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Article

The Potential of Fibroblast Growth Factors to Stimulate Hair Growth *In Vitro*

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SUMMARY

There is a great need for studying mechanisms of hair follicle cycles and designing small molecular drugs, biologics, formulation, and surgical treatment for androgenic alopecia (AGA, a common form of hair recession). While minoxidil has served as a solution in the past, it is a vasodilator and can react heavily with alcohol. Furthermore, minoxidil must be used at night, as it requires 6 hours for the product to settle and perform its function. Therefore, alternative approaches are needed to treat AGA. Numerous clinical trials are studying the use of fibroblast growth factors (FGFs) for tissue regeneration applications. Additionally, FGFs have been proven to stimulate stem cell growth and tissue regeneration. In the present study, we tested the effects of four FGFs, namely FGF1 (aFGF), FGF2 (bFGF), FGF10 (KGF-2), and FGF21, as compared to the gold standard, minoxidil, on isolated primary mouse hair follicle dermal papilla (DP) cells. We also used plain cell culture media (without growth factors or serum) as a negative control. Cell proliferation and viability were measured using both a biochemical assay and fluorescent microscope examinations. Our results indicate that several FGFs can promote DP cell proliferation. Interestingly, our results show that minoxidil promotes DP cell proliferation in the absence of a vascular system. In addition, DP cells cultured in FGFs exhibited a more organized cytoskeleton as compared to the ones cultured with minoxidil, suggesting the potential advantages of FGFs in general cellular health. In that regard, we hypothesize that FGFs can stimulate hair growth and treat AGA.

INTRODUCTION

Patterned hair loss (androgenic alopecia, or AGA, being the most common type of hair loss) can affect all ethnicities and genders at different ages (1). Statistically, more than 50% of all men over the age of 50 will develop hair loss while the same holds true for women after menopause (2). In the United States alone, there are an estimated 50 million men and 30 million women affected by hair loss (2). Hair loss can be a result of family history, hormonal changes, medical conditions, medication and supplements, radiation therapy, stressful events, hairstyles, or disease treatments (e.g., chemotherapy). Hair loss can also be dependent on age, gender, and climates and lead to emotional distress and anxiety.

There are three phases of hair growth: anagen phase, catagen phase, and telogen phase (3). The anagen phase is the hair growth period consisting of 90-95% of the total hair mass, lasting around 2 to 8 years with the constant growth of hair. Dermal Papilla (DP) cells are created in this stage and actively proliferate (3). Without proliferation in this stage, the strands are too short and the hair sheds extremely quickly. The catagen phase is the transition phase where the hair stops growing, lasting around 4 to 6 weeks, and the telogen phase is when the hair falls out, lasting around 2 to 3 months (4). The cause of AGA starts with variations in one gene (the AR gene), which has been confirmed in recent scientific studies (5). The AR gene provides instructions for making proteins called androgen receptors. When these receptors are disrupted, the body does not respond appropriately to dihydrotestosterone and other androgens. AR genes are usually activated during the anagen phase, or growth phase, of the normal hair growth cycle. In AGA, however, excessive activation from these genes leads to follicular miniaturization and a shorter anagen phase, resulting in thinner and shorter hair follicles (5).

Although minoxidil (marketed as Rogaine®) was commercialized in the 1980s and became the main hair growth product, working by dilating capillaries to increase blood flow to the scalp region, there are many side effects (6). When taken orally, minoxidil can treat high blood pressure, and when taken topically, minoxidil can benefit certain types of baldness (7). However, minoxidil may interact with alcohol and lead to other side effects associated with its role as a vasodilator, including dizziness and chest pain (8). Furthermore, minoxidil lacks a confirmed mechanism for hair growth, and it is unknown whether minoxidil can directly promote hair cell growth (8). Thus, there is a need to find alternative approaches for treating hair loss. Despite its side effects and other FDA-approved drugs including ruxolitinib and tofacitinib for treating general hair loss, minoxidil has risen as the most popular product for hair loss treatment (9-11). Nevertheless, minoxidil has side effects affecting a certain percentage of users. Previous studies suggest minoxidil produces side effects in 13.7% of users in the high dose (5%) group and 10.3% of users in the low dose (2%) group, including itching, burning, and flaking (12).

We sought to investigate safer alternatives and safer strategies to stimulate hair growth by using fibroblast

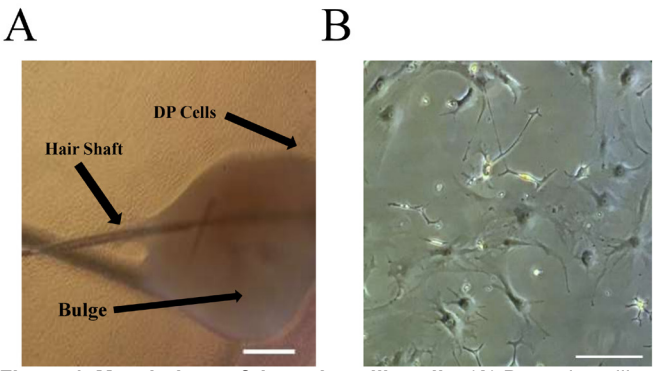

Figure 1. Morphology of dermal papilla cells. (**A**) Dermal papilla cells derived from a hair follicle of C57BL6 mouse. Scale bar, 500 µm. Taken with a white light inverted microscope. (**B**) Passage one dermal papilla cell images. Scale bar, 50 µm.

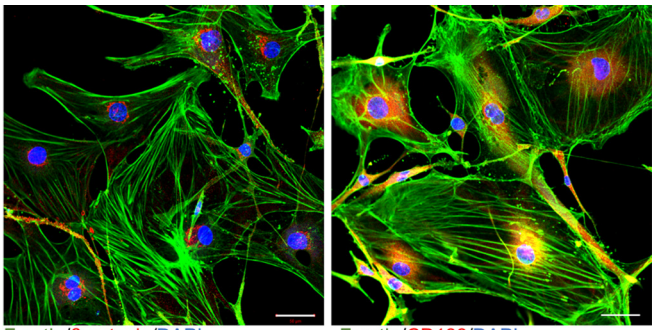

Figure 2. Representative immunofluorescent staining of dermal papilla cells. Green represents the cytoskeleton (F-actin) and blue for the cell nuclei (DAPI). The cells were also stained with two dermal papilla cell markers (β-catenin and CD133), shown red in the images, respectively. Scale bar, 50 µm.

growth factors (FGFs), which are a family of cell signaling proteins, naturally present in the body, that regulate a wide variety of biological processes (13). FGFs play a role in cell replication as well as tissue repair and cranial structure (14-15). Dysregulated expression of the FGFs is associated with multiple conditions, including unregulated cell growth and cell decline (as seen in AGA) (16). Previous studies show FGFs' positive effects on Type 2 Diabetes (through regulation of insulin cells), targeting cell signaling involved with the action on skin aging, and reducing tumors produced naturally in the body (17). There are 18 mammalian FGFs, which are grouped into six subfamilies based on differences in sequence homology and phylogeny, each specializing in a specific role: 1. FGF1 and FGF2; 2. FGF3, FGF7, FGF10, and FGF22; 3. FGF4, FGF5, and FGF6; 4. FGF8, FGF17, and FGF18; 5. FGF9, FGF16 and FGF20; 6. FGF19, FGF21, and FGF23 (18). Additionally, since FGFs are already present throughout the body, we hypothesized that directing these growth factors to the targeted area at an optimal dose would cause less toxicity and side effects (20, 21). FGFs may be a promising stimulant for hair cell growth since many types of FGFs have been established as sources of cell growth for regenerative medicine applications (19, 22). However, studies on their effects on hair regrowth have been sparse. Over the last two decades, FGFs have been reported to be major players in both embryonic development and tissue repair (23, 24). Many studies also point to somatic stem cells as major targets of FGF signaling in both tissue homeostasis and repair (25). FGFs appear to promote self-renewing proliferation and inhibit cellular senescence, further increasing the reliability of these factors (26-28).

In the present study, we chose four types of FGFs and tested their potential in promoting hair cell growth in vitro. The four FGF types are acidic fibroblast growth factor (aFGF), basic fibroblast growth factor (bFGF), keratinocyte growth factor-2 (KGF-2), and fibroblast growth factor 21 (FGF21) along with two controls. The reason we chose the particular FGFs is that previous literature had examined the effects of these FGFs, namely aFGF (29), bFGF (30), KGF-2 (31), and FGF21 (32) on cell growth and their applications for regenerative medicine. We used mouse hair follicle DP cells as model systems in our study, because access to human hair follicle models or in vivo animal studies are more difficult for high school students. We used cell proliferation and cell viability of DPs as indicators of hair growth stimulation. We hypothesized that FGFs may be beneficial in promoting hair cell growth in vitro. We found that FGFs were beneficial as compared to the gold standard, minoxidil. Although we have not yet tested in vivo, our study finds that FGFs may be a promising choice for improved APA treatments. This study serves as a proof of concept which paves the ground for future preclinical studies in vitro and in vivo.

RESULTS

Since it is difficult to do animal experiments and more so for human clinical trials, we sought to use human DP cell culture as a cellular model to study the effects of FGFs. Known for their regenerative abilities, mouse skin dermis tissues were cultured in 100 mm dish (**Figure 1**). DP cell growth can benefit hair regrowth, while DP cell loss can result in baldness, justifying our use of DP proliferation in vitro as a proxy for hair growth in vivo. Moreover, it was previously reported that cultured DP cells also have hair-forming activity and do not lose the activity even after long-term cultivation (33). We used CD133 and β-catenin to identify DP cells from the other cell types in the skin tissue using immunohistochemistry. Therefore, we were able to demonstrate that DP cells can be identified and isolated from dermis tissue for experimentation (**Figure 2**) (34).

To determine cell viability and proliferation, we used Cell Counting Kit-8 (CCK-8), which provides a convenient and sensitive method to measure cell proliferation and toxicity. The kit utilizes a highly water-soluble tetrazolium salt, WST-8, which produces a water-soluble formazan dye upon reduction. The amount of the formazan generated by dehydrogenases is relative to the numbers of living cells. We utilized CCK-8 to measure cell proliferation with FGFs and minoxidil and FBS controls (**Figure 3**). The raw and normalized data indicate that DP cells in 0% FBS media

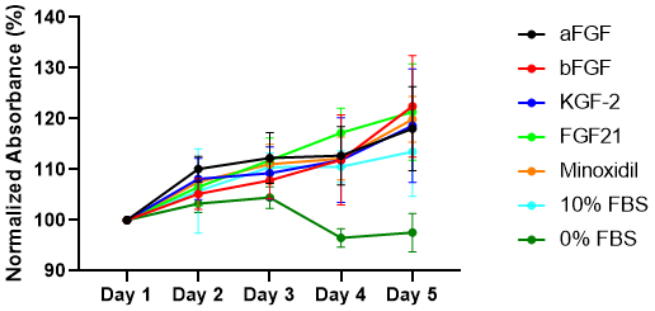

Figure 3. Normalized CCK-8 absorbance between different hair growth factors. Normalized values from each of the triplicate samples to their Day 1 values then calculated the means and standard deviations in form of the normalized percentage (%) since Day 1. Each point represents the mean and error bars of +/- 1 standard deviation.

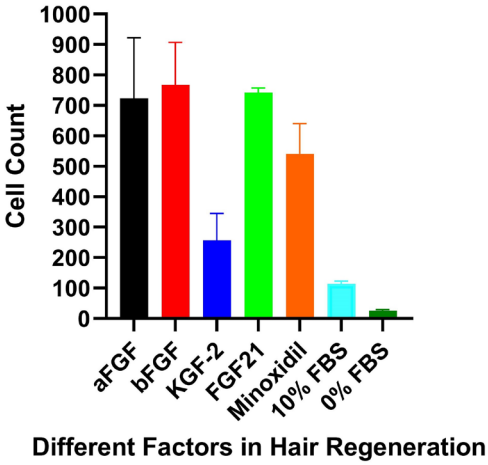

Figure 4. Cell number counting by Hoechst staining and microscopic observation. Total cell count (raw number) of the different groups after the 5-day incubation. Each point represents the mean and error bars of +/-1 standard deviation.

proliferated poorly, with a 3.5% cell decrease over the time course. DP cells in all other groups proliferated more than in 0% FBS media based on statistical analysis, ranging from 15% to 22% growth in various conditions (**Figure 3**). The cell growth of minoxidil-treated cells remained steady through the 5-day experiment, proliferating around 15% of the original culture. The percentages shown were derived from a simple mathematical equation between the normalized starting cell number for all factors (100) and the varying end numbers (**Table 1**). For instance, minoxidil's normalized cell number grew from around 100 to around 115, demonstrating healthy proliferation of around 15%. While the FGFs and minoxidil performed relatively differently, bFGF (bFGF vs. Minoxidil; p=0.9967) and FGF21 (FGF21 vs. Minoxidil; p=0.9999) demonstrated a trend present with minoxidil, but the results were not statistically significant. Because it is difficult to count cells using a white light microscope, we used Hoechst staining to visualize the nuclei of cells so that we could clearly count the numbers of DP cells cultured in different FGF groups for 5 days. Similar to previous experiments, we used a negative control (0% FBS media) and a standard cell culture control (10% FBS media). Our data demonstrated that cells cultured in bFGF, FGF21, and aFGF had the highest cell numbers after the 5-day culture with average cell counts of 768.0, 742.0, and 723.7 cells respectively under a 4x objective. These average numbers were greater than that of minoxidil-treated cells (541.7 cells) but was not shown to be statistically significant. (**Figure 4**) By contrast, the negative control (0% FBS media) had an average of 25.3 cells and the positive control (10% FBS media) had an average of 113.7 cells. Literature has shown 0% FBS plain media is ineffective in culturing DP cells, so we used that as a negative control for

	aFGF Average	aFGF SD	bFGF Average	bFGF SD	KGF-2 Average	KGF-2 SD	FGF21 Average	FGF21 SD	Minoxidil Average	Minoxidil SD	10% FBS Average	10% FBS SD	0% FBS Average	0% FBS SD
Day 1	100.000	6.359642	100.000	5.956147	100.000	5.696448	100.000	3.040194	100.000	3.695829	100.000	3.759712	100.000	3.878311
Day 2	110.096	7.505923	105.187	2.542156	108.165	8.018366	106.571	7.301391	107.550	8.860022	105.786	6.070046	103.256	6.307554
Day 3	112.265	4.069416	113.447	6.156205	109.286	4.520595	111.802	7.319399	111.070	7.357326	110.489	4.692698	104.488	6.224015
Day 4	112.711	5.388877	111.914	7.486792	111.858	4.763786	117.246	8.423589	112.158	6.358416	110.569	7.204507	96.506	3.911018
Day 5	118.058	3.577029	122.468	7.282990	118.653	7.082773	121.357	8.699317	119.899	5.343272	113.514	3.981630	97.554	3.359776

Table 1. Cell proliferation assay by CCK-8. Mean values and standard deviations (SD) for normalized data. Normalized data units are absorbances normalized to 100 by setting up a ratio of the total number of cells at the end versus the beginning. The normalized data both have 3 samples per time point.

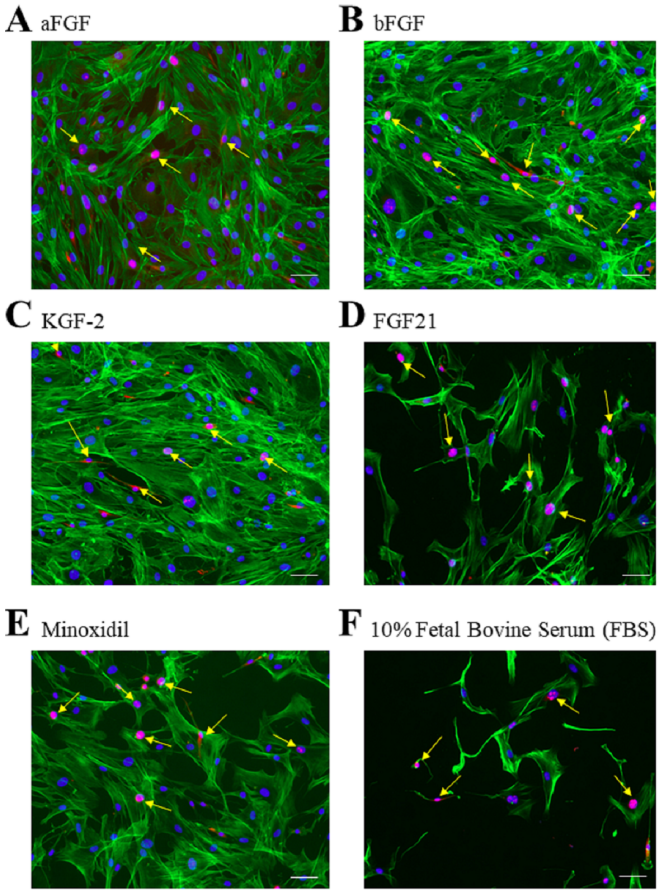

Figure 5. Dermal papilla cell proliferation and cytoskeleton. Representative fluorescent images from aFGF- (**A**), bFGF- (**B**), KGF-2- (**C**), FGF21- (**D**), minoxidil- (**E**) and 10% FBS- (**F**) treated DP cells after 5 days or treatment in the most densely populated areas. The pink dots represent Ki67-positive (proliferating) cells, blue represents cell nuclei, while green represents the cytoskeleton of the cells (phalloidin). The arrows are pointing to the Ki67-positive cells. Scale bar, 100 μm.

our study (35). These data indicate the cell counts for different factors differed at the end of experiment. Although bFGF (bFGF vs. Minoxidil; p=0.9967), FGF21 (FGF21 vs. Minoxidil; p=0.9999), and aFGF (aFGF vs. Minoxidil; p=0.9995) did not perform significantly better than minoxidil, the difference in cell count suggests that the FGFs may have positive effects on cell proliferation.

Following the quantification of cell number, we used Ki67 staining to visualize the proliferation of DP cells in various treatment groups. Ki67, a marker of cell proliferation, is a non-histone nuclear protein expressed throughout the active phase of cell cycle, except for G0 and early G1 phases. Our data suggests that bFGF promoted the proliferation of DP cells, with the greatest number of Ki67-positive cells (**Figure 5A-F**); pink nuclei indicated with yellow arrows). In addition, cells in the FGF groups (except for FGF21) exhibited a healthy cytoskeleton (phalloidin staining, green), which is detailed below. There is a certain degree of cell proliferation in the minoxidil-treated groups, but the morphology of cytoskeleton of the DP cells indicates some abnormality as seen in **Figure 5**. According to Fletcher et al., absence of visible fibers stained by phalloidin is an indicator of unhealthy cytoskeleton (36). A stronger example of the cytoskeleton can be demonstrated in the bFGF group, where most of the fibers were interwoven and thickly connected.

DISCUSSION

The purpose of the study was to test whether growth factors such as FGFs can be used to treat hair loss (or AGA). To address this, we cultured DP cells from mice in various FGF solutions or in 0% FBS media and 10% FBS media. None of the FGFs caused obvious toxicity in DP cells. Among them, bFGF and FGF21 showed a trend in promoting cell growth more potently than the commercial drug minoxidil, as measured by the CCK-8 assay. However, the margins are statistically insignificant. In the cell counting using Hoechst nuclei stain, we discovered that cell cultures with bFGF had a greater cell count than minoxidil. Furthermore, several FGFs increased the numbers of Ki67-positive proliferative cells in the DP cell culture and promoted heathy cytoskeletons as compared to minoxidil treatment. Together, these three methods suggest that FGFs could be further studied and developed as alternative therapeutic agents to treat hair loss.

During the Hoechst staining, we excluded most of the aberrant morphologies but did not find any syncytia cells. If the cells were to be blurred (in the case of 1 or 2 cells) we would have counted it as present — in case we missed any wrongfully marked cells during the staining. Cytoskeletal health is also an important dynamic structure since it allows cells to regulate their structure (size and shape) and provides internal organization. Moreover, the cytoskeleton transports materials such as vesicles around the cell, especially during cell division and proliferation, which we evaluate in this study. We would recommend bFGF over other FGFs due to its proliferation efficiency and data we have seen through both CCK-8 and Hoechst testing. Although data from FGFs did not reach statistical significance when compared to minoxidil, future studies are warranted given the observation on cytoskeleton health.

We acknowledge that our work is preliminary and has limitations. In the future, bigger sample size experiments should be performed to achieve more statistical power. In addition, combining these FGFs with each other or with minoxidil may generate more potent hair regrowth effects. Cell culture conditions we used had a much lower concentration to accommodate for in-vitro studies. The reasoning is that the commercial product is applied topically on the scalp and the drug needs to diffuse into the skin (with considerable reduction in its concentration) but in our cell culture conditions, the cells are directly exposed to the applied concentration of minoxidil. Lastly, to develop a commercially viable solution, more safety and efficacy tests in animal models are necessary before we can start clinical trials. Our research also shows that FGFs may eliminate the side effects of minoxidil, while potentially providing better regeneration than minoxidil.

MATERIALS AND METHODS

Materials

Minoxidil was purchased from Sigma Aldrich (USA). FGFs (aFGF, bFGF, KGF-2, FGF21) were generously provided by Wenzhou Medical University (China). Primary and secondary antibodies were purchased from Abcam (USA).

Isolation of Hair Follicles form Adult Mouse Epidermis and DP cell culture

Aseptic techniques were employed during cell culture. The cell culture for DP cells followed a protocol as described previously (37). A skin biopsy was obtained from a C57BL/6 mouse; afterwards, the skin was placed hair side down on a dissecting mat, and the fat was gently scraped off using a blunt scalpel. The skin was straightened with the dermis side down in a 100 mm dish. After washing with 10 mL of 1× DPBS, the skin sample was incubated in 0.25% Trypsin at 37oC for 30-120 min. Hair was scraped off the skin with curved forceps and a scalpel in an anterior-posterior direction in the trypsin solution. Hair follicles (HFs) were broken down using a scalpel and forceps, then triturated with a 10 mL pipette. The HF suspension was transferred into a 50 mL tube, washed with 1× DBPS, and filtered through a 100 um cell strainer fitted onto a new 50 mL tube. The contents were centrifuged for 5 min at 1.2×100 rpm in an Eppendorf centrifuge. Cells were plated into a 100 mm dish and incubated at 37oC. Cells were cultured in DP medium (IMDM, 10% FBS, 0.5% gentamicin, and 0.2% 2-mercaptoethanol). Once confluent, we passaged cells from the dish to a fibronectin–coated flask (38). We then observed Passage 1 DP cells by using a light microscope (**Figure 1B**). **Figure 1A** shows the cells extracted from the dermal papilla hair follicles while **Figure 1B** shows the microscopic representation of dermal papilla cells.

DP cell culture with various FGFs and minoxidil

DP cells were cultured in 96-well plates with DP medium supplemented with each one of the 4 FGFs (100 ng/mL aFGF, 10 ng/mL bFGF, 100 ng/mL KGF-2, 100 ng/mL FGF21). The marketed minoxidil product has both 2% and 5% stocks. However, in our cell culture conditions, we used a much lower concentration (0.005%). To that end, the three control groups are: DP cells cultured in medium supplemented with 0.005% minoxidil, regular cell culture medium with 10% FBS, and medium without FBS (as negative control). Minoxidil was used for cell culture studies along with aFGF, bFGF, KGF-2, and FGF21 (Wenzhou Medical University, China). Media was changed every 2-3 days.

CCK-8 cell viability assay

10 µl of CCK-8 solution was pipetted into each plate well. 40x10^3 cells were seeded to each well in the 96-well plate. After 24 hours in 10% FBS media, the media was washed once and replaced with serum-free (0% FBS) media. After another 24 hours, the 0% FBS media was removed and the different solutions (10% FBS, 0% FBS, FGFs, Minoxidil) were applied. The plates were incubated for 4 hours in a 37oC incubator. Every day at noon, we measured the absorbance at 450 nm by using a microplate reader. Experiments were run in triplicate. The absorbance readings (raw data) were exported and we normalized the Day 2-5 values to Day 1 value (set as 100) to calculate the normalized data. We used Microsoft Excel and GraphPad Prism to analyze the data. We also used a Biotek plate reader to quantify our formazan dye concentration and used GraphPad Prism to statistically analyze our results (**Table 1**).

Cell proliferation and cytoskeleton immunocytochemistry staining

DP cells were cultured on 4-well slides, washed with 200 µL phosphate-buffered saline (PBS), fixed for 30 minutes with 4% paraformaldehyde (PFA), washed with PBS, and incubated overnight with rabbit anti-Ki67 primary antibody (Abcam, Cat. No. ab66155) and mouse anti-phalloidin primary antibody (Abcam, Cat. No. ab210665) at 1:200 dilution at 4oC overnight. The next day, the samples were washed with PBS and incubated for 1.5 hours with goat anti-rabbit AF647 secondary antibody (Abcam, Cat. No. 150079) and goat anti-mouse AF488 secondary antibody (Abcam, Cat. No. 150113) at 1:200 dilution at room temperature -- diluted in DAKO Protein Block Solution with 0.1% saponin. We then stained with ProLong Gold and DAPI for 10 minutes, before taking representative images with a fluorescent microscope (ECHO Revolve).

Hoechst staining of cell nuclei

To measure cell count, we used 2 µg/mL Hoechst 33342 (ThermoFisher, Cat. No. H3570) in cell media and imaged with a fluorescent microscope (ECHO Revolve). Hoechst imaging was done in 6 well plates, so 1x10^6 cells were seeded to each well for 24hrs in 10% FBS media, 24hrs in 0% FBS media, then 5 days in the different solutions (with a media change on day 3 after the different solutions were applied). Hoechst dye was then applied and 4 independent images were taken at 4x magnification and the cells in view were counted.

After the Hoechst staining was performed, images were taken with an ECHO microscope. We obtained this data by imaging the cells, then manually counting the Hoechst positive nuclei in each group. The confluency of the cells was the same after seeding and before treatments. Counts were performed in triplicates and the numbers were averaged to generate the bar graph.

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