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Understanding the effects of the bovine POLLED variants

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Summary

Horns are paired appendages on the head of bovine species, comprising an inner bony core and outer keratin sheath. The horn bud forms during early fetal development but ossification of the developing horn does not occur until approximately 1 month after birth. Little is known about the genetic pathways that lead to horn growth. Hornless, or polled, animals are found in all domestic bovids. Histological studies of bovine fetuses have shown that the horn bud does not form in polled individuals. There are currently four known genetic variants for polledness in cattle on BTA1. All of the variants are intergenic, but probably affect regulation of nearby genes or long non-coding RNAs. Transcriptomic studies suggest that the expression of two nearby long non-coding RNAs are affected by the Celtic POLLED variant, but further studies are required to confirm these data. Candidate genes located elsewhere in the genome are involved in regulating bone formation and epithelial-to-mesenchymal transition. Expression of one of these candidate genes, *RXFP2*, appears to be reduced in the fetal horn bud of polled animals carrying the Celtic variant compared with horned individuals. Investigating horn ontogenesis and the genetic pathway by which the POLLED variants prevent horn development has implications for cattle breeding. If the genetic basis of horn bud formation and polledness is better understood, then new targets may be identified for precision genome editing to create polled individuals.

Keywords Bovidae, cattle, Celtic, epithelial-to-mesenchymal transition, facial bone, horns, scurs

Introduction

Horns are cranial appendages of bovine species, which include antelope, goats, sheep and cattle. The primary function of horns is male competition for mates (Lundrigan 1996), but they are also used for protection against predators and to aid in competition for resources (Stankowich & Caro 2009), and they may be involved in thermoregulation (Pares-Casanova & Caballero 2014). However, domestic cattle with horns pose a risk to other cattle and handlers (Knierim *et al.* 2015), and can result in economic losses because of damaged hides and bruised tissue which must be trimmed when the meat is processed (Mendonca *et al.* 2016; Youngers *et al.* 2017).

In order to avoid issues related to horns, calves are disbudded using a hot iron, scoop dehorners or caustic paste to prevent horn growth (Animal Health Australia 2014; Cozzi *et al.* 2015). The pain and distress caused to animals by disbudding and dehorning procedures is well documented (Knierim *et al.* 2015). Beyond this distress, there is the potential for the wound site to become infected and compromise animal growth, and the procedure is an additional labour cost to producers (Stafford & Mellor 2005; Bates *et al.* 2015; Bates *et al.* 2016).

Welfare guidelines recommend that preference should be given to breeding hornless, or polled, cattle over dehorning (Animal Health Australia 2014). However, introgression of the genetic variants for polled into specialised breeds (e.g. dairy, beef and tropically adapted breeds) leads to genetic loss of production traits (e.g. milk yield). This is because polledness is usually introduced into a herd by breeding with animals that have lower genetic merit or by crossing with another breed.

Advances in precision genome editing have the potential to introduce variants into the genome without compromising genetic gain. Gene editing allows a desirable phenotype to be introgressed into a population through a known DNA variant. Alternatively, a genetic target can be identified and altered (e.g. by an amino acid substitution) to observe the effect on the phenotype. Although genetic variants for

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polled are known, the pathways that lead to horn formation and the mechanisms by which the complex bovine POLLED variants result in the hornless phenotype are unknown.

Horn morphology, development and inheritance

Horn and scur morphology

Horns of bovids are permanent, paired and symmetrical appendages that vary vastly in morphology between species and even breeds (Davis *et al.* 2011). Horns have two main parts: a 'dead' keratin outer sheath and a bony inner core of 'living' tissue (Zhu *et al.* 2016). Between the keratin sheath and bony core are several layers of tissue: the periosteum (tissue that lines the bones), subcutaneous connective tissue, dermis and epidermis (Davis *et al.* 2011). True horns have a bony core that is attached to the frontal bones and a frontal sinus that extends into the horn spike.

Scurs are horn-like appendages that can occur in bovids, but tend to be shorter than true horns (Capitan *et al.* 2011). The phenotype of scurs varies, ranging from small 'scabs' in the horn bud to appendages as long as 15 cm (Capitan *et al.* 2011). Scurs and horns have two main anatomical differences: (1) the scur is not anchored to the skull and the frontal sinus does not continue into the horn spike; and (2) the bony core of scurs is densely ossified compared with the pneumatised bony core of horns (Capitan *et al.* 2011).

Horn and scur inheritance

The inheritance of horns, polledness and scurs in cattle has been studied since the early 1900s. Understanding the pattern of inheritance was a challenging task for early researchers owing to the epistatic relationship that POLLED has with other loci, and the subsequent difficulties in inferring the genotype of an individual (reviewed by Prayaga 2007). Two types of scurs have been identified in cattle, Type I and Type II, and these have distinct inheritance patterns. In summary:

- Horned (p) is the wt state in cattle and is recessive to POLLED (P).
- Type I scurs are epistatic to POLLED and appear to be sex influenced; however, the inheritance pattern of scurs is unclear (White & Ibsen 1936; Blackwell & Knox 1958; Long & Gregory 1978; Wiedemar *et al.* 2014). Difficulty in determining the inheritance pattern of scurs is attributed to problems with phenotyping, inconsistent age of scur development, sex influence, epistasis with POLLED loci and genetic heterogeneity within breeds (Asai *et al.* 2004; Tetens *et al.* 2015; Grobler *et al.* 2018). Evidence supports the presence of a SCURS locus on BTA19 (Asai *et al.* 2004), and potential loci on BTA2, 9 and 10 (Tetens *et al.* 2015). Early research suggested that homozygous polled males could be polled or scurred (White & Ibsen 1936; Long & Gregory 1978); however, more recent studies have genotyped scurred cattle and found that they were always heterozygous polled (Wiedemar *et al.* 2014; Grobler *et al.* 2018). It was also assumed that scurred females were always homozygous at the SCURS locus (White & Ibsen 1936; Long & Gregory 1978); however, homozygosity mapping of BTA19 in scurred females did not identify a shared homozygous haplotype (Tetens *et al.* 2015).
- Type II scurs are the result of a mutation in *TWIST1* as observed in French Charolais cattle (Capitan *et al.* 2009; Capitan *et al.* 2011). The Type II scur phenotype is dominant over horns, but not over polled (A. Capitan, personal communication). Animals homozygous for the *TWIST1* mutation have not been identified, suggesting embryonic lethality.
- Horns in some zebu cattle breeds may be epistatic to POLLED in males rather than recessive (Smith 1927). In a cross between horned African zebu breeds and Angus, all female progeny were polled, but male progeny had one of three phenotypes: horned, scurred and polled (Smith 1927). This led to the suggestion that another gene is involved in this mode of inheritance, denoted as African horn (Ha) (White & Ibsen 1936). However, the existence of this locus has not been confirmed.

Development of horns

Originally, horn development was thought to be an outgrowth of the skull to form the horn spike. However, horns develop from a separate centre of ossification within the horn bud. Dove (1935) conducted a series of horn bud tissue transplants in young calves and goat kids to identify the origin of horn development and found that horn growth arises from the dermis and hypodermis, and not from the frontal bone. Bony processes develop in the horn bud, and as the neonate ages, the bone attaches to the skull and simultaneously grows outwards to produce the horn spike.

The horn bud was originally reported to be first visible in bovine fetuses at 60 days (Evans & Sack 1973). However, recently, the horn bud was observed by the authors at 58 days of development (Aldersey J.E., Sonstegard T.S., Williams J.L. & Bottema C., unpublished data). At 58 days, there is a ring of depressed tissue at the position where the horn bud develops, which is not visible in polled fetuses of the same age. At 70 days, the horn bud is reported to be well defined and appears as a small, yellowish spot on the fetal head (Wiener *et al.* 2015). By 90 days, the horn bud becomes slightly indented compared with the surrounding smooth skin (Wiener *et al.* 2015).

There are several histological differences between the horn bud and nearby frontal skin throughout bovine fetal development (Table 1) (Capitan *et al.* 2012; Allais-Bonnet *et al.* 2013; Wiener *et al.* 2015). Firstly, the epidermis of the horn bud is thicker than the epidermis of the frontal skin

(Wiener *et al.* 2015). Secondly, hair follicle development occurs later in the horn bud than surrounding tissue; hair follicles are present at 3–4 months of gestation in frontal skin but are not observed in the horn bud until 5–6 months of gestation (Wiener *et al.* 2015). Lastly, the horn bud has thick nerve bundles whereas nerve bundles are absent in frontal skin (Capitan *et al.* 2012; Allais-Bonnet *et al.* 2013; Wiener *et al.* 2015). Similar observations have been made for yak fetuses (Li *et al.* 2018). There is no evidence of ossification in the fetal horn bud (Wiener *et al.* 2015), and horn growth and ossification occur approximately 1 month after birth (Dove 1935). Thus, the horn bud differentiates during early fetal development but horn growth does not occur until after the calf is born.

POLLED genetic variants

The POLLED genetic locus for cattle was first localised to bovine chromosome 1 (BTA1) by linkage mapping (Georges *et al.* 1993), and the position was later refined to the centromeric region in several studies (Schmutz *et al.* 1995; Brenneman *et al.* 1996; Harlizius *et al.* 1997). Four DNA sequence variants have subsequently been identified on BTA1 that are associated with the polled phenotype: Celtic POLLED (P_C), Friesian POLLED (P_F), Mongolian POLLED (P_M), and Guarani POLLED (P_G) (Medugorac *et al.* 2012; Allais-Bonnet *et al.* 2013; Rothammer *et al.* 2014; Medugorac *et al.* 2017; Utsunomiya *et al.* 2019). All known variants are dominant, and cattle carrying a single POLLED variant will be either polled or scurred, depending on their genotype at the SCURS loci. It is likely that other unidentified POLLED variants exist in different populations and breeds (e.g. Shuxuan; Chen *et al.* 2017a).

Table 1 Bovine fetal development of frontal skin and horn bud tissue (Wiener *et al.* 2015).

Gestation length (months)	Frontal skin	Horn bud
2–3	Epidermis has three layers of vacuolated keratinocytes	Epidermis has seven layers of vacuolated keratinocytes
3–4	Epidermis has four layers	Epidermis has 12 layers
	Immature hair follicles present	No hair follicles present
	No nerve bundles present	Nerve bundles present
5–6	Epidermis has six layers	Epidermis has 12 layers
	Hair follicles and sebaceous glands present	Hair follicles and sebaceous glands present
	No nerve bundles present	Nerve bundles present and more pronounced
7–8	Keratinocytes are no longer vacuolated	Keratinocytes are no longer vacuolated
	Hair follicles and sebaceous glands present	Hair follicles and sebaceous glands present

Celtic POLLED variant

The Celtic POLLED variant was first identified in several European beef breeds originating from Celtic geographical areas. The variant is a complex insertion and deletion (indel). A 212 bp sequence (1 705 834–1 706 045 bp)[1] is duplicated and replaces a sequence of 10 bp (1 706 051–1 706 060-bp) that is 6 bp downstream of the original sequence (Fig. 1) (Medugorac *et al.* 2012). Independent association studies found that the indel was the only variant at this site that segregated completely with polledness (Allais-Bonnet *et al.* 2013; Wiedemar *et al.* 2014). The Celtic variant was found to be functionally responsible for polledness by gene editing the variant into wt (horned) crossbred Holstein fibroblasts, which were cloned to produce polled calves (Carlson *et al.* 2016). The progeny of horned dams and the gene-edited Holstein bulls produced from these fibroblasts, which were shown to only carry the Celtic allele and no other unintended edits, were also polled. The Celtic POLLED variant is located between the genes *IFNAR2* and *OLIG1* on BTA1 and does not appear to disrupt any known coding sequence, splice site or intronic region, or any known regulatory regions (Medugorac *et al.* 2012). The variant may interrupt a predicted *HAND1* enhancer site (Nguyen *et al.* 2018), although this is yet to be confirmed experimentally.

Friesian POLLED variant

First identified in Holstein-Friesian cattle, the Friesian POLLED variant is approximately 200 kb downstream of the Celtic variant and is an 80 128 bp duplication of the sequence between 1 909 352 and 1 989 480 bp (Fig. 1) (Medugorac *et al.* 2012; Allais-Bonnet *et al.* 2013; Rothammer *et al.* 2014). The duplicated segment is located immediately after the original sequence and is in the same orientation. It differs from the reference sequence by one T→A transversion at the third position and by a 2 bp deletion (TG) at the 45th position. Further research confirmed that this variant segregated in polled Holsteins that did not carry the Celtic POLLED allele (Wiedemar *et al.* 2014). As with the Celtic POLLED variant, the Friesian POLLED variant does not disrupt any known coding sequence, splice site or intronic region, or any known regulatory regions (Rothammer *et al.* 2014).

Mongolian POLLED variant

A third bovine POLLED variant has been discovered in Mongolian yaks and Mongolian Turano cattle (Medugorac *et al.* 2017). There are horned and polled individuals in these populations, and owing to their isolation, this

[1]All genomic locations refer to the UMD3.1 build. Updated coordinates are available on the OMIA website (omia.org/OMIA000483/9913).

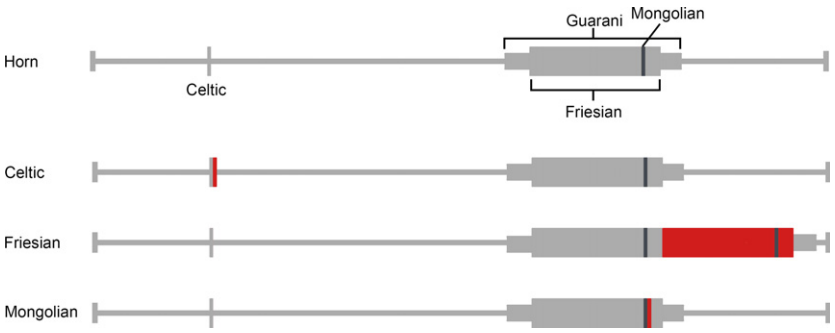

Figure 1 Celtic, Friesian and Mongolian polled variants. The grey rectangles represent the duplicated sequence, and red rectangles represent the insertion site of the duplications. The Guarani variant has not been completely defined at the time of publication.

POLLED variant was suspected to be a spontaneous mutation that had not previously been described.

Whole genome sequencing of a homozygous and heterozygous polled yak localised the Mongolian POLLED locus to an 800 kb region on BTA1 (Medugorac *et al.* 2017). The position of the locus was further refined by genotyping and two variants associated with the polled phenotype in Turano cattle and yaks were identified. The first variant was a 219 bp duplication–insertion 61 bp downstream from the original sequence (P_{219ID} beginning 1 976 128 bp) and the second was a 6 bp deletion and 7 bp insertion 621 bp upstream from P_{219ID} (Medugorac *et al.* 2017) (Fig. 1). Within the 219 bp duplicated sequence, an 11 bp motif (5′-AAAGAAGCAAA-3′) is entirely conserved among Bovidae, and therefore, may be functionally important (Medugorac *et al.* 2017). Intriguingly, the 219 bp sequence is also located within the Friesian variant and Guarani variant (see below), and therefore, the 219 bp sequence (and consequently, the 11 bp conserved motif) is duplicated in the Mongolian, Friesian and Guarani variants (Fig. 1). Haplotype analysis showed that the Mongolian variant is located on a bovine DNA segment, and the variant was introgressed from Turano cattle into Mongolian yaks (Medugorac *et al.* 2017).

Guarani POLLED variant

A fourth variant, Guarani POLLED (P_G), has been recently identified in Nellore cattle (*Bos indicus*) from Brazil (Utsunomiya *et al.* 2019). The polled phenotype in Nellore cattle was traced to a single polled bull, which implies that polledness in the breed is not the result of one of the previously discovered variants. Whole genome sequencing of polled Nellore bulls identified an approximately110 kb sequence (1 893 790–2 004 553 bp) within the POLLED region with increased coverage, indicating a copy number variation caused by an approximately 110 kb duplication. The insertion location of the duplication is yet to be determined. Intriguingly, SNP genotyping of the P_G region in the polled Nellore bulls confirmed that the Guarani variant originated from *Bos taurus* (Utsunomiya *et al.* 2019).

Phenotypes associated with POLLED

Polled fetuses carrying the Celtic variant do not develop horn buds, forming only smooth tissue that is histologically indistinguishable from frontal skin tissue (Allais-Bonnet *et al.* 2013; Wiener *et al.* 2015). Horn bud development is also absent in yak fetuses carrying the Mongolian variant (Li *et al.* 2018), but has not been investigated in fetuses homozygous for the Friesian and Guarani variants.

In addition to the complete absence of horn growth, the POLLED variants are associated with several other phenotypes. The skull morphology of polled cattle is characterised by a narrower and peaked poll (Dove 1935); however, it is unclear whether this phenotype is a result of the POLLED variants affecting skull development or due to the absence of horns, which would cause the outgrowth of the frontal sinus. Polled cattle that carry the Celtic or Friesian variants also have a second row of eyelashes (Fig. 2). Allais-Bonnet *et al.* (2013) examined 78 polled cattle and characterised the phenotype as additional eyelash growth and hypertrichosis (excessive hair growth) of the eyelid. There have been no reports regarding atypical eyelash growth for cattle carrying other variants. There are also no reports that this eyelash phenotype has any detrimental effects on polled individuals.

Bulls from Angus and other polled breeds are more likely to develop a spiral deviation of the penis, a so-called ‘corkscrew penis’ (Blockey & Taylor 1984). The corkscrew penis tends to occur in bulls at least 3 years old and reduces pregnancy rates owing to poor servicing (McDiarmid 1981; Blockey & Taylor 1984). A spiral deviation of the penis has been detected in 11–27% of polled breeds (Angus, Poll Hereford, Poll Shorthorn, Red Poll and Murray Grey) compared with 0–1% of horned Herefords (McDiarmid 1981; Blockey & Taylor 1984). However, it is not known if there is a direct association between the polled phenotype and corkscrew penis.

There have also been reports of preputial abnormalities (preputial prolapse) in polled (P_C) Charolais bulls, caused by poor development or absence of retractor muscles of the prepuce (Prayaga 2007; Allais-Bonnet *et al.* 2013). When assessed for preputial prolapse, two of two homozygous polled (P_C/P_C) Charolais bulls and 11 of 14 heterozygous

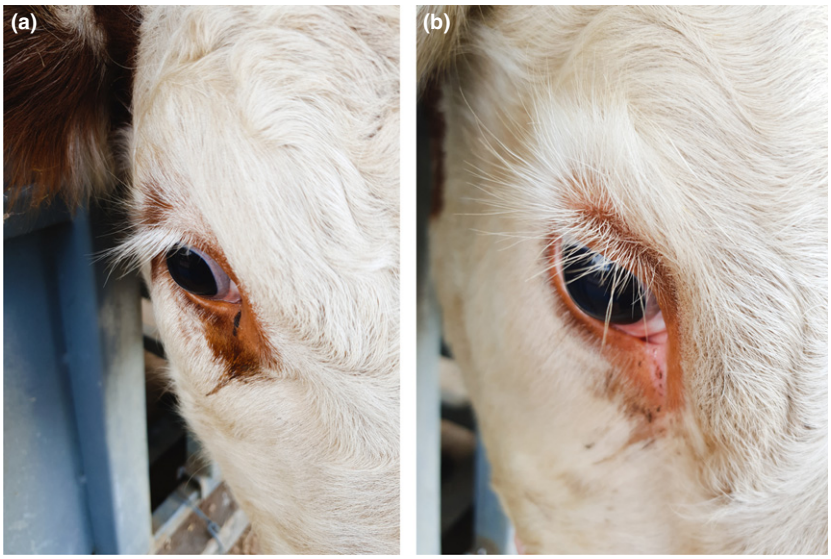

Figure 2 Eyelashes of horned cow (a) and double eyelashes of polled cow (P_CP_C) (b).

polled (P_C/p) Charolais bulls had this defect. However, this abnormality has not been observed in other breeds carrying the Celtic or Friesian POLLED variants or in horned animals. Therefore, the preputial defect appears to be a breed-specific loci interacting with or in LD the POLLED variant (Allais-Bonnet *et al.* 2013). The prepuce defect makes sheath cleaning prior to semen collection difficult; however, it does not appear to affect other reproductive traits or the health of the affected individuals (Allais-Bonnet *et al.* 2013). There have been no reports of other phenotypes associated with polledness in carriers of the Mongolian and Guarani variants.

Candidate genes

As the POLLED variants are not located in any known genes, long non-coding RNAs or microRNAs, it is postulated that the variants affect the expression of genes or non-coding RNAs by disrupting regulatory DNA elements, such as enhancers. The POLLED variants are located within one predicted topologically associating domain (TAD) (1 226 028–2 201 452 bp) containing 23 protein coding genes and non-coding RNAs (Fig. 3) (Wang *et al.* 2018). TADs are regions of a genome where there are more interactions between loci within a domain than between loci located in different domains (Dixon *et al.* 2012; Szalaj & Plewczynski 2018). There is evidence that TAD boundaries act as genetic insulators, ensuring appropriate enhancer–promoter interactions (Dixon *et al.* 2012; Krivega & Dean 2017). Disruption of TAD boundaries can lead to increased interactions between TADs, resulting in an altered phenotype (Yu & Ren 2017; Furlong & Levine 2018).

Two long intergenic non-coding RNAs (lincRNA) have been described within the POLLED predicted TAD, *LincRNA#1* and *LincRNA#2* (*LOC100848368* and *LOC112447133* respectively, in the ARS-UCD1.2 assembly) (Allais-Bonnet *et al.* 2013). LincRNAs are defined as non-coding RNA longer than 200 nucleotides which do not occur within protein coding genes (Deniz & Erman 2017). LincRNAs are expressed at low levels and appear to be tissue or cell type specific (Deniz & Erman 2017). They can regulate gene expression by various methods, including binding to mRNA, miRNA and chromatin modifying complexes, and interacting with transcription factors (Deniz & Erman 2017).

Other candidate genes that may be involved in horn development, outside the POLLED TAD on BTA1, include genes that are (1) associated with the polled phenotype in other bovid species, (2) have variants associated with syndromes that include a polled phenotype or (3) have variants associated with distichiasis (abnormal eyelash growth) in other species (Table 2). One of these candidate genes, *FOXC2*, which is associated with distichiasis in humans, was identified as a horn-specific gene in a study of Bovidae transcriptomes (Wang *et al.* 2019). This study identified 624 horn-specific genes using transcriptomes from 16 tissues, including horn sprouts from goats and sheep, and fetal horn bud and frontal skin from sheep (Wang *et al.* 2019), but no other candidate genes (Table 2) were found to be horn-specific. *FOXC2* is highly expressed in horn tissue and bone (Wang *et al.* 2019). *FOXC2* was also found to be differentially expressed between the horn bud and frontal skin of horned (p/p) bovine fetuses at 90 days of development (Allais-Bonnet *et al.* 2013). These studies suggest that *FOXC2* may be involved in horn development.

Gene and protein expression in horned vs. polled horn bud

Gene expression studies of horn bud tissue from horned and polled cattle can be used to identify genetic pathways

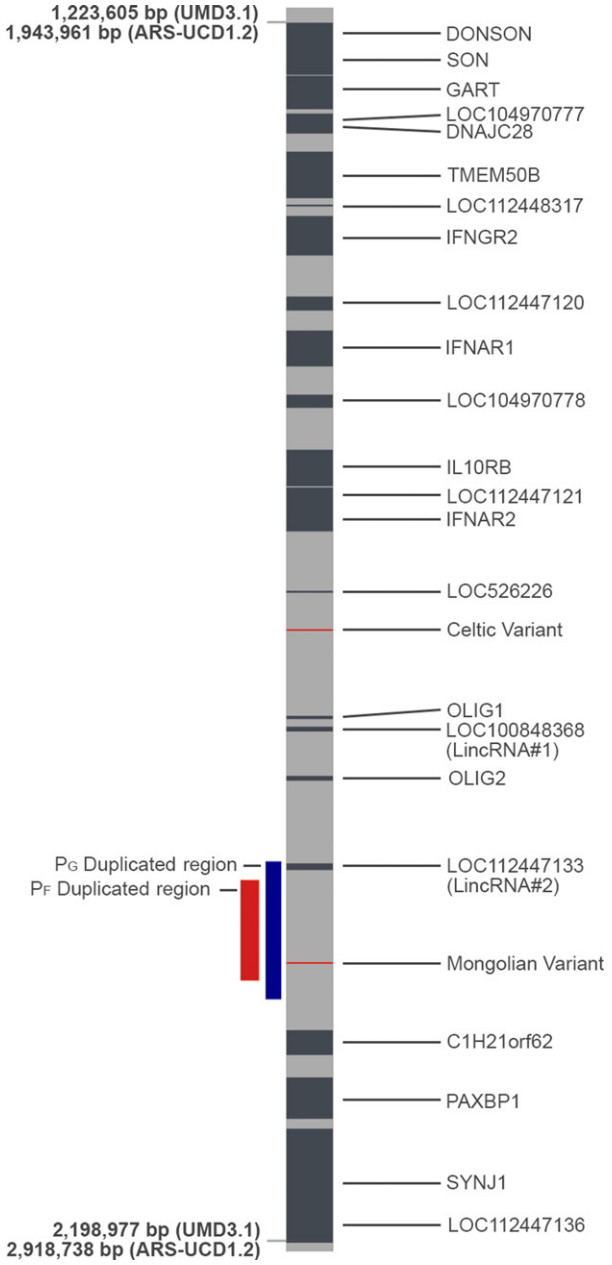

Figure 3 Gene map of predicted topologically associating domain containing POLLED variants on BTA1.

involved in normal horn development and provide clues about the mechanism by which POLLED variants prevent horns. Several studies have investigated gene expression and protein abundance in bovine fetal and neonatal horn bud tissue (Allais-Bonnet *et al.* 2013; Wiedemar *et al.* 2014; Li *et al.* 2018).

Gene expression in fetal horn bud tissue

The first study where gene expression was investigated in the fetal horn bud examined the genes and lincRNA 500 kb upstream and downstream of the Celtic variant: *GART*, *TMEM50B*, *IFNGR2*, *IFNAR1*, *IL10RB*, *IFNAR2*, *OLIG1*, *LincRNA#1*, *OLIG2*, *LincRNA#2*, *C1H21orf62* and *PAXBP1* (Allais-Bonnet *et al.* 2013). Candidate genes *RXFP2*, *FOXL2*, *ZEB2*, *TWIST1*, *TWIST2* and *FOXC2* were also analysed. Biopsies from the horn bud and frontal skin regions of seven polled (P_c/p) and seven horned (p/p) fetuses at 90 days of pregnancy were examined using qRT-PCR. *RXFP2* and *LincRNA#1* were differentially expressed between horned and polled horn bud tissue (Table 3). Expression of *RXFP2* was lower in the polled fetuses than in horned fetuses ($P < 0.05$). The expression of *LincRNA#1* was slightly higher in the horn bud region of polled vs. horned fetuses ($P = 0.052$) (Allais-Bonnet *et al.* 2013). Although the differential expression of *LincRNA#1* was not quite significant between horned and polled horn bud tissue, it should be noted that lincRNAs are difficult to detect. This study also did not assess gene expression in homozygous polled fetuses, in which a larger effect on gene expression may be expected. In addition, differential expression of genes leading to horn bud formation is likely to occur before 90 days of development, as the horn bud is apparent before 60 days of gestation. Therefore, important expression differences in the genes may not have been observed.

An RNAseq study of one horned fetus (150 days post fertilisation) and one polled fetus (158 days post fertilisation) identified significant differences in the gene expression of *OLIG1*, *OLIG2*, *C1H21orf62*, *RXFP2*, *FOXL2* and *LincRNA#2* (Wiedemar *et al.* 2014). These RNAseq results were subsequently examined by qRT-PCR using horn bud and frontal skin biopsies from 21 fetuses that ranged from 70 to 175 days of fetal development. *LincRNA#2*, *RXFP2* and *FOXL2* appeared to be more highly expressed in horned fetuses than polled fetuses at all time points; however, these expression differences were not statistically significant.

The differences in fetal age and uncertainty arising from the small sample size makes it difficult to compare the results from Allais-Bonnet *et al.* (2013) and Wiedemar *et al.* (2014). *RXFP2* was reported to be differentially expressed in both studies, whereas *LincRNA#1*, *LincRNA#2* and *FOXL2* were only reported to be differentially expressed in one of the studies. *RXFP2* had reduced expression in polled horn bud tissue compared with wt horn bud tissue of the fetus. *RXFP2* is on BTA12, and therefore, the mechanism by which the Celtic variant affects *RXFP2* expression is not clear. Interestingly, an insertion in *RXFP2* has been linked with polledness in some European sheep breeds (Wiedemar & Drogemuller 2015; Luhken, *et al.* 2016) and SNPs in *RXFP2* have been associated with ovine horn size and shape (Pan *et al.* 2018). Thus, *RXFP2* may play a role in horn growth and shape, rather than horn bud formation *per se*.

A proteomic study of three polled (P_M) and three-horned yak fetuses at 80–90 days development investigated

Table 2 Candidate genes that may be involved in horn development, but are not located within the POLLED locus on BTA1.

Gene (location)	Function[1]	Association with polledness	Reference
RXFP2 (BTA12)	*Relaxin family peptide receptor 2:* encodes a G-coupled, 7-transmembrane receptor	Variants in *RXFP2* associated with polledness and horn shape in sheep	Wiedemar & Drogemuller (2015); Luhken *et al.* (2016); Pan *et al.* (2018)
FOXL2 (BTA1)	*Forkhead Box L2:* may be involved in ovarian development and function	Loss of function of both *FOXL2* alleles causes Polled Intersex Syndrome in goats	Boulanger *et al.* (2014)
ZEB2 (BTA2)	*Zinc Finger E-Box Binding Homeobox 2:* represses transcription by interacting with activated SMADs	Deletion including *ZEB2* causes Polled and Multisystemic Syndrome in cattle	Capitan *et al.* (2012)
TWIST1 (BTA4)	*Twist Family BHLH Transcription Factor 1:* involved in embryonic development including cranial suture closure	Mutation causing frameshift in *TWIST1* causes Type II scurs in cattle and haploinsufficiency causes craniosynostosis (premature fusion of skull)	Capitan *et al.* (2011)
TWIST2 (BTA3)	*Twist Family BHLH Transcription Factor 2:* may inhibit osteoblast maturation	Mutation in TWIST2 causes Setleis syndrome in humans involving abnormal skull morphology and distichiasis (eyelashes on inner eyelid)	Cervantes-Barragan *et al.* (2011)
FOXC2 (BTA18)	*Forkhead Box C2:* undetermined function but may be involved with mesenchymal tissue development	Mutations in FOXC2 cause syndromes with distichiasis in humans	Sargent *et al.* (2014); Zhang *et al.* (2016)

[1]Gene Cards Suite (2019).

Table 3 Summary of published differentially expressed genes revealed by qPCR comparison of wt and polled fetal horn bud tissue.

Gene	70 day old fetuses (Wiedemar & Drogemuller 2015)	90 day old fetuses (Allais-Bonnet *et al.* 2013)
GART	–	NDE
TMEM50B	–	NDE
IFNGR2	–	NDE
IFNAR1	–	NDE
IL10RB	–	NDE
IFNAR2	–	NDE
OLIG1	–	NDE
LincRNA#1	–	↑[1]
OLIG2	NDE	NDE
LincRNA#2	↓	NDE
C1H21orf62	NDE	NDE
PAXBP1	–	NDE
FOXL2	↓	NDE
RXFP2	↓	↓[2]
TWIST1	–	NDE
ZEB2	–	NDE
TWIST2	–	NDE
FOXC2	–	NDE

–, Not analysed by qPCR; NDE, not differentially expressed; ↓, decreased expression in polled vs. horned horn bud; ↑, increased expression in polled vs. horned horn bud.
[1]Significance = 0.052.
[2]Significance < 0.050.

differentially abundant proteins (DAPs) in tissue from the horn bud region (Li *et al.* 2018). This study identified 29 upregulated proteins and 71 downregulated proteins in the polled fetus compared with horned fetuses. Classification of proteins by Protein Analysis Thorough Evolutionary Relationships (PANTHER) showed that upregulated DAPs were related to metabolic activities, whereas downregulated DAPs were related to cell junction, cytoskeleton formation and cell component organisation. Overall, the DAPs had functions involving cell adhesion, cell motility, keratinocyte differentiation, cytoskeleton organisation, osteoblast differentiation and fatty acid metabolism. Although there were DAPs involved in osteoblast differentiation, bone development in the horn bud does not occur at this stage of fetal development. Proteins associated with cell structure and organisation may be differentially abundant owing to the structural differences between horned and polled fetal horn bud. For example, by 80–90 days of development nerve bundles are present in the wt horn bud and absent in the polled horn bud region.

Gene expression in neonatal horn bud tissue

Gene expression has been also examined in horn bud tissue of neonatal calves (Mariasegaram *et al.* 2010). A study of cDNA from the horn bud tissue of 1–2 week old Brahman calves with polled, scurred and horned phenotypes revealed no difference in expression of genes located within the predicted POLLED TAD region (Mariasegaram *et al.* 2010). The microarray used in the study included *DONSON*, *SON*, *GART*, *TMEM50B*, *IFNGR2*, *IFNAR1*, *IL10RB*, *IFNAR2*, *OLIG1*, *OLIG2* and *PAXBP1*. However, there were no probes for *LOC194970777*, *DNAJC28*, *LOC112448317*, *LOC112447120*, *LOC104970778*, *LincRNA#1*, *LincRNA#2* or *C1H21orf62*. The array included most functional candidate genes outside of the POLLED region, namely *RXFP2*, *TWIST1*, *FOXL2* and *FOXC2*, but not *ZEB2* and *TWIST2*. These functional candidate genes were not differentially expressed. However, the microarray analysis identified 93 other genes that were differentially expressed between horn and polled calves. Genes with greater expression in polled

calves were structural components of cell junctions, and genes with lower expression had functions relating to extracellular regions (Mariasegaram *et al.* 2010).

Candidate pathways

Mammalian embryonic origins of bone and bone formation

As horns are partly bone, pathways involved in bone formation may be disrupted by the POLLED variants. Bone tissue is derived from the mesoderm and cranial neural crest. During embryo development, the mesoderm differentiates into paraxial, intermediate and lateral mesoderm. Only the paraxial and lateral mesoderm form bone; the former is the source of the axial skeleton (ribs, vertebrae and parietal bones of the skull) and the latter creates the appendicular skeleton (limbs) (Jin *et al.* 2016; Sheebaa *et al.* 2016). The cranial neural crest cells migrate to form the frontal and facial bones (Wu *et al.* 2017), and these cells are the most likely candidates to form horns in Bovidae species. In an immunohistochemistry study of sheep fetuses, cells expressing genetic markers for neural crest cells (SOX10 and NFGR) were found in the fetal horn bud at 90 days of development (Wang *et al.* 2019).

A gene within the predicted POLLED locus TAD, *PAXBP1*, potentially plays a role in facial bone development (Blake & Ziman 2014). In humans, PAXBP1 is a binding protein that links transcription factors PAX3 and PAX7 to histone methylation machinery (The UniProt Consortium 2019). The PAXBP1 and PAX3/PAX7 interaction is primarily associated with myogenesis, but there is evidence that PAX3/PAX7 is involved cranial facial development (Blake & Ziman 2014; Monsoro-Burq 2015). A missense mutation in *PAXBP1* leads to dysmorphia in facial bones of humans (Alharby *et al.* 2017) and PAX3 is involved in neural crest specification, delamination, cell survival during migration and differentiation (Monsoro-Burq 2015). Currently, there is no experimental evidence connecting *PAXBP1* to horn growth; however, gene expression of cranial neural crest tissue in horned and polled fetuses has not been assessed. Understanding the lineage of cells that form the horn bud would aid in determining which developmental pathways are disrupted by the POLLED variants.

The cranial facial bones are produced via intramembranous ossification whereby bone tissue forms directly from the condensed mesenchymal cells (Ishii *et al.* 2015; Jin *et al.* 2016; Wu *et al.* 2017). Several candidate genes are involved in regulation of intramembranous ossification. The *TWIST* genes regulate ossification, and mutations in *TWIST1* often cause craniosynostosis, early closure of the cranial sutures (Hayashi *et al.* 2007; Connerney *et al.* 2008; Derderian & Seaward 2012; Huang *et al.* 2014). Additionally, there is evidence that the ligand of RXFP2, relaxin, induces oesteogenic differentiation through the activation of regulators of intramembranous ossification, namely, alkaline phosphatase, RUNX2 and BMP2 (Duarte *et al.* 2014). *RXFP2* expression is lowered in the horn bud region of polled fetuses. Thus, the formation of horn bud bone tissue could be prevented by reduced availability of the RXFP2 receptor. The POLLED variants may affect several other stages of horn bud formation, including blocking the bone precursor cells from successfully migrating to the horn bud or differentiating to bone tissue (Fig. 4). A comparison of the transcriptomes of cranial neural crest and horn bud tissue from horned and polled fetuses pre- and post-neural crest cell migration may resolve the affected pathways.

Epithelial-to-mesenchymal transition

Four of the candidate genes (*TWIST1*, *TWIST2*, *ZEB2* and *FOXC2*; Table 2) encode transcription factors that regulate the epithelial-to-mesenchymal transition (EMT). EMT occurs during embryo implantation, embryogenesis and organ development, and is one of the processes that results in the diversification of cell types and the development of tissues which create organs (Kalluri & Weinberg 2009). During EMT, epithelial cells undergo a series of biochemical changes to become mesenchymal cells (Kalluri & Weinberg 2009). For instance, as part of neural crest cell delamination, the epithelial cells of the neural crest change to migratory mesenchymal cells. Thus, altered gene expression of EMT related transcription factors may contribute to the polled phenotype. Reduced expression of *E-cadherin*, the protein that forms adhesion junctions between cells, is a key event in EMT. Interestingly, expression of the *E-cadherin* gene is directly repressed by the transcription factors encoded by *TWIST1*, *TWIST2*, *ZEB2* and *FOXC2* (Chen *et al.* 2017b). The

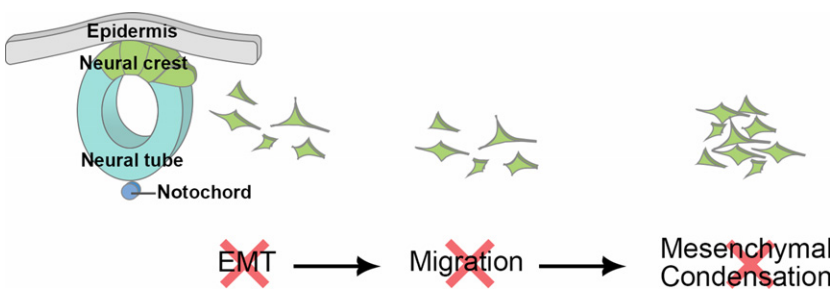

Figure 4 Hypothetical mechanisms whereby the POLLED variants may prevent horn bud formation. EMT, Epithelial-to-mesenchymal transition.

expression of these genes and various EMT markers (*E-cadherin*, *N-cadherin*, *occludin* and *vimentin*) has been examined in the horn bud for bovine fetuses at 90 days of development; however, the gene expression did not differ between horned and polled fetuses (Allais-Bonnet *et al.* 2013). This suggests that EMT is not occurring in the horn bud at 90 days of fetal development. To further explore the effect of the POLLED variants on EMT, expression of EMT candidate genes and markers should be assessed in cranial neural crest cells from the midbrain region in horned and polled fetuses. However, expression may need to studied before the horn bud is visible at 58 days, as the midbrain starts to form between 32 and 41 days of development in *Bos indicus* embryos (Assis *et al*. 2009).

Conclusions

There are four DNA sequence variants currently known to produce the polled phenotype in cattle; however, all of these variants are intergenic. Comparison of gene expression of horn bud tissue in polled and horned fetuses suggests that *LincRNA#1* and *LincRNA#2*, two long intergenic non-coding RNA located near the POLLED variants on BTA1, and *RXFP2* located on BTA12 could be involved in the development of horns. Based on these gene expression studies, the most likely hypothesis is that the POLLED variants affect the regulation of *LincRNA#1* and *LincRNA#2*. However, given there are phenotypic differences between horned and polled fetuses at 58 days of fetal development, the effect of the POLLED variants is likely to have occurred earlier. RNAseq and chromatin interaction studies of tissues from younger horned and polled fetuses would provide information on gene expression differences and regulatory DNA elements within the genomic POLLED region. This information would help to determine whether bone formation, cell migration, EMT and/or other processes are involved in the control of horn development in bovids.

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