RESEARCH ARTICLE

Combination of Cyclopamine and Tamoxifen Promotes Survival and Migration of MCF-7 Breast Cancer Cells – Interaction of Hedgehog-Gli and Estrogen Receptor Signaling Pathways

Maja Sabol 1*, Diana Trnski 1*, Zvonimir Uzarevic 2, Petar Ozretic 1, Vesna Musani 1, Maja Rafaj 1, Mario Cindric 1, Sonja Levanat 1*

1. Division of Molecular Medicine, Rudjer Boskovic Institute, Zagreb, Croatia, 2. Faculty of Education, Josip Juraj Strossmayer University of Osijek, Osijek, Croatia

*levanat@irb.hr

These authors contributed equally to this work.

Abstract

Hedgehog-Gli (Hh-Gli) signaling pathway is one of the new molecular targets found upregulated in breast tumors. Estrogen receptor alpha (ERα) signaling has a key role in the development of hormone-dependent breast cancer. We aimed to investigate the effects of inhibiting both pathways simultaneously on breast cancer cell survival and the potential interactions between these two signaling pathways. ER-positive MCF-7 cells show decreased viability after treatment with cyclopamine, a Hh-Gli pathway inhibitor, as well as after tamoxifen (an ERα inhibitor) treatment. Simultaneous treatment with cyclopamine and tamoxifen on the other hand, causes short-term survival of cells, and increased migration. We found upregulated Hh-Gli signaling under these conditions and protein profiling revealed increased expression of proteins involved in cell proliferation and migration. Therefore, even though Hh-Gli signaling seems to be a good potential target for breast cancer therapy, caution must be advised, especially when combining therapies. In addition, we also show a potential direct interaction between the Shh protein and ERα in MCF-7 cells. Our data suggest that the Shh protein is able to activate ERα independently of the canonical Hh-Gli signaling pathway. Therefore, this may present an additional boost for ER-positive cells that express Shh, even in the absence of estrogen.

OPEN ACCESS

Citation: Sabol M, Trnski D, Uzarevic Z, Ozretic P, Musani V, et al. (2014) Combination of Cyclopamine and Tamoxifen Promotes Survival and Migration of MCF-7 Breast Cancer Cells – Interaction of Hedgehog-Gli and Estrogen Receptor Signaling Pathways. PLoS ONE 9(12): e114510. doi:10.1371/journal.pone.0114510

Editor: Rajeev Samant, University of Alabama at Birmingham, United States of America

Received: July 15, 2014
Accepted: November 10, 2014
Published: December 12, 2014

Copyright: © 2014 Sabol et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper and its Supporting Information files.

Funding: This work was supported by funds from the Croatian Ministry of Science, Education and Sports, Grant No. 098-0982464-2461, and the European Union’s Seventh Framework Programme for Research and Technological Development under grant agreement No. 316289 - InnoMol, FP7-REGPOT-2012-2013-1. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.
Introduction

Breast cancer is a heterogeneous disease divided into three major subtypes with differing response to therapy: the hormone receptor-positive (with either estrogen receptor (ER) or progesterone receptor (PR) expression), the HER-2 amplified, and the triple-negative cancer (ER-negative, PR-negative and HER2-negative). For ER-positive tumors, therapy is mostly based on inhibition of estrogen synthesis or inhibition of estrogen receptor activity, for example tamoxifen is commonly used. However, many of the patients do not respond to endocrine treatment or develop acquired resistance [1].

The Hedgehog-Gli (Hh-Gli) signaling pathway is involved in embryonic development of mammary buds [2], and the pathway genes are expressed in the mammary gland during postnatal development [3]. Aberrant activation of the pathway is associated with tumorigenesis and developmental malformations. The pathway is initiated with binding of the ligand Hedgehog (Sonic, Indian or Desert Hh) to its transmembrane receptor Patched (Ptch). Ptch relieves its repression of Smoothened (Smo), causing a phosphorylation cascade and the release of transcription factor Gli from Suppressor of Fused (SuFu). Gli translocates to the nucleus, where it initiates target gene transcription. Hh-Gli pathway target genes are involved in proliferation and differentiation, cell survival, self-renewal, angiogenesis, and pathway autoregulation [4–6].

Hh-Gli signaling pathway hyperactivation has previously been detected in breast tumors [7–9]. PTCH1 gene was found downregulated due to promoter hypermethylation [10, 11]. SHH promoter is frequently hypermethylated in the normal breast and this methylation is lost in breast tumors [12]. SHH is one of the signature genes associated with poor prognosis of inflammatory breast cancer [13]. Mutations in PTCH1, SMO and SHH genes have been examined in breast cancer: some studies found mutations [14, 15], while others did not [16, 17]. However, biallelic Pro1315Leu (C3944 T) PTCH1 polymorphism was found associated with breast cancer risk when combined with oral contraception [18]. Loss of heterozygosity of the PTCH1 gene is found in 30% of breast cancer patients [10]. The effects of cyclopamine, a Hh-Gli pathway inhibitor, on breast cancer have already been addressed in several studies. It was shown to cause growth inhibition mediated by apoptosis of some breast cancer cell lines [7, 19], while cells derived from normal breast tissue are not responsive to cyclopamine [20]. The Hh-Gli signaling pathway has been implicated in tamoxifen resistance. It was shown that a small molecule SMO inhibitor GDC-0449 can improve the outcome of tamoxifen-resistant tumors. Addition of tamoxifen to GDC-0449 had additional benefits in vitro but not in vivo [21]. Recently, cyclopamine was shown to have anti-proliferative, anti-invasive and anti-estrogenic potency in human breast cancer cells by suppressing the MAPK/ERK signaling pathway. Cyclopamine decreased ERα protein levels in MCF-7 cells and the authors speculate that combining cyclopamine with anti-estrogen therapies could lower the doses and side-effects [22].
Here we show a surprising, unfavorable effect of combined inhibition of Hh-Gli signaling and ERα in human ER-positive breast cancer cells and the potential underlying mechanism. In addition we also show a new, non-canonical interaction between the Hh-Gli and ERα signaling pathways.

Materials and Methods

Cell culture experiments
MCF-7 (ATCC, HTB-22) and SkBr-3 (ATCC, HTB-30) breast cancer cell lines were a kind gift from Dr. Sanja Kapitanović. Both cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and were mycoplasma-free.

MTT assay: cells were plated in 96-well plates 24 hours before treatment, in quadruplicates for each tested concentration: cyclopamine 0.5–7.5 μM (Toronto Research Chemicals, Toronto, Ontario, Canada), tamoxifen 1–10 μM (Toronto Research Chemicals). Combined treatments were with either cyclopamine for 48 h followed by tamoxifen for 48 h, tamoxifen for 48 h followed by cyclopamine for 48 h, cyclopamine + tamoxifen simultaneously for 48 h, cyclopamine + tamoxifen simultaneously for 96 h. Competition experiments: compounds were added simultaneously and MTT assay was performed after 48 h.

Gene expression studies: cells were plated into 6-wells in duplicates 24 h before treatment, and treated with cyclopamine (2.5 μM), Shh protein (3 ng/μl, kind gift from Dr. Anna Kenney) and tamoxifen (1 μM for MCF-7, which is the LD50 dose, or 5 μM for SkBr-3 (LD50 was not reached for SKBr-3, therefore a higher dose was used)) for 24 h or cyclopamine + tamoxifen for 48 and 96 h.

Transfection experiments: cells were transfected with 1 μg of pcDNA4nlSMtGLI1 plasmid expressing the Gli1 transcription factor (kind gift from Dr. Fritz Aberger) using Lipofectamine reagent (Life Technologies, Carlsbad, California, USA). Medium was changed after 5 h and specified wells were treated with Shh protein (3 ng/μl); cells were collected 48 h later.

PTCH1 silencing: cells were transfected with 50 nM Silencer Select siRNA (Life Technologies, s11442) or Silencer Negative Control #1 siRNA (Life Technologies) using siPORT NeoFX (Life Technologies) transfection reagent. Medium was changed after 24 h, and cells were collected after 24 or 48 h.

Wound healing assay
MCF-7 cells were grown to confluence in 24-well plates and serum starved over night. The following day monolayers were wounded with a plastic 200 μl pipette tip and washed with medium to remove detached cells. The wounds were allowed to close in medium without any treatment or in the presence of 10 μM cyclopamine, 10 μM tamoxifen or both drugs together. Images were taken at the 0 and 26 h time points. The wounds were photographed at 10x magnification, on the Olympus CKX41 inverted microscope linked to an Olympus E330 camera.
(Olympus, Shinjuku, Tokyo, Japan). Images were analyzed using the TScratch software, developed by the Koumoutsakos group (CSE Lab), at ETH Zürich [23]. Each time point was normalized to the 0 h image area and reported as the percent of open wound area. For the comparison of open wound areas between different treatments a one-way ANOVA with Newman-Keuls post hoc test for multiple pairwise comparisons was used. Two-tailed p value less than 0.05 was considered statistically significant. Statistical analysis was performed with GraphPad Prism 6 for Windows, version 6.05 (GraphPad Software, San Diego, California, USA).

**Transwell migration assay**
To assay the migration of cells, 5 × 10⁴ cells in 500 µl of serum-free medium were seeded onto 8-µm pore Transwell Inserts (Corning, Corning, NY) in the absence of any treatment or in the presence of 10 µM cyclopamine, 10 µM tamoxifen or a combination of cyclopamine and tamoxifen. The lower chambers were filled with 1 ml of complete medium. After 48 h the cells that had not migrated were wiped off the upper side of the filter using a cotton swab. Migrated cells were fixed with 4% paraformaldehyde/PBS for 10 minutes and subsequently stained with crystal violet for 1 h. Images of five independent fields per insert were taken at 20x magnification using the Olympus BX51 microscope, and the number of migrated cells was counted. For the comparison of the number of migrated cells between different treatments a one-way ANOVA with Newman-Keuls post hoc test for multiple pairwise comparisons was used.

**Quantitative real-time PCR (qRT-PCR)**
RNA extraction and qRT-PCR were performed as previously described [24], with primers ERα F 5’-CAGATGGTCAGTGCCTTGTTGG-3’, R 5’-CCAAGAGCAAGTTAGGACAAACAG-3’ [25] and RPLP0, PTCH1 and GLI1 [26,27]. Expression was normalized using RPLP0 housekeeping gene and relative fold change was calculated using the 2⁻^ΔΔCt formula.

**Immunofluorescent staining**
Immunofluorescent staining and confocal microscopy were performed as previously described [24]. The following primary antibodies diluted 1:100 were used: rabbit polyclonal anti-Hh (Santa Cruz Biotechnology, Dallas, Texas, USA, sc-9024), mouse monoclonal anti-ERα (Santa Cruz Biotechnology, sc-8002). For quantification of nuclear staining, three visual fields of magnification 60–100x were examined and cells were counted (non-treated (NT) N=79; Shh treatment N=124). Quantification of nuclear staining was obtained by determining the percent of cells showing positive ERα nuclear staining. For colocalization analysis of Shh and ERα, confocal images were examined using the Manders’ coefficient plugin of the ImageJ software (v 1.45e) for colocalization of green and red signals.
The difference in nuclear staining and co-localization between untreated samples and each treatment was tested using one-way ANOVA with Dunnett’s post hoc multiple comparisons test.

Co-Immunoprecipitation
For co-immunoprecipitation experiments Protein G Dynabeads (Life Technologies) were coated with 5 µg anti-ERα antibody per sample and cell lysates were immunoprecipitated as per manufacturer’s instructions (Invitrogen, Rev. 005). Dynabeads without bound antibody were used as negative control. Samples were eluted with 1x loading buffer and heated 10 min at 70°C before analysis on Western blot.

Western blot
Fifty µg of protein (determined by Bio-Rad Protein Assay; Bio-Rad, Hercules, California, USA) was loaded on SDS-polyacrylamide gel, transferred to a nitrocellulose membrane and blocked in 5% milk. Primary antibodies (diluted 1:250) for Shh and ERα were the same as for the immunofluorescence experiment, additionally goat polyclonal anti-Ptch1 (Santa Cruz Biotechnology, sc-6147) and rabbit polyclonal anti-Gli1 (Santa Cruz Biotechnology, sc-20687) were used. Actin (Santa Cruz Biotechnology, sc-1616, goat polyclonal, diluted 1:500) was used as loading control. After washing, membranes were incubated with the appropriate secondary HRP-conjugated antibody (Santa Cruz Biotechnology). Proteins were visualized using Super Signal West Pico and Femto reagents (Thermo Fisher Scientific, Waltham, Massachusetts, USA).

Proteomic profiling by 2D-gel electrophoresis and mass spectrometry
Sample preparation
Cells were seeded in four 10 cm dishes for each treatment. After 24 h cells were treated with a combination of 5 µM cyclopamine and 10 µM tamoxifen in culture medium without serum for 48 h. The cells were then harvested at 4000 g (Tehtnica, Centric 400, Železniki, Slovenia) for 6 min, washed five times in 10 mM tris (hydroxymethyl) aminomethane (Tris)-sorbitol buffer, pH 7 and lysed with TissueRuptor (Qiagen, Venlo, Netherlands). The DNA and RNA were removed after treatment with DNase I and RNase A. The reconstituted proteins were precipitated overnight at −20°C with ice-cold acetone and centrifuged for 20 min at 5000 g [29]. The proteins were resuspended in rehydration solution for isoelectric focusing (IEF) containing 7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate hydrate (CHAPS) and 1% dithiothreitol (DTT) (w/v). Protein concentration in solution was estimated with Bradford protein assay.
Two-dimensional electrophoresis

Immobilized pH gradient strips (IPG; 17 cm, non-linear, pH 3–10) were rehydrated for 14 h with 350 mL of rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT (w/v) and 1.5 mg/mL of total protein. The IEF was carried out with a Protean IEF Cell (Bio-Rad) with a low initial voltage and an applied voltage gradient up to 7000 V. The total V x t product applied was 90 000 Vh for each strip at 20°C. The strips were equilibrated in equilibration buffer containing 20 mM DTT, 50 mM Tris adjusted to pH 6.8, 6 M urea, 2% sodium dodecyl sulfate (SDS) (w/v), 30% glycerol (v/v) and 0.01% bromophenol blue (BPB) (w/v) on a tilt table for 15 min. The solution was discarded and the same equilibration buffer solution without the addition of DTT and with the addition of 25 mM iodoacetamide was used for a 15 min protein alkylation reaction. The strips were placed on a 1 mm thick 12% polyacrylamide gel and sealed with 0.1% (w/v) agarose in SDS-electrophoresis buffer containing 0.01% (w/v) BPB. In the second dimension, the electrophoresis was run for 1 h at 15 mA per gel and then at 20 mA for 600 Vh. The electrophoresis was terminated after 30 mA per gel until the BPB reached the bottom of the gel. Tris-glycine running buffer containing 25 mM Tris, 190 mM glycine and 0.1% (w/v) SDS was used in the second dimension. Obtained gels were stained with Coomassie brilliant blue (CBB) G-250 stain [30].

Differential display analysis

Differential display analysis of the gel data sets was undertaken by comparing images of control gel (non-treated cell cultures) with the gel of treated cells (combination of cyclopamine and tamoxifen). Densitometry analysis was performed with image analysis software (Discovery Series PDQuest 2-DE analysis software package version 7.4.0.) integrated with a VersaDoc 4000 Imaging System (Bio-Rad). Master gels were used to obtain the differences between protein profiles of non-terated and treated cell cultures.

In-gel digestion

Differentially displayed protein spots were excised from 2-DE gels into small pieces and subjected to in-gel digestion with trypsin according to Shevchenko et al [31].

Data analysis and protein identification

Samples were mixed with α-cyano-4-hydroxycinnamic acid 1:5, v/v (5 mg/mL; Fluka, Switzerland) and spotted onto a metal plate. MS acquisition was performed with a 4800 Plus MALDI TOF/TOF analyzer (Applied Biosystems, Carlsbad, California, USA) equipped with a 200 Hz, 355 nm Nd:YAG laser. Ions were analyzed in reflectron mode using positive polarity. The instrument parameters were set using the 4000 Series Explorer software (version 3.5.3, Applied Biosystems). Mass spectra were obtained by averaging 1000 laser shots covering a mass range of m/z 900 to 4000. MS/MS of the 10 most intense precursor signals
from MS spectra was achieved by 1 keV collision energy in positive ion mode with air as a collision gas and by averaging 1600 laser shots.

Data were analyzed using ProteinPilot (ProteinPilot™ Software 4.5., 2012 AB SCIEX) [32] for searching against the NCBI database using the Homo sapiens taxonomy. The search parameters allowed for two missed cleavage, trypsin digestion with a peptide tolerance=0.3 Da and MS/MS tolerance=0.5 Da. Only significant scores (greater than 39, p<0.05) for the peptides defined by a Mascot probability analysis were considered to be confidently identified peptides/proteins.

**Results**

MCF-7 and SkBr-3 cells are responsive to cyclopamine and tamoxifen treatment – combination shows unusual adverse effects

Both the ER-positive MCF-7 and the ER-negative SkBr-3 show expression of Hh-Gli pathway components. The major difference between the two cell lines was the expression of Shh and ERx, while the MCF-7 cell line expressed Shh and ERx both on gene and protein (Shh-N, 19 kDa) level, SkBr-3 cells showed low levels of SHH and ERx gene expression and no expression at protein level (Fig. 1A, B). SkBr-3 cells also showed no expression of GLI1 (Fig. 1A).

MCF-7 cells were responsive to both Hh-Gli signaling downregulation with cyclopamine, and ERx inhibition with tamoxifen, which both decreased MCF-7 cell proliferation. Both treatments had a significantly weaker effect on the ER-negative SkBr-3 cell line (Fig. 2A–D). To determine the effects of a combined treatment on cell proliferation, cells were treated with cyclopamine and tamoxifen in four different combinations: cyclopamine for 48 h followed by tamoxifen for 48 h, tamoxifen for 48 h followed by cyclopamine for 48 h, cyclopamine + tamoxifen simultaneously for 48 h and 96 h (Fig. 2E, F). In most cases, the combined effect was very similar to the effect of tamoxifen alone. However, a short-term combined treatment did not cause significantly decreased proliferation in MCF-7 cells (Fig. 2E).

We tested the possible competition of cyclopamine and tamoxifen in both cell lines: cells were treated with a constant concentration of one compound, combined with a range of increasing concentrations of the second compound. For SkBr-3 cell line, there was no significant difference in compound activity (data not shown). In the MCF-7 cell line, however, increasing concentrations of the second compound increased short-term cell survival; regardless of the order of administration (Fig. 3). This suggests that even though cyclopamine and tamoxifen alone show inhibitory effects on MCF-7 cells, when administered together they counter each other’s effects.
Combined cyclopamine and tamoxifen treatment alters Hh-Gli signaling pathway activity in MCF-7 cells and promotes cell migration

Prior to investigating the combined effect of cyclopamine and tamoxifen, we first tested the effect of cyclopamine and tamoxifen alone on the Hh-Gli signaling pathway. Both cell lines showed a similar response when treated with cyclopamine. 24 h after treatment with cyclopamine, PTCH1 and GLI1 expression was downregulated in the MCF-7 cell line and PTCH1 was downregulated in SkBr-3, suggesting pathway inhibition (Fig. 4A). Tamoxifen treatment upregulated PTCH1 and GLI1 expression in MCF-7 cells, while PTCH1 levels remained unchanged in the SkBr-3 cell line (Fig. 4B).

Although some pathway components are expressed, the pathway shows a low level of activity in SkBr-3 cells, but with downregulation possibility with cyclopamine, which may be carried out through other pathway effectors such as Gli2 or Gli3 that were not tested in this study.

Combined treatment with cyclopamine and tamoxifen showed a different effect on ER-positive and ER-negative cell line. ER-positive MCF-7 cell line showed increased Hh-Gli signaling after short-term treatment. Even though the level of PTCH1 mRNA was still elevated after long-term treatment, a decreasing tendency was visible compared with short-term treatment. This is confirmed by the level of PtcH1 protein, which was decreased 40% after long-term combined treatment compared with non-treated cells. ERα protein level showed no change after shorter treatment but declined after longer treatment (Fig. 4C). SkBr-3, however, showed generally downregulated Hh-Gli signaling after combined treatment regardless of treatment duration (Fig. 4D).

Wound induced migration assay was performed to test whether the combination of cyclopamine and tamoxifen has an effect on the ability of MCF-7 cells to migrate, in addition to the effects on Hh-Gli signaling and cell migration.
proliferation. Cyclopamine or tamoxifen alone had no effect on the wound closing rate, compared with the wound closing in the absence of any treatment. On the other hand, combined treatment with cyclopamine and tamoxifen accelerated the wound healing process compared with non-treated conditions and with cyclopamine or tamoxifen alone (Fig. 4E, F). To confirm the obtained results a transwell migration assay was performed. This assay confirmed no effect of either cyclopamine or tamoxifen alone on the migration rate when compared with the non-treated cell migration rate. It also confirmed a higher migration capacity of MCF-7 cells treated with a combination of cyclopamine and tamoxifen compared

Fig. 2. Cell viability after tamoxifen (A,B), cyclopamine (C,D) or combined treatment (E,F) in MCF-7 and SkBr-3 cell lines. Tamoxifen and cyclopamine each inhibit proliferation of MCF-7 cells in a dose dependent manner (A,C). When administered simultaneously, they cause a short term survival effect in MCF-7 cells (C+T 48 h) – pointed out with arrow, whereas long term simultaneous treatment induces strong cell death in these cells (C+T 96 h). Combination treatment of cyclopamine for 48 h followed by tamoxifen for 48 h (C 48 h → T 48 h) or vice versa (T 48 h → C 48 h) showed an effect similar to tamoxifen alone (E). Tamoxifen and cyclopamine show only a mild inhibitory effect on SkBr-3 cell proliferation at longest exposures (B,D) while combined treatment has no pronounced effect (F).

doi:10.1371/journal.pone.0114510.g002
with non-treated cells or cells treated with cyclopamine or tamoxifen alone (Fig. 4G, H). The increase in the migration capacity was even higher when analyzed with the transwell migration assay in comparison with the wound healing assay.

Proteomic profiling of cells treated with cyclopamine and tamoxifen versus non-treated cells

Differential protein expression analysis was conducted to identify the profile of expressed proteins in cells treated with a combination of cyclopamine and tamoxifen. These differentially expressed proteins may explain the effects of the combined treatment with cyclopamine and tamoxifen on cell proliferation and migration. The identified proteins are listed in Table 1. Images of the obtained 2-D gels are shown in S1 Figure. As opposed to cells treated with a combination of drugs, non-treated cells mostly show expression of proteins involved in response to topologically incorrect and unfolded proteins; carbohydrate and amino acid metabolism, gene transcription, RNA processing and translation. Interestingly, the heat shock protein 27 (HSP27) is expressed in both non-treated cells and those treated with a combination of cyclopamine and tamoxifen. However, the protein is shifted in the 2-D gel of treated cells compared with its localization in the 2-D gel of non-treated cells, which could indicate a posttranslational modification after treatment. Additionally, the GRP78 precursor protein, which is a known survival factor [33] that can mediate signaling pathways that lead to proliferation and invasion [33, 34] was expressed only in treated cells. Also, two proteins that can be linked with upregulation of proliferation and migration showed an increase in expression in treated cells, namely prohibitin and keratin 8 [35, 36]. Together these results indicate that certain proteins involved in tumor cell survival and migration are upregulated or possibly activated.
Fig. 4. Effects of cyclopamine (A) and tamoxifen (B) on Hh-Gli pathway gene expression in MCF-7 and SkBr-3 cells. The Hh-Gli pathway is upregulated after short-term combined treatment in MCF-7, but the effect is negated after longer treatment. On the Western blot image, band quantification relative to actin and non-treated cells is denoted below the bands. (C). The effect of combined treatment on SkBr-3 cell line is weak (D). Gene expression levels are shown on graph as relative fold change relative to non-treated conditions with reference value 1 pointed out with emboldened bar. Only combined cyclopamine and tamoxifen treatment induces migration in MCF-7 cells. Representative images of the wound healing assay at 0 and 26 h (after processing with TScratch software [23]) are shown for non-treated conditions (NT; N = 16), cyclopamine treatment (CYC; N = 16), tamoxifen treatment (TAM; N = 14) and combined treatment with cyclopamine and tamoxifen (C+T; N = 12) (E). Quantitative analysis of the percentage of open wound areas is shown on the graph.
Shh regulates ERα expression in MCF-7, but not SkBr-3 cell line
Since inhibition of ERα with tamoxifen affected Hh-Gli signaling we wanted to establish whether there is cross-talk between these two pathways. Therefore, both cell lines were treated with Shh protein. MCF-7 cells responded to stimulation with exogenous Shh protein by Hh-Gli pathway activation (Fig. 5A, C) whereas the ER-negative cell line did not respond to Shh stimulation (Fig. 5B). Interestingly, short-term Shh treatment also had an effect on ERα expression in ER-positive cell line, which was increased (Fig. 5D, F), but this effect was relatively quickly negated 48 h post-treatment (Fig. 5D). In the SkBr-3 cell line there was no upregulation of ERα in response to Shh protein, but rather a slight down-regulation (Fig. 5E).

To check whether the effect of Shh on ERα is mediated via the canonical Hh-Gli signal transduction, cells were transfected with GLI1. After transfection and additional Shh stimulation, Gli1 and Ptch1 gene and protein expressions were elevated in MCF-7 cells (Fig. 6C, S2 Figure), whereas ERα was upregulated in MCF-7 cell line only after exogenous Shh stimulation (Fig. 6A). On the protein level ERα expression decreased after GLI1 transfection, but an increase was visible after Shh addition, compared with only transfected cells (Fig. 6C). This suggests that ERα regulation is not mediated transcriptionally via Gli1 transcription factor, but rather directly by Shh protein.

Even though the transfection was successful in SkBr-3 cells, shown by upregulation of GLI1 and PTCH1 expression (S2 Figure), it had no effect on ERα gene expression which was expected since there is only a low basal level of ERα mRNA expression and no ERα protein production in these cells (Fig. 6B).

To confirm a direct impact of Shh protein on ERα we silenced PTCH1, the primary Shh receptor, which would cause an increase in free, unbound Shh protein that could in turn interact with ERα and increase its activity. The effect was induction of ERα expression in MCF-7 cells, suggesting Shh protein has a direct effect on ERα. (Fig. 6D, F) For SkBr-3 cell line, sufficient knockdown of PTCH1 was achieved 48 h post-transfection (Fig. 6E) and the effect on ERα was downregulation of gene expression (Fig. 6G).

Shh protein interacts with ERα
To verify whether Shh has a direct effect on ERα, cells were treated with Shh protein, for 48 h and localization of Shh and ERα was visualized. Non-treated cells showed Shh staining in a granular pattern in the cytoplasm, mostly surrounding the nucleus, while ERα was scattered in the cytoplasm and stronger in the nuclei. Shh treatment caused an interesting effect: co-localization of Shh

(*) P<0.05 (F). Transwell migration assay confirmed increased migration capacity of cells after combined cyclopamine and tamoxifen treatment. Representative images of migrated cells after 48 h are shown for non-treated conditions (NT; N=15), cyclopamine treatment (CYC; N=15), tamoxifen treatment (TAM; N=15) and combined treatment (C+T; N=15) (G). Quantitative analysis of the relative number of migrated cells (analyzed relative to non-treated cells) is shown on graph, (*) P<0.0001 (H).
### Table 1. Differentially expressed proteins in MCF-7 cells treated with cyclopamine and tamoxifen compared with non-treated control cells.

| No | Protein Description | GI Accession | Score | General Functions |
|----|---------------------|--------------|-------|-------------------|
| 1  | Heat shock protein 90-alpha | gi|32488 | 142 | ・ Molecular chaperone that promotes the maturation, structural maintenance and proper regulation of specific target proteins involved i.e. in cell cycle control and signal transduction |
| 2  | Ezrin | gi|11276938 | 110 | ・ Involved in connections of major cytoskeletal structures to the plasma membrane ・ In epithelial cells, required for the formation of microvilli and membrane ruffles on the apical pole |
| 3  | KHSRP protein | gi|54648253 | 145 | ・ Role in mRNA trafficking ・ Gene expression activation |
| 4  | Heat shock protein 75 | gi|2865466 | 100 | ・ Involved in maintaining mitochondrial function and polarization ・ Negative regulator of mitochondrial respiration able to modulate the balance between oxidative phosphorylation and aerobic glycolysis |
| 5  | TATA-binding protein-associated factor 2N isoform 2 | gi|4507353 | 52 | ・ RNA and ssDNA-binding protein with roles during transcription initiation at distinct promoters |
| 6  | Alpha-tubulin | gi|340021 | 232 | ・ Tubulin is the major constituent of microtubules |
| 7  | Pyrroline-5-carboxylate dehydrogenase | gi|1353248 | 81 | ・ Irreversible conversion of delta-1-pyrroline-5-carboxylate (P5C), derived either from proline or ornithine, to glutamate |
| 8  | UDP-glucose 6-dehydrogenase isoform 1 | gi|4507813 | 72 | ・ Involved in the biosynthesis of glycosaminoglycans |
| 9  | Translation initiation factor 4A–III | gi|496902 | 144 | ・ Core component of the splicing-dependent multiprotein exon junction complex ・ mRNA processing ・ mRNA splicing ・ mRNA transport ・ Nonsense-mediated mRNA decay ・ RNA processing ・ Translation regulation |
| 10 | Glutamate dehydrogenase 1, mitochondrial precursor | gi|4885281 | 64 | ・ Cellular amino acid biosynthetic process ・ Converts L-glutamate into alpha-ketoglutarate |
| 11 | Alpha-enolase isoform 1 | gi|4503571 | 119 | ・ Multifunctional enzyme that, as well as its role in glycolysis, plays a part in various processes such as growth control, hypoxia tolerance and allergic responses |
| 12 | Laminin-binding protein | gi|34234 | 170 | ・ Required for the assembly and/or stability of the 40 S ribosomal subunit ・ Also functions as a cell surface receptor for laminin ・ Plays a role in cell adhesion to the basement membrane and in the consequent activation of signaling transduction pathways |
| 13 | Keratin 10 | gi|28317 | 51 | ・ Structural protein which forms the intermediate filament |
| 14 | Heat shock protein 27 | gi|35182 | 124 | ・ Involved in stress resistance and actin organization ・ Negative regulation of apoptotic process ・ Positive regulation of angiogenesis ・ Positive regulation of blood vessel endothelial cell migration |

### Proteins with $\geq$2 times lower expression in MCF-7 cells treated with cyclopamine + tamoxifen compared with control cells

| No | Protein Description | GI Accession | Score | General Functions |
|----|---------------------|--------------|-------|-------------------|
| 18 | far upstream element-binding protein 1 | gi|17402900 | 172 | ・ Regulates MYC expression |
| 19 | far upstream element-binding protein 1 | gi|17402900 | 172 | ・ Regulates MYC expression |
| 20 | Heterogeneous nuclear ribonucleoprotein H | gi|5031753 | 116 | ・ Component of the heterogeneous nuclear ribonucleoprotein (hnRNP) complexes which provide the substrate for the processing events that pre-mRNAs undergo before becoming functional ・ pre-mRNA alternative splicing regulation |
| 21 | Elongation factor 1 alpha | gi|31092 | 40 | ・ Promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis |
| 22 | Tu translation elongation factor, mitochondrial, isoform CRA_b | gi|119572383 | 148 | ・ Promotes the GTP-dependent binding of aminoacyl-tRNA to the A-site of ribosomes during protein biosynthesis |
| 23 | C protein | gi|306875 | 97 | ・ Protein C is a vitamin K-dependent serine protease that regulates blood coagulation by inactivating factors Va and VIIIa in the presence of calcium ions and phospholipids ・ negative regulation of apoptotic process ・ post-translational protein modification |
| 24 | Triosephosphate isomerase | gi|136066 | 75 | ・ carbohydrate metabolic process |
| No | Protein Description                  | GI Accession  | Score | General Functions                                                                                                                                                                                                 |
|----|-------------------------------------|---------------|-------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 28 | GRP78 precursor, partial gi|386758       | 133   | • Involved in the correct folding of proteins and degradation of misfolded proteins • Cellular protein metabolic process • Cellular response to antibiotic • Cellular response to glucose starvation • Negative regulation of apoptotic process • Positive regulation of cell migration |
| 29 | Heat shock protein 27 gi|662841        | 91    | • Involved in stress resistance and actin organization • Negative regulation of apoptotic process • Positive regulation of angiogenesis • Positive regulation of blood vessel endothelial cell migration                                           |

Proteins with ≥2 times higher expression in MCF-7 cells treated with cyclopamine + tamoxifen compared with control cells

| No | Protein Description                  | GI Accession  | Score | General Functions                                                                                                                                                                                                 |
|----|-------------------------------------|---------------|-------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 31 | Keratin 8, isoform CRA_a gi|119617057     | 76    | • Plays a role in maintaining cellular structural integrity and also functions in signal transduction and cellular differentiation                                                                                       |
| 32 | Prohibitin isoform 1 gi|4505773       | 308   | • Role in human cellular senescence and tumor suppression • Antiproliferative activity is reported to be localized to the 3’ UTR • Positive regulation of cell proliferation and migration                                          |

General Functions are obtained from the UniProt and NCBI Gene databases. Protein numbers correspond to the numbers marked on the 2-D gels (Figure S1). Numbers in the table correspond to spot numbers denoted on the 2-D gel images; missing numbers in the table are unidentified proteins or proteins with score less than 39.

doi:10.1371/journal.pone.0114510.t001

---

**Fig. 5. Effect of stimulation with Shh protein on pathway activity in MCF-7 (A,C) and SkBr-3 cells (B).** Gene expression levels are shown on graph as relative fold change relative to non-treated conditions with reference value 1 pointed out with emboldened bar. Relative gene expression of ERα after treatment with Shh protein (D,E). Non-treated cells (NT) have a relative value 1. ERα protein expression in MCF-7 cells increases after treatment with Shh protein for 48 h (F) Protein bands were quantified and normalized relative to actin and non-treated conditions and the relative values are denoted below each band.

doi:10.1371/journal.pone.0114510.g005
and ERα in the cytoplasm of the cells (Fig. 7A). There was very little co-localization of ERα and Shh in untreated cells, but after 48 h-treatment with Shh protein there is significantly less nuclear staining of ERα (P<0.0003) and ERα and Shh co-localized in the cytoplasm (P<0.0001) (Fig. 7B). This suggests that Shh acts directly on ERα, modifying its activity. Co-immunoprecipitation results however, indicate an interaction of Shh and ERα proteins in general, regardless of treatment with exogenous Shh protein (Fig. 7C). This is not unusual as the MCF-7 cells produce high amounts of Shh protein. These results undoubtedly show an interaction between Shh and ERα proteins, which is the first mention of direct interaction between these two proteins. However, adding exogenous Shh protein did not increase this interaction, as would be expected from the immunofluorescence data. It is possible that, since the MCF-7 cells already produce high amounts of Shh protein, addition of exogenous protein has no influence on the interaction rate. However, the fact that there is an obvious interaction between these two proteins is a new and intriguing finding that needs to be investigated further as it opens new possibilities in the aspect of Hh-Gli signaling research in ER-positive breast cancer.
Fig. 7. Immunofluorescent staining of MCF-7 cell line in non-treated cells (NT) and treated with Shh protein detected by confocal microscopy. ERα is stained green (column 1), Shh is stained red (column 2), nuclei are stained blue with DAPI (column 3), and the last column shows the overlay of signals. Yellow
Discussion

The role of Hh-Gli signaling in breast cancer is still unclear, especially regarding their association with steroid receptor signaling. To date the findings of Hh-Gli component expression in breast cancer cell lines is contradictory, particularly for Shh and Gli1. We found expression of Gli1 and Shh in the ER-positive cell line (MCF-7), but Ramaswamy et al. on the other hand found no expression of Shh in MCF-7 cells [21]. This inconsistency may be due to the fact that the authors looked only at the expression of unprocessed Shh protein (45 kDa). This is supported by the expression of SHH at the mRNA level which they did find. Two other studies, on the other hand, did find Shh expression in MCF-7 cells [7, 37]. Also, some studies show high expression of GLI1 in ER-negative cell lines, including SkBr-3 [20, 38], but in our hands GLI1 expression was not detectable in SkBr-3 cells. Recently a study showed a positive correlation between ERα and GLI1 expression [39], supporting lower levels of GLI1 in the ER-negative cell line. Even though these authors did find very low GLI1 expression in SkBr-3 it was much lower than in MCF-7. Given the lower levels of GLI1 in MCF-7 cells that we detected it is not surprising it was undetectable in SkBr-3.

Cyclopamine has been tested together with gefitinib in prostate cancer cell lines, where the combined treatment induced a supra-additive inhibitory growth effect on serum-free and serum-stimulated cell lines. This effect is established through cell cycle arrest in G1 phase and increased apoptosis. Cyclopamine and gefitinib-treated cells showed a decreased ability for invasion, and this effect was amplified in combined treatment [40]. In other studies on prostate cancer cells cyclopamine used in combination with ErbB inhibitors gefitinib or lapatinib showed a synergistic effect [41, 42] and combination of docetaxel+cyclopamine+gefitinib induced more intensive cell death compared to either treatment alone [43]. In cholangiocarcinoma treatment with cyclopamine and MEK inhibitor U0126 showed an additive effect, especially in cells with KRAS mutation [44].

Our results regarding the effect of cyclopamine on breast cancer cells are in agreement with previous studies that have shown that cyclopamine inhibits human breast cancer cell growth by increased apoptosis [19]. In a study by Che et al. [22] cyclopamine was reported to have anti-proliferative, anti-invasive and anti-estrogenic potency in human breast cancer. This is similar to our findings which also showed the anti-estrogenic effect of cyclopamine, ERα gene expression was downregulated after cyclopamine treatment.

In the ER-positive breast cancer cell line, however, combined treatment with cyclopamine and tamoxifen increased cell viability after short-term treatment, but it was not seen in ER-negative cells. This effect was dose-dependent, and
competition experiments have shown that higher concentrations of both compounds are required for the survival effect. Short-term combined treatment of MCF-7 cells upregulated the Hh-Gli signaling pathway and promoted cell migration (Figs. 2–4).

To elucidate the effect of the combination of these two drugs on the profile of expressed proteins we performed proteomic profiling of cells treated with a combination of cyclopamine and tamoxifen as well as control non-treated cells. This analysis revealed that a small but unique set of proteins is upregulated upon combination treatment in comparison with non-treated cells. All of them have been linked to cell proliferation and migration (Table 1). GRP78, a known survival factor, has been known to mediate signaling pathways that lead to proliferation and migration [33, 34]. Prohibitin was initially shown to block cell proliferation [45], but this ability was attributed to its 3’ untranslated region [46]. However, there is emerging evidence that prohibitin as a protein is required for cell proliferation and adhesion [47]. This protein is also known for activating the Raf-MEK-ERK signaling pathway and inducing cell migration [36, 48]. Another protein found to be upregulated after treatment with cyclopamine and tamoxifen is keratin 8. The data on the role of keratin 8 in cancer are inconsistent. Some studies show that keratin 8 overexpression correlates with lower tumorigenicity, invasiveness and motility [49], while others found it to be correlated with poor prognosis, invasiveness and cell migration [35, 50, 51]. HSP27, which is expressed under stressful conditions, is found both in treated cells and non-treated cells, but the protein was shifted in relation to the protein in non-treated cells suggesting it was modified. It has been found that the phosphorylated form of this protein participates in stress resistance and act as a negative regulator of apoptosis and a positive regulator of proliferation and migration [52–55]. This suggests that a combination of these drugs potentially enhances the migration ability of these cells, which is consistent with the results obtained by the wound healing and transwell migration assays, showing that cells treated with the combination of drugs have a higher migration capacity than the non-treated ones. Whether this effect is related to the upregulation of the Hh-Gli signaling pathway remains to be investigated. It should be looked into whether the Hh-Gli signaling pathway can directly or indirectly affect the expression of these proteins.

Apart from Hh-Gli pathway being regulated by compounds affecting ERα (tamoxifen), the communication works also in the other direction, from Hh-Gli signaling to ERα. The link between ERα and Hh-Gli signaling pathways has been addressed in previous studies. It was shown that upregulation of ERα by E2 also upregulated Shh which canonically activated Hh-Gli signaling and Gli1 expression in human breast cancer cells [37]. The same link was observed in ERα positive gastric cancer [56]. In both studies the vice versa link was not observed. We on the other hand, show a potential mechanism of ERα regulation through Hh-Gli signaling. Although there may be a transcriptional link between Hh-Gli and estrogen signaling via FoxM1 [25, 57], this does not seem to be the case here. Transfection of GLI1 does not automatically induce transcription of ERα, like it does of PTCH1; suggesting ERα expression is not regulated transcriptionally via
Gli1. Only after exogenous addition of Shh protein there is an induction in ERα, regardless of GLI1 levels. Our co-immunoprecipitation assay confirmed a direct link between Shh and ERα proteins (Figs. 5–7). It is possible that the cholesterol modification of the Shh protein plays a role in this interaction since cholesterol is the precursor molecule for steroid hormones, but this remains to be analyzed. This interaction may be the cause of upregulation of ERα activity and consequently upregulation of ERα gene and protein expression. Silencing of PTCH1 leads to a reduced number of receptor molecules on the membrane, allowing increased binding of endogenous Shh to the ERα, which leads to upregulation of ERα expression (Fig. 6), since ERα autoregulates its own expression [58].

The mechanism which is responsible for the increased viability of ER-positive cell line after combined treatment with cyclopamine and tamoxifen, in comparison with either treatment alone, is not clear. We show that the Hh-Gli signaling is upregulated and proteins involved in proliferation and migration enhancement are expressed, but the link between them and the Hh-Gli signaling remains to be elucidated. Although Hh-Gli signaling seems to be a good potential target for breast cancer therapy, caution must be advised, especially when combining therapies. We have demonstrated that combined treatment of cyclopamine and tamoxifen may induce an opposite effect, providing cells with short-term survival and increased ability to migrate, which may be deleterious for the patient. On the other hand, we show a potential direct link between Shh and ERα proteins. According to our results Shh can bind ERα and activate it. This might be a mechanism that enhances survival of breast cancer cells with expression of Shh, even in estrogen deficient conditions.

Supporting Information

S1 Figure. 2-D gels of non-treated control MCF-7 cells (A) and MCF-7 cells treated with cyclopamine and tamoxifen (B). 2-D gel of MCF-7 cells treated with a combination of cyclopamine and tamoxifen with indicated spots that have ≥2 times higher expression compared with control cells (C). 2-D gel of MCF-7 cells treated with a combination of cyclopamine and tamoxifen with indicated spots that have ≥2 times lower expression compared with control cells (D). Indicated spots were used for further MS analysis. Results are shown in Table 1. doi:10.1371/journal.pone.0114510.s001 (TIF)

S2 Figure. GLI1 and PTCH1 gene expression levels after transfection with GLI1 plasmid in ER-positive MCF-7 cells (A, C) and ER-negative SkBr-3 cells (B, D). doi:10.1371/journal.pone.0114510.s002 (TIF)

Acknowledgments

The authors wish to thank Lucija Horvat, B.Sc. for help with confocal microscopy and Mirela Levacic Cvok, B.Sc. for all the help with the laboratory work. We thank
Dr. Marijeta Kralj for use of the Olympus CKX41 microscope linked to the Olympus E330 camera and Dr. Mirko Hadzija and Dr. Marijana Popovic Hadzija for use of the Olympus BX51 microscope.

Author Contributions
Conceived and designed the experiments: MS SL. Performed the experiments: MS DT ZU PO VM MR. Analyzed the data: MS DT PO VM MC SL. Contributed reagents/materials/analysis tools: MC. Contributed to the writing of the manuscript: MS DT SL.

References
1. Higgins MJ, Baselga J (2011) Targeted therapies for breast cancer. J Clin Invest 121: 3797–3803. doi:10.1172/JCI57152.
2. Hatsell SJ (2006) Gli3-mediated repression of Hedgehog targets is required for normal mammary development. Development 133: 3661–3670. doi:10.1242/dev.02542.
3. Lewis MT, Ross S, Strickland PA, Sugnet CW, Jimenez E, et al. (1999) Defects in mouse mammary gland development caused by conditional haploinsufficiency of Patched-1. Development 126: 5181–5193.
4. Cohen MM (2003) The hedgehog signaling network. Am J Med Genet A 123A: 5–28. doi:10.1002/ajmg.a.20495.
5. Mazumdar T, DevVecchio J, Ting S, Jones J, Agyeman A, et al. (2010) Hedgehog signaling drives cellular survival in human colon carcinoma cells. Cancer Res.
6. Stecca B, Ruiz i Altaba A (2010) Context-dependent Regulation of the GLI Code in Cancer by HEDGEHOG and Non-HEDGEHOG Signals. J Mol Cell Biol 2: 84.
7. Kubo M, Nakamura M, Tasaki A, Yamanaka N, Nakashima H, et al. (2004) Hedgehog signaling pathway is a new therapeutic target for patients with breast cancer. Cancer Res 64: 6071–6074. doi:10.1158/0008-5472.CAN-04-0416.
8. Moraes RC, Zhang X, Harrington N, Fung JY, Wu M-F, et al. (2007) Constitutive activation of smoothened (SMO) in mammary glands of transgenic mice leads to increased proliferation, altered differentiation and ductal dysplasia. Development 134: 1231–1242. doi:10.1242/dev.02797.
9. Ten Haaf A, Bektas N, von Serenyi S, Losen I, Arweiler EC, et al. (2009) Expression of the glioma-associated oncogene homolog (GLI) 1 in human breast cancer is associated with unfavourable overall survival. BMC Cancer 9: 298. doi:10.1186/1471-2407-9-298.
10. Sinha S, Singh RK, Alam N, Roy A, Roychoudhury S, et al. (2008) Alterations in candidate genes PHF2, FANCC, PTCH1 and XPA at chromosomal 9q22.3 region: pathological significance in early- and late-onset breast carcinoma. Mol Cancer 7: 84. doi:10.1186/1476-4598-7-84.
11. Wolf I, Bose S, Desmond JC, Lin BT, Williamson EA, et al. (2007) Unmasking of epigenetically silenced genes reveals DNA promoter methylation and reduced expression of PTCH in breast cancer. Breast Cancer Res Treat 105: 139–155. doi:10.1007/s10549-006-9440-4.
12. Cui W, Wang L-H, Wen Y-Y, Song M, Li B-L, et al. (2010) Expression and regulation mechanisms of Sonic Hedgehog in breast cancer. Cancer Sci 101: 927–933. doi:10.1111/j.1349-7006.2010.01495.x.
13. Bièche I, Lerebours F, Tozlu S, Espie M, Marty M, et al. (2004) Molecular profiling of inflammatory breast cancer: identification of a poor-prognosis gene expression signature. Clin Cancer Res Off J Am Assoc Cancer Res 10: 6789–6795. doi:10.1158/1078–0432.CCR-04-0306.
14. Oro AE, Higgins KM, Hu Z, Bonifas JM, Epstein Jr EH, et al. (1997) Basal cell carcinomas in mice overexpressing sonic hedgehog. Science 276: 817.
15. Xie J, Johnson RL, Zhang X, Bare JW, Waldman FM, et al. (1997) Mutations of the PATCHED gene in several types of sporadic extracutaneous tumors. Cancer Res 57: 2369.
16. Vorechovský I, Benediktsson KP, Toftgård R (1999) The patched/hedgehog/smoothened signalling pathway in human breast cancer: no evidence for H133Yn SHH, PTCH and SMO mutations. Eur J Cancer Oxf Engl 1990 35: 711–713.

17. Wicking C, Evans T, Henk B, Hayward N, Simms LA, et al. (1998) No evidence for the H133Y mutation in SONIC HEDGEHOG in a collection of common tumour types. Oncogene 16: 1091–1093. doi:10.1038/sj.onc.1216044.

18. Chang-Claude J, Dunning A, Schnitzbauer U, Galimbacher P, Tee L, et al. (2003) The patched polymorphism Pro1315Leu (C3944 T) may modulate the association between use of oral contraceptives and breast cancer risk. Int J Cancer J Int Cancer 103: 779–783. doi:10.1002/ijc.10889.

19. Mukherjee S, Frolova N, Sadlonova A, Novak Z, Steg A, et al. (2006) Hedgehog signaling and response to cyclopamine differ in epithelial and stromal cells in benign breast and breast cancer. Cancer Biol Ther 5: 674.

20. Zhang X, Harrington N, Moraes RC, Wu M-F, Hilsenbeck SG, et al. (2008) Cyclopamine inhibition of human breast cancer cell growth independent of Smoothened (Smoo). Breast Cancer Res Treat 115: 505–521. doi:10.1007/s10549-008-0093-3.

21. Ramaswamy B, Lu Y, Teng KY, Nuovo G, Li X, et al. (2012) Hedgehog Signaling Is a Novel Therapeutic Target in Tamoxifen-Resistant Breast Cancer Aberrantly Activated by PI3 K/ AKT Pathway. Cancer Res 72: 5048–5059. doi:10.1158/0008–5472.CAN-12–1248.

22. Che J, Zhang F-Z, Zhao C-Q, Hu X-D, Fan S-J (2013) Cyclopamine is a novel Hedgehog signaling inhibitor with significant anti proliferative, anti invasive and anti estrogenic potency in human breast cancer cells. Oncol Lett. Available: http://www.spandidos-publications.com/10.3892/ol.2013.1195. Accessed 23 June 2014.

23. Gebäck T, Schulz M, Koumoutsakos P, Detmar M (2009) TScratch: a novel and simple software tool for automated analysis of monolayer wound healing assays. Biotechniques 46: 265–274.

24. Sabol M, Car D, Musani V, Ozretic P, Oreskovic S, et al. (2012) The Hedgehog signaling pathway in ovarian teratoma is stimulated by Sonic Hedgehog which induces internalization of Patched. Int J Oncol. 41(4):1411–8.

25. Madureira PA, Varshochi R, Constantiniou D, Francis RE, Coombes RC, et al. (2006) The Forkhead box M1 protein regulates the transcription of the estrogen receptor alpha in breast cancer cells. J Biol Chem 281: 25167–25176. doi:10.1074/jbc.M603906200.

26. Leovic D, Sabol M, Ozretic P, Musani V, Car D, et al. (2012) Hh-Gli signaling pathway activity in oral and oropharyngeal squamous cell carcinoma. Head Neck 34: 104–112. doi:10.1002/hed.21696.

27. Maurac I, Sabol M, Musani V, Car D, Ozretic P, et al. (2012) A low-grade ovarian carcinoma case with coincident LOH of PTCH1 and BRCA1, and a mutation in BRCA1. Int J Gynecol Pathol Off J Int Soc Gynecol Pathol 31: 264–271. doi:10.1097/PGP.0b013e31823b6f0f.

28. Manders E, Verbeek F, Aten J (1993) Measurement of co-localization of objects in dual-colour confocal images. J Microsc 169: 375–382.

29. Antonioli P, Bachi A, Fasoli E, Righetti PG (2009) Efficient removal of DNA from proteomic samples prior to two-dimensional map analysis. J Chromatogr A 1216: 3606–3612. doi:10.1016/ j.chroma.2008.11.053.

30. Panfoli I, Calzia D, Santucci L, Raverà S, Bruschi M, et al. (2012) A blue dive: from “blue fingers” to “blue silver”. A comparative overview of staining methods for in-gel proteomics. Expert Rev Proteomics 9: 627–634.

31. Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins silver-stained polyacrilamide gels. Anal Chem 68: 850–858.

32. Shilov IV, Seymour SL, Patel AA, Loboda A, Tang WH, et al. (2007) The Paragon Algorithm, a next generation search engine that uses sequence temperature values and feature probabilities to identify peptides from tandem mass spectra. Mol Cell Proteomics 6: 1638–1655.

33. Lee AS (2007) GRP78 Induction in Cancer: Therapeutic and Prognostic Implications. Cancer Res 67: 3496–3499. doi:10.1158/0008-5472.CAN-07-0325.

34. Misra UK, Deedwania R, Pizzo SV (2006) Activation and Cross-talk between Akt, NF-B, and Unfolded Protein Response Signaling in 1-LN Prostate Cancer Cells Consequent to Ligation of Cell Surface-associated GRP78. J Biol Chem 281: 13694–13707. doi:10.1074/jbc.M511694200.
35. Alam H, Kundu ST, Dalal SN, Vaidya MM (2011) Loss of keratins 8 and 18 leads to alterations in 6 integrin-mediated signalling and decreased neoplastic progression in an oral-tumour-derived cell line. J Cell Sci 124: 2096–2106. doi:10.1242/jcs.073585.

36. Rajalingam K, Wunder C, Brinkmann V, Churin Y, Hekman M, et al. (2005) Prohibitin is required for Ras-induced Raf–MEK–ERK activation and epithelial cell migration. Nat Cell Biol 7: 837–843. doi:10.1038/ncb1283.

37. Koga K, Nakamura M, Nakashima H, Akiyoshi T, Kubo M, et al. (2008) Novel link between estrogen receptor alpha and hedgehog pathway in breast cancer. Anticancer Res 28: 731–740.

38. Zhao J, Chen G, Cao D, Li Y, Diao F, et al. (2009) Expression of Gli1 correlates with the transition of breast cancer cells to estrogen-independent growth. Breast Cancer Res Treat 119: 39–51. doi:10.1007/s10549-009-0323-3.

39. Sun Y, Wang Y, Fan C, Gao P, Wang X, et al. (2014) Estrogen promotes stemness and invasiveness of ER-positive breast cancer cells through Gli1 activation. Mol Cancer 13: 137.

40. Mimeault M, Moore E, Moniaux N, Hénichart JP, Depreux P, et al. (2006) Cytotoxic effects induced by a combination of cyclopamine and gefitinib, the selective hedgehog and epidermal growth factor receptor signaling inhibitors, in prostate cancer cells. Int J Cancer 118: 1022–1031.

41. Hu W, Liu T, Xiong J, Wang C (2007) Blockade of sonic hedgehog signal pathway enhances antiproliferative effect of EGFR inhibitor in pancreatic cancer cells. Acta Pharmacol Sin 28: 1224–1230. doi:10.1111/j.1745-7254.2007.00620.x.

42. Shaw G, Prowse DM (2008) Inhibition of androgen-independent prostate cancer cell growth is enhanced by combination therapy targeting Hedgehog and ErbB signalling. Cancer Cell Int 8: 3. doi:10.1186/1475-2867-8-3.

43. Mimeault M, Johansson SL, Henichart J-P, Depreux P, Batra SK (2010) Cytotoxic effects induced by docetaxel, gefitinib, and cyclopamine on side population and nonside population cell fractions from human invasive prostate cancer cells. Mol Cancer Ther 9: 617–630. doi:10.1158/1535-7163.MCT-09-1013.

44. Jinawath A, Akiyama Y, Sripa B, Yuasa Y (2007) Dual blockade of the Hedgehog and ERK1/2 pathways coordinately decreases proliferation and survival of cholangiocarcinoma cells. J Cancer Res Clin Oncol 133: 271–278. doi:10.1007/s00432-006-0166-9.

45. Nuell MJ, Stewart DA, Walker L, Friedman V, Wood CM, et al. (1991) Prohibitin, an evolutionarily conserved intracellular protein that blocks DNA synthesis in normal fibroblasts and HeLa cells. Mol Cell Biol 11: 1372–1381.

46. Jupe ER, Liu X-T, Kiehlbauch JL, McClung JK, Dell’Orco RT (1996) Prohibitin in Breast Cancer Cell Lines: Loss of Antiproliferative Activity Is Linked to 3 Untranslate Region Mutations. Cell Growth Differ 7: 871–878.

47. Sievers C, Billig G, Gottschalk K, Rudel T (2010) Prohibitins are required for cancer cell proliferation and adhesion. PLoS One 5: e12735.

48. Xu Z, Wu J, Zha X (2011) Up-regulation of prohibitin 1 is involved in the proliferation and migration of liver cancer cells. Sci China Life Sci 54: 121–127. doi:10.1007/s11427-010-4130-1.

49. Iyer SV, Dange PP, Alam H, Sawant SS, Ingle AD, et al. (2013) Understanding the Role of Keratins 8 and 18 in Neoplastic Potential of Breast Cancer Derived Cell Lines. PLoS ONE 8: e53532. doi:10.1371/journal.pone.0053532.

50. Chu Y-W, Seftor EA, Romer LH, Hendrix MJ (1996) Experimental coexpression of vimentin and keratin intermediate filaments in human melanoma cells augments motility. Am J Pathol 148: 63.

51. Fillies T, Werkmeister R, Packeisen J, Brandt B, Morin P, et al. (2006) Cytokeratin 8/18 expression indicates a poor prognosis in squamous cell carcinomas of the oral cavity. BMC Cancer 6: 10.

52. Guay J, Lambert H, Gingras-Breton G, Lavoie JN, Huot J, et al. (1997) Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27. J Cell Sci 110: 357–368.

53. Kwon S-M, Kim S-A, Yoon J-H, Ahn S-G (2010) Transforming Growth Factor B1–Induced Heat Shock Protein 27 Activation Promotes Migration of Mouse Dental Papilla–derived MDPC-23 Cells. J Endod 36: 1332–1335. doi:10.1016/j.joen.2010.04.010.
54. Song IS, Kang S-S, Kim E-S, Park H-M, Choi CY, et al. (2014) Heat shock protein 27 phosphorylation is involved in epithelial cell apoptosis as well as epithelial migration during corneal epithelial wound healing. Exp Eye Res 118: 36–41. doi:10.1016/j.exer.2013.11.002.

55. White SR, Tse R, Marroquin BA (2005) Stress-Activated Protein Kinases Mediate Cell Migration in Human Airway Epithelial Cells. Am J Respir Cell Mol Biol 32: 301–310. doi:10.1165/rcmb.2004-0118OC.

56. Kameda C, Nakamura M, Tanaka H, Yamasaki A, Kubo M, et al. (2010) Oestrogen receptor-α contributes to the regulation of the hedgehog signalling pathway in ERα-positive gastric cancer. Br J Cancer 102: 738–747. doi:10.1038/sj.bjc.6605517.

57. Teh M-T, Wong S-T, Neill GW, Ghali LR, Philpott MP, et al. (2002) FOXM1 is a downstream target of Gli1 in basal cell carcinomas. Cancer Res 62: 4773–4780.

58. Eeckhoute J, Keeton EK, Lupien M, Krum SA, Carroll JS, et al. (2007) Positive Cross-Regulatory Loop Ties GATA-3 to Estrogen Receptor Expression in Breast Cancer. Cancer Res 67: 6477–6483. doi:10.1158/0008-5472.CAN-07-0746.