TGF-beta signalling in bovine mammary gland involution and a comparative assessment of MAC-T and BME-UV1 cells as in vitro models for its study

Charlotte Alexandra Mitz [1] , **Alicia Mercedes Viloria-Petit** Corresp. [1]

[1] Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada

Corresponding Author: Alicia Mercedes Viloria-Petit
Email address: aviloria@uoguelph.ca

The goal of the dairy industry is ultimately to increase lactation persistency, which is the length of time during which peak milk yield is sustained. Lactation persistency is determined by the balance of cell apoptosis and cell proliferation; when the balance is skewed toward the latter, this results in greater persistency. Thus, we can potentially increase milk production in dairy cows through manipulating apoptogenic and antiproliferative cellular signalling that occurs in the bovine mammary gland. Transforming growth factor beta 1 (TGFβ1) is an antiproliferative and apoptogenic cytokine that is upregulated during bovine mammary gland involution. Here we discuss possible applications of TGFβ1 signalling for the purposes of increasing lactation persistency. We also compare the features of MAC-T and BME-UV1 cells, two popular bovine mammary epithelial cell lines, to assess their appropriateness for the study of TGFβ1 signalling. TGFβ1 induces apoptosis and arrests cell growth in BME-UV1 cells, and this was reported to involve suppression of the somatotropic axis. Conversely, there is no proof that exogenous TGFβ1 induces apoptosis of MAC-T cells.In addition to TGFβ1's different effects on apoptosis,hormones and growth factors have distinct effects on TGFβ1 secretion and synthesis in MAC-T and BME-UV1 cell lines. MAC-T and BME-UV1 cells may behave differently in response to TGFβ1 due to their contrasting phenotypes; MAC-T cells have a profile indicative of both myoepithelial and luminal populations, while the BME-UV1 cells exclusively contain a luminal-like profile. Depending on the nature of the research question, the use of these cell lines as models to study TGFβ1 signalling should be carefully tailored to the questions asked.

1 **TGF-beta signalling in bovine mammary gland involution**
2 **and a comparative assessment of MAC-T and BME-UV1**
3 **cells as in vitro models for its study**

4 Charlotte Alexandra Mitz[1] and Alicia Mercedes Viloria-Petit[1]
5 [1] Department of Biomedical Sciences, Ontario Veterinary College, University of Guelph,
6 Guelph, ON, Canada

7 Corresponding Author:
8 Alicia Viloria-Petit
9 Email address: aviloria@uoguelph.ca

ABSTRACT

11   The goal of the dairy industry is ultimately to increase lactation persistency, which is the

12   length of time during which peak milk yield is sustained. Lactation persistency is determined by

13   the balance of cell apoptosis and cell proliferation; when the balance is skewed toward the latter,

14   this results in greater persistency. Thus, we can potentially increase milk production in dairy

15   cows through manipulating apoptogenic and antiproliferative cellular signalling that occurs in

16   the bovine mammary gland. Transforming growth factor beta 1 (TGFβ1) is an antiproliferative

17   and apoptogenic cytokine that is upregulated during bovine mammary gland involution. Here we

18   discuss possible applications of TGFβ1 signalling for the purposes of increasing lactation

19   persistency. We also compare the features of MAC-T and BME-UV1 cells, two popular bovine

20   mammary epithelial cell lines, to assess their appropriateness for the study of TGFβ1 signalling.

21   TGFβ1 induces apoptosis and arrests cell growth in BME-UV1 cells, and this was reported to

22   involve suppression of the somatotropic axis. Conversely, there is no proof that exogenous

23   TGFβ1 induces apoptosis of MAC-T cells. In addition to TGFβ1's different effects on apoptosis,

24   hormones and growth factors have distinct effects on TGFβ1 secretion and synthesis in MAC-T

25   and BME-UV1 cell lines. MAC-T and BME-UV1 cells may behave differently in response to

26   TGFβ1 due to their contrasting phenotypes; MAC-T cells have a profile indicative of both

27   myoepithelial and luminal populations, while the BME-UV1 cells exclusively contain a luminal-

28   like profile. Depending on the nature of the research question, the use of these cell lines as

29   models to study TGFβ1 signalling should be carefully tailored to the questions asked.

INTRODUCTION

31   The dairy industry could gain significant financial advantage by extending the

32 persistency of lactation (Capuco et al., 2003), which is defined as the number of days in which a

33 constant milk yield is maintained (Grossman, Hartz & Koops, 1999). One of the ways in which

34 dairy farmers increase milk yield is through exogenous administration of recombinant bovine

35 growth hormone (rBGH), which is also known as recombinant bovine somatotropin (rBST)

36 (Etherton and Bauman, 1998). While the use of this hormone presents no health concerns to

37 humans (MacLeod et al., 1999), it is costly to the health of dairy cows (Dohoo et al., 2003). To

38 assess the impact of rBGH/rBST on animal health, a meta-analysis was conducted by the

39 Canadian Veterinary Medical Association. The report stated that administration of rBGH/rBST

40 to dairy cows increased the risk of clinical mastitis by up to 25%, of infertility by 40%, and of

41 lameness by up to 55% (Dohoo et al., 2003). The use of rBGH/rBST has been banned in Canada,

42 however this hormone is commonly used on dairy cows in the United States (Forge, 1998;

43 Sechen, 2013). BGH/BST is considered the predominant galactopoietic hormone in dairy cows

44 (Tucker, 2000) and based on studies in rodents (Collier, Annen-Dawson & Pezeshki, 2012) and

45 in bovine mammary gland explants (Palin, Farmer & Duarte, 2017), BGH/BST acts in

46 conjunction with the well-documented galactopoietic hormone, prolactin (PRL). Many of the

47 galactopoietic effects of BGH/BST are thought to be mediated through insulin-like growth factor

48 I (IGF-I; Bauman, 1999). Binding of IGF-I to the IGF receptor (IGFR) activates IGFR intrinsic

49 tyrosine kinase activity, and leads to the activation of the phosphatidylinositol-4,5-biphosphate 3

50 kinase (PI3K)/Akt (Argetsinger et al., 1993). In this review, we will refer to this BGH/IGF-

51 1/PI3K/Akt signalling pathway as the somatotropic axis. One of the ways that IGF-I enhances

52 survival is through suppression of the cytokine transforming growth factor beta 1 (TGFβ1;

53   Gajewska and Motyl, 2004). MAC-T (mammary alveolar cells expressing SV-40 large T

54   antigen) and BME-UV1 (bovine mammary epithelial cells- clone UV1) are two of the most

55   popular immortalized bovine mammary epithelial cell lines used in the study of mammalian

56   lactation (Jedrzejczak and Szatkowska, 2014). TGFβ1 induces apoptosis and arrests cell growth

57   of BME-UV1 cells, and this was reported to involve suppression of the somatotropic axis (Kolek

58   et al., 2003; Gajewska and Motyl, 2004). Interestingly, there is no literature to support that

59   exogenous TGFβ1 induces apoptosis of MAC-T cells, nor suppresses the somatotropic pathway.

60   In this article, we present an overview of TGFβ signaling in the bovine mammary gland, we

61   review and compare the literature on the

62   MACT-T and BME-UV1 cell lines, and we provide possible explanations as to why

63   different responses to TGFβ1 are observed. We also provide suggestions along the way that

64   might aid in improving our understanding TGFβ function □□ the bovine mammary gland, as

65   well as potential means to alter TGFβ signalling as a way of increasing milk production.

66   **SURVEY METHODOLOGY**

67   Relevant journal articles were obtained by performing a search for "MAC-T TGFbeta",

68   "BME-UV1 TGFbeta", "Bovine mammary TGFbeta", "Bovine mammary epithelial TGFbeta",

69   and "Bovine mammary stroma TGFbeta"on the databases Web of Science, Google Scholar,

70   PubMed and JSTOR.

71   **TGFβ1 IN THE BOVINE MAMMARY GLAND**

72   Lactation is followed by involution, which is the process by which the mammary gland

73   transitions from a lactating to a non-lactating state (Hurley, 1989), and is also the initiation of the

74   dry period. The dry period is a term used in the dairy industry and is defined as the period

75   between cessation of milk removal at dry-off and the initiation of milking at the subsequent

76   calving (Hurley and Loor, 2011). In bovine mammary gland involution, there is a decline in

77   systemic hormone (e.g., BGH/BST and PRL) levels, and this is thought to initiate apoptosis of

78   senescent secretory epithelial cells (Pai and Horseman, 2011). These secretory epithelial cells

79   surround the lumen of the alveoli connected to a basement membrane, and are themselves

80   surrounded by a layer of contractile myoepithelial cells (Nickerson and Akers, 2011). During

81   lactation, secretory epithelial cells are responsible for synthesizing and secreting milk into the

82   lumen, which is then drained into the ducts and collected by the lactiferous sinus before milk

83   removal (Nickerson and Akers, 2011). The role of myoepithelial cells is to contract to allow the

84   secretory epithelial cells to secrete milk out of the lumen and into the ducts (Bruckmaier and

85   Blum, 1998). They are also important for the establishment of apical-basal polarity and for

86   cellular survival, as they secrete components of the basement membrane, which by binding

87   integrins on the basal site of luminal epithelial cells, activate cellular signalling that direct these

88   events (Weaver et al., 2002).

89   Transforming growth factor beta (TGFβ) belongs to a family of growth factors of at least

90   33 members, where TGFβ □□□□□□ exists in three different isoforms, TGFβ1, 2 and 3

91   (Gilbert, Vickaryous & Viloria-Petit, 2016). TGFβ1 plays a key role in bovine mammary gland

92   involution by inducing apoptosis and autophagy in bovine mammary luminal epithelial cells

93   (Kolek et al., 2003; Gajewska, Gajkowska & Motyl, 2005; Zarzyńska, Gajewska & Motyl,

94   2005). Our review of the bovine literature did not yield any reports of TGFβ1's effects on

95   myoepithelial cells, specifically. Unlike some other species, alveolar structures remain intact and

96   secretory epithelial and myoepithelial cells remain in the same position throughout bovine

97   mammary involution and the dry period (Holst, Hurley & Nelson, 1987). Morphologically,

98   bovine involution is characterized by reduced alveolar lumen area (Holst, Hurley & Nelson,

99   1987), as well as reduced integrity and increased permeability of tight junctions (Pai and

100   Horseman, 2011). Polarized human and murine mammary luminal epithelial cells with well-

101   established tight and adherens junctions as well as hemidesmosomes are resistant to apoptosis

102   (Weaver et al., 2002; Avery-Cooper et al., 2014), and this might be the case for bovine

103   mammary epithelial cells, as supported by a study by Singh et al. (2005) demonstrating that cell-

104   ECM communication is important in regulating bovine mammary epithelial cell survival. In

105   other species such as mice, TGFβ1 is reported to dysregulate tight junctions causing mammary

106   epithelial cells to undergo apoptosis (Avery-Cooper et al., 2014). Whether TGFβ1 disrupts tight

107   junctions in the bovine mammary gland, and whether this is responsible for TGFβ1-induced

108   apoptosis in the involuting bovine mammary gland, remains to be addressed.

109   TGFβ1 and its receptors are upregulated during bovine involution (Plath et al., 1997), and

110   in vitro findings suggest that elevated TGFβ1 expression during involution is due in part to its

111   increased secretion by bovine mammary cells during the transition from lactation to the dry

112   period (Zarzyńska, Gajewska & Motyl, 2005), as well as through enhanced release of TGFβ1

113   from the extracellular matrix (ECM) (De Vries et al., 2011). TGFβ1 arrests bovine epithelial cell

114   growth (Kolek et al., 2003) and such effect of TGFβ1 was shown to be important for the

115   development of fully functional mammary glands in mice. In particular, TGFβ1 inhibited ductal

116   branching and lateral extension via induction of Wnt5a expression and release (Roarty and Serra,

117   2007), and thus is currently considered a major regulator of ductal patterning in the mammary

118   gland. It is unclear whether other isoforms, in addition to TGFβ1, are involved in lactation and/or

119   involution in the bovine mammary gland. In this review, we focus specifically on the TGFβ1

120   isoform because it is the most commonly studied in the literature, but it is important to note that

121   all three isoforms are present and differentially expressed in the bovine mammary gland (Maier

122   et al., 1991). TGFβ1 and TGFβ3 are expressed by both the alveolar epithelium and the

123   subepithelial stroma, while TGFβ2 is only expressed in epithelial cells (Maier et al., 1991).

124   Bovine studies involving TGFβ3 should be encouraged in the future, given its demonstrated role

125   in the involuting murine mammary gland, including repression of mammary luminal epithelial

126   cell differentiation via induction of apoptosis (Nguyen & Pollard, 2000), and clearance of the

127   dead epithelial cells via induction of phagocytic activity in the neighboring epithelial cell

128   survivors (Fornetti et al., 2016).

129   In recent years, there has been a greater research focus on the mammary stroma and its

130   influence on epithelial function. The stroma primarily consists of adipocytes and fibroblasts, but

131   also contains endothelial cells, ECM, and inflammatory cells (Kass et al., 2007). TGFβ1 is a

132   potent mediator of ECM synthesis and protects the ECM from degradation (Lasky and Brody,

133   2000; Woodward et al., 2005). Moderate amounts of fibrous tissue is a normal component of the

134   involuting bovine mammary gland, and plays a major role in remodeling and in preparing the

135   gland for the subsequent lactation period (De Vries et al., 2010). However, excessive TGFβ1

136   correlates with extensive tissue fibrosis, and this can interfere with the normal function of the

137   bovine mammary gland, namely milk production (Andreotti et al., 2014). Excessive tissue

138   fibrosis characterizes bovine mastitis (Andreotti et al., 2014), which is defined as inflammation

139   of the mammary gland, and this is a major cause of financial losses to the dairy industry

140   (Heikkilä et al., 2012). Furthermore, TGFβ1 is overexpressed in mastitic mammary glands in

141   parallel with increased fibrosis and apoptosis of epithelial cells (Andreotti et al., 2014), and has

142   been shown to enhance adhesion and invasion of *Staphylococcus aureus (S. aureus)* to bovine

143   mammary fibroblasts (Zao et al., 2017), which makes TGFβ an attractive target of future

144   research aimed to understand and control bovine mastitis. Further, with the strong evidence

145   supporting TGFβ1's effects on both the stromal and parenchymal compartments of the mammary

146   gland, a more holistic approach to studying TGFβ1 signalling incorporating both stromal and

147   epithelial cells may be necessary to study novel approaches to increasing milk production. In

148   vitro treatment of whole tissue explants (De Vries et al., 2011, Magro et al., 2017) is an attractive

149   possibility, as they may convey key mechanistic information impossible to obtain by analysis of

150   biopsies. Another alternative is the use of three-dimensional co-culture models that incorporate

151   ECM and stromal cells of interest in addition to the epithelial cells, as those recently developed

152   by our group (Pallegar et al., 2018).

153 **TGFβ1 SIGNALLING IN THE BOVINE MAMMARY GLAND**

154 TGFβ1 classically signals via a receptor serine/threonine kinase hetero-tetramer,

155 comprised of equal parts T̲GFβ r̲eceptors I̲ (TβRI) and I̲I̲ (TβRII). TGFβ1 ligands have high

156 affinity for the type II but not type I TGFβ receptors (Massagué, 1998). Upon TGFβ1 ligand

157 binding, the constitutively active TβRII dimer binds and phosphorylates TβRI, which becomes

158 activated and phosphorylates receptor-associated s̲mall m̲others a̲gainst d̲ecapentaplegic (R-

159 Smad) transcription factors, specifically Smad2 and Smad3. These R-Smads form a complex

160 with the co-Smad, Smad4, which translocates to the nucleus, where it associates with other

161 transcriptional elements to regulate gene transcription (Figure 1). Among the factors affecting

162 the outcome of canonical (Smad-mediated) TGFβ1 signalling are the type of R-Smad activated,

163 the nature of the interacting transcriptional co-activators and co-repressors, as well as the

164 phosphorylation site, whether at the carboxy terminus or the central linker region (Gilbert,

165 Vickaryous & Viloria-Petit, 2016).

166 In bovine mammary epithelial cells, TGFβ1 is documented to induce apoptosis and cell

167 growth arrest through canonical Smad signalling (Kolek et al., 2003). In non-bovine cells,

168 TGFβ1-induced apoptosis has also been reported to occur through non-canonical pathways such

169 as m̲itogen-a̲ctivated p̲rotein k̲inase (MAPK)/Erk, p38, c-Jun N-Terminal Kinase, PI3K/Akt, and

170 Par6 signalling pathways (reviewed by Zhang 2009; Avery- Cooper et al., 2014). As mentioned

171 above, TGFβ1 has also been shown to inhibit mammary ductal branching in mice via Wnt

172 signalling activation (Roarty and Serra, 2007). Not all of these pathways have been explored in

173 bovine mammary epithelial cell apoptosis; however, downregulation of the PI3K/Akt and the

174 MAPK/Erk pathways occurs in parallel with TGFβ1-induced apoptosis and growth arrest of

175 bovine mammary epithelial cells *in vitro* (Gajewska and Motyl, 2004; Di et al., 2012).

176   Contrary to its inhibitory effect on the bovine mammary epithelium, TGFβ1 promotes

177   stromal development in the mammary gland (Musters et al., 2004). TGFβ1 enhances bovine

178   fibroblast proliferation through the MAPK/Erk pathway (Gao et al., 2016), and promotes the

179   transition of fibroblasts to myofibroblasts (De Vries et al., 2011); the latter are key mediators of

180   ECM protein synthesis and tissue fibrosis (Phan, 2008). Furthermore, TGFβ1 is known to cause

181   bovine mammary epithelial cells to switch to a mesenchymal phenotype through a process

182   known as epithelial-to-mesenchymal transition (EMT) (Chen et al., 2017). In bovine mammary

183   epithelial cells, EMT was reported to occur (at least in part) through the Smad pathway (Chen et

184   al., 2017). In non-bovine cells, other Smad-independent pathways have been implicated in TGF-

185   β-induced EMT such as the MAPK/Erk pathway, p38 MAPK, c-Jun N-terminal kinases, Par6

186   signaling and the PI3K/Akt pathway (Xu, Lamouille, & Derynck, 2009; Avery Cooper et al.,

187   2014). A study exploring the use of tamoxifen as an inhibitor of tissue fibrosis, recently

188   demonstrated that TGFβ induces a mammary fibroblast to myofibroblast transition via activation

189   of MAPK/Erk signalling (Carthy et al., 2015). There is no current evidence on the involvement

190   of specific non-canonical signalling pathways in TGFβ1-induced EMT or fibroblast to

191   myofibroblasts conversion in bovine cells. Further research is necessary to understand the

192   contribution of non-canonical signalling pathways to TGFβ1-induced apoptosis, EMT, activation

193   of fibroblast into myofibroblasts, growth arrest, and mammogenesis in the bovine gland.

194   **TGFβ1 IMPACT ON APOPTOSIS AND GROWTH ARREST**

195   One of the first studies to investigate TGFβ signalling in bovine mammary epithelium

196   assessed the effect of serum starvation on TGFβ1 synthesis and secretion. Zarzyńska, Gajewska

197   & Motyl (2005) mimicked the withdrawal of lactogenic hormones, growth factors, and nutrients

198   that occurs at the end of lactation and the beginning of involution by reducing fetal bovine serum

199   (FBS) content in the growth media from 10% to 0.5%, and analyzed TGFβ1 expression

200   (Zarzyńska, Gajewska & Motyl, 2005). In media containing 0.5% FBS but not 10% FBS, TGFβ1

201   protein and mRNA expression was increased (measured by laser scanning cytometry and RT-

202   PCR, respectively), and reached its maximum levels after 24 hours in BME-UV1 cells or 48

203   hours in MAC-T cells. These results suggested that a factor in FBS suppresses TGFβ1

204   expression. The authors demonstrated that IGF-I (a key player in the somatotropic axis mediated

205   by GH) suppresses TGFβ1 expression in BME-UV1 cells and MAC-T cells (Zarzyńska,

206   Gajewska & Motyl, 2005). The percentage of apoptotic cells was also measured (using the sub-

207   G1 region of a DNA histogram), and this reflected the patterns of TGFβ1 expression in MAC-T

208   and BME-UV1 cells (Zarzyńska, Gajewska & Motyl, 2005). The investigators noted that there

209   was a positive relationship between endogenous TGFβ1 expression and apoptosis, which

210   provides support for the hypothesis that endogenous TGFβ1 induces apoptosis in these two cell

211   lines. BME-UV1 cells were also reported to undergo apoptosis and growth arrest in response to

212   exogenous (2 ng/mL) TGFβ1, and this was found to occur mainly through the intrinsic pathway

213   of apoptosis (Kolek et al., 2003). Gajewska, Gajkowska & Motyl (2005) demonstrated the

214   formation of a Smad-DNA complex in the nucleus 2 hours after TGFβ1 treatment in BME-UV1

215   cells, indicating that the effects of TGFβ1 are transcriptionally mediated.

216   In 1995, Woodward et al. studied the influence of TGFβ1 on cell proliferation and cell

217   death of MAC-T cells (Woodward et al., 1995). This study found that maximal reduction in

218   proliferation, as determined by total DNA measurements and thymidine incorporation, was

219   obtained at 40 pM of TGFβ1. However, cytotoxicity (cell death) was not demonstrated by the

220   trypan blue exclusion method using a TGFβ1 concentration as high as 40 nM (1000-fold greater

221   than 40 pM) (Woodward et al., 1995). This result contrasts the idea that TGFβ1 induces

222   apoptosis of MAC-T cells, suggested by the Zarzyńska, Gajewska & Motyl (2005) study, which

223   found that maximum levels of apoptosis paralleled maximum levels of TGFβ1 ligand after 48

224   hours of serum starvation in MAC-T cells (Zarzyńska, Gajewska & Motyl, 2005). Thus, the

225   evidence of TGFβ1-induced apoptosis of MAC-T cells is weak if not inexistent, compared to that

226   for BME-UV1 cells.

227   In addition to the studies discussed above with immortalized bovine MEC lines, an *ex*

228   *vivo* study on bovine mammary gland explants revealed increased caspase 3 and cleaved Parp in

229   parallel with increased levels of TGFβ1 and TβR2 during the dry period (Zarzyńska et al., 2007).

230   These results provide indirect evidence that TGFβ1 induces apoptosis of bovine mammary

231   epithelial cells *in vivo*. Furthermore, the group of Di et al. (2012) demonstrated that exogenous

232   TGFβ1 induces apoptosis of primary bovine mammary epithelial cells isolated from the bovine

233   mammary gland. This study revealed that levels of the death receptor ligand Fas ligand (FasL)

234   were reduced in parallel with increased levels of TGFβ1-induced apoptosis, which supports the

235   findings by Kolek et al. (2003) demonstrating that TGFβ1-induced apoptosis in bovine

236   mammary epithelial cells occurs through the intrinsic mitochondrial pathway. The results of this

237   *ex vivo* assay demonstrate that primary bovine mammary epithelial cells undergo TGFβ1-

238   induced apoptosis, similar to the BME-UV1 cells (Kolek et al., 2003), and this provides strong

239   support for the idea that BME-UV1 mimic the behavior of cells directly isolated from the bovine

240   mammary gland.

241   **CROSSTALK BETWEEN TGFβ1 AND THE SOMATOTROPIC AXIS**

242   Gajewska and Motyl (2004) found that suppression of PI3K/Akt signalling pathway was

243   involved in the apoptotic response to TGFβ1 in BME-UV1 cells. In these cells, TGFβ1 was

244   found to increase the expression of insulin-like growth factor binding protein (IGFBP)-3 and

245   IGFBP-4, which led to the sequestration of IGF-I from the IGF-I receptor, reduction of PI3K/Akt

246   signalling, and increased expression of the pro-apoptotic protein Bcl-2-associated death promoter

247   (Bad) (Gajewska and Motyl, 2004). A separate study found that exogenous IGF-I administration

248   to BME-UV1 cells completely blocked TGFβ1 expression and apoptosis, and this effect was

249   reversed through the use of a PI3K inhibitor (Zarzyńska and Motyl, 2005). In agreement with the

250   studies performed on BME-UV1 cells, Di et al. (2012) demonstrated that increased levels of Bad

251   mediated the apoptotic response to TGFβ1 in primary non-immortalized bovine mammary

252   epithelial cells isolated from the bovine mammary gland, which lends support to the hypothesis

253   that TGFβ1-induced apoptosis in bovine mammary epithelial cells is mediated in part by a

254   reduction of PI3K/Akt signalling. Altogether, this provides additional support for the argument

255   that BME-UV1 cells closely resemble the responses to TGFβ1 that are seen in primary bovine

256   mammary epithelial cells.

257   Zarzyńska, Gajewska & Motyl (2005) compared the effects of GH and somatostatin (a

258   negative regulator of GH) on TGFβ1 expression in MAC-T and BME-UV1 cells (Table 1). GH

259   significantly reduced TGFβ1 levels in BME-UV1 cells supplemented with 10% FBS (nutrient-

260   rich conditions), while it significantly increased TGFβ1 levels in BME-UV1 cells supplemented

261   with 0.5% FBS (nutrient-poor conditions). Treatment with somatostatin, a negative regulator of

262   GH, was only evaluated in BME-UV1 cells supplemented with 10% FBS, in which it increased

263   TGFβ1 protein levels. This suggests that in nutrient-rich conditions such as those expected

264   during lactation, GH suppresses TGFβ1 expression in the bovine mammary gland, an effect that

265   is blocked by somatostatin. In MAC-T cells, GH and somatostatin did not affect TGFβ1 protein

266   levels (Zarzyńska, Gajewska & Motyl, 2005). Thus, TGFβ1 expression is altered by hormones of

267   the somatotropic axis such as GH and somatostatin in BME-UV1 cells, but not in MAC-T cells

268   (Zarzyńska, Gajewska & Motyl, 2005). Taken together with the demonstrated role of the

269   somatotropic axis in the control of survival in bovine mammary epithelial cells (Gajewska and

270   Motyl, 2004), the results discussed above suggest that the modulation of TGFβ1 levels and

271   subsequent activity by the somatotropic axis is a mechanism to control survival/apoptosis in

272   BME-UV1 cells but not in MAC-T cells.

273   The above results are also supported by *in vivo* evidence. Zarzyńska et al. (2007)

274   evaluated the expression of TGFβ1, TGFβRII, IGF-I Receptor α (IGF-IRα), IGF-I Receptor β

275   (IGF-IRβ), GH-R, IGFBP-3, -4, and -5, as well as biochemical markers of apoptosis (cleaved

276   Parp and caspase 3) in bovine mammary gland explants at early lactation, late lactation, and

277   during dry off (Zarzyńska et al., 2007). This study revealed that increased apoptosis, TGFβ1,

278   TGFβRII, IGFBP-4, and-5 expression was accompanied by downregulation of GH-R and IGF-

279   IRα during dry off (Zarzyńska et al., 2007). This suggests that TGFβ1 exerts its pro-apoptotic

280   effects through suppression of the somatotropic pathway (Figure 2).

281   As discussed above, IGF-I somatogenic effects have been shown to be caused, at least in

282   part, by inhibition of TGFβ1 signalling (Zarzyńska and Motyl, 2005). Thus, IGF-I administration

283   might be an effective way to block TGFβ1 signalling and its pro-apoptogenic and

284   antiproliferative effects *in vivo*; in fact, this has already been done in an *in vitro* study by

285   Zarzyńska and Motyl (2005). *In vivo* studies examining the effect of IGF-I on milk production

286   have yielded inconsistent results. Davis et al. (1989) compared the effects of administering

287   systemic infusions of IGF-I or GH to lactating goats and measured the effects on milk production

288   over a 10-day period. After 36 hours, goats receiving GH had a milk yield increased by an

289   average of 24%, while goats receiving IGF-I had no significant increase in milk yield compared

290   to saline-infused goats (Davis et al., 1989). In agreement with these results, intramammary

291   infusion of IGF-I had no significant effect on milk protein expression or milk yield in dairy cows

292   after a 4-day treatment interval (Mackle et al., 2000). Conversely, infusion of IGF-I for 6 hours

293   via an external pudic arterial catheter in lactating goats resulted in milk yield significantly

294   increased by 9% 2 hours post-infusion, and this was associated with significantly increased

295   mammary blood flow by 50-80% (Prosser et al., 1994).

296   Taken together, the above evidence does not consistently support an impact of IGF-I

297   administration (and possibly indirect TGFβ1 inhibition) on acute milk yield following IGF-I

298   administration. One limitation of these studies is that only short periods of time were used to

299   examine milk production, rather than measuring the amount of milk produced over the entire

300   lactation period, which has an average length of 225 days in goats and 305 days in cattle (Ahuya

301   et al., 2009; Bachman & Schairer, 2003). It is possible that IGF-I administration could delay the

302   onset of apoptosis and growth arrest that occurs in secretory epithelial cells leading to the decline

303   in lactation and the beginning of involution without increasing peak milk yield. We propose that

304   exogenous administration of IGF-I would extend the time in which peak yield is maintained

305   compared to control cows. This area of research has not been recently investigated and many

306   unknown questions still remain regarding harnessing TGFβ1 signalling for the purposes of

307   extending lactation. For instance, many studies have focused on TGFβ1's effect on cell number,

308   but have not evaluated TGFβ1's possible effect of metabolic activities of secretory epithelial

309   cells during lactation.

PHENOTYPIC DIFFERENCES IN MAC-T VS. BME-UV1 CELLS

310

311   In the previous section, we provided evidence of TGFβ1-induced apoptosis in BME-

312   UV1, but not MAC-T cells (Kolek et al., 2003; Woodward et al., 1995), and we also discussed

313   how the somatotropic axis controls TGFβ1 expression in the former but not the latter (Zarzyńska,

314   Gajewska & Motyl, 2005). Comparing the literature on MAC-T and BME-UV1 cells yielded

315   some very important differences that should be taken into account before using these cell lines to

316   study TGFβ1 signalling.

317   The cell populations that compose the MAC-T and BME-UV1 cell lines were previously

318   identified via determination of cell surface marker and cytokeratin expression under standard

319   adherent (ADH), ultra-low adherence (ULA) and three-dimensional (3D) laminin-rich

320   (Matrigel^{TM}) culture conditions (Arévalo Turrubiarte et al., 2016). Staining of the luminal-

321   specific cytokeratin (CK) CK19 and myoepithelial-specific CK14 (both human-specific

322   antibodies) (Borena et al., 2013) was used to identify luminal and myoepithelial cell populations,

323   respectively. Staining of CK19 and CK14 was supplemented by evaluating epithelial cell

324   adhesion molecule (EpCAM) expression using a mouse-specific antibody. EPCAM is a cell-

325   surface marker more highly expressed in bovine luminal-alveolar progenitor cells compared to

326   other mammary epithelial cell types (Perruchot et al., 2016). To further support the luminal cell

327   classification, Arévalo Turrubiarte et al. (2016) examined aldehyde dehydrogenase (ALDH)

328   activity under standard adherence conditions, as ALDH is highly expressed in bovine luminal

329   progenitor cells compared to other bovine mammary epithelial cell populations (Martignani et

330   al., 2010).

331   In order to assess basal phenotype, Arévalo Turrubiarte et al. assessed cluster of

332   differentiation (CD) markers: CD49f (integrin α6 chain) using a rat-specific antibody and CD10

333   (neutral endopeptidase) using a mouse-specific antibody. CD49f is responsible for cell-matrix

334   adhesions and for transmitting signals between mammary epithelial cells and the ECM (Giancotti

335   and Ruoslahti, 1999). CD49f is expressed in cells of both luminal and basal origin; however, it is

336   more highly expressed in basal cells (Finot, Chanat & Dessauge, 2018; Rauner and Barash,

337   2012; Perruchot et al., 2016). CD10, on the other hand, is normally used as a marker of basal

338   cells (Safayi et al., 2012), but it is also enriched in mammosphere-forming cell populations;

339   hence CD10 is also used as a marker of stem cells (Maguer-Satta, Besançon & Bachelard-

340   Cascales, 2011). The ambiguity surrounding the significance of CD10 staining is addressed by

341   staining for multiple markers in order to not misidentify cell phenotypes. For example, an

342   additional marker of basal cells that can be used is CK14 (Borena et al. 2013), which was also

343   included in the panel by the aforementioned researchers (Arévalo Turrubiarte et al., 2016).

344   Arévalo Turrubiarte et al. (2016) marker expression assessment in BME-UV1 cells led to the

345   conclusion that they have a luminal phenotype based on their high expression of EpCAM and

346   CK19, and their high ALDH activity. Interestingly, the BME-UV1 cells also express CD10. In

347   the absence of any other basal markers, these investigators interpreted the high CD10 expression

348   and high ALDH activity of BME-UV1 cells as having a greater "stem-like" phenotype compared

349   to the MAC-T cells (Arévalo Turrubiarte et al., 2016; Table 2).

350   The MAC-T cells were found by the same investigators to have a higher CD49f

351   expression and lower ALDH activity compared to the BME-UV1 cells under standard adhesion

352   culture conditions. In addition, MAC-T cells expressed CK14, a cytokeratin characteristic of

353   myoepithelial cells, while the BME-UV1 cells did not (Arévalo Turrubiarte et al., 2016). The

354   high expression of CD49f, the low ALDH activity, and the expression of CK14 suggest that

355   MAC-T cells contain a myoepithelial cell population (Arévalo Turrubiarte et al., 2016; Table 2).

356 Although the MAC-T cells contain characteristics indicative of myoepithelial cells, these cells

357 were previously reported to lack oxytocin responsiveness and smooth muscle actin expression

358 (Zavizion, Gorewit & Politis, 1995), which are two hallmarks of myoepithelial cells (Gudjonsson

359 et al., 2005). Further, Arévalo Turrubiarte et al (2016) found MAC-T cells to lack CD10, which

360 is a marker also expressed by mature bovine myoepithelial cells (Perruchot et al., 2016). It is

361 important to note that the assessment of oxytocin and smooth muscle actin in MAC-T was

362 performed following their culture as monolayers on glass (Zavizion, Gorewit & Politis, 1995).

363 Instead, a 3D culture model on reconstituted basement membrane, such as Matrigel^{TM}, could be

364 employed in order to examine the myoepithelial potential of the MAC-T cells. We based this

365 suggestion in studies by Mroue et al. (2015), in which a role for the gap junction protein

366 Connexin 43 was demonstrated in the contractile response of myoepithelial cells to oxytocin via

367 assessment of mouse derived mammary epithelial cell organoids under 3D culture conditions on

368 Matrigel^{TM}. Appropriate ECM interactions under these conditions might promote differentiation

369 into a myoepithelial phenotype capable of proper assembly of functional gap junctions and

370 consequently contractibility following oxytocin exposure. Another thing to note about the study

371 by Arévalo Turrubiarte et al. (2016) is the use of non-bovine specific antibodies (human-specific

372 CK14 and CK19, rat-specific EpCAM, and mouse-specific CD49f and CD10), which does not

373 take into account interspecies differences. To illustrate this concern, the UniProt Consortium

374 (2017) revealed that human and bovine CK14 and CK19 have 91.1% and 88.8% protein

375 sequence homology, respectively, while rat and bovine EpCAM and CD49f share 78.1% and

376 91.9% protein homology, respectively. Lastly, mouse and bovine CD10 share 91.3% protein

377 homology (The UniProt Consortium, 2017). These differences in protein sequences between

378   bovine, and the species against which the antibody was directed, can lead to misleading results

379   not necessarily reflecting the true levels of expression or a given protein.

380   Another interesting feature of the MAC-T cells is their capability of synthetizing α- and

381   β-casein, which is indicative that there are luminal/alveolar cells present within this cell line

382   (Huynh, Robitaille & Turner, 1991). One explanation to the bi-phenotypic features of MAC-T

383   cells, is that they contain bi-potent progenitor cells that gave rise to cells of both basal and

384   luminal origin (Rauner and Barash, 2012). One study, which investigated the ability of MAC-T

385   cells to form a functional mammary gland *in vivo*, suggests that this might be the case. MAC-T

386   cells were mixed with the reconstituted ECM Matrigel™, and were implanted into the dorsal

387   tissue of 8-week-old BALB/C nude male mice (Park et al., 2016). After 6 weeks, the

388   transplanted tissue of these mice was dissected and analyzed for bovine mammary protein

389   expression (Park et al., 2016). At this time, the MAC-T transplants had the characteristic alveolar

390   structures of a female mammary gland (Park et al., 2016). These investigators evaluated the

391   expression of CK18 and CK14 (luminal and myoepithelial cytokeratins, respectively), both in the

392   mammary tissue sections, as well as *in vitro*. The tissue sections expressed CK18, mainly

393   localized in the luminal cells, and CK14, which was mainly localized in the ductal and

394   myoepithelial cells. The MAC-T cell line grown in monolayer culture stained positively for both

395   CK14 and CK18 (Park et al., 2016). These results suggest that the MAC-T cell line contains a

396   heterogeneous population. The above findings are additionally supported by a previous study in

397   which MAC-T cells were subcloned into three distinct cell lines (Zavizion, Gorewit & Politis,

398   1995).

399   Both MAC-T and BME-UV1 cells are derived from lactating mammary epithelium,

400   however there were differences in the generation of these cell lines that should be considered.

401   Although both cell lines were immortalized via transfection with simian virus 40 (SV40) large T

402   antigen (the temperature sensitive tsA58 mutant T antigen, specifically) and they were generated

403   from mammary tissue obtained at slaughter from Holstein cows; BME-UV1 were obtained from

404   a pregnant lactating cow (Zavizion et al., 1996), while MAC-T cells were obtained from a non-

405   pregnant lactating cow (Huynh, Robitaille & Turner, 1991). Further, the transfection to generate

406   BME-UV1 cells was carried out on a homogenous population of cells generated by subcloning a

407   luminal-enriched cell population (Zavizion et al., 1996), while the transfection to generate MAC-

408   T cells was performed on cells of epithelial morphology derived from serial dilution cloning of

409   the original heterogeneous population of mammary cells (Huynh, Robitaille & Turner, 1991).

410   Thus, differences between the MAC-T and BME-UV1 cells may be a consequence of the

411   differing physiological statuses of the animals that were sampled and/or the cell population that

412   was immortalized. It is also possible that the cells acquired different phenotypes due to lab-

413   specific culture conditions since different culture conditions were used to generate the cells

414   (Arévalo Turrubiarte et al., 2016). Such effect of culture conditions on the phenotype acquired

415   by mammary epithelial cells has indeed been reported for human mammary cells (Ince et al.,

416   2007).

417 **SUMMARY AND FUTURE DIRECTIONS**

418   Using laser-scanning cytometry, Zarzyńska, Gajewska & Motyl (2005) demonstrated that

419 endogenous levels of TGFβ1 in the bovine mammary gland epithelial cell line MAC-T reached

420 their maximum in parallel with apoptosis. Apart from this study, there is no other evidence

421 supporting that TGFβ1 induces apoptosis in MAC-T cells. Future experiments could use TβRI/II

422 kinase inhibitors and/or TGFβ1 blocking antibodies, in order to conclusively demonstrate that

423 apoptosis of MAC-T cells under starving conditions in induced by TGFβ1. Suppression of

424 PI3K/Akt activity was reported to mediate TGFβ1-induced apoptosis of BME-UV1 cells

425 (Gajewska and Motyl, 2004); however, the role of PI3K/Akt in TGFβ1-induced apoptosis of

426 MAC-T cells has never been explored. Individual and combination treatments of exogenous

427 TGFβ1, a TβRI/II kinase inhibitor, and a PI3K/Akt inhibitor would reveal the contribution of the

428 PI3K/Akt pathway to the antiproliferative and apoptogenic effect of TGFβ1 in MAC-T cells.

429 From the available *ex vivo* studies demonstrating TGFβ1-induced apoptosis in primary bovine

430 mammary epithelial cells (Di et al., 2012; Zarzyńska et al., 2007) and the similar capacity of

431 BME-UV1 cells to undergo apoptosis in response to TGFβ (Kolek et al., 2003), we can infer that

432 BME-UV1 cells resemble the *in vivo* bovine mammary epithelium in relation to apoptosis.

433   The literature suggests that MAC-T cells may be composed of a bi-potent progenitor

434 population based on the presence of both luminal and basal phenotypes (Arévalo Turrubiarte et

435 al., 2016; Park et al., 2016). It would first be necessary to prove this by performing a clonal

436 assay on MAC-T cells to demonstrate these cells give rise to both luminal and basal cells. A

437 further step would be to compare the effect of TGFβ1 on MAC-T cells to that on bi-potent

438 progenitors derived from bovine mammary explants, in order to evaluate if cell line

439 immortalization is a confounding variable. In recent years, several different models of epithelial

440   cell hierarchy in the bovine mammary gland have been proposed (Rauner and Barash, 2012;

441   Perruchot et al., 2016; Finot, Chanat & Dessauge, 2018). A better understanding of lineage

442   commitment to basal or luminal phenotypes may shed light on the different origins of MAC-T

443   and BME-UV1 cells, and thus guide their appropriate use in studies aimed to elucidate the role

444   of TGFβ in bovine mammary epithelium apoptosis and bovine mammary gland biology in

445   general.

446   In relation to the inverse relationship between the somatotropic and the TGFβ signalling

447   pathway, studies in the past have found that IGF-I administration to dairy animals resulted in no

448   change in milk production (Davis et al. 1984; Mackle et al, 2000). We suggest that these results

449   may reflect the study design. When evaluating the efficacy of a treatment, it is important that we

450   address lactation persistency in terms of peak milk production as well as what happens before

451   and after, by measuring milk production over the entire lactation period. IGF-I may not

452   necessarily increase peak milk production in dairy cows, but it may still reduce the decline in

453   milk production that inevitably follows peak milk yield. Another approach to reducing TGFβ1's

454   potent apoptogenic and anti-proliferative effects on the epithelium and stimulatory effects on the

455   stroma is to modify the dairy cow's diet. For example, Gao et al. (2016) demonstrated that dairy

456   cows fed a diet of corn stover had higher serum levels of TGFβ1 compared to cows fed a diet of

457   alfalfa. Furthermore, mammary glands of cows fed the diets of corn stover and alfalfa were

458   examined *ex vivo*, and the former demonstrated increased levels of TGFβ1 and increased levels

459   of vimentin (a marker of mammary stroma). This suggests that we can indirectly affect TGFβ1

460   signalling through modifying nutrition of dairy cows. This may be the most cost-efficient and

461   practical approach to modifying TGFβ1 signalling in order to increase milk production, rather

462   than the administration of growth factors or hormones such as IGF-I and rBST, respectively.

463   An important concern to note is that inhibition of TGFβ1 signalling can result in

464   undesired side effects such as delayed wound healing and chronic inflammation (Herbertz et al.

465   2015). Furthermore, TGFβ1 plays an important role in mammary gland remodeling during

466   involution since it is likely responsible for, at least in part, replacing senescent secretory

467   epithelial cells (Di et al., 2012; Zarzyńska et al., 2007), and for promoting stromal development

468   (Musters et al., 2004). Therefore, inhibiting TGFβ1 completely to prevent involution and the dry

469   period would not be well-advised. In fact, De Vries et al. (2011) suggested that, rather than

470   inhibiting TGFβ □□□□□□□□□□, exogenous TGFβ1 could be used during the first week of

471   the dry period to hasten remodeling, shorten the dry period, and thus maximize milk production

472   in the subsequent lactation period. It would be interesting to test this hypothesis *in vivo* by

473   administering TGFβ1 during early dry period and comparing the effects to inhibition of TGFβ1

474   during the same time window, or comparing the same contrasting approaches during the late dry

475   period. The length of the treatment is an important factor to take into consideration when

476   designing these experiments, given the potential enhancement of mastitis susceptibility by

477   TGFβ. A shorter treatment time might be required, based on the above-discussed evidence that

478   TGFβ1 enhances *S. aureus* adhesion and invasion into bovine mammary fibroblasts (Zhao et al.,

479   2017). Taking this into account, any studies administering TGFβ1 in dairy cows should report

480   prevalence of mastitis in different treatment groups to ensure that TGFβ1 is not predisposing

481   dairy cows to intramammary infections.

482   Most researchers studying involution and lactation focus on the mammary epithelium, but

483   recent work has demonstrated that TGFβ1 equally affects the mammary stroma. Future research

484   is necessary to understand how TGFβ1's effect on the stroma affects the epithelial compartment.

485   This would be particularly useful in determining if stromal-epithelial interactions affect how

486   closely MAC-T and BME-UV1 cells match the response of *in vivo* bovine mammary epithelial

487   cells to TGFβ1. We can evaluate the effect of epithelial-stromal interactions on TGFβ1

488   signalling by employing bovine mammary gland explants (De Vries et al., 2011, Magro et al.,

489   2017), or a 3D culture system with re-constituted ECM (such as Matrigel^{TM}) to more accurately

490   mimic the *in vivo* mammary gland (Lee et al., 2007). Another option is to use a co-culture system

491   containing stromal cells (fibroblasts, adipocytes, or both) in addition to the epithelial cells

492   (Zhang et al., 2002) or in addition to the epithelial cells and the ECM (Pallegar et al., 2018).

493   Taking this approach will allow scientists to gain a better understanding of the interaction

494   between epithelial and stromal compartments, and how this affects cell behavior.

CONCLUSIONS

495

496   TGFβ1 signalling is a research interest in the field of mammalian lactation and

497   involution, and can potentially be manipulated in order to increase lactation persistency. In the

498   bovine mammary gland, MAC-T and BME-UV1 cell lines have different responses to TGFβ1,

499   and these responses should be carefully considered before employing them to study TGFβ1

500   signalling. BME-UV1 cells have been documented to undergo apoptosis in response to

501   exogenous and endogenous TGFβ1, and this involved suppression of the somatotropic pathway

502   (Kolek et al., 2003; Zarzyńska, Gajewska & Motyl, 2005; Gajewska and Motyl, 2004).

503   Conversely, TGFβ1-induced apoptosis of MAC-T cells has never been demonstrated, and no

504   involvement of the GH/IGF-I/PI3K/Akt pathway has been reported. Furthermore, hormones of

505   the somatotropic pathway (GH and somatostatin) alter TGFβ1 expression in BME-UV1, but not

506   in MAC-T cells (Zarzyńska, Gajewska & Motyl, 2005).

507   The different responses of BME-UV1 and MAC-T cells to TGFβ1 is possibly a result of

508   their differing phenotypes. BME-UV1 cells express a luminal phenotype, while MAC-T cells

509   possess a phenotype indicative of both luminal and myoepithelial populations (Arévalo

510   Turrubiarte et al., 2016). For researchers wishing to study interactions between TGFβ1 signalling

511   and the somatotropic pathway in alveolar bovine mammary epithelial cells, the current evidence

512   suggests that BME-UV1 cells better reflect the physiology of milk-secreting bovine alveolar

513   mammary epithelial cells.

514   Taking into account TGFβ1's effect on mammary stroma and the role of stromal-

515   epithelial interactions in mammary gland physiology, the influence of stromal cells is an

516   important factor to consider when testing how closely bovine mammary cell lines (including

517   MAC-T and BME-UV1) resemble the *in vivo* mammary gland. The experimental design for

518   future research involving MAC-T and BME-UV1 cells should keep this in mind.

519   **ACKNOWLEDGMENTS**

520   We would like to recognize all the support and suggestions we received from members of

521   the Viloria-Petit, Coomber, and Mutsaer laboratories, as well as from a number of other

522   members of the Department of Biomedical Sciences at the University of Guelph. Our special

523   thanks to Charles Mitz and Mary Ellen Cybulski for their critical review of the manuscript. We

524   dedicate this work to the memory of C.A.M. grandfather, Larry Mitz.

525   **REFERENCES**

526   Ahuya C, Ojango J, Mosi R, Peacock C, Okeyo A. Performance of Toggenburg dairy goats in
527   smallholder production systems of the eastern highlands of Kenya. *Small Ruminant Research* 83:
528   7-13 DOI: 10.1016/j.smallrumres.2008.11.012.

529   Arévalo Turrubiarte M, Perruchot M, Finot L, Mayeur F, Dessauge F. 2016. Phenotypic and
530   functional characterization of two bovine mammary epithelial cell lines in 2D and 3D models.
531   *American Journal of Physiology - Cell Physiology* 310:C348-C356 DOI:
532   10.1152/ajpcell.00261.2015.

533   Andreotti C, Pereyra E, Baravalle C, Renna M, Ortega H, Calvinho L, Dallard B. 2014.
534   Staphylococcus aureus chronic intramammary infection modifies protein expression of
535   transforming growth factor beta (TGF-β) subfamily components during active involution.
536   *Research in Veterinary Science* 96:5-14 DOI: 10.1016/j.rvsc.2013.11.002.

537   Argetsinger L, Campbell G, Yang X, Witthuhn B, Silvennoinen O, Ihle J. Carter-Su C. 1993.
538   Identification of JAK2 as a growth hormone receptor-associated tyrosine kinase *Cell* 74:237-244
539   DOI: 10.1016/0092-8674(93)90415-M.

540   Avery-Cooper G, Doerr M. Gilbert R, Youssef M, Richard A, Huether P, Viloria-Petit A. 2014.
541   Par6 is an essential mediator of apoptotic response to transforming growth factor beta in
542   NMuMG immortalized mammary cells. *Cancer Cell International* 14:19 DOI: 10.1186/1475-
543   2867-14-19.

544   Bachman K, Schairer M. 2003. Invited review: bovine studies on optimal lengths of dry Periods.
545   *Journal of Dairy Sciences* 86:3027-3037 DOI:10.3168/jds.S0022-0302(03)73902-2

546   Bauman D. 1999. Bovine somatotropin and lactation: from basic science to commercial
547   application. *Domestic Animal Endocrinology* 17:101-116 DOI: 10.1016/S0739-7240(99)00028-
548   4.

549   Borena B, Bussche L, Burvenich C, Duchateau L, Van de Walle G. 2013. Mammary stem cell
550   research in veterinary science: An Update. *Stem Cells and Development* 22:1743-1751 DOI:
551   10.1089/scd.2012.0677.

552   Bruckmaier R, Blum J. 1998. Oxytocin release and milk removal in ruminants. *Journal of Dairy*
553   *Science* 81(4):939-949 DOI:10.3168/jds.S0022-0302(98)75654-1.

554   Capuco A, Ellis S, Hale S, Long E, Erdman R, Zhao X, Paape M. 2003. Lactation persistency:
555   insights from mammary cell proliferation studies. *Journal of Animal Science* 81:18-31 DOI:
556   10.2527/2003.81suppl_318x.
557

558   Carthy J, Sunqvist A, Heldin A, van Dam H, Kletsas D, Heldin C, Moustakas A. 2015.
559   Tamoxifen inhibits TGF-β-mediated activation of myofibroblasts by blocking non-Smad

560   signaling through ERK1/2. *Journal of Cellular Physiology* 230:3084-92 DOI:
561   10.1002/jcp.25049.

562

563   Chen Q, Yang W, Wang X, Li X, Qi S, Zhang Y, Gao M. 2017. TGF-β1 Induces EMT in Bovine
564   Mammary Epithelial Cells Through the TGFβ1/Smad Signaling Pathway. *Cellular Physiology*
565   *and Biochemistry* 43:82-93 DOI: 10.1159/000480321.

566   Collier R, Annen-Dawson E, Pezeshki A. 2012. Effects of continuous lactation and short dry
567   periods on mammary function and animal health. *Animal 6*:403-414
568   DOI:10.1017/S1751731111002461

569

570   Davis S, Gluckman P, Hodgkinson S, Farr V, Breier B, Burleigh B. 1989. Comparison of the
571   Effects of Administration of Recombinant Bovine Growth Hormone or N-Met Insulin- like
572   Growth Factor-I to Lactating Goats. *Journal of Endocrinology.* 123:33-39.

573   De Vries L, Casey T, Dover H, VandeHaar M, Plaut K. 2011. Effects of transforming growth
574   factor-β on mammary remodeling during the dry period of dairy cows. *Journal of Dairy Science*
575   94:6036-6046 DOI: 10.3168/jds.2011-4590.

576   De Vries L, Dover H, Casey T, VandeHaar M, Plaut K. 2010. Characterization of mammary
577   stromal remodeling during the dry period. *Journal of Dairy Science* 93:2433–2443 DOI:
578   10.3168/jds.2009-2764.

579   Di H, Wang L, Wang G, Zhou L, Yang Y. 2012. The signaling mechanism of TGF-β1 induced
580   bovine mammary epithelial cell apoptosis. *Asian-Australasian Journal of Animal Sciences*
581   25:304-310 DOI: 10.5713/ajas.2011.11251.

582   Dohoo I, DesCôteaux L, Leslie K, Fredeen A, Shewfelt W, Preston A, Dowling P. 2003. A meta-
583   analysis review of the effects of recombinant bovine somatotropin. *Canadian Journal of*
584   *Veterinary Research* 67 252–264 PMID: 1462861.

585   Etherton T, Bauman D. 1998. Biology of somatotropin in growth and lactation of domestic
586   animals. *Physiological Reviews* 78:745-61 DOI: 10.1152/physrev.1998.78.3.745.

587   Finot L, Chanat E, Dessauge F. 2018. Bovine mammary gland development: new insights into
588   the epithelial hierarchy. *bioRxiv 251637* [Preprint] DOI: 10.1101/251637.

Forge, Frédéric. 1998. RECOMBINANT BOVINE SOMATOTROPIN (rbST). Library of
Parliament – Parliamentary Research and Information Service. Available at:
https://lop.parl.ca/Content/LOP/ResearchPublicationsArchive/bp1000/prb981-e.asp (accessed
May 14, 2018).

Fornetti J, Flanders K, Henson P, Tan A-C, Borges V, Schedin P. 2016. Mammary epithelial cell
phagocytosis downstream of TGF-β3 is characterized by adherens junction reorganization. *Cell*
Death and Differentiation 23:185–196 DOI:10.1038/cdd.2015.82.

589   Gajewska M, Gajkowska B, Motyl T. 2005. Apoptosis and autophagy induced by TGF- β1 in
590   bovine mammary epithelial BME-UV1 cells. *Journal of Physiology and Pharmacology* 56:143-
591   157.

592   Gajewska M, Motyl T. 2004. IGF-binding proteins mediate TGF-beta 1-induced apoptosis in
593   bovine mammary epithelial BME-UV1 cells. *Comparative Biochemistry and Physiology Part C:*
594   *Toxicology & Pharmacology* 139:65-75 DOI: 10.1016/j.cca.2004.09.006.

595   Gao Y, Wang Y, Li Y, Xia X, Zhao S, Che Y, Sun Y, Lei L. 2016. TGF-β1 promotes bovine
596   mammary fibroblast proliferation through the ERK 1/2 signalling pathway. *Cell Biology*
597   *International* 40: 750-760 DOI: 10.1002/cbin.

598   Giancotti F, Ruoslahti E. 1999. Integrin signalling. *Science* 285:1028–1032 DOI:
599   10.1126/science.285.5430.1028

600   Gilbert R, Vickaryous M, Viloria-Petit A. 2016. Signalling by transforming growth factor beta
601   isoforms in wound healing and tissue regeneration. *Journal of Developmental Biology* 4:21 DOI:
602   10.3390/jdb4020021.

603   Grossman M, Hartz S, Koops W. 1999. Persistency of lactation yield: a novel approach. *Journal*
604   *of Dairy Science* 82: 2192-2197 DOI: 10.3168/jds.S0022-0302(99)75464-0.

605   Gudjonsson T, Adriance M, Sternlicht M, Petersen O, Bissell M. 2005. Myoepithelial cells: their
606   origin and function in breast morphogenesis and neoplasia. *Journal of Mammary Gland Biology*
607   *and Neoplasia* 10: 261-272 DOI: 10.1007/s10911-005-9586-4.

608   Heikkilä A, Nousiainen J, Pyörälä S. 2012. Costs of clinical mastitis with special reference to
609   premature culling. *Journal of Dairy Science* 95:139 –150 DOI: 10.3168/jds.2011-4321.

610   Holst B, Hurley W, Nelson D. 1987. Involution of the bovine mammary gland: histological and
611   ultrastructural changes. *Journal of Dairy Science* 70:935-944 DOI: 10.3168/jds.S0022-
612   0302(87)80097-8.

613   Herbertz S, Sawyer J, Stauber A, Gueorguieva I, Driscoll K, Estrem S, Cleverly A, Desaiah D,
614   Guba S, Benhadji K, Slapak C, Lahn M. 2017. Clinical development of galunisertib (LY2157299
615   monohydrate), a small molecule inhibitor of transforming growth factor-beta signaling pathway.
616   *Drug Design, Development and Therapy* 9:4479-99 DOI: 10.2147/DDDT.S86621.
617
618   Hurley W, Loor J. 2011. Mammary gland | growth, development and involution. In: Fuquay JW,
619   ed. *Encyclopedia of Dairy Sciences*. San Diego: Academic Press. 338-345 DOI: 10.1016/B978-
620   0-08-100596-5.21408-X.

621   Hurley W. 1989. Mammary gland function during involution. *Journal of Dairy Science* 72:1637-
622   1646 DOI: 10.3168/jds.S0022-0302(89)79276-6.

623   Huynh H, Robitaille G, Turner J. 1991. Establishment of bovine mammary epithelial cells

624   (MAC-T): An in vitro model for bovine lactation. *Experimental Cell Research* 197:191-199
625   DOI: 10.1016/0014-4827(91)90422-Q.

626   Ince T, Richardson A, Bell G, Saitoh M, Godar S, Kamoub A, Iglehart J, Weinberg R. 2007.
627   Transformation of different human breast epithelial cell types leads to different tumor
628   phenotypes. *Cancer Cell* 12:160-170 DOI: 10.1016/j.ccr.2007.06.013.

629   Jedrzejczak M, Szatkowska I. 2014. Bovine mammary epithelial cell cultures for the study of
630   mammary gland functions. *In Vitro Cellular & Developmental Biology – Animal* 50:389-398
631   DOI: 10.1007/s11626-013-9711-4.

632   Kass L, Erler J, Dembo M, Weaver V. 2007. Mammary epithelial cell: influence of extracellular
633   matrix composition and organization during development and tumorigenesis. *The International*
634   *Journal of Biochemistry & Cell Biology* 30:1987–1994 DOI: 10.1016/j.biocel.2007.06.025.

635   Kolek O, Gajkowska B, Godlewski M, Motyl T. 2003. Antiproliferative and apoptotic effect of
636   TGF-β1 in bovine mammary epithelial BME-UV1 cells. *Comparative Biochemistry and*
637   *Physiology Part C: Toxicology & Pharmacology* 134:417-430 DOI: 10.1016/S1532-
638   0456(02)00249-1.

639   Lasky J, Brody A. 2000. Interstitial fibrosis and growth factors. *Environmental Health*
640   *Perspectives* 108:751-762 DOI: 10.1289/ehp.00108s4751.

641   Lee G, Kenny P, Lee E, Bissell M. 2007. Three-dimensional culture models of normal and
642   malignant breast epithelial cells. *Nature Methods* 4: 359–365 DOI: 10.1038/nmeth1015.

643   Mackle T, Dwyer D, Bauman D. 2000. Intramammary infusion of insulin or long R3 insulin-like
644   growth factor-I did not increase milk protein yield in dairy cows. *Journal of Dairy Science*
645   83:1740-1749 DOI: 10.3168/jds.S0022-0302(00)75044-2.

646   MacLeod S, Gougeon R, Marks G, Pollak M, Tenenbein M, Woodland C. 1999. Report of the
647   Royal College of Physicians and Surgeons of Canada Expert Panel on Human safety of RBST.
648   Ottawa (ON): Health Canada. Available at: https://www.canada.ca/en/health-
649   canada/services/drugs-health-products/veterinary- drugs/other-issues/recombinant-bovine-
650   somatotropin-rbst/executive-summary-report- royal-college-physicians-surge)ns-canada-expert-
651   panel-human-safety-rbst-health- canada-1999.html (accessed 2017 Dec 7).
652
653   Magro G, Brevini TAL, De Maglie M, Minozzi G, Scanziani E, Piccinini R. 2017. An explant of
654   heifer mammary gland to study the immune response of the organ. *Research in Veterinary*
655   *Science* 114:44-50 DOI: 10.1016/j.rvsc.2017.03.002.

656   Maguer-Satta V, Besançon R, Bachelard-Cascales E. 2011. Concise review: neutral
657   endopeptidase (CD10): a multifaceted environment actor in stem cells, physiological
658   mechanisms, and cancer. *Stem Cells* 29:389-396 DOI: 10.1002/stem.592.

659 Maier R, Schmid P, Cox D, Graeme B, McMaster G. 1991. Localization of transforming growth
660 factor-beta1,- beta 2 and -beta 3 gene expression in bovine mammary gland. *Molecular and*
661 *Cellular* Endocrinology 82:191–198 DOI: 10.1016/0303-7207(91)90031-M.

662 Martignani E, Eirew P, Accornero P, Eaves C, Baratta M. 2010. Human milk protein production
663 in xenografts of genetically engineered bovine mammary epithelial stem cells. *PLoS ONE* 5:
664 e13372 DOI: 10.1371/journal.pone.0013372.

665 Massagué J. 1998. TGF-beta signal transduction. *Annual Review of Biochemistry* 67:753-791
666 DOI: 10.1146/annurev.biochem.67.1.753.

Mroue R, Inman J, Mott J, Budunova I, Bissell M. 2015. Asymmetric expression of connexins
between luminal epithelial- and myoepithelial- cells is essential for contractile function of the
mammary gland. *Developmental Biology* 399: 15-26 DOI: 10.1016/j.ydbio.2014.11.026.
667
668 Musters S, Coughlan K, McFadden T, Maple R, Mulvey T, Plaut K. 2004. Exogenous TGF-β1
669 promotes stromal development in the heifer mammary gland. *Journal of Dairy Science* 87:896-
670 904 DOI: 10.3168/jds.S0022-0302(04)73234-8.
671
672 Nguyen A, Pollard J. 2000. Transforming growth factor β3 induces cell death during the first
673 stage of mammary gland involution. *Development* 127: 3107-3118 PMID:10862748.

Nickerson S, Akers R. 2011. Mammary gland | anatomy. In: Fuquay J, ed. *Encyclopedia of Dairy
Sciences*. 2nd ed. San Diego: Academic Press. 328-337.
674
675 Pai V, Horseman N. 2011. Mammary Gland Involution: events, regulation and influence on
676 breast disease. In: Carrasco J, Mota M, eds. *Endothelium and epithelium: composition, functions
677 and pathology*. 1st ed. Cincinnati: Nova Science Publishers. 247-284.
678

Palin M-F, Farmer C, Duarte C. 2017. Triennial lactation Symposium/BOLFA: adipokines affect
mammary growth and function in farm animals. *Journal of Animal Sciences* 95:5689–5700
DOI:10.2527/jas2017.1777.

Pallegar N, Garland C, Mahendralingam M, Viloria-Petit A, Christian S. 2018. A novel 3-
dimensional co-culture method reveals a partial mesenchymal to epithelial transition in breast
cancer cells induced by adipocytes. *J Mammary Gland Biol Neoplasia*. In Press. DOI:
10.1007/s10911-018-9420-4.

679 Park H, Lee W, Jeong H, Song H. 2016. regeneration of bovine mammary gland in
680 immunodeficient mice by transplantation of bovine mammary epithelial cells mixed with
681 Matrigel. *International Journal of Stem Cells* 9:186-191 DOI: 10.15283/ijsc16044.

682 Perruchot M, Arévalo-Turrubiarte M, Dufreneix F, Finot L, Lollivier V, Chanat E, Mayeur F,
683 Dessauge F. 2016. Mammary epithelial cell hierarchy in the dairy cow throughout lactation. *Stem
684 Cells and Development* 25:1407-1418 DOI: 10.1089/scd.2016.0098.

685   Phan S. 2008. Biology of fibroblasts and myofibroblasts. *Proceedings of the American Thoracic*
686   *Society* 5:334-337 DOI:10.1513/pats.200708-146DR.

687   Plath A, Einspanier R, Peters F, Sinowatz F, Schams D. 1997. Expression of transforming
688   growth factors alpha and beta-1 messenger RNA in the bovine mammary gland during different
689   stages of development and lactation. *Journal of Endocrinology* 155:501-511 DOI:
690   10.1016/S0301-6226(97)00072-9.

691   Prosser C, Davis S, Farr V, Moore L, Gluckman P. 1994. Effects of close-arterial (external
692   pudic) infusion of insulin-like growth factor-II on milk yield and mammary blood flow in
693   lactating goats. *Journal of Endocrinology* 142:93-99 PMID: 7964289.
694
695   Rauner G, Barash I. 2012. Cell hierarchy and lineage commitment in the bovine mammary
696   gland. *PLoS ONE* 7: e30113 DOI: 10.1371/journal.pone.0030113.

697   Roarty K, Serra R. 2007. Wnt5a is required for proper mammary gland development and TGF-
698   beta-mediated inhibition of ductal growth. *Development* 134:3929-3939 DOI:
699   10.1242/dev.008250.

700   Safayi S, Korn N, Bertram A, Akers R, Capuco A, Pratt S, Ellis S. 2012. Myoepithelial cell
701   differentiation markers in prepubertal bovine mammary gland: effect of ovariectomy. *Journal of*
702   *Dairy Science* 95:2965-2976 DOI: 10.3168/jds.2011-4690.

703   Sechen, S. 2013. Bovine Somatotropin (bST) – Possible increased use of antibiotics to treat
704   mastitis in cows. Food and Drug Administration. Available at:
705   https://www.fda.gov/downloads/AnimalVeterinary/SafetyHealth/ProductSafetyInformation/UC
706   M383073.pdf (accessed May 14, 2018).

707   Singh K, Dobson J, Phyn C, Davis V, Farr A, Molenaar A, Stelwagen K. 2005. Milk
708   accumulation decreases expression of genes involved in cell–extracellular matrix communication
709   and is associated with induction of apoptosis in the bovine mammary gland. *Journal of Dairy*
710   *Science* 98:67-68. DOI: 10.1016/j.livprodsci.2005.10.016.

711   Tucker H. 2000. Hormones, mammary growth, and lactation: a 41-year perspective. *Journal of*
712   *Dairy Research* 83:874-878 DOI: 10.3168/jds.S0022-0302(00)74951-4.
713
714   The UniProt Consortium. 2017. UniProt: the universal protein knowledgebase. *Nucleic Acids*
715   *Research* 45:D158–D169 DOI: 10.1093/nar/gkw1099.
716
717   Weaver V, Lelièvre S, Lakins J, Chrenek M, Jones J, Giancotti F, Werb Z, Bissel M. 2002. β4
718   integrin-dependent formation of polarized three-dimensional architecture confers resistance to
719   apoptosis in normal and malignant mammary epithelium. *Cancer Cell* 2:205-216 PMID:
720   12242153.

721   Woodward T, Dumont N, O'Connor-McCourt M, Turner J, Philip A. 1995. Characterization of
722   transforming growth factor-β growth regulatory effects and receptors on bovine mammary cells.
723   *Journal of Cellular Physiology* 165:339-348 DOI: 10.1002/jcp.1041650215.

724   Woodward J, Rennie I, Burn J, Sisley K. 2005. A potential role for TGFbeta in the regulation of
725   uveal melanoma adhesive interactions with the hepatic endothelium. *Investigative*
726   *Ophthalmology & Visual Science* 46:3473-3477 DOI: 10.1167/iovs.04-1311.

727   Xu J, Lamouille S, Derynck R. 2009. TGF-beta-induced epithelial to mesenchymal transition.
728   *Cell Research* 19:156–172 DOI: 10.1038/cr.2009.5.

729   Zarzyńska J, Gajewska M, Motyl T. 2005. Effects of hormones and growth factors on TGF-β1
730   expression in bovine mammary epithelial cells. *Journal of Dairy Research* 72:39-48 DOI:
731   10.1017/S0022029904000639.

732   Zarzyńska J, Gajkowska B, Wojewódzka U, Dymnicki E, Motyl T. 2007. Apoptosis and
733   autophagy in involuting bovine mammary gland is accompanied by up-regulation of TGF-beta1
734   and suppression of somatotropic pathway. *Polish Journal of Veterinary Sciences* 10:1-9. PMID:
735   17388018.

736   Zarzyńska J, Motyl T. 2005. Dissimilar effects of LY 294002 and PD 098059 in IGF-I- mediated
737   inhibition of TGF-beta1 expression and apoptosis in bovine mammary epithelial cells. *Journal of*
738   *Physiology and Pharmacology* 56:181-193. PMID: 16077202.

739   Zavizion B, Gorewit RC, Politis I. 1995. Subcloning the MAC-T bovine mammary epithelial cell
740   line: morphology, growth properties and cytogenetic analysis. *Journal of Dairy Science* 78:515-
741   527 DOI: 10.3168/jds.S0022-0302(95)76662-0.

742   Zavizion B, van Duffelen M, Schaeffer W, Politis I. 1996. Establishment and characterization of
743   a bovine mammary epithelial cell line with unique properties. *In Vitro Cellular & Developmental*
744   *Biology- Animal* 32:138-148 DOI: 10.1007/BF02723679.

745   Zhang H, Bennett J, Smith K, Sunil N, Haslam S. 2002. Estrogen mediates mammary
746   epithelial cell proliferation in serum-free culture indirectly via mammary stroma-derived
747   hepatocyte growth factor. *Endocrinology* 143:3427–3434 DOI: 10.1210/en.2002-220007.

748   Zhang Y. 2009. Non-Smad pathways in TGF-β signaling. *Cell research* 19:128-139
749   DOI:10.1038/cr.2008.328.

750   Zhao S, Gao Y, Xia X, Che Y, Wang Y, Liu H, Sun Y, Ren W, Han W, Yang J, Lei L. 2017.
751   TGF-b1 promotes Staphylococcus aureus adhesion to and invasion into bovine mammary
752   fibroblasts via the ERK pathway. *Microbial Pathogenesis* 106:25-29 DOI:
753   10.1016/j.micpath.2017.01.044.

Effect of hormones on TGFβ1 expression in BME-UV1 and MAC-T cells

Hormone	Cell Lines	
	MAC-T	**BME-UV1**
Growth Hormone	No significant effect	↓ TGFβ1 protein levels in 10% FBS but ↑ TGFβ1 protein levels in 0.5% FBS
Somatostatin	No significant effect	↑ TGFβ1 protein levels in 10% FBS

1   Table summarizes select data from Zarzyńska, Gajewska & Motyl, 2005.

Comparison of MAC-T and BME-UV1 luminal and basal marker expression in relation to one another

	MAC-T	**BME-UV1**
CD10 Expression	Absent	Present
CD49f Expression	Present (higher in ADH)	Present
EpCAM Expression	Present (lower in ADH and ULA)	Present
CK14 Expression	Present	Absent
CK19 Expression	Absent	Present
ALDH Activity (ADH)	Lower	Higher

1   Table summarizes select data from Arévalo Turrubiarte et al., 2016. Unless otherwise indicated,
2   the expression indicated reflects results obtained under three different culture conditions:
3   standard adhesion (ADH), ultralow adherence (ULA) and Matrigel™.

Figure 1

Canonical TGFβ signalling

TGFβ1 binding to the constitutively active TβRII Ser/Thr kinase promotes its re-localization and the formation of a tetrameric complex with the TβRI, leading to phosphorylation and activation of TβRI ser/thr kinase. The latter in turn phosphorylates the Smad2/3 transcription factors, permitting their association with Smad4. The Smad2/3/4 complex translocates to the nucleus where in association with other transcription factors (not shown) modulates the expression of target genes that, among other effects, promote apoptosis and inhibit cell proliferation in normal mammary epithelial cells.
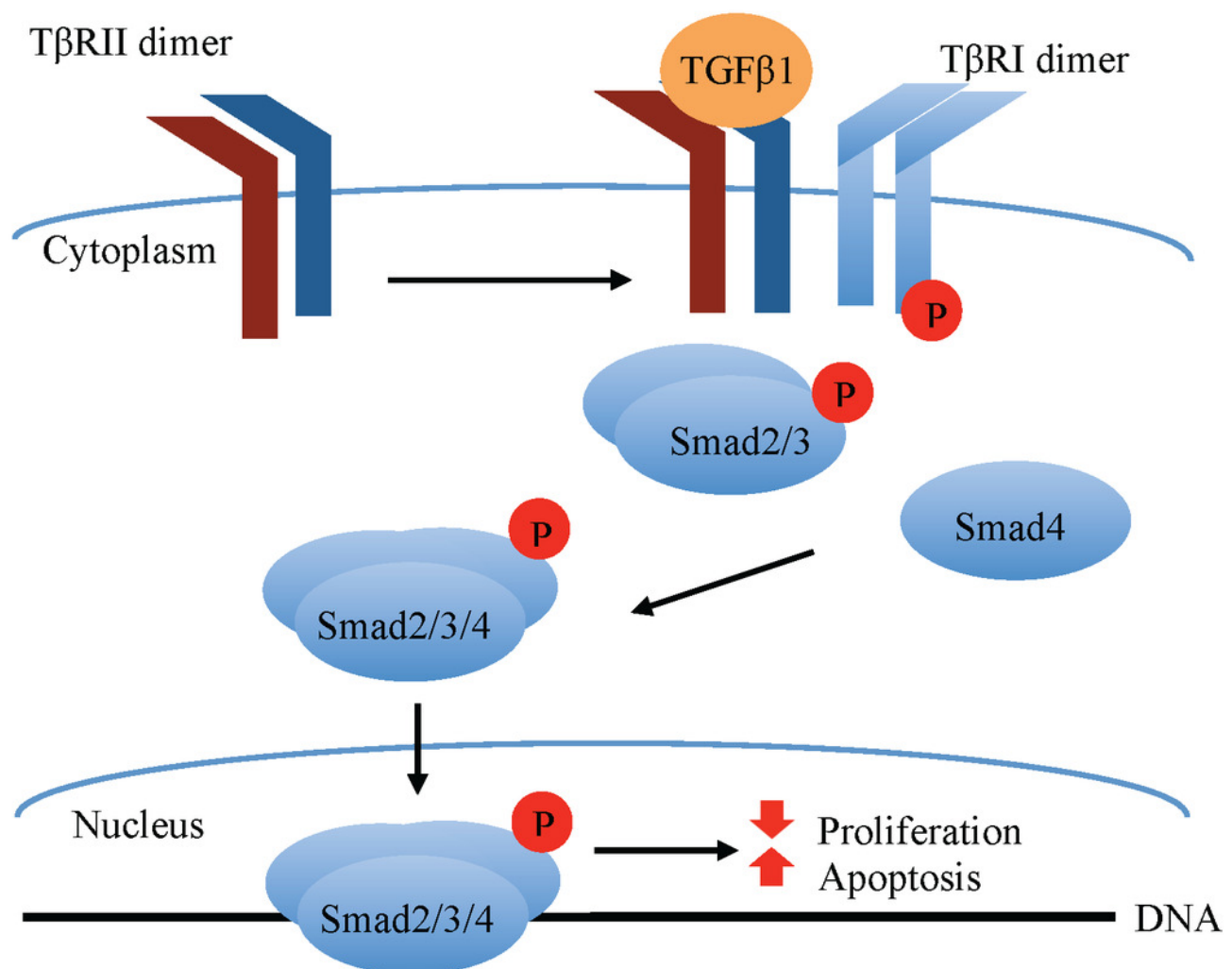
Figure 2

Crosstalk between TGFβ1 and the somatotropic pathway

TGFβ1 and IGF-1 repress each other' signalling and consequent celular response (apoptosis and survival, respectively) in bovine mammary epithelial cells. This involves the inhibition of TGFβ1 expression by IGF-1 and the blockade of IGF-1 signalling activation by TGFβ1-induced IGF binding proteins (IGFBPs). The exact mechanisms by which these occur have not been described for bovine mammary epithelium.