

# Histone H3 Phosphorylation in Human Skin Histoculture as a Tool to Evaluate Patient's Response to Antiproliferative Drugs

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## Supplementary Issue: Gene and Protein Expression Profiling in Disease

**ABSTRACT:** Evaluation of patient's response to chemotherapeutic drugs is often difficult and time consuming. Skin punch biopsies are easily accessible material that can be used for the evaluation of surrogate biomarkers of a patient's response to a drug. In this study, we hypothesized that assessment of phosphorylated histone H3 in human skin punch biopsies could be used as a pharmacodynamics biomarker of patient's response to the kinesin spindle protein inhibitor SCH2047069. To test this hypothesis, we used a human skin histoculture technique that allows culturing intact human skin in the presence of the drug. Human melanoma and skin histocultures were treated with SCH2047069, and the effect of the drug was assessed by increasing histone H3 phosphorylation using immunohistochemistry. Our results demonstrate that SCH2047069 has a significant effect on cell proliferation in human melanoma and skin histoculture and justify using human skin punch biopsies for evaluation of the pharmacodynamic changes induced by SCH2047069.

**ACRONYMS:** Histone subunit H3 (H3), Kinesin spindle protein (KSP), 5-ethynyl-2'-deoxyuridine (EDU), Dimethyl sulfoxide (DMSO), Formalin-fixed paraffin embedded (FFPE).

**KEYWORDS:** KSP, skin histoculture, phosphorylated histone H3, biomarker

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## Introduction

Skin punch biopsy is a simple surgical procedure that can be used to obtain surrogate tissue for the assessment of target engagement and drug response biomarkers. The fundamental differences in the structure of human and mouse skin offer a challenge in evaluating skin as a surrogate tissue for biomarker research using mouse models. In order to assess the feasibility of using patient skin biopsies to monitor the activity of chemotherapeutic agents that target the cell cycle, we utilized a human skin histoculture model<sup>1,2</sup> treated with the kinesin spindle protein (KSP) inhibitor SCH2047069. In this model, human skin specimens obtained from plastic surgery are cultured *ex vivo* and treated with drugs to evaluate its mechanism of action and potential toxicity.<sup>3,4</sup> The main advantage of this method is the ability to preserve unique skin architecture.

Most chemotherapy drugs target cancer cell proliferation by damaging DNA structure or by interfering with the processes of DNA replication and mitosis to induce cell death. An interesting group of antimetabolic drugs are the KSP (*Eg5*) inhibitors.<sup>5,6</sup> KSP is an essential motor protein required for bipolar spindle formation and the separation of duplicated chromosomes during mitosis.<sup>7</sup>

Several small-molecule inhibitors targeting KSP have been developed, such as SCH2047069, Monastrol,<sup>8</sup> Ipsenib,<sup>9</sup> MK-0731,<sup>10</sup> ARRY-520,<sup>11</sup> and KPYB10602.<sup>12</sup> SCH2047069 was previously shown to effectively induce mitotic arrest in a panel of 57 human tumor cell lines from many different tissue origins.<sup>13</sup> It was also demonstrated that SCH2047069 has significant activity *in vivo* using human tumor xenograft models.<sup>13</sup>

Histone H3 phosphorylation (phospho H3) is a reliable and commonly used marker of mitosis.<sup>14</sup> Early in mitosis, during prophase, phosphorylation of histone H3 correlates with the condensation of chromatin into discrete chromosomes and the formation of the mitotic spindle. Histone H3 phosphorylation continues throughout prometaphase and metaphase and then disappears by the end of anaphase.<sup>15</sup> This particular pattern of histone H3 phosphorylation makes it a specific marker for cells arrested in mitosis, which could be easily analyzed by traditional immunohistochemistry (IHC) techniques.

In this study we used human skin histoculture to validate the hypothesis, that assessment of phosphorylated histone H3 in human skin could be used as a pharmacodynamics biomarker of a patient's response to the KSP inhibitor SCH2047069.

## Material and Methods

**Histoculture conditions.** Four fresh human skin explants were purchased from Cureline Inc. The specimens were obtained from healthy female subjects undergoing abdominoplasty surgery. One melanoma specimen taken from a patient with stage IV metastatic skin lesion was obtained from the University of Rochester. Because all tissue samples used in this study were existing, deidentified samples, the research was exempted from seeking Internal Review Board approval under §46.101, b, (4). After removing subcutaneous fat, the skin specimens were cut into small (2–3 mm) pieces, placed onto Matrix 0.4  $\mu\text{m}$  Millicell-CM insert (Millipore), and cultured at the air–liquid interface with the epidermis side facing upward at 37 °C in 5% CO<sub>2</sub> incubator in Dulbecco's modified Eagle medium supplemented with 4.5 g/L glucose, L-glutamine, sodium pyruvate (Mediatech, Inc.), 10% fetal bovine serum (SAFC Biosciences), 100  $\mu\text{g}/\text{mL}$  penicillin, and 100  $\mu\text{g}/\text{mL}$  streptomycin.<sup>2,3,16</sup>

The skin explants were incubated with vehicle (dimethyl sulfoxide [DMSO] 0.1%) or SCH2047069 at 0.025, 0.1, 1, 4, 8, and 10  $\mu\text{M}$  concentrations for 16 and 24 hours. Selection of the drug concentration was based on the previously published data.<sup>13</sup> Human melanoma histocultures were treated similar to the skin cultures described earlier. Following drug treatment, four pieces per condition were collected and processed into formalin-fixed paraffin-embedded (FFPE) blocks for IHC analysis. To analyze cells in S-phase, 5  $\mu\text{M}$  of 5-ethynyl-2'-deoxyuridine (EDU) was added to the culture 18 hours prior to tissue harvesting.

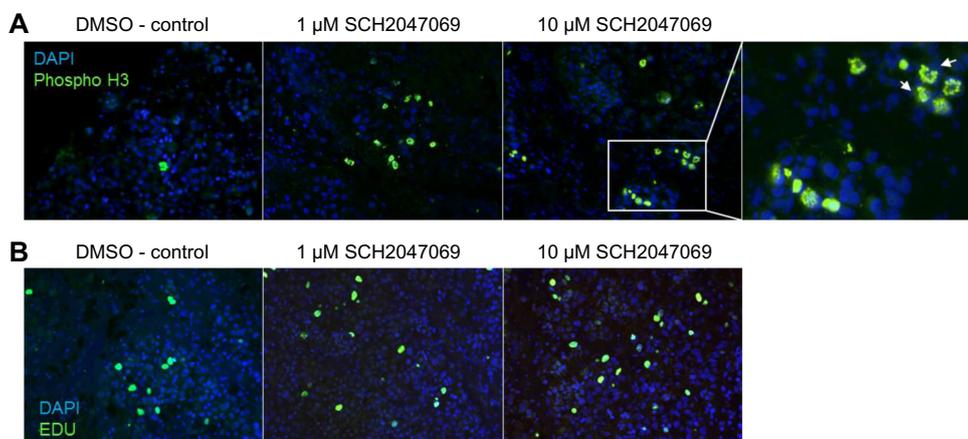
**Immunohistochemistry.** FFPE tissue blocks containing human skin or melanoma tissue were sectioned, deparaffinized, and rehydrated with serial passage through changes in xylene, graded ethanols, and deionized water. All slides were subjected to heat-induced epitope retrieval in target

retrieval solution (pH 6.1) (cat S1699; Dako). Slides were then incubated with antiphospho H3 – Alexa488 (#9701; Cell Signaling) for 60 minutes at room temperature in a dark room. EDU staining was done using Click-iT EDU imaging kit following the manufacturer's instructions (Life Technologies, Cat# C10337). Slides were washed twice in TBS-T, mounted using Vectashield Mounting Medium with DAPI (Vector Laboratories, Cat# H-1500), and analyzed using Leica DMRE Fluorescent microscope and Nikon Cool-snap HQ camera. Phospho H3-positive cells were manually counted from two skin sections. Each section had ~3 mm<sup>2</sup> of epidermis surface. Statistical analyses were conducted using a one-way analysis of variance (ANOVA) test with GraphPad Prism software.

## Results and Discussion

In order to evaluate the feasibility of using human skin punch biopsies to monitor patient's response to the antimetabolic drug SCH2047069, we utilized a previously described tissue histoculture model.<sup>1</sup> Human skin biopsies were obtained from healthy donors, and tumor tissue was derived from a melanoma patient. Histoculture sections were incubated with SCH2047069 or DMSO control, and its effects on cell cycle were evaluated 24 hours after treatment.

**Phosphorylation of histone H3 in human melanoma histoculture.** Analysis of phospho H3 in a melanoma histoculture treated with DMSO control for 24 hours showed occasional positive cells undergoing mitosis (Fig. 1A). Upon treatment with SCH2047069, a significant increase in the number of phospho H3-positive cells was observed, demonstrating that KSP inhibition can induce cell cycle arrest in mitosis in melanoma tissue cultured *ex vivo*. Detailed visualization of phospho H3-positive cells (Fig. 1A, right panel) showed a typical rosette chromosome conformation, previously



**Figure 1.** KSP inhibitor SCH2047069 induces mitotic arrest in human melanoma histoculture. **(A)** Phospho H3 immunostaining in melanoma histocultures. Analysis of phospho H3 showed an accumulation of cells arrested in mitosis upon treatment with SCH2047069. Phospho H3-positive cells have impaired chromosome segregation with a distinct rosette conformation (white arrows). **(B)** Analysis of cell proliferation by EDU incorporation. Cells in S-phase were visualized using EDU incorporation assay following treatment with SCH2047069 and DMSO control. SCH2047069 treatment has no effect on the number of proliferating EDU+ cells under the described conditions. Representative images are shown.

described to be the consequence of KSP deficiency and incorrect chromosome segregation.<sup>13</sup>

To determine the overall extent of cell proliferation in the melanoma histoculture, changes in total number of cells in S-phase were analyzed using EDU incorporation. The thymidine analog, EDU, was added to the melanoma histoculture for 18 hours, and EDU-positive cells were visualized by immunofluorescence. A substantial number of EDU-positive cells in the melanoma histoculture sample were detected demonstrating active proliferation of cells under histoculture conditions (Fig. 1B). Notably, addition of SCH2047069 to the culture for 24 hours did not impact the amount of EDU-positive cells, confirming that KSP inhibition arrests cells in mitosis and does not affect DNA replication and transition through the S-phase of cell cycle.<sup>6</sup>

Altogether, these results demonstrate feasibility of using immunostaining of phospho H3 to measure the effects of the KSP inhibitor SCH2047069 on human melanoma samples treated *ex vivo*.

**Assessment of human skin histocultures response to SCH2047069.** To examine the feasibility of using phospho H3 immunostaining in skin punch biopsies as a potential pharmacodynamic biomarker, the effect of SCH2047069 on human skin histocultures was evaluated. A significant

increase in the number of cells arrested in mitosis was observed after 16 and 24 hours of culture in the presence of SCH2047069 at 0.1, 4, and 8  $\mu\text{M}$  concentrations (Figs. 2A and B). Notably, the assay showed reproducible results in all the four donors tested. The effect of the drug was more prominent after 24 hours than after 16 hours of treatment (2.48-fold increase in phospho H3 at 8  $\mu\text{M}$  concentration of SCH2047069,  $P < 0.01$ , Fig. 2A). Whether the effect of SCH2047069 can be quantified after longer exposures to the drug remains to be determined.

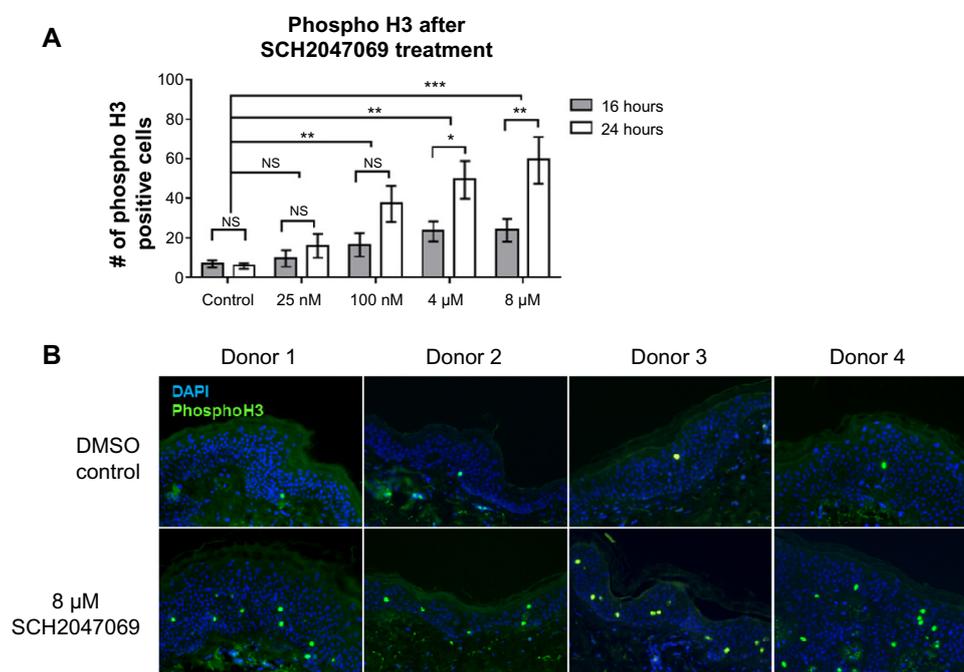
Our study demonstrates the utility of the human skin histoculture model to evaluate the feasibility of using phospho H3 immunostaining in skin punch biopsies as a pharmacodynamic biomarker of response to KSP inhibitors and potentially other chemotherapeutic drugs that affect cell division.

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### Author Contributions

Conceived and designed the experiments: SS, KP. Analyzed the data: FU, SS. Wrote the manuscript: FU. Jointly developed the structure and arguments for the paper: FU, SS.



**Figure 2.** Quantification of histone H3 phosphorylation in response to KSP inhibition in human skin biopsies. **(A)** Quantification of phospho H3-positive cells from skin histoculture sections. Human skin histocultures derived from abdominoplasty specimens of four human donors were treated with SCH2047069 or DMSO control, and phospho H3-positive cells were manually counted from two skin sections. Each section had  $\sim 3 \text{ mm}^2$  of epidermis surface. A significant enrichment on phospho H3-positive cells was observed at 100 nM, 4  $\mu\text{M}$ , and 8  $\mu\text{M}$  concentrations of SCH2047069 compared with DMSO control at 16 and 24 hours. Phospho H3 expression at 24 hours post treatment was significantly higher than that at 16 hours with SCH2047069 at concentrations 4 and 8  $\mu\text{M}$ . Statistical analysis was conducted using two-way ANOVA test. Symbols \*\*\* and \*\* indicate significance at  $P < 0.05$ ,  $P < 0.01$ , and  $P < 0.001$ , respectively. Error bars represent standard error of the mean. **(B)** Representative images of phospho H3 staining after 24 hours of treatment with 8  $\mu\text{M}$  SCH2047069 or DMSO control. Representative images of skin histocultures from four healthy human donors illustrate the accumulation of phospho H3-positive cells following SCH2047069 treatment.



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