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Research Article

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Gene Expression Under Combined Hypoxia And Acidosis In Chondrosarcoma

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Abstract

Chondrosarcomas are the second most common cause of bone cancer and are removed surgically with wide margins. On recurrence, they are resistant to chemo and radiation therapy and new treatment options are critically required. This tumor type produces hyaline cartilage, a cartilage normally formed under hypoxic and acidic environment due to lack of vasculature in cartilage. Paradoxically, chondrosarcomas arise in the well vascularized, oxygen rich environment of the bone. Hypoxia and acidosis are two stressors where the cellular effects are typically reported separately even though cells experience combined effects of hypoxia and acidosis. Given the mechanistic links between hypoxia and acidosis we hypothesized that gene expression profiles will be differentially changed when chondrosarcoma cells were exposed to individual compared to combined stressors. We investigated expression of four genes expressed during cartilage and cartilage tumor formation in primary chondrocytes and two grade II chondrosarcoma cell lines, SW1353 and JJ012. Two genes, PTH1R and SOX9 are known to respond to hypoxia and acidosis separately. Two genes, IDH1 and IDH2, are mutated in chondrosarcoma cell lines JJ012 and SW1353 respectively. These mutations confer a condition of false hypoxia on the cells through stabilization of HIF-1α. The result is chondrosarcoma cells metabolize glycolytically through aerobic glycolysis. How the cells respond to hypoxia and acidosis is of considerable interest as metabolically the cells are molecularly predisposed to these conditions. Our gene expression data found that combined hypoxia and extracellular acidosis alter gene expression compared to either stressor alone. Cells showed gene specific responses to stressors that were cell type specific likely indicating influence on gene expression regulatory sequences. The importance of this work is highlighting that conditions under which cells are investigated is crucial and should be considered when measuring cell response to in vitro treatment exposures.

Keywords: Chondrosarcoma, Hypoxia, Acidosis, Gene Expression, IDH

1. Introduction

The study of sarcomas is important because their extremely poor prognosis results in a high mortality rate. One particularly aggressive sarcoma, the chondrosarcoma, is the second most common form of bone cancer. Most chondrosarcomas are resistant to chemo-and radiation therapy with surgery being the treatment regimen of choice for primary tumors. If tumors reoccur, they are extremely aggressive. Treatment has not advanced, and metastasis is still an important burden for patient survival. It is estimated that one third of patients will not survive sarcomas due to metastatic spread.

Although the origins of chondrosarcoma are open to debate, they arise in bone, a blood and oxygen rich environment, and produce hyaline-like cartilage. Paradoxically, hyaline cartilage is normally devoid of a blood supply and chondrocytes, the cells that produce cartilage, exist in a hypoxic environment. In response to hypoxia, cells increase anaerobic metabolism which subsequently leads to an excess of lactic acid accumulation as a byproduct of glycolytic metabolism [1]. Cellular response to acidosis in chondrocytes in not well understood, but studies suggest acid-sensitive ion channels (ASICs) and intracellular calcium transit may cause changes in gene transcription [2].

Parathyroid hormone-1-receptor (PTH1R) is a G-protein receptor that mediates the effects of parathyroid hormone (PTH) and is a key regulator of articular chondrocytes, maintaining them in a non-hypertrophic state both at baseline and following injury [3]. Its ligand parathyroid hormone related protein (PTHrP) is known to be induced by hypoxia [4]. A mutation in PTH1R has been reported in enchondromatosis, a dominant disorder characterized by multiple cartilage tumors, frequently associated with skeletal deformity [5]. PTHrP/PTH1R prevent chondrocyte differentiation, keeping them in a proliferative state. *PTHrP/PTH1*R is known to positively regulate *SOX9* and persistent elevated levels would keep chondrocytes in a proliferative state [6].

SOX9 is a master regulatory gene which modifies the structure of chromatin at downstream target genes, thus orchestrating developmental transitions in a variety of tissues, including bone

[7]. In chondrocytes its expression is activated by hypoxia in a HIF1-α dependent manner, however, under acidic conditions *SOX9* expression is downregulated in chondrocyte culture [8,9].

Resident chondrocytes in human cartilage are exquisitely adapted to hypoxia and use it to regulate tissue-specific metabolism [10]. Two metabolic genes of interest that have been reported to undergo mutations in many cancer cells are *IDH1* and *IDH2* and are observed in chondrosarcoma, gliomas, Acute Myeloid Leukemia (AML), and melanoma [11]. They are present in 85% of enchondromatosis (multiple enchondromas), and in 52-59% of conventional and 57% of dedifferentiated chondrosarcomas [12]. Normal IDH produce the metabolite α-ketoglutarate which indirectly act to label HIF1-α for proteosomal degradation. Conversely, the oncometabolite D-2-hydroxyglutarate produced via mutant IDH does not label HIF-1α for degradation. Stabilized HIF-1α under aerobic conditions results in aerobic glycolytic metabolism. HIF-1α is a potent transcriptional factor involved in many cancer pathways and its presence is key in the tumorigenic process [13,14]. Therefore, although cartilage forming tumors exist in an oxygen rich environment, the presence of mutated IDH genes and stabilized HIF-1α create pseudo-hypoxia and the cells metabolize glycolytically by aerobic glycolysis.

The effects of combined hypoxia and acidosis are not fully understood, but models within solid breast tumor environments found hypoxia and lactic acidosis synergistically upregulate the unfolded protein response genes, increase inflammatory response genes, and inhibit canonical hypoxia response pathways. In this model, hypoxia regulator proteins such as HIF-1α were upregulated under hypoxic conditions. However, the tumor cells downregulated transcription of the HIF-1α gene in the presence of combined mild extracellular lactic acidosis and hypoxia [15]. Hypoxia/acidosis is a destructive manifestation of pathology for most cells, yet some cell types are particularly adept at functioning under these conditions and are found in solid tumors, corneal stroma, and cartilage. While some hypoxia-inducible genes (*VEGF* and *IL-8*) can be stimulated by acidosis, several other hypoxia-induced genes are repressed by acidosis (*CA9, GLUT1, OP, LDH*A), dependent on the cancer cell line [16-19]. However, hypoxia and acidosis are two stressors where the cellular effects are typically reported separately even though cells are experiencing the combined effects of hypoxia and acidosis. Given the mechanistic links between hypoxia and acidosis we hypothesized that gene expression profiles will be differentially changed when chondrosarcoma cells were exposed to individual compared to combined stressors. Our gene expression data found that both hypoxia and extracellular acidosis alter gene expression not only separately but also when combined. This study further highlights the importance of these stressors in cartilage and the cartilage forming tumors.

2. Materials And Methods

2.1. Cell Culturing, Morphology and Proliferation

JJ012 cells were kindly donated by Dr. J Block (Rush University Medical Center, Chicago, IL USA). SW1353 were obtained from the ATCC (MD USA). Both are grade two chondrosarcomas with different growth characteristics. Primary chondrocytes were obtained from costal cartilage of an apparently healthy 25-year-old female. All cells were grown in Dulbecco's modified eagle medium (DMEM), 10% fetal bovine serum, and 1% penicillin/streptomycin. Primary chondrocytes were grown at low pass (p4) due to dedifferentiation under continued cell culture.To examine cell proliferation in JJ012, SW1353, and the primary chondrocyte CON5, 10^4 cells were plated for each cell type in individual wells of a 6-well. Each cell type was trypsinized from their plate after 2-5 days of growth and counted using a hemocytometer. Data for the growth curve was plotted as cell number/time.

2.2. Growth under Hypoxia and Acidosis

Approximately $1x10^6$ cells were suspended in the following conditions: normoxia/pH=7.4 DMEM; normoxia/pH=5.5; hypoxia/pH=7.4 and hypoxia/pH=5.5. Normoxia- treated flasks were incubated at 37 ºC in humidified 5% CO_2 and 20% O_2 for 48 hours. Hypoxic- treated flasks were incubated at 37 ºC in humidified 5% CO_2 and 5% O_2 (balanced with 90% N_2) for 48 hours. All media used for hypoxic treatments were equilibrated for 48 hours by keeping the media in the hypoxic chamber. The media was adjusted to acidic level of pH=5.5 by adding 1M HCl solution to DMEM.

2.3. Reverse Transcription and Real-Time PCR Analysis

RNA was directly isolated from cells in tissue culture dishes, and genomic DNA eliminated using a Direct-zol™ RNA MiniPrep (Zymo Research, Irvine, CA, USA). Complimentary DNA (cDNA) was generated using an RT-First Strand Kit (Bioline, Swedesboro, NJ, USA). RNA and cDNA concentrations were measured using the Nanovue Plus Nanodrop (GE Healthcare, Little Chalfont, UK). Polymerase chain reactions (PCRs) were performed using SYBR green detection (Qiagen) using primers for Human PTH1R (NM_000316; PPH00740A); Human SOX9 (NM_000346; PPH02125A); Human IDH1 Human IDH1 (NM_005896; PPH06067A); Human IDH2 (NM_002168; PPH07326A); and Human ACTB (NM_001101; PPH00073G) (Qiagen) in a BioRad CFX96 system (BioRad, Hercules, CA, USA). Manufacturer guidelines were used for PCR reaction volumes and cycle parameters. The cycling parameters were 95ºC for 10 minutes to activate the hotstart polymerase, then 40 cycles of 95ºC for 15 seconds and 60ºC for 60 seconds. Reaction specificities were assessed with a melt curve of 65ºC to 95ºC in 0.2ºC increments. Gene expression was calculated as $2^{-(CqGOI - CqRef)}$, where CqGOI is the Cq value of the gene of interest and CqRef, is the Cq value for the reference gene. The delta Cq of each sample was normalized by subtracting the ΔCq cutoff set at a Cq of 30 ($2^{-(Cqcutoff-CqRef)}$). Fold expression values were normalized in log2 relative to their respective gene expressions in normoxia/pH=7 to determine which genes were up (+) and down (-) regulated +/- standard error of mean.

2.4. D-2-Hydroxyglutarate Assay (Colorimetric)

D-2-hydroxygluterate concentration levels in SW1353, JJ012, and primary chondrocytes (CON5) cell lysate were measured by using a colorimetric assay kit (Abclonal ab211070) according to manufacturer's guidelines. *IDH1/IDH2* mutants in JJ012 and SW1353 were indirectly confirmed due to elevated D-2-HG

elevated concentration levels when compared to the IDH wild type CON5. The cell lysates were derived by cultures that were grown in normoxia/pH=7.

2.5. Data Analysis

A two-way analysis of variance ANOVA (nonparametric) was conducted for each gene followed up by Bonferroni post-test to pinpoint the source of variance between the treatments within each cell line. For D-2-Hydoxygluterate data, a one-way ANOVA (nonparametric) was applied followed by Tukey's test. The significance value was set at $p<0.05$. GraphPad Prism software was used to generate the graphs with mean and standard error of mean (SEM).

3. Results

3.1 Growth Characteristics

SW1353 and JJ012 are both classified as primary human chondrosarcoma grade II yet they exhibit distinct morphological features (Figure 1). The JJ012 are mainly polyhedral inshape, contain enlarged hyperchromatic nuclei, and are often binucleated. Mitotic figures are common, and there is distinct cell atypia and pleomorphism. This has led some researchers to treat this cell line as more closely related to dedifferentiated chondrosarcoma [20]. SW1353 resembles CON5 cells. Unlike CON5, SW1353 exhibit moderate cell atypia and pleomorphic characteristics. Lastly, the CON5 primary cells exhibit cell nuclei that are considerably smaller than those of SW1353 and JJ012, and there is a lack of mitotic activity and cellular atypia.

Growth characteristics of JJ012 are more aggressive with a short doubling time (~24hrs) compared to SW1353 and CON5 (both ~48hrs) (Figure 2).

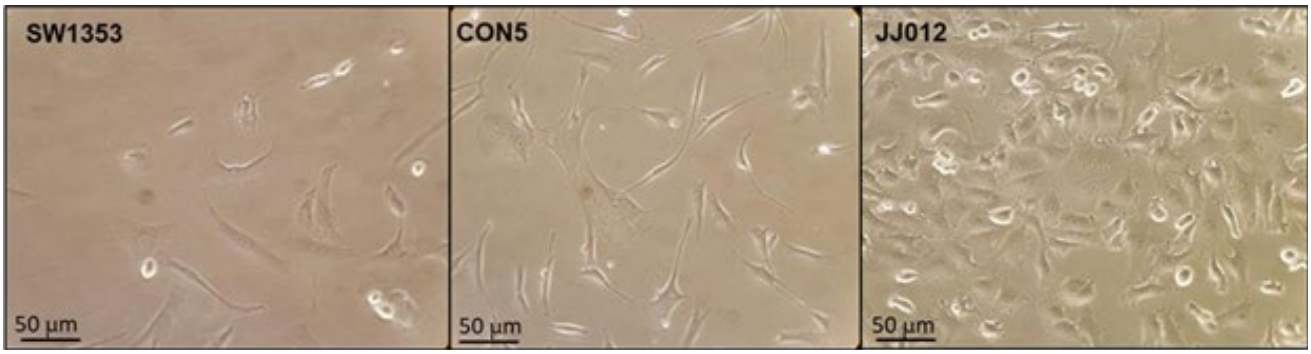

Figure 1: SW1353/CON5/JJ012 phenotypical profile. Enlarged nuclei and mitotic figures can be observed in JJ012 and SW1353. Morphologically, SW1353 cells appear very similar to primary chondrocytes.

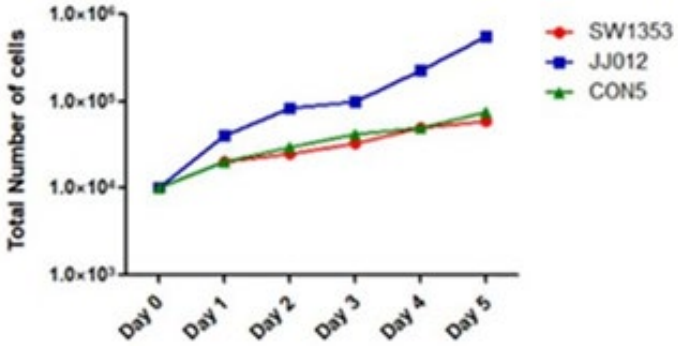

Figure 2: Cell proliferation under normoxia/pH7.4. JJ012 cells display an aggressive growth pattern with a doubling time of approximately 23-24 hours compared to SW1353 and primary chondrocytes CON5. For these cell lines the doubling time is approximately 46-48 hours.

3.2 Gene Expression under Hypoxia, Acidosis, and Combined Hypoxia/Acidosis

3.2.1. *PTH1R* Expression

In hypoxia, expression of *PTH1R* is marginally downregulated in SW1353 (-0.18 ± 0.25) compared to JJ012 (+1.85 ± 0.31) and CON5 (+1.29 ± 0.04) that both show upregulation.
Differences in expression between cell lines is significant: JJ012 versus SW1353 ($p<0.001$) and CON5 versus SW1353 ($p<0.05$).

Under acidosis, CON5 and SW1353 are slightly upregulated (+0.84 ± 0.03 and +1.00 ±0.31 respectively) while JJ012 is downregulated (-1.04 ± 0.12). The difference in expression between JJ012 and SW1353is significant ($p<0.01$) and JJ012 compared to CON5 ($p<0.01$).

In hypoxia /acidosis, CON5 and SW1353 are upregulated (+2.10 ± 0.84 and +1.77 ± 0.37 respectively). JJ012 is slightly downregulated (-0.33 ± 0.08). Expression is significantly different between JJ012 versus CON5 $p<0.001$ and JJ012 versus SW1353 $p<0.01$).

Overall, *PTH1R* was upregulated in primary chondrocyte under all conditions, with the largest expression in the combined conditions. Hypoxia had an upregulating effect on JJ012, while acidosis induced downregulation. The synergistic effect under combined conditions was one of marginal downregulation. The effects on SW1353 were similar to CON5 under acidic exposure, whether in combination with hypoxia or alone, whereas hypoxia alone resulted in downregulation (Figure 3).

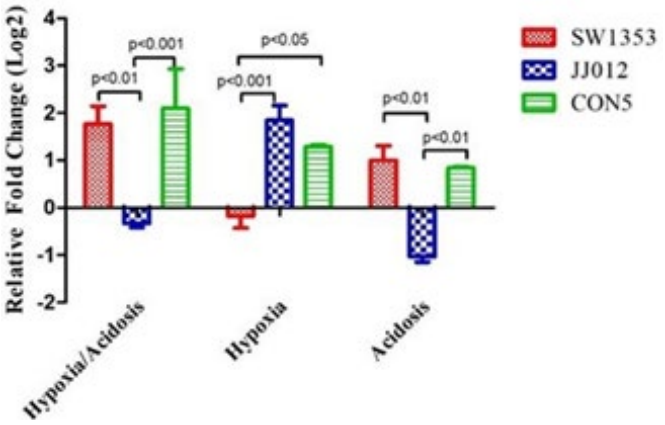

Figure 3: *PTH1R* relative gene expression. The log2 fold change in *PTHIR* expression is shown for each cell line in all three experimental conditions. Relative fold changes were normalized to expression under normoxia/pH=7.4. Positive values represent upregulation and negative values downregulation from the control.

3.2.2. *SOX9* Expression

Hypoxia alone (Figure 4) induces marginal changes in *SOX9* expression (JJ012 +0.44 ± 0.20; SW1353 -0.7 5 ± 0.19; and CON5 -0.74 ± 0.09). The difference in expression between cells is significant (JJ012 vs SW1353 p<0.01; JJ012 vs CON5 p<0.001).

In acidosis, *SOX9* expression in primary chondrocyte is upregulated (+2.60 ± 0.90) whereas SW1353 and JJ012 are only marginally changed (JJ012 -0.11 ± 0.39; SW1353 +0.55 ± 0.04). The expression is significantly different between chondrocyte and chondrosarcoma cells (CON5 vs JJ012 p<0.001; CON5 vs SW1353 p<0.001).

In hypoxia /acidosis, primary chondrocyte is significantly upregulated (+2.36 ± 0.09) and that expression is significant when compared to JJ012 and SW1353 (p<0.001 for both respectively). Moreover, SW1353 shows negligible difference from normoxia/pH=7.4 (+0.05 ± 0.16), while JJ012 is downregulated (-0.84 ± 0.06). Acidosis appears to be the overriding stimulus in chondrocytes whereas in SW1353 and JJ012 the individual changes induced by a single stimulus appear to cancel under combined conditions.

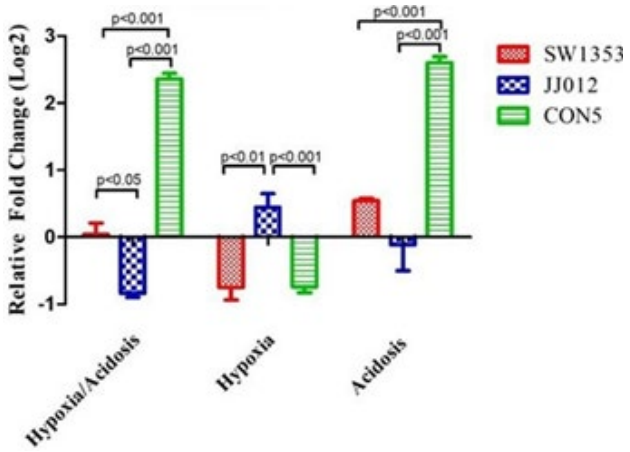

Figure 4: *SOX9* relative gene expression normalized to expression at normoxia/pH 7.4. The log2 relative fold change in *SOX9* expression is shown for each cell line in all three experimental conditions. The most notably significant difference is the acidosis dependent (>2.0 log fold) upregulation of *SOX9* expression in CON5 cells, and largely independent relationship from hypoxia condition.

3.2.3. *IDH1* Expression

Under hypoxia alone, *IDH1* expression in SW1353 was marginally upregulated from the control (+0.04 ± 0.10); in JJ012, it was slightly more upregulated (+0.50 ± 0.18) and in CON5, it was slightly downregulated (-0.15 ± 0.04). There was no statistically significant difference of *IDH1* expression between the cells in hypoxia alone.

When acidosis is the only stressor, *IDH1* expression in SW1353 was slightly upregulated (+0.37 ± 0.16); in JJ012, it was downregulated (-0.83 ± 0.29) and in CON5, it was minimally downregulated (-0.07 ± 0.08). There is a significant difference in expression between JJ012 versus SW133 (p<0.001) and JJ012 versus CON5 (p<0.05).

In the combined conditions of hypoxia/acidosis, *IDH1* expression in SW1353 was slightly downregulated (-0.15 ± 0.20); in JJ012 it was upregulated (+1.19 ± 0.31) and in CON5 it showed slight upregulation (+0.14 ± 0.23). This expression was significantly different between JJ012 and SW1353 (p<0.001) as well as between JJ012 and CON5 (p<0.01).

Hypoxia and acidosis, alone or combined, had little effect on expression of *IDH1* in primary chondrocytes and SW1353 cells. JJ012 cells showed downregulation under acidosis, a marginal increase under hypoxia, and a synergistic upregulation under combined conditions.

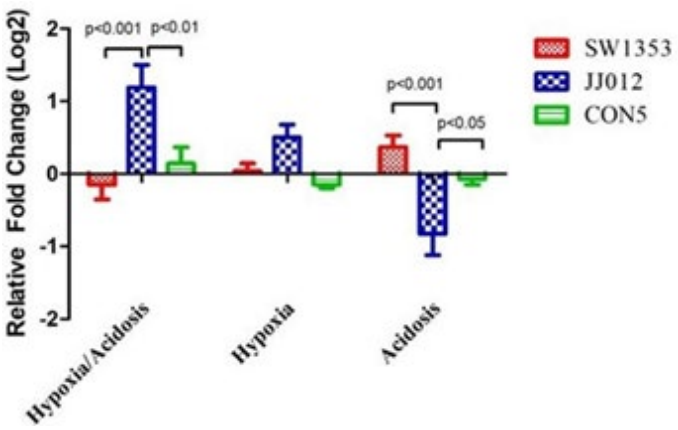

Figure 5: *IDH1* relative gene expression. The log2 fold change in *IDH1* expression is shown for each cell line in all three conditions: Hypoxia/acidosis, hypoxia, and acidosis. Notice the negligible change of expression in SW1353 and CON5 from the control in all conditions. JJ012 is an *IDH1* mutant, while CON5 and SW1353 are *IDH1* wild type.

3.2.4. *IDH2* expression

Hypoxia alone upregulates *IDH2* expression in JJ012 (+1.36 ± 0.42) and SW1353 (+0.22 ± 0.12) while it is downregulated in CON5 (-0.46 ± 0.08). The expression is significantly different between JJ012 and SW1353 ($p<0.01$) as well as between JJ012 and CON5 ($p<0.001$).

When acidosis is the only stressor, the *IDH2* expression is upregulated in all cells SW1353 (+1.58 ± 0.15); JJ012 (+1.47 ± 0.28) and CON5 (+2.02 ± 0.10) with no statistically significant difference in IDH2 expression between the lines. *IDH2* expression in all three cell lines is upregulated under combined conditions: SW1353 (+0.85 ± 0.15); JJ012 (+1.54 ± 0.19) and CON5 (+2.10 ± 0.84). There is no statistically significant difference in *IDH2* expression between the cells.

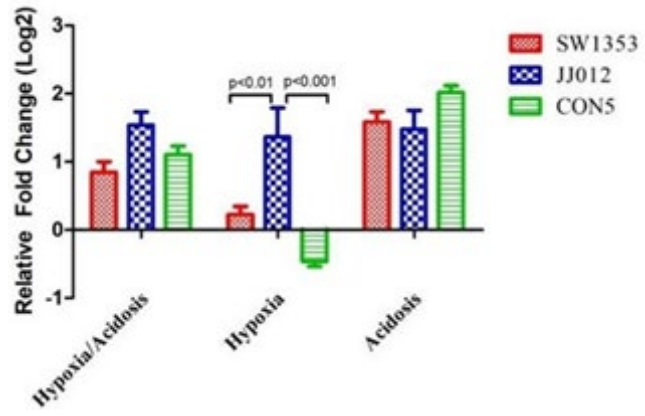

Figure 6: *IDH2* relative gene expression. The log2 fold change in *IDH2* expression is shown for each cell line in all three conditions. Notice the consistent and significant upregulation of all three cell lines under acidosis alone.

3.3. Elevated D-2-Hydroxygluterate concentration levels in chondrosarcoma

D-2-Hydroxygluterate (D-2-HG) is an oncometabolite and is produced in cells that harbor *IDH1/IDH2* mutations. IDH wild-type converts isocitrate into α-ketoglutarate, therefore D-2-HG levels should be minimal. D-2-HG was the most elevated in JJ012, followed by SW1353 and lowest in CON5 (Figure 7). One-way ANOVA and Tukey's posttest established a significant difference in D-2-HG concentration levels between JJ012 and SW1353 ($p<0.01$). The difference between SW1353 and CON5 was also significant ($p<0.01$). The most pronounced significance was observed between JJ012 and CON5 ($p<0.001$). Indeed, the D-2-HG level in JJ012 was 10.5-fold higher than that in CON5.

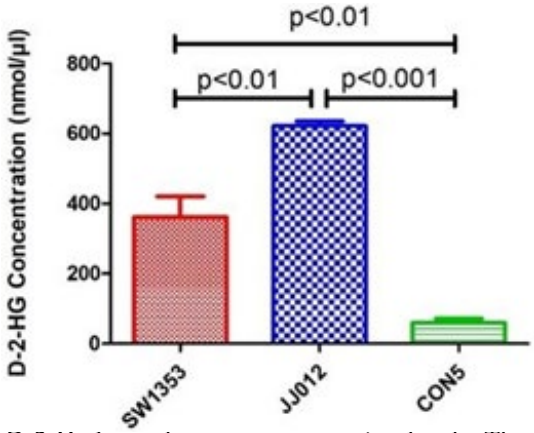

Figure 7: D-2-Hydroxygluterate concentration levels. There is a significant increased concentration of D-2-HG in JJ012 (621.9 nmol/µl) and SW1353 (361.6 nmol/µl) in comparison to CON5 (59.6 nmol/µl). D-2-HG concentration was measured from cells grown in normoxia/pH=7.4.

4. Discussion

Chondrocytes, the cartilage forming cells, reside under an environment toxic to most cells due to lack of vasculature, persistent hypoxia (1-5%), and related acid production through glycolytic metabolism. Paradoxically, chondrosarcoma, the cartilage forming tumors often arise in bone, a well vascularized tissue with normoxia and physiological pH. We chose relatively severe acidosis as chondrocytes are known to experience these levels and we have previously measured expression of *ASIC2* in costal chondrocytes, an ion channel known to be active at low pH [2]. In pathological conditions of articular cartilage such as osteoarthritis and rheumatoid arthritis, the tissue pH become acidic, falling to approximately pH 5.5 [21].

The *IHH-PTHrP* signaling pathway is implicated in the pathogenesis of many types of benign cartilage tumors. Enchondromas, osteochondromas and chondroblastomas all express PTHrP and its receptor PTHR1 [22]. Parathyroid hormone related protein (PTHrP) activates expression of its receptor PTHR1, delaying hypertrophy in chondrocytes and keeping them in a proliferative mode. Human chondrocytes cultured under hypoxia enhance PTHrP expression but its effect on PTHR1 under these and combined conditions is not well described [23].

Our data shows increased expression of *PTHR1 in* chondrocytes under hypoxia and acidosis, with a further increase under combined conditions suggesting an additive effect. Chondrosarcoma cells appear to have different responses that may reflect the genetic heterogeneity in cartilaginous tumors.

Morphologically, SW1353 cells appear more chondrocyte-like and show a similar response to primary chondrocytes under acidosis and combined hypoxia and acidosis. JJ012, a more undifferentiated cell type shows a rigorous response under hypoxia alone that is cancelled in the presence of acidosis.

SOX9 is expressed throughout the life of a permanent chondrocyte and is repressed as a chondrocyte differentiates towards hypertrophy. Aggrecan and collagen type II production is partly under control of *SOX9* and make up the bulk of extracellular matrix. Our data shows normal chondrocytes upregulate *SOX9* as a response to acidosis but not hypoxia. Responses to individual stressors can be expected, and acidosis has been shown to override oxygen deprivation and induce mitochondria remodeling in mouse cortical neurons to preserve ATP production although this has not been shown for chondrocytes at low pH. Interestingly, low levels of expression of *SOX9* in chondrosarcoma cells may be related to mutations in *IDH1 and IDH2* [24].

The accumulation of D-2-HG in chondrosarcoma cells because of IDH mutations has recently been shown to reduce expression of *SOX9* through higher hypermethylation of the promoter region in MSC induced chondrocytes [25].

There is considerable interest in the expression of *IDH1* and *IDH2*, the use of mutant IDH inhibitors, and the demonstration of anti-tumor activity. Many cell culture experiments continue to use normoxia/physiologically normal pH when investigating expression of these mutant genes. Our data shows sensitivity of these genes to hypoxia and acidosis. JJ012 has mutated *IDH1* gene and SW1353 a mutated *IDH2* gene [26]. Expression of *IDH1* in JJ012 exposed to hypoxia and acidosis certainly appears to exhibit sensitivity. There is significant decrease in expression under acidosis, a slight increase under hypoxia, yet under combined conditions there is a significant increase. Contrary to this, SW1353 showed little change in expression of *IDH1* except under acidosis. Why different cell types differ in their response to hypoxia is an unanswered question. The mutated *IDH1* gene in JJ012 may be subject to changed methylation at its promoter region, there is evidence for rapid induction of histone methylation in many cell lines induced by hypoxia that is HIF-1α independent [27]. Hypoxia induces HIF-1α expression, however hypoxia induced changes in methylation may also affect binding of HIF-1α and subsequent expression of downstream genes [28]. An investigation of the methylation status of *IDH* promoter sequences would of great interest in different chondrosarcoma cell lines. There is little evidence for acidosis induced changes to DNA methylation status in humans, therefore changes in expression under acidosis may involve different, yet unidentified, mechanisms.

The tumor pH is low due to glycolytic metabolism and production of lactic acid. Low pH is known to affect tumor cell function, including local invasiveness and metastatic spread [29-31]. Acidosis exposure increased expression of *IDH2* in all cell types. Under hypoxia only JJ012 showed significant increase in expression, whereas SW1353 showed a small increase and control cells a small decrease in expression. Combined conditions showed an increase in *IDH2* expression in all cell types. Although JJ012 and SW1353 are both grade II chondrosarcoma, there are many differences in cell morphology and growth characteristics. Differences in expression of *IDH1* and *IDH2* under hypoxia and acidosis may clearly be due to individual tumor characteristics, albeit they are both classified as grade II chondrosarcoma.

5. Conclusions

This work underlines the effect of culture conditions on chondrosarcoma and chondrocyte activity and that studying chondro-related biology at physiological levels of oxygen and pH may be inappropriate. Ambient values of 21% O_2 represent abnormally high levels of oxygen and lead to potentially inappropriate responses. This may be particularly important in the response of cells to gene targeted therapies where expression under relevant physiological conditions is more meaningful. Although we hypothesize that combined hypoxia and acidosis will modulate gene expression it is apparent that there are different mechanisms responsible for modulation under different conditions, and how these operate under combined stress remains to be determined. Mutant IDH genes result in accumulation of D-2- hydroxyglutarate, stabilization of HIF-1α, and aerobic glycolytic metabolism. This is a confounding factor where cells are still responding to oxygen on one hand while metabolizing as if under hypoxia and dissecting the mechanisms for gene expression profiles will remain a challenge under these conditions.

Acknowledgements

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