Oocyte peptides as paracrine tools for ovarian stimulation and oocyte maturation

David G. Mottershead¹ and Andrew J. Watson²,³,⁴

¹Robinson Institute, School of Paediatrics and Reproductive Health, Medical School, The University of Adelaide, Adelaide 5005, Australia
²Department of Obstetrics and Gynaecology, Children’s Health Research Institute-Lawson Health Research Institute, The University of Western Ontario, London, ON, Canada
³Department of Physiology and Pharmacology, Children’s Health Research Institute-Lawson Health Research Institute, The University of Western Ontario, London, ON, Canada
⁴Correspondence address. E-mail: awatson@uwo.ca

ABSTRACT: Recent studies report the production and isolation of a stable bioactive recombinant human bone morphogenetic protein 15 (rhBMP15) that is appropriately processed in HEK-293 cells and activates the SMAD 1/5/8 pathway in mouse granulosa cell cultures. Further, the purified rhBMP15 induces the expression of genes associated with cumulus expansion. Thanks to recent research, we have a greater understanding of the importance of the dialogue that occurs between the oocyte and the granulosa cell layer with regard to regulating folliculogenesis and the acquisition of oocyte developmental competence and maturation. BMP15 is one of the critical components of these intra-follicular communication pathways. The production of recombinant human BMP15 is important for understanding the biochemistry of this specific pathway and for also fully understanding its functional contributions to mediating oocyte development. The production of a stable recombinant human BMP15 is also important for use in experiments aimed at optimizing ovarian stimulation protocols and in vitro oocyte maturation methods. This is required to improve oocyte and embryonic developmental competence and increase our ability to effectively use in vitro methods for animal production and the treatment of human infertility.

Key words: growth factors / protein biochemistry / folliculogenesis / assisted reproductive technology / embryo transfer

Introduction

Controlled ovarian stimulation with gonadotrophins is an important method of stimulating follicular development, and for collecting more than the normal numbers of maturing oocytes from a wide variety of mammalian species (Cantineau et al., 2007; Horcajadas et al., 2007; Loutradis et al., 2008; Delvigne, 2009; Klemmt et al., 2009; Lussiana et al., 2009; Verberg et al., 2009). This method has been applied to literally dozens of species although perhaps most routinely to the mouse (for research purposes), the cow, sheep and pig (for animal production purposes) and the human (for addressing infertility; Cantineau et al., 2007; Fauzdar et al., 2009; Figueira et al., 2009; Klemmt et al., 2009; Raziel et al., 2009; Sismanoglu et al., 2009).

In all cases, however, the outcome from all ovarian stimulation protocols is unpredictable. This occurs due to a number of factors including ovarian cycle synchronization or suppression, ovarian reserve, subject age and variation in pharmacokinetics between subjects and genetic factors to name a few (Cantineau et al., 2007; Aboulghar, 2009; Delvigne, 2009; Fauzdar et al., 2009; Figueira et al., 2009; Klemmt et al., 2009; Raziel et al., 2009; Sismanoglu et al., 2009).

An additional and quite likely a predominant factor that is perhaps not fully appreciated by all concerned is that the dialogue between the oocyte and the associated follicle may be suboptimal at the time of retrieval after gonadotrophin stimulation (Eppig et al., 1997; Eppig, 2001; Matzuk et al., 2002). In fact, the number and quality of the follicles and their oocytes that develop may not be determined by the hypothalamus and the pituitary, but instead be regulated by intra-ovarian factors (McNatty et al., 2004). Therefore, to standardize ovarian stimulation protocols and to achieve reproducible and reliable outcomes, it will be necessary to ensure the levels of intra-ovarian factors are optimal and if not, supplement those levels by administering recombinant peptides.

The study by Li et al. (2009) brings us closer to that goal as they describe the production and isolation of a stable bioactive recombinant human bone morphogenetic protein 15 (rhBMP15). The rhBMP15 peptide is appropriately processed in HEK-293 cells and the purified protein activates the SMAD 1/5/8 pathway in primary cultures of mouse granulosa cells. Further, the purified rhBMP15 induces the expression of genes associated with cumulus expansion, namely Ptx3, Has2, Tnfalfp6 and Pigs2. Thus, in total, this new purified rhBMP15 protein mimics a great number of known BMP15 functions and represents an important new agent for regulating folliculogenesis and oocyte developmental competence.
**Intra-ovarian factors**

Although the hypothalamic–pituitary–gonadal axis plays an indisputable and vital role in regulating folliculogenesis, the concerns regarding the variability in response following the administration of gonadotrophin-based stimulation protocols extend well beyond the simple variation in follicle or oocyte numbers (Cantineau et al., 2007; Horcajadas et al., 2007; De Roover et al., 2008; Loutridis et al., 2008; Aboughar, 2009; Fauzdar et al., 2009; Figueira et al., 2009; Verberg et al., 2009; Vloeberghs et al., 2009). There is an increasing concern that oocytes generated from ovarian stimulation protocols do not achieve a normal developmental competence likely due to variations in oocyte mRNA and protein pools, and possibly due to abnormal variations in epigenetic patterning (Fauque et al., 2007; Sato et al., 2007; Meng et al., 2008). Since these components of oocyte developmental competence are likely influenced or regulated by intra-ovarian factors, it may well be necessary to employ recombinant intra-ovarian factors in combination with gonadotrophins to achieve optimal oocyte developmental competence in vivo and in vitro.

**Growth differentiation factor 9 and BMP15 as key oocyte-derived factors**

Research in recent years has focused on defining the molecular dialogue that occurs between the oocyte and the granulosa cells as the oocyte grows (Gilchrist et al., 2008). Oocyte paracrine growth factors acting on neighboring granulosa cells are important regulators of follicular growth (Eppig, 2001). Attention has primarily focused on the members of the transforming growth factor-β (TGF-β) family, including members such as growth differentiation factor 9 (GDF9) and BMP15. The deletion or mutation of Gdf9 or Bmp15 can greatly affect fertility (Dong et al., 1996; Galloway et al., 2000). BMP15 null mice display cumulus cell dysfunction, whereas GDF9 null mice display failed folliculogenesis (Dong et al., 1996; Yan et al., 2001). Thus, these factors are not only required for early folliculogenesis, but they are also potent mediators of granulosa cell differentiation (Gilchrist et al., 2004a; Juengel and McNatty, 2005; McNatty et al., 2007). There are important differences between species, e.g. mice are able to function without BMP15, however this is an essential factor for both human and sheep fertility (Montgomery et al., 2001; Yan et al., 2001; Teixeira Filho et al., 2002; McNatty et al., 2003; Di Pasquale et al., 2004; Hanrahan et al., 2004; Moore et al., 2004; Dixit et al., 2006). Interestingly, Gdf9 or Bmp15 heterozygosity results in increased fertility in sheep (Montgomery et al., 2001; Hanrahan et al., 2004), but once again no obvious effect is observed on mouse fertility (Dong et al., 1996; Yan et al., 2001). In humans, abnormal expression of GDF9 has been linked to polycystic ovarian syndrome (Teixeira Filho et al., 2002), and mutations in Gdf9 and Bmp15 are associated with premature ovarian failure (Di Pasquale et al., 2004; Dixit et al., 2006) and dizygotic twinning (Montgomery et al., 2004; Palmer et al., 2006).

As with all members of the TGF-β family, GDF9 and BMP15 are produced in a pro-form which is proteolytically processed during synthesis and secretion (Massagué, 1990). This means that the final product consists of an amino-terminal pro-region and a smaller biologically active carboxy-terminal mature region. A particular characteristic of GDF9 and BMP15 is that they lack the fourth conserved cysteine residue which is normally present in TGF-β family members to enable the formation of an inter-monomer disulphide bridge (McPherron and Lee, 1993; Dubé et al., 1998; Laitinen et al., 1998). Thus, GDF9 and BMP15 form non-covalently associated homodimers and intriguingly they may be able to form heterodimers in culture (Liao et al., 2003; McNatty et al., 2003, 2004), which raises the possibility of greater molecular interactions in vivo. These factors signal via TGF-β receptors and activate SMAD cascades (Kaivo-oja et al., 2006). Within the ovary, the granulosa cells express most components of the TGF-β family signaling system, including type-II receptors and ALK type-I receptors, co-receptors such as β-glycan, binding proteins such as follistatin and the SMAD and co-SMAD intracellular messengers (Juengel and McNatty, 2005; Kaivo-oja et al., 2006). Clearly, GDF9 and BMP15 are among the most important intra-ovarian factors as their roles in mediating oocyte–granulosa cell interactions during folliculogenesis would indicate. It is thus critical that we develop molecular tools that will enable a full understanding of their biochemistry and function.

**Recombinant GDF9 and BMP15**

GDF9 was the first of these two oocyte-secreted factors to be produced as a recombinant protein (Elvin et al., 1999; Hayashi et al., 1999). However, neither of these two reports were the recombinant protein purified, being used in various bioassays as an impure preparation. This situation, and the fact that impure preparations have been used throughout the published literature (Elvin et al., 1999; Hayashi et al., 1999; Kaivo-oja et al., 2003, 2005; Gilchrist et al., 2004b; Hickey et al., 2005; McNatty et al., 2005a, b), may well be the prime cause for the differing results that have been reported over the years concerning the bioactivity of GDF9 (Elvin et al., 1999; Dragovic et al., 2005). Indeed, only in 2008, the characterization of a purified recombinant GDF9 has been published (Mottershead et al., 2008). There have also been sporadic reports of the use of a purified commercially available bacterially produced GDF9 (MARTINS et al., 2008; HUANG et al., 2009; SHI et al., 2009). The issue with any bacterially produced TGF-β family member is the question of what percentage of the refolded protein is correctly folded to give biologically active protein. Further, a bacterially produced protein will lack post-translational modifications, such as glycosylation and phosphorylation, which are of particular relevance for BMP15 and GDF9 (McMAHON et al., 2008; Sato et al., 2008). Hence, any bacterially produced GDF9 or BMP15 should preferably be compared with a purified mammalian cell produced version of the protein, before being confident of having a correctly folded protein with full bioactivity.

The purification and initial characterization of a recombinant BMP15 were first published in 2000 by the Shimasaki laboratory (OTSUKA et al., 2000). This material was a version of the human BMP15 protein with a carboxy-terminal FLAG tag, instrumental for the purification of the protein via immunoaffinity chromatography. However, as pointed out by Li et al. (2009), there has been a concern over the effect of the carboxy-terminal FLAG tag on BMP15 protein stability and bioactivity, as previously it has been found that a carboxy-terminal tag fused to TGF-β (WAKEFIELD et al., 1991), or GDF9 (MOTTERSHEAD et al., 2008) is detrimental for bioactivity. The recombinant human BMP15
produced by Di Pasquale and associates (Di Pasquale et al., 2004; Bodin et al., 2007; Rossetti et al., 2009) also suffers from the same issue; in this case, the carboxy-terminal tag is even longer, consisting of both the Myc and His6 tags in tandem. The new report from the Matzuk laboratory (Li et al., 2009) has placed the affinity purification tag (in this case, the FLAG tag) close to the amino-terminus of the mature region of hBMP15, leaving the carboxy-terminus unmodified. Further, and most importantly, in this current study, the authors have compared their purified N-FLAG tagged hBMP15 with a recently commercially available untagged hBMP15 mature region. Hence, we can be confident that the bioactivity of the epitope-tagged growth factor represents that also of the untagged native sequence growth factor. The effect of an epitope tag on the bioactivity of both human and mouse GDF9 has been clearly described previously (Mottershead et al., 2008).

Apart from one recent study (McIntosh et al., 2008), the issue of the role/effect of the pro-region of BMP15 or GDF9 on the bioactivity of the corresponding mature region is yet to be tackled by the workers in this field. Within the TGF-β family, the effect of the pro-region on the bioactivity of the corresponding mature region covers the full spectrum from potentiation (AMH/MIS; Wilson et al., 1993) to no effect (BMP9; Brown et al., 2005) to inhibition (TGF-β; McMahon et al., 1996). This is why it will be important to determine the situation for GDF9 and BMP15, by comparing the bioactivity of the purified (under native conditions) pro-mature complex for each growth factor with that of the corresponding purified mature region. Currently, commercially available mammalian cell produced forms of mGDF9 and hBMP15 contain just the mature regions of the respective proteins. Therefore, data based solely on experiments using these proteins should be interpreted cautiously given that we do not know how the pro-region affects the bioactivity of the mature region. To date, all the published, well-characterized purified recombinant BMP15 proteins (Saito et al., 2008; Li et al., 2009) have contained both the pro-region as well as the mature region. The situation for the published recombinant GDF9s (Elvin et al., 1999; Hayashi et al., 1999; Kaivo-Oja et al., 2003, 2005; Gilchrist et al., 2004b; Liao et al., 2004; Hickey et al., 2005; McNatty et al., 2005a, b) is most likely to be similar, although one study has produced and characterized the purified GDF9 mature region (Mottershead et al., 2008), and a side-by-side comparison with a pro-mature complex awaits future studies.

In the current study by Li et al. (2009), the purified rhBMP15 protein induces the expression of a number of genes in murine granulosa cells, a subset of which are associated with cumulus expansion, namely Ptx3, Has2, Tnfip6 and Pgs2. This raises the issue of the involvement of GDF9 and BMP15 in the process of cumulus expansion, a controversial area where differing results have been obtained by groups using their own versions of unpurified (Elvin et al., 1999) or partially purified (Dragovic et al., 2005; Dragovic et al., 2007) recombinant GDF9, as well as one study using a purified BMP15 (Yoshino et al., 2006). Although any extensive discussion as to the nature of the cumulus cell expansion enabling factor (CEEF) is beyond the scope of this review, it is hoped that now with the availability of purified recombinant BMP15 and GDF9 (Li et al., 2009; Sugiuara et al., 2009) that some of the issues surrounding the identity of the CEEF (Pangas and Matzuk, 2005) will be addressed. A further issue that should be investigated now that these proteins are available is the presence or absence of various post-translational modifications on the recombinant BMP15 and GDF9 proteins, both of which recently have been reported to be phosphorylated (McMahon et al., 2008). This modification is crucial to investigate further, as it is the first time that any member of the TGF-β family has been reported to be phosphorylated, and further, it was stated that this particular modification was necessary for biological activity.

**BMP15 and GDF9 as reagents for in vitro oocyte maturation**

In vitro oocyte maturation has been employed for producing preimplantation embryos from predominantly agricultural species (cow, sheep, goat and pig) for over 25 years. It is now increasingly being employed as a method to treat human infertility. The main, well-documented concern with this approach is that embryos produced from oocytes matured in vitro display a reduced developmental capacity than embryos produced from in vivo matured oocytes (Banwell and Thompson, 2008; Duranthron et al., 2008; Rinaudo and Lamb, 2008; Watkins et al., 2008; Watkins and Fleming, 2009; Wells et al., 2009). At the molecular level, variations in gene expression patterns and abnormalities in epigenetic programming are increasingly being documented in embryos and fetuses derived from oocytes matured in vitro (Banwell and Thompson, 2008; Duranthron et al., 2008; Rinaudo and Lamb, 2008; Watkins et al., 2008; Watkins and Fleming, 2009; Wells et al., 2009). Our understanding of the reasons for these differences in embryos produced from in vitro and in vivo matured oocytes is rudimentary at best but likely lies in important variations in the environment in which the oocyte develops. The most obvious difference is that in vitro matured oocytes are removed from the follicular environment and are therefore likely missing or are underexposed to the critical intra-ovarian factors that control the acquisition of oocyte developmental competence (Banwell and Thompson, 2008; Duranthron et al., 2008; Rinaudo and Lamb, 2008; Watkins et al., 2008; Watkins and Fleming, 2009; Wells et al., 2009).

Recent studies have indicated that the addition of BMP15 (Hussein et al., 2006) or GDF9 (Hussein et al., 2006; Yeo et al., 2008) increases developmental competence when added within an in vitro maturation setting. In particular, Yeo et al. (2008) demonstrated that addition of GDF9 to oocyte in vitro culture medium has a dramatic effect on the number of day 15 mouse fetuses, indicating that if the oocyte is in good shape at the start of the procedure, the chances of obtaining a viable and healthy pregnancy increase as well. This beneficial effect on the fetal development rate by supplementing in vitro oocyte culture medium with ovarian paracrine factors is quite likely to apply to BMP15 supplementation as well, given the effect of BMP15 on the blastocyst rate and quality (Hussein et al., 2006). However, a limitation of both of these studies (Hussein et al., 2006; Yeo et al., 2008) is that they involved the use of only partially purified recombinant BMP15 and GDF9, and it is very likely that some of the beneficial effects of GDF9 and BMP15 have been masked by inhibitory components from the 293H cell conditioned media.

Thus, the production of a purified recombinant human BMP15 will allow for studies aimed at optimizing in vitro oocyte maturation protocols. The expected benefits include an increase in oocyte
developmental competence, improved embryo quality and pregnancy rates following embryo transfer, and likely even increased fetal viability.

Conclusions

In the past 10 years, research has revealed great insight into the dialogue that occurs between the oocyte and the granulosa cell layer, raising our understanding of the importance of paracrine and autocrine intra-ovarian factors in regulating folliculogenesis and acquisition of oocyte-developmental competence and maturation. BMP15 is one of the critical components of these intra-follicular communication pathways. The production of recombinant human BMP15 will afford an important opportunity to investigate the biochemistry of this specific pathway and will provide an opportunity to fully understand its functional contributions to mediating oocyte development. In addition, the production of a stable recombinant human BMP15 provides an important agent for experiments aimed at optimizing ovarian stimulation and in vitro oocyte maturation protocols. Such optimization should provide for improved embryo developmental competence and increase our ability to use in vitro methods for increasing animal production and the treatment of human infertility. Finally, the production of recombinant BMP15 will allow for experiments aimed at rescuing follicular defects in BMP15 null mice as a prelude to demonstrating the therapeutic value that recombinant BMP15 could have on the health of human patients.

References


Hanrahan JP, Gregan SM, Mulans P, Mullen M, Davis GH, Powell R, Galloway SM. Mutations in the genes for oocyte-derived growth factors GDF9 and BMP15 are associated with both increased


Submitted on August 26, 2009; resubmitted on September 30, 2009; accepted on September 30, 2009.