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Initial assessment of Id protein antagonist (aHLH) shows anti-proliferative activity on HEK293T, LS174T and HCT116 cells

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ABSTRACT

Id proteins (1–4) which are dominant-negative antagonists of the basic helix-loop-helix (bHLH) family of transcription factors function in a variety of cellular processes. They bind to the ubiquitously expressed 'E box' protein of the class A bHLH proteins (E12, E47, E2–2, and HEB). Each Id protein has distinct preferences and specificities for their E protein targets. A mutant version of E47 protein, an Id protein antagonist known as aHLH which is defective in DNA binding functions is known to suppress growth in human carcinoma cell lines. This work investigates the effects of the Id protein antagonist aHLH on growth and on pro-apoptotic functions *in vitro* using immortalized (highly tumorigenic) transformed human embryonic kidney cells (HEK293T), human epithelial colon adenocarcinoma cell line (LS174T) and human epithelial colon carcinoma cell line (HCT116). Plasmid encoding the aHLH proteins was transiently over-expressed in cultured HEK293T, LS174T and HCT116. Cell viability assay with MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was performed. Apoptotic and cell cycle assays were also performed on the transfected cells thereafter. Here, the results demonstrate that Id inhibition by aHLH impairs cell viability, inhibits cell proliferation and induces apoptosis in HEK293T, LS174T and HCT116 cell lines. These findings on the role of aHLH in cell growth and survival could help manipulate their functions as targets for developing anti-cancer agents and could also serve as molecular markers of human cancers.

Introduction

Id proteins (Id1, Id2, Id3, and Id4) are helix-loop-helix (HLH) transcriptional regulators. They function by binding to and sequestering basic HLH (bHLH) transcription factors (*e.g.* the E protein E47). Id proteins function basically through antagonism of bHLH transcriptional regulators and the bHLH proteins are known to positively regulate genes during cell fate determination and differentiation [1]. The Id proteins form heterodimers with the basic helix-loop-helix (bHLH) proteins. Id proteins bind to the ubiquitously expressed 'E box' protein of the class A bHLH proteins (E12, E47, E2–2, and HEB) [2,3]. Accumulating evidence suggests that although the Id proteins interact with each of the four E proteins, each Id protein has

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distinct preferences and specificities for their E protein targets [4]. Id proteins are expressed during development and inhibit bHLH transcription factors to block differentiation and maintain self-renewal. Id protein expression is silenced in adult tissues; however they can be re-activated in several disease processes, including cancer [5,6].

The class A bHLH proteins function by repressing or activating specific genes involved in cell development. Among this group of transcription factors, E47 protein is recognised as the strongest stimulator of cyclin-dependant kinase inhibitors (CDKIs). The expression of E47 protein suppresses tumor cells by activating CDKIs to stop cells from entering the S-phase and also induce apoptosis [7]. Studies show that over-expression of the E47 protein in human adenocarcinoma cell lines in co-immunoprecipitation and sub-cellular colocalization studies resulted in the sequestration of Id proteins as Id-bHLH heterodimers and led to suppression of growth [8].

Also a mutant version of E47, an Id protein antagonist known as aHLH which is defective in DNA binding functions suppresses growth in human adenocarcinoma cell lines [8]. However, little is known about the growth-inhibitory and cell survival activities of the Id protein antagonist (aHLH) in tumor cells and the mechanisms of these effects are not well understood. Hence this research aims at investigating the consequences of loss-of-function of Id protein on growth and on pro-apoptotic functions in different cellular contexts using selective ablation with aHLH on immortalised (highly tumourigenic) transformed human embryonic kidney cells (HEK293T), human epithelial colon adenocarcinoma cell line (LS174T) and human epithelial colon carcinoma cell line (HCT116).

Materials and methods

Adherent cell culture

Immortalized (highly tumorigenic) transformed human embryonic kidney cells (HEK293T), human epithelial colon adenocarcinoma cell line (LS174T) and human epithelial colon carcinoma cell line (HCT116) (American Type Culture Collection, USA) were cultured as described previously [9].

Transfection of adherent cells

8×10^4/well of HCT116 cells were plated the day before transfection in a 24-well plate. Same was done for LS174T and HEK293T cells in different plates. Cells were transfected with negative control vector (pcDNA3), aHLH and E47 (positive control). The procedures followed are described earlier [10].

Cell proliferation assay

MTT (3-(4, 5-Dimethylthiazol-2-yl)−2, 5-diphenyltetrazolium bromide) assay (Sigma-Aldrich, USA) was performed to assess the proliferative activity of HCT116, LS174T and HEK293T cells. The methodology followed is elaborated in earlier study [9].

Cell cycle assay

Cycling cells were quantified using Hoechst dye stain and EdU (5-ethynyl-2-deoxyuridine) ("Click-It EdU", Invitrogen, USA) to evaluate cell cycle parameters in HCT116, LS174T and HEK293T. The analysis was performed as outlined previously [9].

Cell death analysis

The apoptotic, necrotic and viable cell populations of the transfected HCT116, LS174T and HHEK293T were determined by DiOC6 (3, 3′-dihexyloxacarbocyanine iodide) (Invitrogen, USA), counter-stained with Propidium Iodide (PI) as previously described [9].

Data from the flow cytometer were also analysed using Windows Multiple Document Interface Software version 2.9.

Statistical analyses

Statistical analysis was done using the online available tool http://faculty.vassar.edu/lowry/VassarStats.html. The probability (P) values were then estimated for the significance of the difference between the test and control. Differences were considered to be statistically significant at $P < 0.05$ (*), highly significant at $P < 0.001$ (**), very highly significant at $P < 0.0001$ (***).

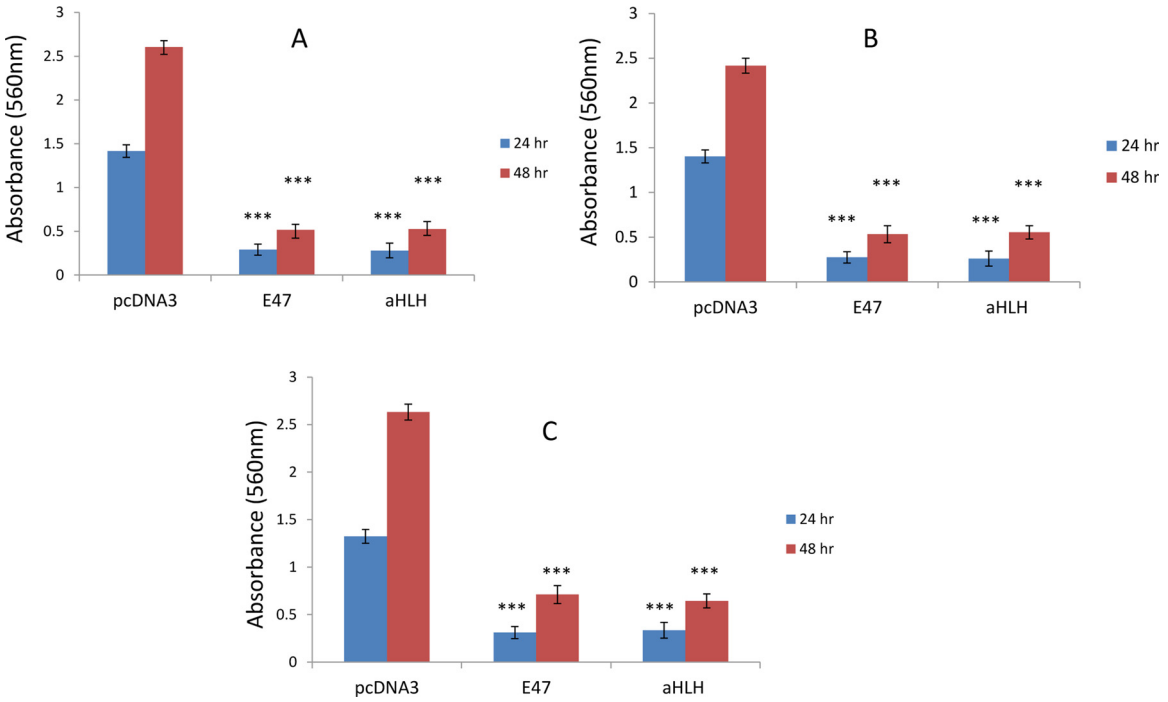

Fig. 1. Cell viability analysis with MTT assay showing relative viable cell numbers at 24 and 48 h post-transfection on HEK293T cells (A), LS174T cells (B) and HCT116 cells (C) transfected with either negative control vector (pcDNA3) or E47 (positive control) and aHLH. The corresponding absorbance values when cells were incubated in MTT assay for two hours and solublised in DMSO after 24 and 48 h of transfection were measured at 560 nm. Mean absorbance ± S.E.M of 4 separate/independent samples respectively for each transfection type.

Results

aHLH and E47 reduce viability in HEK293T, LS174T and HCT116 cells

An Id protein antagonist (aHLH) and E47 were expressed in 8×10^4/well each of HEK293T, LS174T and HCT116 cells seeded in 24-well plates. MTT assay was performed on the cells at 24 and 48 hour post-transfection. The absorbance values were then measured spectrophotometrically. Results showed a significant reduction in the number of viable HEK293T, LS174T and HCT116 cells transfected with aHLH at both 24 and 48 h post-transfection. Similar results were observed with the E47 transfectants (Fig. 1).

aHLH and E47 suppress proliferation of HEK293T, LS174T and HCT116 cells

Cell cycle analysis was done to investigate the effects of Id protein antagonist (aHLH) and the E47 protein on growth of HEK293T, LS174 and HCT116 at 48 h post transfection. This was also to confirm if the observations in section 3.1 (Fig. 1) could be attributed to the suppression of S-phase cells. 8×10^4/well of each cell type was plated in 12-well plates and transfected with either negative control pcDNA3 or aHLH and E47. Results showed growth arrest by the aHLH and E47 proteins in HEK293T LS174T and HCT116 cells (Fig. 2).

aHLH and E47 induce apoptosis in HEK293T, LS174T and HCT116 cells

Earlier observations made in sections 3.1 revealed that aHLH and E47 protein significantly reduce HEK293T, LS174T and HCT116 viability (Fig. 1). Next was to investigate whether the reduction in populations of viable HEK293T, LS174T and HCT116 cells with MTT could also be attributed to cell death. An independent cell death marker DiOC6 (3, 3′-dihexyloxacarbocyanine iodide), counter-stained with Propidium Iodide (PI) was used to evaluate the populations of apoptotic and necrotic cells at 24 and 48 h post-transfection. The results showed that aHLH and the E47 protein significantly induced apoptosis at both 24 and 48 hour post-transfection (Fig. 3).

Discussion

This study explored the consequences of loss-of-function of Id protein on its pro-apoptotic functions in different cellular contexts by selective ablation with Id protein antagonist (aHLH) on) immortalised (highly tumorigenic) transformed human

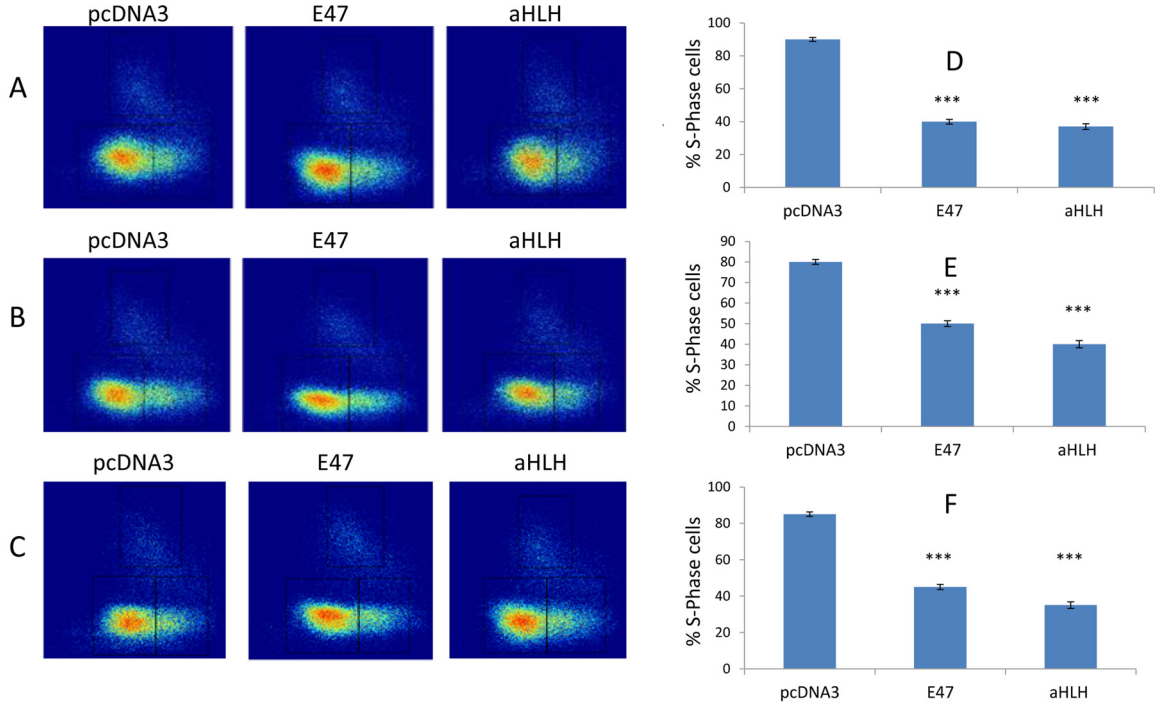

Fig. 2. Cell cycle analysis showing cell cycle parameters (G_1, S and G2/M) in HEK293T (A), LS174T (B) and HCT116 (C) transfected with either negative control vector (pcDNA3) or E47 (positive control) and aHLH proteins using Saint 18 reagent. Cells were pulsed for EdU S-phase detection at 48 h post-transfection. D: Percentage S-phase cells in HEK293T cell population. E: Percentage S-phase cells in LS174T cell population. F: Percentage S-phase cells in HCT116 cell population. Digital images were taken at X10 magnification with BX-41 microscope for automated image analysis using CellProfiler.

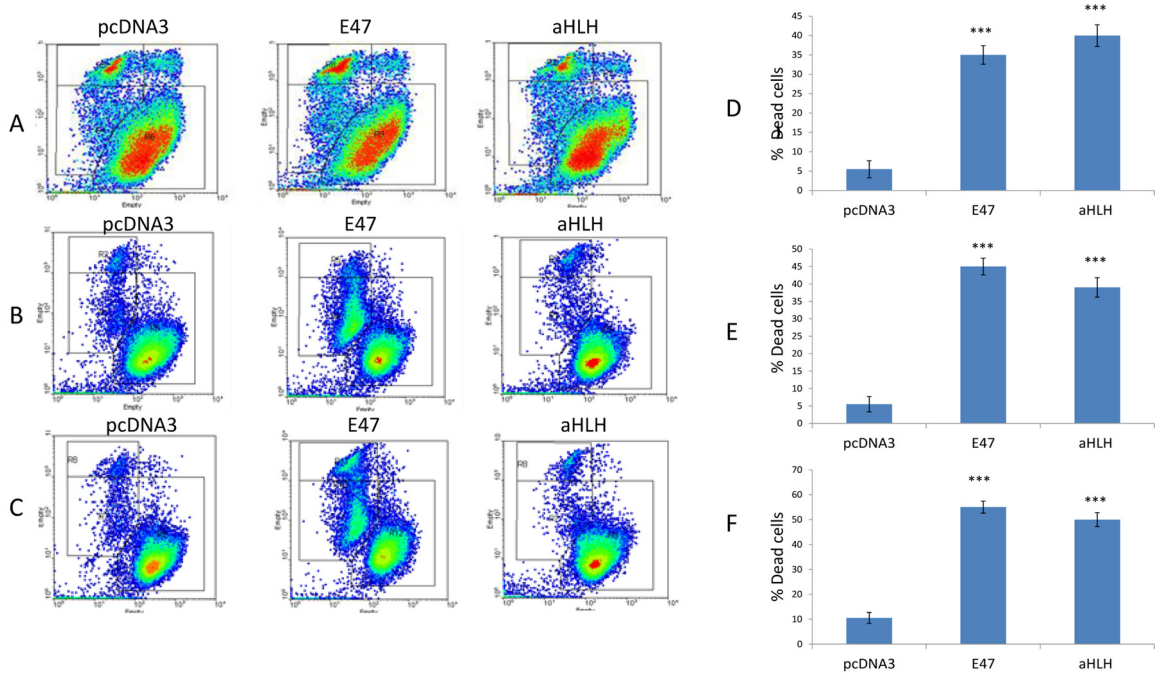

Fig. 3. Flow cytometric analysis of HEK293T cells (A), LS174T cells (B) and HCT116 cells for viable, apoptotic and necrotic cell population using a mitochondrial membrane-potential marker of viable cells (DiOC6) and Propidium Iodide (PI) as a marker of non-viable necrotic cells at 48 h post-transfection. Cells were transfected with either negative control vector (pcDNA3), E47 (positive control) or aHLH using Saint 18 reagent. Cells were incubated with DiOC6 at 50 nM for 15 min. Cells were counter-stained with PI at 2.5 mg/ml on ice and processed through the flow. D: Percentage apoptotic populations in HEK293T cells. E: Percentage apoptotic populations in LS174T cells. F: Percentage apoptotic populations in HCT116 cells.

embryonic kidney cells (HEK293T), human epithelial colon adenocarcinoma cell line (LS174T) and human epithelial colon carcinoma cell line (HCT116).

MTT assay showed a significant reduction in the number of viable HEK293T cells when transfected with aHLH at both 24 and 48 h post-transfection (Fig. 1A). Similar effects of aHLH on LS174T and HCT116 cells were observed with the antagonist reducing the populations of viable cells after 24 and 48 h post-transfection (Fig. 1B and 1C).

Cell cycle analysis (Fig. 2D, 2E and 2F) to determine the effects of aHLH on S-phase cells in all three cell lines (HEK293T, LS174T and HCT116) showed the arrest of S-phase cells. The above findings suggest that the reduction in viable HEK293T, LS174T and HCT116 numbers when transiently expressed with aHLH may be due to suppression of growth. Research has shown that the oncogenic properties of Id genes enhance cell proliferation like the tumor suppressor characteristics of some bHLH proteins which are antagonised by Id proteins [1]. This is exemplified by the deregulation of bHLH such as E47 in cell lines which results in the suppression of growth by inducing cell cycle arrest in G1 phase [11].

When the study was extended to further investigate whether the reduction in cell viability was also mediated through apoptosis with an independent apoptotic marker (DiOC6), the results showed that aHLH induced apoptosis in HEK293T, LS174T and HCT116 cells (Fig. 3D, 3E and 3F respectively). The aHLH gene therefore exhibits a growth suppressive as well as pro-apoptotic effects on transformed human embryonic kidney cells (HEK293T), human epithelial colon carcinoma cell line (HCT116) and human epithelial colon adenocarcinoma cell line (LS174T). Evidence available suggests that a mutant version of E47 (aHLH) which is defective in DNA binding functions as well as transactivation suppresses growth in human adenocarcinoma cell lines [8]. In addition, the E47 protein was also found to arrest growth in all three cell lines.

As Id proteins act as positive regulators of cell growth, they are also actively involved in cell cycle progression. It is now evident that Id proteins play essential role in cell proliferation and cell cycle control *in vivo* from *Drosophila* sp. and mice models [1]. Id expression is maintained throughout G1 phase of the cell cycle and upregulated as the cells progress to the S-phase following mitogenic stimulation [3,12]. They control cell cycle by regulating the transcription of several target genes and also by direct association with non-bHLH proteins.

The Id protein antagonist (aHLH) is a classic example of the E47 protein mutant. The E47 protein which is an E2A protein is well-known to exhibit growth suppressive effect. The E2A-encodes the E47 bHLH transcriptional regulator [11] whose functions are antagonized through hetero-dimerization with Id proteins, are involved in mediating a G1 cell cycle arrest when ectopically over-expressed in cell line models. The pro-growth abilities of an Id protein involves the inhibition of E2A bHLH-regulated expression of the gene encoding the cyclin dependent kinase (CDK) inhibitor $p21^{Cip1/Waf\ 1}$ and this results in pRB phosphorylation. Consequently the phosphorylation of pRB causes its dissociation from E2F-DPI complexes into S phase [13].

The findings of this work clearly support the fact that the aHLH protein suppresses growth in human cancer cell lines as observed in our immortalized (highly tumorigenic) transformed human embryonic kidney cells (HEK293T), human epithelial colon adenocarcinoma cell line (LS174T) and human epithelial colon carcinoma cell line (HCT116). As a result, the aHLH protein could serve as an anti-cancer agent to suppress the growth of cancerous cells, thereby providing an avenue for the treatment of cancers.

Conclusion

This study investigated the effects of the Id protein antagonist aHLH on growth and on pro-apoptotic functions *in vitro* using HEK293T, LS174T and HCT116. The reduction in viable cell populations of HEK293T, LS174T and HCT116 cells when ectopically overexpressed with aHLH was due to the arrest of S-phase cells and cell death. The above findings on the role of aHLH in cell growth and survival could help manipulate their functions as targets for developing anti-cancer agents and could also serve as molecular markers of human cancers.

Data availability

All data used to support the results of this research are available from Foster Kyei upon request.

Author's contribution

FK conceived of and designed the study, performed the experiment and research, analyzed the data and wrote the manuscript.

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Declaration of competing interest

The author declares that there is no conflict of interest

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