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PTHR1/SOX9 and *IDH1/IDH2* Relative Expression in Primary Chondrocyte and Chondrosarcoma Cells Under the Synergistic Influence of Inducible Hypoxia and Extracellular Acidosis

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PTHR1/*SOX9* AND *IDH1*/*IDH2* RELATIVE EXPRESSION IN PRIMARY CHONDROCYTE AND CHONDROSARCOMA CELLS UNDER THE SYNERGISTIC INFLUENCE OF INDUCIBLE HYPOXIA AND EXTRACELLULAR ACIDOSIS

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ABSTRACT

PTHR1/*SOX9* AND *IDH1*/*IDH2* RELATIVE EXPRESSION IN PRIMARY CHONDROCYTE AND CHONDROSARCOMA CELLS UNDER THE SYNERGISTIC INFLUENCE OF INDUCIBLE HYPOXIA AND EXTRACELLULAR ACIDOSIS

Kostika Vangjeli
Old Dominion University, 2020
Director: Dr. Christopher Osgood

Cartilage cells (Chondrocytes) grow in rather unique environmental conditions in the human body. Cartilage is avascular tissue and lacks innervation. Its main source of nutrients is derived from the synovial fluid and/or perichondrium. Consequently, these cells must survive and thrive under hypoxic and acidic stressors. Published data suggests that there are a multitude of genes affected from either one of these two stressors or both. However, these factors are frequently overlooked in cartilage research, and results are reported in either normoxia/pH=7.0 conditions, or they only account for one of the conditions. The scope of this study is to examine how these stressors affect gene expression in primary chondrocytes and chondrosarcomas. In this study, one primary chondrocyte cell line (CON5) and two chondrosarcoma grade II cell lines, JJ012-*IDH1* mutant and SW1353- *IDH2* mutant, were grown in four experimental conditions: hypoxia (5% O_2), acidosis (pH=5.5), hypoxia and acidosis, and normoxia/(pH=7). Four genes of interest were analyzed via RT-qPCR relative to the *ACTB* housekeeping gene: parathyroid hormone receptor-1 (*PTHR1*), SRY-box transcription factor 9 (*SOX9*), and isocitrate dehydrogenase 1 and 2 (*IDH1/IDH2*). *PTHR1* and *SOX9* keep chondrocytes in a proliferative state and delay their hypertrophy. On the other hand, *IDH1* and *IDH2* are metabolic enzymes that convert isocitrate to α-ketoglutarate (α-KG). Their mutant counterparts further convert α-KG into a competitive oncometabolite D-2-Hydroxygluterate (D-2-HG). Our colorimetric assay data suggest that D-2-HG concentration levels are 10.5-fold and 6-fold more elevated in JJ012/SW1353 respectively than in the *IDH* wild type CON5. Our gene expression data indicates that both inducible 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 biology research.

This thesis is dedicated to those patients who have been affected by cartilage-related diseases and especially from chondrosarcoma.

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CHAPTER 1
INTRODUCTION

1.1 Effects of Inducible Hypoxia and Extracellular Acidosis in Chondrocytes

Cartilage cells are unique compared to "traditional" cells in the human body in that they have no direct access to a vascular bed and therefore rely on metabolic pathways to survive. As a result, they operate in a hypoxic environment (<5% O_2) marked by the expression of hypoxia inducible factors (HIFs.) These transcriptional factors help chondrocytes adapt to hypoxia and are known to induce parathyroid hormone-related protein (PTHrP) expression and its main receptor and mediator parathyroid hormone receptor-1 (PTHR1) (Manisterski, 2010). Primary chondrocytes have tightly regulated cycles of proliferation, differentiation, and hypertrophy. This process is governed by a negative feedback loop between PTHrP and Indian hedgehog proteins (IHH) (Fig. 1). However, when this loop is thrown off balance, it can result in an overexpression of PTHrP, which is a hallmark of many bone cancers (Mak I.W., 2013; Johnson, 2011).

As a result of lack of oxygen, chondrocytes metabolize glucose via glycolysis. One of the by-products of this reaction is lactate, leading to acidosis. Cancerous cells also share a similar acidosis environment. They not only operate in hypoxic microenvironments, but often rely on glycolytic instead of oxidative metabolism. In the 1920s, Otto Heinrich Warburg and his colleagues observed that cancer cells uptake significantly more glucose than normal cells and they do so anaerobically even when oxygen is present (Warburg, 1927).

1.2 IHH-PTHrP Loop – *PTHR1* Gene

Parathyroid hormone (PTH) plays a crucial role in bone formation by maintaining Ca^{2+} homeostasis. PTH is largely produced by chief cells in response to low Ca^{2+} in blood, which triggers a sequence of signals that lead to bone resorption. Parathyroid Hormone-related proteins (PTHrP) are comparable to PTH in that they bind to similar receptors, mainly PTHR1. These peptides are key regulators in the proliferation, differentiation, and death of many cell types. In the case of chondrocytes, they prevent their differentiation, keeping them in a proliferative state which results in delayed hypertrophy. The IHH-PTHrP is a negative feedback loop that keeps the rate of chondrocyte differentiation tightly regulated (Vortkamp, 1996). The IHH proteins are produced by chondrocytes in the postmitotic pre-hypertrophic zone (Iwasaki M, 1997). PTHrP,

on the other hand, are produced by chondrocytes in the resting and proliferating zones. A drop in the PTHrP levels signals the chondrocytes in the pre-hypertrophic zone to diffuse IHH in the resting zone and produce PTHrP. However, as the PTHrP levels increase, they downregulate the IHH, explaining their polarized expression and localization (Fig. 1).

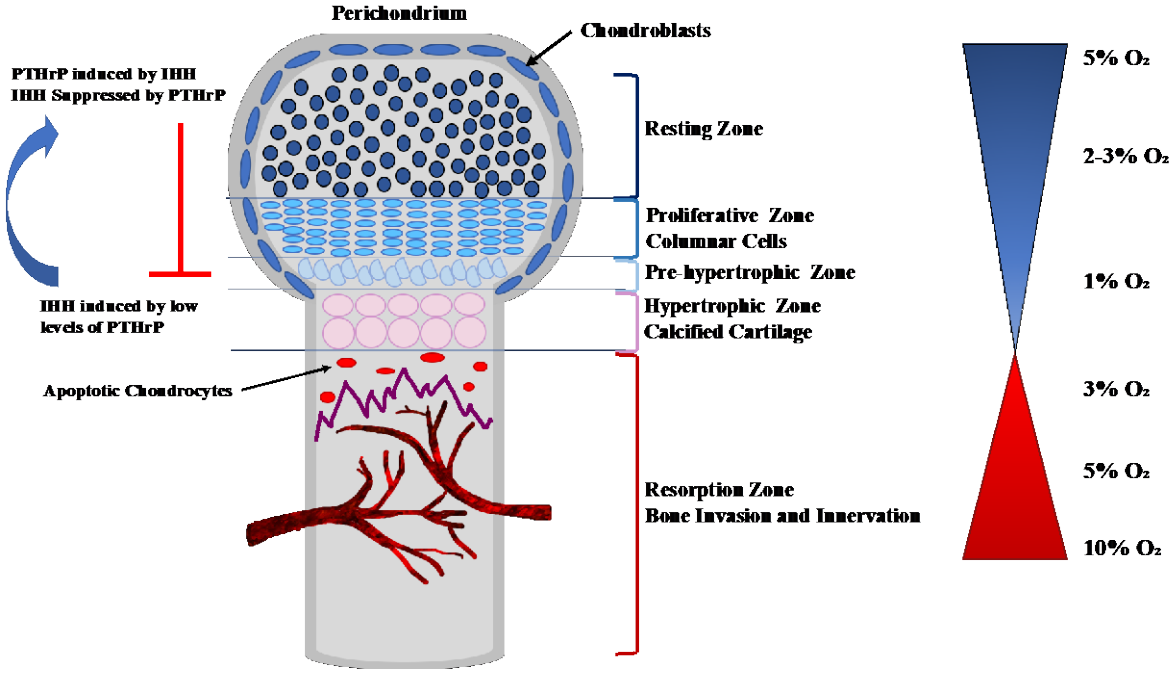

Fig. 1. Cartilage zones in epiphysial plate of long bone with oxygen permeation levels and (IHH/PTHrP loop). IHH protein secreting chondrocytes are found in the postmitotic pre-hypertrophic region. They induce PTHrP production in the Resting Zone (RZ) in response to a reduced level of PTHrP in the pre-hypertrophic zone. PTHrP secreting chondrocytes are in the RZ and as their levels increase, they gradually downregulate IHH expression in the RZ. In the proliferating zone (PZ), chondrocytes develop a very distinct columnar shape and arrangement in the direction of the bone growth. On the right, Gradient decrease of oxygen levels from the perichondrium (5%) to the hypertrophic zone approximately (1%). Resorption/Ossification Zone, oxygen levels gradually increase as the cartilage is calcified and replaced with bone matrix and blood vessels penetrate the haversian system along with innervation.

PTHrP and their associated receptors are also known to be induced under hypoxic conditions via *HIF-1α* and *HIF-2α* genes (Zhao H. L.-L., 2009; Manisterski, 2010).

The following is the cellular cascade on the effects of PTHrP on delaying chondrocyte differentiation. This process is regulated by the phosphorylation of SOX9:

→*PTHrP* ↥ →*PTHR1* ↥

→cAMP & Protein Kinase A production ↥

→Phosphorylation of *SOX9* transcriptional factor ↥

→Inhibition of *p57* (apoptotic gene) ↓

→Expression of anti-apoptotic *Gli3*, *Bcl*-2 ↥

→*Runx2* and *Runx3* (crucial genes in differentiation.) ↓

1.3 *SOX9* gene

SOX 9 is a multi-functional transcriptional factor, and a chief regulator in sex determination (Jakob, 2011). What is of interest to our study is that *SOX9* is involved in chondrogenesis and is highly expressed in chondrocytes (Asou, et al., 2006). However, this expression is not uniform throughout the chondrogenic process. *SOX9* expression is initially required to commit undifferentiated mesenchymal stem cells to the osteochondrogenic process. It is further required to transition those cells to condensed mesenchymal cells. At this stage, *SOX9* activates *SOX5* and *SOX6* and their co-expression is required to commit these cells to differentiate into chondrocytes. During the proliferation phase, *SOX9* prevents the conversion of chondrocytes into hypertrophy. As expected, *SOX9* expression is nonexistent past the hypertrophic zone (Zhao, et al., 1997).

Extensive studies in mice embryos have shown that deletion of *SOX9* results in drastic skeletal chondroplasia, where the cells did not go past the condensation phase (Akiyama, et al., 2002). Moreover, *SOX9* is part of the cascade that blocks apoptotic gene p53 and halts chondrocytes from undergoing hypertrophy (Ikegami, et al., 2011). The rationale for picking this gene is that *PTHrP*/*PTHR1* is known to positively regulate *SOX9* and persistent elevated levels would keep chondrocytes in a proliferative state (Huang, et al., 2000).

1.4 *IDH1* and *IDH2* genes

Two metabolic genes of interest that have been reported to undergo mutations in many cancer cells are *IDH1* and *IDH2*. In healthy cells, isocitrate dehydrogenase (IDH) is an enzyme involved in the conversion of isocitrate into α-ketoglutarate (α-KG) (Fig. 2). One of the byproducts of this reaction is the conversion of NAD+ to NADPH, which among a multitude of

other functions also plays a role in producing reactive oxygen species (ROS) in neutrophils and reducing oxidative stress. This is a bidirectional reaction which depending on cellular needs can result in α-KG production or consumption (Metallo, 2011). There are three known forms of IDH. One is located strictly in mitochondria (IDH3) and is not known to undergo any clinically relevant mutations (Bhagavan, 2011). Different from *IDH1* and *IDH2*, *IDH3* is involved in the conversion of NAD^+ to NADH.

Mutant IDH are observed in many clinical conditions. They are present in 85% of enchondromatosis [multiple enchondromas (ENC) which are benign tumors], in 52-59% of conventional CS, and 57% of dedifferentiated CS (Amary, et al., 2011). In the case of mutated IDH1 and IDH2, the reaction does not end with the production of CO_2 and α-KG but the latter is further converted into D-2-hydoxygluterate (D-2-HG), which is an oncometabolite and considered to be an α-KG antagonist (Yang, 2012).

D-2-HG has a variety of pathways that can lead to oncogenesis, but one that is of interest is that it induces the HIF-1α by blocking the prolyl hydroxylase (PHDs) from marking HIF-1α for degradation by the von Hippel-Lindau (VHL) proteins. PHDs are oxygen-sensing enzymes that under normoxia can hydroxylate both HIF-1α/2α (Yong, 2018).

1.5 Chondrosarcoma Grade Characterization

Primary bone cancers are the rarest (<1%) of all bone cancers. Different from the (secondary) metastatic bone tumors that spread from elsewhere from the body to the bones, the primary cancers originate in the cartilage and bones themselves. Despite its rarity, as of 2019 it was estimated that there were 3,500 new diagnoses of primary bone cancer in United States. Nearly half (approximately 1660) of the above-stated number are expected to result in death (American Cancer Society, 2019). Chondrosarcomas (CS) are the second most common form of primary bone cancer with osteosarcomas topping the list (Siegel, 2018). This high-grade CS exhibits an aggressive nature marked by resistance to radiation and chemotherapy. As a result, the best treatment option is via surgical resection. However, low-grade CS have been successfully treated by clearing the tumor via curettage, followed by liquid nitrogen cryotherapy, slow thawing, and final bone grafting and fixation (Gelderbloma, 2008). CS are categorized into three grades, which consider multiple factors (Fig. 3; Fig. 4).

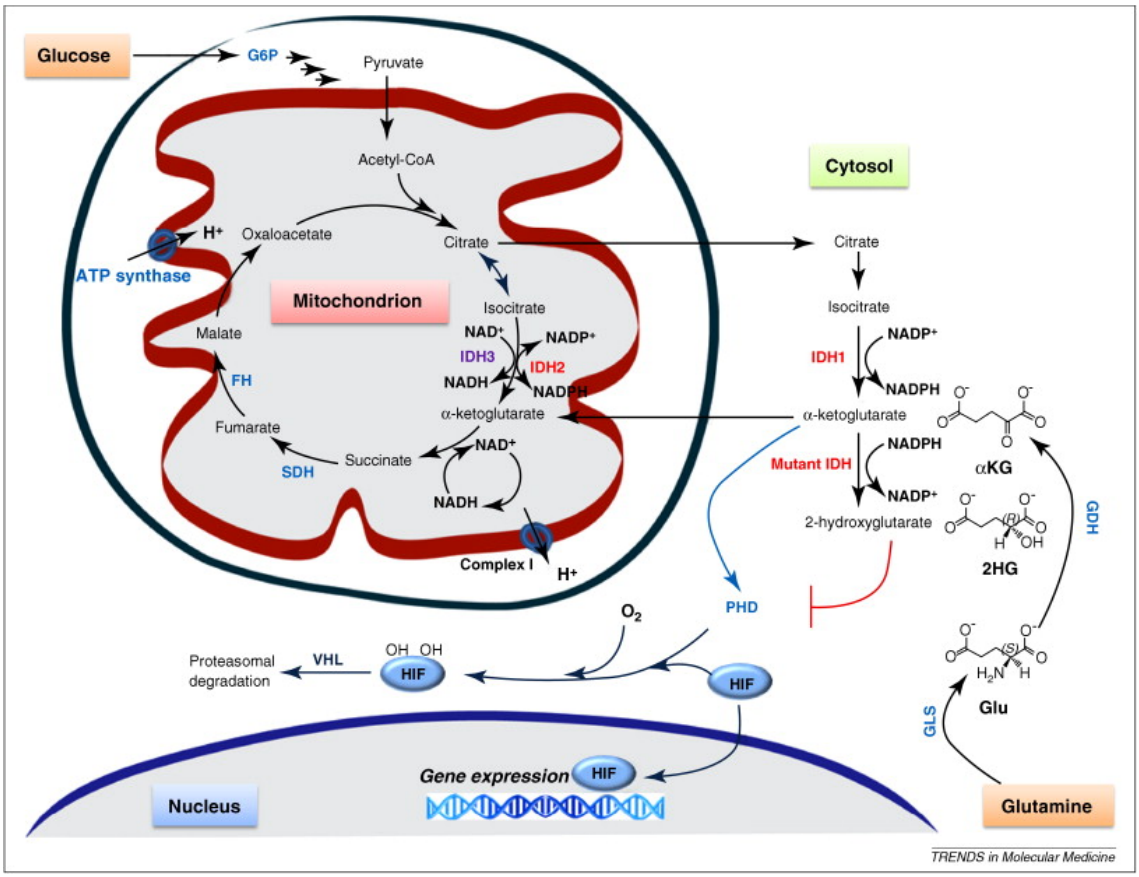

Fig. 2. The role of IDH in cancer metabolism of gliomas and AML.
Enzymes of the TCA cycle in the mitochondria and mutated IDH in the cytosol are represented. The hypothesis of how 2HG produced by mutated IDH might act through the accumulation of HIF1α via PHD inhibition leading to cancer development is shown schematically.
Abbreviations: FH-fumarate hydratase; SDH-succinate dehydrogenase; PHD-prolyl hydroxylase; GLS-glutaminase; GDH-glutamate dehydrogenase; G6P- glucose 6-phosphate. The IDH1/IDH2/IDH3 is a bidirectional synthesis of α-KG and isocitrate (not shown in picture). IDH1 mutant pathway plays a pivotal role in further converting α-KG into D-2-HG. Notice the difference in structure between the two compounds is the sole replacement of a single oxygen in α-KG on the second carbon with a hydroxyl group which gives rise to D-2-HG.
*The above picture has been properly cited and re-printed with the author's written permission. (Dang, 2010)

*Enchondroma – (ENC) benign cartilage forming tumor

*Chondrosarcoma – (CS) malignant cartilage forming tumors tumor

*Primary or Conventional Chondrosarcoma – (CCS) arise in pre-existing normal bone and are common

*Non-Conventional Chondrosarcoma – include dedifferentiated CS, clear cell CS and Mesenchymal CS: less common

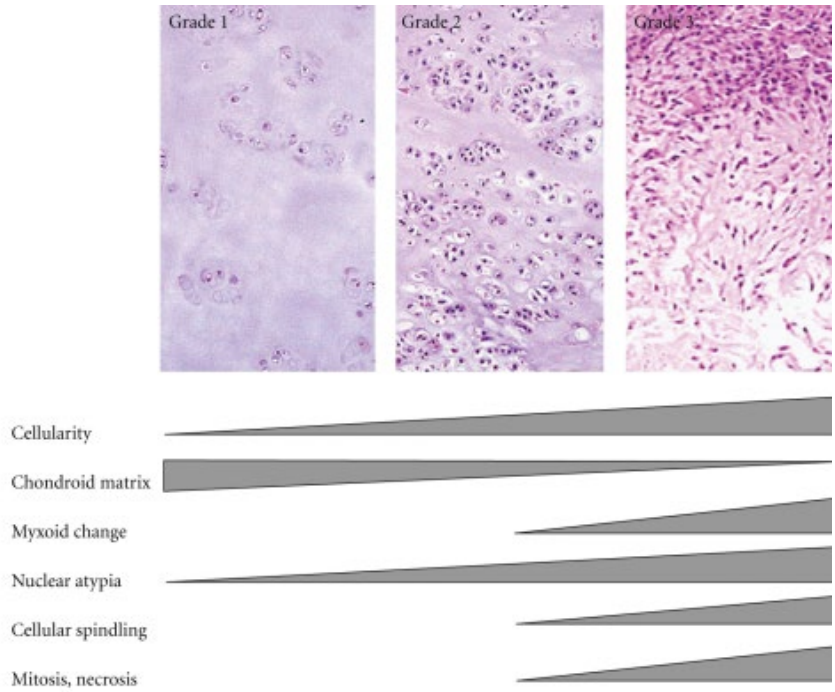

Fig. 3. Chondrosarcoma grade classification. Multiple criteria are taken in consideration when determining the grade classification of chondrosarcoma. Cellularity increases from lower grade to higher ones. Chondroid matrix decreases. Myxoid matrix becomes present in grade II chondrosarcoma and more pronounced in grade III but is missing in grade I. Nuclear atypia is increasingly more observable as we move from grade I-III. Cellular spindling, mitosis and necrosis are observable mostly in grade II and III and absent in grade I.
 * (Kim et al., 2010)

Note the degree of cellularity with the tumor grade progression (Fig. 3). Hyaline cartilage is hypocellular exhibiting low cellularity. Chondroid matrix is abundantly being secreted. As the tumor progresses from grade I-III the amount of chondroid matrix decreases while cellularity increases. The nature of ECM changes with the grade progression. In grade II and higher, it consists of part-hyaline chondroid cartilage, part-myxoid. Myxoid cartilage is defined as when the chondrocytes no longer reside in lacunae spaces and develop cytoplasmic processes. In addition, mitotic activity is mainly absent in ENC and low-grade CS. It progressively becomes more present and hypercellular in grades II and III. Nuclear atypia strictly refers to the size of

cell nucleus. Grade I chondrosarcoma cells have nuclei that are 2-3 times larger than those of normal chondrocytes. With grade progression those differences become even more pronounced. In grade II and III, cells take a stellate shape like extensions, and irregular contours are also present as we progress to higher grade levels.

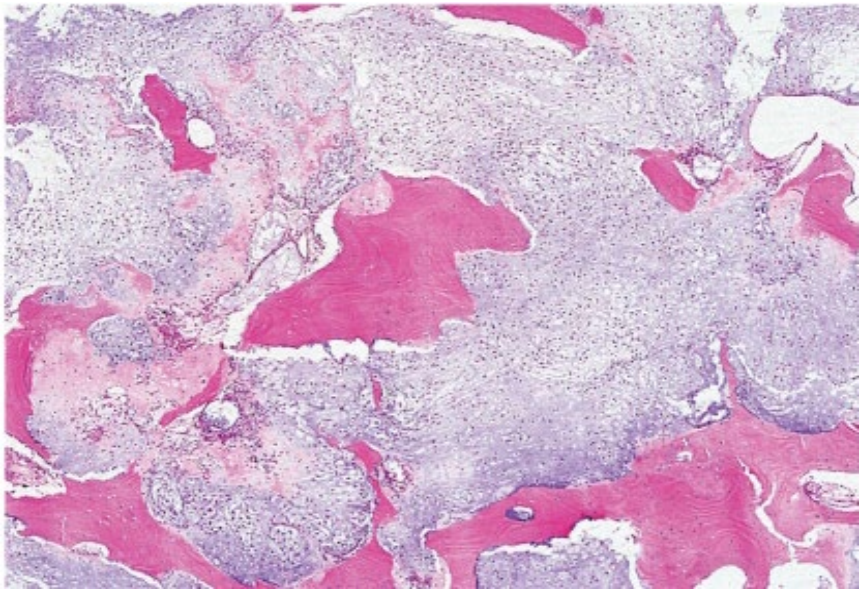

Fig. 4. Grade II chondrosarcoma in tissue (H&E staining). Several features of chondrosarcoma are present that set them apart from enchondromas. Increased cellularity; myxoid change in the matrix; and most importantly the invasive nature of cartilage growth around pre-existing trabecular bone.

One can observe the degree of cartilage infiltration (blue) into pre-existing bone trabeculae (red) in Fig. 4. This is one of the most distinct characteristics that differentiates any grade CS from ENC. In aggressive tumors, the rate of tumor growth is faster than the rate of osteoclasts being able to form and resorb bone. Thus, one can observe the infiltration pattern as depicted in Fig. 4. Benign slow growing tumors, such as ENC grow in nodules that are well contoured and delineated by bone tissue. In such cases the tumor growth is slower than the rate of osteoclasts being able to absorb bone. Cartilage growth will be well defined and lack infiltration characteristics, hence explaining the growth pattern.

Lastly cartilage tumors have the ability to "scallop" (not shown in picture). Cartilage nodules will impinge the inner layer of the circumferential lamellae of the bone cortex. This results in osteoclast resorption. Once the cortex has been pierced, the tumor can metastasize elsewhere in the body.

1.6 Significance and Hypotheses

Since the cartilage milieu is one of hypoxia and acidosis, it stands to reason you should minimize confounding factors that can significantly alter data interpretation and conclusions. This can be achieved by using hypoxic/acidic fixed media that mimics the chondrocyte's naturally occurring environment. However, there is a paucity of research analyzing the synergistic effect that both these stressors have on relative gene expression or cell-to-cell communication. Often, results are reported under normoxia/pH=7.0 media conditions. Considering the significance of hypoxia and acidosis in cartilage biology, any study that analyzes the HIF group of proteins and its effect on downstream targets (*PTHRrP/PTHR1/SOX9* among them), must take in account the above stated facts in experimental design. Of note, in ambient conditions of 21% O_2, the half-life of *HIF-1α* is less than 5 minutes and it is marked for rapid degradation (Elkins, 2002).

IDH1/*IDH2* mutants in CS are the most likely contributors to further induce *HIF-1α* and prevent them from being marked for proteasomal degradation. Consequently, *PTHR1* and *SOX9* will also be upregulated, keeping cells in a proliferative state and delaying their eventual hypertrophy. In order to elucidate the above-mentioned relationships, we analyze the relative gene expression of four genes of interest: *PTHR1*, *SOX9*, *IDH1*, and *IDH2* in reference to the actin beta (*ACTB*) housekeeping gene in all four conditions: hypoxia/acidosis, hypoxia only, acidosis only, and normoxia/pH=7.0. Both *18SrRNA* and *GAPDH* were excluded as possible housekeeping candidates due to their active role in hypoxia. **We hypothesize that *IDH1/IDH2* will be more upregulated in CS, specifically *IDH1* in JJ012 and *IDH2* in SW1353 in comparison to *IDH* expression in primary chondrocyte (CON5). Moreover, combined hypoxia/acidosis positively upregulates expression of *PTHR1* compared to both stressors alone in primary chondrocytes (CON5), and expression is further exacerbated in chondrosarcoma grade II: JJ012 (*IDH1* mutant) and SW1353 (*IDH2* mutant).**

CHAPTER 2
MATERIALS AND METHODS

Work undertaken for this thesis was approved by ODU Institutional Biosafety Committee #1182126.4.

2.1 Cell Culturing

JJ012 cells were kindly donated by Dr. J Block (Rush University Medical Center, Chicago, IL USA).

Newly thawed SW1353 and JJ012 cells were plated in T75 flasks with Dulbecco's modified eagle medium (DMEM); 10% chondrocyte growth supplement and 1% streptomycin. Once the cells reached 80-90% confluency, they were trypsinized, resuspended in normoxia/pH=7 DMEM, and 0.5ml of the cell suspension (approximately $1x10^6$ cells) was plated in 4.5ml of normoxia/pH=7 DMEM in a T25 flask. The same process was repeated for three additional experimental conditions: normoxia/pH=5.5 (acidosis); hypoxia/pH=7 and hypoxia/pH=5.5. The normoxia-treated flasks were incubated at 37ºC in humidified 5% CO_2 and 20% O_2 for 48 hours. The hypoxic-treated flasks were incubated at 37ºC in humidified 5% CO_2 and 5% O_2 (balanced with 90% N_2) for 48 hours. In order to equilibrate the oxygen tension, all media used for hypoxic treatments were de-gassed 48 hours prior to plating by keeping the media in the hypoxic induced chamber. Moreover, the T25 empty flasks were de-gassed one hour before plating the cells. The media was adjusted to acidic level of pH=5.5 by adding 1M HCl solution in the DMEM and measured by the AB15 pH meter probe (Thermo Fisher Scientific).

2.2 RNA Extraction and cDNA Synthesis

After 48 hours, the JJ012 cells were lysed inside the flasks by adding 350µL Trizol and left at room temperature for 10 minutes. An equal volume of 99.5% ethanol was added to the lysate. The RNA extraction for SW1353 was completed using ISOLATE II RNA MiniKit (Bioline, Swedesboro, New Jersey. Cat No. BIO-52071) following the manufacturer's guidelines. The RNA concentration for both cell lines was measured using Nanovue Plus Nanodrop (GE Healthcare, Little Chalfont, UK). cDNA synthesis was achieved using SensiFAST^{TM} cDNA Synthesis Kit (Bioline, Swedesboro, New Jersey. cat. No.BIO-65053)

following the manufacturer's recommended steps. cDNA concentration was measured using the Nanovue Plus Nanodrop.

2.3 RT-qPCR

RT-qPCR was performed to measure the relative fold expression of each gene for each cell line. The PCR Mastermix (PM) was prepared first by adding 12.5µL SYBR Green Mastermix multiplied by the number of wells needed. Next, 10.5µL nuclease-free grade water was added multiplied by the number of wells needed. Each well contained the following components: 23µl of PM; 1µl of the appropriate primer and 1µl of cDNA for each of the assigned conditions. In total, each well contained 25µl of solution and was run in triplicates for each treatment. Additionally, each primer had its own quality control (QC) well that received 23µl PM; 1µl primer and 1µl nuclease-free water for a total of 25µl.

Primers used:

RT2 PCR Primer Set for Human PTH1R (NM_000316). RT² qPCR Primer Assay (Qiagen, PPH00740A-200)

RT² qPCR Primer Assay for Human SOX9 (NM_000346). RT² qPCR Primer Assay (Qiagen, PPH02125A-200)

RT² qPCR Primer Assay for Human IDH1 (NM_005896). RT² qPCR Primer Assay (Qiagen, PPH06067A-200)

RT² qPCR Primer Assay for Human IDH2 (NM_002168). RT² qPCR Primer Assay (Qiagen, PPH07326A-200)

RT² qPCR Primer Assay for Human ACTB (NM_001101). RT² qPCR Primer Assay (Qiagen, PPH00073G-200)

2.4 Data Analysis

Relative gene expression for *PTHR1*, *SOX9*, *IDH1* and *IDH2* were measured relative to the *ACTB* housekeeping gene expression. Cq values were generated in the RT-qPCR for each gene for all four treatments: hypoxia/acidosis, hypoxia only, acidosis only, and normoxia/pH=7. Since there were a minimum of three data points for each experiment, the standard error of mean was calculated for each Cq value, which was negligible. Next, ΔΔCq values were calculated for each gene of interest in relation to *ACTB*. The fold expression values were normalized in log2 in

relation to their respective gene expression in normoxia/pH=7 to determine which genes were up- and down-regulated.

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 built-in mean and standard error of mean (SEM).

2.5 Cell Proliferation Assay

In order to further elucidate the differences between the JJ012/SW1353 and the primary chondrocyte CON5, 10^4 cells were initially plated for each cell line in every individual well of a 6-well plate on three different plates. Each well was assigned a specific day 1-5. The wells labeled as "day 1" were washed with PBS and trypsinized after 24 hours. The cells were centrifuged, resuspended in 1ml of DMEM, and counted using a hemocytometer for each cell line. After 48 hours the wells labeled as "day 2" underwent the same process. This procedure was repeated up to day 5. Data for the growth curve was plugged in Prism to generate a graph with all three cell lines including their doubling rate.

2.6 D-2-Hydroxyglutarate Assay (Colorimetric)

D-2-hydroxygluterate concentration levels in SW1353, JJ012, and CON5 cell lysate were measured by using a colorimetric assay kit (ab211070) according to manufacturer's recommendations (Abclonal). The *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. In primary chondrocytes, D-2HG levels are minimal. The cell lysates were derived by cultures that were grown in normoxia/pH=7.

2.7 Puromycin Selection and Lentiviral Transfection

The lentiviral clone, CAG-GFP lentivirus, was used to transfect JJ012 and SW1353 cells. One of the genes included in the vector is PuroR, which confers puromycin resistance to the cells once they have been successfully transfected. The minimum puromycin level needed to kill each cell line was determined prior to transfection. Cancer cells respond differently to puromycin

selection, with reports from 200-400ng/ml in fibrosarcoma (Zhong, 2007) to as high as 5000ng/ml in human breast cancer cells (Johnstone, 2008). To our knowledge, no published report exists on the puromycin concentration needed for chondrosarcoma cells (Thermo Fisher Scientific, 2019). Following manufacturer's recommendations, serial dilutions were performed to determine the optimal puromycin concentration for JJ012 and SW1353 chondrosarcoma cells. In each well, 160,000 cells were plated in a 96-well plate in triplicates for each dilution and each separate chondrosarcoma cell line. Then, 120µl of Dulbecco's modified eagle medium (DMEM) with 10% chondrocyte growth supplement and no antibiotic were added to each well. The plate was incubated overnight at 37ºC at 5% CO_2 and 20% O_2. The puromycin stock, 10mg/ml in distilled water, sterile filtered (alfa aesar by Thermo Fisher Scientific, J67236) was serially diluted in increments of 1000ng/ml from 10,000ng/ml to 1000ng/ml. The following day, the media was aspirated and replaced with 120µl per well of the puromycin-rich DMEM at the appropriate concentrations. The plate was incubated at 37ºC at 5% CO_2 and 20% O_2. Results were observed and photographs captured using BX-51 phase contrast microscope at the two-day mark and at the four-day mark with the media replenished on the second day (data not included). In the end of the fourth day, cell death was observed in all concentrations except the control cells with no puromycin. This was an indicator that the minimum puromycin concentration needed was at sub 1000ng/ml. In order to obtain precise results, the titration was repeated with finer increments of 100ng/ml ranging from 1000ng/ml to 100ng/ml and control cells with no antibiotic added. Pictures were taken using phase contrast BX-51 microscope at the two- and four-day marks. Based on those results, it was determined that the minimum concentrations of puromycin that result in complete cell death for JJ012 and SW1353 are 400ng/ml and 500ng/ml respectively (Fig. 5).

Once the minimum puromycin concentration was determined for SW1353 and JJ012, the cells were transfected with the lentiviral clone, CAG-GFP (Cellomics Technology, Halethorpe, MD). On day 1, 10^4 cells per well were plated on DMEM with 10% growth factors and 1% penicillin/streptomycin in a 24-well plate. Four rows (2 wells per row) were plated representing four distinct multiplicities of infection (MOI=10,5,1 and 0). On day 2, the transduction media was prepared at the right dilution for each MOI. Polybrene was used to increase the efficacy of the viral infection. However, since it can be toxic for the cells, it was diluted from its original

concentration of 10mg/ml (10µg/µl) to 5.0µg/µl in up to 5ml of growth media (0.5ml/well x 10 wells.) The formula used to calculate the amount of lentivirus needed/MOI was:

Total transducing unit (TU) needed = number cells/well x desired MOI

Total volume of lentivirus = TU/ (10^8 TU/ml constant value)

MOI=10; (2 wells x 10^4 cells x 10)/10^8= 0.002ml (2.0µl) lentivirus/well (4 µl lentivirus in 1ml polybrene media for 2 wells)

MOI= 5; (2 wells x 10^4 cells x 5)/10^8= 0.001ml (1.0µl) lentivirus/well (2 µl lentivirus in 1ml polybrene media for 2 wells)

MOI=1; (2 wells x 10^4 cells x 1)/10^8= 0.0002ml (0.2µl) lentivirus/well (0.4 µl lentivirus in 1 ml polybrene media for 2 wells)

The plate was centrifuged at 800g for 30 minutes and incubated at 37ºC at 5% CO_2. On day 3, the media was aspired from the wells and new media with no antibiotic was added to allow enough time for the expression of the puromycin-resistant gene. On day 5, the media was aspirated, and puromycin-rich media was introduced at the appropriate determined concentrations for JJ012 and SW1353. Then the cells were checked for survivability. All cells under MOI 10, 5 and 1 survived and expressed GFP, indicating a successful transfection, while the control cells without the antibiotic-resistant gene died after the second round of treatment with the puromycin-rich media. Images of the successful transfection of the cells were captured using a BX-51 phase contrast microscope in Fig. 10. Cells from MOI 10, 5, and 1 were pooled together and plated in a T-25 flask for each cell line. Cells were kept under puromycin selection for approximately one month, then they were frozen at -80ºC for 24 hours in DMSO, followed by further freezing and storage in liquid nitrogen at -196ºC.

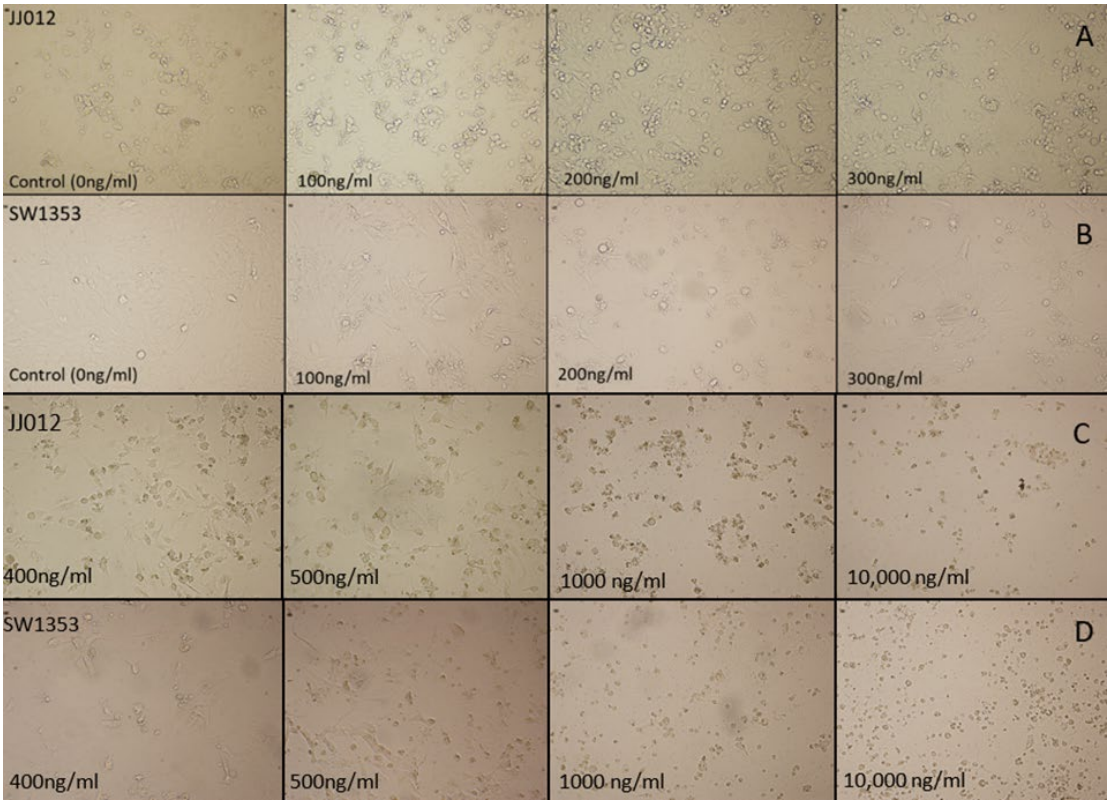

Fig. 5. Puromycin selection for JJ012 (row A/C) and SW1353 (row B/D).
Cells were evaluated every two days and the minimum puromycin concentration that resulted in complete cell death after 4 days was the concentration used for the lentiviral experiment. Based on the phase contrast images at 10X magnification, complete cell death for JJ012 was observed at 400ng/ml (0.4μg/ml). This is marked with distinctly darker and shrinking cells as opposed to healthy-looking ones, which appear bright from the inside. See higher concentrations for a comparison basis. Using the same parameter, for SW1353 the lowest concentration under which complete cell death was observed was at 500ng/ml (0.5μg/ml).

CHAPTER 3
RESULTS

3.1 RT-qPCR Cycles, Melt Curves and Melt Peaks

Denaturation is the first step in the PCR reaction. The thermocycler in the CFX Connect Real-Time System (BIO-RAD; Hercules, CA) reaches approximately 95ºC, which causes the double stranded DNA helix to unwind into two, single stranded (ssDNA) chains. This phase lasts for 10 minutes. During the annealing phase, the temperature drops to 60ºC which allows the primers to attach to the appropriate end of the target sequence for the next minute. At the onset of the extension phase (5 seconds), the temperature slightly increases from 60ºC to 65ºC. DNA polymerase attaches to the primers and allows the addition of nucleotides which are complementary to the DNA template. This results in extended double stranded DNA (dsDNA). SYBR Green binds to the newly synthesized DNA and fluoresces as the reaction progresses. The fluorescence accumulates and is measured after each cycle. The Cq value measured represents the intensity of the fluorescence above the background level. The above steps were repeated for 40 cycles, followed by melt curve analysis from 65ºC to 95ºC in 0.2ºC increments to ensure melt point temperature consistency in each primer set (Fig. 6; Fig. 7).

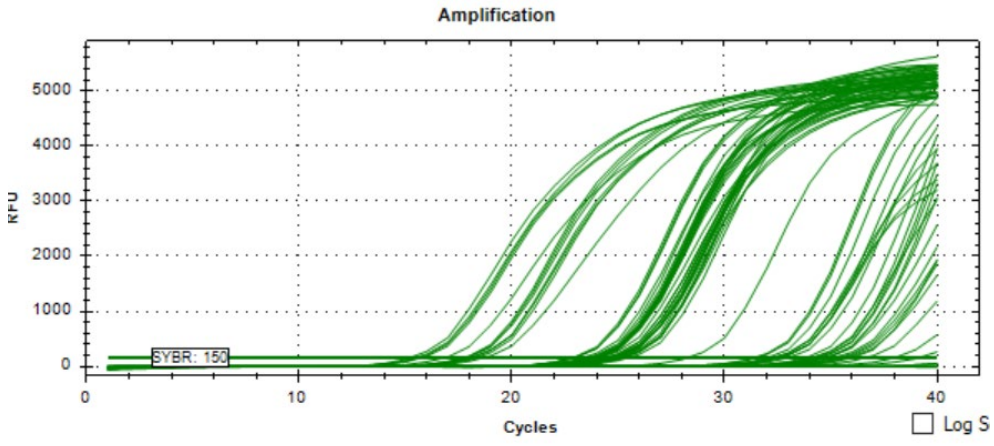

Fig. 6. RT-qPCR amplification (CON5). SYBR green value amplification in Relative fluorescence units (RFU) in relation to how many cycles are needed for each primer set to generate a signal in primary chondrocyte cells (CON5) The same steps were followed for the two CS: JJ012 and SW1353 to generate Cq values.

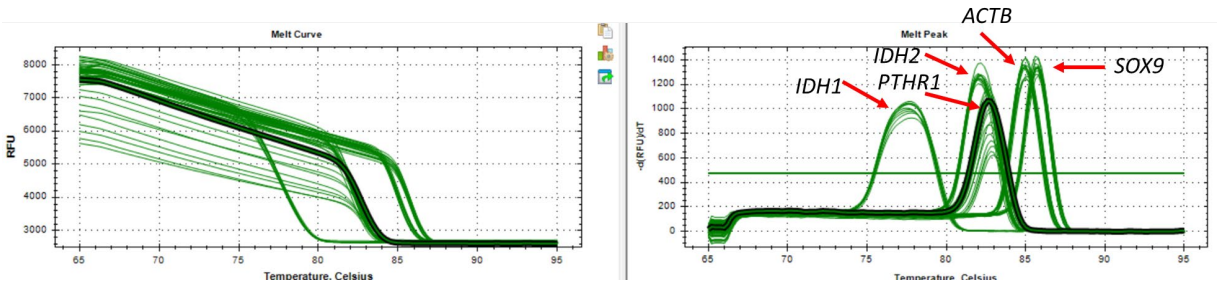

Fig. 7. RT-qPCR Melt Curve and Melt Peak (CON5). Each prominent spike represents melting peaks of a given gene: To ensure that the primers melting peak was consistent the temperature range was determined along with the standard deviation (SD) and Standard error of mean (SEM): SOX9 – 85.6°- 85.8° (SD=0.1/ SEM = 0.029); ACTB – 84.8° - 85.0° (SD=0.16 / SEM =0.04); PTHR1 – 82.6° - 83° (SD=0.13 / SEM=0.04); IDH1- 77.2°- 77.8° (SD=0.21 / SEM=0.06); IDH2 – 82.0° - 82.2° (SD=0.09 / SEM=0.028)

Cq values were generated at the end of each RT-qPCR experiment.

Relative fold expression $\Delta\Delta Cq = 2^{-(\text{Gene of interest Cq - } ACTB \text{ Cq})}$ was calculated for all 4 experimental conditions: hypoxia/acidosis; hypoxia/pH=7; normoxia/pH=5.5; normoxia/pH=7.

The ratio value was calculated in relation to our experimental control normoxia/pH=7 and the value normalized on log2: $\Delta\Delta Cq_{\text{(experimental condition)}} / \Delta\Delta Cq_{\text{(Control normoxia/pH=7)}}$

3.2 Aggressive Growth of JJ012 vs SW1353 and CON5

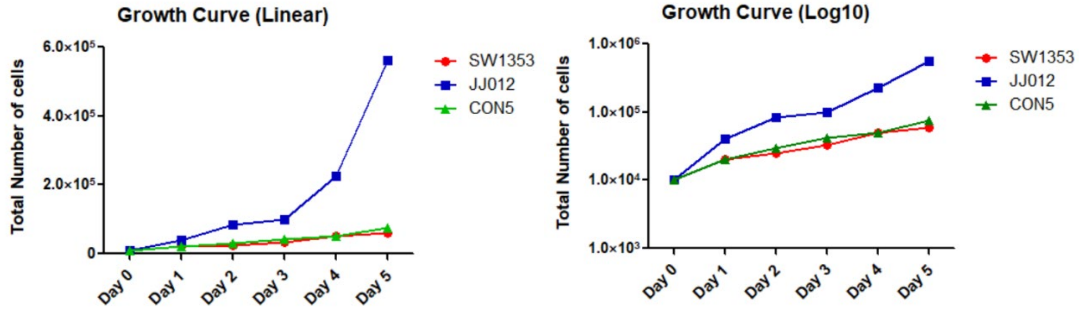

Fig. 8. Cell proliferation linear and log graph. JJ012 grade II chondrosarcoma cell line displays a rather aggressive growth pattern (exponential). Doubling time was approximately 23-24 hours (1 day). In contrast, the same grade chondrosarcoma SW1353 is comparatively identical to the primary chondrocyte CON5 cell line. For both cell lines, the doubling time is approximately 46-48 hours (2 days).

SW1353 and JJ012 are both classified as primary human chondrosarcoma grade II even though they exhibit distinct morphological features. Not to be confused with tumor stage, the tumor grade takes into consideration the cell's abnormality and its pleomorphic qualities under a microscope. Stage tumor defines the metastatic ability of these tumors to spread elsewhere in the body. In our case, the grade of these cell lines was easily traceable, but not the stage due to confidential patient information and IRB guidelines.

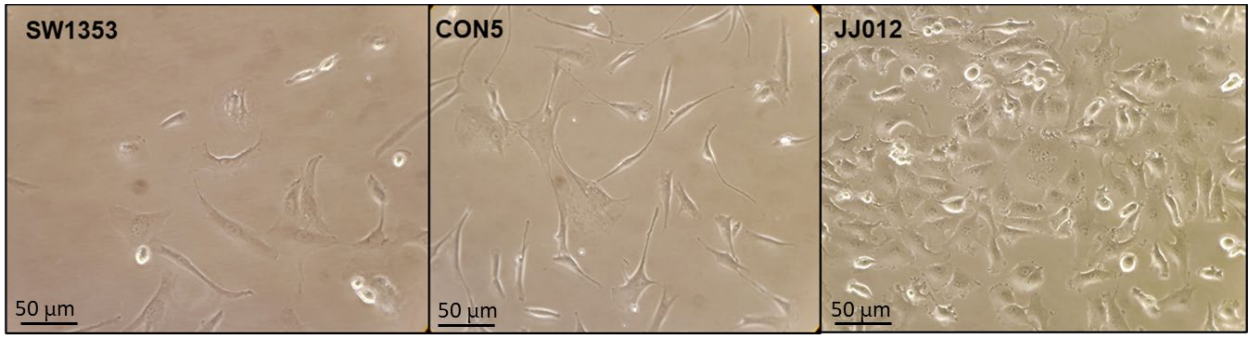

Fig. 9. SW1353/CON5/JJ012 phenotypical profile. The enlarged nuclei can be observed in JJ012 and SW1353. In addition, there are mitotic figures present in both cell lines. Due to their aggressive nature, JJ012 can be more closely associated with dedifferentiated chondrosarcoma than conventional grade II CS. As a reference point, CON5 provides distinct morphological differences with hypo chromatic nuclei that are 2-3 times smaller than those of CS.

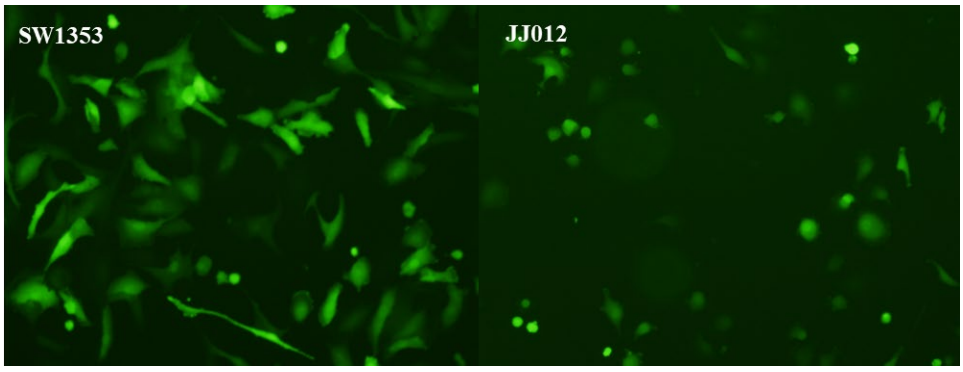

Fig. 10. Chondrosarcoma-CAG-GFP lentivirus clone transfection**.** Successful viral transfection was confirmed by the survival of SW1353/JJ012 under puromycin selection as well as expression of GFP under BX-51 phase contrast microscope FITC setting. JJ012 showing clear distinct atypia and increased nucleus size. SW1353 phenotypically are closer to primary chondrocytes.

The JJ012 cell line was obtained from a 39-year-old male. Its aggressive nature can be highlighted by the fact that it has a doubling time of approximately 24 hours (Fig. 8). They are mainly polyhedral in shape, contain enlarged hyperchromatic nuclei, and are often binucleated. Mitotic figures are common, and there is distinct cell atypia and pleomorphism (Fig. 9). They have developed eosinophilic cytoplasmic extensions since most of their metabolism is directed not at producing extra cellular matrix (ECM), but multiplying (Fig. 9; Fig. 10). This has led some researchers to treat this cell line as more closely related to dedifferentiated chondrosarcoma (Nakagawa, et al., 2019). On the other hand, SW1353 originated from the right humerus of a 72-year-old female in 1977. In appearance and doubling time, they resemble CON5 cells. Unlike CON5, SW1353 exhibit moderate cell atypia and pleomorphic characteristics. Lastly, the CON5 primary cells were isolated from a healthy 25-year-old female. Size of the cell nuclei are considerably smaller than those of SW1353 and JJ012, and there is a lack of mitotic activity and cellular atypia.

3.3 Hypoxia/acidosis *PTHR1* upregulation in CON5

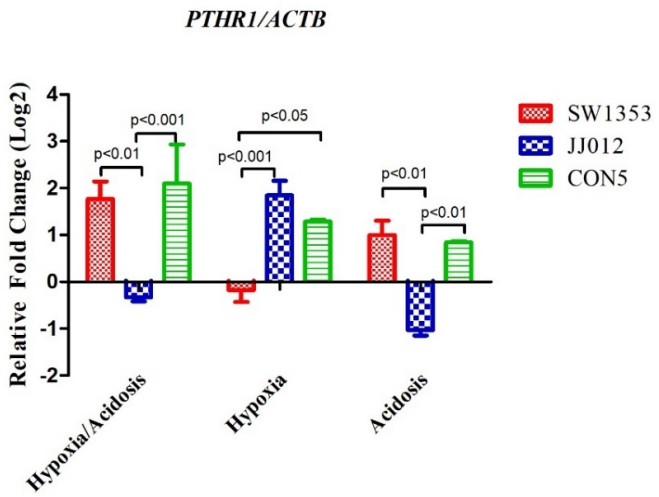

Fig. 11. *PTHR1* relative gene expression. The log2 fold change in *PTHR1* expression is shown for each cell line in all three conditions: hypoxia/acidosis, hypoxia, and acidosis. Since the relative fold change was normalized in log2, positive values represent upregulation from the control condition in normoxia/pH=7, while negative values show downregulation from the control. Due to that data transformation, gene expression values between 0-1 in either direction are described as marginally/slightly downregulated or upregulated depending on the direction. Whereas, expression values greater or smaller than 1 are described as significantly upregulated or downregulated from the control.

There is a difference in *PTHR1* expression between the cell lines in each condition: In hypoxia /acidosis, CON5 and SW1353 are significantly upregulated (2.10 ± 0.835 SEM and 1.77 ± 0.37 SEM respectively). JJ012 is slightly downregulated (-0.33 ± 0.08 SEM). This expression is significantly different: JJ012 versus CON5 $p<0.001$ and JJ012 versus SW1353 $p<0.01$).

In hypoxia only, SW1353 is slightly downregulated (-0.18 ± 0.2533 SEM); JJ012 upregulated (+1.85 ± 0.31) and CON5 is upregulated (1.29 ± 0.04). This expression between cell lines is significant: JJ012 versus SW1353 ($p<0.001$) and CON5 versus SW1353 ($p<0.05$). (Table 1).

Lastly, when acidosis is the only stressor, CON5 and SW1353 are slightly upregulated (0.84±0.03 SEM and 1.00±0.31 SEM respectively) while JJ012 is downregulated (-1.04±0.12 SEM). The difference in expression is significant ($p<0.01$ for both cell lines) when compared to JJ012 (Table 1). Overall, *PTHR1* was upregulated in all conditions in the primary chondrocyte, with the largest expression in the combined conditions. Hypoxia had an upregulating effect on JJ012, while acidosis induced downregulation. Their synergistic effect was one of marginal downregulation. These stressors had opposite effects on SW1353 and resulted in significant upregulation in combined conditions in Fig. 11.

3.4 Acidosis dependent upregulation of *SOX9* in CON5

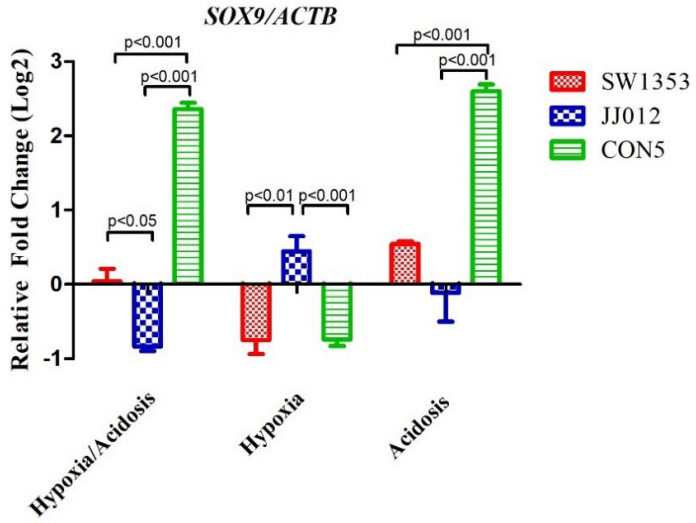

Fig. 12. *SOX9* relative gene expression. The log2 relative fold change in *SOX9* expression is shown for each cell line in all three conditions: Hypoxia/acidosis, hypoxia, and acidosis. The most notably significant difference is the acidosis dependent (>2.0 log fold change) upregulation of *SOX9* expression in CON5 cells, and largely independent relationship from hypoxia condition.

There is a difference of *SOX9* expression between the cell lines in each condition: In hypoxia /acidosis, primary chondrocyte is significantly upregulated (2.36 ± 0.085 SEM) and that expression is significant when compared to JJ012 and SW1353 ($p<0.001$ for both) (Table 2). Moreover, SW1353 shows negligible difference from normoxia/pH=7 with (0.05±0.16 SEM) log fold upregulation, while JJ012 is downregulated (-0.84±0.06 SEM) (Fig. 2).

Hypoxia alone, induces marginal *SOX9* expression in JJ012 (0.44±0.20 SEM) and downregulates it in the other two cell lines: (-0.75±0.19 SEM) in SW1353 and (-0.74±0.0889 SEM) in CON5. Moreover, the difference in expression between cells is significant (JJ012 vs SW1353 $p<0.01$; JJ012 vs CON5 $p<0.001$) (Table 2).

In acidosis only, *SOX9* expression in primary chondrocyte is similarly over expressed (2.60 ± 0.90 SEM) log fold change just like in the combined condition. In the other two chondrosarcomas that expression shows marginal variation from the base. In JJ012, *SOX9* expression is slightly downregulated (-0.11±0.39 SEM) and in SW1353 slightly upregulated (0.55 ±0.035 SEM). The expression is significantly different between cells: (CON5 vs JJ012 $p<0.001$; CON5 vs SW1353 $p<0.001$) (Table 12).

3.5 *IDH1* unaffected by hypoxia/acidosis in SW1353/CON5 and sensitive to both hypoxia and acidosis in JJ012

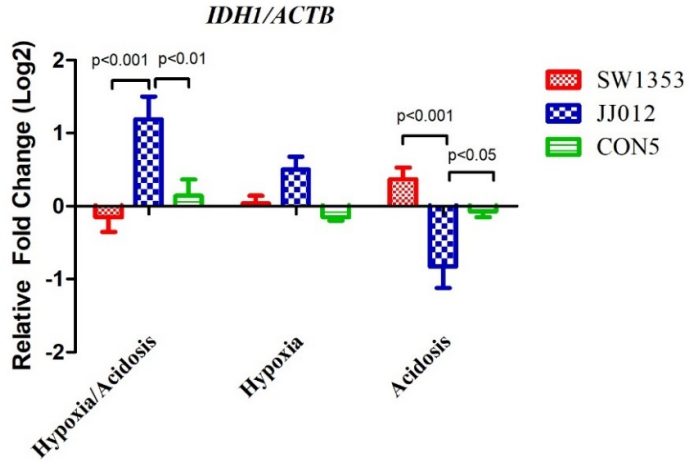

Fig. 13. *IDH1* relative gene expression. The log2 fold change in *IDH1* expression is shown for each cell line in all three conditions: Hypoxia/acidosis, hypoxia, and acidosis. Notice the negligible change of expression in SW1353 and CON5 from the control in all conditions. JJ012 is an *IDH1* mutant, while CON5 is an *IDH* wild type.

In the combined conditions of hypoxia/acidosis, *IDH1* expression in SW1353 was slightly downregulated (-0.15 ± 0.2025 SEM); in JJ012 it was upregulated (1.19 ± 0.31 SEM) and in CON5 it showed slight upregulation (0.14 ± 0.2255). This expression was significantly different between JJ012 and SW1353 ($p<0.001$) as well as between JJ012 and CON5 ($p<0.01$).

Under the effects of hypoxia alone, *IDH1* expression in SW1353 was marginally upregulated from the control (0.04 ± 0.1015 SEM); in JJ012, it was slightly more upregulated (0.50 ±0.18 SEM) and in CON5, it was slightly downregulated (-0.15 ± 0.0441 SEM). There was no statistically significant difference of *IDH1* expression between the cells in hypoxia alone.

Lastly, when acidosis is the only stressor, *IDH1* expression in SW1353 was slightly upregulated (0.37 ± 0.1572 SEM); in JJ012, it was downregulated (-0.83 ± 0.29 SEM) and in CON5, it was minimally downregulated (-0.07 ± 0.0808 SEM). There is a significant difference in expression between JJ012 since it was downregulated more than the other cell lines. JJ012 versus SW133 ($p<0.001$) and JJ012 versus CON5 ($p<0.05$).

Overall, in SW1353 and CON5 there was a minimal up/down regulation of *IDH1* expression across all three experimental conditions from the control (normoxia/pH=7).

3.6 Acidosis driven *IDH2* expression in SW1353/CON5 and upregulated synergistic effect in JJ012

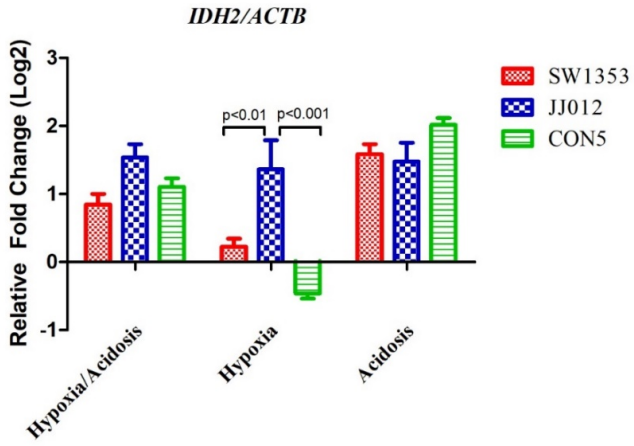

Fig. 14. *IDH2* relative gene expression. The log2 fold change in *IDH2* expression is shown for each cell line in all three conditions: Hypoxia/acidosis, hypoxia, acidosis. Notice the consistent and significant upregulation of all three cell lines under acidosis alone.

IDH2 expression in all three cell lines is upregulated under the combined conditions: SW1353 (0.85 ± 0.1530 SEM); JJ012 (1.54 ± 0.19 SEM) and CON5 (2.10 ± 0.835 SEM). There is no statistically significant difference in *IDH2* expression between the cells.

Hypoxia alone, upregulates *IDH2* expression in JJ012 (1.36 ± 0.42) while it downregulates it in CON5 (-0.46 ± 0.0781 SEM) and slightly upregulates it in SW1353 (0.22 ± 0.1172 SEM). The expression is significantly different between JJ012 and SW1353 ($p<0.01$) as well as between JJ012 and CON5 ($p<0.001$).

Lastly, when acidosis is the only stressor, the IDH2 expression is upregulated in all cells : SW1353 (1.58 ± 0.1495 SEM); JJ012 (1.47 ± 0.28 SEM) and CON5 (2.02 ± 0.0984 SEM). The upregulation was similar in all three cell lines therefore there was no statistically significant difference in *IDH2* expression.

3.7 D-2-Hydroxygluterate elevated concentration levels in Chondrosarcoma

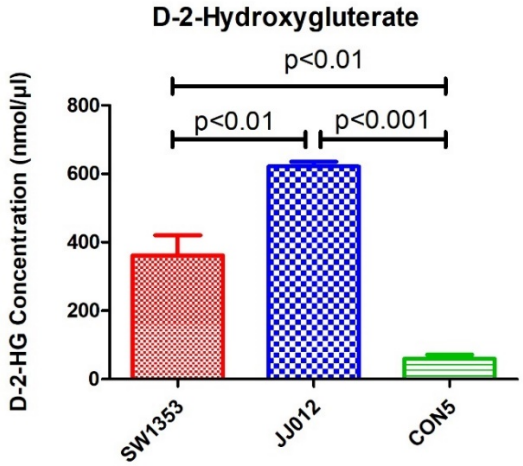

Fig. 15. D-2-Hydroxygluterate concentration levels. There is a significant increased concentration of D-2-HG in JJ012 (621.9 nmol/µl) and SW1353 (361.6 nmol/µl) in comparison to CON5 (59.6 nmol/µl). The cell lysate from where the D-2-HG concentration was measured was harvested in cultures grown in normoxia/pH=7.

D-2-HG is an oncometabolite and is highly produced in cells that harbor *IDH1*/*IDH2* mutations. *IDH* wildtype will convert isocitrate into α-ketoglutarate, therefore D-2-HG levels should be minimal. The difference between α-ketoglutarate and D-2-HG consist of a single

replacement of an oxygen atom with a hydroxyl group. D-2-HG was the most elevated in JJ012, followed by SW1353 and lowest in CON5 (Fig. 15). There is a significant difference in concentration level between the cell lines: (JJ012 vs CON5 $p<0.001$); SW1353 vs CON5 $p<0.01$ and JJ012 vs SW1353 $p<0.01$) (Table 5).

3.8 Statistical Analysis

Table 1

PTHR1 two-way ANOVA. Statistical analysis of *PTHR1* expression followed by Bonferroni post-test to compare replicate means by row (the source of variance of ANOVA between cell lines compared to each other). The (p) value interpretation: ns (not significant); * ($p<0.05$); ** ($p<0.01$); *** ($p<0.001$).

Two-way ANOVA	Table Analyzed:	*PTHR1/ACTB*		
Source of Variation	% of total variation	P value		
Interaction	63.46	< 0.0001		
Column Factor	20.8	0.0018		
Row Factor	12.15	0.0127		
Source of Variation	P value summary	Significant?		
Interaction	***	Yes		
Column Factor	**	Yes		
Row Factor	*	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	4	16.41	4.102	16.26
Column Factor	2	5.376	2.688	10.66
Row Factor	2	3.141	1.571	6.226
Residual	13	3.28	0.2523	
Bonferroni posttests				
SW1353 vs JJ012				
Row Factor	SW1353	JJ012	Difference	95% CI of diff.
Hypoxia/Acidosis	1.77	-0.3333	-2.103	-3.624 to -0.5822
Hypoxia	-0.1767	1.85	2.027	0.6661 to 3.387
Acidosis	0.9967	-1.035	-2.032	-3.553 to -0.5105
Row Factor	Difference	t	P value	Summary
Hypoxia/Acidosis	-2.103	4.587	P<0.01	**
Hypoxia	2.027	4.942	P<0.001	***
Acidosis	-2.032	4.431	P<0.01	**
SW1353 vs CON5				
Row Factor	SW1353	CON5	Difference	95% CI of diff.
Hypoxia/Acidosis	1.77	2.095	0.325	-1.341 to 1.991
Hypoxia	-0.1767	1.29	1.467	-0.05449 to 2.988
Acidosis	0.9967	0.84	-0.1567	-1.678 to 1.364
Row Factor	Difference	t	P value	Summary
Hypoxia/Acidosis	0.325	0.6471	P > 0.05	ns
Hypoxia	1.467	3.199	P < 0.05	*
Acidosis	-0.1567	0.3417	P > 0.05	ns
JJ012 vs CON5				
Row Factor	JJ012	CON5	Difference	95% CI of diff.
Hypoxia/Acidosis	-0.3333	2.095	2.428	0.9072 to 3.949
Hypoxia	1.85	1.29	-0.56	-2.081 to 0.9612
Acidosis	-1.035	0.84	1.875	0.2087 to 3.541
Row Factor	Difference	t	P value	Summary
Hypoxia/Acidosis	2.428	5.296	P<0.001	***
Hypoxia	-0.56	1.221	P > 0.05	ns
Acidosis	1.875	3.733	P<0.01	**

For *PTHR1* expression, Bonferroni posttest shows a significant row factor relationship between SW1353 and JJ012 in the combined conditions ($p<0.01$), in hypoxia ($p<0.001$), in acidosis ($p<0.01$). Between SW1353 and CON5 there is no significant difference in the combined conditions ($p>0.05$) and acidosis ($p>0.05$). However, in hypoxia the difference is significant ($p<0.05$). Between JJ012 and CON5, there is a significant difference in *PTHR1* expression in the combined conditions ($p<0.001$), and in acidosis only ($p<0.01$). While no difference was observed due to hypoxia alone ($p>0.05$).

Table 2

SOX9 two-way ANOVA. Statistical analysis of *SOX9* expression followed by Bonferroni post-test to compare replicate means by row (the source of variance of ANOVA between cell lines compared to each other). The (p) value interpretation: ns (not significant); * ($p<0.05$); ** ($p<0.01$); *** ($p<0.001$).

Two-way ANOVA	Table Analyzed:	*SOX9/ACTB*		
Source of Variation	% of total variation	P value		
Interaction	43.68	< 0.0001		
Column Factor	33.31	< 0.0001		
Row Factor	18.44	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	***	Yes		
Column Factor	***	Yes		
Row Factor	***	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	4	17.33	4.332	45.04
Column Factor	2	13.22	6.608	68.7
Row Factor	2	7.316	3.658	38.03
Residual	15	1.443	0.09619	
Bonferroni posttests				
SW1353 vs JJ012				
Row Factor	SW1353	JJ012	Difference	95% CI of diff.
Hypoxia/Acidosis	0.045	-0.84	-0.885	-1.801 to 0.03080
Hypoxia	-0.75	0.4433	1.193	0.2775 to 2.109
Acidosis	0.545	-0.1133	-0.6583	-1.574 to 0.2575
Row Factor	Difference	t	P value	Summary
Hypoxia/Acidosis	-0.885	3.126	P < 0.05	*
Hypoxia	1.193	4.215	P<0.01	**
Acidosis	-0.6583	2.325	P > 0.05	ns
SW1353 vs CON5				
Row Factor	SW1353	CON5	Difference	95% CI of diff.
Hypoxia/Acidosis	0.045	2.36	2.315	1.399 to 3.231
Hypoxia	-0.75	-0.74	0.01	-0.9058 to 0.9258
Acidosis	0.545	2.6	2.055	1.139 to 2.971
Row Factor	Difference	t	P value	Summary
Hypoxia/Acidosis	2.315	8.177	P<0.001	***
Hypoxia	0.01	0.03532	P > 0.05	ns
Acidosis	2.055	7.258	P<0.001	***
JJ012 vs CON5				
Row Factor	JJ012	CON5	Difference	95% CI of diff.
Hypoxia/Acidosis	-0.84	2.36	3.2	2.381 to 4.019
Hypoxia	0.4433	-0.74	-1.183	-2.002 to -0.3642
Acidosis	-0.1133	2.6	2.713	1.894 to 3.532
Row Factor	Difference	t	P value	Summary
Hypoxia/Acidosis	3.2	12.64	P<0.001	***
Hypoxia	-1.183	4.673	P<0.001	***
Acidosis	2.713	10.71	P<0.001	***

For *SOX9* expression, Bonferroni posttest shows a row factor relationship between SW1353 and JJ012 in the combined conditions ($p<0.05$) and hypoxia ($p<0.01$). While no significant difference is observed under acidosis ($p>0.05$). Between SW1353 and CON5 there is no significant difference of *SOX9* expression in hypoxia. However, in combined conditions and acidosis the difference is significant ($p<0.001$). Between JJ012 and CON5, there is a significant difference in all three conditions ($p<0.001$).

Table 3

IDH1 two-way ANOVA. Statistical analysis of *IDH1* expression followed by Bonferroni post-test to compare replicate means by row (the source of variance of ANOVA between cell lines compared to each other).

Two-way ANOVA	Table Analyzed:	*IDH1/ACTB*		
Source of Variation	% of total variation	P value		
Interaction	63.03	< 0.0001		
Column Factor	5.31	0.1558		
Row Factor	17.28	0.0069		
Source of Variation	P value summary	Significant?		
Interaction	***	Yes		
Column Factor	ns	No		
Row Factor	**	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	4	4.943	1.236	12.33
Column Factor	2	0.4166	0.2083	2.079
Row Factor	2	1.355	0.6776	6.761
Residual	17	1.704	0.1002	
Bonferroni posttests				
SW1353 vs JJ012				
Row Factor	SW1353	JJ012	Difference	95% CI of diff.
Hypoxia/Acidosis	-0.1533	1.19	1.343	0.4262 to 2.260
Hypoxia	0.04	0.5	0.46	-0.3603 to 1.280
Acidosis	0.37	-0.83	-1.2	-2.020 to -0.3797
Row Factor	Difference	t	P value	Summary
Hypoxia/Acidosis	1.343	4.648	P<0.001	***
Hypoxia	0.46	1.78	P > 0.05	ns
Acidosis	-1.2	4.642	P<0.001	***
SW1353 vs CON5				
Row Factor	SW1353	CON5	Difference	95% CI of diff.
Hypoxia/Acidosis	-0.1533	0.14	0.2933	-0.5270 to 1.114
Hypoxia	0.04	-0.1533	-0.1933	-1.014 to 0.6270
Acidosis	0.37	-0.07	-0.44	-1.260 to 0.3803
Row Factor	Difference	t	P value	Summary
Hypoxia/Acidosis	0.2933	1.135	P > 0.05	ns
Hypoxia	-0.1933	0.748	P > 0.05	ns
Acidosis	-0.44	1.702	P > 0.05	ns
JJ012 vs CON5				
Row Factor	JJ012	CON5	Difference	95% CI of diff.
Hypoxia/Acidosis	1.19	0.14	-1.05	-1.967 to -0.1329
Hypoxia	0.5	-0.1533	-0.6533	-1.474 to 0.1670
Acidosis	-0.83	-0.07	0.76	-0.06029 to 1.580
Row Factor	Difference	t	P value	Summary
Hypoxia/Acidosis	-1.05	3.633	P<0.01	**
Hypoxia	-0.6533	2.528	P > 0.05	ns
Acidosis	0.76	2.94	P < 0.05	*

For *IDH1* expression, Bonferroni posttest shows a row factor relationship between SW1353 and JJ012 in the combined conditions ($p<0.001$) and acidosis ($p<0.001$). While no significant difference is observed under hypoxia ($p>0.05$). Between SW1353 and CON5 there is no significant difference of *IDH1* expression in any of the conditions ($p>0.05$). Between JJ012 and CON5, there is a significant difference in the combined conditions ($p<0.01$) and acidosis ($p<0.05$). While no significant expression was observed under hypoxia ($p>0.05$).

Table 4

IDH2 two-way ANOVA. Statistical analysis of *IDH2* expression followed by Bonferroni post-test to compare replicate means by row (the source of variance of ANOVA between cell lines compared to each other).

Two-way ANOVA	Table Analyzed:	*IDH2/ACTB*		
Source of Variation	% of total variation	P value		
Interaction	26.35	0.0005		
Column Factor	11.87	0.004		
Row Factor	47.75	< 0.0001		
Source of Variation	P value summary	Significant?		
Interaction	***	Yes		
Column Factor	**	Yes		
Row Factor	***	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Interaction	4	4.354	1.088	8.457
Column Factor	2	1.961	0.9804	7.618
Row Factor	2	7.889	3.945	30.65
Residual	18	2.317	0.1287	
Bonferroni posttests				
SW1353 vs JJ012				
Row Factor	SW1353	JJ012	Difference	95% CI of diff.
Hypoxia/Acidosis	0.8467	1.537	0.69	-0.2323 to 1.612
Hypoxia	0.2233	1.363	1.14	0.2177 to 2.062
Acidosis	1.583	1.473	-0.11	-1.032 to 0.8123
Row Factor	Difference	t	P value	Summary
Hypoxia/Acidosis	0.69	2.356	P > 0.05	ns
Hypoxia	1.14	3.892	P<0.01	**
Acidosis	-0.11	0.3755	P > 0.05	ns
SW1353 vs CON5				
Row Factor	SW1353	CON5	Difference	95% CI of diff.
Hypoxia/Acidosis	0.8467	1.107	0.26	-0.6623 to 1.182
Hypoxia	0.2233	-0.46	-0.6833	-1.606 to 0.2390
Acidosis	1.583	2.017	0.4333	-0.4890 to 1.356
Row Factor	Difference	t	P value	Summary
Hypoxia/Acidosis	0.26	0.8876	P > 0.05	ns
Hypoxia	-0.6833	2.333	P > 0.05	ns
Acidosis	0.4333	1.479	P > 0.05	ns
JJ012 vs CON5				
Row Factor	JJ012	CON5	Difference	95% CI of diff.
Hypoxia/Acidosis	1.537	1.107	-0.43	-1.352 to 0.4923
Hypoxia	1.363	-0.46	-1.823	-2.746 to -0.9010
Acidosis	1.473	2.017	0.5433	-0.3790 to 1.466
Row Factor	Difference	t	P value	Summary
Hypoxia/Acidosis	-0.43	1.468	P > 0.05	ns
Hypoxia	-1.823	6.225	P<0.001	***
Acidosis	0.5433	1.855	P > 0.05	ns

For *IDH2* expression, Bonferroni posttest shows a row factor relationship between SW1353 and JJ012 in hypoxia ($p<0.01$). While no significant difference is observed under combined conditions and acidosis only ($p>0.05$). Between SW1353 and CON5 there is no significant difference of *IDH2* expression in any of the conditions ($p>0.05$). Between JJ012 and CON5, there is a significant difference in hypoxia ($p<0.001$) but not in acidosis and combined conditions ($p<0.05$).

Table 5

One-way ANOVA (nonparametric) and Tukey's comparison test. The one-way ANOVA and Tukey's Multiple Comparison Test established the statistical difference between D-2-HG concentration levels between each cell line.

One Way ANOVA of Data 1					
One-way analysis of variance					
P value	< 0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	3				
F	62.41				
R squared	0.9541				
ANOVA Table	SS	df	MS		
Treatment (between columns)	475200	2	237600		
Residual (within columns)	22840	6	3807		
Total	498000	8			
Tukey's Multiple Comparison Test	Mean Diff.	q	Significant? P < 0.05?	Summary	95% CI of diff
SW1353 vs JJ012	-260.4	7.309	Yes	**	-414.9 to -105.8
SW1353 vs CON5	302	8.476	Yes	**	147.4 to 456.5
JJ012 vs CON5	562.3	15.79	Yes	***	407.7 to 716.9

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.

Table 6

Basic statistics on gene expression. The Mean and Standard Error of Mean (SEM) was calculated for each gene and cell line under combined conditions, hypoxia only and acidosis only.

		SW1353			JJ012			CON5		
Gene	Experimental Conditions	Mean	SEM	N	Mean	SEM	N	Mean	SEM	N
PTHR1	Hypoxia/Acidosis	1.77	0.3700001	2	-0.3333333	0.08412952	3	2.095	0.835	2
	Hypoxia	-0.1766667	0.2533333	3	1.85	0.3080584	3	1.29	0.04000002	2
	Acidosis	0.9966667	0.31168	3	-1.035	0.115	2	0.84	0.03	2
SOX9	Hypoxia/Acidosis	0.045	0.165	2	-0.84	0.05773502	3	2.36	0.085049	3
	Hypoxia	-0.75	0.19	2	0.4433333	0.2034153	3	-0.74	0.08888195	3
	Acidosis	0.545	0.035	2	-0.1133333	0.3886015	3	2.6	0.09	3
IDH1	Hypoxia/Acidosis	-0.1533333	0.202512	3	1.19	0.31	2	0.14	0.2254625	3
	Hypoxia	0.04	0.1014889	3	0.5	0.1769181	3	-0.1533333	0.04409586	3
	Acidosis	0.37	0.1571623	3	-0.83	0.2920616	3	-0.07	0.08082904	3
IDH2	Hypoxia/Acidosis	0.8466667	0.1530069	3	1.536667	0.1946222	3	1.106667	0.1225198	3
	Hypoxia	0.2233333	0.1172367	3	1.363333	0.4248268	3	-0.46	0.07810249	3
	Acidosis	1.583333	0.1494806	3	1.473333	0.2783483	3	2.016667	0.09837571	3

CHAPTER 4
DISCUSSION

To interpret which stressor has the most significant influence on gene expression for a given cell line, they are first evaluated separately. If one downregulates expression while the other upregulates it, the stressor that has the most significant effect is the one that matches with the direction of the combined effect.

PTHR1 – The two main objectives of analyzing *PTHR1* were to observe whether the PTHrP-IHH pathway is involved or disrupted in any way to the point where it creates a cascade of effects leading to carcinogenesis. If *PTHR1* is significantly upregulated in chondrosarcoma and downregulated in the primary chondrocyte, that would be an indicator that cells are remaining in a proliferative state rather undergoing hypertrophy. The second objective was to establish that hypoxia does induce *PTHR1* expression and can become a confounding factor if overlooked. Our results indicate that in the combined condition, *PTHR1* is most upregulated in CON5 when compared to JJ012 ($p<0.001$) and SW1353 (Table 1). Not only that, but *PTHR1* expression remains upregulated in all three experimental conditions in the primary chondrocyte: hypoxia/acidosis (2.10± 0.835 SEM); hypoxia (1.29 ± 0.04) and acidosis (0.84 ± 0.03).

In JJ012, *PTHR1* upregulated expression is sensitive to hypoxia since it is the only condition under which it is significantly upregulated by (1.85 ± 0.31 SEM) log fold change. Under acidic conditions, it was downregulated (-1.04 ± 0.12). Their cumulative effect resulted in marginal downregulation from control (-0.33 ± 0.08).

In SW1353, the *PTHR1* upregulation was mainly driven by acidosis (1 ± 0.3117 SEM) since in hypoxia it was marginally downregulated (-0.18 ± 0.2533 SEM) and their combined effects resulted in upregulation (1.77 ± 0.37 SEM).

Our results show that the relative gene expression was not as elevated in chondrosarcoma as in the primary chondrocyte. However, the PTHrP-IHH loop pathway should not be ruled out. We have taken in consideration only half of the loop. Indeed, there is evidence that points to Indian hedgehog proteins as the culprit. Tiet, et al. (2006) suggests that in enchondromas and chondrosarcomas, the PTHrP-IHH pathway is largely missing. After treating normal growth plates with PTHrP, the IHH levels were downregulated as expected. However, when the same

treatment was applied to enchondroma and chondrosarcoma cultures, the IHH remained unchanged at large. IHH can be a good candidate gene to be taken in consideration for future studies. Additionally, the process of chondrocyte differentiation and maturation is a complex one that involves many pathways that often intertwine with each another, not limited to: wingless-int family members (wnts), fibroblast growth factors (FGFs), insulin-like growth factors (IGFs) and hormone molecules such as: androgen, estrogen and glucocorticoids.

SOX9 – In the primary (CON5) chondrocyte, the narrative that merged from our relative gene expression results indicate that *SOX9* is mainly sensitive to acidosis and independent from hypoxia. The synergistic effect of hypoxia and acidosis is mainly driven by acidosis alone since hypoxia by itself induced slight downregulation (-0.74 ± 0.0889 SEM), acidosis had the most profound effect (2.60 ± 0.09 SEM) and the combined stressors induced upregulation (2.36 ± 0.085 SEM) (Fig. 12). This is consistent with published data from other sources (Das, et al., 2009). However, there are conflicting reports in the published literature on *SOX9* expression in cartilage. Some report downregulation (Gebauer, et al., 2005) some report both up- and downregulation dependent on exposure time to acidosis (Das, et al., 2009). *SOX9* plays a crucial role in chondrogenesis and has direct control on a multitude of genes involved in ECM secretion, not limited to *COL2A1*, *COL10A1*, and *ACAN*. The latter ones establish a predominant hypocellular homeostatic environment with ample matrix secretion. In addition, SOX9 induces mesenchymal stem cells to differentiate into chondrocytes, and once they have differentiated it delays their eventual hypertrophy (Akiyama, Chaboissier, Martin, Schedl, & de Crombrugghe, 2002).

In chondrosarcoma cells, *SOX9* expression was at large either downregulated in most conditions or experienced no significant difference from the control (Table 6). This is in line with published research. For example, Tang, et al. (2010) showed that SW1353 display low *SOX9* and *Col2A1* expression, and those levels are reversed in dedifferentiated chondrosarcoma. It might seem counterintuitive considering that *SOX9* delays chondrocyte hypertrophy, but this points to the direction that the carcinogenesis of these chondrosarcoma does not lie with *SOX9* mutation but with some other genes. In our case, *IDH1*/*IDH2* are potential frontrunners. From a scientific standpoint this is supported due to the increased hypercellularity, especially in JJ012. Chondrosarcoma cells, particularly grade II and III direct much of their metabolic energy not at

producing ECM but rather multiplying, so *SOX9* expression is not as essential to their expansion and evasive nature. A question that stands is: What causes *SOX9* to be significantly downregulated in chondrosarcoma ($p<0.001$) in comparison to healthy cartilage in combined conditions? This leads us to *IDH1*/*IDH2* analysis.

IDH1*/*IDH2 - Nakagawa, et al. (2019) showed that once the *IDH1* mutant was inhibited by DS-1001b inhibitor in grade III chondrosarcoma, the *SOX9* expression went back up. This can elucidate why *SOX9* levels were depressed in the chondrosarcomas. However, the mechanism by which it does so needs further investigation and is a good direction for future studies. JJ012 heterozygous *IDH1* mutation is somatic in origin in the R132G allele region via (CGT>GGT) missense mutation while SW1353 harbor *IDH2* gene mutation in the R172S region (Suijker, 2015). Indeed, our results showed that *IDH1* was upregulated in the combined condition in JJ012 (1.19 ± 0.31 SEM). (Fig. 13). The expression was significant compared to SW1353 ($p<0.001$) and to CON5 ($p<0.01$) (Table 3). This further highlights the conclusion that JJ012 is sensitive to both these stressors due to the *IDH1* mutation that it harbors. Interestingly, acidosis and hypoxia on their own had opposite effects, with the former downregulating it (-0.83 ± 0.29 SEM) and the later upregulating it (0.50 ± 0.18 SEM). Additionally, D-2-HG concentration levels in JJ012 cell lysate were significantly more elevated ($p<0.001$) or 10.5-fold higher when compared to that of CON5 (Table 5). Lastly, SW1353 and CON5 expressed no meaningful difference in any of the conditions compared to the control since they are not *IDH1* mutants.

Our expectation for *IDH2* expression was that it was going to be significantly more upregulated in SW1353 when compared with the other two cell lines. Our results indicate that *IDH2* is heavily driven by acidosis and that is observable in all three cell lines (Fig. 14). The key data that helped to elucidate the acidosis dependent relationship was derived by the gene expression in CON5. Hypoxia slightly downregulated the expression (-0.46 ± 0.0781 SEM); acidosis significantly upregulated it (2.02 ± 0.0984 SEM) and their combined effect resulted in upregulation (1.58 ± 0.1495 SEM). In some ways it was irrelevant whether a cell line was an *IDH2* mutant or a wild type because they were all induced by acidosis.

IDH1/*IDH2* expression were expected to be elevated in JJ012 and SW1353 but the question that emerged from our gene expression data is: why does *IDH* wild type CON5 show elevated expression of *IDH2* expression in acidosis? Especially since the D-2-HG concentration

levels in CON5 were 10.5-fold and 6-fold lower than those of JJ012 and SW1353 respectively. As described in the procedure, the cell lysate used to measure the D-2-HG were obtained from cells that were grown in normoxia/pH=7. The D-2-HG concentration levels produced in hypoxia and acidosis were not measured. This would be an excellent direction and quantifiable data point to further support the gene expression data. Indeed, there is some evidence that indirectly supports the idea that acidosis induces decreased levels of α-KG. Intlekofer, et al. (2017) showed that in response to acidosis (pH=6.0, similar to what hypoxic cells experience), L-2-HG was elevated, and α-KG was reduced by about four-fold. To my knowledge, D-2-HG has not been tested under acidic condition. However, D/L-2-HG are chiral (enantiomer) molecules with similar function to inhibit α-KG. One would expect elevated D-2-HG and downregulated α-KG levels in acidosis just like the ones recorded for L-2-HG.

Hypoxia/Acidosis – From our gene data, we can conclude that inducible hypoxia and extracellular acidosis play a significant role in altering gene expression in all three cell lines under investigation whether individually or combined. Our experiment design included two chondrosarcoma grade II and one primary chondrocyte cell line grown in four experimental conditions: hypoxia (5% oxygen), acidosis(pH=5.5), normoxia/pH=7.0 and hypoxia/acidosis. For future experiment designs, I would suggest that instead of having one fixed pH of acidosis to incorporate a pH range. For example, pH from 6.0-7.0 divided into increments of 0.2. Moreover, to develop a better understanding of the effects of hypoxia, cells can be grown in 1% oxygen in addition to the 5% and 21% oxygen levels. As previously discussed, chondrocytes are exposed to an oxygen gradient that changes in deeper layers of cartilage. 5% oxygen inducible hypoxia used in our study can be applicable more to the resting chondrocytes but not mirror the oxygen exposure in cells found in deeper cartilage layers such as in the proliferating and hypertrophic zones. This would provide further data to build a comprehensive comparison between experimental conditions, cells, and genes of interest.

In our study, the three samples represent three distinct age groups. CON5 –female, mid 20s; JJ012 – male, late 30s; and SW1353 – female, early 70s. We know that age plays an important role in chondrosarcoma. The predominant number of cases are reported between the ages of 30 and 60 at a 1:1,000,000 incidence. However, the age of the third group (70+) are even more susceptible to develop it (Hogendoorn, et al., 2010). Chondrogenesis is a highly regulated

process by many sex hormones not limited to PTHrP, the female hormones of estrogen and progesterone, and the male hormone androgen. As we age, these hormone steroid levels can deplete or be imbalanced for a multitude of reasons, exposing cartilage to many possible diseases including chondrosarcoma and osteoarthritis (Wolff, et al., 2012). Such steroids can affect *SOX9* levels which are crucial at birth for normal cartilage development (Mork, 2010). But as we stop growing and continue to age, those levels can be at risk of being downregulated and induce cartilage frailties. Since SW1353 was isolated from a 72-year-old woman, that can possibly introduce a new confounding factor. However, a larger pool sample of cells from a variety of age groups would be required to elucidate that relationship.

CHAPTER 5
CONCLUSION

Inducible hypoxia and extracellular acidosis play a significant role in gene expression in cartilage biology. This is true due to the hypoxic (5%-1% oxygen) and slightly acidic environment in which chondrocytes reside. Four genes of interest were analyzed. On one side, *PTHR1* and *SOX9* were considered. They are known to delay chondrocyte differentiation while delaying their hypertrophy. *PTHR1* is involved in the PTHrP-IHH loop and is induced by *HIF-1α*. On the other side, *IDH1* and *IDH2* were chosen for relative gene analysis since they are crucial metabolic enzymes that convert isocitrate into α-ketoglutarate in the TCA cycle. Since *IDH1*/*IDH2* have been reported to mutate in cancer cells, two chondrosarcoma grade II cell lines JJ012 and SW1353 were chosen in addition to one *IDH* wildtype primary chondrocyte – CON5. The grade characterization was described for each cell line and morphological differences defined. The presence of *IDH1*/*IDH2* mutations in the chondrosarcomas was further supported by measuring the D-2- Hydroxyglutarate concentration levels in all three cells lines. D-2-HG levels were 10.5-fold (JJ012) and 6-fold (SW1353) higher than in CON5. The effects of acidosis on D-2-HG should be further investigated since our colorimetric assay was conducted in normoxia/pH=7. Our results indicate that *IDH2* is sensitive to acidosis in all three cell lines analyzed. Similarly, *SOX9* is acidosis dependent and it is most upregulated in primary chondrocyte (2.60 ± 0.09 SEM). Our data showed that the only cell line under which *PTHR1* was consistently upregulated in all three conditions was in primary chondrocyte: hypoxia/acidosis (2.10± 0.835 SEM); hypoxia (1.29 ± 0.04) and acidosis (0.84 ± 0.03). The *PTHR1* expression was not as elevated in chondrosarcoma as we expected it to be. This indicates that *PTHR1* is largely missing and other aspects of PTHrP -IHH loop or other signaling pathway need to be analyzed.

These results show that these stressors alternate gene expression when individually applied as well as combined, re-emphasizing the importance of including them in cartilage research. Their synergistic effect can be further elucidated by incorporating a wider range of pH and oxygen levels.

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5115 Hampton Blvd, Norfolk, VA 23529

EDUCATION

	GPA	DATE
• Master's in Biology - Old Dominion University	3.88	2018-Pres.
• Bachelor of Science in Biology– Worcester State University	3.78	2012-2015
• Associates Degree in Liberal Arts - Quinsigamond Community College	3.89	2008-2010

WORK EXPERIENCE

Old Dominion University, Norfolk, VA **08/18 -Pres.**
Teacher Assistant – Microbiology (BIOL 317)

- Teach students valuable techniques such as: isolating, sub culturing, and identifying bacterial cultures; aseptic technique; Gram staining; determining MIC, MBC; interpret APIs and bacterial growth curves.
- Assist and guide students to grow and succeed in their knowledge of microbiology.

Graduate (Thesis) student in Biology – Research topic: PTHR1/SOX9 and IDH1/IDH2 relative gene expression in Chondrosarcoma cells under the synergistic influence of Hypoxia and Acidosis

- Analyze gene expression via RT-qPCR under a variety of experimental conditions.
- Perform Lentiviral transduction in primary Chondrocyte and Chondrosarcoma cell lines. Establish appropriate MOI for each cell line.
- Experienced with cell culturing under aseptic technique while upholding cGMP practices.

New England Newborn Screening (NBS), Worcester, MA **02/17 – 06/18**
Laboratory Technician I – Endocrinology State Lab at UMASS Medical School

- Conduct ELISA assays for a variety of endocrine congenital diseases not limited to: Hypothyroidism (T4 and TSH markers), Congenital Adrenal Hyperplasia (17OHP markers) and Cystic Fibrosis (IRT testing).
- Provide analytical interpretation/reporting of blood markers of newborn specimens to follow-up.
- Operate and maintain daily Genetic Screening Processors (GSPs), AutoDELFIAs, and Pantheras.
- Conduct monthly quality control (QC) reports and analysis for internal review and the Center for Disease Control (CDC).

SGS ACCUTEST, Marlborough, MA **05/16 - 12/16**
Lab Technician / Environmental Chemist- Organic Extractions / Concentrations

- Well-versed in all phases of organic preparatory methods according to US EPA methodologies and SOPs
- Conduct complete extraction of all matrices for PCB/Soxhelt, pesticides, herbicides, ABN/SIM, EPH, TPH, DRO/ORO according to MCP/RCP, DoD, and client specific technical specs.
- Operate and maintain Buchi-Syncore concentrator and various water bath or evaporators such as Organomation, S-EVAP, N-EVAP. Calibrate equipment/sonicators.
- Concentrate samples and QC to be analyzed at GC, GC/MS labs
- Prepare standards (surrogates, spikes, acid/base solutions.) Measure Ph of solutions.
- Proficient in utilizing LIMS, to review work list and schedule the workflow efficiently.