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High Throughput Screening of Biologically Functional Small Molecules for Modulating the Expression of FGFR1OP2/wit3.0 in Fibroblasts

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High Throughput Screening of Biologically Functional Small Molecules for
Modulating the Expression of FGFR1OP2/wit3.0 in Fibroblasts

A thesis submitted in partial satisfaction of the
requirements for the degree Master of Science in Oral Biology

by

William Joseph Cheng

2012

ABSTRACT OF THE THESIS

High Throughput Screening of Biologically Functional Small Molecules for Modulating the Expression of FGFR1OP2/wit3.0 in Fibroblasts

by

William Joseph Cheng

Master of Science in Oral Biology

University of California, Los Angeles, 2012

Professor Ichiro Nishimura, Chair

Objectives: In skin wounds, inflammation associated cytokines and growth factors induce fibroblasts to undergo transdifferentiation allowing them to contract the wound. Though wounds in the oral cavity heal with a lesser degree of inflammation, they contract at a faster pace and exhibit reduced scarring compared to the skin. FGFR1OP2/wit3.0 is a small cytoplasmic peptide highly expressed by oral wound fibroblasts, but not by skin wound fibroblasts. In this study we aim to determine the exogenous factors possibly inducing FGFR1OP2/wit3.0 expression in skin fibroblasts through the development of a high throughput screening assay.

Methods: This study examined the use of direct mRNA measurement assays for use in high throughput applications to find chemical agents that up or downregulate FGFR1OP2/wit3.0

expression. Using mouse clonal fibroblastic cell lines we characterized genetic expression, phenotype, and si-RNA knockdown of wit3.0. We then developed and optimized a protocol for high throughput screening on a bDNA platform. A pilot screening was performed at the UCLA MSSR.

Results: Confocal microscopy revealed that the cytoskeletal arrangement of actin and wit3.0 were phenotypically different in the heterozygous knockout stem cell derived fibroblasts. wit3.0 (+/-) cells exhibited disorganized actin fibers, while the wildtype showed a more organized archetype. We found that wit3.0 was expressed in both L-929 and C3H/10T1/2 and appears in immunostaining as a cytoplasmic, granular protein. We were able to successfully perform a high throughput screening of 320 chemicals as a pilot study, and identified 7 compounds as modulators of wit3.0.

Conclusion: This pilot study has identified biologically functional chemical compounds which could provide a novel clue in elucidating the mechanism of fibroblast-driven wound closure, and presents a new therapeutic potential in the field of wound healing.

The thesis of William Joseph Cheng is approved.

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University of California, Los Angeles

2012

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INTRODUCTION

We know today that a skin wound heals through three stages: inflammatory, tissue formation, and tissue remodeling. After platelets form clots to stop the loss of blood, inflammatory cells migrate to the wound and release factors which activate molecular cascades and transdifferentiate cells. In tissue formation, cellular proliferation and extracellular matrix secretions lead to formation of granulation tissue to fill the wound. Fibroblasts in particular, play an important role in the this stage of wound healing by laying down ground substance and collagen, secreting fibronectin, and signaling to local cells causing them to differentiate. The tissue then enters the remodeling stage and becomes stronger via wound contraction. In the remodeling stage fibroblasts continue to lay down a more organized collagen network, and in skin wounds, transdifferentiate into myofibroblasts which help contract wound edges through expression of alpha smooth muscle actin (α -SMA). Left to heal without intervention, wounds will go through secondary healing, in which scar tissue is forms to fill the wound. Primary healing, or healing with minimal scar tissue, can be attained by approximating the wound edges and subcutaneous wound by applying mechanical force. Over the last 4000 years, humankind has worked to manage wounds in order to achieve wound management techniques that promote primary healing without infection.(1)

Although the first mention of wound healing exists in 2000BC on cuneiform tablets found in the Sumerian civilization, it was not until the Egyptians in 1550 that attention was paid to dressing wounds with linen and honey, which acted as an antibacterial. Fast forward to 200 AD, Galen found that moist environments were important for wound healing and described closing wounds with gut and silk, both of which are still used today. In 400 AD, Indian surgeon Susruta created the first medical suture with an eyed needle. A large problem with wounds prior

to the 19th century was attributed to infection, and the discovery of bacteria and germs by Pasteur and Lister eventually led to antibiotics. Although monofilament synthetic sutures were created in the 20th century, silk and gut sutures held using an eyed needle is still used today to attain primary closure 1700 years after its first discovery. Today much of the research done on wound closure focuses at the molecular level and how inflammatory factors come together and effect local and migrating cells to orchestrate wound closure.(1)

Interestingly, compared to wounds in the skin, oral wounds close rapidly and heal with minimal scarring.(2) Fibroblasts found in the oral wound have expressed a lower level of α -smooth muscle actin, which is considered an integral part of the wound contraction mechanism in skin.(3, 4) Isolated cDNA libraries from oral wounds revealed that wound inducible transcript-3.0 (wit3.0) also known as fibroblast growth factor receptor 1 oncogene partner 2 (FGFR1OP2) was highly expressed.(5) It has been shown that upregulation of FGFR1OP2/wit3.0 increases fibroblast cell mobility, *in vitro* collagen gel contraction, and wound closure in mouse skin wounds *in vivo*.(4, 6) We have hypothesized that exogenous factors can modulate the expression of FGFR1OP2/wit3.0 in fibroblasts, and that those factors can be used to characterize oral and skin wound closure at a molecular level.

We started towards our end goal by characterizing the phenotypic and genetic expression of FGFR1OP2/wit3.0 in different fibroblasts. This was followed by the establishment of a branched DNA (bDNA) assay using siRNA knockdown of FGFR1OP2/wit3.0 in mouse clonal fibroblasts as the system optimization platform. Finally, as a pilot study, we applied our developed protocol to screen 320 biologically active small chemical molecules, and studied their effects on FGFR1OP2/wit3.0 expression in fibroblasts.

METHODS

Cell Cultures of Mouse Fibroblasts: CCL-1 and CCL-226

Clonal adult mouse skin fibroblasts (CCL-1) were cultured at 37 degrees Celsius and 5% CO₂ in t75 flasks in complete fibroblast culture medium: Dulbecco's modified Eagle's medium, penicillin streptomycin 1% solution, and 10% of fetal bovine serum (Invitrogen, Grand Island, NY). After incubation for 3-4 days, the cells were passaged. Separately, clonal mouse embryonic derived fibroblasts (CCL-226) were cultured in the same manner, except they required Basal Eagle Medium (BME), replacing DMEM. Cells from passage 4-8 were used in this study.

Fibroblastic Cells Derived From Mouse Embryonic Stem Cells Carrying FGFR1OP2/wit3.0 (+/-) Mutation

Six mouse embryonic stem (ES) cell lines were identified in the database of the International Gene Trap Consortium (www.genetrap.org, accessed December 21, 2008) containing a gene trap mutagenesis in the FGFR1OP2/wit3.0 allele. The C57Bl/6J (B6) ES cell line with IST10830D12 mutation (Texas A&M Institute for Genomic Medicine, Houston, TX) and wild-type B6 mouse ES cells were expanded on feeder cells following a standard protocol. The trypsinized ES cells were cultured in nonadherent plates (BD Biosciences, Bedford, MA) to form embryoid bodies in differentiation medium consisting of knockout Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA), 20% Heat Inactivated FBS, 1 mmol/L L-glutamine, 0.1 mmol/L B-mercaptoethanol, 1% nonessential amino acids, and 1% penicillin streptomycin (Invitrogen). The aggregates were then transferred into gelatin-coated plates and the outgrowing cells were serially

passaged every 5 to 7 days. Cells passaged in passage 1 appeared to have a robust fibroblast and epithelial layered morphology. Cell genotype was verified against C57/BL mouse tail DNA using PCR.

Immuocytology and Confocal Laser Scanning Microscopy

Fibroblasts were seeded at a low concentration on glass microscopy chambered slides (Labtek Fischer, Rochester, NY) with 2ml culture medium. After two days, cells are fixed in 4% formaldehyde and treated with Triton X-100 (Sigma, St. Louis, MO). Cells are then incubated for 1 hour with the primary antibody for FGFR1OP2/wit3.0 (Abnova, Walnut, CA) and 30 min with Alexa Fluor 488 secondary antibody (Invitrogen). Cells are subsequently incubated with the Phalloidin Alexa Flour 568 (Invitrogen) for 20 min for Actin. Cells are mounted with DAPI containing mounting media (Vectorlabs, Burlingame, CA). Using a Confocal Laser Scanning Microscope (Leica Microsystems, Richmond, IL), multi-fluorescent images were scanned using 40x and 60x oil immersion at a resolution of 512x512 to 2048x2048 pixels with an average of 6 scans per section and 10 sections per scan.

Gene Expression Assays of FGFR1OP2/wit3.0, col1, ph4, a-SMA, krt-8

Gene expression assays on cultured cells were carried out by detaching cultures using trypsin 0.05% (Invitrogen) for 3 minutes at 37 degrees Celcius. Cells were centrifuged for 5 minutes at 1500 rpm and media was aspirated. Cell pellet was then treated with lysis buffer (Qiagen, Valencia, CA) and homogenized using QIA shredder (Qiagen). RNA was extracted using Easy RNA Kit (Qiagen), and stored at -80c. RNA concentrations were measured using NanoDrop

1000 Spectrophotometer (NanoDrop, Wilmington, DE), and Reverse Transcriptase completed using Clontech Advantage RT-for-PCR Kit (Clontech, Mountain View, CA) to obtain cDNA. qPCR was completed using Taqman (Applied Biosystems, Foster City, CA) gene expression primers: (Mm99999915_g1 (gapdh), Mm00801666_g1 (col1a1), Mm01243184_m1 (p4hb), Mm00835759_m1 (krt8), Mm00470836_m1 (fgfr1op2), and Mm01204962_gH (acta2)). Experiments were carried out in triplicate.

Quantigene 2.0 wit 3.0 Assay – Cell number Optimization

CCL-1 and CCL-226 cells at varying concentrations were grown overnight in a 96 well plate (BD Biosciences). Lysis buffer and probes (Affymetrix, Santa Clara, CA) were then added to the cells for an incubation period of 15 min at 37 degrees Celsius. 100ul of lysate was transferred from each well to the bDNA capture plate (Affymetrix) and left to hybridize overnight at 53 degrees Celsius. 100ul of Amplifier, label probe and substrate was added in separate wash and incubation steps of 1 hour each at 46 degrees Celsius. Plate was read with a Luminometer.

Small-Interfering RNA Knockdown of FGFR1OP2/wit3.0 and Branch DNA (bDNA) assay

Small-interfering RNA (siRNA) targeting FGFR1OP2/wit3.0 (Invitrogen) was transfected with Lipofectamine 2000 (Invitrogen) in OPTI-MEM (Invitrogen) and CCL-1 or CCL-226 cells were incubated for 6 hours at 37 degrees Celsius. The media was then exchanged with fresh media, and cells were further incubated for 48 hours. The siRNA knockdown results were examined using Taqman-based real time RT-PCR. Separately, the branched DNA (bDNA) assay (Quantigene 2.0 oligonucleotide assay, Affymetrix, Santa Clara, CA) were used to examine the

expression of FGFR1OP2/wit3.0. Cells were treated with lysis buffer and the custom-made bDNA probes for FGFR1OP2/wit3.0 (Affymetrix). 100ul of cell lysate was transferred from each well to the bDNA capture plate (Affymetrix) and left to hybridize overnight at 53 degrees Celsius. 100ul of Amplifier, label probe and substrate was added in separate wash and incubation steps of 1 hour each at 46 degrees Celsius. The resultant chemiluminescence was quantified with a luminometer. The Z-factor, a measure of statistical size, was calculated for each group using the formula: $Z = 1 - 3 \cdot (\text{SD of sample} + \text{SD of Control}) / \text{ABS}(\text{mean of Sample} - \text{mean of control})$

High Throughput Screening

CCL-1 was grown in the method mentioned above to 80% confluence and suspended. Media and chemicals from the screening library were added to 4 plates in duplicate using a custom built automated high throughput screening device. Using a Titan automated multipipetter (Titertek-Berthold, Huntsville, AL), 25ul of CCL-1 cells were added giving a final concentration of 7.5×10^4 in eight 96 well plates (BD Biosciences). For the first set of 4 plates, bDNA assay was performed as above. To the second set of 4 plates, 25ul of ATPLite cell viability reagent (PerkinElmer, Santa Clara, CA) was added and read within 30 minutes.

RESULTS

Characterization of FGFR1OP2/wit3.0 in Fibroblasts

Immunocytology was completed on four groups of fibroblasts: B6 ES cell-derived wild type and FGFR1OP2/wit3.0 (+/-) knockout fibroblasts, as well as CCL-1 skin fibroblasts and CCL-226 embryonic fibroblasts. Cells were stained for F-actin (Red), FGFR1OP2/wit3.0 (green) and Nucleus (Blue) (Fig. 1). In ES cell-derived fibroblasts, FGFR1OP2/wit3.0 protein was found to be expressed as a granular cytoplasmic protein, which appeared to co-localize with actin fiber near cell surface (Fig. 1A). On the contrary, B6 ES-derived FGFR1OP2/wit3.0 (+/-) mutant fibroblasts exhibited more pronounced F-actin fibers that appeared disorganized (Fig. 1B). The loss of FGFR1OP2/wit3.0 seemed to result in the loss of fibroblastic morphology. CCL-1 and CCL-226 cells showed the cytoplasmic FGFR1OP2/wit3.0 molecules with minimal association with F-actin fibers (Fig. 1C and 1D, respectively).

Genetic Expression and siRNA Knockdown of FGFR1OP2/wit3.0 in Fibroblasts

Taqman-based RT-PCR was utilized to elucidate the genetic expression of FGFR1OP2 in CCL-1 and CCL-226 fibroblast cell lines along with the expression of krt8, p4h, col1, and a-SMA. (Fig. 2A) FGFR1OP2/wit3.0 was shown to be expressed in both CCL-1 and CCL-226, producing a genetic expression (relative to B6 fibroblasts) of 5.28 and 3.43 respectively. Krt-8, a marker for epithelial cells, was not significantly expressed by either cell line. P4h genetic expression for CCL-1 and CCL-226 was 2.6 and 1.67 respectively. Col1 genetic expression differed greatly between CCL-1 and CCL-226 with genetic expression being 5.09 and 175 respectively. a-SMA was not significantly expressed by CCL-1, but achieved a genetic expression of 11.7 by CCL-

226 fibroblasts. siRNA knockdown of CCL-1 and CCL-226 cell lines produced genetic expressions of 20% and 46% when compared to their respective negative siRNA control groups. (Fig. 2B)

Assessment of FGFR1OP2/wit3.0 Expression and Z-factor in Fibroblasts using the bDNA Assay

We utilized siRNA targeting FGFR1OP2/wit3.0 as a mock screening on our cell lines. The siRNA knockdown was performed on CCL-1 and CCL-226 cell types and FGFR1OP2/wit3.0 expression was measured using the bDNA assay system (Fig. 3A). Both methods indicated that siRNA treatment was successful, and induced between 20-50% knockdown in CCL-1 and CCL-226 cells. The bDNA assay generated highly consistent data, resulting in the desired Z-factor of 0.84 with CCL-1 and 0.89 with CCL-226 (Fig. 3B). This established that utilizing either fibroblast cell line, a large enough change in expression could be measured in FGFR1OP2/wit3.0, with bDNA assay as the measurement platform.

High Throughput Assay Optimization

Most chemicals stored in the biochemical screening library are dissolved in 3% DMSO, which can be cytotoxic to cells. We incubated both CCL-1 and CCL-226 in 3%DMSO media for 48 hours, and found that only CCL-1 survived to be normal in morphology and proliferation (Fig. 4A). The flow of the high throughput experiment was determined to be: 1- Chemicals and Media Delivery, 2- Seeding of 7.5×10^5 cells in each well, 3- 48 hour incubation, and 4- lysis and mRNA quantification by bDNA assay (Fig. 4B). The experiment was determined to be done in

duplicate, so that viability could be measured using ATPLite. The high throughput facility programed the delivery of 96 well plates into their custom automated assay system, which is capable of automating the entire protocol for the screening of up to 100,000 chemicals (Fig. 4C).

High Throughput Chemical Screening for modulators of FGFR1OP2/wit3.0

Utilizing our developed procedure, we screened 320 chemicals for their potential effects on the expression of FGFR1OP2/wit3.0 as a pilot study. Color coordinated hit maps were generated from taking the ratio of bDNA assay and ATPLite (Fig. 5A). The ratios served as our normalized gene expression, and therefore we used it as our primary method of selection for potential FGFR1OP2/wit3.0 regulators. 15 potential chemicals were selected using this method through a selection threshold of 1.75 standard deviation. The secondary stage of selection utilized scatterplots of Quantigene (bDNA) vs ATPLite. Several chemicals had downregulating and upregulating effects on FGFR1OP2/wit3.0 while staying within the normal viability range (Fig5.B). The 15 chosen chemicals were narrowed down to 7 by eliminating those that did not fall within this viability range of +/- 1 standard deviation.

Upregulators & Downregulators of FGF1OP2/wit3.0

In our screening, several promising chemicals were identified as potential regulators of FGFR1OP2/wit3.0 expression. 2-Aminopurine, Dihomo-gamma-linolenoyl glycine, D-Asparagine D-Amino Acid, and Genistein were identified as upregulators of FGFR1OP2/wit3.0 activity. Hypericin, 5-Iodotubercidin, and Veratridine were identified as downregulators of FGFR1OP2/wit3.0 activity. (Fig. 6)

DISCUSSION

It has been shown that FGFR1OP2/wit3.0 is highly expressed in fibroblasts during oral wound closure.(4) This study investigated the (+/-) knockout FGFR1OP2/wit3.0 phenotype using Gene Trap Mutagenesis ESC. The ES-derived FGFR1OP2/wit3.0 (+/-) mutant cells exhibited unusual cell morphology, as compared to other fibroblastic cells (Fig. 1). Cell motility plays an important mechanism of tissue contraction at the wound site, and normally requires cells to be polarized. The loss of FGFR1OP2/wit3.0 appears to be related to the loss of cell polarity in the fibroblast.

Real time PCR of the C3H cell lines CCL-1 and CCL-226 has established that FGFR1OP2/wit3.0 is expressed constitutively in both cell lines. CCL-1, a skin fibroblast, expresses p4h and col1 (both fibroblastic markers), but not α -SMA. This is expected as elevated α -SMA expression has been shown to be associated with transdifferentiated fibroblasts called “myofibroblasts” which are present during wound closure in skin.(7-9) CCL-226 was shown to express a similar level of p4h, and expressed a larger value of col1 while expressing α -SMA. This may be due to the fact that CCL-226 is a fibroblast-like cell from embryonic origins. Cells that have not progressed as far, or committed completely to a mature cell type, often express certain genes at a higher level. CCL-226 could be an example of one type of ES-like cell that has been said to circulate the blood stream and aid in wound healing. Successful siRNA knockdown of either cell line was essential in providing us with a model on which to test the bDNA assay with. The significant knockdown of both cell lines allowed us to later apply them to the Quantigene 2.0 assay in order to achieve a Z-factor value.

In order to find molecular regulators of unknown genes, a research team may have to run thousands of experiments. The gold standard today for measuring mRNA expression level in cells is through Real Time Polymerase Chain Reaction (RT-PCR). This process can prove to be long and tedious due to the necessity of isolating genetic material, reverse transcribing mRNA into DNA, and performing a real time PCR. The objective of our study is to develop a high throughput assay so that we may more efficiently screen for the effect of exogenous factors on the expression of FGFR1OP2/wit3.0 in mouse fibroblasts. In order to achieve these goals, we utilized bDNA assay, a direct mRNA measurement system. This bDNA system, the Quantigene 2.0 system, does not require cell sample processing, nor is reverse transcription necessary. Following a mRNA stabilizing lysis, the rest of the assay occurs in 1 plate over less than 24 hours. By utilizing FGFR1OP2/wit3.0 siRNA as a mock knockdown chemical, we were able to simulate the effects of a downregulating chemical in our final high throughput assay platform. Success was determined in part by calculating a Z score, which is a measure of statistical size that conveys the assay's ability to discern changes in genetic expression. The obtained z-score of $>.5$ was promising; it represents our assay's sensitivity and reproducibility, and shows that it is suitable for use in high throughput drug screening (Fig. 2).(10)

We cooperated with the University of California Los Angeles' Molecular Screening Shared Resource laboratory which houses a custom built robot that can automate screening assays to screen its 100,000 available chemicals. As of now, the workflow starts by plating chemicals and media into blank 96 well plates in duplicate. The cells are then plated using the automated Titan multidrop and incubated at 37 degrees for 48 hours before being subjected to either the Quantigene or ATPlite assay. Currently, the robot is capable of automating almost the entire workflow, including incubation and application of the Quantigene and ATPLite assays.

We have obtained a promising set of bioactive small chemical compounds that await further characterization (Fig. 4).

After receiving data from the screening, we explored many different methods to identify which chemicals were FGFR1OP2/wit3.0 modulating chemicals. Ultimately, we decided on using a stringent two-pass system that identifies the chemicals based on both genetic expression and cell viability. Utilizing the ratio of Quantigene/ATPlite for the first pass selection, we ensured that we normalized for cell viability when calculating genetic expression. Second pass selection, based on cell viability alone, allowed us to exclude those chemicals that caused excessive hyper or hypoplasia of cells. The result is that the chemicals identified are those that could be possibly utilized as a therapeutic agent in the future.

Through our pilot study, we discovered 4 chemicals that upregulated the expression of FGFR1OP2/wit3.0 over 1.75 standard deviations versus the mean. The chemicals identified were: 2-Aminopurine, Dihomo-gamma-linolenoyl glycine (DGLG), D-Asparagine, and Genistein.

2-Aminopurine is known as an inhibitor of double stranded RNA-dependent protein kinase R (PKR)(11), but is also studied for use as a probe in studying DNA as it is naturally fluorescent.(12) It pairs with Thymine as an Adenine analogue and could lead to mutagenesis in some types of cells.(13) It has also been shown to inhibit interferon, c-Fos and cmyc gene expression.(14) c-Fos is a proto-oncogene that dimerises with C-jun to form the AP-1 complex which has been shown to be involved in the communication between cells is speculated to hold a large role in wound repair.(15, 16) It may be through the disruption of the AP-1 complex that FGFR1OP2/wit3.0 expression is upregulated as a response.

DGLG is a chemical in the MSSR library that has not had much literature based studies conducted on it thus far it is a 363.5 MW compound dissolved in DMSO categorized in the endocannabinoid library. Due to its structure, it may be somewhat similar to the endogenous dihomogamma-linolenic acid (DGLA) which is known to form metabolites that cause anti-inflammatory and anti-proliferatory affects effects through interaction with the COX-1 and COX-2 pathways.(17) It has also been known to block the transformation of arachidonic acid to leukotrienes and is speculated to produce anti-thrombotic effects.(18) n-3 and n-6 fatty acids and the precursor to DGLA, Linoleic Acid, has been shown to positively affect a cellular migration wound healing model. (19)

D-Asparagine, an amino acid, is essential in producing asparagine linked glycosylation. It has been shown that asparagine linked glycoproteins are necessary for migration of corneal epithelial sheets.(20) It possible that asparagine linked glycoproteins, maybe on the cell surface, modulate FGFR1OP2/wit3.0 expression.

The last upregulator found was Genistein (GEN), an isoflavones found in plants (soybeans and coffee). Genistein has been found to mimic the effects of estrogen by being able to bind to the estrogen receptor (mostly estrogen receptor beta)(21), which is a downregulator of cutaneous wound healing in younger organisms. However in aged organisms, estrogen has recently been shown to promote wound healing through the receptor ERbeta.(22) In fact there has been much interest and study in estrogen and wound healing, with research indicating that estrogen levels affect wound healing though anti-inflammatory and downregulation of macrophage inhibiting factor. Topical application of Estrogen has been shown to reduce wound size and stimulate matrix deposition.(23) In 2010, Genistein was found to promote wound healing through acceleration of keratinocyte migration and re-epithelialization via the IGF

receptor.(24) The researchers attributed the anti-inflammatory activity of GEN to activation of the MAPK pathway.

Through our pilot study, we discovered 3 chemicals that downregulated the expression of FGFR1OP2/wit3.0 more than 1.75 SD versus the mean. The chemicals identified to be downregulators were Hypericin, 5-iodotubercidin, and veratridine.

Hypericin, along with hyperforin is one of the principle active agents of St. John's Wort. It is believed to act as an antibiotic, antiviral(25) and non-specific kinase inhibitor. Since the days of ancient Greece Hypericin has been used topically for healing of wounds and in surgery. It has been shown that Hypericin has anti-inflammatory effects, and that it may be one of the reasons that St. Johns Wort aids wound healing.(26) Recently, a solution of St. Johns Wort fractionated using column chromatography was utilized on wounds. They found that there is a possible synergistic property to the chemicals contained in St. Johns Wort. No fractionated chemical alone had greater wound healing activity than the entire extract.(27) Hypericin downregulates FGFR1OP2/wit3.0 expression, and may do so to promote healing through a pathway independent of FGFR1OP2/wit3.0. It might also downregulate FGFR1OP2/wit3.0 activity alone, but cause upregulation when paired with its synergistic partners in St. John's Wort.

The second downregulator, 5-iodotubercidin, is a potent inhibitor of the enzyme adenosine kinase. Activation of adenosine receptors have been demonstrated to promote more rapid healing in dermal wounds and in diabetics.(28) It has been shown that activation of Adenosine A2A receptor is especially involved in every phase of wound healing: inflammatory, angiogenesis and fibroblast matrix production.(29-31) As 5-iodotubercidin inhibits adenosine kinase, levels of adenosine should be higher. In this case, it would be possible that adenosine

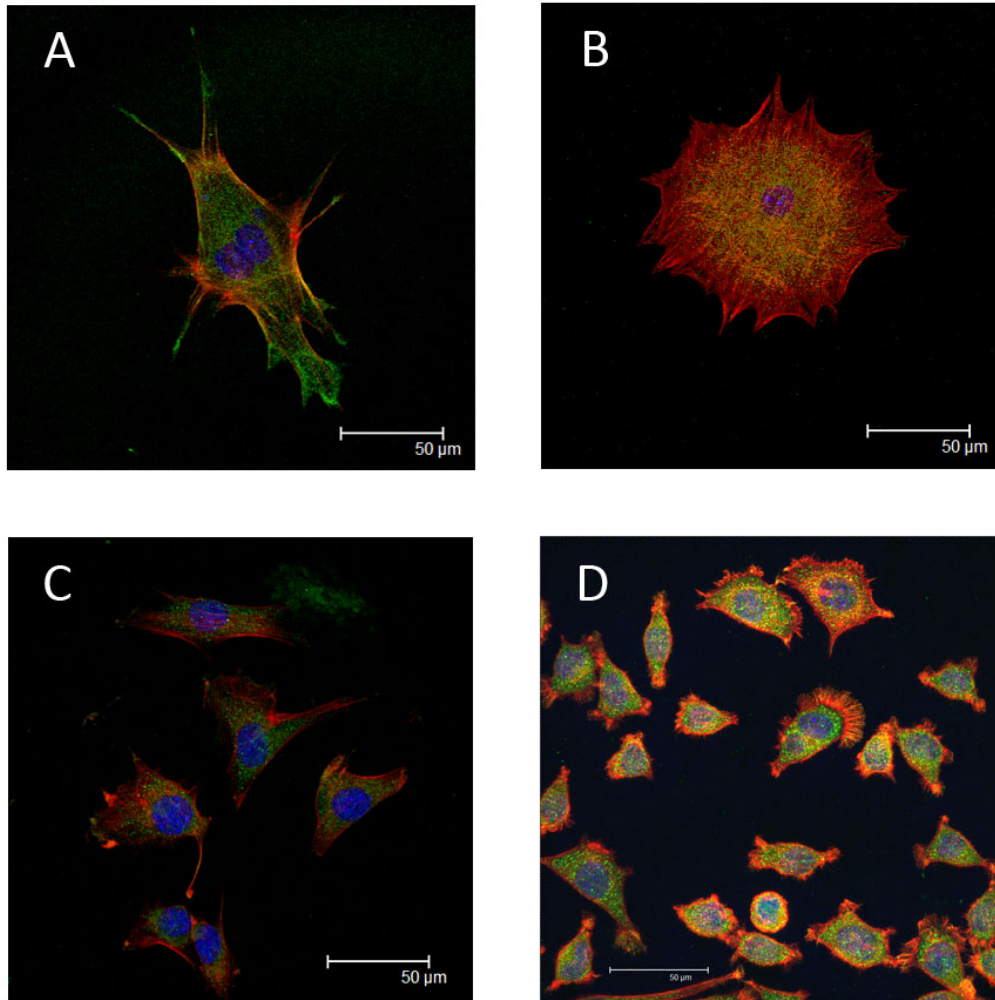
mediated healing pathway is completely different than that of FGFR1OP2/wit3.0, or that there is a negative feedback type association with FGFR1OP2/wit3.0 and the activation (or over-activation) of adenosine receptors. As these drug screenings are done *in vitro*, it is also entirely possible that the relationship we see here between 5-iodotubercidin and FGFR1OP2/wit3.0 may not hold true *in vivo*.

The last FGFR1OP2/wit3.0 downregulator, Veratridine, is a steroid derived alkaloid from plants. It functions as a neurotoxin by activating sodium ion channels (which increases sodium concentration inside the cell).(32) There have been studies showing that the depolarization of the membrane through Na⁺, is responsible for the formation of the actin cable.(33) Other studies have focused on the requisite of the B2 voltage gated sodium channels for cell-cell adhesion and migration.(34) As Veratridine has been shown to play a role in cell-cell adhesion and migration, it could be related to the pathway in FGFR1OP2/wit3.0 role in wound healing.

High throughput drug screening on fibroblasts has helped us elucidate several chemical compounds that may help us discover the molecular role of FGFR1OP2/wit3.0 and give us better understanding of the oral wound repair process. Discovery of these chemicals that upregulate FGFR1OP2/wit3.0 could provide faster wound closure in the oral cavity, or induce an oral wound phenotype in skin wounds. Chemicals that downregulate FGFR1OP2/wit3.0 could provide surgeons a method to control wounds that heal too rapidly, and a means to study the relationship of soft tissue and alveolar bone in the oral cavity. We have shown in this study that FGFR1OP2/wit3.0 exists constitutively in both skin and embryonic derived fibroblasts, and that its heterozygous knockout produces disorganizing effects on the fibroblast cytoskeleton. We have developed and optimized a high throughput assay for the chemical screening of FGFR1OP2/wit3.0 modulators in fibroblasts. Lastly, our pilot study has identified 7 genes, all of

which have been linked to wound healing, and may play a role in the molecular control of FGFR1OP2/wit3.0. In the near future, a full scale screening of chemicals would be greatly beneficial to gather empirical evidence on FGFR1OP2/wit3.0's molecular role.

FIGURE 1

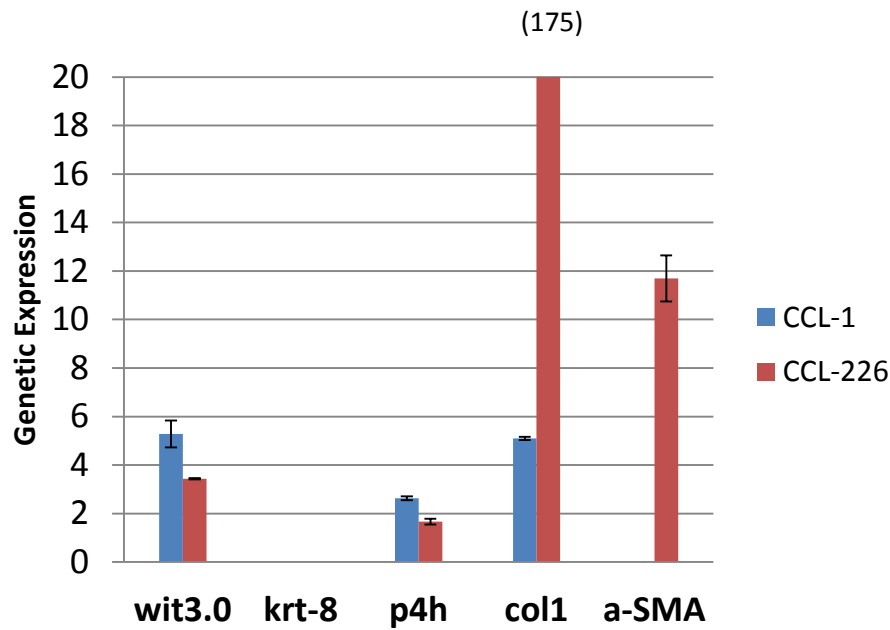


Immunocytohistology of AES-derived B6 wt (+/+) (A) and wit3.0 heterozygous (+/-) (B) cells using confocal microscopy. Overlay consists of three different layers. Red staining represents F-Actin. Green staining represents wit 3.0. Blue staining is DAPI stain. the mutant (+/-) cells exhibit less expression of wit3.0, lack wit3.0 expression at the periphery, and presents with disorganized actin fibers. Immunocytohistology of CCL-226 C3H mouse skin fibroblast (C) and

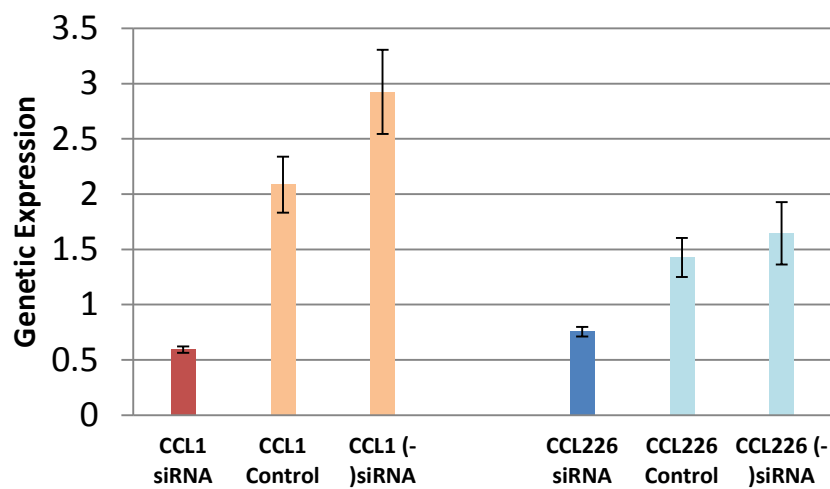
CCL-1 C3H mouse embryonic derived fibroblast cells (D) using confocal microscopy. In all cell types, wit3.0 does not co-localize with actin, and is present as a granular cytoplasmic peptide.

FIGURE 2

A



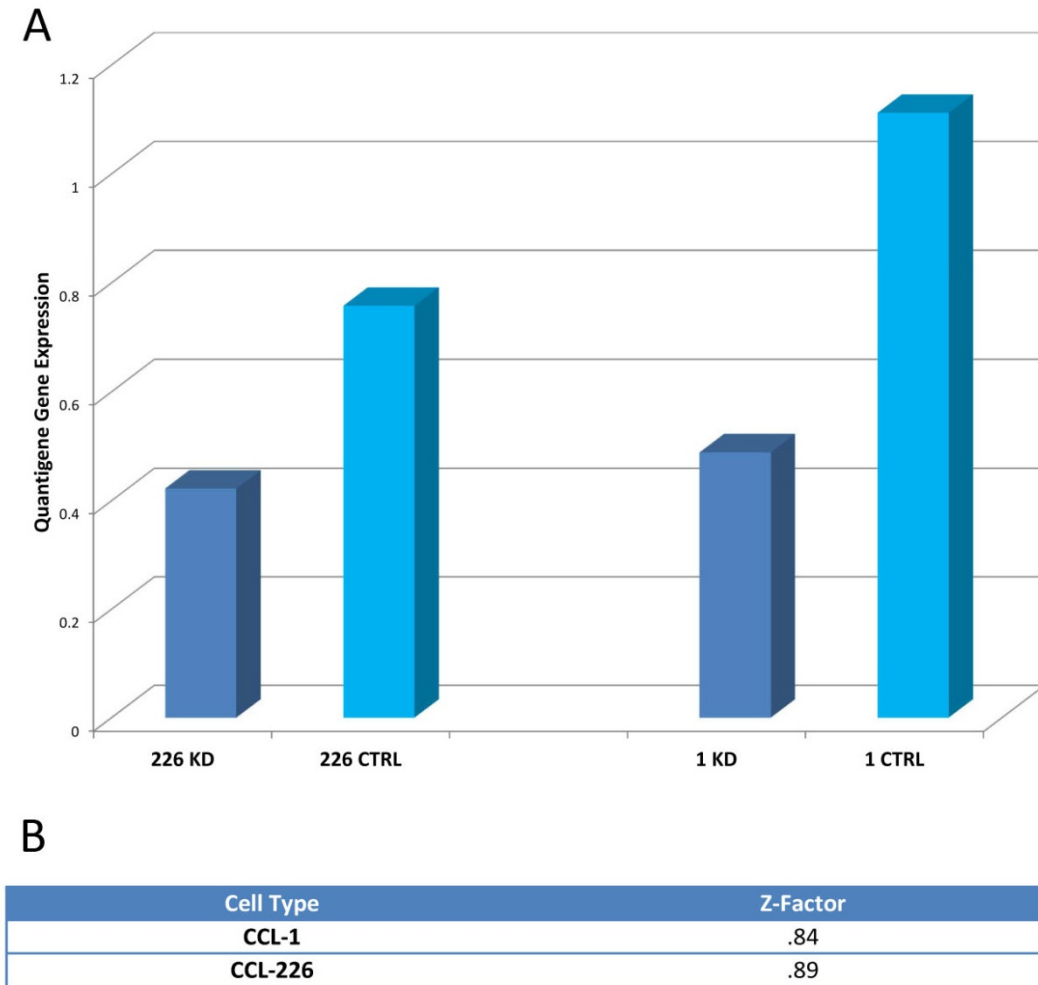
B



(A) Taqman-based RT-PCR was used to find the genetic expression of FGFR1OP2, krt8, p4h, col1, and a-SMA in CCL1 (blue) and CCL-226 (red) cells. Wit3.0 and p4h

was shown to be expressed in both CCL-1 and CCL-226. Krt-8 was not significantly expressed by either cell line. Col1 genetic expression differed greatly between CCL-1 and CCL-226 with genetic expression being 5.09 and 175 respectively (graph scale does not reflect the 175). α -SMA was not significantly expressed by CCL-1, but achieved a genetic expression of 11.7 by CCL-226 fibroblasts. (B) siRNA knockdown of CCL-1 (red) and CCL-226 (blue) cell lines produced genetic expressions of 20% and 46% when compared to their respective negative siRNA (yellow, light blue) control groups.

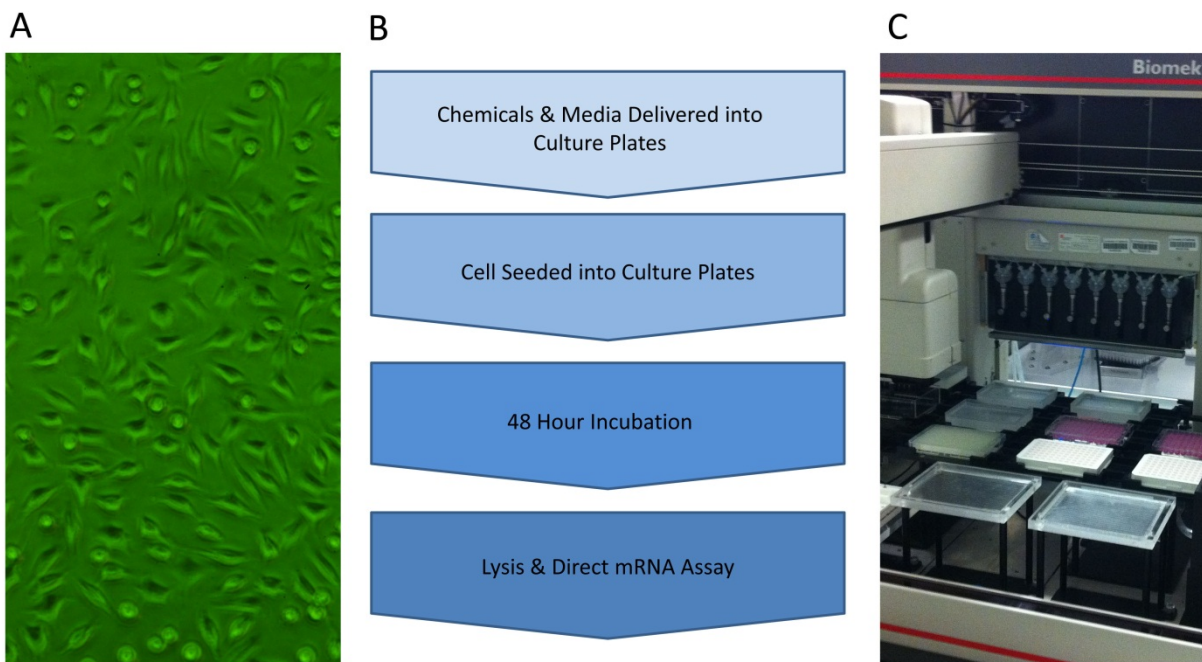
FIGURE 3



(A) Wit3.0 siRNA knockdown performed on the Affymetrix Quantigene (bDNA) direct mRNA detection platform. 226 KD (CCL-225 Knockdown) and 1 KD (CCL-1 Knockdown) originated from cells treated with siRNA targeting wit3.0. 226 CTRL (CCL-226 Control) and 1 CTRL (CCL-1 Control) are control groups treated with negative siRNA. CCL226 was knocked down by 55% while CCL-1 was knocked down

by 44% versus the control. (B)Table showing Z-factor, a statistical measurement of effect size used in high throughput screening to judge the power of pilot screenings. A Z-factor above .5 denotes an excellent high throughput assay response. CCL – 1 at 4x and CCL-226 at 1x dilution produce Z-factors of .84 and .89 respectively.

FIGURE 4



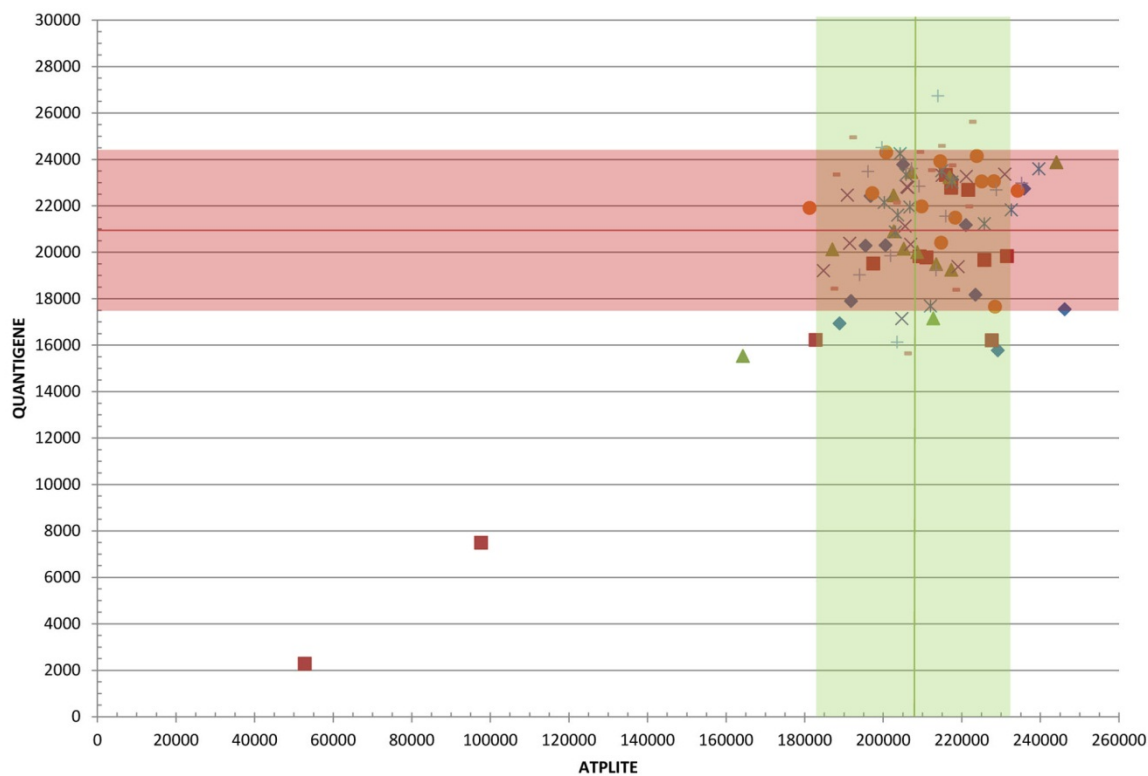
(A) Light microscopy photograph of CCL-1 cells 48 hours after automatic seeding into a 96 well plate with media containing DMSO. Cell morphology, and growth patterns are normal. (B) Flowchart showing the sequence of events in the developed high throughput assay. Over 30,000 chemicals can be automatically delivered with media into culture plates. Cells are then seeded and a 48 hour incubation takes place. In the last step, lysis and direct measurement of mRNA levels take place over 24 hours. (C) Photograph of one section of the high throughput automated screening device. This device can automate chemical deliverance, incubation and the direct mRNA assay.

FIGURE 5

A

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.068844	0.089627	0.093317	0.103701	0.09642	0.095768	0.106249	0.115948	0.113909	0.071229	0.101159	0.081294
B	0.076692	0.087093	0.098812	0.094708	0.043278	0.085641	0.104805	0.10802	0.102328	0.071158	0.093727	0.088702
C	0.095847	0.103071	0.107522	0.110769	0.113185	0.091273	0.097794	0.094538	0.107112	0.080616	0.088538	0.098169
D	0.098214	0.10272	0.106463	0.108396	0.110451	0.103919	0.101147	0.110722	0.105208	0.083712	0.088425	0.11764
E	0.093818	0.098461	0.106033	0.113393	0.118672	0.093991	0.109394	0.106097	0.105807	0.083403	0.102759	0.110553
F	0.096676	0.111489	0.10236	0.121	0.107852	0.098406	0.104739	0.101048	0.114295	0.077263	0.095054	0.120828
G	0.097629	0.12272	0.113874	0.119661	0.124962	0.090107	0.099117	0.109242	0.099803	0.079225	0.098093	0.09829
H	0.09937	0.12464	0.111212	0.130257	0.115386	0.109202	0.116527	0.114784	0.109488	0.076098	0.084389	0.098642

B



(A) 96 well plate “hit map” of the ratio of Quantigene/ATPLite. Results are from the incubation of CCL-1 cells with enzyme inhibiting chemicals performed using the high throughput screening protocol. The plate map is color coded from high (green) to low (red) genetic expression. (B) Scatterplot of ATPLite vs Quantigene values. Possible up

and down-regulatory candidates are located in the green regions, above or below the red zone. These hits represent those that stayed within 1 standard deviation of the mean viability, but are more than 1 standard deviation away from the mean gene expression level.

FIGURE 6

% Change	Compound	Action
151%	2-Aminopurine	PKR, Interferon, c-Fos, cmc inhibitor
142%	Dihomo-gamma-linolenoyl glycine	Unknown
141%	D-Asparagine	Asparagine Linked Glycosylation
139%	Genistein	Mimics Estrogen, IGFR Agonist

% Change	Compound	Action
41%	Hypericin	Kinase Inhibitor, Anti- Inflammatory
59%	5-Iodotubercidin	Adenosine kinase inhibitor
76%	Veratridine	Sodium Channel Activator

The above list of chemicals was generated through a two pass selection system from data generated from the screening. The first pass depended on chemicals that were identified to be above 1.75 above or below the mean. The second pass required the chemical to be within 1 standard deviation of the mean. All of the 7 chemicals discovered that modulated the level of wit3.0 had previously been studied in the field of wound healing.

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