EXPERIMENTAL CELL RESEARCH 328 (2014) 186–196

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/yexcr

Research Article

# Xanthosine administration does not affect the proportion of epithelial stem cells in bovine mammary tissue, but has a latent negative effect on cell proliferation

*Gat Rauner*[a,b], *Itamar Barash*[a,*]

[a]*Institute of Animal Science, ARO, The Volcani Center, P.O. Box 6, Bet-Dagan, 50250, Israel*
[b]*The Robert H. Smith Faculty of Agriculture, Food and Environment, The Hebrew University of Jerusalem, Israel*

ARTICLE INFORMATION

*Article Chronology:*
Received 27 April 2014
Received in revised form
1 June 2014
Accepted 22 June 2014
Available online 30 June 2014

*Keywords:*
Stem cell
Mammary gland
Bovine
Xanthosine
IMPDH

ABSTRACT

The challenge in manipulating the proportion of somatic stem cells lies in having to override tissue homeostasis. Xanthosine infusion via the teat canal has been reported to augment the number of label-retaining cells in the mammary gland of 3-month-old bovine calves. To further delineate xanthosine's effect on defined stem cells in the mammary gland of heifers—which are candidates for increased prospective milk production following such manipulation—bovine mammary parenchymal tissue was transplanted and integrated into the cleared mammary fat pad of immunodeficient mice. Xanthosine administration for 14 days did not affect the number of label-retaining cells after 10- and 11-week chases. No change in stem cell proportion, analyzed according to CD49f and CD24 expression, was noted. Clone formation and propagation rate of cultured cells, as well as expression of stem cell markers, were also unaffected. In contrast, a latent 50% decrease in bovine mammary cell proliferation rate was observed 11 weeks after xanthosine administration. Tumor development in mice was also limited by xanthosine administration. These effects may have resulted from an initial decrease in expression of the rate-limiting enzyme in guanine synthesis, IMPDH. The data indicate that caution should be exerted when considering xanthosine for stem cell manipulation.



## Introduction

Postnatal mammary gland development progresses toward a functional morphology for the synthesis and transfer of nutrients to newborns. A rudimentary ductal network is established during sexual maturation, and regulated synchronization with embryo growth during pregnancy secures the generation of a dynamic architecture for successful lactation [1,2]. The expansion and branching of the ductal network, and the development of pregnancy-induced lobuloalveolar structures and then milk-secreting alveoli, are supported by epithelial cell hierarchy. Two concepts have been established for the initiation of cell hierarchy in the adult mouse mammary gland, which

*Abbreviations*: LREC, label-retaining epithelial cell

*Corresponding author. at: Institute of Animal Science, ARO, The Volcani Center, P.O.Box 6, Bet-Dagan 50250, Israel. Fax: +972 8 0475075.
E-mail addresses: gat.rauner@mail.huji.ac.il (G. Rauner), itamar.barash@mail.huji.ac.il (I. Barash).

http://dx.doi.org/10.1016/j.yexcr.2014.06.017
0014-4827/

differ with respect to the presence of bipotent versus unipotent stem cells at the top of the hierarchy ([3,4] and reviewed in [5]). This discrepancy was recently reconciled by clonal cell-fate mapping [6], which suggests that adult mammary gland contains bipotent mammary stem cells as well as distinct long-lived unipotent progenitor cells generated from the stem cells by asymmetric division. Recent studies have also defined a heterogeneity among stem cells, which comprise long-term and short-term repopulating cells [7], as well as slow-cycling stem cells [8]. Together, these studies have added a level of complexity to the initial basal, $ER^-/PR^-$ characteristics of mammary stem cells [9] that have been shown capable of generating an entire functional gland upon transplantation [10,11], and are indirectly regulated by progesterone levels [12] as well as by the Notch [13,14], Hedgehog [15] and Wnt [9,16] pathways. This relatively high resolution of stem-cell characteristics was established in the mouse mammary gland. It was only partly recapitulated in human studies, and to a lesser extent in the mammary gland of farm animals [17,18]. Nevertheless, altering bovine mammary gland production capabilities via cellular manipulation is highly desirable [19], and successful attempts may be applicable for tissue-regeneration studies in humans [20,21].

In an attempt to manipulate bovine mammary cell composition toward a higher rate of stem cells, Capuco et al. [22] applied a methodology reported to augment symmetric cell division in cultures of adult hepatic stem cell lines [23]. Administration of the purine nucleoside xanthosine to these cultures circumvented p53-induced repression of IMPDH, a rate-limiting enzyme in the guanine-synthesis pathway, and shifted the liver cells from p53-dependent asymmetric cell division to symmetric cell division. Consequently, in bovines, xanthosine was infused via the teat canal into the mammary gland of 3-month-old female calves for 5 consecutive days [22] and a twofold induction in the number of 5-bromo-2′-deoxyuridine (BrdU)-label-retaining cells after a 40-day chase was reported. Label-retaining cells maintained the suggested characteristic of somatic stem cells, i.e. selective segregation of their template DNA strands to themselves during division, and passing the newly synthesized chromatids to their more differentiated daughters during asymmetric divisions [24,25]. However, label-retaining cells constitute a mixed population in the mammary gland [26], and doubts have been recently raised concerning their compatibility with stem cells which, in the small intestine, segregate their chromosomes randomly [27].

The current study was devoted to further delineating the long-term effect of xanthosine on mammary cell populations and its ability to override homeostasis. A “bovinized” mouse model was established, and cells were subjected to recently established cell-sorting methodology and analysis of lineage-commitment parameters of bovine mammary epithelial cells [28]. We report that xanthosine administration did not affect the number of label-retaining epithelial cells (LRECs) in heifer mammary implants. No long-term effect was observed on either the proportion or activity of the epithelial cell population, including stem cells. However, a significant decrease in cell proliferation was observed 11 weeks after xanthosine injection.

## Materials and methods

### Mice

FVB/N mice were housed under a 12-h light/dark cycle and given *ad libitum* access to food and water. After tumor-cell injections, they were inspected for palpable tumor development twice a week. NOD-SCID mice were purchased from Harlan Laboratories (Jerusalem, Israel). They were kept in sterilized cages under a 12-h light/dark cycle and supplemented with sterile water and irradiated food *ad libitum*. For all surgical procedures, mice were anesthetized with isoflurane (Abbott Laboratories, Maidenhead, England) mixed with $O_2$ using a veterinary anesthesia machine. All animals used in this study were treated humanely. Study protocols were in compliance with the regulations of the Israeli Ministry of Health and local institution policies (approval no. IL-202/09).

### Bovine mammary tissue transplantation and xanthosine administration

Mammary biopsies were harvested from the parenchymatic region of the udder of individual 7- to 10-month-old Holstein heifers that had been commercially slaughtered. Freshly isolated tissue pieces of approximately 3 $mm^3$ (i.e. implants) were transplanted bilaterally into the ♯4 mammary fat pads of 3-week-old NOD-SCID female mice cleared of their endogenous epithelium [29,30]. A single implant was transplanted per fat pad. After a 7-day establishment period in the mouse fat pad, xanthosine administration was initiated. Each mouse received 14 consecutive daily doses of 9.6 mg xanthosine dehydrate (Sigma, St. Louis, MO) dissolved in 300 μL PBS pH 10 by IP injection. Xanthosine was not administered to control animals, which instead received IP injections of PBS adjusted to pH 10. Transplantation of bovine implants into NOD-SCID mice was performed in five individual experiments with bovine tissue excised from different heifers. Xanthosine treatment was applied in all but the first experiment, which was designed to follow the dynamics of BrdU-label retention by the implanted cells.

### Histological analysis and immunostaining

After excision from the mouse fat pad, bovine implants were dissected into pieces of approximately 1 $mm^3$, fixed in Bouin's solution, dehydrated in a graded ethanol series (50% to 100%), cleared in xylene and embedded in paraffin. Paraffin sections (5 μm) were stained with hematoxylin and eosin (H&E; Sigma) to visualize the morphology of the epithelial structures within the stroma. For immunofluorescence analyses, the paraffin sections were boiled in 0.01 M citrate buffer for 10 min and incubated with the primary antibody overnight at 4 °C. Tissue sections were then incubated with a secondary antibody for 1 h at room temperature. Nuclei were stained with DAPI (10 μg/mL, Santa Cruz Biotechnology, Santa Cruz, CA). The antibodies and their dilutions are listed in Table 1. Stained sections were visualized and photographed under an inverted fluorescence microscope (Eclipse Ti, Nikon Instruments, Melville, NY) equipped with NIS-Elements AR 3.2 imaging software (Nikon Instruments).

### Long-term BrdU-label retention

For BrdU chase analysis of LRECs, mice received 14 consecutive daily doses of 720 μg BrdU (Sigma) dissolved in 300 μL PBS by IP injection. The procedure was begun 1 week after bovine implant transplantation and was performed simultaneously with the xanthosine treatment. LRECs in the bovine implants were monitored for up