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LIFE SCIENCE AND BIOMEDICINE
REPLICATION
SUPPLEMENTARY-RESULT

All-trans retinoic acid (ATRA) reduces proliferative capacity and Brachyury levels in the chordoma cell line UCH-1

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Abstract

Chordoma is a rare bone cancer for which there are no approved drugs. Surgery is the principle treatment but complete resection can be challenging due to the location of the tumours in the spine and therefore finding an effective drug treatment is a pressing unmet clinical need. A major recent study identified the transcription factor Brachyury as the primary vulnerability and drug target in chordoma. Previously, all-trans retinoic acid (ATRA) has been shown to negatively influence expression of the Brachyury gene, *TBXT*. Here we extend this finding and demonstrate that ATRA lowers Brachyury protein levels in chordoma cells and reduces proliferation of the chordoma cell line U-CH1 as well as causing loss of distinctive chordoma cell morphology. ATRA is available as a generic drug and is the first line treatment for acute promyelocytic leukaemia (APL). This study implies ATRA could have therapeutic value if repurposed for chordoma.

Keywords: Chordoma; Brachyury; All-trans retinoic acid (ATRA); Drug repurposing.

Introduction

Chordoma is a rare bone cancer with an annual incidence of 1 in 1,000,000 people and a poor prognosis. It occurs mostly in the spine and whilst surgical resection is currently the most effective treatment this is difficult due to proximity to important structures. Chordomas are largely refractory to current chemotherapy, therefore, there is a pressing unmet clinical need for effective treatments (Stacchiotti & Sommer, 2015). Recently, the transcription factor Brachyury was identified as a primary drug target in chordoma and has been the subject of drug discovery efforts (Robinson et al., 2020; Sharifnia et al., 2019).

All-trans retinoic acid (ATRA) is available as a generic drug and is used as a differentiation agent to treat acute promyelocytic leukaemia (APL; Fey & Buske, 2013). U-CH1 chordoma cells treated with ATRA have reduced levels of *TBXT* (Brachyury gene) mRNA and reduced proliferative capacity (Aydemir et al., 2012). This study extends and validates these findings offering further insight into the mechanisms of ATRA as a potential chordoma therapeutic agent.

Objective

The finding that ATRA reduces levels of *TBXT* (Brachyury) mRNA and reduces proliferative capacity opens three important questions. Firstly, does the reduction in mRNA levels feed through to measurable

reduction in Brachyury protein? Secondly, is this effect of ATRA specific to the previously tested chordoma cell line, or is it a universal feature of chordoma cells? Finally, is the reduced proliferative capacity induced by ATRA in chordoma cells, which correlates to Brachyury loss, accompanied by the morphological changes observed following specific siRNA-mediated Brachyury depletion? The objectives of this current study are to address these outstanding and important questions.

Methods and materials

Cell culture and drug treatment. U-CH1 and JHC7 were obtained from ATCC and cultured as per ATCC guidelines. For all experiments described, 7.75×10^4 cells were seeded in each well of a 6 well plate. ATRA (Abcam) and DMSO (Sigma Aldrich) were added to the concentrations specified. Drug and media were refreshed every 3 days. Cell counting was performed using a TC20 cell counter (Biorad). Cells were imaged using an Evos Core microscope (AMG).

Protein extraction and western blot. Protein was extracted using M-PER buffer (ThermoFisher Scientific) according to the manufacturer's instructions. Samples were denatured in Bolt LDS Sample buffer and Reducing Agent and run on an SDS-polyacrylamide pre cast gel (Bolt 4–12% Bis-Tris Plus, Life Technologies). Samples were transferred onto PVDF membrane in Towbin buffer (10% methanol) and the membrane was blocked in blocking solution (10% skimmed milk in PBS 0.1% Tween20). The membrane was incubated with primary antibody in blocking solution overnight and the membrane was incubated with secondary antibody at room temperature for 2 hours. Primary antibodies used were anti-Brachyury (Abcam, ab209665) and anti-GapDH (Santa Cruz, sc365062). Secondary (HRP linked) antibodies used were: anti-rabbit IgG (CST, 7074S), anti-mouse IgG (CST, 7076S).

Results

Treatment of chordoma cells with ATRA reduces Brachyury levels. JHC7 and U-CH1 chordoma cells were treated with 10 or 20 μM ATRA. ATRA caused a reduction in Brachyury levels in both lines, although this was less pronounced in JHC7 (Fig.1).

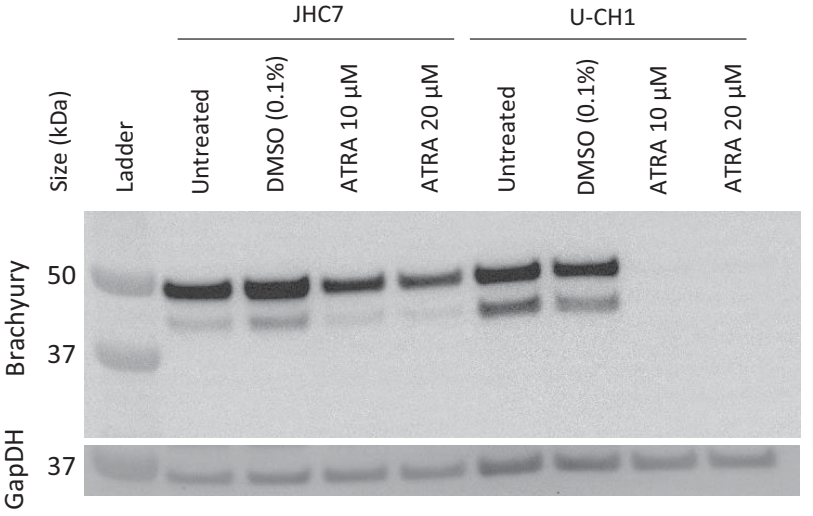

Figure 1. Western blot showing Brachyury levels in two chordoma cell lines following no treatment or treatment with DMSO or ATRA. U-CH1 cells were treated for 6 days and JHC7 cells were treated for 9 days. The same membrane was reprobed with anti-GAPDH antibody as a loading control. The blots were imaged using a Biorad Chemidoc. Colorimetric and chemiluminscent images were combined to show ladder and protein detection in the same image. This western blot is representative of 2 independent repeats.

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Treatment of chordoma cells with ATRA causes reduced proliferative capacity and morphological change. U-CH1 cells treated with ATRA have reduced proliferative capacity (Aydemir et al., 2012). We treated U-CH1 cells with 20 μM ATRA and this resulted in the cell culture failing to increase cell numbers, thus validating the original study (Fig. 2). This was postulated to be a proliferative inhibition but the possibility of cell death or senescence were not excluded. To extend this we assessed whether there were any morphological changes apparent commensurate with those previously observed following specific siRNA-mediated Brachyury depletion (Hsu et al., 2011). ATRA treatment resulted in a loss of the physaliferous phenotype and the cells became more elongated and branching (Fig. 3).

Discussion

Brachyury has emerged as a target for the treatment of chordoma. The finding that ATRA results in the reduction of *TBXT* (Brachyury) mRNA brings ATRA into consideration as a repurposed therapeutic intervention. Here we have added additional insight into the relationship between the ATRA response pathway and Brachyury in chordoma cells. Our findings support a model in which ATRA triggers morphological change consistent with differentiation, which involves the shutdown of Brachyury activity. Indeed, such a model is supported by work in zebrafish notochord development, where ATRA

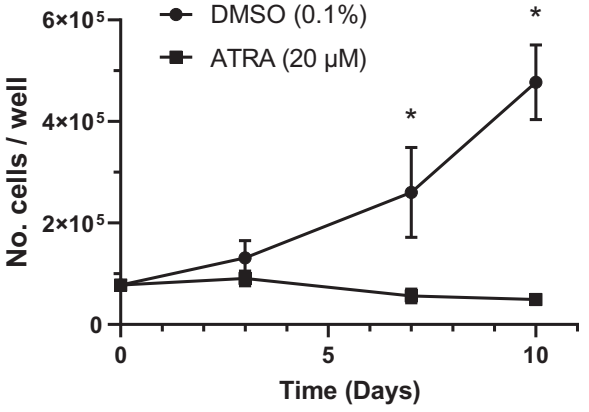

Figure 2. Cell number plot for U-CH1 with and without ATRA treatment. Values shown are the mean of two independent repeats. Error bars show standard error of the mean. Asterisks denote statistical significance <p = 0.05 (multiple t-tests using the Holm-Sidak method, equal variance, no. t tests = 4).

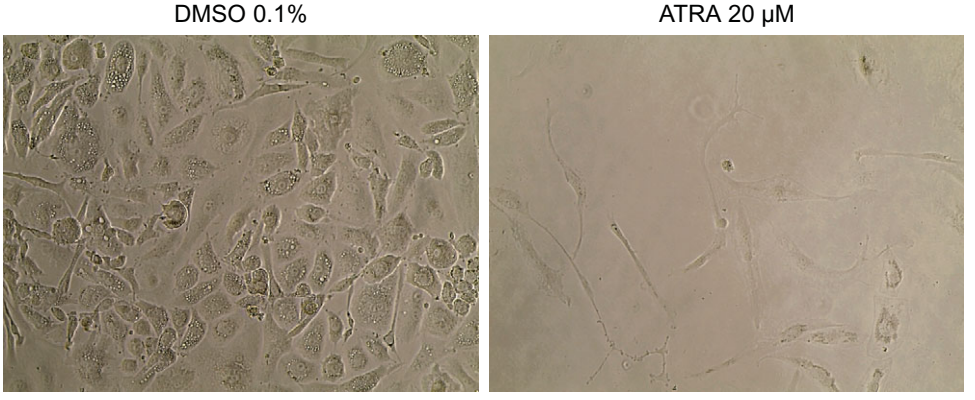

Figure 3. Brightfield images of U-CH1 cells after treatment with either DMSO or ATRA 20 μM for 10 days, illustrating the morphology changes observed for the whole cell population. The pictures are representative of two independent repeats. Images captured using × 20 objective.

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treatment rapidly reduces levels of transcripts from the zebrafish orthologue gene, *ntl* (Martin & Kimelman, 2010).

Conclusions

This study demonstrates that ATRA treatment lowers Brachyury levels, consistent with mRNA studies (Aydemir et al., 2012). The extent to which ATRA influences Brachyury levels varies between chordoma cells types, so targeting Brachyury up-stream regulatory pathways with ATRA might not prove to be universally effective. It remains unclear if Brachyury is directly regulated by an ATRA response pathway, but we can conclude that response to ATRA cellular changes are highly similar to those observed for specific Brachyury depletion. Thus, repurposing of ATRA to target a Brachyury activating pathway is an important consideration.

Author Contributions. HR and JAW conceived and designed the study. HR conducted data gathering. HR performed statistical analyses. HR, JAW and RJM wrote the article.

Data Availability Statement. The data that support the findings of this study are openly available in the Open Science Framework at http://doi.org/10.17605/OSF.IO/C2V6E, reference number [C2V6E].

Conflicts of Interest. HR, RJM and JAW declare no conflicts of interest.

Financial Declaration. This work was supported by Cancer Research Wales (HR, JAW) and Life Sciences Research Network Wales (HR, JAW).

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Peer Reviews

Reviewing editor: Prof. Martin Michaelis
University of Kent, School of Biosciences, Canterbury, United Kingdom of Great Britain and Northern Ireland, CT2 7NJ

This article has been accepted because it is deemed to be scientifically sound, has the correct controls, has appropriate methodology and is statistically valid, and met required revisions.

doi:10.1017/exp.2020.31.pr1

Review 1: All-trans retinoic acid (ATRA) reduces proliferative capacity and Brachyury levels in chordoma cells

Reviewer: Dr. Michael Kelley
Duke University School of Medicine, Duke Cancer Institute, Durham, United States

Date of review: 24 January 2020

Conflict of interest statement. Reviewer declares no conflicts of interest.

Comments to the Author: While the discussion recognizes the potential lack of generalizability, it is not clear why the authors elected to repeat the published work in only the same cell line. The discussion could also recognize that additional preclinical investigation is needed to assess specificity and sufficiency of ATRA in chordoma treatment.

Score Card

Presentation

5.0 /5

Is the article written in clear and proper English? (30%)	5/5
Is the data presented in the most useful manner? (40%)	5/5
Does the paper cite relevant and related articles appropriately? (30%)	5/5

Context

5.0 /5

Does the title suitably represent the article? (25%)	5/5
Does the abstract correctly embody the content of the article? (25%)	5/5
Does the introduction give appropriate context? (25%)	5/5
Is the objective of the experiment clearly defined? (25%)	5/5

Analysis

Does the discussion adequately interpret the results presented? (40%)	5/5
Is the conclusion consistent with the results and discussion? (40%)	4/5
Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%)	4/5

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doi:10.1017/exp.2020.31.pr2

Review 2: All-trans retinoic acid (ATRA) reduces proliferative capacity and Brachyury levels in chordoma cells

Reviewer: Dr. Charles Lin

Date of review: 04 June 2020

Conflict of interest statement. Dr. Charles Y. Lin is employed by Kronos Bio, is a consultant for Jnana Therapeutics and is a shareholder of and inventor of intellectual property licensed to Syros Pharmaceuticals.

Comments to the Author: Here the authors use ATRA, a compound previously shown to decrease brachyury levels in the chordoma cell line JHC7, and show it decreases brachyury in UCH-1 cells. The authors also show that ATRA treatment decreases chordoma cellular growth and that the morphology of the cells change (here authors insinuate cellular differentiation).

It is overstated to say that ATRA decreases brachyury levels and chordoma cell proliferation, when really the authors have replicated the study done by Aydemir et al., and extended this to one other cell line. They should make this clear in the title (All-trans retinoic acid (ATRA) reduces proliferative capacity and Brachyury levels in the UCH-1 chordoma cell line), or, alternatively, test this hypothesis in multiple other chordoma cell lines. Furthermore, the authors state that UCH-1 cells are differentiating with ATRA treatment, yet they show no actual evidence that this is the case other than the change in morphology (do the expression of specific differentiation markers change?). The pictures of cells +/- ATRA treatment show far fewer cells with ATRA treatment. Are these cells undergoing cell death (rather than differentiation) upon treatment that that is why they are reducing in numbers?

Brachyury seems to be minimally reduced with ATRA treatment in JHC7. Can the authors quantify the western? Is this reduction significant? Furthermore, the pictures of cells + ATRA should be made clearer. Finally, the authors should show full concentration-response curves with ATRA so we understand the dosing chosen (20μM) for both JHC7 and UCH-1 cells.

Score Card

Presentation

Is the article written in clear and proper English? (30%)	5/5
Is the data presented in the most useful manner? (40%)	4/5
Does the paper cite relevant and related articles appropriately? (30%)	2/5

Context

Does the title suitably represent the article? (25%)	2/5
Does the abstract correctly embody the content of the article? (25%)	4/5
Does the introduction give appropriate context? (25%)	3/5
Is the objective of the experiment clearly defined? (25%)	5/5

Analysis

2.6 /5

Does the discussion adequately interpret the results presented? (40%)	2/5
Is the conclusion consistent with the results and discussion? (40%)	4/5
Are the limitations of the experiment as well as the contributions of the experiment clearly outlined? (20%)	1/5

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