BMP4 induces terminal differentiation of primary trophoblast cells and increases chorionic gonadotrophin secretion.

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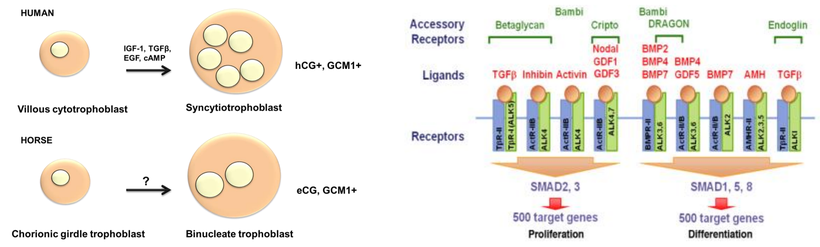
Supported by wellcome trust

Introduction

• Terminal differentiation of trophoblast cells and subsequent chorionic gonadotrophin production is critical to normal placental development and pregnancy outcome.

• The chorionic girdle (CG) is a discrete annular structure of the early equine conceptus that gives rise to the endometrial cups[1]. Rapidly proliferating trophoblast cells of the CG differentiate into eCG-secreting binucleate cells beginning at around day 32 of pregnancy[2].



• TGFβ proteins are abundantly expressed at the human and equine fetal-maternal interface. Specifically TGFβ1 is upregulated in the mare pregnant endometrium corresponding to the window of CG development[3,4].

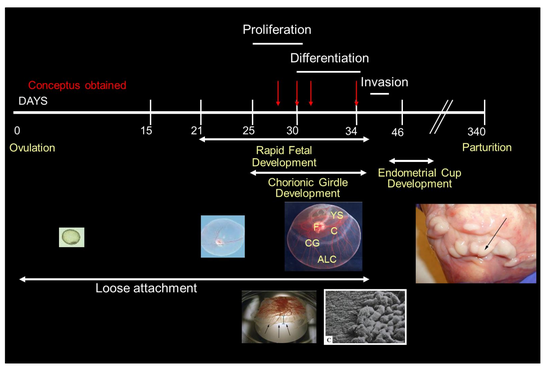
• The TGFβ superfamily SMAD2/3 pathway regulates placental function but the activity of the alternative pathway through SMAD1/5/8 in the placenta is unknown[3].

• TGFβ, activin and bone morphogenic protein 4 (BMP4) are implicated in differentiation of human embryonic stem cells that when treated are driven down a trophoblast lineage[4,5].

• **The aim of this study was to investigated the role of TGFβ signalling pathways in regulation of terminal differentiation of trophoblast cells of the CG.**

Materials and Methods

1. CG and chorion was isolated from day 27-34 equine conceptuses and either snap frozen or pure populations of chorionic girdle trophoblast cells were isolated. Cells were grown in the presence or absence of 1, 10, or 100 ng/ml BMP4 +/- 1μM A83-01 (SMAD2/3 pathway inhibitor) for up to 5 days.



2. Media from the cells was collected, spun down and the supernatant stored for subsequent eCG ELIZA (DRG International).

3. Differentiation was determined following labelling of the cells with CellTrace™ BODIPY® TR methyl ester and nuclear stain Hoeschst.

4. Uni-, and binucleated cells number was quantified in 5 randomly selected fields under an inverted fluorescent microscope, counted *post-hoc* with the use of Image J Cell Counter.

5. Equine specific primers were used to determine Type I and Type II and accessory receptor mRNA expression[1]. qRT-PCR and RT-PCR was conducted using standard methods as previously described[2].

6. Western blotting was used to detect expression of phospho- and Total SMAD5 and phospho- and Total SMAD2 (as control) in CG or chorion tissue lysates using antibodies directed against the human proteins (Cell Signaling, Technology, MA).

7. All data was subjected a one-way ANOVA statistical test with *post-hoc* Dunnetts Multiple Comparison Test or Bonferroni.

Results

Receptors for TGFβ superfamily ligands are tightly regulated in the chorionic girdle.

	Gene Name	Gene Symbol	Ligand	Fold Change CG vs CHR	Hyb Value CG
Type I receptors	Activin A receptor, type2 -like 1	ALK1	TGFB	NRD	0.35
	Activin A receptor, type I	ALK 2	BMP7	1.1 down	9.54
	Bone morphogenetic protein receptor, type IA	**ALK3**	**BMP4**	**1.1 up**	**6.03**
	Activin A receptor, type IB	ALK4	Activin	1.3 down	8.28
	Transforming growth factor, beta receptor 1	ALK5	TGFB	NRD	2.61
	Bone morphogenetic protein receptor, type IB	ALK6	BMP4	NRD	4.09
	Activin A receptor, type IC	ALK7	Nodal, GDF1, Vg1	40.5 up	5.47
Type II receptors	Transforming growth factor, beta receptor II	TGFBR2	TGFB	NRD	4.31
	Activin receptor type 2	ActR-IIB	Activin	NRD	3.29
	Bone Morphogenetic Protein Receptor type 2	**BMPR-II**	**BMP4**	**2.3 up**	**6.99**
Accessory Receptors	Transforming growth factor, beta receptor III	Betaglycan	TGFB	NRD	2.36
	Endoglin	Eng	TGFB	NRD	3.73
	RGM domain family, member B	**Dragon**	**BMP4**, BMP2	**1.2 up**	**6.67**
	Teratocarcinoma-derived growth factor 1	Cripto	Nodal , GDF3, GDF1	1.3 down	6.27
	BMP and activin membrane-bound inhibitor homolog	**Bambi**	**BMP4** Activin,	**1.3 down**	**12.36**

Table 1. Relative expression of TGFβ receptors in d34 CG compared to d34 chorion tissue. Data generated with a 44K gene probe equine expression array. NRD= expression in CG too low to reliably determine fold change.

Results

SMAD1,5 signalling is activated during chorionic girdle development *in vivo.*

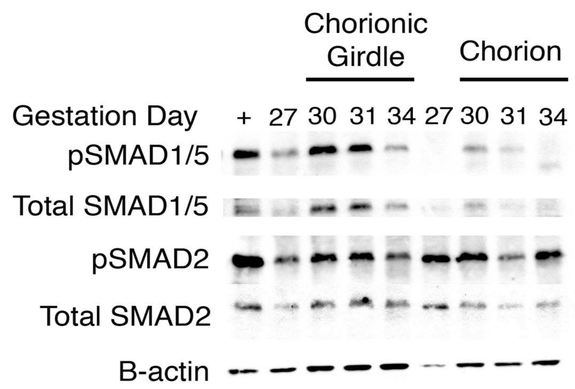


Figure 2 Western blot analysis of phospho- and Total SMAD1/5 and and phospho- and Total SMAD2 (as a control) in chorionic girdle and chorion tissue isolated from day 27, 30, 31 and 34 conceptuses. + = mouse spleen.

BMP4 receptors are expressed in CG trophoblast prior to and during the period of binucleate cell differentiation *in vivo.*

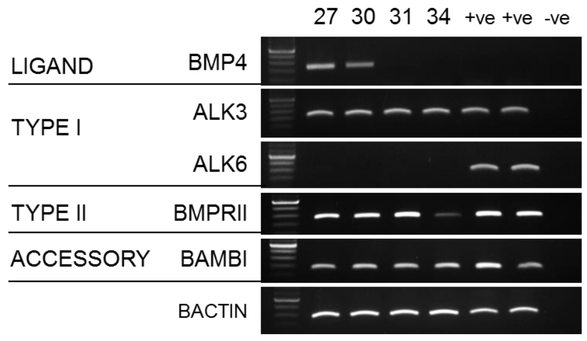


Figure 3. Representative RT-PCR gels showing temporal expression of type I, type II and accessory receptors specific for the ligand BMP4 in chorionic girdle tissue. 27, 30, 31 and 34 = days post ovulation, n=3. Testis and uterus used as positive controls.

BMP4 stimulates terminal differentiation of CG trophoblast cells *in vitro.*

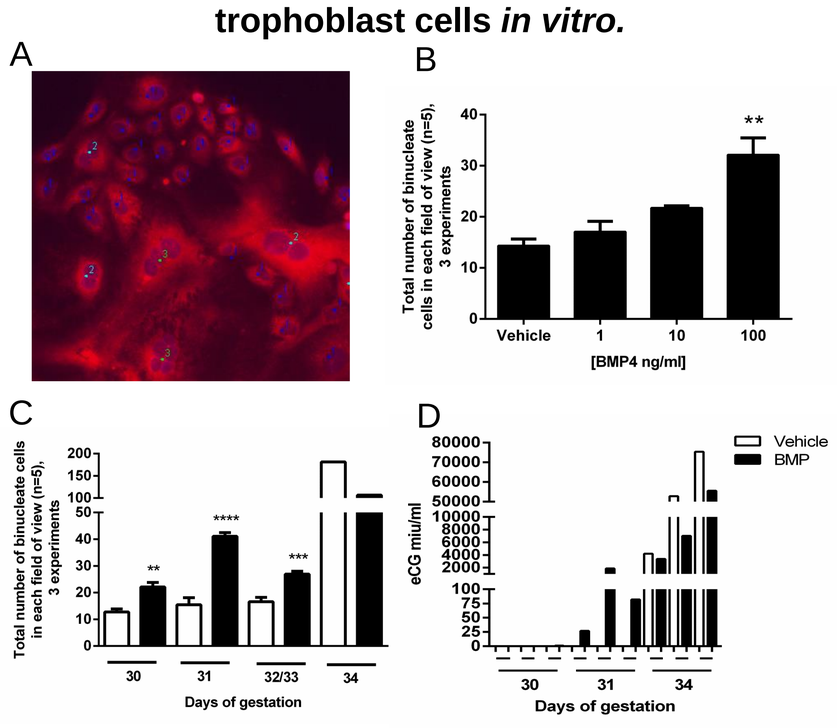


Figure 4 A. Representative images of CG trophoblast cells in culture. **B.** Quantification of binucleate CG trophoblast cells following culture in absence or presence of 1, 10, or 100 ng/ml BMP4 for 72h. **C.** Quantification of binucleate cells following culture in the presence (black bars) or absence (white bars) of 100 ng/ml BMP4 for 72h, at four different stages of CG development. **D**. eCG secretion from CG trophoblast cells treated in the absence or presence of 100 ng/ml BMP4 for 72h measured by eCG ELIZA.. ** p≤0.01, *** p≤0.001, **** p≤0.0001 relative to corresponding vehicle cells.

Inhibition of SMAD2/3 pathway with daily BMP4 stimulation leads to further increases in terminal differentiation of CG trophoblast cells *in vitro.*

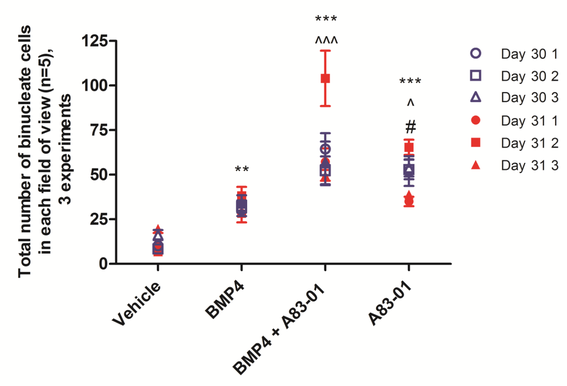


Figure 5. Quantification of binucleate cells following culture in the presence or absence of 100 ng/ml BMP4, 1μM A83-01 inhibitor, treated daily, for 5 days, at day 30 and 31 of gestation. ** p≤0.01 *** p≤0.001 relative to corresponding vehicle cells . ^ p≤0.05 ^^^ p≤0.001 relative to 100ng/ml BMP4. # p≤0.05 relative to 100ng/ml BMP4 plus 1μM inhibitor.

BMP4 expression indicates the ligand may act on the chorionic girdle primarily though paracrine signalling *in vivo.*

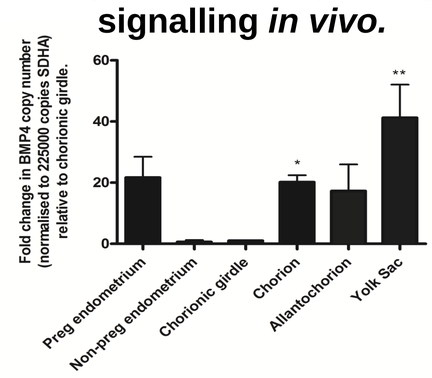


Figure 6. Spatial expression of the ligand BMP4 in endometrium (pregnant and non-pregnant), chorion, allantochorion and yolk sac relative to chorionic girdle tissue in day 27, 30 and 31 conceptuses. Data expressed as fold change over BMP4 copy number in the chorionic girdle. Copy numbers normalised to 225000 copies of the housekeeping gene SDHA.

BMP4 receptors are expressed in first trimester human placental tissue.

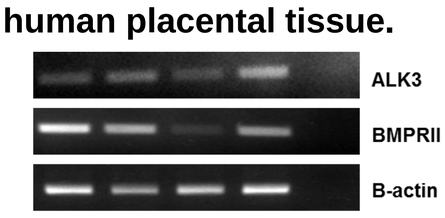


Figure 7. Representative RT-PCR gels showing temporal expression of ALK3 and BMPRII receptors specific for the ligand BMP4 in human placental tissue. 12+0, 6+2 and 11+0 = weeks and days of gestation, n=3. SAOS-02 = human sarcoma osteogenic cell line as positive control.

Summary and Conclusion

1) TGFβ receptor expression in the CG was tightly regulated. CG preferentially expresses receptors ALK3 and BMPR-II, BAMBI and Dragon, that bind the ligand BMP4.

2) SMAD1/5 signalling is activated during chorionic girdle development *in vivo* and peaks at Day 31 corresponding with the initiation of binucleate cell differentiation.

3) Type I, type II and accessory receptors specific to the ligand BMP4 are expressed in the CG trophoblast.

4) BMP4 significantly increased total binucleate cell number and eCG secretion at 100 ng/ml in day 31 CG trophoblast *in vitro*. Further, the response was dependent on the development stage.

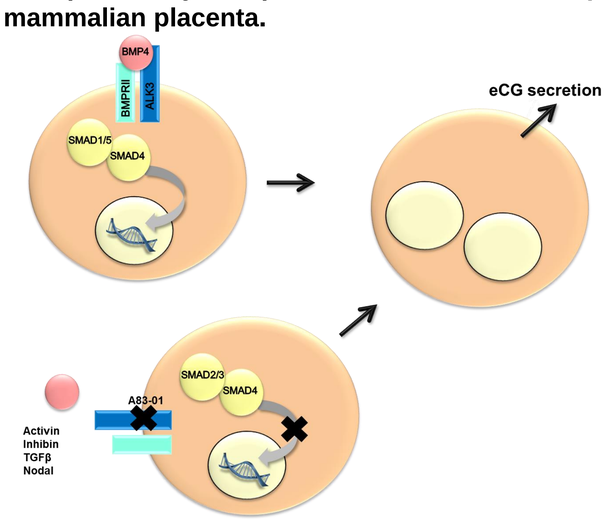
5) Inhibition of the SMAD2/3 signalling pathway in combination with 100ng/ml BMP4 further increases the differentiation rate of CG trophoblast *in vitro*.

6) The high expression of BMP4 in the chorion, allantochorion and yolk sac, the structures that abut the CG, suggests a level of paracrine regulation on the trophoblast cells.

7) Type I, type II and accessory receptors specific to the ligand BMP4 are expressed in early human placenta suggesting this pathway may also be active in syncytiotrophoblast differentiation.

In conclusion, our findings support a role for BMP4 signalling in the regulation of terminal differentiation of primary equine trophoblast cells via binding to BMPR-II and ALK3 and subsequent activation of SMAD1/5 pathway.

The observation of BMP4 signalling in primary trophoblast provides a previously unreported mechanism of TGFβ signalling in the mammalian placenta.



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Work is supported by a Wellcome Trust project grant to AdM and travel funded by the Society for Endocrinology.