL.** *Analysis of longitudinal data.* Oxford: Oxford University Press, 2002.
35. **Dionne CA, Jaye MC, and Schorb W.** Angiotensin II is mitogenic in neonatal rat cardiac fibroblasts. *Circulation Research* 72: 7-19, 1993.
36. **Dugina V, Fontao L, Chaponnier C, Vasiliev J, and Gabbiani G.** Focal adhesion features during myofibroblastic differentiation are controlled by intracellular and extracellular factors. *J Cell Sci* 114: 3285-3296., 2001.
37. **Duranteau J, Chandel NS, Kulisz A, Shao Z, and Schumacker PT.** Intracellular signaling by reactive oxygen species during hypoxia in cardiomyocytes. *Journal of Biological Chemistry* 273: 11619-11624, 1998.
38. **Efron B and Tibshirani RJ.** *An introduction to bootstrap.* New York: Chapman and Hall, 1993.
39. **Eghbali M, Czaja MJ, Zeydel M, Weiner FR, Zern MA, Seiffter S, and Blumenfeld OO.** Collagen chain mRNAs in isolated heart cells from young and adult rats. *Journal of Molecular & Cellular Cardiology* 20: 267-276, 1988.
40. **Elphic RC, Hirahara M, Terasawa T, Mukai T, and Zhu H.** Signal transduction. How do cells sense oxygen? [letter; comment.]. *Science* 291: 1939-1941, 2001.
41. **Elsasser A, Schlepper M, Klovekorn WP, Cai WJ, Zimmermann R, Muller KD, Strasser R, Kostin S, Gagel C, Munkel B, Schaper W, and Schaper J.** Hibernating myocardium: an incomplete adaptation to ischemia. *Circulation* 96: 2920-2931, 1997.
42. **Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, and Ratcliffe PJ.** C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 107: 43-54, 2001.

43. **Eriksson M and Leppa S.** Mitogen-activated protein kinases and activator protein 1 are required for proliferation and cardiomyocyte differentiation of P19 embryonal carcinoma cells. *Journal of Biological Chemistry* 277: 15992-16001, 2002.
44. **Fandrey J, Frede S, and Jelkmann W.** Role of hydrogen peroxide in hypoxia-induced erythropoietin production. *Biochemical Journal* 303: 507-510, 1994.
45. **Feld Y, Melamed-Frank M, Kehat I, Tal D, Marom S, and Gepstein L.** Electrophysiological modulation of cardiomyocytic tissue by transfected fibroblasts expressing potassium channels: a novel strategy to manipulate excitability. *Circulation* 105: 522-529., 2002.
46. **Feugate JE, Li Q, Wong L, and Martins-Green M.** The cxc chemokine cCAF stimulates differentiation of fibroblasts into myofibroblasts and accelerates wound closure. *Journal of Cell Biology* 156: 161-172, 2002.
47. **Foletta VC.** Transcription factor AP-1, and the role of Fra-2. *Immunology & Cell Biology* 74: 121-133, 1996.
48. **Frangogiannis NG, Michael LH, and Entman ML.** Myofibroblasts in reperfused myocardial infarcts express the embryonic form of smooth muscle myosin heavy chain (SMemb). *Cardiovascular Research* 48: 89-100, 2000.
49. **Friedman N, Linial M, Nachman I, and Pe'er D.** Using Bayesian Networks to Analyze Expression Data. *Journal of Computational Biology* 7: 601-620, 2000.
50. **Fukuda K.** Development of regenerative cardiomyocytes from mesenchymal stem cells for cardiovascular tissue engineering. *Artificial Organs* 25: 187-193, 2001.
51. **Ganesh SK, Skelding KA, Mehta L, O'Neill K, Joo J, Zheng G, Goldstein J, Simari R, Billings E, Geller NL, Holmes D, O'Neill WW, and Nabel EG.** Rationale and study design of the CardioGene Study: genomics of in-stent restenosis. *Pharmacogenomics* 5: 952-1004, 2004.

52. **Gaynor JS, Turner AS, Johnson SM, Horwitz LD, Whitehill TA, Harken AH, and Hafizi S.** Expression of functional angiotensin-converting enzyme and AT1 receptors in cultured human cardiac fibroblasts. *Circulation* 98: 2049-2054, 1998.
53. **Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, and Zhang J.** Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* 5: R80. Epub 2004 Sep 2015., 2004.
54. **Goldberg PL, MacNaughton DE, Clements RT, Minnear FL, and Vincent PA.** p38 MAPK activation by TGF-beta1 increases MLC phosphorylation and endothelial monolayer permeability. *American Journal of Physiology - Lung Cellular & Molecular Physiology* 282: L146-154, 2002.
55. **Goldstein DR, Ghosh D, and Conlon EM.** Statistical issues in the clustering of gene expression data. *Statist Sinica* 12: 219--240, 2002.
56. **Gong W, Hao B, and Chan MK.** New mechanistic insights from structural studies of the oxygen-sensing domain of Bradyrhizobium japonicum FixL. *Biochemistry* 39: 3955-3962, 2000.
57. **Gonschior P, Gonschior GM, Conzen PF, Hobbhahn J, Goetz AE, Peter K, and Brendel W.** Myocardial oxygenation and transmural lactate metabolism during experimental acute coronary stenosis in pigs. *Basic Research in Cardiology* 87: 27-37, 1992.
58. **Gurantz D, Cowling RT, Villarreal FJ, Greenberg BH, and Carmeliet P.** Tumor necrosis factor-alpha upregulates angiotensin II type 1 receptors on cardiac fibroblasts
Fibroblast growth factor-1 stimulates branching and survival of myocardial arteries: a goal for therapeutic angiogenesis? [letter; comment.]. *Circulation Research* 85: 272-279, 1999.

59. **Hannigan M, Zhan L, Ai Y, and Huang CK.** The role of p38 MAP kinase in TGF-beta1-induced signal transduction in human neutrophils. *Biochemical & Biophysical Research Communications* 246: 55-58, 1998.
60. **Hart CE, Stetler-Stevenson WG, Clowes AW, and Ohkubo N.** Angiotensin type 2 receptors are reexpressed by cardiac fibroblasts from failing myopathic hamster hearts and inhibit cell growth and fibrillar collagen metabolism. *Circulation* 96: 3555-3560, 1997.
61. **Hartemink A, Gifford D, and Jaakkola T.** Maximum Likelihood Estimation of Optimal Scaling Factors for Expression Array Normalization. *Microarrays: Optical Technologies and Informatics*, edited by Bittner M, Chen Y, Dorsel A and Doughterty E, 2001, p. 132-140.
62. **Heckerman D.** A tutorial on learning Bayesian networks. In: *Learning in graphical models*, edited by Jordan M. Cambridge, MA: MIT Press, 1999, p. 301-354.
63. **Herrera B, Fernandez M, Roncero C, Ventura JJ, Porras A, Valladares A, Benito M, and Fabregat I.** Activation of p38MAPK by TGF-beta in fetal rat hepatocytes requires radical oxygen production, but is dispensable for cell death. *FEBS Letters* 499: 225-229, 2001.
64. **Herskowitz A, Choi S, Ansari AA, and Wesselingh S.** Cytokine mRNA expression in postischemic/reperfused myocardium. *American Journal of Pathology* 146: 419-428, 1995.
65. **Ideker T, Ozier O, Schwikowski B, and Siegel AF.** Discovering regulatory and signalling circuits in molecular interaction networks. *Bioinformatics* 18: S233-S240, 2002.
66. **Imoto S, Higuchi, T, Goto, T, Tashiro, K, Kuhara, S, Miyano, S.** Combining microarrays and biological knowledge for estimating gene networks via bayesian networks. *J Bioinform Comput Biol* 2: 77-98, 2004.
67. **Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, and Kaelin Jr. WG.** HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O₂ sensing. *Science* 292: 464-468, 2001.

68. **Jaakkola P.** Targeting of HIF- α to the von Hippel-Lindau ubiquitylation complex by O₂-regulated prolyl hydroxylation. *Science* 292: 468-472, 2001.
69. **Jewell UR, Kvietikova I, Scheid A, Bauer C, Wenger RH, and Gassmann M.** Induction of HIF-1 α in response to hypoxia is instantaneous. *FASEB Journal* 15: 1312-1314, 2001.
70. **Kaab S, Barth AS, Margerie D, Dugas M, Gebauer M, Zwermann L, Merk S, Pfeufer A, Steinmeyer K, Bleich M, Kreuzer E, Steinbeck G, and Nabauer M.** Global gene expression in human myocardium-oligonucleotide microarray analysis of regional diversity and transcriptional regulation in heart failure. *J Mol Med* 82: 308-316, 2004.
71. **Kanehisa M.** A database for post-genome analysis. *Trends Genet* 13: 375-376, 1997.
72. **Kanehisa M, Goto, S.** KEGG: Kyoto Encyclopedia of Genes and Genomes. *Nucleic Acids Res* 28: 27-30, 2000.
73. **Kardami E, Cattini PA, and Farivar RS.** Salicylate or aspirin inhibits the induction of the inducible nitric oxide synthase in rat cardiac fibroblasts. *Circulation Research* 78: 126-136, 1996.
74. **Kawano H, Do YS, Kawano Y, Starnes V, Barr M, Law RE, and Hsueh WA.** Angiotensin II has multiple profibrotic effects in human cardiac fibroblasts. *Circulation* 101: 1130-1137, 2000.
75. **Kimura SH, Ikawa M, Ito A, Okabe M, and Nojima H.** Cyclin G1 is involved in G2/M arrest in response to DNA damage and in growth control after damage recovery. *Oncogene* 20: 3290-3300, 2001.
76. **Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG, Jr., and Vasconcelles MJ.** Identification and characterization of a low oxygen response element involved in the hypoxic induction of a family of *Saccharomyces cerevisiae* genes.

Implications for the conservation of oxygen sensing in eukaryotes. *Science* 292: 464-468, 2001.

77. **Kreutzer U and Jue T.** Critical intracellular O₂ in myocardium as determined by ¹H nuclear magnetic resonance signal of myoglobin. *American Journal of Physiology* 268: H1675-1681, 1995.

78. **Kucich U, Rosenbloom JC, Shen G, Abrams WR, Hamilton AD, Sebti SM, and Rosenbloom J.** TGF-beta1 stimulation of fibronectin transcription in cultured human lung fibroblasts requires active geranylgeranyl transferase I, phosphatidylcholine-specific phospholipase C, protein kinase C-delta, and p38, but not erk1/erk2. *Archives of Biochemistry & Biophysics* 374: 313-324, 2000.

79. **Landeem LK, Shepherd BR, Naughton GK, Ratcliffe A, Williams SK, and Feld Y.** Electrophysiological modulation of cardiomyocytic tissue by transfected fibroblasts expressing potassium channels: a novel strategy to manipulate excitability. *Circulation* 104: 2063-2068, 2001.

80. **Lando D.** Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science* 295: 858-861, 2002.

81. **Lappi DA, Baird A, Majack RA, Reidy MA, and Carver W.** Collagen expression in mechanically stimulated cardiac fibroblasts. *Circulation Research* 68: 106-113, 1991.

82. **Larkin J, Frank, BC, Gaspard, RM, Duka, I, Gavras, H, Quackenbush, J.** Cardiac transcriptional response to acute and chronic angiotensin II treatments. *Physiol Genomics* 18: 152-166, 2004.

83. **Lavoisier A.** Memoir on the combustion of candles in atmospheric air and in respirable air, edited by Sciences FAd. Paris, 1777.

84. **Lazzeroni L and Owen A.** Plaid models for gene expression data. *Statist Sinica* 12: 61--86, 2002.

85. **Lee T, Rinaldi, NJ, Robert, F, Odom, DT, Bar-Joseph, Z, Gerber, GK, Hannett, NM, Harbison, CT, Thompson, CM, Simon, I, Zeitlinger, J, Jennings, EG, Murray, HL, Gordon, DB, Ren, B, Wyrick, JJ, Tagne, JB, Volkert. TL, Fraenkel. E, Gifford. DK, Young. RA.** Transcriptional regulatory networks in *Saccharomyces cerevisiae*. *Science* 298: 799-804, 2002.
86. **Li C and Hung Wong W.** Model-based analysis of oligonucleotide arrays: model validation, design issues and standard error application. *Genome Biol* 2: RESEARCH0032, 2001.
87. **Li P, Li J, Feng X, Li Z, Hou R, Han C, and Zhang Y.** Gene expression profile of cardiomyocytes in hypertrophic heart induced by continuous norepinephrine infusion in the rats. *Cell Mol Life Sci* 60: 2200-2209, 2003.
88. **Liang J and Kachalo S.** Computational analysis of microarray gene expression profiles: Clustering, classification, and beyond. *Chemometrics and Intelligent Laboratory Systems* 62: 199-216, 2002.
89. **Long CS and Brown RD.** The cardiac fibroblast, another therapeutic target for mending the broken heart?[comment]. *Journal of Molecular & Cellular Cardiology* 34: 1273-1278, 2002.
90. **Long X, Boluyt MO, Hipolito ML, Lundberg MS, Zheng JS, O'Neill L, Cirielli C, Lakatta EG, and Crow MT.** p53 and the hypoxia-induced apoptosis of cultured neonatal rat cardiac myocytes. *J Clin Invest* 99: 2635-2643, 1997.
91. **Lundgren P, Chen YB, Kupper H, Kolber Z, Bergman B, Falkowski P, and Bruick RK.** Transcription. Oxygen sensing gets a second wind. [letter; comment.]. *Science* 294: 1534-1537, 2001.

92. **Maltseva O, Folger P, Zekaria D, Petridou S, and Masur SK.** Fibroblast growth factor reversal of the corneal myofibroblast phenotype. *Investigative Ophthalmology & Visual Science* 42: 2490-2495, 2001.
93. **Mamchaoui K and Saumon G.** A method for measuring the oxygen consumption of intact cell monolayers. *American Journal of Physiology - Lung Cellular & Molecular Physiology* 278: L858-863, 2000.
94. **Mathur A and Martin JF.** Stem cells and repair of the heart. *Lancet* 364: 183-192, 2004.
95. **McCabe LR, Banerjee C, Kundu R, Harrison RJ, Dobner PR, Stein JL, Lian JB, and Stein GS.** Developmental expression and activities of specific fos and jun proteins are functionally related to osteoblast maturation: role of Fra-2 and Jun D during differentiation. *Endocrinology* 137: 4398-4408, 1996.
96. **McCord JM and Fridovich I.** Superoxide dismutase. An enzymic function for erythrocyte hemocuprein (hemocuprein). *J Biol Chem* 244: 6049-6055, 1969.
97. **McCord JM and Fridovich I.** The utility of superoxide dismutase in studying free radical reactions. I. Radicals generated by the interaction of sulfite, dimethyl sulfoxide, and oxygen. *J Biol Chem* 244: 6056-6063, 1969.
98. **Meyyappan M, Wong H, Hull C, and Riabowol KT.** Increased expression of cyclin D2 during multiple states of growth arrest in primary and established cells. *Molecular & Cellular Biology* 18: 3163-3172, 1998.
99. **Miller LD, McPhie P, Suzuki H, Kato Y, Liu ET, and Cheng SY.** Multi-tissue gene-expression analysis in a mouse model of thyroid hormone resistance. *Genome Biol* 5: R31, 2004.

100. **Muller P, Parmigiani G, Robert C, and Rousseau J.** Optimal sample size for multiple testing: the case of gene expression microarrays. *Journal of the American Statistical Association* 99: 990-1001, 2004.
101. **Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, Pasumarthi KB, Virag JI, Bartelmez SH, Poppa V, Bradford G, Dowell JD, Williams DA, and Field LJ.** Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts.[see comment]. *Nature* 428: 664-668, 2004.
102. **Nabel EG.** CDKs and CKIs: molecular targets for tissue remodelling. *Nature Reviews Drug Discovery* 1: 587-598, 2002.
103. **Novotna J, Bibova J, Hampl V, Deyl Z, and Herget J.** Hyperoxia and recovery from hypoxia alter collagen in peripheral pulmonary arteries similarly. *Physiological Research* 50: 153-163, 2001.
104. **O'Farrell PH and Semenza GL.** Perspectives on oxygen sensing. *Cell* 98: 105-114, 1999.
105. **Outinen PA, Sood SK, Pfeifer SI, Pamidi S, Podor TJ, Li J, Weitz JI, and Austin RC.** Homocysteine-induced endoplasmic reticulum stress and growth arrest leads to specific changes in gene expression in human vascular endothelial cells. *Blood* 94: 959-967, 1999.
106. **Packer L and Fuehr K.** Low oxygen concentration extends the lifespan of cultured human diploid cells. *Nature* 267: 423-425, 1977.
107. **Pathak M, Sarkar S, Vellaichamy E, and Sen S.** Role of myocytes in myocardial collagen production. *Hypertension* 37: 833-840., 2001.
108. **Pedoux R, Lefort K, Cuenin C, Cortes U, Kellner K, Dore JF, and Nakazawa H.** Specific induction of gadd45 in human melanocytes and melanoma cells after UVB irradiation. *Int J Cancer* 98: 811-816., 2002.

109. **Pellequer JL, Brudler R, and Getzoff ED.** Biological sensors: More than one way to sense oxygen. *Current Biology* 9: R416-418, 1999.
110. **Penn MS, Zhang M, Deglurkar I, and Topol EJ.** Role of stem cell homing in myocardial regeneration. *International Journal of Cardiology* 95 Suppl 1: S23-25, 2004.
111. **Pollard KS and van der Laan MJ.** Choice of a null distribution in resampling-based multiple testing. *J Statist Plann Inference* 125: 85--100, 2004.
112. **Porwol T, Ehleben W, Brand V, and Acker H.** Tissue oxygen sensor function of NADPH oxidase isoforms, an unusual cytochrome aa3 and reactive oxygen species. *Respiration Physiology* 128: 331-348, 2001.
113. **Potapova I, Plotnikov A, Lu Z, Danilo P, Jr., Valiunas V, Qu J, Doronin S, Zuckerman J, Shlapakova IN, Gao J, Pan Z, Herron AJ, Robinson RB, Brink PR, Rosen MR, and Cohen IS.** Human mesenchymal stem cells as a gene delivery system to create cardiac pacemakers. *Circulation Research* 94: 952-959, 2004.
114. **Prabhakar R, Petrashevskaya N, Schwartz A, Aronow B, Boivin GP, Molkentin JD, and Wieczorek DF.** A mouse model of familial hypertrophic cardiomyopathy caused by a alpha-tropomyosin mutation. *Mol Cell Biochem* 251: 33-42, 2003.
115. **Prentice H and Webster KA.** Genomic and proteomic profiles of heart disease. *Trends Cardiovasc Med* 14: 282-288, 2004.
116. **Priestly J.** *Experiments and observations on different kinds of air.* London: J. Johnson in St. Paul's Church-yard, 1775.
117. **Quackenbush J.** Microarray data normalization and transformation. *Nat Genet* 32 Suppl: 496-501, 2002.
118. **R Development Core Team.** R: A language and environment for statistical computing. Vienna, 2004.

119. **Rangappa S, Fen C, Lee EH, Bongso A, and Wei ES.** Transformation of adult mesenchymal stem cells isolated from the fatty tissue into cardiomyocytes. *Annals of Thoracic Surgery* 75: 775-779, 2003.
120. **Rhaleb NE, Peng H, Harding P, Tayeh M, LaPointe MC, and Carretero OA.** Effect of N-acetyl-seryl-aspartyl-lysyl-proline on DNA and collagen synthesis in rat cardiac fibroblasts. *Hypertension* 37: 827-832., 2001.
121. **Rodt SA, Ahlen K, Berg A, Rubin K, and Reed RK.** A novel physiological function for platelet-derived growth factor-BB in rat dermis. *Journal of Physiology* 495: 193-200, 1996.
122. **Rorth M and Jensen PK.** Determination of catalase activity by means of the Clark oxygen electrode. *Biochimica et Biophysica Acta* 139: 171-173, 1967.
123. **Roy S, Khanna S, Bickerstaff AA, Subramanian SV, Atalay M, Bierl M, Pendyala S, Levy D, Sharma N, Venojarvi M, Strauch A, Orosz CG, and Sen CK.** Oxygen sensing by primary cardiac fibroblasts: a key role of p21(Waf1/Cip1/Sdi1). *Circ Res* 92: 264-271, 2003.
124. **Roy S, Khanna S, and Sen CK.** Perceived hyperoxia: oxygen-regulated signal transduction pathways in the heart. *Methods in Enzymology* 381: 133-139, 2004.
125. **Roy S, Khanna S, Wallace WA, Lappalainen J, Rink C, Cardounel AJ, Zweier JL, and Sen CK.** Characterization of perceived hyperoxia in isolated primary cardiac fibroblasts and in the reoxygenated heart. *Journal of Biological Chemistry* 278: 47129-47135, 2003.
126. **Rumsey WL, Pawlowski M, Lejavardi N, and Wilson DF.** Oxygen pressure distribution in the heart in vivo and evaluation of the ischemic "border zone". *American Journal of Physiology* 266: H1676-1680, 1994.
127. **Sa G, Fox PL, and Sartore S.** Contribution of adventitial fibroblasts to neointima formation and vascular remodeling: from innocent bystander to active participant. *Circulation Research* 74: 1149-1156, 1994.

128. **Salceda S and Caro J.** Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *Journal of Biological Chemistry* 272: 22642-22647, 1997.
129. **Sawyer DB and Colucci WS.** Mitochondrial oxidative stress in heart failure: "oxygen wastage" revisited. *Circulation Research* 86: 119-120, 2000.
130. **Schadt EE, Li C, Ellis B, and Wong WH.** Feature extraction and normalization algorithms for high-density oligonucleotide gene expression array data. *J Cell Biochem Suppl* Suppl 37: 120-125, 2001.
131. **Schadt EE, Li C, Su C, and Wong WH.** Analyzing high-density oligonucleotide gene expression array data. *J Cell Biochem* 80: 192-202, 2000.
132. **Scharpf R, Iacobuzio-Donahue C, and Parmigiani G.** When should one subtract background fluorescence in cDNA microarrays. Baltimore, MD: Department of Biostatistics, Johns Hopkins University, 2004.
133. **Schrantz N, Bourgeade MF, Mouhamad S, Leca G, Sharma S, and Vazquez A.** p38-mediated regulation of an Fas-associated death domain protein-independent pathway leading to caspase-8 activation during TGFbeta-induced apoptosis in human Burkitt lymphoma B cells BL41. *Molecular Biology of the Cell* 12: 3139-3151, 2001.
134. **Schroen B, Heymans S, Sharma U, Blankesteyn WM, Pokharel S, Cleutjens JP, Porter JG, Evelo CT, Duisters R, van Leeuwen RE, Janssen BJ, Debets JJ, Smits JF, Daemen MJ, Crijns HJ, Bornstein P, and Pinto YM.** Thrombospondin-2 is essential for myocardial matrix integrity: increased expression identifies failure-prone cardiac hypertrophy. *Circ Res* 95: 515-522, 2004.
135. **Schwartz Y and Kornowski R.** Autologous stem cells for functional myocardial repair. *Heart Failure Reviews* 8: 237-245, 2003.

136. **Semenza GL.** HIF-1 and tumor progression: pathophysiology and therapeutics. *Trends in Molecular Medicine* 8: S62-67, 2002.
137. **Semenza GL.** HIF-1, O(2), and the 3 PHDs: how animal cells signal hypoxia to the nucleus. *Cell* 107: 1-3, 2001.
138. **Semenza GL.** Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology. *Trends in Molecular Medicine* 7: 345-350, 2001.
139. **Semenza GL.** Perspectives on oxygen sensing. *Cell* 98: 281-284, 1999.
140. **Sharma UC, Pokharel S, van Brakel TJ, van Berlo JH, Cleutjens JP, Schroen B, Andre S, Crijns HJ, Gabius HJ, Maessen J, and Pinto YM.** Galectin-3 marks activated macrophages in failure-prone hypertrophied hearts and contributes to cardiac dysfunction. *Circulation* 110: 3121-3128, 2004.
141. **Siaghy EM, Devaux Y, Sfaksi N, Carreaux JP, Ungureanu-Longrois D, Zannad F, Villemot JP, Burlet C, and Mertes PM.** Consequences of inspired oxygen fraction manipulation on myocardial oxygen pressure, adenosine and lactate concentrations: a combined myocardial microdialysis and sensitive oxygen electrode study in pigs. *Journal of Molecular & Cellular Cardiology* 32: 493-504, 2000.
142. **Silverman HS, Wei S, Haigney MC, Ocampo CJ, and Stern MD.** Myocyte adaptation to chronic hypoxia and development of tolerance to subsequent acute severe hypoxia. *Circulation Research* 80: 699-707, 1997.
143. **Simkhovich B, Kloner, RA, Poizat, C, Marjoram, P, Kedes, LH.** Gene expression profiling--a new approach in the study of myocardial ischemia. *Cardiovasc Pathol* 12: 180-185, 2003.
144. **Sitte N, Huber M, Grune T, Ladhoff A, Doecke WD, Von Zglinicki T, and Davies KJ.** Proteasome inhibition by lipofuscin/ceroid during postmitotic aging of fibroblasts. *FASEB J* 14: 1490-1498, 2000.

145. **Smyth G.** Linear Models and empirical Bayes methods for assessing differential expression in microarray experiments. *Statist Appl in Genetics and Mol Biol* 3, 2004.
146. **Smyth GK, Yang, Y.H., Speed, T.** Statistical issues in cDNA microarray data analysis. *Methods Mol Biol* 224: 111-136, 2003.
147. **Staversky RJ, Watkins RH, Wright TW, Hernady E, LoMonaco MB, D'Angio CT, Williams JP, Maniscalco WM, and O'Reilly MA.** Normal remodeling of the oxygen-injured lung requires the cyclin-dependent kinase inhibitor p21(Cip1/WAF1/Sdi1). *American Journal of Pathology* 161: 1383-1393, 2002.
148. **Stekel D.** *Microarray Bioinformatics*: Cambridge University Press, 2003.
149. **Sun Y and Weber KT.** Infarct scar: a dynamic tissue. *Cardiovascular Research* 46: 250-256, 2000.
150. **Teichert-Kuliszewska K, Monge JC, Mohamed F, Bendeck MP, Stewart DJ, and Tsutsumi Y.** Angiotensin II type 2 receptor is upregulated in human heart with interstitial fibrosis, and cardiac fibroblasts are the major cell type for its expression. *Circulation Research* 82: 1007-1015, 1998.
151. **Tibshirani R, Hastie T, Narasimhan B, Eisen M, Sherlock G, Brown P, and Botstein D.** Exploratory screening of genes and clusters from microarray experiments. *Statist Sinica* 12: 47--59, 2002.
152. **Ueno S, Ohki R, Hashimoto T, Takizawa T, Takeuchi K, Yamashita Y, Ota J, Choi YL, Wada T, Koinuma K, Yamamoto K, Ikeda U, Shimada K, and Mano H.** DNA microarray analysis of in vivo progression mechanism of heart failure. *Biochem Biophys Res Commun* 307: 771-777, 2003.
153. **Urabe Y, Tsutsui H, Kinugawa S, Sugimachi M, Takahashi M, Yamamoto S, Tagawa H, Sunagawa K, Takeshita A, and Dubey RK.** Exogenous and endogenous

adenosine inhibits fetal calf serum-induced growth of rat cardiac fibroblasts: role of A2B receptors. *Circulation* 96: 2501-2504, 1997.

154. **Valiunas V, Doronin S, Valiuniene L, Potapova I, Zuckerman J, Walcott B, Robinson RB, Rosen MR, Brink PR, and Cohen IS.** Human mesenchymal stem cells make cardiac connexins and form functional gap junctions. *Journal of Physiology* 555: 617-626, 2004.

155. **van der Laan MJ, Dudoit S, and Pollard KS.** Multiple Testing Part II: Step-down procedures for control of the family-wise error rate. *Statistical Applications in Genetics and Molecular Biology* 3, 2004.

156. **van der Laan MJ, Dudoit S, and Pollard KS.** Multiple Testing Part I: Single-step procedures for control of general type I error rates. *Statistical Applications in Genetics and Molecular Biology* 3, 2004.

157. **Vanden Hoek TL, Becker LB, Shao Z, Li C, and Schumacker PT.** Reactive oxygen species released from mitochondria during brief hypoxia induce preconditioning in cardiomyocytes. *Journal of Biological Chemistry* 273: 18092-18098, 1998.

158. **Virmani R, Correa R, Yu ZX, Farb A, Leon MB, Elami A, Fu YM, Casscells W, Epstein SE, and Villarreal FJ.** Identification of functional angiotensin II receptors on rat cardiac fibroblasts. *Circulation* 88: 2493-2500, 1993.

159. **Vozenin-Brotans MC, Sivan V, Gault N, Renard C, Geffrotin C, Delanian S, Lefaix JL, and Martin M.** Antifibrotic action of Cu/Zn SOD is mediated by TGF-beta1 repression and phenotypic reversion of myofibroblasts. *Free Radical Biology & Medicine* 30: 30-42, 2001.

160. **Wagers AJ and Kansas GS.** Potent induction of alpha(1,3)-fucosyltransferase VII in activated CD4+ T cells by TGF-beta 1 through a p38 mitogen-activated protein kinase-dependent pathway. *Journal of Immunology* 165: 5011-5016, 2000.

161. **Wenger RH.** Mammalian oxygen sensing, signalling and gene regulation. *Journal of Experimental Biology* 203 Pt 8: 1253-1263, 2000.
162. **Wenzel S, Taimor G, Piper HM, and Schluter KD.** Redox-sensitive intermediates mediate angiotensin II-induced p38 MAP kinase activation, AP-1 binding activity, and TGF-beta expression in adult ventricular cardiomyocytes. *FASEB Journal* 15: 2291-2293, 2001.
163. **Whalen WJ.** Intracellular PO₂ in heart and skeletal muscle. *Physiologist* 14: 69-82, 1971.
164. **Wharton J, Morgan K, Allen SP, Chester AH, Catravas JD, Polak JM, Yacoub MH, and Kellar RS.** Scaffold-based three-dimensional human fibroblast culture provides a structural matrix that supports angiogenesis in infarcted heart tissue. *Circulation* 98: 2553-2559, 1998.
165. **Winegrad S, Henrion D, Rappaport L, and Samuel JL.** Self-protection by cardiac myocytes against hypoxia and hyperoxia. *Circulation Research* 85: 690-698, 1999.
166. **Wittenberg BA and Wittenberg JB.** Oxygen pressure gradients in isolated cardiac myocytes. *Journal of Biological Chemistry* 260: 6548-6554, 1985.
167. **Xu W, Zhang X, Qian H, Zhu W, Sun X, Hu J, Zhou H, and Chen Y.** Mesenchymal stem cells from adult human bone marrow differentiate into a cardiomyocyte phenotype in vitro. *Experimental Biology & Medicine* 229: 623-631, 2004.
168. **Yamamoto T, Kozawa O, Tanabe K, Akamatsu S, Matsuno H, Dohi S, and Uematsu T.** Involvement of p38 MAP kinase in TGF-beta-stimulated VEGF synthesis in aortic smooth muscle cells. *Journal of Cellular Biochemistry* 82: 591-598, 2001.
169. **Yang YH, Dudoit S, Luu P, Lin DM, Peng V, Ngai J, and Speed TP.** Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. *Nucleic Acids Res* 30: e15, 2002.

170. **Zhang X, Azhar G, Nagano K, and Wei JY.** Differential vulnerability to oxidative stress in rat cardiac myocytes versus fibroblasts. *J Am Coll Cardiol* 38: 2055-2062., 2001.
171. **Zhao L and Eghbali-Webb M.** Release of pro- and anti-angiogenic factors by human cardiac fibroblasts: effects on DNA synthesis and protection under hypoxia in human endothelial cells. *Biochim Biophys Acta* 1538: 273-282., 2001.
172. **Zhao M, Chow A, Powers J, Fajardo G, and Bernstein D.** Microarray analysis of gene expression after transverse aortic constriction in mice. *Physiol Genomics* 19: 93-105, 2004.
173. **Zhu H and Bunn HF.** How do cells sense oxygen? *Science* 292: 449-451, 2001.
174. **Zhu J, Lum, PY, Lamb, J, GuhaThakurta, D, Edwards, SW, Thieringer, R, Berger, JP, Wu, MS, Thompson, J, Sachs, AB, Schadt, EE.** An integrative genomics approach to the reconstruction of gene networks in segregating populations. *Cytogenet Genome Res* 105: 363-374, 2004.

TABLES

	Multiple Testing Adjustment		
	None	Bonferroni	FDR
Number of significant genes	601	23	75
p-value of Cappa2 gene	0.000014	0.171	0.002

Table 1. The chosen multiple testing adjustment has a profound effect on the conclusions. In this example a linear model (145) was fit to the microarray data from Roy et al. (125), after filtering out all genes that were not present either in all 3% or all 20% samples, and the p-value for the null hypothesis of no differential expression was computed using no adjustment, a Bonferroni adjustment, and the FDR-controlling technique of (9). The first row gives the number of genes that were declared significant at level $\alpha = 0.01$. The second row gives the p-value of the Cappa1 gene. All computations were done using the Bioconductor R software (53, 118).

<i>m</i>	Probability of at least one false positive
1	0.05
10	0.40
50	0.92
100	0.994

Table 2. Illustrating the need to adjust p-values for multiple comparisons: The probability of at least one false positive increases rapidly as the number *m* of hypotheses increases.

	glycolysis, gluconeogenesis	carbohydrate metabolism	carbon fixation
Aldh1a1	+	+	
Aldoa	-	-	-
Pgam1	-	-	
Pfkf	-	-	
Pgk1	-	-	-
Ldh1	-	-	
Tpi1	-	-	-

Table 3. KEGG pathways over-represented by genes showing significant change between cardiac fibroblasts exposed to 3% or 20% O₂. +/- indicates up/down regulation in 20% *versus* 3% O₂ groups.

	glycolysis	glucose catabolism	alcohol catabolism	monosaccharide catabolism	hexose catabolism	carbohydrate catabolism	energy derivation by oxidation of organic compounds	glucose metabolism	energy pathways	main pathways of carbohydrate metabolism	hexose metabolism	monosaccharide metabolism	alcohol metabolism	carbohydrate metabolism
Aldoa	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pgam1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pfkl	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pgk1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ldh1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gys1							-		-					
Tpi1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ldlr												+		
Pgm2														-

Table 4. Biological processes over-represented by genes showing significant change between 3% and 20% O₂ groups. +/- indicates up/down regulation in 20% vs 3% O₂ groups.

	intramolecular transferase activity, phosphotransferases	intramolecular transferase activity	oxidoreductase activity	catalytic activity
Aldh1a1				+
Pcna				-
Tap1				+
Enpp1				-
Pgm2	-	-		-
Aldoa				-
Pgam1	-	-		-
Ptp4a3				+
Igtp				-
NA			-	-
Pfkl				-
Cp				-
H19				-
Sgrp23				+
Pgk1				-
Ptp4a1				+
NA				+
Nars				+
Egln1			-	-
Ldh1				-
Ndr2				-
Hp				-
Mdm2				+
Gys1				-
Mmp3				-
P4ha2			-	-
Tpi1				-

Table 5. Molecular functions over-represented by genes showing significant change between cardiac fibroblasts grown under 3% or 20% O₂ ambience. +/- indicates up/down regulation in 20% *versus* 3% O₂ groups.

target Gene	candidate activator(+)/inhibitor(-) genes
CDKN1A	Gpi1(-), Hig1(-), Cd81(+), Anxa11(+), Wdr26(+), Bgn(-), Psma3(+), Dnajc5(-), Capza1(+), Hbxip(+), 3300001H21Rik(-), 1500034E06Rik(-), 4932431F02Rik(+)
Tob1	Prnp(+), Tpt1(-), Mapk6(+), Slc7a5(+), Eps15(+), Serpina3n(-), Hdac5(-), 1200013P24Rik(+), Msn(+), Pgk1(-), 2010008E23Rik(-), D10627(-), EglN1(-), Rbm3(+), Atp6v1d(+), Mdm2(+), Gnb2-rs1(-)
Wig1	Ddt(+), Kif4(+), Dnajc7(+), D1Ucla4(+), Gpaa1(+), Pfn2(+), Taf9(+), 2310065K24Rik(+), Crsp3(+), Sat1(+), 2010100O12Rik(+), Mcfd2(+), Phlda3(+), D5Ertd606e(+), Sept7(+), 0610007P06Rik(-), Sars1(+)

Table 6. Candidate activator (+) / inhibitor (-) genes for CDKN1A, Tob1 and Wig1. Each candidate together with TP53, are capable of explaining better the variance of the target gene than TP53 alone does. The proportion of the variance of the target gene that can be explained by TP53 and each of the candidates is $R^2=99\%$.

FIGURE LEGENDS

Figure 1. Cellular responses to reoxygenation following chronic hypoxia. Chronic hypoxia results in cellular adjustments such that reoxygenation causes hyperoxic insult as evident in part in the form of oxidative damage in numerous studies. *ischemic*, diagram of ischemic tissue, with focus of insult represented by the blue center (region **i**). Focal ischemia is known to be associated with graded oxygenation from near-zero status at focus to levels increasing with distance from focus. The concentric circles represents regions (i-iv) of the tissue with increasing graded distance from the focus of insult (blue); *reperfused*, diagram of the reperfused tissue with corrected pO_2 state represented by change of color from a shade of blue (hypoxic) to red. Important elements triggered by oxygen, during the course of reoxygenation-associated remodeling include: in region **i** cell death or fatal oxidative injury at the focal point of insult, making room for regenerating tissues; in region **ii** non-fatal cellular stress, triggering reparative responses; in region **iii** survival of phenotypically altered cells that favor remodeling (physiological or pathological / fibrogenic). Fibrosis denies room to regenerating healthy cells; and in region **iv** correction of pO_2 of mildly hypoxic cells localized beyond a critical distance from the focus of insult, favoring regeneration and restoration of physiological functioning of the organ. Δ , represents ΔpO_2 in response to reoxygenation. ΔpO_2 : **i>ii>iii>iv**. While a large ΔpO_2 causes ROS mediate injury in reoxygenated region **i**, perceived hyperoxia supports remodeling in regions **ii** and **iii**.

Figure 2. Elevated oxygen sensitive signaling in adult primary cardiac fibroblasts. ROS generation is enhanced followed by growth-inhibition and differentiation. TGF β 1 and downstream p38MAPK is activated. Cell cycle check-points known to be associated with differentiation eg p21, cyclins and Fra-2 are up-regulated. Broken lines represent effects

where intermediary steps are expected. Grey solid lines represent association with differentiation phenotype.

Figure 3: The effect of normalization on microarray data. On the left are boxplots of un-normalized expression measures for the arrays. On the right are boxplots of the normalized expression measures for the same arrays. The data are from Roy et al. (125) The three boxplots on the left of each panel are from cells at 20% O₂; the three boxplots on the right of each panel are from cells at 3% O₂. The quantile normalization algorithm of (12), implemented in the Bioconductor R (53, 118) software package, was used.

Figure 4. An illustration of the Benjamini-Hochberg algorithm for controlling FDR. Since the third (ordered) p-value is the last to fall below the line, the Benjamini-Hochberg algorithm rejects the hypotheses corresponding to the three smallest p-values.

Figure 5. Heatmaps for 67 genes differentially expressed in cardiac fibroblasts under 10% and 21% oxygen conditions. a. unclustered genes and subjects; b. clustering using Manhattan metric and complete linkage; c. clustering using differences in mean expression for each group.

Figure 6: Pathway construction based on GeneChip™ expression data investigating oxygen-sensitive genes in adult primary cardiac fibroblasts. To obtain insights on perceived hyperoxia induced changes in cell growth and differentiation on a pathway sense the results of GeneChip™ analysis were mapped onto known pathways associated with cell-cycle/death and cytoskeletal changes. GenMAPP, KEGG and gene ontology (GO) were used

to create the pathways. Genes shown in red ellipse are candidates identified using GeneChip™ assay that were up-regulated in 20%O₂ compared to 3%O₂. Green ellipses are genes that were down-regulated under conditions mentioned above. The expressions of candidates shown in red ellipse with blue outline have been independently verified using either real-time PCR or ribonuclease protection assay (6). APAF, apoptotic protease activating factor; Bmp, bone morphogenetic protein; BAX, Bcl2-associated X protein; BID, BH3 interacting domain death agonist; Catn, catenin; Cappa, capping protein alpha; CASP, caspase; ccng, cyclin G; Cdc61, cell division cycle; CDK, cyclin-dependent kinase; CDKN1A, cyclin-dependent kinase inhibitor 1A (p21); Clu, clusterin; Cx43, gap junction membrane channel protein; GADD, growth arrest and DNA-damage-inducible; IQGAP1, IQ motif containing GTPase activating protein 1; Inhba, inhibin beta-A; Klf, Kruppel-like factor 4 (gut); Lsp1, lymphocyte specific 1; MAPK, mitogen activated protein kinase; Mdm2, transformed mouse 3T3 cell double minute 2; N-Cdh, cadherin 2; Pfn, profilin 2; PXN, paxillin; TGF, transforming growth factor; Tob, transducer of ErbB-2.1; TP53, transformation related protein 53; Vcl, vinculin; Wig, wild-type p53-induced gene 1. Reprinted with permission from Roy et al. *J. Biol. Chem.* 2003.

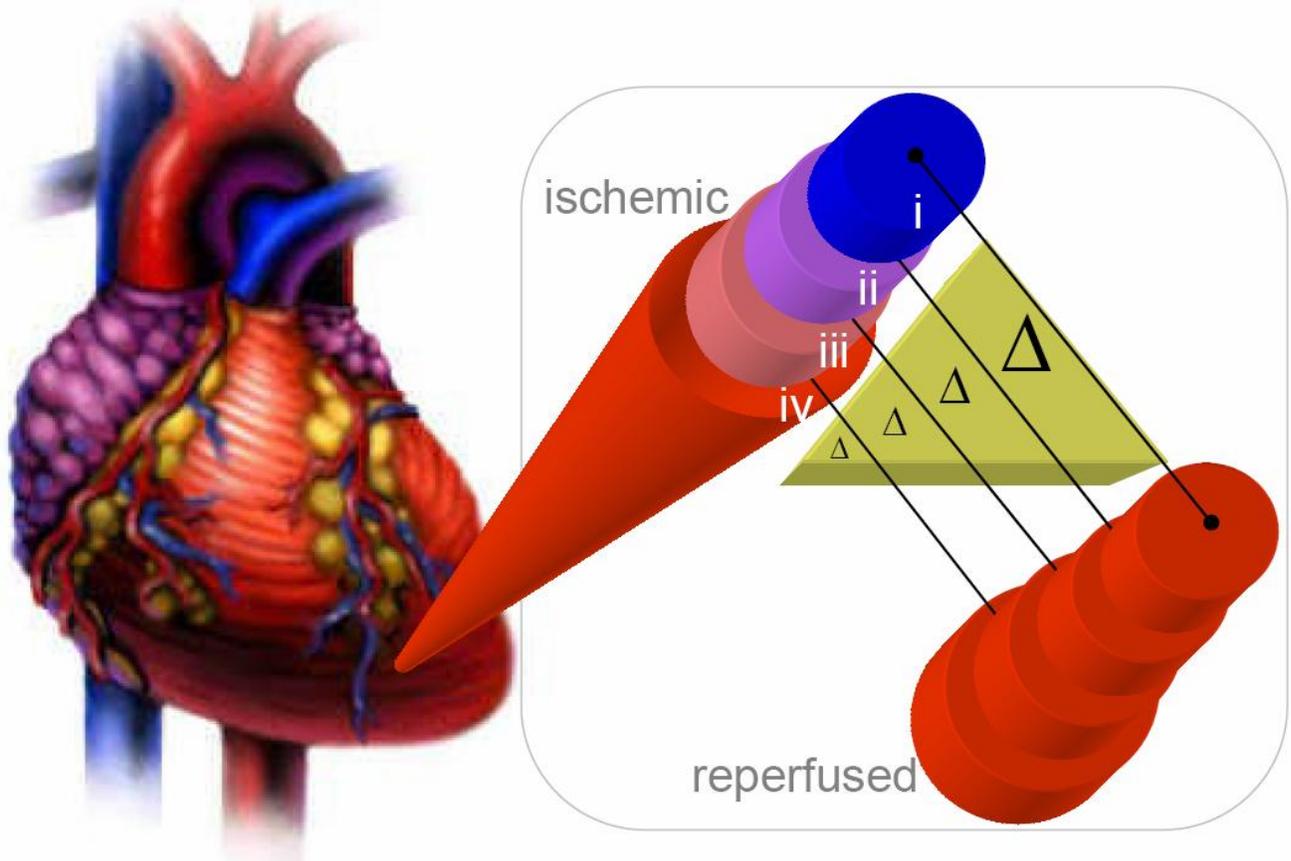


Figure 1
Sen et. al.

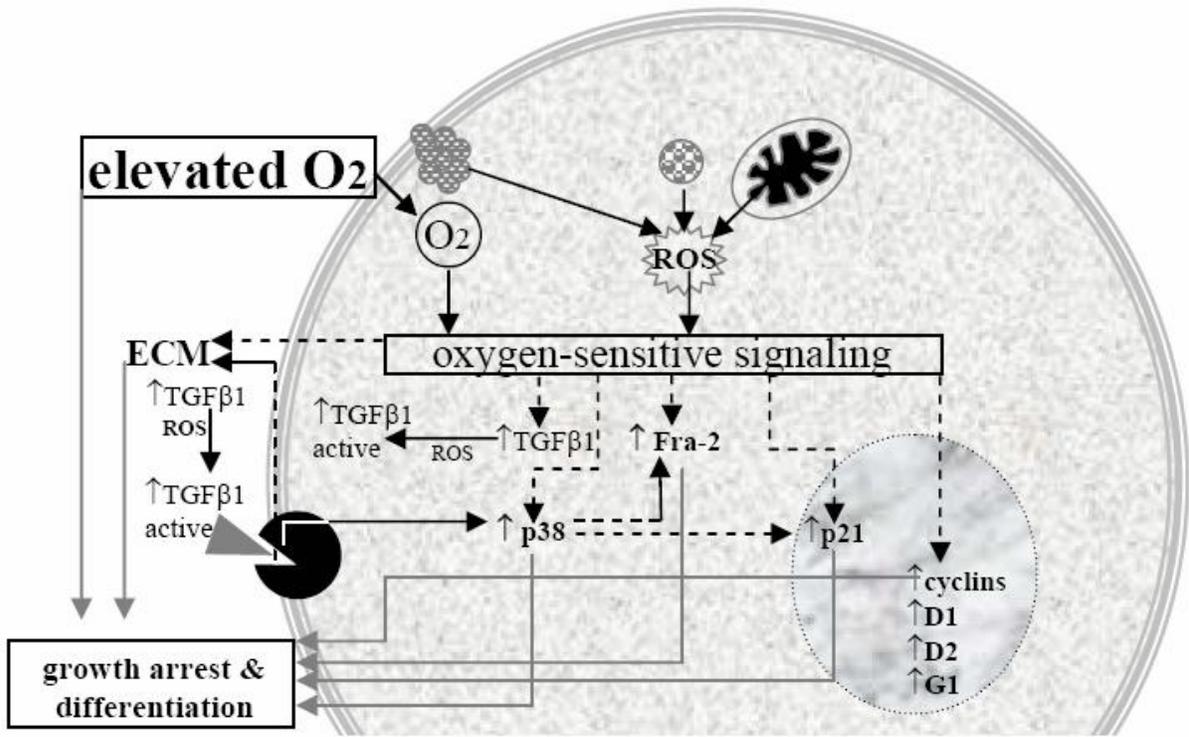


Figure 2
Sen et. al.

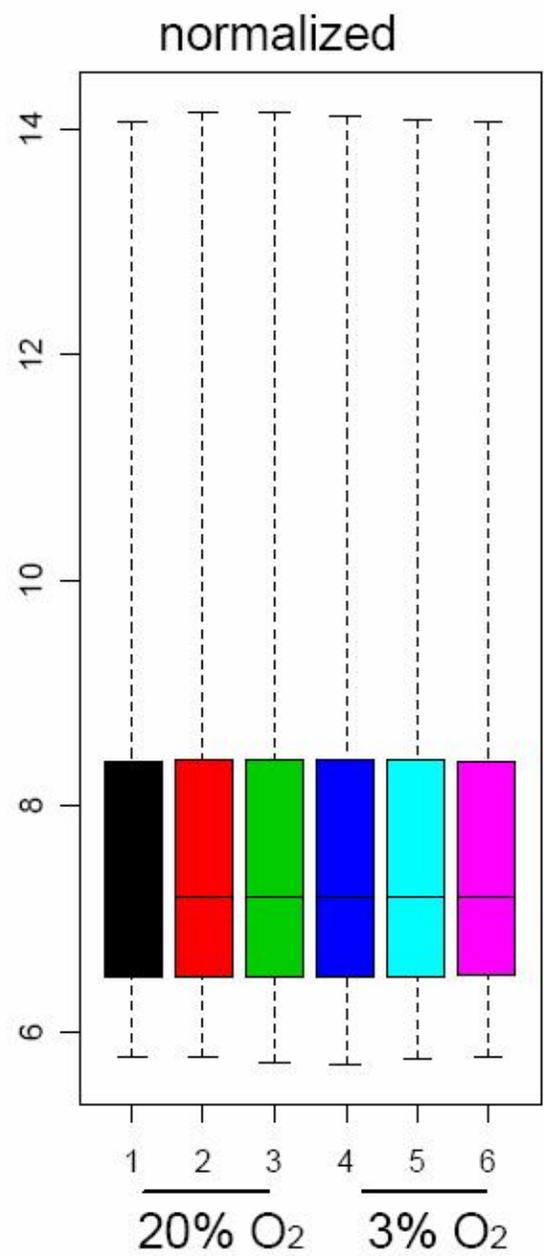
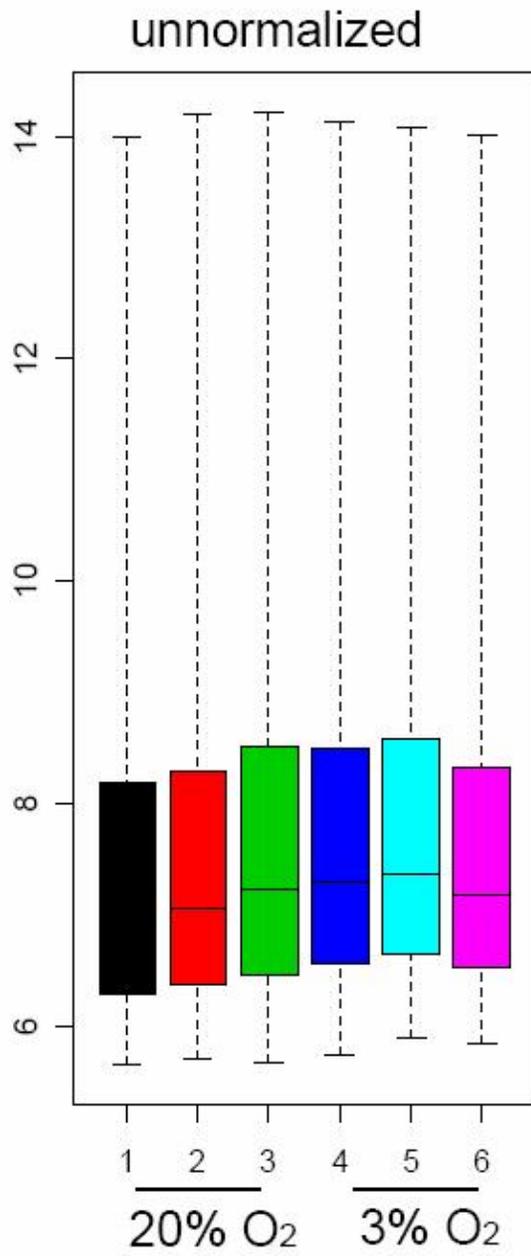


Figure 3
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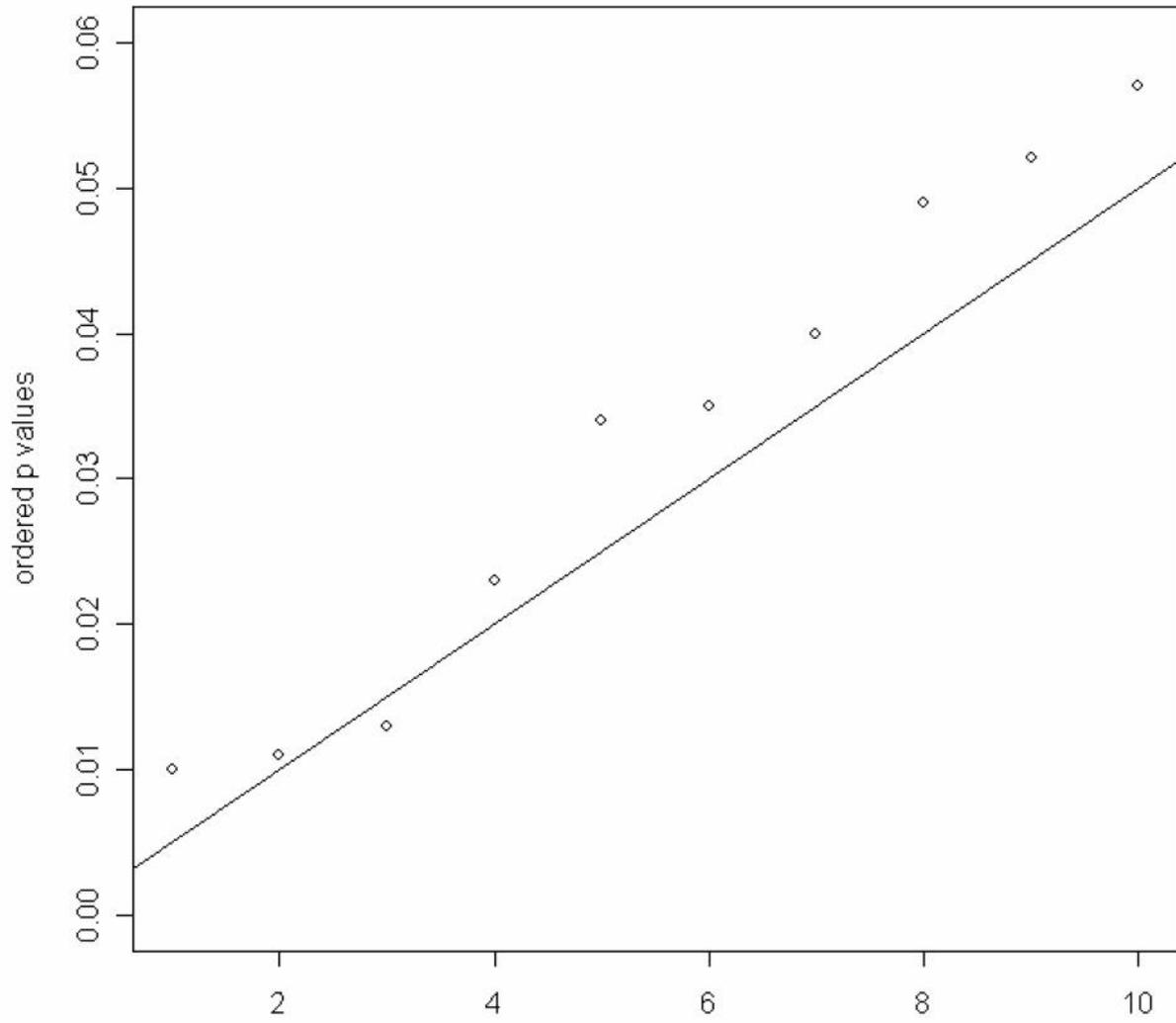


Figure 4
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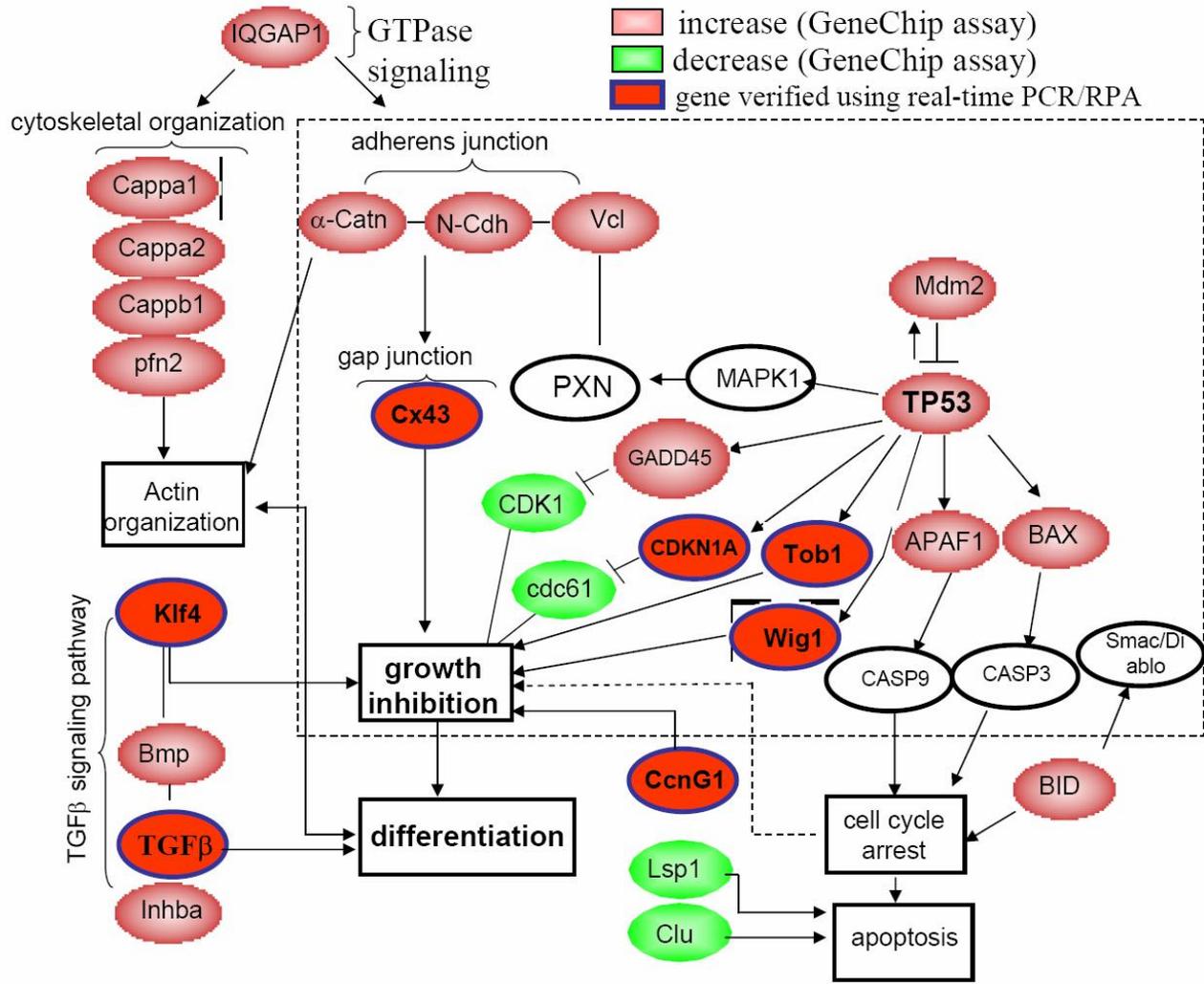


Figure 6
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