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Regulation of epithelial cell turnover and macrophage phenotype by epithelial cell-derived transforming growth factor beta1 in the mammary gland

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ABSTRACT

Transforming growth factor beta1 (TGFB1) is a multi-functional cytokine that regulates cell proliferation, apoptosis and immune system responses. In the breast, the mammary epithelium is the primary source of TGFB1 and increased expression is associated with increased breast cancer risk. This study was conducted to investigate the roles of epithelial cell-derived TGFB1 in regulation of epithelial cell activity and macrophage phenotype in the mammary gland. *Tgfb1* null mutant and wildtype mammary epithelium was transplanted into contra-lateral sides of the cleared mammary gland of TGFB1 replete scid mice. Transplanted tissue was analysed for markers of proliferation and apoptosis to determine the effect of *Tgfb1* null mutation on epithelial cell turnover, and was analysed by immunohistochemistry to investigate the location, abundance and phenotype of macrophages. The number of proliferating and dying ductal epithelial cells, determined by BrdU and TUNEL, was increased by 35% and 3.3-fold respectively in mammary gland transplanted with *Tgfb1* null epithelium compared to wildtype epithelium ($p < 0.05$). Abundance of F4/80+ macrophages in between *Tgfb1* null epithelial cells compared to wildtype epithelial cells was increased by 50%. The number of iNOS+ and CCR7+ cells in the stroma surrounding *Tgfb1* null alveolar epithelium was increased by 78% and 2-fold respectively, and dendriform MHC class II+ cells within ductal epithelium were decreased by 30%. We conclude that epithelial cell-derived TGFB1 in the mammary gland has two functions: (1) regulation of cellular turnover of epithelial cells, and (2) regulation of local macrophage phenotype. These findings shed new light on the diversity of roles of TGFB1 in the mammary gland which are likely to impact on breast cancer risk.

1. Introduction

Transforming growth factor beta1 (TGFB1) is a multifunctional cytokine that controls many aspects of cellular function, including proliferation, differentiation, migration, apoptosis, and the immune response [1–3]. A strong association between breast cancer risk and the TGFB1 L10P gene polymorphism has been reported by the Breast Cancer Association Consortium [4,5]. The TGFB1 L10P gene is linked to both increased cellular expression of TGFB1 and elevated circulating TGFB1 [5,6] suggesting that TGFB1 expression is an important determinant of breast cancer risk.

TGFB1 has both stimulatory and inhibitory roles in regulating tissue homeostasis, development, remodelling, and cancer progression [3,7]. TGFB1 acts as a tumour suppressor in the early phase of cancer development, and promotes invasion and metastasis during the later stages of cancer progression [7]. TGFB1 can suppress tumour development through inhibition of cell proliferation, induction of apoptosis, and suppression of growth factor, cytokine and chemokine production [8,9]. However, as tumours progress, resistance to TGFB1 is acquired through mutations or inhibition of TGFB1 signalling pathways. At this time, tumour cells begin to secrete large quantities of TGFB1 which appears to further promote tumour progression [7,10]. This surge in TGFB1 production by tumour cells mediates epithelial-mesenchymal transition, increased angiogenesis and impairs immune surveillance, thereby promoting tumour invasion and metastasis [7–10].

TGFB1 is produced by many cell types. It is secreted as an inactive latent complex (LTGFB1) consisting of the active mature TGFB1 dimer non-covalently bound to a latency associated peptide (LAP) [11–13]. This large complex is associated with latent TGFB binding protein (LTBP) [12]. Active TGFB1 has a half life of 2 min, whereas TGFB1 associated with its latent complex is substantially more stable, with a half life of 90 min [14]. Activation of latent TGFB1 through heat, acid or alkaline treatment, proteolysis or irradiation [11,12,15,16] is necessary before TGFB1 can exert

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biological effects [11]. Therefore, activation of TGFB1 from the latent form is a key regulatory event that controls its biological availability [13,14,17].

Latent TGFB1 is expressed by mammary epithelium during puberty, in cycling virgin mice, and during pregnancy [17–19]. The highest expression of both latent and active TGFB1 is observed at the diestrus phase of the ovarian cycle [17]. TGFB1 has a largely inhibitory role in regulating mammary gland epithelial cell proliferation and ductal development. Exogenous TGFB1 released from pellets implanted into the developing mammary gland causes cessation of DNA synthesis in the highly proliferative terminal end buds, which results in complete inhibition of ductal growth. Transgenic mice expressing constitutive active TGFB1 exhibit significant retardation in ductal development [20,21]. On the other hand, transgenic mice carrying a dominant negative receptor in the mammary epithelium exhibit mammary epithelial hyperplasia together with inappropriate alveolar development [22] and delayed epithelial cell death during involution [23]. Accelerated ductal development during puberty is observed in heterozygous *Tgfb1* null mutant mice [17], and *Tgfb1* homozygous null mutant epithelium transplanted into wildtype hosts also results in accelerated ductal development [24]. Thus TGFB1 has an important autocrine function in constraining inappropriate epithelial cell proliferation, development and survival, which is likely to affect breast cancer risk. However, as a multi-functional cytokine, it is highly likely that TGFB1 in the mammary gland acts on other cell types within the stroma, which in turn may also influence breast cancer risk, and these effects are yet to be investigated.

Key target cells for TGFB1 action are macrophages, which are bone-marrow derived cells present in the mammary gland stroma that regulate epithelial cell function in normal development. In estrous cycling adult mice, macrophages promote epithelial cell proliferation, alveolar development, phagocytose dying epithelium and promote tissue remodelling [25]. In addition to their function in regulation of epithelial cell turnover during the ovarian cycle, colony-stimulating factor1 (CSF1)-regulated macrophages contribute to development during puberty [26,27], pregnancy [28] and the switch to a lactational state [28]. Macrophages have also been shown to support stem cell activity in the mammary gland [29]. In breast cancer, macrophages play multiple roles in metastasis, and promote growth and survival of tumour cells, angiogenesis and cell invasion [8,30].

Tgfb1 and *Csf1* null mutant mice have strikingly similar reproductive phenotypes, including perturbation in mammary gland development and function [31], suggesting the possibility of a mechanistic link between TGFB1 and macrophages in the mammary gland. Furthermore, in established mammary gland tumours, TGFB signalling in the epithelium regulates recruitment of macrophages and expression of inflammatory markers [32]. This study seeks to investigate cross-talk between epithelial cell-derived TGFB1 and macrophages, to better understand the role of TGFB1 in regulation of normal mammary gland function. Through analysis of mammary gland tissue from *Tgfb1* null mutant and wildtype mice transplanted into TGFB1 replete recipients, we have shown that epithelial cell-derived TGFB1 regulates both the rate of epithelial cell turnover, and macrophage phenotype in the mammary gland. These findings provide new information on the diversity of roles of TGFB1 in the mammary gland which potentially contribute to breast cancer risk.

2. Methods and materials

2.1. Animals

All animal experiments were approved by the University of Adelaide Animal Ethics Committee and were conducted in accordance with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (7th ed., 2004). All mice were maintained in specific pathogen-free conditions with controlled light (12 h light, 12 h dark cycle) and temperature at the Laboratory Animal Services Medical School facility.

2.1.1. FVB and BalbC Prkdc^{scid} mice

Female FVB mice and 24 day old, wild-type BalbC *Prkdc*^{scid} female mice were obtained from the Waite campus of the University of Adelaide, South Australia. The diestrus stage of the estrous cycle in FVB mice was determined by daily histological analysis of vaginal smears [33].

2.1.2. Tgfb1 null mutant and wild-type mice

Heterozygous (*Tgfb1*+/−) breeding pairs on a mixed CF1/129/C3H background produced progeny that were homozygous for a targeted null mutation in the *Tgfb1*gene (*Tgfb1*−/−), heterozygous (*Tgfb1*+/−), or homozygous wild type (*Tgfb1*+/+). All mice in the colony are homozygous for the *Prkdc*^{scid} mutation. The *Tgfb1* genotype of each mouse was determined by diagnostic PCR of tail DNA, using the forward primer 5′-GAGAAGAACTGCTGTGCG together with the reverse primers (1) 5′-GTGTCCAGGCTCCAAATATAGG to detect the intact *Tgfb1* gene, or (2) 5′-CTCGTCCTGCAGTTCATTCA, to detect the mutant *Tgfb1* null gene [34].

2.2. Mammary gland transplants

Donor mammary gland tissue was obtained from 8-week-old *Tgfb1*+/+ and *Tgfb1*−/− female mice. The mice were killed and the inguinal pair of mammary glands was dissected. Single tissue fragments (1-mm^3) isolated from the donor mammary glands were rinsed and maintained in ice-cold PBS. Under 2% Avertin anaesthesia, donor mammary gland tissue was transplanted into the cleared fat pad of recipient 24 day old BalbC *Prkdc*^{scid} female mice. To clear the fat pad, the portion of mammary gland from the nipple to the mammary gland lymph node was dissected and removed. In each recipient, donor tissue fragments were inserted into the cleared fat pad, one side of the fat pad was transplanted with *Tgfb1*+/+ mammary tissue; the contra-lateral side of the fat pad was transplanted with *Tgfb1*−/− mammary tissue [24]. The transplants were performed using *Tgfb1*−/− and *Tgfb1*+/+ donors ($n = 3$ per genotype) to generate a total of 20 recipient mice.

Expression of TGFB1 by mammary gland epithelium is highest at diestrus [17], and therefore this stage was selected to analyse the effect of epithelial cell-derived TGFB1 on macrophage function. However, between mouse variation in progesterone secretion at diestrus could confound these experiments, as progesterone also regulates macrophage phenotype and function [25]. In order to standardise the hormonal environment and mimic diestrus, a model employing hormone replacement in ovariectomised mice was utilised. This model has been shown to support mammary gland ductal and alveolar development and mimics the diestrus phase of the ovarian cycle [35], (Hodson and Ingman, in preparation). The mice were ovariectomised (OVX) at the age of 12 weeks (i.e. 9 weeks after transplantation) and allowed to recover for 1 week. The mice were injected daily for 3 days with 17β-estradiol (10 μg/ml in sesame oil) (Sigma, MO, USA) and progesterone (10 mg/ml in sesame oil) (Sigma) administered subcutaneously (OVX + PE) to mimic the diestrus stage of the natural cycle. The OVX + PE mice were killed 1 day after the final hormone injection (diestrus-like stage) and the transplanted mammary glands were dissected. Some mice were injected with 100 μl i.p. of a 10 mg/ml solution of bromodeoxyuridine (BrdU) (Sigma) before they were killed. The mammary glands were either embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen at −80 °C, or fixed in 4% paraformaldehyde and embedded in paraffin.