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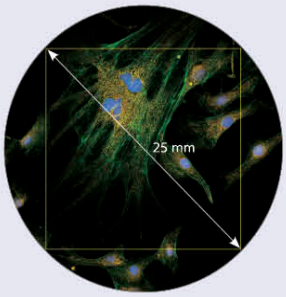

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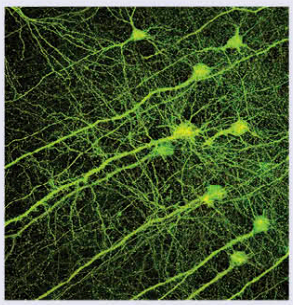

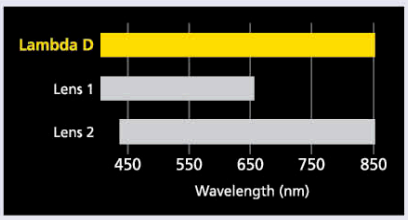

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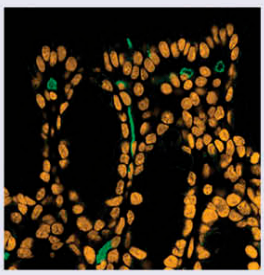

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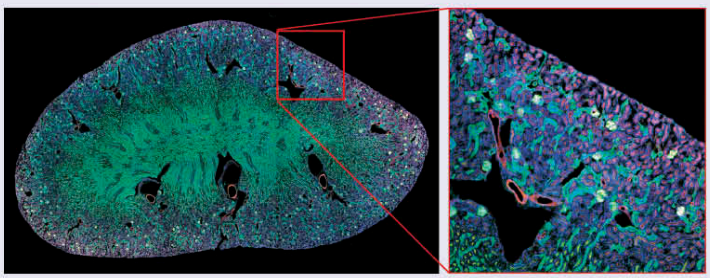

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Journal of Cellular Physiology

Decreased Oxygen Tension Lowers Reactive Oxygen Species and Apoptosis and Inhibits Osteoblast Matrix Mineralization Through Changes in Early Osteoblast Differentiation

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Accumulating data show that oxygen tension can have an important effect on cell function and fate. We used the human pre-osteoblastic cell line SV-HFO, which forms a mineralizing extracellular matrix, to study the effect of low oxygen tension (2%) on osteoblast differentiation and mineralization. Mineralization was significantly reduced by 60–70% under 2% oxygen, which was paralleled by lower intracellular levels of reactive oxygen species (ROS) and apoptosis. Following this reduction in ROS the cells switched to a lower level of protection by down-regulating their antioxidant enzyme expression. The downside of this is that it left the cells more vulnerable to a subsequent oxidative challenge. Total collagen content was reduced in the 2% oxygen cultures and expression of matrix genes and matrix-metabolizing enzymes was significantly affected. Alkaline phosphatase activity and RNA expression as well as RUNX2 expression were significantly reduced under 2% oxygen. Time phase studies showed that high oxygen in the first phase of osteoblast differentiation and prior to mineralization is crucial for optimal differentiation and mineralization. Switching to 2% or 20% oxygen only during mineralization phase did not change the eventual level of mineralization. In conclusion, this study shows the significance of oxygen tension for proper osteoblast differentiation, extra cellular matrix (ECM) formation, and eventual mineralization. We demonstrated that the major impact of oxygen tension is in the early phase of osteoblast differentiation. Low oxygen in this phase leaves the cells in a premature differentiation state that cannot provide the correct signals for matrix maturation and mineralization.

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Bone strength is heavily dependent on the quality of bone matrix formation and its subsequent mineralization. This specialized extra cellular matrix (ECM) is produced by differentiating osteoblasts and consists out of collagens, non-collagenous proteins (NCPs), mineral and water (Clarke, 2008).

Osteoblast differentiation can be divided in three stages. First there is a rapid proliferative phase, followed by a matrix deposition phase and finally the matrix mineralization phase. Osteoblast differentiation and subsequent matrix mineralization can be influenced by the already deposited ECM through cell–ECM interaction and the secretion or activation of cell signaling molecules (Franceschi and Iyer, 1992).

Collagens play an important part in this process. Without the deposition of a correctly structured collagen matrix, osteoblast differentiation is impaired (Vetter et al., 1991; Quarles et al., 1992; Franceschi et al., 1994; Wenstrup et al., 1996). NCPs, like osteocalcin (OC), osteopontin (OP), bone sialoprotein (BSP), and MEPE might also influence osteoblast differentiation (Ducy et al., 1996) but seem to have an additional and more predominant effect on matrix mineralization. Many NCPs function as nucleation factors and regulate hydroxyapaptite crystal formation and size (Boskey et al., 1993; Hunter and Goldberg, 1994; Rowe et al., 2005; Qin et al., 2007).

Most of what we know about the process of matrix maturation and mineralization has been investigated in cells cultured under regular (20% oxygen) culture conditions. Oxygen levels in the bone marrow and the surrounding bone tissue however, have been measured and modeled to be somewhere between 1% and 10% (Chow et al., 2001; Harrison et al., 2002). Oxygen tension can influence gene expression and modulate cell–cell interactions (Park et al., 2002; Haase, 2009). In addition low oxygen levels might influence cell metabolism and induce stress, modulating the levels of reactive oxygen species (ROS) and the scavenging mechanisms cells employ. High levels of ROS can cause DNA and protein damage, usually followed by the activation of regulated apoptosis (Giorgio et al., 2005). Lower levels of ROS however play a role in intra-cellular signaling cascades (Janssen-Heininger et al., 2008). In order to regulate ROS levels in the cell, several ROS scavenging mechanisms are in place amongst which a number of antioxidant enzymes. These antioxidant enzymes can be divided into three groups; superoxide dismutases (SODs), catalase (CAT), and glutathione peroxidases (GPXs). SODs reduce superoxide radicals into H_2O_2, which can be further reduced into H_2O by either CAT or any of the GPXs (Mates and Sanchez-Jimenez, 1999).

Previous studies using MSCs or murine osteoblast cell lines show a decrease in ALP expression and a severe inhibition of mineralization in cells cultured under low oxygen conditions (Tuncay et al., 1994; D'Ippolito et al., 2006; Grayson et al., 2006; Utting et al., 2006). Little is known about what happens to the interplay between osteoblasts and the matrix, cell metabolism, or the induction of apoptosis during low oxygen conditions as well as the antioxidant enzyme expression and protection against ROS.

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We investigated the effects of low oxygen tension on osteoblast metabolism, ROS production, and ROS protection as well as on matrix formation and mineralization, focusing on a number of known regulatory mechanisms that might affect these processes in order to determine whether low oxygen levels affect ECM–osteoblast interactions that regulate osteoblast differentiation and matrix formation.

Materials and Methods

Cell culture

Human SV-HFO cells are pre-osteoblasts (Chiba et al., 1993; Eijken et al., 2006) and were seeded in a density of 5×10^3 vital cells/cm^2 and pre-cultured for 1 week in α-MEM (GIBCO, Paisley, UK) supplemented with 20 mM HEPES, pH 7.5 (Sigma, St. Louis, MO), streptomycin/penicillin, 1.8 mM $CaCl_2$ (Sigma), and 10% heat-inactivated FCS (GIBCO) under standard conditions at 37°C and 5% CO_2 in a humidified atmosphere. During pre-culture cells remained in an undifferentiated stage. At this point cells were seeded in a density of 1×10^4 vital cells/cm^2 in the presence of 2% charcoal-treated FCS. For induction of osteoblast differentiation and mineralization the basal medium was freshly supplemented with 10 mM β-glycerophosphate (Sigma) and 100 nM dexamethasone (Sigma). The medium was replaced every 2–3 days. Cells were cultured at 37°C under standard conditions or in hypoxia chambers (Billups Rothenberg Inc., Delmar, CA) gassed with a mixture of 2% oxygen, 5% CO_2, and 93% nitrogen at days of medium refreshment.

DNA, mineralization, protein, and proliferation assays

DNA and calcium measurements were performed as described previously (Eijken et al., 2006). Briefly, for DNA measurements cell lysates were incubated with heparin (81U/ml in PBS) and ribonuclease A (50 mg/ml in PBS) for 30 min at 378°C. DNA was stained by adding ethidium bromide (25 mg/ml in PBS). Analyses were performed by using a Victor2 plate reader (PerkinElmer, Waltham, MA) with an extinction filter of 340 nm and an emission filter of 590 nm. For calcium measurements, cell lysates were incubated overnight with 0.24 M HCl at 48°C. Calcium content was determined colorimetrically with a calcium assay kit (Sigma) according to the manufacturer's description. Results were adjusted for DNA content of the cell lysates. For Alizarin Red S staining cell cultures were fixed for 60 min with 70% ethanol on ice. After fixation, cells were washed twice with PBS and stained for 10 min with Alizarin Red S solution (saturated Alizarin Red S in demineralized water adjusted to pH 4.2 using 0.5% ammonium hydroxide). For protein measurement 200 ml of working reagent (50 volumes BCATM reagent A, 1 volume BCATM reagent B; PierceQ5) was added to 10 ml of sonicated cell lysate. The mixture was incubated for 30 min at 378°C, cooled down to room temperature and absorbance was measured, using a Victor2 plate reader at 595 nm.

Proliferation was measured using a BrdU labeling kit (Roche, Basel, CH) to load, incorporate, wash, and release BrdU which was measured using a plate reader (Victor^2 1420 multilabel counter, Wallac, Waltham, MA) according to the manufacturer's protocol.

Collagen staining and quantification

Cells were fixed for 1 h using 10% formaldehyde. After fixation cells are washed with PBS and stained with Sirius Red Staining Solution (Direct Red, Sigma) for 1 h, followed by three wash steps with 0.01% HCl to get rid of excess dye. The dye was extracted and quantification took place using a Victor2 plate reader at 550 nm.

Apoptosis assay

Apoptosis was measured through the binding of annexin V and the uptake of propidium iodide (IQ products) by flow cytometry. For analysis 10,000 cells were counted (FACS Canto II, BD Biosciences, Breda, NL) and the percentage of apoptotic cells was determined by counting vital (unstained), early apoptotic (annexin V stained), necrotic (PI stained), and late apoptotic cells (double stained).

qPCR analysis

Total RNA was isolated using TRIzol reagent (Invitrogen, Breda, NL) according to the manufacturer's protocol. RNA was isolated as previously described (Eijken et al., 2006). Total RNA amount was determined using a spectrophotometer (ND1000, NanoDrop, Wilmington, DE). For cDNA synthesis 1 μg of total RNA was reverse transcribed using a cDNA synthesis kit according to the manufacturer's protocol (MBI Fermentas, St. Leon-Rot, GER). qPCR analysis was performed using a ABI 7500 Fats Real-Time PCR detection system (Applied Biosystems, Carlsbad, CA). Reactions were performed in 25 μl volumes using a qPCR core kit (for assays using a probe) or a qPCR kit for SYBR green I (for assays using SYBR Green) (Eurogentec, Maastricht, NL). Primer and probe sets were designed using Primer Express software (version 2.0, Applied Biosystems).

Stress induction and ROS measurement

Cells were incubated with 1 mM H_2O_2 (30%, Merck, Haarlem, NL) in order to induce oxidative stress at various time points to induce oxidative stress. In order to visualize and measure superoxide radical production, cells were incubated with MitoSox Red (Invitrogen) after which pictures were taken on a fluorescent microscope (Axiovert 200 MOT, Zeiss, Sliedrecht, NL) for quantification using Cell Profiler cell image analysis software (http://www.cellprofiler.org, Broad Institute, Cambridge, MA). Total ROS production was measured after incubating the cells with DCF-DA (Sigma), after which fluorescence was measured on a plate reader (Victor^2 1420 multilabel counter, Wallac).

Statistics

All experiments have been performed three times with biological triplicates for each condition in each experiment. Differences between experimental and control conditions have been statistically analyzed by using two-tailed *t*-tests.

Results

Osteoblast mineralization

Human pre-osteoblast SV-HFO cells were cultured for 3 weeks under normal (20% O_2) or low (2% O_2) oxygen conditions. SV-HFO cells undergo a 3-week differentiation and mineralization process in which the first week consists mostly out of proliferation and matrix production, followed by a second week of matrix maturation which is followed by a third week of matrix mineralization. Alizarin red staining of mineralized matrix on day 19 of culture, showed strong reduction of matrix mineralization by 2% O_2 (Fig. 1A). Staining quantification showed that mineralization still occurred but to a significantly reduced extent (Fig. 1B).

ROS production

We assessed the ROS production of cells cultured under high or low O_2. Total ROS production was significantly lower in cells cultured under 2% O_2 (Fig. 2A). Next we determined the amount of superoxide radicals produced in both cell systems using the specific fluorescent stain mitosox red (Fig. 2B). Quantification showed a significant decrease of superoxide radicals in cells cultured under 2% O_2, indicating reduced mitochondrial activity and a down-scaled cellular metabolism (Fig. 2C).

Since ROS production was strongly decreased, we examined the state of the cell's protection mechanism against oxidative stress. We determined the expression levels of several antioxidant enzymes in cells cultured under high or low O_2

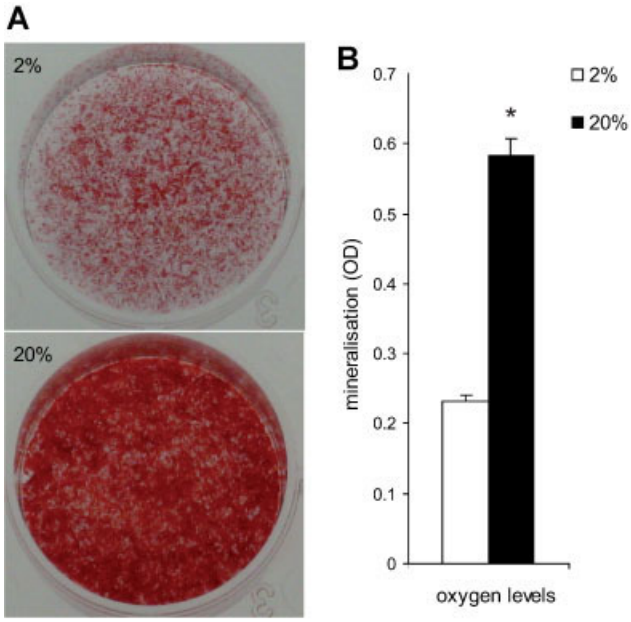

Fig. 1. Inhibition of osteoblast matrix mineralization by 2% oxygen. Cell cultures grown under 2% O_2 mineralize to a lesser extend compared to cells cultured under 20% O_2. A: Alizarin Red staining (ARS) of SV-HFO cultures on day 19, cultured under 2% (upper part) or 20% O_2 (lower part). B: Quantification of ARS on day 19 cells cultured under 2% (white) or 20% O_2 (black). *$P < 0.05$ 20% versus 2%. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcp]

levels at day 12, around the onset of mineralization (Fig. 2D,E). Both cytoplasmic (SOD1) and mitochondrial (SOD2) SOD expression was significantly down-regulated in cells cultured under 2% O_2, suggesting a down-scaled protection mechanism. This was supported by the significantly down-regulated expression of the H_2O_2 radical scavenger CAT under low oxygen conditions (Fig. 2D). Surprisingly, SOD3, which is localized extracellular to scavenge ROS in the ECM (Marklund, 1984; Carlsson et al., 1995), was significantly up-regulated. Expression of the various GPX genes was not significantly altered by O_2 (Fig. 2E).

Proliferation and apoptosis

Next, we examined the effects of oxygen tension on proliferation and apoptosis. For both conditions, proliferation significantly decreased between days 3 and 10. At day 3 of culture the proliferation rate of cells cultured under 2% O_2 was significantly lower compared to cells cultured under 20% O_2. However, at day 10 of culture the situation was reversed, with higher proliferation rates in cells cultured under low oxygen tension. This shows that cells cultured under low oxygen condition retained a higher proliferative capacity while under high oxygen tension their proliferative capacity decreased with about 50% (Fig. 3A). Up to the onset of mineralization at day 12 of culture there was no significant difference in apoptosis between 2% and 20% O_2 cultured osteoblasts. A strong and significant increase in apoptotic cells and a concomitant decrease in living cells was observed after culture for 19 days under 20% but not under 2% O_2 (Fig. 3B). This difference in apoptosis was also reflected in the difference in expression of apoptosis-related genes. Both the caspases 3 and 6 as well as cytochrome *c* were significantly lower in the 2% O_2 condition while BAX expression stayed the same (Fig. 3C).

Induced oxidative stress

Next, we investigated the effect of induced oxidative stress on the osteoblasts cultured under either 2% or 20% O_2, and having different levels of radical protection. To this end we induced oxidative stress by treatment with 1 mM H_2O_2 for 24 h followed by analyses of apoptosis. In line with the reduced expression of antioxidant enzymes, cells cultured under 2% O_2 had an increased susceptibility to oxidative stress as demonstrated by the significant decrease in living and increase in apoptotic cells (Fig. 4A,B). This was supported by gene expression levels showing a significant increase in cytochrome *c* and caspase 3 expression in cells cultured under 2% O_2 (Fig. 4C,D). Cells cultured under 20% O_2 displayed a smaller but significant increase in caspase 3 levels as well, but lacked a response in cytochrome *c* levels (Fig. 4C,D).

ROS formation and increased apoptosis have been linked to increased matrix mineralization and may explain the increased mineralization seen for 20% oxygen (Fig. 1) (Proudfoot et al., 2000; Clarke et al., 2006; Liberman et al., 2008; Bruedigam et al., 2010). Therefore, we questioned whether the reduced antioxidant enzyme expression (Fig. 2D,E) and increased apoptosis (Fig. 4B) after H_2O_2 treatment in the 2% O_2 condition would lead to enhanced mineralization. In order to test this, we induced oxidative stress using 1 mM H_2O_2 at the onset of mineralization (day 12) in osteoblasts cultured on either 2% (Fig. 4E) or 20% O_2 (Fig. 4F). Although we measured increased levels of apoptosis in 2% O_2 cultured cells (Fig. 4B), this had no effect on mineralization as measured on day 19 (Fig. 4E). Most interestingly, induction of oxidative stress by H_2O_2 significantly enhanced mineralization in 20% O_2 osteoblast cultures (Fig. 4F). This implicates that there must be additional differences in the 2% and 20% O_2 cultures in osteoblast differentiation which are important to explain the differences in matrix mineralization. Strong induction of oxidative stress by continuous treatment with H_2O_2 from the start of osteoblast differentiation was detrimental for mineralization, irrespective of O_2 regimen (Fig. 4E,F).

Matrix production and turnover

To study effects on the collagenous ECM we performed Sirius Red Collagen staining on day 19 after culture under 2% O_2 or 20% O_2. Osteoblasts cultured under 2% O_2 showed less collagen staining (Fig. 5A) which was confirmed by extraction and quantification demonstrating a significant decrease in the total amount of collagen (Fig. 5B). In contrast, expression of various collagen genes, including COL1A1, was significantly increased on days 5 and 19 in the 2% oxygen condition (Fig. 5C). Next we examined the expression of matrix-metalloproteinases (MMPs) and their inhibitors, tissue-inhibitor-of-metalloproteinases (TIMPs), which both are involved in breakdown of ECM and can regulate cell differentiation and apoptosis (Bond et al., 2002; Buxton et al., 2008). MMP1 and MMP23B were expressed by the human osteoblasts and were reduced in the low oxygen condition at day 19, but not at day 5 (Fig. 5D). The expression levels of TIMP3 were significantly lower in the 2% O_2 cultured osteoblasts at day 19. TIMP1 expression was slightly but not significantly lower as well (Fig. 5D). These data demonstrate that oxygen tension is of significance for the formation and turnover of the ECM.

Osteoblast differentiation

Changes in ECM composition, could lead to a delay in differentiation, leaving the cells in an immature stage at the onset of mineralization. We investigated cell differentiation

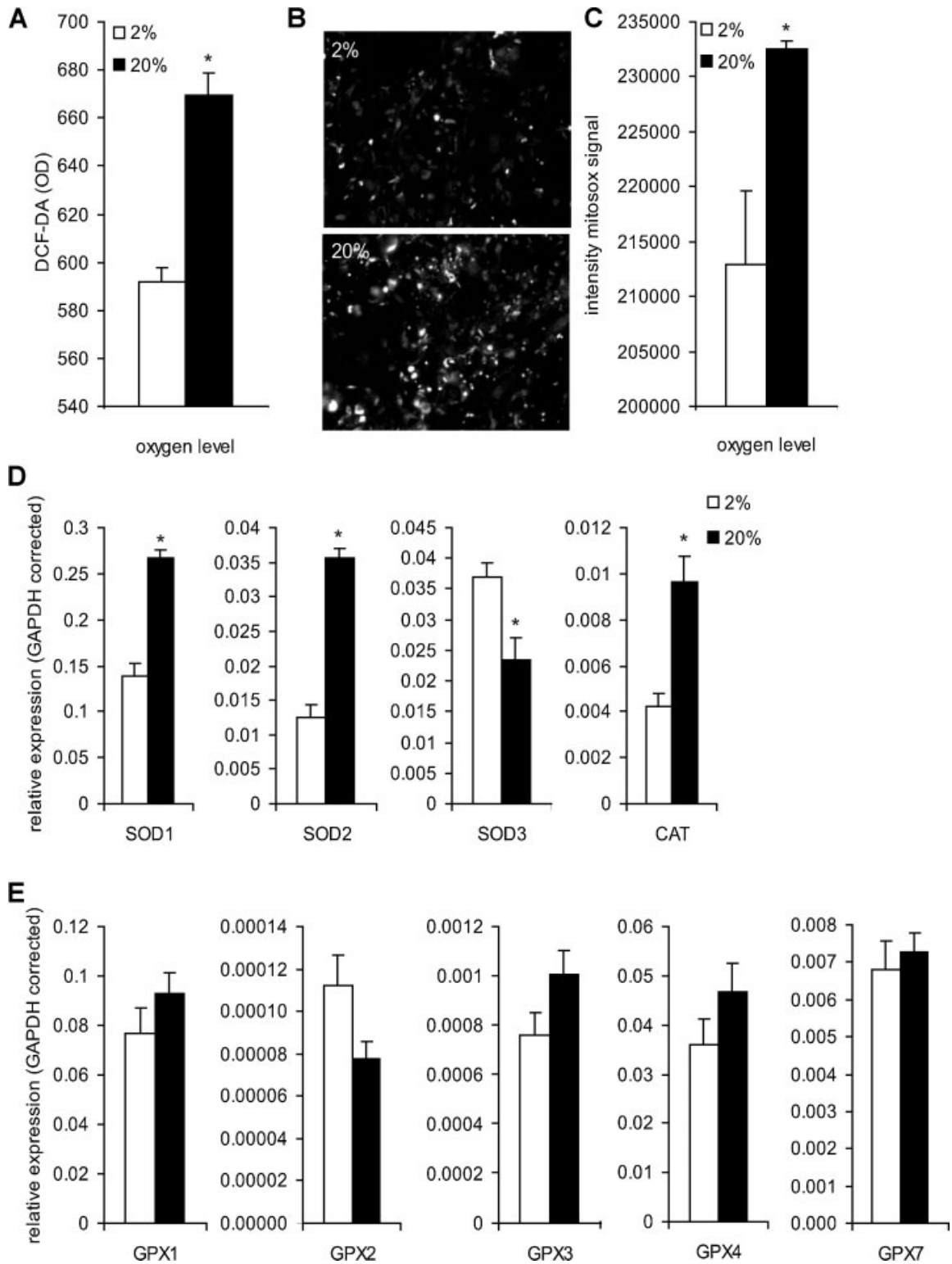

Fig. 2. Effect of 2% and 20% oxygen on ROS production and gene expression of antioxidants. A: Total radical production on day 12 of culture in cells cultured on high (black) or low (white) oxygen. B: Pictures of mitosox red super oxide radical staining in day 12 cells cultured on high (lower part) or low (upper part) oxygen. C: Quantification of mitosox red super oxide staining on day 12 in cells cultured on high (black) or low (white) oxygen. D,E: Relative gene expression levels of anti oxidant scavengers, corrected for GAPDH. *$P < 0.05$ 20% versus 2%.

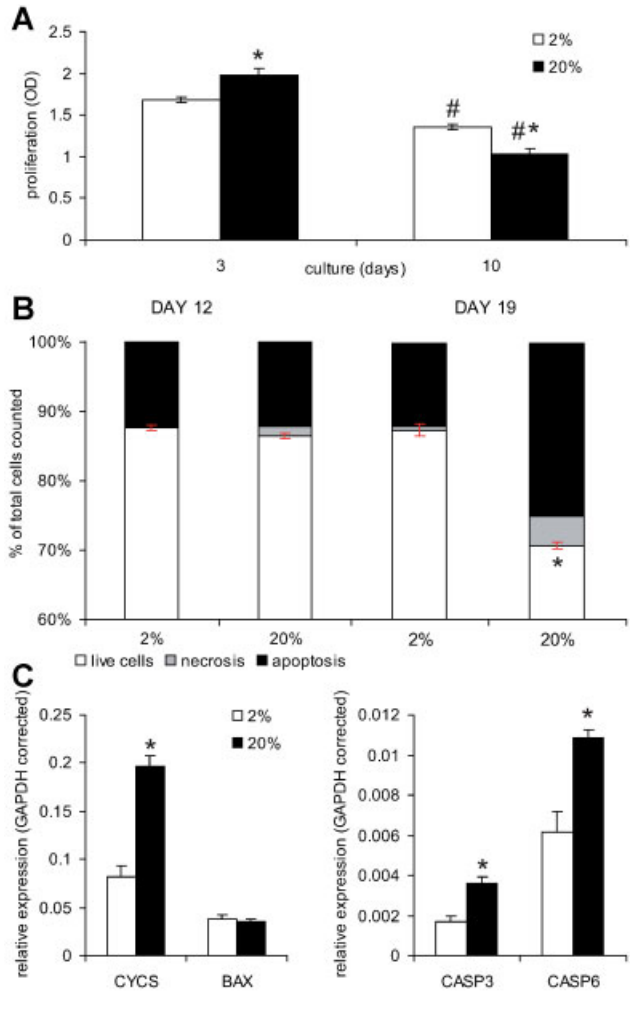

Fig. 3. Effects of 2% and 20% oxygen on osteoblast cell proliferation and apoptosis. A: Cell proliferation on days 3 and 10 of culture, measured by BrdU incorporation in cells cultured on high (black) or low (white) oxygen. B: FACS analysis of cells cultured on high or low oxygen tension on day 12 (left) or day 19 (right). Cells are sorted in living cells (white), apoptotic cells (black), and necrotic cells (gray). C: Relative gene expression levels corrected for GAPDH of apoptosis related genes measured in cells cultured on high (black) or low (white) oxygen tension. *$P < 0.05$ 20% versus 2%, #$P < 0.05$ day 3 versus day 10.

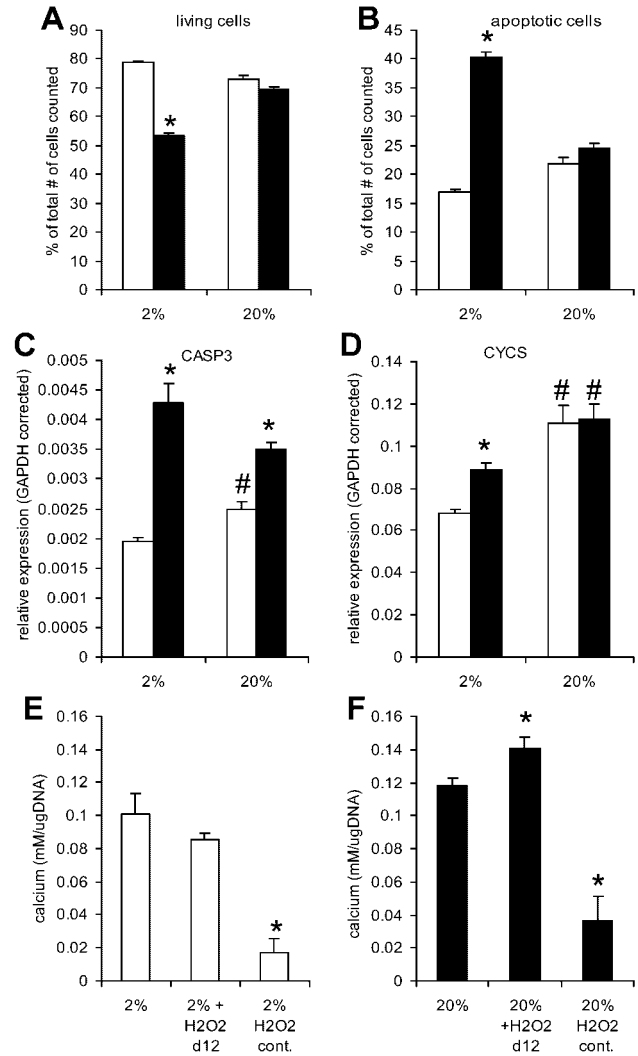

Fig. 4. Effect of oxygen radical challenge on cells cultured under 2% and 20% oxygen. A,B: FACS analysis of (A) living (left panel) and (B) apoptotic (right panel) cells, 24 h after stress induction (1 mM H_2O_2) cultured on high or low oxygen. C,D: Relative gene expression levels corrected for GAPDH measured 24 h after stress induction (1 mM H_2O_2) of apoptosis related genes measured in cells cultured under 2 or 20% oxygen. In parts A–D the open bars indicate vehicle and the solid bars 1 mM H_2O_2 treatment. E,F: The effect of continuous (days 0–19) or timed (days 12–19 = mineralization phase) induction of apoptosis (1 mM H_2O_2) on matrix mineralization measured on day 19 in cells cultured under 2% (E) or 20% (F) oxygen. # $p < 0.05$ 20% versus 2%, * $p < 0.05$ control versus H_2O_2.

throughout the entire culture period by measuring alkaline phosphatase (ALP) activity in the cells. In the 20% O_2 condition, ALP activity peaked at the onset of mineralization after which it declined. In the osteoblasts cultured under 2% oxygen, we saw a similar pattern, with a peak at day 12 albeit that at all stages of differentiation the ALP levels were significantly reduced in 2% O_2 osteoblasts (Fig. 6A). This was supported by the significant reduction in ALP gene expression levels (Fig. 6B). Inhibition of osteoblast differentiation was further demonstrated by reduced RUNX2 expression (Fig. 6C). These data show that differences in O_2 may cause differences in osteoblast differentiation already at an early stage.

To further test this we cultured osteoblasts for various periods during differentiation on either 2% or 20% oxygen and then switched the percentage oxygen (see Fig. 6D left part for schematic representation). These analyses demonstrated that 20% O_2 in the first week of osteoblast differentiation is crucial to observe the increased mineralization after 3 weeks compared to 2% O_2 (Fig. 6D). Longer incubation with 20% O_2 did not result in any further increase in mineralization. These data corroborate the observations on early changes in ALP activity and expression and RUNX2 expression (Fig. 6A–C) and that the effect of oxygen on eventual mineralization originates in the early phase of osteoblast differentiation.

Discussion

The current study demonstrates the significance of oxygen tension for osteoblast proliferation, differentiation, apoptosis, and mineralization as well as the level of ROS production and the expression of antioxidant enzymes. Low oxygen tension

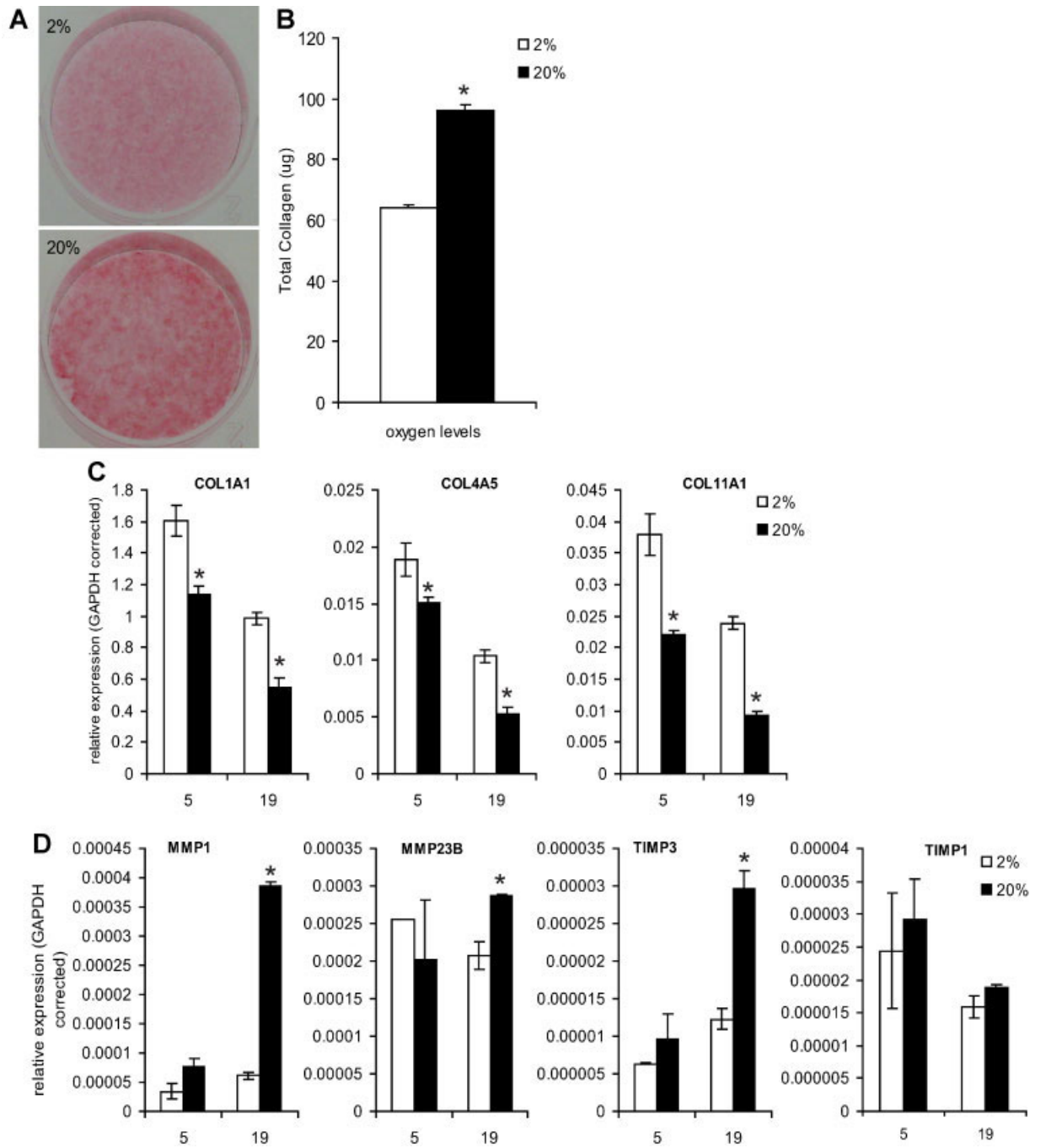

Fig. 5. Impact of oxygen on collagen production and ECM-related genes. A: Amount of collagen in the ECM is lower in 2% (upper part) compared to 20% (lower part) oxygen cultured osteoblasts as assess by Sirius red staining at day 19 cells. B: Quantification of the Sirius red staining of cells cultured on 20% (black) or 2% (white) oxygen. C: Relative expression levels of several collagen genes corrected for GAPDH on days 5 and 19 of culture for cells cultured on high (solid squares) or low (open squares) oxygen. D: Relative gene expression levels on days 5 and 19 corrected for GAPDH for a number of MMPs and TIMPs produced by the cells when cultured on high (black) or low (white) oxygen. *$P < 0.05$ 20% versus 2%. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcp]

inhibits osteoblast-mediated mineralization due to an inhibition of osteoblast differentiation. Time phase studies demonstrated that this inhibition is time dependent and caused by effects in the early phase of osteoblast differentiation. We propose that oxygen tension affects osteoblast differentiation via effects on genes like RUNX2 and ALP and alters osteoblast differentiation via changes in the ECM composition. We observed changes in ECM and genes involved in ECM production and turnover in cells cultured under low oxygen. These would lead to alterations in matrix formation and may eventually inhibit osteoblast differentiation and matrix mineralization. It has been shown that osteoblast differentiation and subsequent matrix mineralization can be influenced by the already deposited ECM through cell–ECM interaction and the secretion or activation of

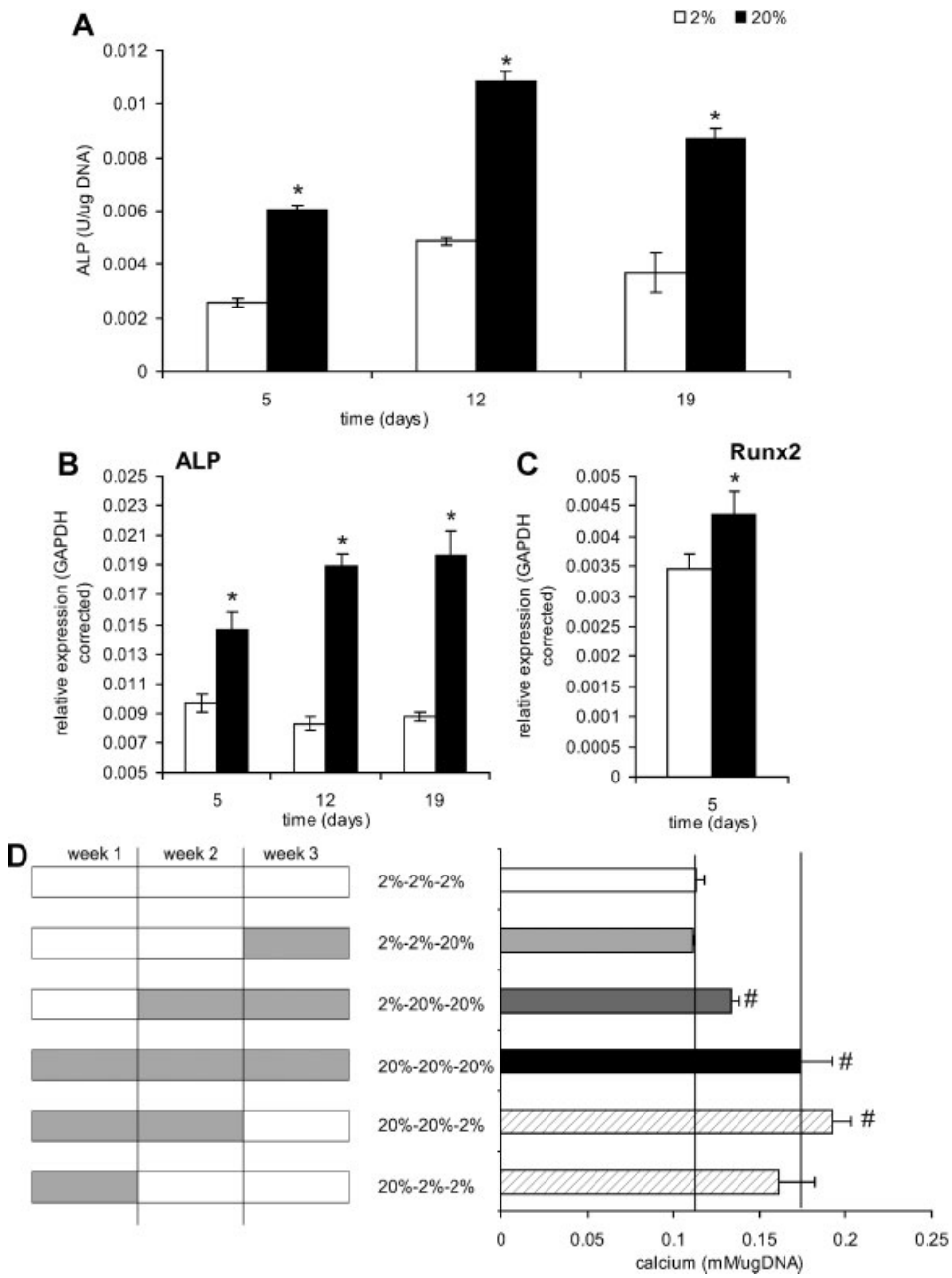

Fig. 6. Effect of oxygen on ALP, RUNX2 and timing of effects on mineralization. Low oxygen tension inhibits osteoblast differentiation. A: ALP activity corrected for DNA amount measured on day 5, 12 and 19 of culture grown on high (black) or low (white) oxygen tension. B,C: Relative gene expression levels corrected for GAPDH of ALP throughout the culture and Runx2 on day 5 in cells cultured on high (black) or low (white) oxygen. D: Schematic representation of oxygen tension (left part) during culture and matrix mineralization measured as the amount of calcium corrected by DNA (corresponding right part). Cells were cultured on low (white squares in the schematic) or high (grey squares in the schematic) oxygen for varying amounts of time during the three week culture period each depicted by another color. Striped bars depict matrix mineralization in cells cultured on 20% oxygen for varying amounts of time. *$p < 0.05$ 20% versus 2%, #$p<0.05$ versus the 2%–2%–2% condition.

cell signaling molecules (Franceschi and Iyer, 1992). Collagens play an important part in this process. Without the deposition of a correctly structured collagen matrix, osteoblast differentiation is impaired (Vetter et al., 1991; Quarles et al., 1992; Franceschi et al., 1994; Wenstrup et al., 1996). In parallel with the decrease in differentiation, under low oxygen tension the osteoblasts retain a more proliferative phenotype while high oxygen tension decreased the proliferation rate by 50% in the first 10 days of culture. This is in line with the general observation that low levels of ROS are associated with cells in a proliferative state (Bell et al., 2007).

Our current observations on low oxygen and osteoblast differentiation are in line with previous studies on osteoblast cell lines and human MSCs (Salim et al., 2004; D'Ippolito et al., 2006; Grayson et al., 2006; Utting et al., 2006). We extent these observations by demonstrating that the oxygen sensitivity for

regulating differentiation and ECM mineralization is limited to the early phase of osteoblast differentiation. Also we provide for the first time data about the impact of oxygen tension on the metabolic state with respect to ROS production. We focused on ROS production and scavenging mechanisms and were interested in their production and expression in relation to osteoblast differentiation and ECM mineralization. ROS are produced by mitochondria and play a role in numerous cellular signaling pathways like the Pi_3K pathway, FOXO signaling and TNFα mediated cell death (Manolagas, 2010). ROS levels in the cell can determine its fate, from proliferation to immediate cell death (Hamanaka and Chandel, 2010). The cellular response to hypoxia requires the mitochondrial generation of ROS to propagate signaling events that regulate transcription, calcium stores, and energy stores at the cellular level (Chandel et al., 1998; Hamanaka and Chandel, 2010). We observed a decrease in total ROS production as well as a reduction in super oxide radicals under 2% O_2. This indicates that the osteoblasts do not necessarily recognize this 2% level of oxygen tension as a severe state of hypoxia.

The reduced ROS production in cells cultured under 2% O_2 is followed by a decrease in gene expression of the protective antioxidant enzymes SOD1 and SOD2 and CAT. This indicates a decrease in ROS and H_2O_2 radical scavenging in cells cultured on low oxygen tension. This would be an expected adjustment in line with the cell's decreased oxygen consumption and metabolic state. In other words the osteoblasts switch from using energy for a high protection state to a low protection state. At the same time safeguarding a minimum level of ROS needed for cell survival and proliferation. Although there might be a trend toward lower levels, the GPX subfamily of scavengers was never significantly different between 2% and 20% oxygen. A drawback of this switch to lower ROS protection under low oxygen condition is that this leaves the cells vulnerable to oxidative stress and ROS challenges, leading to increased apoptosis as shown by the H_2O_2 studies.

An interesting observation from an ECM and bone formation perspective is the fact that, in contrast to SOD1 and 2, SOD3 expression is significantly up-regulated in osteoblasts cultured under low oxygen tension. A major difference with SOD1 and 2 is that these SODs are located intracellular, while SOD3 is excreted and can be found in extracellular fluids (Marklund et al., 1982). After secretion it can bind heparin sulfate proteoglycans found on cell surfaces or in the ECM from where it can scavenge radicals in the extracellular environment (Sandstrom et al., 1992). Little is known about its role in bone formation, but there is a direct link between SOD3 levels and the occurrence of osteoarthritis (Regan et al., 2005), which suggests that SOD3 in the extracellular space is involved in skeletal processes. It is interesting to link this difference in SOD3 expression under 2% O_2 to the changes in ECM and ECM metabolizing enzymes and thereby osteoblast differentiation as discussed above. The concept that ECM, ECM turnover and SOD3 play a role in the control of osteoblast differentiation is subject to ongoing studies.

ROS levels also affect cell survival. High levels can activate signaling pathways that lead to the induction of apoptosis (Giorgio et al., 2005). Here we show that a decrease in ROS levels is accompanied by a decrease in apoptotic cells and a down regulation of the expression of apoptotic genes. In vivo, osteoblast apoptosis occurs frequently and plays a role in controlling bone metabolism (Weinstein et al., 1998). Apoptosis has also been shown to be actively involved in mineralization (Lynch et al., 1998). We also recently provided evidence that an increase in apoptosis leads to enhanced matrix mineralization (Bruedigam et al., 2010). Here we show that the osteoblast cultures with the highest level of apoptosis (20%) produce more mineralized matrix than cultures with less apoptotic cells (2%). To test the relationship between 2% and 20% oxygen, apoptosis and mineralization we increased ROS and induced apoptosis by adding H_2O_2. In this experiment, an increase in apoptosis was, however, not sufficient to overrule the inhibition of matrix mineralization in cells cultured under 2% O_2 (Fig. 4E). An explanation for this might be that in the 2% O_2 condition the cells are not properly differentiated and ECM maturation is inappropriate to lead to full mineralization.

Following this we focused on matrix formation. Overall collagen protein analyses in the ECM demonstrated a significant decrease in the 2% oxygen condition. However, 2% oxygen led to a significant increase in the expression of various collagen genes, an observation that fits with data found in rat calvarial osteoblasts after Northern analysis (Warren et al., 2001). A discrepancy between protein and gene expression is not unique and has been described before. These observations on collagen may be related to a negative auto-feedback system. In other words the level of collagen protein may regulate the expression of the collagen gene (Fouser et al., 1991). Alternatively, it has been reported that intracellular collagen fragments suppress collagen gene expression (Fouser et al., 1991; Ingber, 2006) but yet a definitive explanation for the protein–mRNA discrepancy remains elusive.

We hypothesized that an explanation for the differences in ECM between 2% and 20% oxygen may lie in differences in collagen metabolism. MMPs are the major enzymes involved in matrix remodeling and collagen break down and influence a great number of signaling processes. While degrading matrix, MMPs may release bioactive breakdown products, such as stored pools of growth factors, and disrupt matrix adherence (Giannelli et al., 1997; Lochter et al., 1997; Petitclerc et al., 1999; Alexander et al., 2001). In addition, they also can act directly on cell surfaces by releasing bioactive cell surface molecules (Suzuki et al., 1997; Powell et al., 1999) or cleaving surface signaling molecules (Levi et al., 1996; Sheu et al., 2001). MMP expression was significantly reduced by 2% O_2 indicating that it is highly unlikely that the difference in ECM mineralization can be explained by increased ECM turnover in the 2% O_2 condition.

Alternatively, it is tempting to speculate that MMP activity limited ECM turnover, is important for maturation of ECM and thereby mineralization. Thereby a reduction in MMP expression might partially contribute to the inhibition of bone formation and mineralization. This is supported by data from several MMP knock out models (Holmbeck et al., 1999; Zhou et al., 2000; Stickens et al., 2004; Mosig et al., 2007). Also mutations in the human MMP2 gene have been linked to multicentric osteolysis with arthritis (MOA), an osteolytic bone disease (Martignetti et al., 2001). All these data indicate that a decrease in MMP expression can have a negative influence on matrix formation and mineralization as observed in the 2% oxygen condition. Activity of MMPs is regulated by TIMPs. These proteins have been reported to inhibit MMPS by binding their catalytic domain (Willenbrock and Murphy, 1994), but also to activate MMPs (Strongin et al., 1995; Butler et al., 1998) and having growth promoting activity (Gomez et al., 1997). Interestingly, TIMP-3 may contribute to the increased apoptosis in the 20% oxygen condition as its expression is significantly higher in this condition and TIMP-3 has been shown to induce apoptosis of osteoblasts (Yuan et al., 2008).

The studies shown in Figure 6D are important for understanding the overall effect of oxygen on bone formation and mineralization. These data demonstrate that the oxygen concentration in the first week of osteoblast differentiation is crucial for a proper differentiation and eventual mineralization after 3 weeks. In other words, at low (2%) oxygen tension osteoblast differentiation is delayed compared to high (20%) oxygen tension leading to diminished mineralization after 3 weeks. This is supported by the observations on RUNX2 and ALP activity. Under 2% O_2 cells have not reached a certain state

of differentiation, produced the correct proteins, and created the proper environment to jump start mineralization at the correct point in time. It appears that cultures that have been switched back from 2% in the first week of culture to 20% O_2 thereafter try to catch up but they do not reach the full mineralization as seen when they were exposed to 20% in the first week.

In conclusion, this study shows the significance of oxygen tension for proper osteoblast differentiation, ECM formation, and eventual mineralization. Low oxygen tension affects the interplay between osteoblast differentiation and matrix formation at multiple levels, changing key players in the process. It affects apoptosis as well as matrix production, but also directly influences osteoblast differentiation and gene expression. We have demonstrated that the major impact of oxygen tension is in the early phase of osteoblast differentiation. Finally, induction of oxidative stress at non-toxic levels leads to a much higher increase in apoptosis in cells cultured under 2% O_2 and having reduced antioxidant enzymes protection compared to those cultured on 20% O_2 (Fig. 4B). These observations hold implications for human tissue regeneration, where cells are often moved from the body (low O_2) into a laboratory culture environment (high O_2) and back into the body (low O_2). This might affect not only their chances of survival but also their capacity to differentiate and form qualitative bone matrix.

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