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# Physiological skin oxygen levels: An important criterion for skin cell functionality and therapeutic approaches

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## ABSTRACT

The skin is made up of different layers with various gradients, which maintain a complex microenvironment, particularly in terms of oxygen levels. However, all types of skin cells are cultured in conventional incubators that do not reproduce physiological oxygen levels. Instead, they are cultured at atmospheric oxygen levels, a condition that is far removed from physiology and may lead to the generation of free radicals known to induce skin ageing. This review aims to summarize the current literature on the effect of physiological oxygen levels on skin cells, highlight the shortcomings of current *in vitro* models, and demonstrate the importance of respecting skin oxygen levels. We begin by clarifying the terminology used about oxygen levels and describe the specific distribution of oxygen in the skin. We review and discuss how skin cells adapt their oxygen consumption and metabolism to oxygen levels environment, as well as the changes that are induced, particularly, their redox state, life cycle and functions. We examine the effects of oxygen on both simple culture models and more complex reconstructed skin models. Finally, we present the implications of oxygen modulation for a more therapeutic approach.

## 1. Introduction

Oxygen is essential for the life of mammalian cells as it is their energy source, and its distribution in the different organs regulates their homeostasis. As the skin lies at the boundary between the outside and inside of the body, it is subject to heterogeneous oxygenation, with oxygen coming from both the atmosphere and the microcirculation. Therefore, oxygen is present in the form of gradients, leading to very low levels, particularly at the base of the epidermis [1]. Although these data are well known, *in vitro* cultures of skin cells are commonly grown at oxygen levels from the air, which are much higher than physiological levels and are considered to be hyperoxia for the cells. In addition, while oxygen is essential for cellular activity, it is also responsible for deleterious effects through the generation of reactive oxygen species (ROS). The latter are specifically involved in the progression of skin ageing. In the last decade or so, the scientific community has only started to take an interest in the consequences of oxygen levels for cellular activity. However, this criterion still needs to be given more consideration. With the general recommendation to reduce, or even ban the use of animals in cosmetics in Europe, the development of *in vitro* models for studying the skin has greatly evolved, and the demand for models that most

accurately represent the behavior of skin cells *in situ* is growing. In fact, *in vitro* models of skin cells are widely used to decipher fundamental mechanisms of skin biology, evaluate the effects of drugs and dermo-cosmetic products, and reconstruct skin for grafting *in vivo* in the case of severe burns. Understanding how different levels of oxygen modulate the metabolism and activity of the skin is essential in defining appropriate study models to reproduce the physiological microenvironment as closely as possible and thus obtain relevant results. This review aims to provide an overview of the existing literature regarding the effects of physiological oxygen on skin cells. We begin by clarifying the terminology used about oxygen levels. Indeed, each of the terms “normoxia”, “hyperoxia”, and “hypoxia” is not always used with the same meaning in the literature, depending on the authors. We review the specific and heterogeneous distribution of oxygen in the different layers of the skin tissue and the current understanding of how skin cells adapt their oxygen consumption and metabolism to oxygen levels. We further address the changes in skin cell activity as a function of oxygenation, including their redox state, activity and functionality. Finally, we discuss the implications of increasing physiological oxygen levels in the context of therapeutic approaches.

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## 2. Terminology for different levels of oxygen

Over the last decade or so, there has been an increasing number of studies on the role of oxygen in cellular physiology. However, the authors have used different terms such as hypoxia and normoxia, which can be confusing when they do not refer to the same meaning. For example, the term ‘hypoxia’ can refer either to a lack of oxygen or to a level below the oxygen content of the air. To avoid any confusion, it is essential to define and clarify the different oxygen-related terms and to provide clear definitions (Fig. 1).

### 2.1. Normoxia

Normoxia is a term that usually refers to the oxygen level present in the air, which is typically around 21 %. In *in vitro* cell culture conditions, it corresponds to approximately 18.6 % oxygen in standard incubators equilibrated with 5 % carbon dioxide at 37 °C in a humidified atmosphere [2]. However, this oxygen condition is sometimes referred to as “hyperoxia,” indicating that cells are exposed to a higher level of oxygen than is physiologically normal for them. It is worth noting that some studies have also used the term “normoxia” or “physiological normoxia” to describe the physiological oxygen level observed in tissues *in vivo* [3, 4].

### 2.2. Hypoxia

The term “hypoxia” is commonly used to describe an oxygen level significantly lower than the physiological norm. This is particularly the case in pathological conditions such as within cancerous tumors or following ischemia. However, in some cases, hypoxia has also been used to refer to physiological oxygen levels, i.e. “physioxia”, below “normoxia” [5].

### 2.3. Physioxia

Our team first introduced the new term, “physioxia”, in 2011 [1] to describe the partial pressure of oxygen in each organ under normal physiological conditions. As oxygenation varies between organs in the body, physioxia refers to different levels of oxygen. Since then, the term

has been widely used in many studies to describe the physiological oxygen levels. The emergence of the term “physioxia” allows us to avoid the confusing use of “normoxia” (meaning for the authors a normal condition for the body) or “hypoxia” (meaning for the authors a lower oxygen level than in the air) to describe the physiological conditions.

### 2.4. Other oxygen-related terms

The term “anoxia” is used unambiguously to refer to the absence of oxygen. The term “hyperoxia” refers to an oxygen level higher than normoxia, without specifying how much. However, it has already been used to describe an oxygen level higher than physioxia, i.e. “normoxia” [6,7].

To summarize this part of the terminology, the introduction of the term “physioxia” therefore removes the ambiguity in the use of the terms “normoxia”, “hypoxia” and “hyperoxia”, which can lead to confusion. Based on these definitions, we will use, and we encourage scientists to use, “physioxia” to refer to physiological oxygen levels, “normoxia” to describe atmospheric levels (whether in the air or in a cell culture incubator), and “hypoxia” to refer to oxygen deficiency when compared to physiological conditions, “physioxia”.

## 3. Skin cell oxygenation

### 3.1. Physiological oxygen levels in skin layers and appendages

Oxygen makes up 21 % or 160-mm Hg of the gases in the atmosphere. When we breathe, oxygen enters our lungs where it is warmed and humidified by the pulmonary tract. This process reduces the partial pressure of oxygen to about 100 mm Hg (13.1 % O<sub>2</sub>). Oxygen then enters the blood capillaries, binds to haemoglobin which allows it to be transported in the liquid phase, and is distributed to all the body organs *via* the microvasculature. Within an organ, oxygen diffuses from the capillaries into the tissues and cells *via* a diffusion gradient.

The skin is characterized by a dense microcirculation, that is irregularly distributed throughout its various layers. The hypodermis, the deepest layer, contains arteries and veins that form the subdermal plexus at the base of the dermis. From there, the vessels branch out to form the dermal plexus at the papillary dermis below the epidermis. The latter,

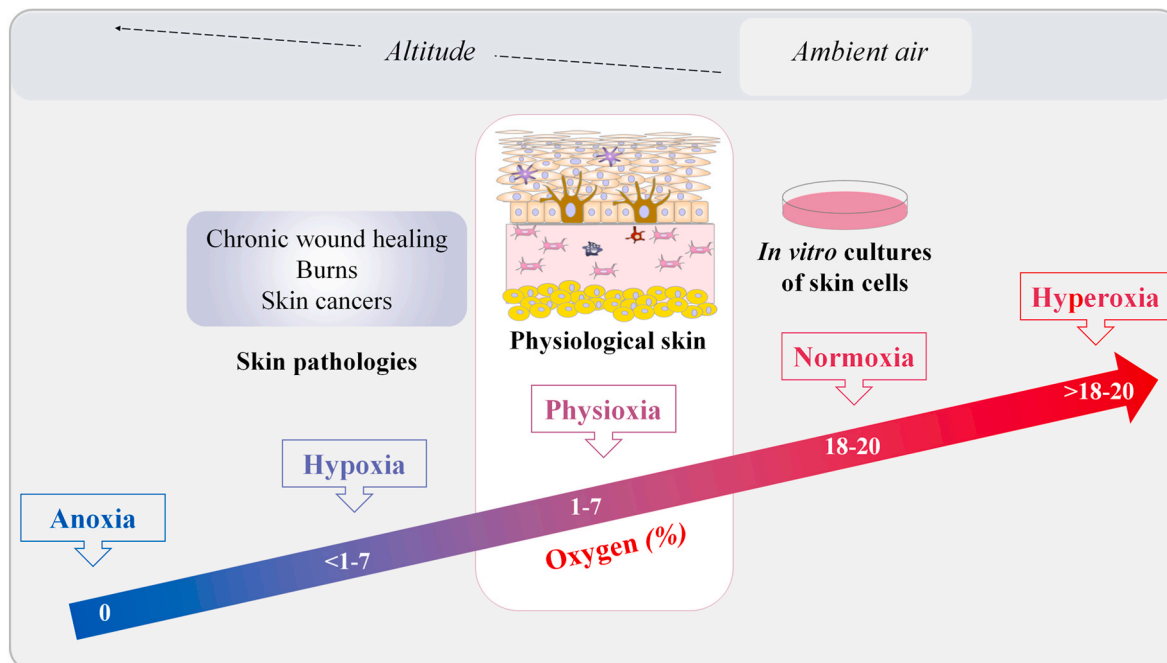


Fig. 1. Terminology for different levels of oxygen.

which is the superficial layer of the skin, has no blood vessels [8]. As a result of this heterogeneous vascularization, skin layers are characterized by the formation of cutaneous oxygen gradients which have been clearly reviewed by Grillon et al. [9,10]. This gradient overlaps with another one caused by the atmospheric oxygen diffusing through the epidermis (Fig. 2). The oxygen diffuses into the skin tissue to a depth of 266–375  $\mu\text{m}$  and contributes significantly to the oxygen supply of the skin [11]. In the basal epidermis, hair follicles and sebaceous glands, the oxygen tension is low, ranging from 19 to 0.76 mmHg (2.5 % and 0.1 %  $\text{O}_2$ , respectively) [12,13].

The amount of oxygen in the skin can vary depending on various factors, such as age. During embryogenesis, stem cells are in a hypoxic microenvironment, with an oxygen level around 15 mm Hg (2 %  $\text{O}_2$ ), which promotes an increase in proliferation potential, and helps the development of multiple organs [14]. Once the placenta has formed, it forms a network with the mother's blood which increases the oxygen level to around 60 mm Hg. This optimal transfer of oxygen and nutrients to the foetus is crucial for its development [15]. As the embryo develops, its skin layers and its vascularization continue to grow in a low-oxygen microenvironment [16]. Foetal oxygenation is between 15 and 45 mm Hg depending on the gestational age [3,17]. After birth, skin development is almost complete, and the superficial layers of the skin are exposed to atmospheric oxygen and complete their maturation. Skin microcirculation is also affected as we age, ultimately leading to skin oxygenation changes. More specifically, the density of the microcirculation decreases as the skin ages, affecting the control of blood flow and reducing oxygen levels [18,19].

### 3.2. About skin cells in *in vitro* culture

As the oxygen level in the skin under physiological conditions is significantly lower than that in the atmosphere, *in vitro* cell culture models grown in a classical incubator under standard oxygen conditions do not reflect the microenvironment found in the living tissue. The oxygen concentration is 18.6 %, i.e. 4 to 10 times higher than in physiological skin conditions, resulting in a state of hyperoxygenation for the

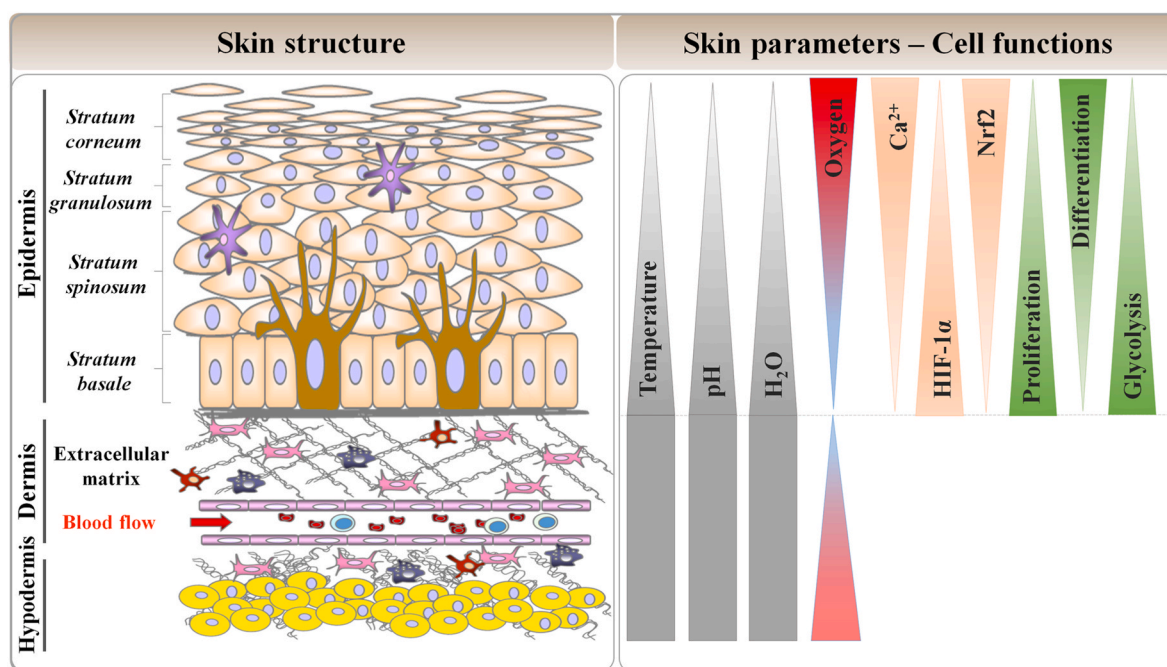
cells. In addition, numerous studies attempting to mimic physioxia, particularly when culturing keratinocytes, fibroblasts, melanocytes, or adipocytes, have used different oxygen levels as physioxia, which can lead to inconsistent findings (see part 5.1 below). Furthermore, skin physioxia depends on oxygen gradients, which are difficult to reproduce accurately in laboratory tests. Selecting the appropriate oxygen levels for each cell type is a particularly challenging task.

The development of 3D culture models with reconstructed epidermis or full-thickness skin has brought significant advances in reproducing the skin microenvironment, providing models that are much closer to physiology. However, progress still needs to be made in reproducing oxygen gradients. Most of these models are prepared in conventional incubators with 18.6 %  $\text{O}_2$ , where oxygen only reaches the tissue by diffusion from the air and culture medium. Recent advances, including the introduction of the cutaneous endothelial component, combined with microfluidic systems for perfusion [20–22], and associated with controlled oxygen incubators should allow us to approach physiological oxygen levels in the coming years.

## 4. Physioxia and cutaneous cell metabolism

### 4.1. Effect of physioxia on oxygen consumption rate (OCR) by skin cells

When oxygen reaches the tissues, its partial pressure drops to about 40 mm Hg (5.3 %  $\text{O}_2$ ). And when it enters the cell, it passively diffuses across the lipid membranes into the mitochondria, where respiration occurs. The mitochondria use about 95 % of the cellular oxygen through oxidative phosphorylation. They remain operational across a range of oxygen levels until it falls below a critical threshold, typically around 0.1 % [23]. The respiratory chain complex, which includes cytochrome C oxidase, transfers electrons and reduces oxygen to water. Cytochrome C oxidase exhibits a high affinity for oxygen, resulting in its continuous activity. This affinity is regulated by the presence of nitric oxide, which competes with oxygen for binding to this enzyme [24]. In this chain, oxygen is the final acceptor [25,26]. The oxygen consumed by the cells is determined using OCR parameters, which are calculated by dividing



**Fig. 2. Maintaining epidermal homeostasis: A complex interplay of physical, chemical and functional gradients.** The complex structure of the skin is associated with several physical and chemical gradients within the epidermis, including temperature, pH,  $\text{H}_2\text{O}$ , and oxygen, which define a specific microenvironment. As a consequence, oxygen-related factors such as Nrf2 and HIF-1 $\alpha$  that maintain skin balance are differentially expressed in the epidermis, and cellular activities, such as metabolism, proliferation and differentiation of keratinocytes occur along these gradients.

the global oxygen consumption rate (in  $\text{amol.s}^{-1}$ ) by the number of cells. Evaluating the cutaneous OCR is crucial because it can provide information on a deregulation at the metabolic level, especially during disease or aging [27]. *In vivo*, in human skin, the OCR of the basal layer of the epidermis is around 10  $\text{amol/cell.s}$ , while the cells in the upper layers have lower OCR levels [28,29]. This suggests that skin keratinocytes have much lower metabolic activity when compared to organs such as the liver, where the OCR is between 200 and 400  $\text{nmol/cell.s}$  [30]. In addition, the OCR tends to increase with age, particularly in older fibroblasts, due to the deregulation of mitochondrial enzymes [31]. Cellular OCR is influenced by the culture model. In fact, the OCR of adipose stem cells in a 3D spheroid configuration is higher than the OCR of the same cells in monolayer culture [32].

The control of how much oxygen skin cells take up is thought to be influenced by oxygen availability. Therefore, we would expect to see a change in OCR when moving from normoxia to physioxia. In normoxia, oxygen uptake by skin cells is mainly limited by oxygen diffusion from the microenvironment. In addition, the cytochrome C oxidase in the mitochondria has a strong affinity for oxygen and it is suggested that this affinity keeps the cells metabolically active [23]. Indeed, recent research has shown that keratinocytes grown in normoxia consume more oxygen and produce more ATP than those grown in a low-oxygen environment [33]. Similarly, a study of dermal fibroblasts found that the rate of oxygen consumption decreased at 3 % oxygen. At the same time, extracellular acidification increased, indicating a shift in cellular metabolism towards a higher rate of glycolysis [34].

#### 4.2. Effect of physioxia on skin cell metabolism

The two main processes within cells are oxidative phosphorylation and glycolysis, which are responsible for producing ATP, the primary energy source for cell activities. Cells can adapt their metabolism and switch between these mechanisms, depending on a number of factors, one of which is the availability of oxygen. Changes in oxygen levels can affect the various systems involved in these processes, such as the mass and density of mitochondria as well as the expression and activity of key enzymes involved in these metabolic pathways.

The skin epidermis exhibits a higher metabolic activity than the dermis due to the continuous cell renewal and robust defence mechanisms. Keratinocytes, the predominant cells in the epidermis, rely primarily on the glycolysis pathway for energy production, utilising approximately 70 % of the glucose content of this layer. Even when oxidative phosphorylation is functional, it contributes only 2 % to glucose utilisation [35–37].

The basal layer of the epidermis, enriched with glucose from the dermis, promotes glycolysis, aided by the overexpression of the primary glucose transporter, GLUT1 [38,39]. On the other hand, suprabasal keratinocytes, which have limited access to glucose, lean towards lipid metabolism *via* the pentose phosphate pathway, particularly due to the significant activity of glucose 6-phosphate dehydrogenase in the granular layer. This enzyme serves as the primary initiator of this pathway [40]. Several studies suggest that glycolysis helps to maintain the proliferative potential of cells [32,41–43], as depriving keratinocytes of glucose promotes their differentiation [44]. However, mitochondrial metabolism plays a crucial role in the differentiation of epidermal keratinocytes [45]. Like adipocyte stem cells, their metabolism may be altered to become more aerobic during differentiation. This is shown by an increase in OCR and a decrease in extracellular acidification rate [46].

It is clear that the oxygen gradient across the skin plays an important role in modulating these metabolic pathways and controlling the homeostasis between keratinocyte renewal and differentiation. Recently, much attention has been paid to the maintenance of physioxia and its relationship with the metabolism of skin cells in *in vitro* cultures (Table 1).

A recent work analysed the metabolites produced by keratinocytes

**Table 1**  
Effect of physioxia on skin cell metabolism.

Cell types	Effect of physioxia <i>versus</i> normoxia	Culture conditions	References
<i>Keratinocytes</i>			
Human oral keratinocytes	↑ glycolysis-related metabolites ↑ Phosphate pentose pathway-related metabolites	24 h and 72 h, 2 % O <sub>2</sub>	[47]
Human skin keratinocytes	↑ PDK1 (mRNA)	3 % O <sub>2</sub>	[5]
Keratinocytes from external auditory canal	↓ ATP production = lactate production ↓ mitochondrial mass ↓ mitochondria density	24 h, 5 % O <sub>2</sub>	[48]
<i>Fibroblasts</i>			
Human oral fibroblasts	↑ αketoglutaric acid (less effects on metabolites than on keratinocytes)	24 h and 72 h, 2 % O <sub>2</sub>	[47]
Human skin fibroblasts	↑ GLUT1, PDK1, VEGF-A (mRNA) ↑ Glucose uptake ↑ Lactate production ↑ GLUT1, lactate deshydrogenase A (mRNA, protein) ↑ glycolytic enzymes (mRNA, protein, activity) ↑ mitochondrial mass ↑ mitochondrial potential ↓ mitochondrial complex-1 activity	3 % O <sub>2</sub>  12 h (mRNA), 24 h (protein), 3% O <sub>2</sub>  24 h, 3% O <sub>2</sub>	[5] [34] [34]

and primary fibroblasts under different oxygen levels and culture times. The keratinocytes displayed a marked change in metabolites when exposed to physioxia, compared to hypoxia and normoxia. These cells overexpressed metabolites involved in the phosphate pentose pathway and glycolysis. On the other hand, fibroblasts were less sensitive to changes in oxygen. However, they increased the production of α-ketoglutaric acid, an activator of prolyl hydroxylase that induces hypoxia-inducible factor 1 (HIF-1α) degradation. The increased production of this enzyme also acts as an antioxidant [47]. In addition, another study found that the cells grown in low oxygen conditions (3 %) showed overexpression of key genes related to glycolysis, including GLUT1 and vascular endothelial growth factor A (VEGFA) for fibroblasts, and the pyruvate dehydrogenase kinase 1 (PDK1), for both fibroblasts and keratinocytes. All these genes are regulated by HIF-1α. This suggests that these cells begin to adapt metabolically to low oxygen levels [5]. Besides, Wang et al. reported an increase in glucose uptake and lactate production when fibroblasts were cultured at 3 % O<sub>2</sub>. They also observed an increase in the mRNA and protein levels of the enzymes GLUT1 and lactate dehydrogenase under physioxia conditions, as well as an upregulation of the expression and activity of hexokinase 2, 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3, phosphoglycerate kinase 1, enolase 1, and pyruvate kinase M2, other key enzymes in glycolysis [34].

In a study of external auditory canal keratinocytes, Shih et al. reported a reduction in cell metabolism, with a significant decrease in both OCR levels and ATP production after 24 h under 5%O<sub>2</sub> compared to 21% O<sub>2</sub>. However, lactate levels did not increase, under these conditions, which could be attributed to the short incubation time with the cells being in the transition phase between normoxia and physioxia [33].

The dynamics of the mitochondrial network are closely linked to oxygen level. The shape and mass of mitochondria are related to the state of the cell, for example, during apoptosis, there is a cellular reduction in mitochondrial membrane potential [49]. When fibroblasts are cultured under physioxia conditions, there is an increase in both



mitochondrial mass (after 48 h exposure) and membrane potential (after 24 h exposure) compared to normoxia. It should be noted that under these physiological conditions, the mitochondrial complex I activity was decreased [34]. In contrast, the study of keratinocytes by Shih et al. reported a reduction in mitochondrial mass, as well as mitochondrial density, at 5 % O<sub>2</sub> after 24 h of exposure [33].

These results reveal that oxygen levels do indeed modulate cellular metabolism. Cells undergo metabolic adaptations in response to reduced oxygen levels, favouring the glycolysis pathway, the decrease in OCR and in ATP levels, and also changes in mitochondria. Although there are some differences between studies, they all show an effect of oxygen levels on skin cell metabolism. Further research into the role of oxygen on skin cell metabolism would provide a better understanding of the influence of each cell type and the time of exposure to oxygen in order to explain the differences observed.

## 5. Physioxia and redox signalling in skin cells

### 5.1. Effect of physioxia on ROS production and on antioxidant efficacy

In cellular metabolism, oxygen consumption is accompanied by the generation of secondary reduced metabolites, ROS. Approximately 1–2% of the oxygen used by the mitochondria is converted to superoxide radical (O<sub>2</sub><sup>•-</sup>) [50], a precursor of other ROS such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hydroxyl radical (OH<sup>-</sup>) and singlet oxygen (<sup>1</sup>O<sub>2</sub>). Several organelles and oxygen-depend enzymes in mammalian cells are capable of producing ROS, such as mitochondria, NADPH oxidase (NOX), cyclooxygenase, lipoxygenase, xanthine oxidase/oxidoreductase, heme oxygenase 1 (HO-1) and nitric oxide synthases (NOS). In addition, exogenous stressors such as pollutants, smoke, and ultraviolet radiations (UV) can also generate ROS in high levels and are major contributors to skin photo-aging. In fact, oxidative stress accounts for approximately 80 % of the process of skin aging.

ROS are naturally present in cells at reasonable levels and play a crucial role in cell signalling [51]. The balance of ROS concentrations is maintained by antioxidant enzymes, including superoxide dismutase (SODs), glutathione peroxidase (GPX), and catalase (CAT). These enzymes work together to prevent oxidative reactions to cellular components, thus protecting cells from oxidative stress damage.

The amount of ROS in cells is influenced by several factors, including the level of oxygen in the environment. For a long time, it was thought that ROS production increased automatically with the cellular oxygen levels. However, this principle has been challenged and studies have shown that ROS production only increases with oxygen levels if the cells adapt by increasing their metabolism and mitochondrial activity [52]. In mammalian cells, ROS increase in normoxia compared to physioxia, leading therefore, to an upregulation of antioxidant expression, as the cells adapt to a higher degree of oxidative stress. For example, muscle

cells have higher levels of ROS than those grown in physioxia, leading to a permanent state of oxidative stress. To adapt and cope with this oxidative stress, muscle cells tend to overexpress antioxidant enzymes such as CAT and SOD [53]. This phenomenon has also been observed in epidermoid carcinoma A431 cells [54], macrophages [55], and endothelial cells [56].

In skin cells, the effect of oxygen levels on basal ROS production is not yet fully understood. Indeed, several studies have reported very different findings, and Table 2 provides a non-exhaustive summary of these results. On the one hand, an increase in the basal ROS levels has been observed in cultures of dermal fibroblasts and keratinocytes submitted to normoxia, leading to the induction of oxidative stress [57–59]. On the other hand, the opposite effects have been reported on the same cell types but also on adipocytes, associated with the stimulation of cell proliferation and migration [6,34,60,61]. Moreover, other studies did not observe any significant changes in keratinocytes and melanocytes [60,62].

Interestingly, after induction of oxidative stress with hydrogen peroxide treatment, primary keratinocytes and the HaCaT cell line showed an increased level of ROS in normoxia compared to physioxia [62]. This effect was confirmed by the higher activity of the antioxidant enzymes, SODs and CAT in physioxia, thus providing better antioxidant defences [62]. It has to be noted that, in epithelial carcinoma A431, the reverse has been observed with the decrease in CAT amount and activity in physioxia versus normoxia [63].

The impact of oxygen levels on ROS production can also be assessed by investigating the activity and expression of cellular enzymes responsible for ROS production. Several studies have been carried out in different cell types, and according to the results summarized by Stuart et al., it is suggested that mitochondrial ROS production is not strongly correlated with oxygen levels. Instead, the sensitivity to oxygen of certain enzymes, such as NOX4, NOS, and ROS transporters such as aquaporins can modulate ROS production through their activity and expression [64].

In the context of skin, the variation observed in cellular ROS levels may be related to several factors, including the use of different cell types or origins, variations in oxygen conditions and differences in experimental procedures, such as the selection of ROS probes or the culture duration. In addition, most studies are based solely on the use of dye oxidation, which is known to be biased by other intracellular oxidation reactions not directly related to ROS, and they should benefit from complementary or more specific methodologies as stated by Forman et al. [65]. Therefore, a thorough understanding of these variations requires careful attention to experimental parameters and methodological choices. In addition, deciphering variations such as enzyme expression and transporter activity could provide valuable insights to better understand the consequences of oxygen levels on oxidative stress and antioxidant defences in skin cells.

**Table 2**

Effects of low oxygen levels on ROS production by skin cells.

Cell Type	% oxygen in the culture	Incubation time	ROS detection probe	ROS production in physioxia versus normoxia	References
<b>Keratinocytes</b>	1,50 %	1 day	CM-H <sub>2</sub> DCFDA	++	[60]
	2 %	3 days	ROS-ID	-	[58]
	3 %	4 days	CM-H <sub>2</sub> DCFDA	=	[62]
<b>Fibroblasts</b>	1–10 %	3 days	CM-H <sub>2</sub> DCFDA	-	[66]
	5 %	3 days	OxiSelect	-	[59]
	5 %	3 days	MitoSOX	++	[61]
	3 %	3 days	H <sub>2</sub> DCFDA	++	[34]
	2 %	>10 days	MitoSOX H <sub>2</sub> DCFDA	-	[57]
<b>Melanocytes</b>	1,50 %	1 day	CM-H <sub>2</sub> DCFDA	=	[60]
<b>Adipocytes</b>	2 %	>10 days	H <sub>2</sub> DCFDA	++	[6]

++: higher ROS production in physioxia than in normoxia; -: lower ROS production in physioxia than in normoxia; = : non-significant effect between physioxia and normoxia.

5.2. Physioxia and Nrf2 pathway

The changes in the antioxidant system observed in physioxia may also be related to the Nrf2-mediated signalling pathway. In response to oxidative stress, cells activate the Keap1-Nrf2 signalling pathway, which is an essential mechanism for detoxification through antioxidant action. The transcription factor Nrf2 plays a central role in regulating the expression of many antioxidant genes. Its role in skin healing was highlighted in 2002 [67]. Under basal conditions, Nrf2 is held in the cytoplasm by its inhibitor, Keap1, which is sensitive to redox changes due to its cysteine-rich nature (Fig. 3B). This complex, anchored to actin filaments also facilitates Nrf2 degradation via the E3 ubiquitin ligase. During oxidative stress, specific cysteines in Keap1 are oxidized by ROS, resulting in the dissociation of the complex. As a consequence, Nrf2 accumulates in the cytoplasm and translocates to the nucleus, forming a heterodimer with the MAF small protein. In the nucleus, this complex binds to antioxidant response element, ARE, sequences in the promoter regions of target genes, regulating the expression of nearly 500 genes. These genes include antioxidant enzymes (HO-1, SOD, CAT, GPX), detoxification enzymes, stress response proteins, and metabolic enzymes [68].

In the skin, the expression and activity of Nrf2 are detected more in the superficial layers of the epidermis. Then, it decreases with depth and is less active in the keratinocytes of the basal layer, forming a gradient of expression of its target genes [69,70]. So, its expression is associated with differentiated keratinocytes which are located in the most oxygenated layers of the epidermis (Fig. 2). Maintaining the basal levels of Nrf2 is essential for the barrier function of the epidermis. Indeed, transgenic mice deficient or overexpressing Nrf2 in the epidermis show an alteration of the lipid barrier, inflammation in the epidermis and hyperproliferation of keratinocytes [71,72].

The interactions between oxygen and the expression or activity of Nrf2 have been little explored, and not yet in normal skin. A link

between Nrf2 and oxygen levels has been reported in primary endothelial cells [56]. This work found that physioxia did not induce a difference in the nuclear accumulation of Nrf2, and its binding to DNA remained unchanged compared to normoxia. However, the expression of its target genes decreased, especially that of HO-1, and is reversed upon reoxygenation. An explanation has been brought involving the transcription factor, BaCH1, which is overexpressed in physioxia and is also present in the nucleus. It competes with Nrf2 for binding to specific promoters of the same antioxidant genes, but, unlike Nrf2, BaCH1 inhibits their transcription [56]. In the case of the squamous cell carcinoma A431, exposure to physioxia (2 % O<sub>2</sub>) induced a reduction in Nrf2 mRNA expression compared to normoxia, which was associated with a decrease in the expression of the antioxidant genes, CAT, glutathione peroxidase 1, superoxide dismutase 1, and heme oxygenase [54].

Extensive research has been conducted to investigate the role of Nrf2 in regulating oxidative processes in skin cells. However, re-evaluating its function in skin cells under physioxia may be necessary. Additionally, studying its interactions with other protein partners while excluding ambient air as a confounding variable is essential. Doing so will provide a clearer understanding of the role of Nrf2 in skin cells.

5.3. Physioxia and HIF-1 signalling pathway

HIF-1 is well known as the master regulator of the cellular adaptive response to hypoxia, as it regulates many genes that promote survival, metabolism, angiogenesis, etc [73]. HIF-1 is a heterodimer composed of two subunits: HIF-1 $\alpha$  and HIF-1 $\beta$ . HIF-1 $\alpha$  levels are regulated by oxygen (Fig. 3A). In normoxia, it is expressed and continuously degraded. This degradation involves hydroxylation by prolyl hydroxylase domain-2, an oxygen-dependent enzyme. Hydroxylated HIF-1 $\alpha$  binds to the von-Hippel Lindau protein, pVHL, leading to its ubiquitination and degradation. In hypoxia, HIF-1 $\alpha$  degradation is inhibited, allowing its accumulation and translocation into the nucleus. There, it forms a

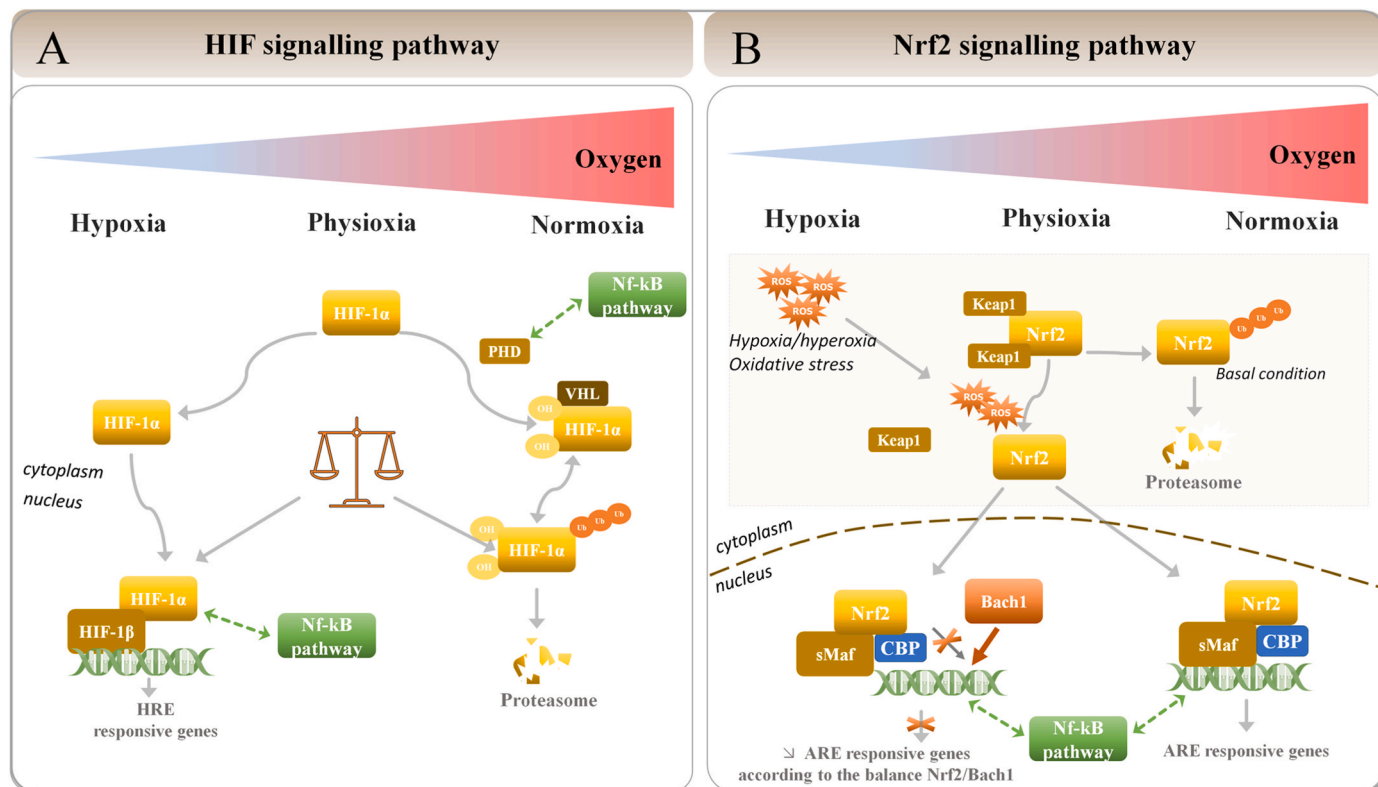


Fig. 3. Physioxia and main signalling pathways. (A) HIF signalling pathway according to oxygen levels and interactions with NF-kB pathway. (B) Nrf2 signalling pathway according to oxygen levels and interactions with NF-kB pathway.

complex with HIF-1 $\beta$  and binds to the HRE sequence in the promoters of its target genes [74,75].

Other isoforms of HIF-1 $\alpha$  exist and play a role in hypoxia cascade. HIF-2 $\alpha$  shares about 40 % similarity with HIF-1 $\alpha$  and binds to most of the same HRE sequences of target genes. The main distinction lies in their expression patterns: while HIF-1 $\alpha$  is ubiquitously expressed, HIF-2 $\alpha$  is specific to certain tissues such as kidneys, lungs, heart, and intestine [76]. Concerning the regulation of antioxidants, it has been demonstrated that SOD2 is among the genes regulated by HIF- $\alpha$ 2 [77]. Another isoform, HIF-3 $\alpha$ , is also tissue specific. Known for its negative regulation of HIF-1 $\alpha$  and HIF-2 $\alpha$  activity, HIF-3 $\alpha$  also targets numerous genes involved in metabolism, including GLUT1, which is also targeted by HIF-1 $\alpha$  and HIF-2 $\alpha$  [78]. Nevertheless, transcriptomic studies have identified a set of genes that are specifically regulated by HIF-3 $\alpha$  [79]. Until now, the roles of the two latter HIF-1 isoforms in the skin have been poorly investigated.

In normal skin, HIF-1 $\alpha$  is primarily present in the epidermis especially in the basal layer where oxygen levels are low [80,81], and also abundant in hair follicles, sebaceous glands, and sweat glands [81]. Conversely, HIF-2 $\alpha$  is rare and mostly found in the dermis, mainly in capillary endothelial cells [81]. It has also been found in keratinocytes [82]. These distinct patterns suggest different roles in regulating specific functions across different regions of normal human skin. Notably, HIF-1 $\alpha$  plays a crucial role in various skin physiological processes, such as pigmentation, cell proliferation, migration, and extracellular matrix production [80].

The stabilization of HIF-1 $\alpha$  is well-documented during hypoxia: it quickly accumulates (within 2–24 h) in hypoxic cells. Subsequently, it is the HIF-2 $\alpha$  isoform that stabilizes and persists in hypoxia for up to 72 h [1,83]. However, data regarding the stabilization of HIFs under physioxia are still unclear. Indeed, a study on primary adipocytes cultured in physioxia from their isolation from *in vivo* tissue, thus never having been in normoxia, shows the expression and stabilization of HIF-1 $\alpha$ , consequently inducing the transcription of target genes such as GLUT1 [6]. Similarly, a higher number of HIF-1  $\alpha$ -expressing keratinocytes was found in physioxia compared to normoxia [58]. Conversely, other data reveal that HIF-1 $\alpha$  is not activated or accumulated in physioxia [84]. These latter findings have been confirmed by the presence of a balance between its accumulation and degradation in physioxia, as in normoxia [85]. In keratinocytes, a slight accumulation of HIF-1 $\alpha$  has been demonstrated on the first day of cell adaptation to physioxia (5 % O<sub>2</sub>), and subsequently, no trace has been identified, even after four days in culture [86].

In addition to its response to low oxygen, HIF-1 $\alpha$  is also sensitive to changes in ROS levels. ROS generated directly in the cytoplasm by UV radiation of keratinocytes inhibit HIF-1 $\alpha$  expression, whereas those induced later due to mitochondrial alteration stimulate its expression [80]. Indeed, it has been demonstrated that the accumulation of HIF-1 $\alpha$  leads to a decrease in ROS [87], typically mitochondrial ROS [88]. However, there are conflicting data that HIF-1 $\alpha$  accumulation is either not associated with ROS regulation, or instead leads to an increase in ROS [6,89].

The involvement of HIF in the adaptive response to oxidative stress highlights a crucial connection with Nrf2, two oxygen-sensitive transcription factors. A study revealed that HIF-1 $\alpha$  is activated in response to ROS, through a direct interaction between Nrf2 and a conserved antioxidant response element located approximately 30 kilobases upstream of the HIF1A gene, which upregulates its expression [90]. Moreover, in hypoxic conditions, a direct and physical interaction between the two proteins HIF-1 $\alpha$  and Nrf2 has also been observed in the nucleus of pancreatic cells [91].

Although this complex regulation has been demonstrated, it remains largely unexplored in skin tissue, in particular for intermediate oxygen levels, thus offering a vast field of investigation to understand its role both physiologically and pathologically.

#### 5.4. Physioxia and NF- $\kappa$ B signalling pathway

NF- $\kappa$ B, originally identified as the “nuclear factor binding near the  $\kappa$  light-chain gene in B cells” [92], is a key transcription factor that regulates inflammatory and immune responses, as well as cell proliferation and survival. It also activates genes encoding pro-inflammatory cytokines, chemokines, adhesion molecules, and enzymes responsible for ROS production [93]. In the skin, NF- $\kappa$ B is crucial for various cellular processes and contributes to both normal functions and disease development. It is essential for the formation of hair follicles and exocrine glands [94]. In the epidermis, NF- $\kappa$ B contributes to the establishment of the skin protective barrier by regulating essential genes involved in keratinocyte differentiation, such as those encoding involucrin and loricrin [95]. In addition, NF- $\kappa$ B inhibits keratinocyte apoptosis which is essential for maintaining skin integrity and supporting wound healing. NF- $\kappa$ B is activated by stimuli such as UV radiation, pathogens, and stress [94]. The inflammatory response mediated by NF- $\kappa$ B is essential for fighting infections and facilitating wound healing. Furthermore, emerging evidence suggests that NF- $\kappa$ B may influence skin ageing through persistent low-grade inflammation that degrades collagen and elastin, resulting in wrinkles and sagging skin [96]. However, excessive or prolonged NF- $\kappa$ B activation can lead to skin pathologies such as psoriasis and atopic dermatitis but can also promote the survival of damaged or cancerous cells, contributing to skin cancer.

Oxygen levels can influence the activity of NF- $\kappa$ B as it becomes activated in hypoxia, typically below 5 % oxygen [92]. NF- $\kappa$ B is involved in cellular adaptation to low oxygen levels by targeting genes responsible for maintaining oxygen homeostasis [97]. In hyperoxia, NF- $\kappa$ B is also activated in the cellular response to oxidative stress, which may include the activation of protective mechanisms or, if the stress is severe, programmed cell death [98]. There are currently no studies on the role of NF- $\kappa$ B in physioxia, but NF- $\kappa$ B doesn't work in isolation, it has been found to interact with other important signalling pathways in the skin, such as HIF-1 and Nrf2 which are themselves modulated during physioxie (Fig. 3).

The interaction between NF- $\kappa$ B and HIF-1 has been extensively studied due to its importance in hypoxia inflammation and tissue oxygen homeostasis, and this is clearly reviewed by Korbecki et al. [97]. Indeed, both HIF-1 and NF- $\kappa$ B are critical transcription factors that are activated in response to hypoxia, through different mechanisms. On the one hand, HIF-1 activation is primarily regulated by the availability of oxygen on which PHD activity depends and thus the degradation or stabilization of HIF-1 $\alpha$ . On the other hand, NF- $\kappa$ B activation during hypoxia is more complex as it occurs through multiple pathways: inhibition of I $\kappa$ B degradation leading to NF- $\kappa$ B release and activation; activation of kinases such as ERK, p38 MAPK, and Akt/PKB which directly phosphorylate and activate NF- $\kappa$ B; and indirect activation of NF- $\kappa$ B through HIF-1 induced expression of proteins such as PHD2 and PHD3 which enhances NF- $\kappa$ B transcriptional activity. The specific outcome of HIF-1 and NF- $\kappa$ B crosstalk is likely to be highly dependent on the cell type, the duration and severity of oxygen changes, and other stimuli.

NF- $\kappa$ B and Nrf2 are both critical regulators in the body response to oxidative stress and inflammation [99]. The Nrf2 pathway promotes cell protection against oxidative stress and inflammation, whereas the NF- $\kappa$ B pathway promotes pro-inflammatory responses and tissue damage [99, 100]. The interplay between Nrf2 and NF- $\kappa$ B represents a complex “crosstalk” with multiple levels of influence. Firstly, both Nrf2 and NF- $\kappa$ B compete for resources, such as the transcriptional co-activator CREB-binding protein (CBP), suggesting that heightened activity in one pathway may suppress the other [101]. In addition, NF- $\kappa$ B can directly inhibit Nrf2 activity through its p65 subunit which prevents Nrf2 from binding to DNA, thereby preventing activation of Nrf2 target genes [99]. These pathways are interconnected. For example, Nrf2 activation can reduce oxidative stress and in turn moderate NF- $\kappa$ B activation.

The interaction between NF- $\kappa$ B, HIF-1, and Nrf2 pathways plays a



crucial role in cellular responses to oxidative stress, particularly in the effects of H<sub>2</sub>O<sub>2</sub>. H<sub>2</sub>O<sub>2</sub> demonstrates dual effects on HIF-1: it stabilizes HIF-1α by inhibiting PHD enzymes, leading to its activation, yet it can also deactivate HIF-1 through alternative pathways such as PI3K [102]. Moreover, H<sub>2</sub>O<sub>2</sub> activates the NF-κB pathway by promoting IκB phosphorylation and degradation, allowing for the nuclear translocation of NF-κB and activation of its target genes [102]. In addition, It acts also as a potent Nrf2 activator by oxidizing specific cysteine residues on Keap1, preventing Nrf2 degradation and enabling its nuclear translocation to induce antioxidant gene expression [103]. In situations of oxidative stress, such as those caused by H<sub>2</sub>O<sub>2</sub>, these pathways work together to control cellular responses, ensuring the maintenance of homeostasis and protection against oxidative damage.

This section has attempted to highlight the links between physiological oxygen levels and redox signalling. It appears that the relationship between oxygen levels and ROS production by skin cells is still unclear. The interactions between the main redox signalling pathways, NF-κB, HIF-1 and Nrf2, in the skin remain complex and multifaceted (Fig. 3). Furthermore, the lack of studies under conditions of microenvironmental oxygen levels further complicates the precise understanding of these interactions. More research is needed to fully understand the cross-talk between these pathways and their impact on skin homeostasis and disease processes.

## 6. Physioxia and life cycle of skin cells

### 6.1. Effects on skin cell proliferation

It has long been observed that skin cells proliferation is influenced by the availability of oxygen [104]. In the last fifteen years, several works have investigated the effect of physiological oxygen levels on cellular activities compared to normoxia. Table 3 summarises the main data from the literature reporting the effects of physiological oxygen levels (physioxia has been considered to be between 2 and 6 % O<sub>2</sub> in the skin, according to the authors) on the proliferation of different skin cell types. Most of the studies demonstrated a higher rate of cell proliferation in physioxia compared to normoxia, whatever the cell type, mainly for a culture period between 3 and 14 days.

For keratinocytes, the increase in the proliferation rate can be up to 12 times faster than in normoxia as shown for freshly isolated

keratinocytes at 5 % oxygen [105]. The higher cell proliferation in physioxia conditions has been demonstrated for primary keratinocytes of different sources (Table 3) but also for the HaCaT cell line in physioxia at 3 % oxygen [62]. Besides the effect on cell proliferation in monolayer, physioxia was also shown to improve the colony-forming efficiency of keratinocytes [105,106], with a higher proportion of large holoclone-like colonies [58], indicating an enrichment in stem cells and a low proportion of differentiated keratinocytes. The effect of physioxia on the proliferation of basal keratinocytes in 3D models was also investigated. In agreement with the previous data, the proliferation index determined by the percentage of cells expressing the cell proliferation biomarker, Ki67, in the basal layer was shown to be higher in physioxia than in normoxia in reconstructed epidermis prepared from neonatal keratinocytes [62].

Regarding fibroblasts, primary fibroblasts from individuals of different ages decrease their proliferation when oxygen levels are high, and conversely, cells grow faster when oxygen levels are lower than 15 % O<sub>2</sub> [107]. Other studies have shown that dermal papilla cells proliferate faster and display less senescence under 2 % oxygen compared to 21 % O<sub>2</sub> [57].

The same effect of oxygen level on proliferation has been observed in other skin cell types such as melanocytes and adipocyte stem cells (Table 3). Interestingly, choroidal melanocytes exposed to low physiological oxygen showed larger and rounder nuclei than those of melanocytes grown in normoxia, consistent with an increase in DNA synthesis [108].

Several studies have attempted to understand the mechanisms involved. The higher proliferative potential observed in primary oral keratinocytes in physioxia (6 days, 2 % O<sub>2</sub>) was shown to be associated with low levels in p21 expression, a cell cycle inhibitor, and in β-galactosidase activity, a marker of senescence, but also with a high level in HIF-1α [58], which has also been reported in adipocytes [6]. Furthermore, a significant upregulation of the expression of HIF-1α target genes involved in metabolic reprogramming, VEGFA, GLUT1, PDK1, was observed in both keratinocytes and fibroblasts under 3 % O<sub>2</sub> [5], confirming the activation of the HIF-1α pathway in physioxia.

However, few works contradict the idea that skin cells proliferate better under physioxia. In fact, it has been reported that keratinocytes show a significant decrease in cell proliferation rate during physioxia, lasting 3 or 4 days in monolayer [5]. In the same study, the authors

**Table 3**  
Effects of low oxygen levels on skin cell proliferation.

Cell type	% oxygen in the culture	Incubation time	Cell proliferation in physioxia versus normoxia	Cell origin	References
<b>Keratinocytes</b>	4 %	2 days	=	Adults/Old	[109]
	3 %	3 days	-	Adults	[5]
	5 %	4 days	++	Adults	[48]
	3 %	4 days	++	Neonatal	[62]
	2 %	6 days	++	Adults (oral keratinocytes)	[58]
	5 %	8 days	++	Adults	[105]
	5 %	14 days	++	Neonatal	[110]
<b>Fibroblasts</b>	5 %	3 days	-	Adults	[61]
	3 %	3 days	++	Adults	[34]
	6 %	5 days	++	Adults	[111]
	4 %	7days	++	Neonatal/Adults	[107]
	2 %	10 days	++	Adults	[112]
	2 %	>10 days	++	Adults (dermal papillary cells)	[57]
<b>Melanocytes</b>	5 %	3 days	++	Neonatal	[59]
	1-5%	6 days	++	Neonatal	[113]
	3 %	>10 days	++	Adults	[108]
	5 %	(np)	++	Adults (hair follicle)	[114]
<b>Adipocytes</b>	2 %	3 days	++	Adults	[6]
<b>Keratinocytes in 3D models</b>	3 %	14–15 days	-	Adults	[5]
	3 %	15 days	++	Neonatal	[62]

++: higher proliferation in physioxia than in normoxia; -: lower proliferation in physioxia than in normoxia; = : non-significant effect between physioxia and normoxia; np: not provided in the publication.

described a full-thickness skin model derived from adult keratinocytes with a lower proliferative index in physioxia than in normoxia. Young fibroblasts were also shown to decrease their proliferation index at 5 % O<sub>2</sub> whereas old fibroblasts were not affected by oxygen levels over 3 days [61]. Finally, in short-term culture (2 days) neither old nor young keratinocyte proliferation rates were affected by physioxia versus normoxia [109].

To fully understand these findings, it is important to consider how long the cells were exposed to physioxia and to monitor their growth for several days, not just during the initial adaptation period. Furthermore, additional factors, such as the origin of the cells and their specific culture conditions, may also influence the results.

### 6.2. Effects on skin cell differentiation

In the epidermis, skin renewal depends on the differentiation process of basal keratinocytes. However, in the basal layer of the epidermis, a group of proliferating keratinocytes is maintained in a low oxygen microenvironment, which helps them remain undifferentiated [14]. Low-oxygen levels (5 % and 2 %) have been shown to significantly reduce the epidermal differentiation and stratification in colonies formed by human epidermal cells, and to reduce the expression of differentiation markers such as K1, K10, and involucrin in post-confluent cells [106]. In addition, the transferrin receptor, a marker of actively cycling cells, VEGFA and cyclooxygenase-2 were overexpressed in the cells at lower oxygen levels. A recent research by Koh et al. confirmed these findings, demonstrating greater differentiation in normoxia with a higher expression of differentiation/stress markers such as K10, involucrin, filaggrin, HMOX1, the malic enzyme 1, the thioredoxin reductase 1, and the NAD(P)H quinone dehydrogenase 1. Cultures in normoxia also inhibits the expression of K7 and K15, which are potential biomarkers of stem cells in the epidermis [105]. Furthermore, keratinocyte growth in low oxygen levels perturbed the binding of the activator protein 1 (AP1) transcription factor to the AP1 response element, thereby altering the transcription of keratinocyte differentiation genes and prooxidant enzyme genes such as cyclooxygenase 2 which are involved in early differentiation [106,115].

The effect of oxygen levels on keratinocyte differentiation is easily seen in 3D models. A decrease in RHE thickness was observed as the oxygen level decreases from 21 to 2 % [105] and confirmed by comparing growth at 3 % and 21 % oxygen in a skin equivalent model [62] and in a full thickness skin model [5]. Interestingly, in RHE prepared with keratinocytes only, the differentiation occurs but, below 10 % oxygen, no stratum corneum was observed [105]. In contrast, in skin equivalents obtained with a fibroblast sub-layer under the RHE [62], and in full-thickness skin model [5], a stratum corneum is formed, suggesting a role for fibroblasts in the final differentiation of keratinocytes in physioxia. This was confirmed in the last two models by the presence of late differentiation biomarkers, loricrin, filaggrin or involucrin, localized in the upper layers of the epidermis but in lower levels in physioxia. In addition, differences in lipid composition [5] and in lipid-related enzymes [105] also suggest changes in the terminal differentiation of keratinocytes in physioxia.

### 6.3. Effects on skin cell senescence

During their life cycle, skin cells undergo a natural or induced process called cellular senescence, in which they stop dividing and undergo significant changes in their metabolic activity, their structure and their communication with neighbouring cells through the secretion of senescence-associated secretory phenotype factors (SASP). The senescence process is closely associated with skin ageing. Very low levels of oxygen, hypoxia, have been shown to delay the entry of normal cells into senescence [116]. However, the effect of oxygen on this senescence process in the skin has been poorly studied and therefore remains largely unknown.

For keratinocytes, physioxia cultures with 2 % O<sub>2</sub> induce a reduction in senescence-associated beta-galactosidase (SA-β-Gal) activity, a typical activity of senescent cells, and a decrease in the expression of p21<sup>WAF1/CIP1</sup> and p16<sup>INK4A</sup>, two main markers of senescence [58].

A similar effect was observed in human dermal fibroblasts. Their growth in normoxia has been shown to induce premature senescence compared to physioxia (5 % O<sub>2</sub>) after successive passages. Senescence markers such as SA-β-gal activity and p16 expression were increased in both young and old cells cultured in normoxia compared to physioxia. In addition, miRNA-181a, a senescence marker, was significantly increased in older cells exposed to 21 % oxygen compared to those exposed to 5 % oxygen [61]. Furthermore, dermal papilla cell senescence induced by an exposure to 21 % O<sub>2</sub> can be rescued by switching back to 2 % O<sub>2</sub> exposure, demonstrating the reversibility of the senescence induction by oxygen modulation [57].

Skin adipose stem cells also show an effect of low oxygen levels. Chen and colleagues have demonstrated that skin adipose stem cells, when cultured under physioxia conditions with 2 % oxygen since their isolation, have fewer senescent cells with reduced SA-β-Gal activity and an increased viability, compared to the same cells cultured under normoxia conditions. Furthermore, this work demonstrates that the survival rate of these cells is better in ischemia models both *in vitro* and *in vivo* after transplantation [6], suggesting that culture in normoxia causes the cells to lose their potential. These results confirm previous findings that stem cells of different origins can maintain their proliferative potential and delay senescence under physiological conditions [117,118].

To date, no direct studies have reported an effect of oxygen on melanocyte senescence. However, this process is closely linked to glycolysis [119] and glycolysis is well known to be associated with the expression of the HIF-1α factor, which is itself dependent on oxygen levels and is expressed in skin cells under physioxia. So, this area remains to be explored as well as an effect mediated *via* neighbouring senescent cells [120].

In fact, besides a direct effect of oxygen on skin cells in the process of senescence, an indirect impact may occur between a senescent cell and its neighbouring cells *via* SASP factors. The composition and function of SASP are very heterogeneous and depend mainly on factors such as epigenetic modifications and microenvironmental conditions [121]. Research has shown that oxygen levels can affect SASP, as demonstrated in studies of human and mouse fibroblasts that failed to develop robust SASP when grown under normoxia [122]. Furthermore, a comparative analysis between hypoxia (1 % O<sub>2</sub>) and physioxia (5 % O<sub>2</sub>) by van Vliet on human skin fibroblast cultures reports that the pro-inflammatory factor composition of SASP is reduced in hypoxia due to the activation of the MAPK pathway, which inhibits the mTOR factor [4], a key regulator of senescent cell SASP [123].

No studies have compared the effects of physioxia and normoxia on SASP heterogeneity in skin cells. However, this effect is evident due to oxygen-sensitive signalling factors that play a role in skin cell senescence. The HIF-1α pathway plays a crucial role in regulating the proliferation capacity and cell cycle of skin cell. When HIF-1α is suppressed in keratinocytes, the reconstructed epidermis shows a decrease in cell proliferation potential. This leads to a reduction in the presence of laminin 322 and integrin α6, followed by an increase in cellular apoptosis [124]. The research conducted by Welford et al. directly demonstrated that HIF-1α plays a crucial role in cell senescence. They found that HIF knockout fibroblasts significantly accelerated the onset of cell senescence in normoxia and reduced proliferation in hypoxia. This was due to the macrophage migration inhibitory factor (MIF), a key HIF-1α effector that delays senescence [125]. HIF-1α can directly cause cellular senescence or indirectly affect other cellular processes, such as regulating the activity of the mTOR NF-κB axis and controlling the transcription of various pro-inflammatory factors involved in the SASP [126]. Furthermore, HIF-1α is crucial in regulating the p53 pathway, the key mediator of cellular senescence. This regulation occurs through a direct interaction that leads to the degradation of HIF-1α [127]. In

addition, the effectors of HIF, mainly MIF, are also linked to p53 and can inhibit its pro-senescence effect, as shown in a study by Jung et al. [128].

Understanding the effects of oxygen on the skin cell senescence remains an emerging field that needs to be explored in greater depth in order to gain a better understanding of skin ageing in particular.

As oxygen levels are likely to modulate the production of ROS and affect certain signalling pathways, it was expected that cell life would also be disrupted. As shown in this section, with the exception of very few studies, it is clear that proliferation of the different skin cell types is enhanced and senescence is delayed under physioxia conditions. In addition, the process of keratinocyte differentiation is slowed down. Thus, the transition from physiological conditions to *in vitro* normoxic cell culture favours the commitment of cells to senescence and, in the case of keratinocytes, to differentiation, thus allowing the strengthening of the skin barrier, but to the detriment of the population of keratinocyte precursors required for skin regeneration.

## 7. Physioxia and skin cell functions

### 7.1. Effects on skin cell migration

Skin cell migration is essential for wound healing and skin repair. Several studies have shown that the mobility of keratinocytes, fibroblasts and melanocytes is significantly affected by variations in oxygen levels. When cultured in low oxygen (1–2% O<sub>2</sub>), skin keratinocytes tend to display a higher level of mobility, as compared to normoxia [129, 130]. Interestingly, this effect is also age dependent. Primary keratinocytes from younger subjects tend to migrate faster when grown in a 4 % oxygen environment than in normoxia conditions. However, older keratinocytes show an inverse response, migrating faster in normoxia than in physioxia [109]. These results suggest a strong correlation between migration, oxygen levels, and age. Therefore, they provide an interesting approach to studying healing in the elderly. For fibroblasts, faster migration in low oxygen conditions has also been reported for dermal papilla cells at 2 % O<sub>2</sub> [57] and for dermal fibroblasts at 3 % O<sub>2</sub> [34]. For melanocytes, the research of Abreu et al. demonstrated a similar effect using primary cells cultured for short periods in physioxia at 5 % O<sub>2</sub> [59].

This rapid migration of skin cells in a low oxygen environment is influenced by several factors. One of the key factors is the microtubule-associated protein 4 (MAP4), which is a cytoskeletal protein that regulates microtubule dynamics and promotes faster cell migration, as observed in HaCaT cells [131]. Additionally, the process of migration is associated with increased production of adhesion proteins, such as integrin  $\beta$ 1. Integrin  $\beta$ 1 is highly sensitive to oxygen, and its expression is higher in hypoxia, which is believed to contribute to the movement of keratinocytes during wound healing [132]. Blocking the Notch1 signalling pathway not only reverses the effects on migration but also on the expression of  $\beta$ 1 integrin. This pathway is downregulated at low oxygen levels, highlighting its crucial role in migrating keratinocytes under hypoxic conditions [132].

### 7.2. Effects on skin pigmentation

Melanogenesis is a fundamental function of the epidermis to protect skin cells from damage caused by UV exposure. As early as the 1990s, Dieckmann et al. reported the role of oxygen levels on the activity of human primary melanocytes *in vitro*. Between 1 and 5 % O<sub>2</sub>, both melanin content and tyrosinase enzymatic activity were unchanged, whereas at higher oxygen levels, 10–18 % O<sub>2</sub>, they progressively decreased [114,133]. Two recent studies have partially confirmed these results. Tyrosinase activity in human melanocytes was higher at 5 % O<sub>2</sub> but only after a short culture period, 3 days versus 5 days [59]. The increase in melanin content was also observed in choroidal melanocytes at 3 % O<sub>2</sub> but was not associated with an increase of the microphthalmia-associated transcription factor, tyrosinase,

tyrosinase-related protein 1 and the premelanosome protein PMEL gene expression, the main markers of melanogenesis [108]. Therefore, given the paucity of results on the relationship between melanocytes and oxygen, further work is therefore needed to better understand the role of oxygen levels in the mechanisms of melanogenesis.

### 7.3. Effects on skin cell angiogenesis

Skin microvascularization has a critical function directly related to oxygenation as it delivers oxygen to the skin tissue. A direct effect of oxygen on the angiogenic potential of skin endothelial cells has been demonstrated with a 30 % increase in the number of pseudo-microvessels when cells were in hypoxia (1 % O<sub>2</sub>) compared to normoxia [1]. It is well known that in the wound healing process, the hypoxic microenvironment induces the production of pro-angiogenic factors by skin cells, thus helping skin regeneration by stimulating angiogenesis (for review see Ref. [134]). However, very few studies have investigated this activity in physioxia. The production of the proangiogenic factor VEGFA was shown to be higher in physioxia in keratinocytes [5,106] and in fibroblasts [135]. Besides, a paracrine effect on angiogenesis has recently been demonstrated *via* dermal fibroblasts. When cultured at 2 % O<sub>2</sub>, human primary fibroblasts produced proangiogenic factors, such as epidermal growth factor and fibroblast growth factor-2, which activate endothelial cell angiogenesis [136].

The influence of oxygen on skin endothelial cells extends beyond the simple role of vessel formation. In fact, several adhesion molecules expressed on skin endothelial cells, such as CD31, CD34, CD102 or CD106 have been shown to be modulated by oxygen (physioxia 3,5 % O<sub>2</sub> and/or hypoxia 1 % O<sub>2</sub>). This modulation correlates with a decrease in endothelial cell interactions and a significant inhibition of immune cell adhesion [1,9]. These findings suggest potential effects of oxygen in the immune regulation within the skin, as reviewed in Ref. [9] and need to be further investigated.

As oxygen is essential for cellular life, but also influences the fate of skin cells, it was important to focus on its role in the fundamental processes of the skin carried out by these cells. As reported here, the protection of the skin through pigmentation, but also the healing process, including cell migration and angiogenesis, are all more effective in physioxia than in *in vitro* normoxic cultures. There is no doubt that further work in the future will show that other activities, such as skin immunity as suggested here, are modulated by oxygen levels. This raises the problem of choosing the right *in vitro* culture conditions to study the mechanisms involved in the different activities of the skin or to evaluate the effectiveness of compounds for pharmaceutical or dermocosmetic purposes.

## 8. Increasing physiological oxygen for therapeutic approaches

Knowing that oxygen is essential for cell life, the idea of enhancing physiological oxygen was considered to boost skin cell activity or to restore physiological oxygen levels when necessary. These hyperoxygenation treatments have been carried out by increasing the oxygen level in the local atmosphere, either under normobaric or under hyperbaric conditions to facilitate oxygen penetration into the skin, mainly for *in vivo* applications. Besides, several oxygen-delivery agents have also been developed to deliver oxygen locally by topical application. It has to be noticed that in most of the *in vitro* studies, hyperoxia was compared to normoxia, and very rarely to physioxia.

### 8.1. *In vivo* hyperbaric oxygenation

Hyperbaric oxygen treatments have long been recognized for their beneficial effects on wound healing and burns (see review by Ref. [137]), but the understanding of the mechanisms involved has only recently been reported (see part 8.2 and 8.3). In fact, both chronic wounds, particularly in diabetic patients, and major skin burns lead to a

lack of microvascular regeneration and therefore to a chronic hypoxia. Although the establishment of hypoxia is a positive signal for the initiation of angiogenesis and subsequent tissue regeneration, its chronic establishment deprives cells of oxygen and therefore of the energy they need to function.

Besides this effect on skin repair in chronic wound healing and burns, very few studies have investigated the effect of hyperbaric oxygen treatments on healthy and/or mature skin. In the context of skin aging, Hachmo et al. conducted a prospective clinical trial on a healthy aging population treated daily for 3 months. They reported an increase in collagen density, in elastic fiber length and in the number of blood vessels, as well as a decrease in fiber fragmentation and the number of senescent cells in the tissue. They saw no effect on epidermal thickness but a decrease in papillary dermis depth. This suggests that oxygen is involved in the pathophysiology of skin aging *in vivo*, through mechanisms such as angiogenesis and senescent cell clearance [138]. In addition, skin elasticity has been shown to be improved in irradiated patients with late radiation tissue injuries [139].

### 8.2. *In vitro* normobaric hyperoxygenation

Few studies have focused on the effect of hyperoxygenation on skin cells *in vitro* under normobaric conditions. Both human primary keratinocytes and melanocytes show that their proliferation is progressively inhibited by increasing the oxygen level from 20 % to 30 %, 50 % and 90 % over a 14-day culture period [110,133]. However, when the oxygen treatment is fractionated (90 min/day for 10 days) using 40 % O<sub>2</sub> (316 mm Hg), a lack of effect on these cells was observed. But the effect of such treatment depends on the cell type. In fact, human primary fibroblasts showed an increased proliferation under the same conditions [140].

Cell migration has also been shown to be affected by hyperoxia. Indeed, HaCaT keratinocytes exposed to 40 % and 60 % O<sub>2</sub> present an increase in migration, both in wound scratch and in Transwell invasion, that is associated with a decrease in microtubule density near the nucleus and is mediated by the regulation of MAP4 [131].

In addition to these effects, skin pigmentation has also been reported to be modulated by hyperoxygenation. Increasing oxygen levels from 18 % to 31 % and 47 % induced the inhibition of tyrosinase activity. As the tyrosinase affinity constant *K<sub>m</sub>* was unchanged, this suggests a change in the native structure of tyrosinase [114]. Moreover, the final melanin content was decreased at 31 % O<sub>2</sub> but surprisingly increased greatly at 47 % O<sub>2</sub>, suggesting that melanin is formed by a mechanism independent of tyrosinase activity at the higher oxygen level [114].

Supplying oxygen is also of interest when oxygen is lacking. *In vitro* reoxygenation experiments performed on fibroblasts and endothelial cells have been shown to reverse the detrimental effects of hypoxia. Reoxygenation could attenuate the expression of hypoxia-induced inflammatory markers (interleukin-6 and tumor necrosis factor), increase angiogenesis (smooth muscle actin) and collagen I synthesis, and thus promote wound healing [141].

### 8.3. *In vitro* hyperbaric oxygenation

Several attempts have been made to increase oxygen levels *in vitro* under hyperbaric conditions to mimic the *in vivo* treatments forcing oxygen to enter into the body. These protocols used intermittent treatments of hyperbaric oxygen, with 100 % O<sub>2</sub> and 1–3 atm, usually for 90 min/day. In *in vitro* studies, oxygen levels in the culture medium reached approximately 316 mm Hg (40 % O<sub>2</sub>) at 1 atm, 869 mm Hg (80 % O<sub>2</sub>) at 2 atm, and 991 mm Hg (~100 % O<sub>2</sub>) at 2,5 atm [140].

After a single treatment with hyperbaric oxygen, the proliferation of keratinocytes was not altered [142] whereas the number of fibroblasts were increased [143]. With repeated treatment, human dermal fibroblasts show an increase in cell proliferation associated with a decrease in collagen and glycosaminoglycan synthesis [140], but also with a higher

ROS production, a higher expression of HIF-1 $\alpha$ , VEGFA and the stromal cell-derived factor 1, and the stimulation of fibroblast migration [135]. An effect on cell metabolic activity was also observed. After a short treatment with hyperbaric oxygenation, human dermal fibroblasts have mitochondria with reduced size and decreased motility, but also changes in respiration, indicating that the treatment induces changes in the bioenergetic functions of fibroblast [144].

Reconstruction of human skin equivalents subjected to hyperbaric oxygen treatment resulted in a thicker epidermis when compared to normoxic conditions [140] and also to a thicker stratum corneum [145]. This increased epidermal differentiation was confirmed by immunohistochemical analysis showing the early expression of the p63 marker, keratins 1/10/11 in the upper layers and keratin 14 in the basal layer. In addition, the lateral migration of keratinocytes over the deepithelialized dermal substrate was faster in hyperbaric oxygen-treated skin equivalent compared to untreated samples, and collagen IV production was improved [145].

### 8.4. Oxygen-releasing materials

In order to achieve hyperoxygenation and facilitate this type of treatment, many oxygen-releasing materials have been developed particularly during the last five years. Their advantages are that they release oxygen continuously over the time and only require a topical application to the skin. These materials are of different types, associated with other polymers and come in different forms (emulsions, hydrogels, patches). They have been shown to enhance cell proliferation, cell activity, and therefore to improve angiogenic activity, wound healing and may have antibacterial properties (for recent reviews see Refs. [146, 147]). The problem with this type of material is its ability to release oxygen for long enough to allow wound healing *in vivo* and how to monitor and control oxygen delivery. Typically, they increase oxygen levels by few percent and at a lower rate than normobaric or hyperbaric oxygenation. To date, very few studies have reported the mechanism of oxygen-releasing materials at the cellular level. In a recent paper, Guan et al. sought to unravel the underlying mechanisms of oxygen-releasing microspheres on cutaneous cells *in vitro*. In addition to the ability of this material to deliver oxygen into the culture medium, they demonstrated a significant increase in intracellular oxygen levels and ATP level, indicating energy generation upon oxygen delivery. Furthermore, ROS levels were increased as well as several factors involved in re-epithelialization and angiogenesis, VEGFA, the fibroblast growth factor-2 and the platelet-derived growth factor subunit B. Finally, they reported that continuous oxygenation promoted cell survival, migration, and angiogenesis [148]. Several other publications have reported *in vitro* and/or *in vivo* effect of different oxygen-releasing materials on the stimulation of angiogenesis, migration, collagen deposition and reduction of inflammation, all of which contribute to improved wound healing [149–151]. It remains to be understood how signalling pathways are activated by released oxygen to produce these factors and improve cutaneous functions.

Besides, oxygen-releasing materials, alone or in combination with other agents, have shown an interest in skin tissue engineering. Azari et al. developed a multilayer oxygen-releasing electrospun scaffold and demonstrated *in vitro* promotion of dermal differentiation and epidermal proliferation by examining gene expression of various markers [152]. Similarly, a scaffold capable of releasing both oxygen and ciprofloxacin was able to improve *in vivo* wound healing with collagen deposition and reduce necrosis [153]. Thus, oxygen-releasing scaffolds offer a potential strategy to accelerate skin tissue regeneration either *in vitro* or *in vivo*.

### 8.5. Hyperoxygenation: benefits and risks

As outlined in this section, hyperoxygenation therapy has been shown to present several beneficial effects on both healthy and damaged skin. It improves and accelerates skin repair by inducing cellular



metabolism and several pathways and participates in skin regeneration through its effect on the extracellular matrix. In particular, repeated intermittent hyperbaric oxygenation is able to trigger a cellular cascade including HIF-1 $\alpha$  signaling pathway, the same as hypoxia, but providing at the same time the oxygen required for cell activity, resulting in the stimulation and proliferation of stem cells. This phenomenon has been described by Hadanny and Efrati as the “hypoxic-hyperoxic paradox” [154]. Future *in vitro* investigations in hyperoxygenation mechanisms should consider the physiological oxygen level as reference to avoid misinterpretation because normoxia is not representative of skin cell activity *in situ*.

In this oxygen-rich microenvironment and particularly with prolonged exposure, cells must cope with the increased levels of ROS, leading to oxidative damage and potentially to cell toxicity. A proper balance must be maintained between ROS and scavengers, antioxidant enzymes and molecules. However, it has recently been shown that the antioxidant defenses of keratinocytes decrease as oxygen levels increase from physioxia to normoxia [62]. Further studies are needed to understand how antioxidant enzymes and molecules are regulated in hyperoxia and to assess how long skin cells can regulate ROS excess without damage to optimize hyperoxia treatments.

It is known that low oxygen levels help to maintain cell stemness [155]. In the skin, the stem cells are located in the basal layer of the epidermis, with the lowest oxygen level. Under hyperoxic conditions, stem cells are stimulated and forced to proliferate and then differentiate. This is necessary to achieve skin repair or regeneration. However, prolonged treatment could result in cell exhaustion with loss of stem cells and therefore of skin regeneration potential, particularly in the epidermis where the stem cells are the key players in regulating the formation of the skin barrier.

In order to reap the benefits of hyperbaric treatments, particularly for skin regeneration, it is necessary to find conditions that limit the detrimental effects. Several strategies could be proposed and studied: combining an antioxidant treatment or stimulating antioxidant defences to prevent excessive ROS production and limit oxidative stress; limiting the duration of treatments; optimizing the duration, the frequency and

the oxygen level in hyperbaric intermittent treatments so as not to deplete the stem cell pool. Further studies will be required to refine these strategies and to find the optimal balance between the treatment efficacy and the deleterious effects.

### 9. Conclusion and prospects

In recent years, considerable attention has been paid to the importance of oxygen in biological functions (for review, see Refs. [64,156]). This has highlighted the need to improve *in vitro* cell culture conditions to best mimic the physiological conditions. In this review, we have summarized the various findings on the effects of physiological oxygen levels on skin cells. It is now accepted that the behaviour cells cultured in normoxia differs from that of cells in physioxia. The level of oxygen impacts several processes, such as oxygen consumption/metabolism, proliferation, differentiation, and senescence, as well as specific functions, such as migration, pigmentation, and angiogenesis (Fig. 4). However, for other processes, such as ROS production and antioxidant modulation, the effect is less clear and requires further investigation. Various parameters, such as the origin of the cells, their culture time and the methodologies used, should be considered. In addition, all the studies report the level of oxygen used for physioxia, but the level of oxygen seen by the cell is not assessed. In fact, the actual pericellular oxygen level results from the oxygen supply, which is fixed in the incubator, but also from the oxygen consumption by the cells, which depends on the cell type, the state of the cell, its culture time, etc. The mechanisms by which oxygen modifies cellular activities remain to be elucidated. Indeed, the main signalling pathways linked to oxygen and antioxidant defences, HIF-1 $\alpha$  and Nrf2, are still poorly understood in the skin and would likely provide a better understanding of the effects of variations in oxygenation. These findings challenge the data obtained from cultures performed under normoxic conditions. Particularly in the case of skin cells, measurements of pigmentation, oxidative stress and antioxidant defences could be artefacts.

Although we have seen in this review that respecting physiological oxygen levels *in vitro* is fundamental, an increase in physiological

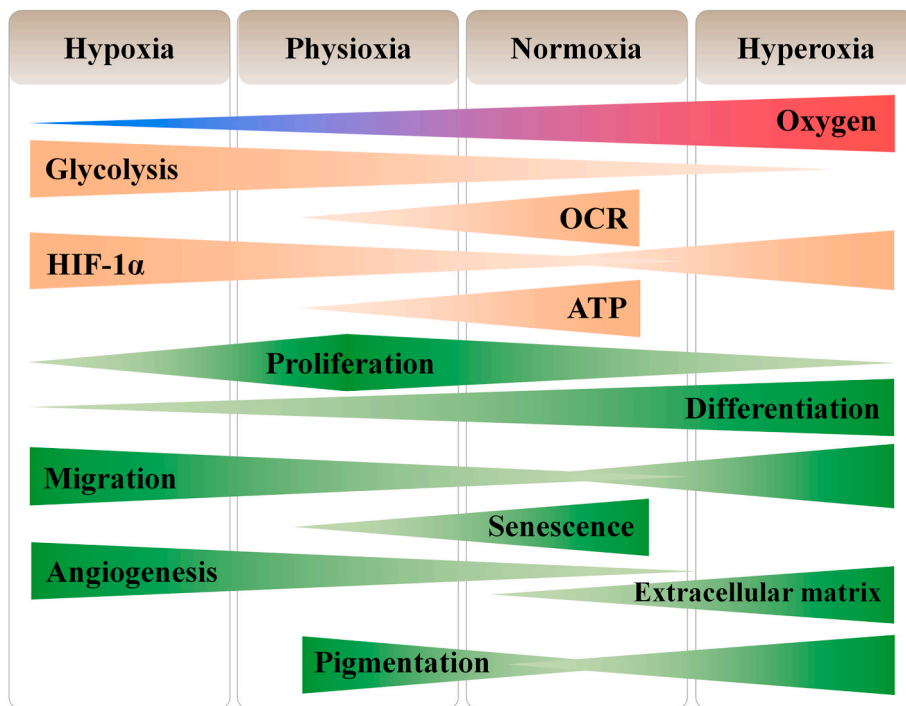


Fig. 4. Effects of oxygen levels on skin cells. Skin cells are naturally in homeostasis in physioxia. Increasing (normoxia, hyperoxia) or decreasing (hypoxia) oxygen levels induce an imbalance with modifications in their metabolism (OCR, ATP production, glycolysis), in their life cycle (proliferation, differentiation, senescence) and in their functions (migration, angiogenesis, pigmentation), forcing them to adapt to this new microenvironment to the detriment of their physiological role.

oxygen levels can bring beneficial effects for the skin, notably in regeneration, and in accelerating healing and burns. However, the consequences of such treatments need to be studied in greater depth to assess the mechanisms involved and also the associated risks. For this purpose and as highlighted in this review, it is necessary to develop cell cultures under physiological conditions to obtain relevant results that reflect what occurs in the skin *in vivo*. In the case of monolayer cultures, this is relatively straightforward but lacks the complexity of the skin microenvironment. Reconstructed epidermis or reconstructed skin models have been widely developed over the last twenty years, but only in normoxia. Few studies have tackled this problem and shown morphological and functional differences in the epidermis. Interestingly, it has recently been demonstrated that 3D models of skin reconstructed in physioxia are closer to a skin explant than the model developed in normoxia, in terms of thickness and state of differentiation [5].

Similarly, the role of cellular interactions merits further investigation under these physiological conditions since fibroblasts appear to be required for stratum corneum formation in physioxia. In contrast, it forms without fibroblasts in normoxia [62,105]. Moreover, there have been no studies of dermis formation in physioxia. Therefore, a large field of research is opening to understand the effects of physiological oxygen levels on skin cells, particularly in reconstructed skin, and to obtain suitable models more compliant with skin *in situ*.

To expand the horizons of this subject, it is also pertinent to consider pathological models and oxygen levels. Most cancer models, including skin cancers, are studied in hypoxia, reflecting the lack of oxygen due to its increased consumption by cancer cells and its low supply. However, all works compare hypoxia with normoxia, whereas physioxia should be compared. This aspect is just beginning to be explored, and, in this review, Adebayo et al. outline the significance of considering cancer models in physioxia to enhance clinical translation [157].

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## CRedit authorship contribution statement

**Nadira Chettouh-Hammas:** Writing – review & editing, Writing – original draft, Visualization, Conceptualization. **Catherine Grillon:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Abbreviations

AP1	Activator Protein 1
ATP	Adenosine Triphosphate
CAT	Catalase
GLUT1	Glucose Transporter 1
GPX	Glutathione Peroxidase
HaCaT	Immortalized human keratinocyte cell line
HIF	Hypoxia Inducible Factor
HO-1	Heme Oxygenase 1

Keap1	Kelch-like ECH-associated protein 1
MIF	macrophage migration inhibitory factor
NF-kB	nuclear factor binding near the k light-chain gene in B cells
NOS	Nitric Oxide Synthases
NOX	NADPH Oxidase
Nrf2	Nuclear factor (erythroid-2)-related factor 2
OCR	Oxygen Consumption Rate
PDK1	Pyruvate Dehydrogenase Kinase 1
PHD	prolyl hydroxylase
ROS	Reactive Oxygen Species
SA-β-Gal	Senescence-Associated Beta-Galactosidase
SASS	Senescence-Associated Secretory Phenotype Factors
SODs	Superoxide Dismutases
VEGFA	Vascular Endothelial Growth FactorA

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