BRIEF REPORT

The zebrafish fibroblast cell line AB9 as a tool to complement gene regulation studies

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The regenerating caudal fin of an adult zebrafish is a great model system to study gene regulation and function. However, manipulation of essential genes in adult fins can be challenging. In this brief report, we suggest that a fibroblast cell line AB9, isolated from regenerating caudal fins of adult zebrafish, can be used to perform pilot studies for gene regulation. We also provide standardized protocols for morpholino and drug treatment of AB9 cells. Our findings suggest that the AB9 cell line is an easily manipulated system that expresses key regulators of the Cx43 dependent growth and patterning pathway, identified from studies in the regenerating fin. We also provide evidence that expression and function of proteins can be easily manipulated, either through targeted morpholino knockdown or by pharmacological inhibition.

Keywords: AB9; zebrafish; morpholino; Cx43

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Introduction

Zebrafish has emerged as an important model system to study growth and development in the last few decades. Large scale mutagenesis studies in zebrafish have identified important genes during vertebrate development. The incredible regenerative capability of both embryonic and adult zebrafish has made it a great model system to examine vertebrate tissue regeneration. However, unlike human and mice, relatively few *in vitro* studies have been carried out in zebrafish to complement the *in vivo* findings.

In the past few decades, a number of stable cell lines from zebrafish embryos (ZEM2S) [1]; (ZF4) [2] and adult caudal fins (AB9 and SJD.1) [3] and liver (ZF-L) [4] have been developed. Cell based assays are important since they can complement findings from *in vivo* studies. Moreover, they are also a simpler system to study gene functions. Zebrafish embryos have been used successfully in toxicology studies [5] and characterizing drug treatment in a stable cell line derived from zebrafish can be an important first step. Here we provide evidence that the zebrafish fibroblast cell line, AB9, can be used to complement regeneration studies in adult fins. Though the zebrafish caudal fin is an excellent system to study regeneration, it has some limitations. It takes about 2 to 4 months to obtain mature zebrafish and manipulation of gene expression in adults can present challenges. Morpholinos are often used for gene knockdown, but off-target effects raise concerns [6]. Examination of essential genes in mutants is not possible during adult stages. The zebrafish caudal fin cell line provides a less complex tissue for pilot studies on gene function that may ultimately be tested in regenerating fins.

Our lab is interested in the significance of Cx43 dependent gap junctional communication in bone growth and patterning

during fin regeneration. We have previously shown that Cx43 promotes cell proliferation/growth and inhibits joint formation/patterning by regulating downstream effectors in regenerating caudal fin. Here we demonstrate that the AB9 cell line can be used to complement our *in vivo* findings. For example, we find that this cell line expresses most of the major players in our pathway such as Connexin43 (Cx43), Semaphorin3d (Sema3d), PlexinA3 (PlxnA3), Neuropilin2a (Nrp2a), Heat shock protein 47 (Hsp47) and Collagen type II (Col II). We also provide evidence that gene expression can be manipulated in this cell line using standard techniques for knock down and pharmacological inhibition.

Materials & Methods

Maintenance of AB9 cells

AB.9 (ATCC® CRL-2298™) is a zebrafish caudal fin-specific fibroblast cell line which grows in Dulbecco's Modified Eagle's medium (DMEM) supplemented with heat-inactivated fetal bovine serum (15%) and antibiotics-antimycotics (Gibco) at 28°C with 5% CO_2. Subculturing can be done once or twice a week depending upon the cell confluency. First, the cells were washed briefly with 1X PBS. To a 100mm culture dish, 2-3 ml of Trypsin-EDTA was added and incubated at 28˚C for approximately 5 minutes or until the cell layer was completely dispersed. To the trypsinized cells, approximately 2-3 ml of growth media was added to inactivate the trypsin. More media was added depending upon the subcultivation ratio (The recommended subcultivation ratio is 1:3 to 1:4) and the cells were aspirated gently with pipetting. Approximately 8-10ml of the culture media containing cells were seeded in 100mm culture dish and incubated for 24-48 hours at 28°C.

Immunofluorescence on AB9 cells

AB9 cells were incubated at 5% CO_2 and 28˚C and grown in tissue culture dishes with minimal essential media (DMEM) supplemented with 15% heat inactivated FBS and antibiotics-antimycotics (Gibco). The cells were seeded onto poly-L-lysine coverglasses and allowed to grow to 80-90% confluency. They were then washed once with cold PBS and fixed with 4% paraformaldehyde in PBS for 30 minutes at room temperature. This was followed by washes in 1X PBS (3X, 5 minutes each), permeabilization with 0.2% triton in PBS for 10 minutes, again multiple washes in 1X PBS and then blocked in room temperature for an hour in 1% BSA in PBS. The following primary antibodies were used: Rabbit anti-Cx43 – (1:200) [7], Rabbit anti-Sema3d (Sigma, 1:200), Rabbit anti-Hsp47 (Abcam, 1:100), Mouse anti-Col II (DSHB-II-II6B3, 1:50), Rabbit anti-Nrp2a (GenScript, 1:200) and Rabbit anti-PlxnA3 (GenScript, 1:200).The coverslips were incubated in primary antibody overnight at 4°C in a wet box. The next day, they were washed in 1X PBS (3X, 5 minutes) and were incubated with secondary antibody for 1 hour at room temperature (RT), protected from light. The following secondary antibodies were used: anti-Rabbit Alexa Fluor 488 or 568 (1:200), anti-Mouse Alexa Fluor 488 or 568 (1:200). DAPI (1:1000) was used to stain the nuclei. It was followed by three 5 minutes washes in 1X PBS and one quick wash in dH2O. They were then mounted with Vectashield and were examined under Nikon Eclipse TE2000-U at 60X.

Morpholino-mediated protein knockdown via electroporation in AB9 cells

AB9 cells were seeded on to 100mm culture dishes and allowed to grow till 80- 90% confluency. On the day of electroporation, the adherent cells were washed briefly with 1x PBS and trypsinized in 0.05% Trpsin-EDTA 1X (Gibco) for 5 min at 28˚C. DMEM media supplemented with 15% heat inactivated FBS, antibiotics-antimycotics (Gibco) were added to inactivate the trypsin. The cells were collected by centrifugation at 750 rpm for 5 minutes. The pellet was resuspended in 1-5ml of HEPES buffer (115mM NaCl, 1.2mM $CaCl_2$, 1.2mM $MgCl_2$, 2.4mM K_2PO_4 and 20mM HEPES with pH adjusted to 7.4) and put on ice. Meanwhile, electroporation cuvettes (Cell Projects) were prechilled on ice and morpholinos were heated at 65˚C for 15 min. 5-10 µM of morpholinos were added to 400µl of resuspended cells in the cuvettes on ice and incubated for 5 minutes. The cells were electroporated at 170V for 6-7 ms using an electroporater (BioRad Gene Pulser X Cell). Electroporated cells were added to 1ml of fresh media in 60mm culture dishes and incubated at 28˚C for 24 hours. The electroporated cells were then used to prepare cell lysates for western blots. Briefly, cells were washed in cold 1X PBS and 1X cold lysis buffer (5mM Tris-HCl, 5mM EDTA, 5mM EGTA with pH 7.5). 500µl of the lysis buffer, along with 100X Halt-TM protease and phosphatase inhibitor cocktail (Thermo Scientific) and 0.6% SDS buffer were added to the culture dishes and the cells were scraped using a sterile rubber cell scraper (Corning Incorporated, Costar). The cell lysate was collected using a 5ml syringe (Beckton Dickinson) with an 18G needle in a fresh tube and heated for 3 minutes at 100˚C. The lysate was passed through a 26G needle and cooled on benchtop. The cell lysates were stored in small aliquots in -80˚C. These cell lysates were used to run western blot as described [7]. Cx43 and Tubulin were detected using anti-Cx43 (1:1000) and anti-α-Tubulin (1:1000) (Sigma) respectively. The primary antibody step was followed by incubation in peroxidase-conjugated Goat anti-Rabbit IgG (1:10,000) for Cx43 blot and peroxidase-conjugated Goat anti-Mouse IgG (1:10,000) for Tubulin. The ECL chemiluminescent reagent (SuperSignal

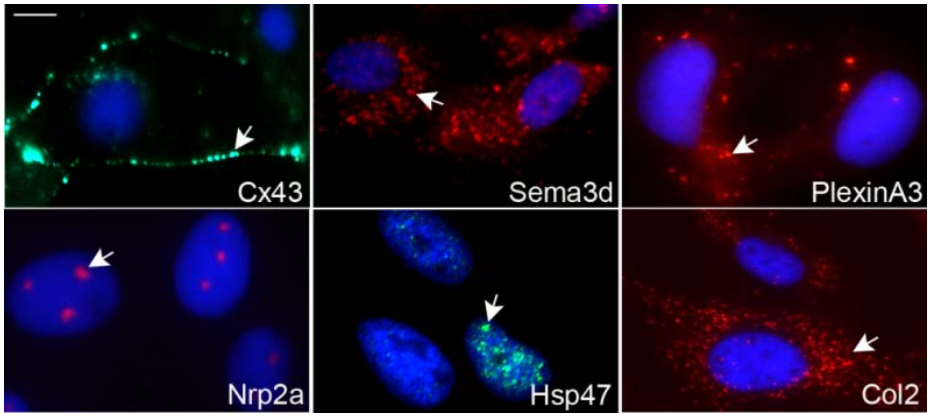

Figure 1. Immunofluorescence analysis on AB9 cells shows expression of various proteins. AB9 cells are stained using antibodies against Cx43, Sema3d, PlxnA3, Nrp2a, Hsp47 and Col II. DAPI (blue) is used for counter-staining nuclei. (Top left panel) Cx43 is expressed as punctate dots in the plasma membrane in the region where two cells are connected. (Top middle panel) Sema3d is observed to be distributed in the cytoplasm. (Top right panel) PlxnA3 is present on the membrane of the cells. (Bottom left panel) Nrp2a is present in the nucleolus of AB9 cells. (Bottom middle panel) Hsp47 is present around the nucleus. (Bottom right panel) Col II is distributed across the cytoplasm. The arrows indicate the specific staining of the respective antibodies. Scale bar is 10µm.

West Femto Maximum Sentivity Substrate, Pierce Rockford, IL) and X-ray films were used for signal detection. Image J software was used for the calculation of relative band intensities [8]. Stripping of the blot was done using stripping buffer (BioRad). The blots were incubated for 5 minutes in a rocker at room temperature and for 20 minutes at 65 ˚C in a water bath with agitation. The blots were washed extensively with 1X TBST and processed for western blot as mentioned [7].

Drug treatment and BrdU staining on AB9 cells

AB9 cells were seeded onto poly-L-lysine coverglasses and were allowed to get 60 – 70% confluent. They were then treated with the 100 µM Hsp47 inhibitor drug (RH01393, ThermoFisher Scientific) or DMSO as control in media for 24 hours. Bromodeoxyuridine (BrdU) staining was done following an immunoflurescence protocol from Cell Signaling Technology. In brief, BrdU (Roche) was diluted in pre-warmed growth media to a final concentration of 0.03 mg/ml. Cells were then incubated with this solution for 30 minutes at 28°C. They were then fixed in cold 70% ethanol for 5 minutes, followed by PBS washes (3X, 5 minutes each). After that, incubation in 1.5M HCl for 30 minutes at RT was followed by PBS washes (2X, 5 minutes each). The cells were then incubated in blocking buffer (5% Goat serum, 0.3% Triton X-100 in 1X PBS) for 1 hour at RT. The coverslips were incubated in Mouse monoclonal anti-BrdU (Roche, 1:50) diluted in antibody dilution buffer (1% BSA in 1X PBS) overnight at 4˚C. It was followed by PBS washes (3X, 5 minutes each) the next day. The coverslips were then incubated in anti-Mouse Alexa Fluor 546 (1:200) and DAPI (1:1000) for 1.5 hours at RT protected from light. It was followed by three 5 minutes washes in 1X PBS and one quick wash in dH2O. They were then mounted with Vectashield and were examined with Nikon Eclipse TE2000-U at 40X.

Results:

For a cell line to be suitable for functional studies, at a minimum it must be easy to visualize protein expression, transfect plasmids, and inhibit protein function for functional assays. Efficient transfection of AB9 cells has been described previously using nucleofection [9]. For our studies, we began by evaluating protein expression through immunofluorescence. We especially focused on proteins related to the Cx43 dependent pathway identified in our lab [8, 10].

AB9 cells express major players in our current model pathway

Immunofluorescence on AB9 cells showed the expression of a majority of proteins in our model pathway (Figure 1). For example, Cx43, a gap junction protein, was expressed as its characteristic punctate dots in the plasma membrane in the region where two cells abut one another [7] whereas Sema3d, a secreted signaling molecule, was observed primarily in secretory vesicles. PlexinA3 and Nrp2a are the two putative receptors of Sema3d [10], and were expected to be present on the membrane. PlxnA3 did show a membrane expression pattern, however we observed robust expression of Nrp2a in the nucleoli of AB9 cells. Interestingly, we have seen a

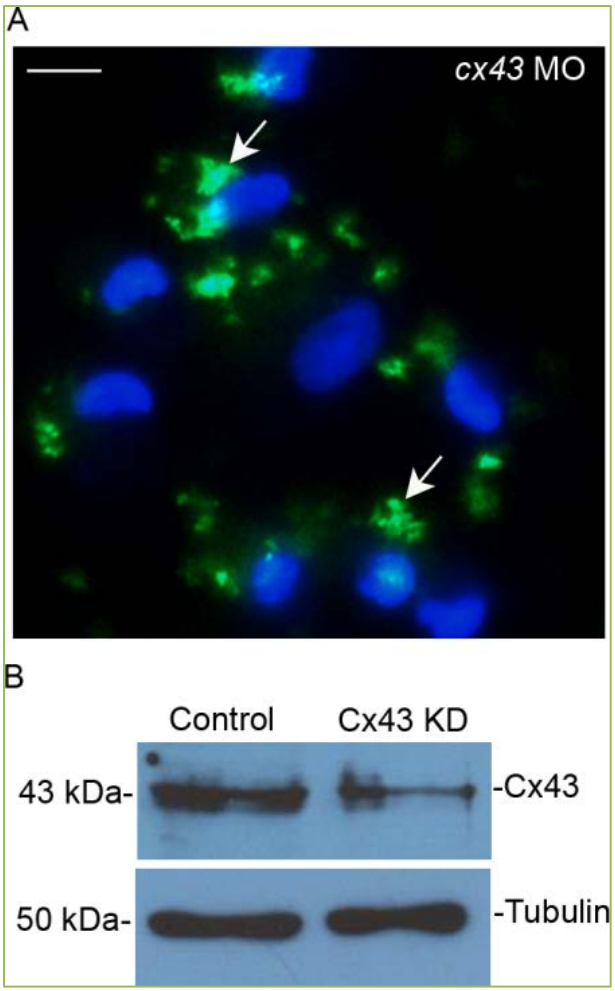

Figure 2. MO-mediated knockdown of Cx43 via electroporation results in reduction of Cx43 protein levels. (A) Representative image of *cx43* morpholino (MO) electroporated in AB9 cells. The arrows indicate the *cx43* MO (green) and was observed 4 hours after electroporation. DAPI (blue) was used for counter-staining nuclei. Scale bar is 10µm. (B) (Top) Western blot image showing a 64% reduction in Cx43 protein levels in Cx43 knockdown cell lysate (*cx43* MO electroporated in AB9 cells) compared to control cell lysate (standard-control morpholino electroporated in AB9 cells). The anti-Cx43 bands are observed at 43 kDa as expected. (Bottom) Western blot image showing the anti-tubulin bands at 50 kDa as the loading control.

similar expression pattern for Nrp2a in regenerating caudal fin as well (data unpublished). Hsp47, a molecular chaperone for collagens, has been recently identified in our lab to be a part of the Cx43 dependent pathway regulating growth and patterning [8]. Hsp47 is an endoplasmic reticulum (ER) resident protein that binds to the collagen triple helix in the ER and transports collagen to the Golgi. In AB9 cells, we observe that Hsp47 is present specifically around the nucleus in a vesicular pattern, where we expect to see ER and Golgi resident proteins. Col II, a secreted protein, is found in vesicles distributed across the cytoplasm. These findings reveal expression and localization of several proteins that function during fin regeneration.

Functional assays in AB9 cells

Knocking down a protein is an important part in studying gene function during development. In our lab, we regularly use antisense morpholinos (MO) to knockdown a protein of interest and study the resulting phenotypes in regenerating caudal fins. To perform knockdown in AB9 cells, we first tested whether electroporation is a reliable method for cellular uptake of MO. We electroporated fluorescein labeled *cx43* MO into AB9 cells, using fluorescein labeled Standard Control MO as a control. In both cases, transfection efficiency was observed between 50% - 70% at approximately 4 hours post electroporation (hpe) (Figure 2A). Next, we tested if MO delivery in AB9 cells led to protein knockdown. Lysates were prepared from Standard Control and *cx43* MO treated cells at 24 hpe. Figure 2B confirms the efficient knockdown of Cx43 by immunoblot. The Cx43 protein levels were reduced (64%) in the knockdown sample (lane 2) compared to the control sample (lane 1). Tubulin was used as a loading control.

We next determined whether we can block protein function by treating AB9 cells with pharmacological agents. For these studies, we chose an inhibitor of Hsp47. In regenerating fins, Hsp47 knockdown causes reduced cell proliferation and growth [8]. In order to determine whether we can replicate a similar downregulation of cell proliferation in AB9 cells, we treated the cells with 100 µM of a chemical inhibitor for Hsp47, RH01393 [11], using DMSO as control. BrdU was used to label proliferating cells. Interestingly, we saw a decrease in cell proliferation following treatment with 100 µM Hsp47 inhibitor (approximately 44%) when compared to DMSO as control (approximately 65%) (Figure 3). Cell viability was 85% following drug treatment (data not shown).

Discussion

The AB9 cell line is an established stable primary fibroblast cell line cultured from the AB strain of adult zebrafish caudal fins [3]. Previous studies have shown high transfection efficiency in these cell lines using nucleofection, thus making it an excellent tool to generate a transgenic cell line [9] or to study overexpression of a particular protein. In this brief report, we provide evidence that the AB9 cell line can also be used for gene knockdown and drug treatment studies. One of the ways to temporarily knockdown a protein in zebrafish is by injecting them with an antisense oligonucleotide targeted against a specific gene. Antisense oligonucleotides have been used to modify the splicing

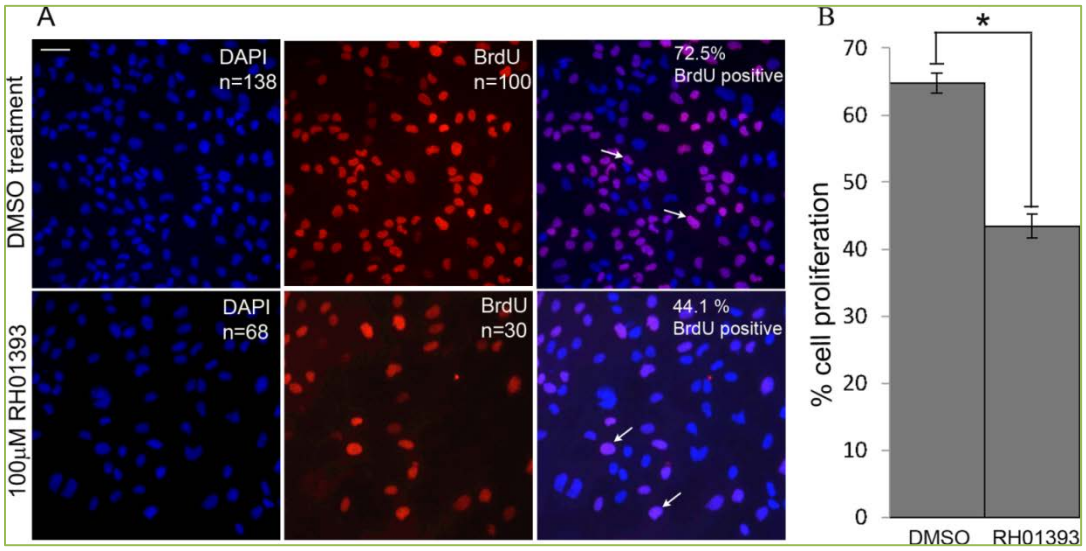

Figure 3. BrdU staining in drug treated AB9 cells reveals significant reduction in cell proliferation. (A) Hsp47 inhibitor Drug (RH01393)/DMSO treatment of AB9 cells for 24 hours. DAPI (blue) is used for counter-staining nuclei indicating total number of cells (n). Top 3 panels represents DMSO treated cells as a mock treatment. Cells are stained with DAPI (n=138), BrdU (n=100) and merged showing 72.5% of BrdU positive cells. Bottom 3 panels represents cells treated with Hsp47 inhibitor drug (RH01393). Cells are stained with DAPI (n=68), BrdU (n=30) and merged showing 44.1% of BrdU positive cells. Scale bar is 20µm. (B) Graph shows the significant reduction (*) in the percentage of BrdU positive cells in Hsp47 inhibitor drug (RH01393) treated cells compared to the DMSO treated control cells. Student's t-test was used for determining statistical significance where $p < 0.05$. Standard error is represented by error bars.

pathway in HeLa cells [12]. The uncharged phosphorodiamidate morpholino oligomers (PMO) are widely used in mammalian cell culture to inhibit RNA or DNA virus amplification [13-20]. Both PMOs and charged fluorescein tagged MOs have been extensively used in zebrafish to study gene function. MOs are especially critical in studying gene function in adult fins, where knocking out an essential gene can be embryonically lethal and generation of an adult specific conditional knockout is not yet possible. AB9 cells can be used to emulate growth and patterning phenotypes from regenerating fins. For example, growth may be evaluated using standard cell proliferation assays, such as BrdU labeling; patterning, on the other hand, may be evaluated by following differentiation markers. This will not only complement MO studies in regenerating fins, but will also help to identify 'on-target' effects in regenerating fins. To our knowledge, this is the first report describing MO uptake through electroporation by a cell line from zebrafish.

The AB9 cell line is also a simpler tool to study the preliminary efficacy of a drug or toxicity. These cells have been used to complement *in vivo* studies in embryos for screening of drugs affecting cell cycle [21]. Many of these compounds were shown to be active in both embryos and AB9 cell lines, thus complementing findings between *in vivo* and *in vitro* studies. We have shown that using the chemical inhibitor for Hsp47 in AB9 cells recapitulated the reduced cell proliferation phenotype observed in regenerating fins following MO mediated Hsp47 knockdown, providing additional evidence that inhibition of Hsp47 contributes to the regulation of cell proliferation. Continued studies in AB9 cells will reveal underlying mechanism of this regulation, which, in turn, may be tested during fin regeneration.

In conclusion, this report aims to promote the fibroblast cell line from zebrafish caudal fin, AB9, as an important tool to complement *in vivo* studies in adult regenerating fin. In particular, this cell line expresses several proteins identified in the Cx43 dependent growth and patterning pathways. Moreover, we believe this cell line will provide a simpler model to study regulation of cell signaling pathways during regeneration, as well as preliminary studies on the efficacy of a particular drug.

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Author's contributions

JB contributed to the conception, design, and drafting of this brief report. She completed immunofluorescence,

standardization of the morpholino knockdown protocol, and BrdU assay following drug treatment protocol. RB completed immunofluorescence on cells, standardization of the morpholino knockdown protocol, and performed immunoblotting following morpholino treatment. She also contributed to writing and editing the report. JS and NS facilitated immunofluorescence studies by standardizing procedures and maintaining AB9 in cell culture; these authors also edited the report. DE standardized cell culture methods and contributed to immunofluorescence studies. She also edited the report. KI contributed to the conception, design, and drafting of this brief report.

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