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Alpha2A adrenoreceptor influence on intestinal epithelial stem cell proliferation

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ABSTRACT

The intestinal epithelium is critical for nutrient absorption, immune function, and hormone release. The mature cell types that are responsible for these functions are produced by crypt base columnar (CBC) stem cells located within the intestinal crypts. These stem cells constitutively divide to produce complete turnover of the entire intestinal epithelial layer every few days. The division of the CBC stem cells can be influenced by a variety of factors, including nutrient availability. There is research that suggests the sympathetic nervous system can contribute to changes in stem cell proliferation. However, it is not known whether this effect is direct via the CBC stem cells, or instead through an indirect mechanism. Thus, we tested whether CBC stem cells express alpha$_{2A}$ adrenoreceptors (α_{2A}-ARs), a receptor subtype utilized by norepinephrine (NE; the primary neurotransmitter of the SNS) and whether NE induces proliferation of intestinal epithelial organoids *in vitro*. Results showed that α_{2A}-ARs mRNA is expressed in CBC stem cells and that there is a trend towards higher expression of α_{2A}-AR mRNA in CBC stem cells compared with other cells of the crypt. We also found a significant decrease in proliferation after application of NE to organoids *in vitro* ($p<.05$). These data support a role for the SNS in regulation of CBC stem cells proliferation, which may influence intestinal epithelial size and function as a whole.

INTRODUCTION

The intestinal epithelium is a single layer of cells that form the inner lining of the small intestine. The mature cells of the intestinal epithelium absorb nutrients (enterocytes), secrete hormones (enteroendocrine cells), and secrete mucus to create an immune barrier (goblet cells) (Denis et al. 2000). To replace these mature cells as they die off, crypt base columnar (CBC) intestinal epithelial stem cells located in the base of the crypt divide constitutively to produce daughter cells of each cell type. The newly produced daughter cells, which are progenitor cells that are referred to as transit amplifying (TA) cells, differentiate and continue to divide as they migrate up the crypt. As these cells reach maturity, they move into the villi. When the cells reach the apex of the villus, they undergo apoptosis and are sloughed off into the lumen of the intestine. Via this process, complete turnover of the entire intestinal epithelial layer occurs every three to five days (Clevers et al. 2009). Consequently, proper CBC stem cell proliferation is critical to maintain the morphology and function of the entire intestinal epithelium.

The sympathetic nervous system (SNS), one of two branches of the autonomic nervous system (ANS), innervates the intestinal epithelium. Data has shown that the SNS may play a role in CBC stem cell proliferation. Specifically, our laboratory has found

that surgical sympathectomy increases intestinal epithelial crypt cell proliferation in rats (Davis et al. 2016). Others have also documented changes in crypt cell proliferation after partial ablation of the SNS nerves innervating the small intestine (Tutton & Helme 1974). Despite these data demonstrating that the SNS is involved in regulating proliferation of crypt cells, it is not known if the SNS directly influences proliferation of the CBC stem cells themselves. The alpha$_{2A}$ adrenoreceptor (α_{2A}-AR), a receptor subtype that binds the primary SNS neurotransmitter norepinephrine (NE) (Jacobowitz 1965), has been localized within the intestinal epithelium, including in the crypt cells (Laburthe et al. 1990). It is not known, though, whether this receptor is expressed by the CBC stem cells, providing a molecular mechanism for direct SNS influence. Thus, we hypothesized that: 1) α_{2A}-AR mRNA is expressed in CBC stem cells and 2) application of NE to intestinal epithelial organoids will alter proliferation rate of intestinal epithelial cells compared to untreated controls. In order to test these hypotheses, we isolated CBC intestinal epithelial stem cells from mice and measured the gene expression of α_{2A}-AR. We compared these mRNA levels of other crypt cells from the same mice in order to evaluate expression of the α_{2A}-AR between intestinal epithelial cell populations. In a separate experiment, crypts were isolated from mice and grown into functioning intestinal epithelial organoids in order to test the proliferation of intestinal epithelial crypt cells in response to NE application. Together, these experiments evaluated SNS influence on intestinal epithelial stem cell proliferation, which contributes to current knowledge of neural control of stem cells.

MATERIALS AND METHODS

Animal housing

Lgr5-GFP (B6.129P2-Lgr5$^{tm1(cre/ERT2)Cle/J}$; The Jackson Laboratory) and C57BL/6J mice were single-housed in shoebox cages. Animals were maintained on a 12:12 light:dark cycle with ad libitum access to food (Teklad 22/5 Rodent Diet 8640 (Envigo, Madison, WI) and tap water. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Illinois Urbana-Champaign.

Crypt isolation

Small intestinal crypts were isolated from adult Lgr5-GFP and wild-type mice using a previously published protocol (Sato & Clevers 2013). Briefly, mice were sacrificed using decapitation under isoflurane (Phoenix, St. Joseph, MO). An incision was made at the base of the abdomen and extended up the sides to open the body cavity. The small intestine was located and removed, cutting at the level of the pyloric sphincter and the ileocecal sphincter. The small intestine was then cut into 10cm-long segments. An 18-gauge needle and syringe were used to flush the intestinal segments with ice-cold 1X phosphate-buffered saline (PBS) (Lonza, Walkersville, MD). The segments were then cut open longitudinally and scraped with glass coverslips to remove the villi. Sections were further cut into 2-4 mm fragments. The fragments were placed in a 1X PBS-filled 50 mL Falcon tube and washed by inverting the tube at least 10 times. The PBS was then removed and 30 mL Crypt Isolation Buffer (Promega, Madison, WI) was added to each tube and rocked for 30 minutes at 4˚C. Samples were washed again with 1X PBS and then passed through a 70-µm cell strainer (Corning, Tewksbury, MA) and collected into 1% BSA-coated (Sigma-Aldrich, St. Louis, MO) 50 mL Falcon tube. Samples were centrifuged at 300 x *g* for 10 minutes at 4°C.

Single cell dissociation

After centrifugation, samples were re-suspended with single cell dissociation medium prepared as described in a previously published protocol (Sato & Clevers 2013). The samples were passed through a 40-µm-cell strainer followed by a 20-µm cell strainer (PluriSelect, Leipzig, Germany). The cells were re-suspended in basal culture medium as described in a previously published primary cell culture protocol (Sato & Clevers 2013). The samples were centrifuged 2x's at 300 x *g* for 5 minutes, and then re-suspended with single cell dissociation medium.

Flow cytometry

The isolated crypt cells were prepared for flow cytometry and FACS as described in a previously published protocol (Sato & Clevers 2013). Briefly, dead cells were excluded using propidium iodide and intestinal crypt cell populations were sorted using a BD FACS ARIA II. Results were analyzed using a BD LSR II. The analyzed cells had a green fluorescent protein (GFP) tag on the Lgr5 receptor, which are highly expressed in CBC stem cells. Samples were sorted into two populations: high GFP expression (CBC stem cells) and low GFP expression (crypt cells

other than CBC stem cells, including the Paneth and transit amplifying cells).

RNA isolation

RNA was extracted from each isolated cell population using the commercially available RNeasy Mini Kit (Qiagen, Valencia, CA). The RNA concentration was measured using a Nanodrop ND-1000 Spectrophotometer.

cDNA synthesis

The isolated RNA was amplified using a commercially available iScript cDNA Synthesis Kit following manufacturer's instructions (Bio-Rad, Hercules, CA). Samples were incubated in the PTC-100 Programmable Thermal Controller for 5 minutes at 25˚C, 30 minutes at 42˚C, then 5 minutes at 85˚C.

Quantitative real-time PCR (qRT-PCR)

Alpha$_{2A}$-AR mRNA expression was quantified by quantitative real time-PCR per manufacturer's instructions of the commercially available Taqman kit (Applied Systems, Foster City, CA). PCR Reaction Mix was made using Taqman Universal PCR Master Mix, Taqman Gene Expression Assay, and cDNA template + H_2O. Expression of the α_{2A}-AR was determined by the TaqMan probe and measured in the Applied Biosystems 7900HT Fast Real-Time PCR System. The expression of GAPDH was utilized a control. Each cell population was plated in triplicate. The following Taqman probes were used: A2AR: Mm00845383_S1 and GAPDH: Mm99999915_g1.

Organoid growth

A wild-type mouse was sacrificed and intestinal epithelial crypts were isolated using the procedures previously described. After crypts were centrifuged for the final time, the supernatant was removed and crypts were re-suspended in Matrigel (Corning Inc., Corning, NY) and plated onto a pre-warmed 24-well plate. Matrigel-crypt mix was applied to the center of each well and then allowed to solidify for 10 minutes in a 37˚C incubator. Once the Matrigel solidified, 500 µL of IntestiCult Organoid Growth Medium (StemCell Technologies, Cambridge, MA) was added per well. Media were changed every 3 days and cells were split after 7 days.

Organoid treatment with norepinephrine

After ten days of growth, organoids received one of three treatments: 1 µM norepinephrine, 10 µM norepinephrine, or a sterile deionized water vehicle control. The drugs were made from norepinephrine bitartrate salt (Sigma Aldrich, St. Louis, MO) dissolved in deionized water. At 0h, 2h, 4h and 6h after administration of the respective drug treatment; proliferation was assayed using the CyQUANT NF Cell Proliferation Assay Kit (Thermo-Fischer, Austin, TX). One hour before the desired time point, a dye binding solution was added in order to bind the cellular DNA. The fluorescence intensity of each of the samples was measured using a Monochromator based absorbance 96 and 384-well plate reader with excitation at ~485 nm and emission detection at ~530 nm. The higher the intensity of the fluorescence, the more DNA is bound indicating higher numbers of cells.

Data analysis

Results were expressed as mean ± SEM. Gene expression for α_{2A}-AR was analyzed using Student's T-test using Number Crunching Statistical Software (NCSS). Proliferation was analyzed using one-way analyses of variance (ANOVA) using NCSS. Each time point of the experiment was analyzed independently and not compared across time points because different cells are used at each time point. Tukey-Kramer post hoc analysis was utilized where appropriate. Differences between treatments were considered statistically significant if $p<0.05$.

RESULTS AND DISCUSSION

Alpha$_{2A}$ adrenoreceptor mRNA expression

Alpha$_{2A}$ adrenoreceptor mRNA is expressed in CBC stem cells (Fig. 1). Although there was no significant difference in α_{2A}-AR mRNA between the CBC stem cells and the other crypt cells (not including CBC stem cells), there is a trend toward increased α_{2A}-AR mRNA expression in the CBC stem cells compared to the other crypt cells (Fig. 1; $p = 0.06$).

Further, we also confirmed the expression of alpha$_{2A}$ adrenoreceptor mRNA in a separate population of crypt cells that were not divided into different cell types (Fig. 2).

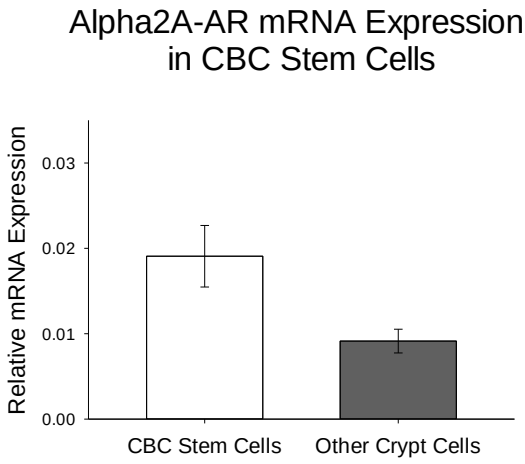

Figure 1: Comparison of α_{2A}-AR relative mRNA expression between CBC stem cells and other crypt cells.

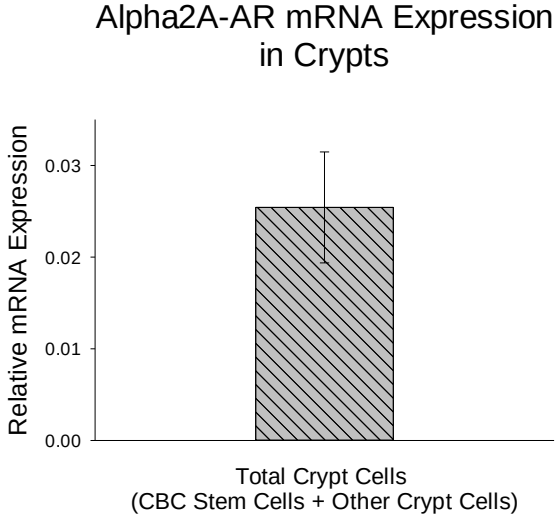

Figure 2: α_{2A}-AR relative mRNA expression in crypt cells.

Proliferative response of organoids to the addition of norepinephrine

Application of NE significantly decreased the proliferation rate of intestinal epithelial organoids at the 2h time point (Fig. 3; $p<.05$). No other time points showed a significant change in the proliferation rate.

Our data revealed that CBC stem cells express α_{2A}-AR mRNA and that intestinal epithelial organoids *in vitro* decrease proliferation in response to NE. Together, these experiments demonstrate a mechanism through which the SNS may be directly influencing intestinal epithelial stem cell proliferation.

When we investigated the expression of α_{2A}-AR mRNA in all crypt cells, we found greater expression of α_{2A}-AR in the stem cells compared with other crypt cells. This finding suggests that the SNS may have a greater influence on the stem cells through this receptor when compared with other crypt cells. Further, a low (1μM) dose of NE decreased proliferation of organoids 2h after application. These data suggest that an increase in SNS influence may lead to a decrease in proliferation rate in the intestinal epithelial cells, perhaps through α_{2A}-AR signaling. Since α_{2A}-ARs are typically defined as inhibitory receptors, activating this receptor with NE would likely suppress proliferation. This idea is consistent with our results showing that intestinal epithelial organoids decrease proliferation in response to NE.

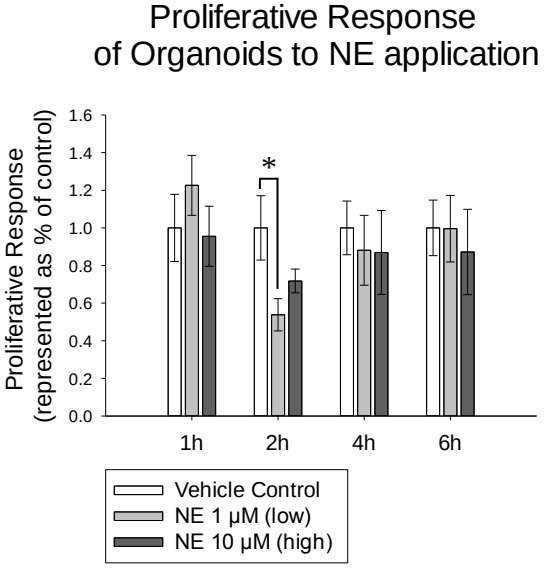

Figure 3: Proliferative response of organoids to the addition of either a 1 μM (low) or 10 μM (high) dose of norepinephrine.

In a separate study, our laboratory has also demonstrated that the *in vivo* denervation of the SNS in rats leads to an increase in intestinal epithelial cell proliferation rate (Davis et al. 2016). This finding complements the present *in vitro* data from the current study because it suggests that decrease in SNS influence leads to an increase in proliferation of intestinal epithelial crypt cells.

Our data reveal interesting trends with respect to the time points and concentration of NE application. After addition of NE to intestinal epithelial organoids, we see a significant decrease in proliferation at the 2h time point. However, there was no significant difference in proliferation at the 4h or 6h time points.

It can be inferred that a significant decrease in proliferation was seen at the 2h time point and not the 4h or 6h time points because neurotransmitters have a short reaction time (Scholmerich et al. 2006). Although normally this reaction time is within seconds of NE application, it takes time for an effect to take place. Signal transduction and activation of cellular pathways involved in decreasing cell proliferation take time to act. Additionally, the time for cell number to significantly decrease requires time. Normally, the intestinal epithelial tissue compensates for cell loss by proliferating, thus the number of cells should remain the same. This trend is displayed in Figure 3, with each of the control populations remaining around a similar value. At the 4h and 6h time points, the organoids have had enough time to recover population size after the decrease in proliferation. This is demonstrated by the lack of significant differences in organoid cell number at these time points. In order to further explore the duration of NE's effect on organoid proliferation, shorter time points can be investigated in future experiments.

Another interesting finding showed that only the application of the 1 μM concentration of NE lead to a significant decrease in proliferation, whereas the 10 μM concentration of NE did not. This may be explained by the fact that there are only a limited number of receptors available for the NE to bind, meaning that a higher concentration of NE does not necessarily yield a larger suppression of proliferation. This trend can be further explored with more trials that utilize multiple different concentrations of NE.

We would like to continue to pursue research on SNS influence of CBC stem cell proliferation by studying intracellular signaling downstream of alpha$_{2A}$ adrenoreceptor activation. The α_{2A}-AR is a G-protein receptor that has been linked to the inhibition of cAMP production (Denis et al. 2000). Inhibition of cAMP production can in turn alter the MAPK pathway, which is linked to regulation of proliferation (Denis et al. 2000). By understanding the molecular mechanism underlying our data showing changes in proliferation rate, it may be possible to manipulate the pathway in order to alter CBC stem cell proliferation for therapeutic purposes.

CONCLUSIONS

Our data support a role for direct neural control of intestinal epithelial stem cells, a topic which has not yet been extensively explored in existing scientific literature. By continuing to evaluate the effects of the SNS on CBC stem cell proliferation, we can better understand how changes in neural function can alter our intestine, further establishing the gut-brain axis.

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