

REDESIGNING NATURE: DEVELOPING A MORE POTENT BMP2 MOLECULE FOR
EXPRESSION IN A TRANSGENIC PUROINDOLINE-RICE EXPRESSION SYSTEM

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ABSTRACT

Bone Morphogenetic Protein 2 (BMP2) is a cytokine growth factor that elicits *de novo* bone formation in adult mammals. The use of BMP2 in surgical applications ranges from spinal fusion procedures to off-label uses such as dental implant augmentation. Currently, 1.5 mg/ml of BMP2 are necessary for these surgical procedures. The use of such relatively high concentrations of BMP2 leads to ectopic bone formation, provokes immune reactions hence rendering treatments ineffective and adds greatly to the overall expense of these therapeutic treatments. An engineered mutant BMP2 designed to have higher biological potency over the current wild type recombinant human BMP2 would reduce both dosages and costs in biomedical applications. The synthesis of a codon optimized DNA sequence designed for expression in rice would ensure high fidelity expression of such recombinant protein products in a biotech rice protein production platform. Although designed for rice recombinant protein expression, the codon optimized DNA sequence produces a fragment that corresponds to the theoretical fragment size of the C-terminal, mature monomeric peptide of BMP2 when expressed in *E. coli*. Results from *in silico* modelling of mutant BMP2 ligands docked with BMP receptors suggested that only certain mutations are tolerated at the L51 and D53 positions. Only certain mutants might have the same affinity for the receptor as the wild type due to steric interactions with other side chains on both the ligand itself and the receptor. For those mutants that did not possess steric conflicts, the recombinant L51-series BMP2 mutants produced a circular dichroism spectrum that was unique and differed from the spectrum of wild-type BMP2. C2C12 alkaline phosphatase activity assays of the wild-type BMP2 protein produced activity similar to previously published results, while the thirteen L51 substituted BMP2 mutant collection samples showed no bioactivity similar to a known negative activity mutant at this position. The expression potential of the codon optimized DNA BMP2 sequence in rice was calculated by comparing several computer generated sequences from online programs. Such models were used to assess whether the recombinant BMP2 possesses similar bioactivity to the BMP2 expressed in mammalian expression systems currently used.

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LIST OF ABBREVIATIONS

BMP2	Bone Morphogenetic Protein 2
rhBMP2	Recombinant Human Bone Morphogenetic Protein 2
BMPRIA	Bone Morphogenetic Protein Receptor IA
CD	Circular Dichroism
C2C12	Murine Promyoblast Cells
DMEM	Dulbecco's Modified Eagle Medium
Dpp	Drosophila Decapentaplegic Protein
EtBr	Ethidium Bromide
Gdn-HCl	Guanidinium Hydrochloride
GRAS	Generally Recognized As Safe
IMAC	Immobilized Metal Affinity Chromatography
LB	Luria Bertani Medium
MSC	Mesenchymal Stem Cells
TGF-β	Transforming Growth Factor Beta
GDF	Growth and Differentiation Factor
PCA	Polymerase Chain Assembly
POIs	Proteins of Interest
RGH6	Regeneration Medium
SMAD	Similar to Mothers against Decapentaplegic
PNPP	para-Nitrophenylphosphate
RG	Rosetta Gami <i>E. coli</i> strain
OD	Optical Density
TCH	Timentin-Claforan-Hygromycin
wt	Wild Type

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INTRODUCTION

1. OVERVIEW OF BONE MORPHOGENETIC PROTEIN 2

1.1 BMP Discovery, Purification and Use

Marshall R. Urist first discovered bone morphogenetic proteins in 1965 [1]. With the discovery that demineralised bovine bone extracts contained active factors which were able to generate *de novo* bone formation in vivo, further examination of the active fraction showed that it contained a combination of growth factors, the majority belonging to the bone morphogenetic protein (BMP) family [2]. One of the major osteogenic factors with the ability to stimulate *de novo* bone formation soon came to be known as BMP2 [3]. The creation of cDNA clones of bovine bone extract-derived peptides revealed that BMP2 alone could elicit *de novo* bone formation upon ectopic implantation within a carrier substance in host mammal tissues, independent of the rest of the components contained within demineralised bone extract [4]. Along with this discovery was that the other BMP related growth factors also possessed the ability to stimulate *de novo* bone formation when ectopically implanted. The discovery of the BMPs enabled a move from the use of mammalian bone extracts to recombinant derived BMP for orthopedic surgical procedures.

1.2 From Allogeneic Transplants to Characterization of the Active Components of Bone Extracts

Before the discovery, isolation and characterization of BMPs, allogeneic bone transplants were the source of exogenous bone tissue for surgical applications, but this method was not

without risks and side effects [5]. The main issues arising from allogeneic bone transplants were pain and infections that arose from the transplantation procedure. These side effects might not only lead to discomfort for the patient, but might also result in rejection of the transplanted tissue or infection, thus rendering treatment efforts ineffective [6, 7]. Since Urist's discovery that bovine bone extracts possessed osteoinductive activity, it was quickly assumed that characterization of the osteoactive components of the demineralised bone extracts might yield the identity of the particular factors involved. Hence, the next direction in the pursuit of the characterization of the bone extract was to identify the individual factors responsible for the osteoactivity of the extracts. Using SDS-PAGE under non-reducing conditions a number of bands of approximately an effective molecular weight ~30 kDa were noted. Under reducing conditions, fragments of 18kDa and smaller were noted, suggesting that these factors were possibly dimers. It was also found that upon reduction that the osteoinductive activity of these factors was lost. Hence, the factors possessed bioactivity only as dimers and the monomeric forms did not possess osteoactivity. Treatment using N-endoglycanases also produced bands <30 kDa, suggesting that some of these factors were glycosylated [8]. However, this strategy was not able to discern the identity of these bands and another approach was needed to uncover the further identity and specific sequences of these factors. The osteogenic action of recombinant BMP2 when mixed and implanted in a carrier matrix in rats resulted in significant cartilage formation within five days of implantation. Resident mesenchymal stem cells migrated into the matrix and differentiated into chondroblast cells, which began laying down a collagenous matrix. Ossification began within a week and by three weeks a fully innervated, vascularised bone was present in place of the implant, containing marrow and cortical and cancellous bone [8].

1.3 Extraction and Purification of BMPs from Animal Bone Sources

Acquiring sufficient amounts of purified BMPs from bovine or porcine sources was a cumbersome extraction process and thus acquiring enough BMP2 for surgical procedures was time consuming and expensive. As bone matrix is a solid material, an adherent tissue marrow free sample would first have to be frozen and pulverized and the organic fraction separated from the bone mineral component, fat and water. This procedure required a large amount of starting material as the organic component of bone makes up only 20-30% of total bone tissue [9]. To obtain a fraction containing 1-2 ug of BMP required a starting material amount of 10 kg of bovine bone [10]. The next major hurdle was to extract soluble BMP from the organic fraction as the collagenous matrix where BMP was located was very impermeable to extraction solvent diffusion, taking at least 16 hours to obtain a minimal yield of BMP2 [11]. The solvents would require high amounts of urea (6-8 M) [12]. The resulting fractions from this extraction process were hard to separate as the BMP proteins are known to precipitate easily and to form aggregates with other non-BMP proteins [13]. Up to six rounds of chromatography or more were required to effectively extract BMP from the organic fraction thus contributing to the expense of BMP purification from animal sources [14].

1.4 Characterization and Cloning of the BMP Active Factors from Bone Extracts

Characterizing the constituents of the organic extract soon led to the identification of the components responsible for the osteogenic activity of the extract, which was found to be a number of related factors which lost their activity under reducing conditions [14]. The

isolation and cloning of the BMP osteogenic components of demineralised bone extracts did not happen until groups working under Wang and Wozney were able to recover proteins from extracts via heparin Sepharose affinity chromatography, subjecting the proteins acquired from the heparin chromatography to tryptic digests, sequencing the resulting tryptic peptides [4]. From these sequences they were able to construct the first cDNA clones of these proteins and hence develop the first recombinant proteins derived from these cDNA clones. Recombinant human BMP2 was found to possess osteogenic activity upon implantation. With this cloning of the BMP2 gene and subsequently the generation of rhBMP2, the promise of larger scale production of BMP2 independent of mammalian bone extracts became a possibility [15].

1.5 Development of Commercial Products of BMP2 and Current Issues

Although it was possible to produce rhBMP2 in bacteria as well as mammalian systems, CHO cell culture derived BMP2 became the backbone of commercial production. A commercial preparation of rhBMP2, namely INFUSE by Medtronic, is approved for use in spinal fusion procedures for patients with degenerative disk syndrome [16]. However, it is also used for a number of off label uses in bone growth enhancement and regeneration [17]. Although commercial preparations of BMP for surgical uses have proven efficacy, they are not without issues and side effects. As the BMP2 solutions are required to soak in a collagen sponge matrix, there is significant leakage producing ectopic bone growth at sites other than the desired implant site. The leakage of these preparations exposes the recombinant BMP2 forms to host immune cells which acquire an intolerance not only towards the implanted

BMP2 but also the host endogenous BMP2 as well. The high doses required are thought responsible for these side effects and multiple treatments with recombinant forms of BMP2 might fail as acquired host immune responses might result in clearance of subsequent treatments using recombinant BMP2 [5].

1.6 Physiological Action of BMPs

Bone formation in mammals occurs mainly through two pathways, the endochondral route and the intramembranous ossification pathway [18]. The endochondral route begins with local, activated mesenchymal stem cells (MSCs) condensing to form a nucleus where they then differentiate into a chondroblast phenotype. Next, they begin to synthesize and secrete a cartilaginous matrix which is mainly composed of collagens and proteoglycans.

Fibroblast-like cells then surround the outer boundary of the cartilaginous matrix and initiate the formation of capillaries which act to feed the growing cartilage cluster [19]. The progressive mineralization of the cartilage limits nutrient diffusion to the chondrocytes and they subsequently die off. Phagocytic cells enter and clear dead cartilage, creating cavities which become occupied by pro-osteoblasts. Once the pro-osteoblasts differentiate to osteoblasts, bone-specific proteins are secreted and ultimately lead to the formation of mature bone [20].

In the process of intramembranous ossification, the MSCs differentiate directly to osteoblasts and begin to generate bone tissue without first establishing a cartilage matrix. Subcutaneous administration of BMP2 induces bone formation by recruiting MSCs and follows the process of the endochondral bone formation pathway [8].

1.7 Physiological Action of Implanted BMP2

In implantation studies, within a span of one to three weeks, a fully formed ossicle of bone is formed, complete with both cortical and cancellous bone layers and fully vascularized and innervated in a rat ectopic assay model [21]. Besides BMP2, the other sequence and structurally related BMPs are also able to illicit bone formation upon implantation. Although there exists a high level of sequence identity between the various BMPs, when activities are compared using the rat ectopic assay there are differences in the degree and onset of bone formation between them. Briefly, the rat ectopic assay consists of subcutaneously implanting a quantity of demineralized rat bone matrix free of osteogenic activity and mixing it with an osteogenic extract or a given recombinant BMP at a known concentration. This implant is removed from the host after some given time, usually within a 1 to 3 week period and assessed for histological changes such as degree of cartilage formation and degree of mineralization of the implant. This assay can be used to assess the relative activity of each of the BMPs and has been a conventional *in vivo* assay to characterize the osteogenic activity of the individual BMP family members [22, 23].

1.8 Relative Activities of the BMPs Derived from the Rat Ectopic Assay

Despite the fact that BMP2 and BMP4 share 92% sequence identity in the mature C-terminal peptide it has been shown the BMP2 possesses an almost 2-fold higher potency in terms of both physical dosage [24]. It is also known that BMP2 is able to generate bone formation faster than BMP4 in this particular assay. BMP5, 6 and 7 share ~60% degree of sequence identity with BMP2 and 4 [25]. In terms of bone formation efficacy between BMP2 and

BMP5, BMP2 possesses a much higher activity in terms of both physical dosage and ossification time [26]. BMPs 6 and 7 like BMP5 can produce bone formation but possess activities similar to BMP5. BMPs 5, 6 and 7 possess bone formation potential, but also possess activities and are known to be involved in the regulation of other physiological functions not related to bone formation. The most abundant BMP in bovine bone extracts, BMP3, is also known to possess no osteogenic activity [27].

1.9 Molecular Details of BMP2

BMP2 is a homodimeric cysteine-knot protein that is structurally related to a larger cytokine family known as the TGF- β superfamily. Sequence homology between TGF- β related superfamily molecules in metazoans is common thus BMP2 shares sequence homology and structural similarity with other BMPs and other family members [28]. The mature C-terminal mature human BMP2 is bioactive when implanted in mouse, rat, porcine and bovine hosts, thus, the mature protein is the same for these species. The members of the TGF- β superfamily have been thought to have arisen from gene duplications [29]. TGF- β superfamily molecules are synthesized as preproteins and with the cleavage of the pre-sequence are stored as biologically inactive proprotein dimers [**FIGURE 1.1**]. Pro-domains of the BMPs are known not to be involved in extracellular signal transduction but are cleaved by furin-like proteases and secreted along with the folded C-terminal bioactive mature proteins. BMPs are inactive not only as monomers but also if not cleaved from their respective pro-domains. The bioactive mature form of BMP2 is a homodimer consisting of two C-terminal cysteine-knot folded peptides linked by a single interchain disulfide bridge

[**FIGURE 1.2**]. The positions of the cysteines are 100% identical in all the known sequenced and structurally related BMPs and even in other TGF- β superfamily members.

FIGURE 1.1: The BMP2 Preproprotein. A) The translated BMP2 preproprotein monomer product before proprotein processing. B) The amino acid sequence of the C-terminal mature sequence, residues 283-396 (www.uniprot.com ID#12643).

A

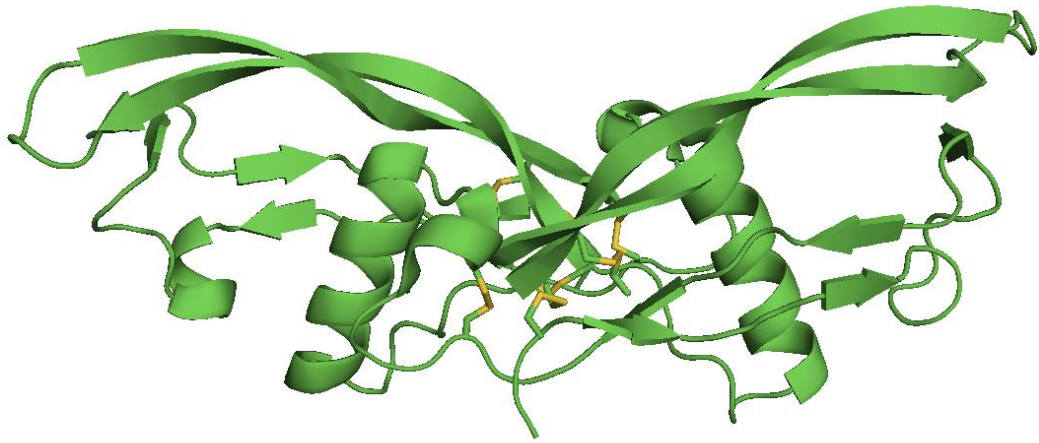


B

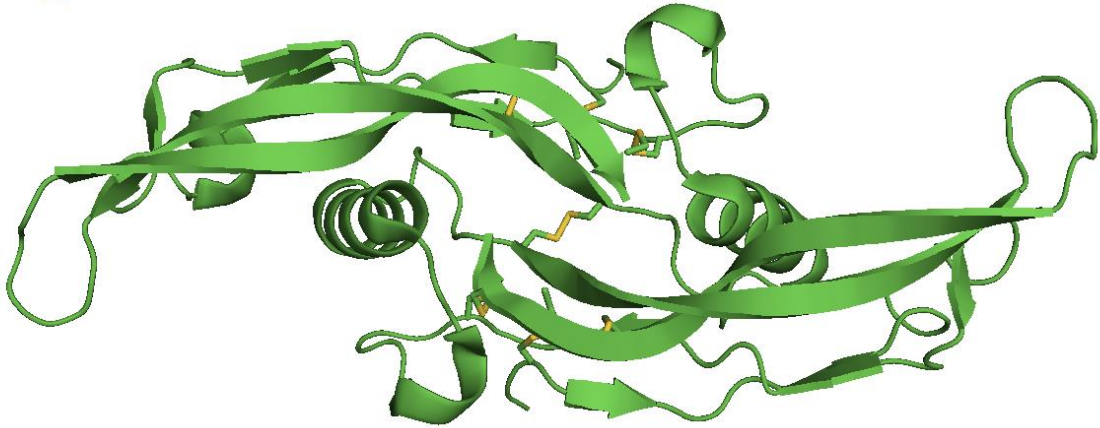
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1 QAKHKQRKRL KSSCKRHPLY VDFSDVGWND WIVAPPGYHA FYCHGECFPF  
51 LADHLNSTNH AIVQTLVNSV NSKIPKACCV PTELSAISML YLDENEKVVV  
101 KNYQDMVVEG CGCR
```

FIGURE 1.2 The Mature BMP2 Ligand. A) Side view of the BMP2 homodimeric ligand.
B) Top view of the BMP2 homodimeric ligand. Structure taken from PDB ID: 1REW.
Disulfide bonds are shown in yellow [51].

A



B



BMP2 shares the highest sequence homology with BMP4 but also shares significant sequence homology with other BMPs such as BMP6 and BMP7 [30]. Other TGF- β superfamily members that share sequence homology are the Growth and Differentiation Factors (GDFs) GDF5, GDF6 and GDF7. Additionally, the *Drosophila* protein Decapentaplegic (Dpp) shares sequence homology with BMP2. BMP2 and Dpp are exchangeable and can compensate for the function of the other, thus showing the large conservation of the sequences in this family of proteins [31] [32]. The number and order of cysteine residues in the TGF- β superfamily C-terminal mature peptides are highly conserved as they are required to form the cysteine-knot architecture and the intrachain disulfide bridge that covalently bonds two mature C-terminal monomer peptides that form an active homodimeric molecule [33]. The cystine-knot fold is thought to give the protein a compact, globular structure through the intertwined bonding scheme of cysteine residues as without the disulfide bonds such peptides would not take on a globular fold spontaneously [33].

1.10 Post-Translational Modifications of BMPs

The BMPs also possess N-linked glycosylations on both the prodomain and the mature C-terminal peptide. In the case of BMP2, each Asn56 possesses an N-linked glycosylation, hence as the mature BMP2 is a homodimer, each mature molecule possesses two N-linked glycosylations at this position. These glycosylations are thought to aid in the retention of mature BMP2 in the extracellular matrix compartment, thus increasing the half-life of these secreted molecules [8]. These glycosylations are not required for activity in BMP2 and 4, but are required for activity in BMP6 [34]. Recombinant forms of BMP2 produced in *E. coli*

are bioactive with little induction difference in the bone tissue composition they generate compared to glycosylated forms produced in CHO cells [35]. When recombinant human BMP2 is produced in a CHO cell expression system, the compositions of the N-linked oligosaccharide structures vary in their mannose content following a formula (rhBMP-2)₂-(GlcNAc)₄(Man)_Z, where Z varies from 10 to 18 [36]. Whether the N-linked glycosylation provides shielding from underlying antigenic epitopes as in the case of the Human GM-CSF N-linked glycosylation has not been shown [37].

1.11 Heparin-Binding Domain of BMPs

The N-terminal portion of BMPs 1, 2, 4 and 3 possesses a heparin binding domain and is thought to aid in the molecular retention in the extracellular milieu, prolonging their half-life and thus activity [38]. This N-terminal heparin binding domain can be utilized in the purification of these BMPs from extracts from host tissues and recombinant systems such as CHO cells and *E. coli* using a heparin-Sepharose matrix. The discovery of these members of the BMP family from bovine bone extracts was facilitated because of this feature of these molecules [14].

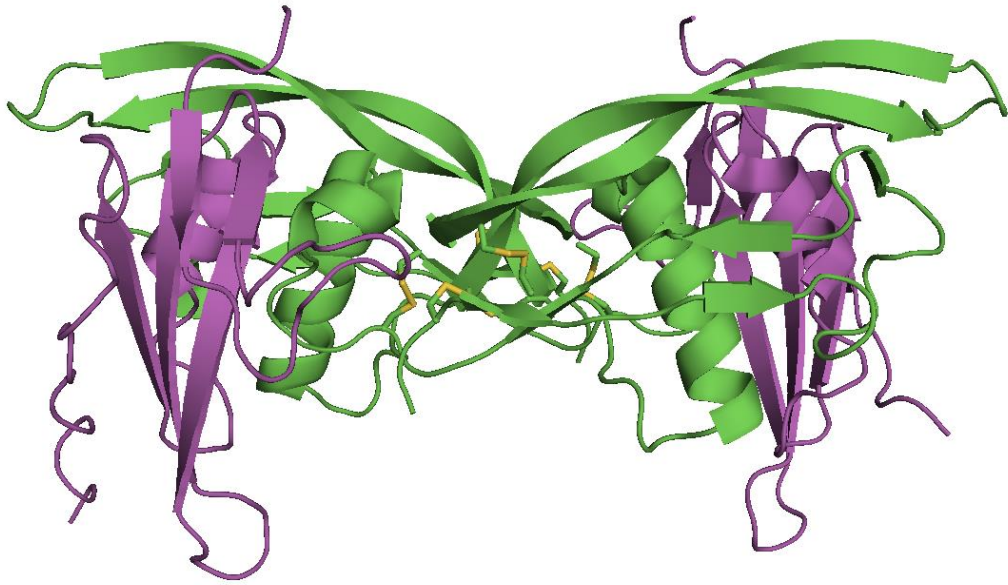
1.12 BMP Signalling Action

The TGF- β superfamily members are all homodimeric proteins but can also form bioactive heterodimers [39]. TGF- β superfamily dimeric ligands signal by first binding either a type I or a type II serine/threonine kinase receptor. TGF- β superfamily ligands display various

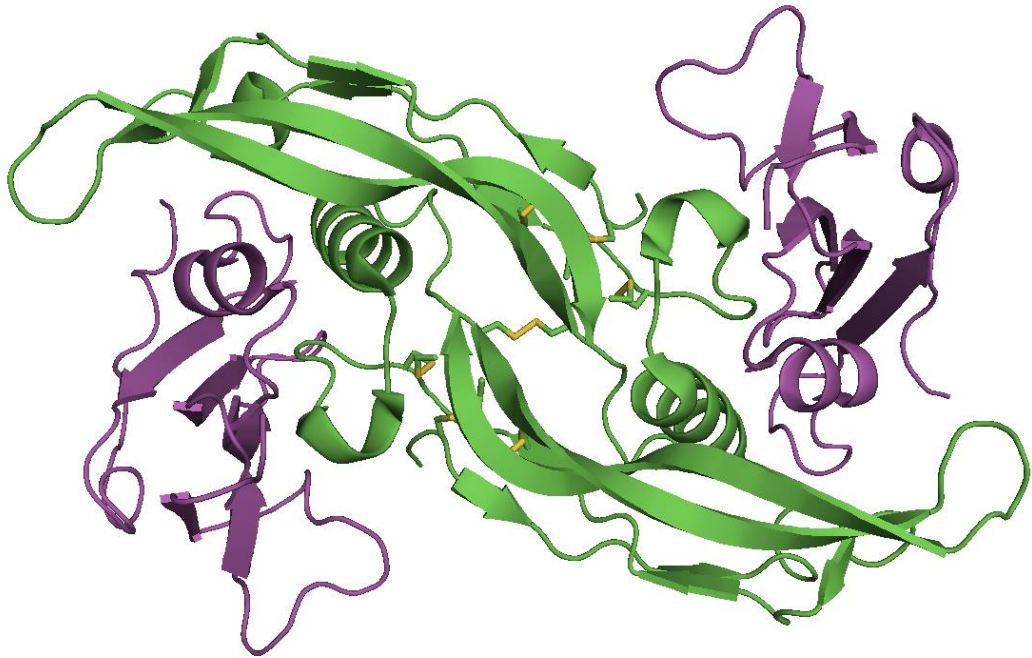
affinities for the different type I and type II receptors. Although there are more than 30 TGF- β superfamily ligands and seven different type I receptors and five different type II receptors, BMP2 has highest binding affinity for the BMPRIA [40] [41]. BMP2 preferentially binds to its type 1A receptors in a 1:2 ratio BMP2-BMPRIA receptor complex [FIGURE 1.3]. Once BMP2 and BMPRIA form a complex, this enables the further binding of BMP type II receptors, resulting in a pentameric signaling complex which activates intracellular signalling.

FIGURE 1.3: The BMP2/BMPRIA Complex. The BMP2 homodimeric ligand is shown in green while the BMPRIA ectodomains are shown in magenta. Structure rendered in PyMol using the PDB ID:1REW crystal structure. Disulfide bonds shown in yellow.

A



B



Following this signaling complex formation, the BMPRIA glycine-serine rich GS domain is phosphorylated by the BMP type II receptors. BMP type II receptors possess constitutive phosphorylase activity when bound and in proximity to the BMPRIA, which in turn, activates the phosphorylase activity of the BMPRIA. A receptor mimic, BAMBI, is able to assemble inactive complexes by sequestering BMP ligands and being able to associate with type II receptors, but not possessing the intracellular domain of *bona fide* type I receptors, thus not relaying the signal into the cytoplasmic compartment. Activation of BMPRIA results in signal transfer across the membrane to the intracellular compartment where the intracellular SMADs become activated [42] [43]. The activation of the SMAD proteins in the cytoplasm results in the formation of a trimeric signalling complex with co-activator SMAD4 and two receptor regulated SMAD2 molecules. These trimeric SMAD signalling complexes are shuttled into the nucleus via the nuclear pore complex. Antagonistic SMADs also exist such as SMADs 6 and 7 also known as the inhibitory SMADs. These SMADs act by blocking the association of SMAD4 with the receptor regulated SMAD2, thus preventing the formation and further shuttling of the SMAD complex, preventing its translocation into the nucleus [43].

As the SMAD complexes accumulate within the nucleus, they associate and interact with transcription factors, some of which are members of the FoxH1 family in *Xenopus* [44]. These complexes are then recruited to cognate promoter sites where they can initiate, repress or permit transcription of their target genes [45].

1.13 Soluble BMP antagonists

Soluble extracellular antagonists are known to sequester mature BMPs and inhibit their activity. These antagonists are known to bind to BMPs at sites other than those sites at which BMPs bind their type I and type II receptors. The formation of a complex between one of these soluble inhibitors and a BMP ligand sequesters the BMPs and hence prevents them from binding to their cognate receptors and thus initiating a transmembrane signal which results in the intracellular activation of the SMAD pathway proteins. The structures of some of the antagonists, notably noggin, have an analogous structure to BMPs, leading some to hypothesize that they evolved from a similar precursor as the BMPs themselves. The sequence of noggin is highly identical to those sequences in mouse and rat, but also in the amphibian *Xenopus* where it was first characterized. Noggin, like the BMPs, is a glycosylated, homodimeric, cystine-knot folded protein which is secreted and able to bind a subset of members of the TGF- β superfamily, although with different affinities depending on the ligand [46]. Experiments using mice have shown that overexpression of noggin produces a phenotype that is highly prone to bone fractures and have reduced bone volume [47]. The presence of the different antagonists depends on which tissues and developmental state is ongoing, noggin is expressed in early development as it is responsible for antagonism of BMP4 activity in the developing notochord, but is known to be expressed in various tissues in the adult. Others such as *Xenopus* chordin bind BMPs in a 2:1 ratio and do not possess the cystine-knot architecture as noggin does [48]. *Xenopus* osteoblasts are known to secrete another antagonist, gremlin. Gremlin is a glycosylated protein that is also known to inhibit a subset of BMPs and produces phenotypic effects similar to noggin [49].

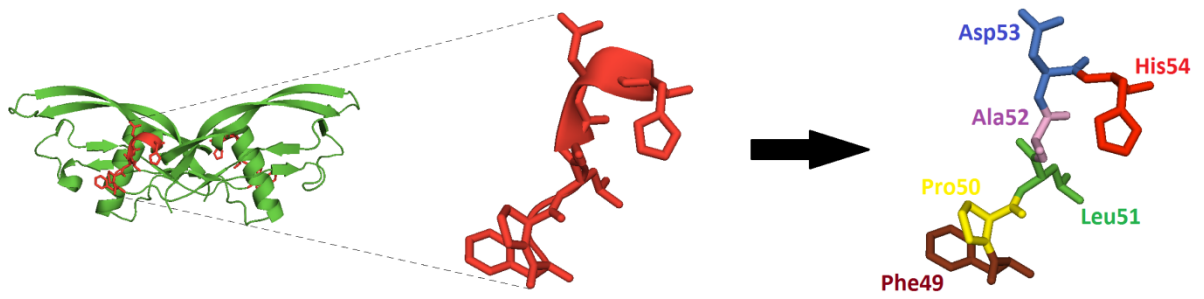
1.14 BMP Binding Interface

The mature homodimeric BMP2 molecule contains binding epitopes which have been termed the “wrist” and “knuckle” epitopes. The wrist epitope comprises residues from both BMP2 monomers and directly interacts with BMPRIA in the binding complex, while the knuckle epitope interacts with the type II receptors [50]. The wrist epitope of BMP2 contains a loop element termed the “pre-helix loop” which is thought to be a free mobile element when unbound in BMP2 but not in all BMP molecules [51]. Upon binding with BMPRIA, the pre-helix loop participates in an induced fit mechanism and adopts a conformation which interacts with the simultaneously induced conformational changes that occur within BMPRIA [52]. The pre-helix loop is comprised of the residues F49, P50, L51, A52, D53 and H54 [FIGURE 1.4]. The residues preceding L51, F49 and P50, were observed to be important for aligning the backbone of the pre-helix loop structure to properly orient the hydrogen bonding network for binding with BMPRIA. Mutation of these residues to alanine showed that the association rate between these mutants and BMPRIA was reduced, suggesting that these residues play a role in orienting the pre-helix loop during binding [51]. When bound to BMPRIA, the BMP2 L51 residue provides main chain hydrogen bonding donor and acceptors to the hydrogen bonding donor and acceptor groups on the side-chain carboxamide group of the Q86 residue of BMPRIA. L51 is a main determinant in BMP2 binding to BMPRIA and the main chain amide and carboxyl groups function as a potential hydrogen bonding contact hotspot [51].

Substitution with proline abrogates this hydrogen bonding scheme therefore disrupting the interaction of BMP2 with BMPRIA leading to no notable bioactivity. Since the main chain of L51 is responsible for providing hydrogen bonding contacts, this residue position may be

tolerant to residue substitutions, as the L51 side-chain plays no direct role in the hydrogen bonding scheme with Gln86 of BMPRIA.

FIGURE 1.4: The Pre-Helix Loop Domain. BMP2 ligand shown in green, the pre-helix loop is shown within the BMP2 homodimeric ligand in red then enlarged and then further color coded. The color coded amino acid residues within the pre-helix loop are shown by their respective residues. Diagram taken from crystal structure PDB ID: 1REW (<http://www.rcsb.org/pdb/explore/explore.do?structureId=1rew>).



It is thought that the side-chain of L56 in the GDF5 molecule, which is analogous to the L51 residue in BMP2, lies within the intramolecular hydrophobic core and plays a structural role [53]. A complete leucine deletion of the analogous residue in the GDF5 molecule, L56, results in no bioactivity in *in vitro* assays using RobC26 and ATDC5 cells [54].

2. RICE WITH PUROINDOLINE FUSION CAPABILITIES FOR RECOMBINANT PROTEIN EXPRESSION

2.1 Puroindolines as N- or C-terminal Fusion Proteins

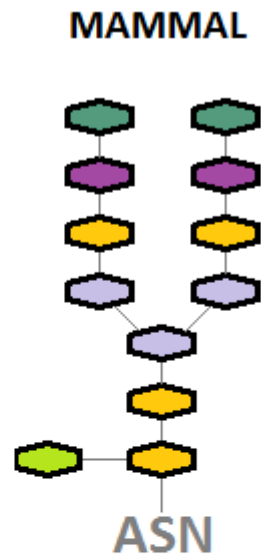
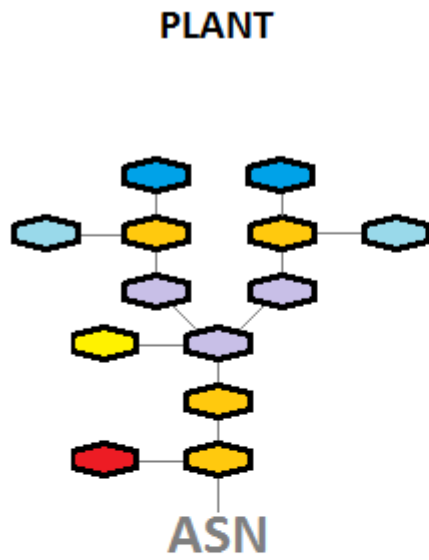
Puroindolines are proteins expressed in wheat (*Triticum aestivum*) which are found associated with the starch granule surfaces. Puroindolines have antimicrobial activity as well as contribute to the structural properties of wheat starch. The role that puroindolines play in wheat starch structure was responsible for changing the properties of the wheat such as to allow modern breadmaking through a mutation in the puroindoline gene [55]. Puroindolines are roughly 12.8 kDa in mass and are disulfide rich proteins [56]. At the starch granule surface, puroindolines are able to associate with polar lipids that cover the starch granule surface. The domain of the puroindoline protein known to associate with these lipids is the tryptophan-rich-domain of which there are two such domains PINA (WKWKWKWK) and PINB (WPTKWKWK) [56]. Wall et al. showed that puroindolines could associate to the starch granules of maize, hence showing that this association occurs with starch granules of other cereal crop species including those species that do not naturally possess puroindolines [57]. Because of its affinity for the starch granule surface, puroindolines can be used as either N- or C-terminal fusion proteins to a POI for expression in cereal crops [58]. The puroindoline fusion system would enable localization to the starch granule surface and thus would target recombinant protein expression to cereal crop endosperm tissue. This would offer facilitated purification as the endosperm is not as populated with complex macromolecules as is the cellular cytoplasm. Once the starch granules are separated, the puroindoline N- or C-terminal fusion could be cleaved off using a proteolytic cleavage using









an enzyme such as enterokinase. This would enable the production of a given POI without the use of IMAC columns or other more complex chromatography methods.

2.2 Rice as a Recombinant Protein Expression Platform

Rice starch is known as a GRAS product. As a cereal crop, rice offers the potential for large scale production of recombinant proteins with the endosperm tissue compartment housing the POIs. Rice plants can be grown on a large scale thus bolstering yields of recombinant proteins. Recombinant protein expression in rice offers the freedom from biological contaminants such as viruses and prions that are a foremost issue for mammalian expression systems [59]. Also the starch endosperm tissue offers relative purity from endotoxins that are often the problem of other plant expression systems such as tobacco [60]. The hypoallergenic properties of rice starch may also be more compatible with immune sensitivities thus giving this system an advantage where immunogenicity is an issue [61]. Plant glycosylation patterns moderately mimic the glycosylation patterns of human proteins, thus enabling post-transcriptional processing close to that of humans [FIGURE 2.1] [62]. Overall, with the ease of purification from rice starchy endosperm, the large scale growth potential and the lack of immunogenic potential over other systems makes rice a desirable expression platform intended for expression of recombinant proteins for use in human surgical applications.

FIGURE 2.1: Comparison of Plant and Mammalian Glycosylation. The N-linked glycosylation structures of plants and mammals are shown. The constituent sugar monomers are shown in the legend. Figure adapted by author from [62].



	MANNOSE		N-ACETYLNEURAMINIC ACID
	N-ACETYLGLUCOSAMINE		ALPHA 1,6-FUCOSE
	BETA 1,4-GALACTOSE		BETA 1,2-XYLOSE
	ALPHA 1,3-FUCOSE		LEWIS X STRUCTURE

3. RATIONALE AND HYPOTHESIS

An engineered BMP2 with a higher biological activity is desired as the current wtBMP2 used in surgical orthopedic procedures is applied at relatively high doses that often lead to unintended side effects and further complications [5]. These high doses are warranted as they are necessary for induction of a notable therapeutic effect. For Medtronic's INFUSE, the recommended dosage is 1.5 mg/ml [63 - 65]. These large doses used in these treatments provoke adverse immune reactions that are detrimental and can render treatments ineffective [66 – 72].

BMP2 has its greatest affinity for BMPRIA over all other type I TGF- β superfamily receptors. As BMP2 is known to have the highest bone forming potential and also the highest affinity for the BMPRIA, a BMP2 molecule engineered with higher affinity for BMPRIA than wt BMP2 could result in increased receptor affinity, prolonged signalling complex lifetime and could result in greater recruitment of the resident MSCs at smaller physical doses [73 – 76].

E. coli produced BMP2 does not include the N56 N-linked glycosylations. Although unglycosylated BMP2 is still biologically functional, absence of N-linked glycosylations is known to contribute to immunogenic effects in certain recombinant proteins [34]. CHO cell produced BMP2 includes the N56 glycosylations but contains an array of N-linked glycosylations and thus yields large batch-to-batch variations [15].

To overcome these obstacles, a plant-based expression host for rhBMP-2 production could improve bioactive protein yields and reduce immunogenicity [20]. Rice offers recombinant protein products that are fully post-translationally processed; rice is known to be hypoallergenic and has a glycosylation pattern which closely mimics that in mammals. A

way to ease purification from rice is to express proteins in the rice endosperm tissue using a puuroindoline (PIN) fusion system [58]. To obtain large amounts of BMP2 with rice is possible as transgenic rice plants can be grown in large quantities, limited only by available acreage [77].

3.1 Purpose: To synthesize a functional and bioactive N-terminal puuroindoline fused engineered BMP2 recombinant protein in rice flour.

3.2 Hypothesis: Since rice is able to express recombinant proteins and binding hotspot positions within the BMP2 molecule have been established [51, 78], I hypothesize that a subset of BMP2 mutant proteins will show greater potency for inducing *de novo* bone formation *in vivo* than wild type BMP2. If BMP2 mutants exhibiting a higher biological potency than wild type BMP-2 are discovered, I further hypothesize that greater potency BMP-2 expressed in the transgenic rice PIN-fusion system will produce proteins with greater efficacy for *de novo* bone formation, will more closely mimic the *wt* glycosylation pattern and enable cheaper purification of BMP2 from rice endosperm.

3.3 Objectives:

1) For expression of recombinant BMP2 in rice, design, synthesis and sub-cloning of a codon optimized BMP2 DNA sequence into appropriate expression vectors suitable for *E. coli* and rice is necessary to establish recombinant protein producing cell lines. Whether such a sequence is functional and whether it is possible to express this mammalian protein in rice is to be assessed.

2) To find residues that would theoretically fit and thus be sterically compatible within the binding interface, an *in silico* screening method was enlisted to reveal those residues with

side chains that would not disrupt the known hydrogen bonding contacts and thus the ligand-receptor binding interaction. Two residue positions that produce notable effects when mutated on the BMP2 are L51 and D53 [51, 79]. These two residue sites have not been thoroughly examined with substitution with the all other sterically compatible standard amino acids and substitutions at these positions may yield mutant BMP2 molecules which possess a higher affinity interaction with BMPRIA.

3) To assess whether these mutations possessed structural differences in comparison to the wild type BMP2 protein, the CD spectra of the mutant protein collection would be determined and used to assess whether these mutations had any effect in terms of structure. Using a conventional assay for BMP2 bioactivity [79], the bioactivity of the mutant BMP2 protein collection would be compared to the activity of the wt BMP2 using alkaline phosphatase activity in a murine promyoblast cell line, C2C12.

4. EXPRESSION OF A PINA-BMP2 FUSION PROTEIN USING A CODON OPTIMIZED DNA SEQUENCE AND A RICE ENDOSPERM TISSUE PUROINDOLINE TETHERED EXPRESSION SYSTEM

4.1 INTRODUCTION

Bone Morphogenetic Protein 2 (BMP2) is a cytokine growth factor which elicits *de novo* bone formation in adult mammals. When implanted ectopically with a carrier substance, BMP2 is able to stimulate *de novo* bone formation which results in the formation of fully functional bone tissue. Although there are other related molecules that also possess osteogenic activity, BMP2 alone is sufficient for *de novo* bone formation. The use of BMP2 in surgical applications is currently widespread and has applications ranging from spinal fusion procedures to off-label uses such as dental implants. Currently large quantities of BMP2 are necessary for these surgical procedures. The main issue with the use of large quantities of BMP2 is that this provokes immune reactions which can render surgical treatments ineffective. In addition, the large quantities necessary for surgical procedures are expensive and add greatly to the overall expense of these surgical treatments. Current expression systems used for BMP2 are mammalian cells which require large amounts of resources to grow purify and maintain and are not without risks in terms of biohazards [80]. Expression of BMP2 in rice endosperm tissue offers a much more biocompatible expression platform with more humanized post-translational modifications and the advantage of large scale production [81]. In addition to expressing BMP2 in rice, to develop a mutant BMP2 with higher biological potency than wt BMP2 would also assist in reducing both dosages and costs for surgical applications. The costs of the production of such mutant proteins could be further reduced if produced in transgenic rice systems as these can be grown on a large scale. To simplify the purification of recombinant BMP2 from rice endosperm tissue, a

BMP2/puroindoline fusion system is used [82]. Puroindolines have affinity for starch granule surfaces and are known to localize at this position [83]. Hence, a higher potency BMP2 produced in a puroindoline fusion system in rice offers a number of advantages of which current systems fall short.

The tRNA pools of each species often differs enough that not all codons are used to the same extent in one organism compared to another. Codon skipping occurs with sequences used in hosts in which there is an insufficient pool of a particular cognate tRNA [84]. To overcome this issue, DNA sequences can be designed such that they are composed of only tRNA pools which are high in abundance for a given particular organism.

Hypothesis: A codon optimized BMP2 DNA sequence designed for expression in rice will be able to express a BMP2 peptide in rice identical to that expressed in mammalian expression systems.

4.2 RESULTS

4.2.1 Synthesis of Codon-Optimized BMP2 Sequence for Rice Expression

To optimize the expression of BMP2 in transgenic rice, a BMP2 codon optimized sequence was designed for expression in rice. This sequence was designed based on the codon usage frequencies tabulated at www.kazusa.co.jp for rice (*O. sativa*) and thus codons were chosen according to their highest usage in *O. sativa*. These codons were used in place of those from the human sequence (NCBI NM_001200.2, mat peptide, nucleotides 1632-1973) for the

mature, C-terminal peptide which spans amino acid residues 283-396 (www.uniprot.org, accession#: P12643). The resulting sequence is shown in **[FIGURE 4.1]**.

FIGURE 4.1: BMP2 codon optimized DNA sequence was designed for expression in rice. The resulting DNA sequence was used in all constructs for all studies for both rice and *E. coli* work. This sequence corresponds to that of the NCBI NM_001200.2, mat peptide, nucleotides 1632-1973 and the C-terminal peptide which spans amino acids residues 283-396 (www.uniprot.org, accession#: P12643). Sequence reads from left to right in the 5'- to -3' direction.

1 caggccaagc acaagcagcg caagcgctc aagtctctct gcaagcgcca cccgctctac
61 gtggacttct cggacgtggg ctggaacgac tggatcgtgg ccccgccggg ctaccacgcc
121 ttctactgcc acggcgagtg cccgttcccg ctcgccgacc acctcaactc caccaaccac
181 gccatcgtgc agaccctcgt gaactccgtg aactccaaga tcccgaaggc ctgctgcgtg
241 ccgaccgagc tctccgcat ctccatgctc tacctcgacg agaacgagaa ggtggtgctc
301 aagaactacc aggacatggt ggtggagggc tgcggctgcc gc

As there were no overt conflicts with codon usage frequencies of such a sequence in the Rosetta strain of *E. coli*, I hypothesized that the *O. sativa* optimized sequence would be compatible for expression in *E. coli* and yield a product that would be of the same molecular weight predicted by using the EXPASY compute pI/Mw tool (http://web.expasy.org/compute_pi/).

As there was no known natural host possessing this particular optimized DNA nucleotide sequence, the synthesis of this DNA fragment with flanking restriction endonuclease sites was carried out using the polymerase chain assembly (PCA) method. A set of DNA oligonucleotides were generated for the PCA experiment from the codon optimized gene sequence using the DNA Works software (<http://helixweb.nih.gov/dnaworks/>). This produced a set of DNA oligonucleotide sequences which were then purchased from Life Technologies (www.lifetechnologies.com) and were assembled using GoTAQ Green DNA Polymerase (www.promega.com) and an Eppendorf Thermocycler using a standard PCR program.

The resulting product was amplified using PCR and flanking primers, forward 5'-
ggtaccggtaccgacgacgacgacaagcaggccaag-3' and reverse 5'-
ggctgccgcactattcctagggagctcgagctc-3'. The resulting PCR product was run on a 1% agarose gel (w/v) and revealed a band corresponding to ~350bp which was the expected size for the BMP2 codon optimized DNA fragment with restriction endonuclease sequences. This fragment was gel purified using a Qiagen Gel Purification Kit and used for subsequent

ligation into a pET21a(+) expression vector to create the expression construct pET21a(+):BMP2 for recombinant protein expression in Rosetta Gami *E. coli*.

4.2.2 Ligation of Codon Optimized BMP2 DNA into pET21a(+) Expression Vector

The gel-purified PCA BMP2 DNA gene product was double digested using *NdeI* and *NotI* (New England Biolabs, www.neb.com) and ligated into a pET21a(+) expression vector also digested with *NdeI* and *NotI*. After ligation and transformation of the BMP2 DNA into the pET21a(+) vector and the RG *E. coli* strain, a subsequent double digest with *NdeI* and *NotI* confirmed the presence of the BMP2 gene in the construct pET21a(+):BMP2 [**FIGURE 4.2**].

This sequence was found to yield a band under reduced conditions in SDS-PAGE that corresponds to the expected fragment size for the reduced monomeric, C-terminal peptide of BMP2 [**FIGURE 4.3**].

FIGURE 4.2: Double Digest of pET21a(+):BMP2. The codon optimized BMP2 DNA sequence was ligated into pET21a(+) and the presence of the BMP2 gene within the construct was confirmed with a *NdeI/NotI* double digest. Lane M: 2-log ladder (NEB). Lane 1: Uncut pET21a(+):BMP2 Lane 2: *NdeI* single digest. Lane 3: *NotI* single digest. Lane 4: *NdeI/NotI* double digested pET21a(+):BMP2. Gel is 1% agarose (w/v) and stained with Ethidium Bromide (EtBr) (1ug/ml).

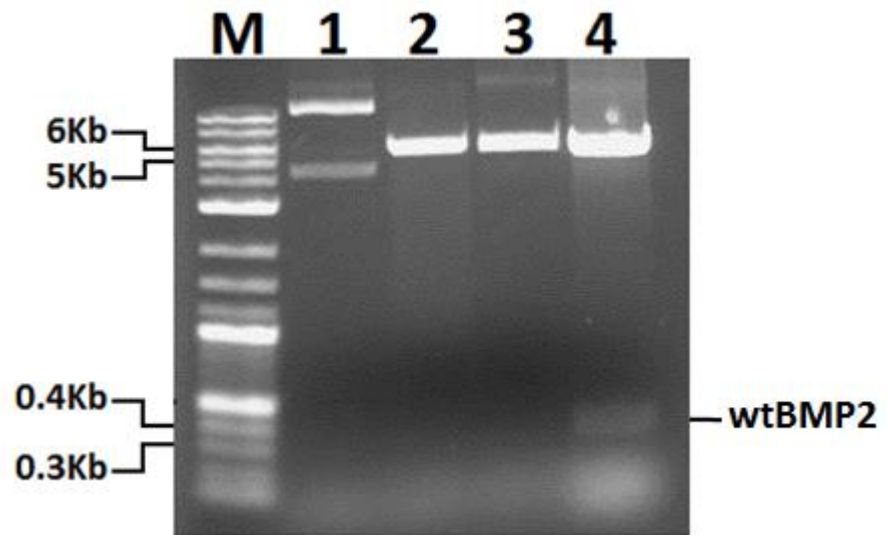
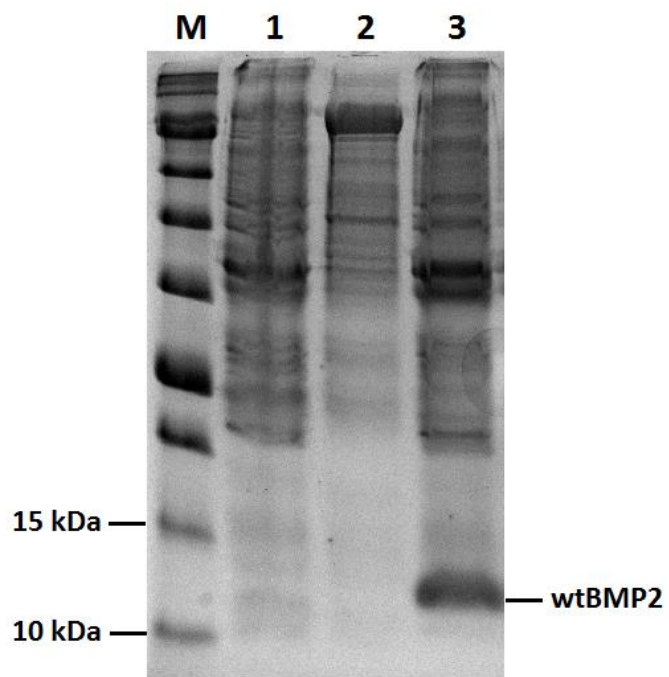


FIGURE 4.3: Expression of wtBMP2 in *E. coli*. Coomassie-stained SDS-PAGE of the codon optimized DNA sequence of wtBMP2 expressed in *E. coli* using the pET21a(+) expression vector. Lane M: Bio-Basic 10-170 kDa Wide Range Pre-Stained Protein Markers. Lane 1: *E. coli* lysate from RG Strain. Lane 2: Lysate from *E. coli* with pET21a(+) in RG. Lane 3: *E. coli* with pET21a(+):BMP2 codon optimized DNA sequence. All samples run under reduced conditions. Gel is 15% acrylamide (w/v).



4.2.3 Ligation of Codon Optimized BMP2 DNA into pCAMBIA Expression Vector

The next step was to clone this sequence into a binary vector suitable for expression in rice [FIGURE 4.4]. The pCAMBIA 1305.1 vector contained a Glutelin-1 promoter, an N-terminal PINA sequence and a Nos-ter terminator [FIGURE 4.5]. The pCAMBIA/BMP2 vector was transformed into *A. tumefaciens* strain LBA4404 using an established method [85]. This construct was digested to confirm the presence of the BMP2 gene within this expression construct [FIGURE 4.6].

FIGURE 4.4: Subcloning Scheme for Synthesis of pCAMBIA/Gt-1/PINA/BMP2 Expression Vector. The codon optimized DNA BMP2 sequence was excised from pGEM using a *KpnI/BamHI* double digest and ligated into a double digested pCAMBIA/Gt-1/PINA/BMP2 expression vector.

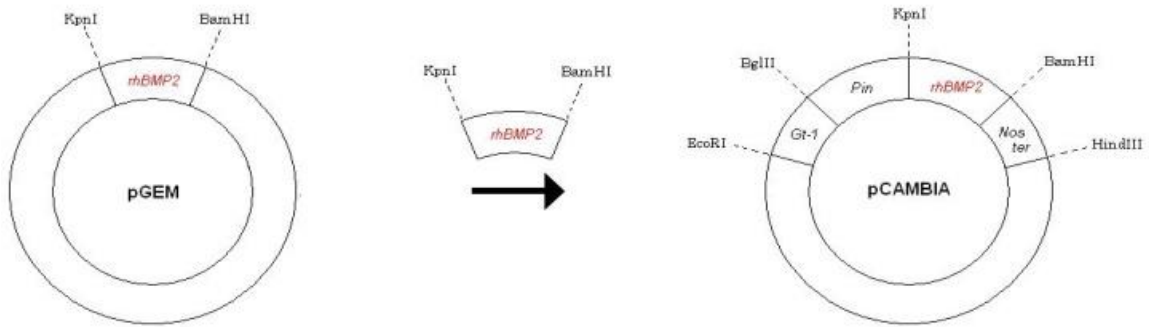


FIGURE 4.5: The pCAMBIA/Gt-1/PINABMP2 Expression Vector Map.

The expression cassette is shown in **A** with the Glutelin-1 promoter, the puromidine sequence, the BMP2 sequence position and the Nos-ter terminator. The exact amplification product is shown with primer sets in **B** and Primer sets in **C**.

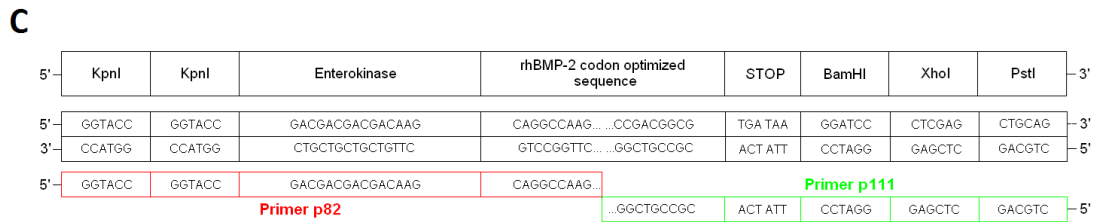
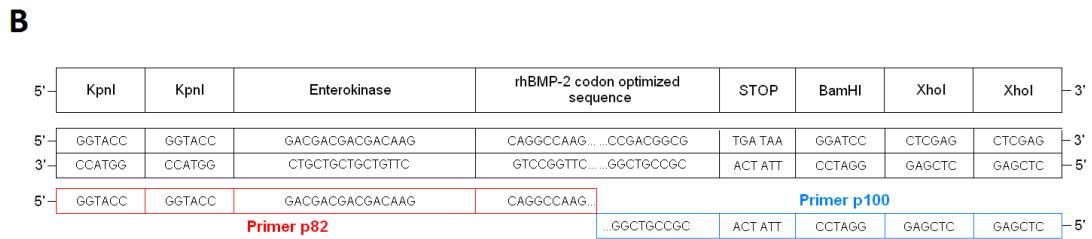
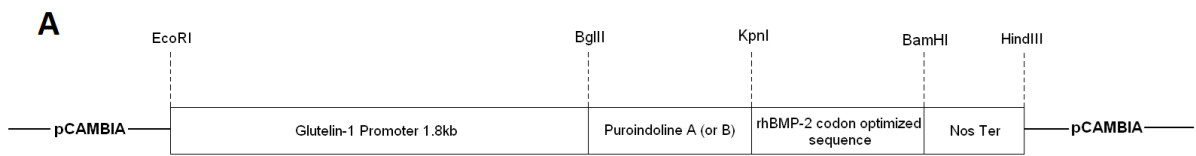
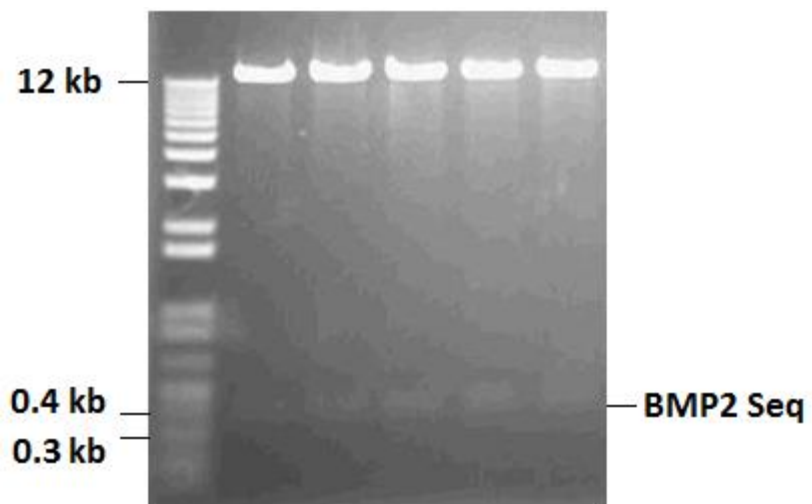


FIGURE 4.6: *KpnI/BamHI* digest of the pCAMBIA/Gt-1/PINA/rhBMP-2 expression vector. The vector containing the BMP-2 sequence was digested to confirm the presence of the optimized BMP-2 gene. The pCAMBIA vector is 11846 bp. Ladder is 1Kb+ from Invitrogen (Life Technologies). The BMP2 gene fragment (Seq) is 350 bp in size. Gel was 1% agarose (w/v) in TAE Buffer.



To transfect rice callus with the pCAMBIA/BMP2 binary vector, we used the protocol outlined in [85]. The rice calli used in these experiments were generated from the Kaybonnet rice cultivar and M202 cultivar. Despite repeated attempts with both cultivars transfection of the pCAMBIA/BMP2 LBA4404 strains with rice callus tissue did not yield any hygromycin resistant transgenic calli. Using X-Gluc staining, calli incubated with the pCAMBIA/BMP2 LBA4404 strain suspensions were stained for GUS activity [86]. The calli had been left on regeneration media as per the CAMBIA rice transformation protocol. After > 4 weeks, GUS activity was evaluated. No calli stained positive for GUS activity (Results not shown).

4.3 DISCUSSION

The PCA-derived BMP2 codon-optimized DNA sequence was able to yield an expected fragment of 13 kDa under reducing conditions with SDS-PAGE. DNA sequencing of this fragment showed that this sequence was identical to the sequence which was designed based on the codon usage tables from www.kazuza.co.jp. Although the codon optimized sequence was shown to produce a fragment corresponding to the expected size of a C-terminal mature BMP2 monomer fragment when expressed in *E. coli*, further subcloning of this fragment into the binary expression vector pCAMBIA 1305.1 and transfection of the LBA4404 harboring this construct with Kaybonnet and M202 callus tissue did not yield any viable transgenic calli. The suspension values for the transfection step range from of an OD₆₀₀ absorbance of 0.2 – 1.0. This relatively large range of values is suited for the Nipponbare cultivar [85] but did not use Kaybonnet or M202, which were the cultivars that we had access to in our lab.

The use of suspensions of pCAMBIA/BMP2 LBA4404 with an OD₆₀₀ of 1 resulted in rice calli covered in bacterial biofilm. We did not assess whether the biofilm was due to the transgenic pCAMBIA/BMP2 LBA4404 or another species introduced via contamination. Further experiments should endeavor to use bacterial suspensions less than an OD₆₀₀ of 1 for callus transfections. For the transfection step, suspensions of OD₆₀₀ absorbance 0.2 – 0.3 were recommended by Dr. Xiongying Cheng (personal communication). Future attempts to assess whether this expression system is viable should also ensure that the glycerol stocks of LBA4404 pCAMBIA/BMP2 are not contaminated and also should employ the use of the Nipponbare cultivar which is used by [85]. As these attempts to create transgenic rice calli and subsequent transgenic rice plants expressing the protein of interest did not yield the desired results, the assessment of whether the pCAMBIA/BMP2 expression vector was able to produce the protein of interest was not possible. Future experiments should be aimed at obtaining a fresh strain of LBA4404 and retransforming this strain with the pCAMBIA/BMP2 vector and reattempting the transfection of rice calli derived from more robust strains such as the Nipponbare rice cultivar.

The transformation of callus tissue with the pCAMBIA PIN-fusion and non-PIN-fusion constructs was unsuccessful. pCAMBIA plasmids contain a GUS gene driven by a CaMV35S promoter so that target plant tissue can be stained to confirm transformation. In all experiments performed, none of the tissue stained positive for GUS, indicating that either the transformation had not occurred or that the plasmid was not present. As *A. tumefaciens* transformed with the pCAMBIA vector was able to proliferate on selective media containing kanamycin and hygromycin (pCAMBIA encodes resistance to both antibiotics), conjugation and transformation of this plasmid construct may not have occurred between *A. tumefaciens*

and the callus tissue. After co-cultivation with liquid suspension of *A. tumefaciens*, calli were noted to lose their proliferating potential. Suspensions of *A. tumefaciens* were diluted to an OD of 1.0 at 600nm, as per [85]. However, this was a relatively dense suspension and may have compromised the callus tissue. Indeed the overgrowth of bacteria on many tissue samples, even in the presence of antibiotics after two weeks was noted. Another issue arising is the contamination of glycerol stocks of *A. tumefaciens* harbouring the pCAMBIA constructs. Here, many samples were found to be contaminated. It could be that both the optical density of the *A. tumefaciens* suspension as well as contamination could be factors in the unsuccessful attempts to produce transgenic rice calli. In the 2008 paper, Hiei and Komari give a range of suspension possibilities, from OD 0.2 to 1.0 at 600nm [85, 86] In a personal communication with a former member of our lab, Dr. Xiongying Cheng, who has successfully transformed rice, it was recommended that a suspension of OD 0.2 be used for future attempts.

4.4 CONCLUSION

The codon optimized DNA sequence of BMP2 designed for expression in rice is able to yield a fragment of an appropriate size when expressed in *E. coli*. However, assessment of whether this DNA sequence was able to express a similar fragment in the pCAMBIA expression vector and produce an analogous protein fragment in transgenic rice harboring this expression cassette failed after repeated attempts.

4.5 METHODS

4.5.1 Synthesis of a Codon-Optimized BMP2 Sequence for Transgenic Rice Expression

To ensure successful expression in rice, codon optimization of the BMP-2 gene was performed. The BMP2 sequence was taken from accession number NM_001200 from the GenBank database. From this DNA sequence, the mature peptide coding sequence was codon-optimized for expression in rice (*Oryza sativa*) according to the Codon Usage Database (<http://www.kazusa.or.jp/codon/>) [FIGURE 4.1]. The optimized coding sequence was submitted to DNA works (<http://helixweb.nih.gov/dnaworks/>) to obtain a set of oligonucleotides suitable for reaction via the PCA method. The oligonucleotide set was obtained from Invitrogen and subjected to PCA reactions.

4.5.2 Construction of pET21a(+):BMP2 Expression Vector

The method of Long et al. [78] was used for construction of the pET21a(+):BMP2 expression construct.

4.5.3 Construction of PIN And Non-PIN Fusion Vectors

To ensure high fidelity transcription in rice, the binary expression vector pCAMBIA 1305.1 was used. The expression cassette contained a Glutelin-1 (*Glu-1*) promoter, which allows targeted protein expression in the rice endosperm tissue compartment. The expression cassette design principles called for a Pin-A sequence immediately to follow the Glu-1 sequence and was connected to the PCA-synthesized codon-optimized rhBMP-2 sequence.

Transcription termination of this transcriptional fusion is accomplished by the Nopaline Synthase terminator (Nos-ter) accessed from a plant pathogen, *Agrobacterium tumefaciens*. For non-PIN-fusion vectors, a different assembly was used. The expression cassette contained a Glu-1 promoter, with the PCA-synthesized codon-optimized rhBMP-2 sequence being flanked by a Glutelin-1 signal sequence [FIGURE 4.3].

4.5.4 *Agrobacterium Transformation with pCAMBIA Constructs*

Agrobacterium tumefaciens LBA4404 was transformed with the PinA::BMP2 expression cassette contained within the binary vector pCAMBIA 1305.1 according to the freeze-thaw method protocol outlined by [85]. Non-PIN fusion constructs were also transformed with *A. tumefaciens*. Transformed *A. tumefaciens* cells were selected on YEP media containing kanamycin (50 mg/L) (Fisher Scientific) and hygromycin (50 mg/L) (Fisher Scientific).

4.5.5 *Transformation of Rice Callus tissue with A. tumefaciens*

Rice callus was harvested from plated rice seeds after 30 days of incubation on 2N6 callus induction media containing 2,4-dichlorophenoxyacetic acid (2 mg/L) [85]. Proliferating callus was removed from the rice seed remnants and was further subcultured on additional 2N6 media for 10 days. After 10 days, subcultured callus was incubated with a suspension of *A. tumefaciens* harbouring the pCAMBIA PINA::BMP2 construct as well as *A. tumefaciens* harbouring the pCAMBIA non-PIN BMP2 construct. The calli were incubated with the suspension of *A. tumefaciens* for 2 min. Following incubation, the calli were blotted

dry on sterile filter paper and plated on 2N6-AS media containing acetosyringone (19.6 mg/L). After three days of incubation, the calli were transferred to 2N6-TCH plates containing timentin (200 mg/L), claforan (250 mg/L) and hygromycin (50 mg/L) for 2 weeks in dark conditions at 28C.

4.5.6 Regeneration of Transgenic Callus Tissue

Once *A. tumefaciens* growth was not visible upon incubating calli, calli were transferred from 2N6-TCH plates to RGH6 media containing 6-Benzylaminopurine (3 mg/L) and 1-Naphthalene acetic acid (0.5 mg/L). Callus plated on RGH6 media was placed in the light at 5000 lux at 25C.

4.5.7 GUS- Staining Method for Confirmation of Transformation of Rice Callus Tissue

To assess whether callus was transformed with the pCAMBIA expression constructs, GUS staining was used as per the method used in Hiei [85].

5. *IN SILICO* ANALYSIS OF BMP2 L51 AND D53 MUTANTS USING THE CRYSTAL STRUCTURE OF THE BMP2/BMPRIA WITH AMBER MINIMIZATION

5.1 INTRODUCTION

Key binding hotspots have been elucidated on the BMP2 molecule. Furthermore, these hotspots have been mutated to residues that have greater affinity for the cognate receptor. Two key amino acid positions, L51 and D53 of the BMP2 molecule have been known to change the affinity of the BMP2 molecule for its cognate receptor for downstream cell signaling [51]. It has been shown that the mutation L > P (L51P) decreases the affinity for the receptor >8000-fold, resulting in no biological activity [51]. Also previously shown is the mutation D > A (D53A) increases the affinity of the molecule for its receptor 17-fold while concomitantly resulting in greater bioactivity *in vitro* [51]. Previous work established the activities of the BMP2 mutants L51P and D53A as well as D53S, D53P and D53R, but did not look at other amino acid substitutions at these residue positions. The exception is with cysteine substitutions as in the TGF- β superfamily, this particular residue is known to play a fundamental structural role that is imperative for function. Whether other amino acid substitutions would have an effect at this position remained unknown and whether such substitutions would be agonistic, antagonistic or would have no effect still remained to be tested.

Because of the large exposure of the D53 side chain to the solvent, side chains with hydrophilic groups may have more direct interaction with the surrounding solvent [87]. In the unbound free crystal structure of the BMP2 ligand, the D53 residue has one of the highest B-factors of all the residues within the PDB ID: 3BMP crystal structure of the free BMP2 molecule with only the exception of the first three amino acid residues of the N-terminus

which fluctuates as a random coil [51]. The direct interaction of the D53 side chain with the bulk solvent may act to dislodge the hydrogen bonding between D53 of the BMP2 ligand and C77 of the BMP type 1A receptor, while further dislodging the pre-helix loop, thus dissociating the BMP2 ligand from the BMP type 1A receptor and hence resulting in the dissociation of the entire signalling complex. Supporting this idea, it has been previously shown that the BMP2 mutant D53A has three-fold greater bioactivity than wild type BMP2 in a C2C12 alkaline phosphatase assay [79]. The current view of residue side chains in this position is that they largely interact with the solvent. This conclusion, however, was based on inference from a limited number of mutants and not all theoretically possible mutant substitutions at this position [50, 51].

To test whether the L51 and D53 positions could tolerate residue substitutions other than those previously characterized, an *in silico* approach was used to assess whether other standard amino acids could be substituted in the L51 and D53 positions using *in silico* mutated BMP2 ligands from the BMP2/BMPRIA crystal structure (PDB ID: 1REW).

Hypothesis: That when tested *in silico*, a subset of mutants of the L51 and D53 positions of BMP2 will be sterically compatible with the receptor-ligand binding interface.

5.2 RESULTS

5.2.1 L51-Series BMP2 mutant crystal structure minimizations

Bond length and angle measurements of the minimized BMP2 L51-series mutant main chain hydrogen bonds were taken to assess whether the known hydrogen bonding contacts were preserved during the minimization step. For *bona fide* hydrogen bonds, known parameters that are characteristic were used to determine whether or not the main chain hydrogen bonds of the L51-position were retained with mutated BMP2 ligands for each residue [FIGURE 5.1]. The bond length and angle measurement function of PyMol molecular viewer was used to determine the bond lengths and angles of the known main chain hydrogen bonds and thus used to assess the steric compatibility of a given substitution. Measurements were made for each BMP2 mutant ligand with minimized wt BMPRIA ectodomains. The mutant minimized ligands were first superimposed on minimized wt BMP2 ligand with BMPRIA ectodomains and the wt ligand was deleted, leaving the mutant with wt receptors [FIGURE 5.3]. All measurements of the hydrogen bonds between the L51-position main chain of the ligand with the Q86 side chain of the receptor were assessed this way. The resulting measurements were compared to known and accepted values for *bona fide* hydrogen bonds from [88]. These values were evaluated as to whether they did meet the criteria for *bona fide* hydrogen bonds. A subset of mutant BMP2 ligands did meet the criteria and are tabulated in [TABLE 5.1]. Ligands which did not fulfill these parameters were not further considered as they might have been sterically incompatible. The mutant BMP2 ligand L51C was excluded due to cysteine being highly conserved in the TGF- β superfamily and possessing an imperative role in the proper folding of the cystine-knot fold of this family of growth factors

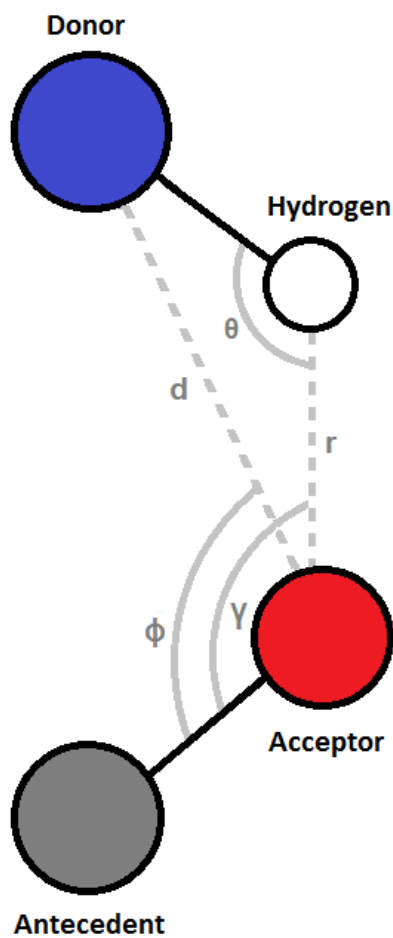
[33]. The addition of another cysteine residue could lead to improper folding rendering such a mutant ligand inactive biologically.

TABLE 5.1: List of Sterically Compatible L51-Series Mutants. Mutants were obtained by minimization of structures using Sybyl and then minimized using AMBER (<http://ambermd.org/>). Measurements were taken of the L51 main chain of BMP2 and the Q86 side chain of BMPRIA. The resulting set of mutants retained the hydrogen bonding scheme of the wild type interaction. Complete measurement parameters are tabulated in the APPENDIX section.

**Sterically Compatible L51-Series
Mutants**

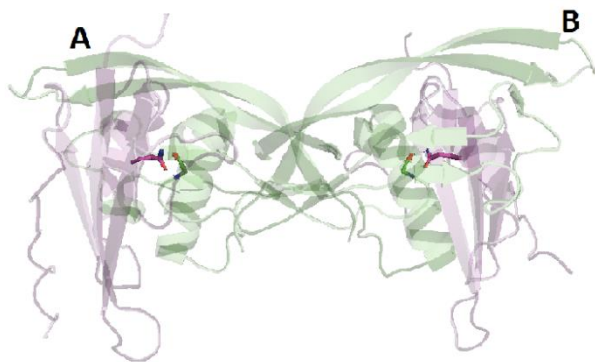
L51M L51Q L51N L51E L51D
L51S L51I L51F L51T L51V
L51A L51G

FIGURE 5.1 Measurement scheme for validation of *bona fide* hydrogen bonds. L51-series mutants were measured according to the scheme outlined. All mutants possessing values outside the ranges shown were not included for further consideration. Values for all minimized mutants are in Appendix Table 1, Figure adapted by author from [88] ©.



- r Distance between Hydrogen and Acceptor ($<2.500 \text{ \AA}$)
- d Distance between Donor and Acceptor ($<3.900 \text{ \AA}$)
- θ Angle of Donor-Hydrogen-Acceptor ($>90.000^\circ$)
- γ Angle of Hydrogen-Acceptor-Antecedent ($>90.000^\circ$)
- ϕ Angle of Donor-Acceptor-Antecedent ($>90.000^\circ$)

FIGURE 5.2 Measurements of Inter Side-Chain Residues of the L51-Series Mutants. For each minimized structure, measurements were taken for both subunits of the BMP2 homodimer. Chains A and B of the BMP2 homodimeric ligand were measured separately according to the *bona fide* hydrogen bond parameter scheme in **FIGURE 5.1**. Hydrogen bonds are shown as black dashes.



CS MN	r	d	θ	γ	ϕ
Leu51A-Gln86					
N-H...O=C	0.988	1.980	161.670	111.401	116.687
C=O...H-N	1.401	2.322	146.648	99.598	113.458
Leu51B-Gln86					
N-H...O=C	1.536	2.540	168.796	124.849	124.612
C=O...H-N	1.643	2.606	155.546	128.414	135.744

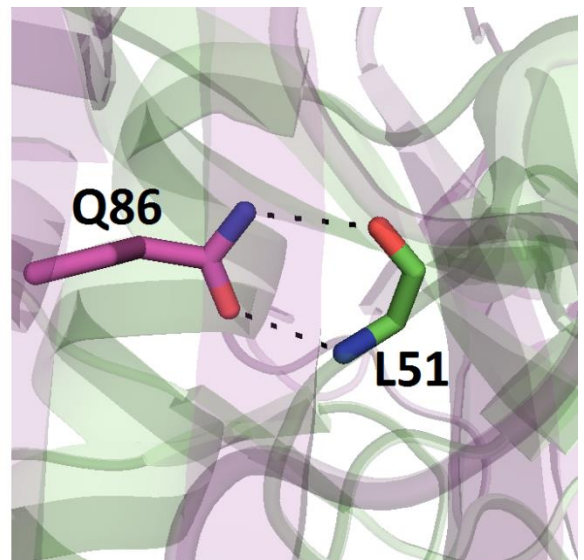
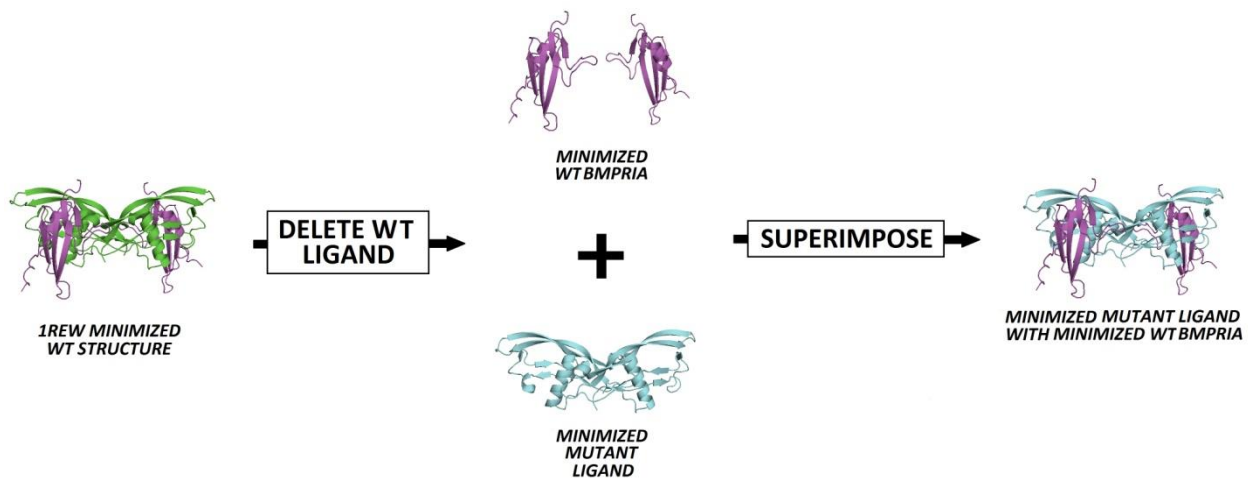


FIGURE 5. 3 Method for measurement of L51-series mutant BMP2 ligands with receptors. The scheme shown was used to make measurements using the measurement functions included with PyMol molecular viewer.



5.2.2 D53-Series BMP2 Mutant Crystal Structure Minimizations

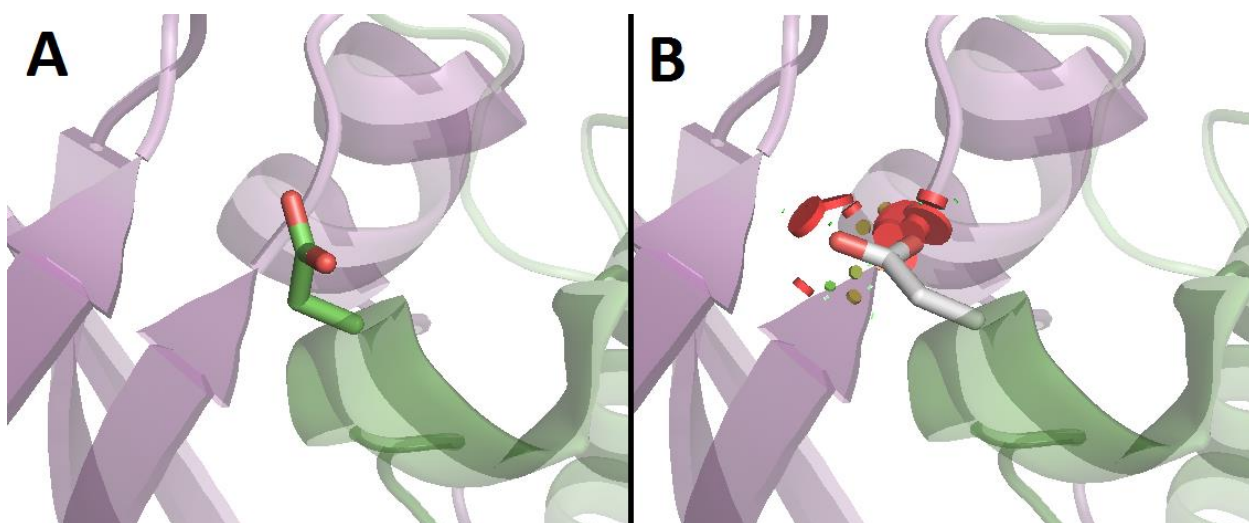
For the BMP2 D53-series of *in silico* mutated BMP2 ligand structures, the ϕ and ψ angles of the substituted amino acid residues were taken and measured using the PyMol plug-in DynoPlot (<http://www.pymolwiki.org/index.php/DynoPlot>). Not included in this series of structures were the substitutions D53C and D53F. As discussed before, cysteine residues are highly conserved in the TGF- β superfamily and are essential for proper structure, so this substitution was again excluded here. The D53F structure did not yield results, so it was not pursued and thus excluded from further analysis. As these mutated ligands were minimized along with the BMPRIA ectodomains from the crystal structures, steric clashes with residues from the spatially opposing residue side chains was taken into account using this approach. The D53 residue of wt BMP2 possesses alpha helix character, thus this parameter was used to compare other residue substitutions to assess whether this alpha helix character was preserved. As is shown in [TABLE 5.2], almost all residue substitutions yielded values within the region of accepted values for right-handed alpha helices as given by Ramachandran [89]. However, there was a large discrepancy when compared to ‘ideal’ alpha helix torsion angles taken from alpha helices in crystal structures [90].

TABLE 5.2 The ϕ and ψ torsion values of the minimized mutant BMP2 D53-series. The dihedral angles ϕ and ψ of each of the D53-series BMP2 mutant structures were measured and tabulated using the PyMol DynoPlot application.

RESIDUE/MUTANT	ϕ	ψ
D53 crys	-49.00	-48.38
D53 min	-46.57	-50.86
D53A	-53.28	-35.43
D53G	-59.00	-32.41
D53V	-43.17	-50.20
D53I	-49.88	-42.33
D53L	-48.58	-45.68
D53S	-56.08	-38.51
D53T	-38.91	-51.60
D53E	-44.62	-46.03
D53N	-49.10	-50.64
D53Q	-42.32	-50.78
D53H	-49.27	-48.07
D53R	-46.30	-24.38
D53K	-43.23	-49.38
D53P	-62.35	-39.99
D53Y	-76.10	-41.95
D53W	-70.38	-21.62

As residue side chains only exposed to solvent are rarely static unless engaged in an interaction where their intrinsic rotational motion is subdued [91], the PyMol wizard mutagenesis rotamer library function was used to assess the number of rotamers recognized by the program for each mutant BMP2 D53-series mutant and whether these rotameric conformations would clash or hinder other residues and their side chains proximal to the mutant residue side chain [FIGURE 5.4]. The number of backbone independent rotameric steric clashes out of the number of total rotameric conformations was assessed. Residues with large side chains of high molecular weight and higher numbers of rotameric conformations recognized by the PyMol program such as the side chains of tryptophan and arginine showed that all the conformations recognized by the program resulted in steric clashes with side chains on the receptor. Many of the mutant BMP2 D53-series ligands did not possess rotameric conformations that were sterically compatible. BMP2 D53-series mutants that did have rotamer conformations that were not sterically permissible were D53G, D53Q, D53A and D53S [See APPENDIX TABLE 2].

FIGURE 5.4 Rotamer Steric Clash Assessment. A) The D53 residue side chain as positioned in the Crystal Structure 1REW. B) The D53 residue side chain using the PyMol wizard mutagenesis function with one of the possible rotamer conformations included in the PyMol rotamer library. The red discs indicate van der Waals overlap with other side chains within the vicinity of the given rotameric conformation. Methods are explained on page 74, Section 5.5.



5.3 DISCUSSION

For the L51 BMP2 mutant pool, residues with side chains of less molecular weight than histidine seem to possess the capability of maintaining the imperative hydrogen bonding scheme with the receptor Q86 side chain carboxamide group in this *in silico* model [FIGURES 5.1 – 5.4]. Mutant BMP2 ligands with L51-substituted amino acid residues such as tryptophan and tyrosine have side chains which were sterically incompatible with the binding interface and thus they did not appear to preserve the main chain hydrogen bonds according to the criteria for *bona fide* hydrogen bonds. Ligands with side chains of higher molecular weight than leucine such as glutamic acid retained the hydrogen bonding scheme of wt BMP2 and thus met the criteria for *bona fide* hydrogen bonding.

For the D53-series, although most minimized mutant BMP2 ligands possessed values that seemed to retain values within the accepted range for retention of the alpha helical structure of the D53 residue in wt BMP2, many residue substitutions at this position seemed to have either ϕ or ψ values that deviated significantly from both the wtBMP2 crystal structure and the minimized crystal structure. D53L, D53N and D53H seemed to possess ϕ or ψ values that were acceptable with the values considered ideal for a right-handed alpha helix [90].

Analysis of steric clashes using the PyMol backbone-independent rotamer library for the BMP2 D53-series revealed that many substitutions might be sterically restricted. Given that this residue position plays a smaller role in binding affinity, certain substitutions may increase or decrease the BMP2-BMPRIA affinity on a smaller level compared to that of mutations at the L51 position. Previous work inferred, through the activity of mutants D53P, D53R and D53S, that as these residues are exposed to solvent, mutations at this position

might be well tolerated and have little to no effect on binding affinity [79]. This residue position may not play a significant role in the interaction between wtBMP2 and BMPRIA but might play a role in BMP2 binding with other TGF- β superfamily receptors. The PyMol rotamer library used here does not factor in differences in possible conformation changes of the main chain backbone. This was chosen as if wide variations in the ϕ and ψ values were to change; this residue position might lose its alpha helical character. Hence only the side chain rotamers in their minimized backbone position were assessed here for each mutant.

5.4 CONCLUSION

The results suggest that certain BMP2 mutants at these two positions, L51 and D53, might be limited if not restricted to a small subset of substitutions and that in agreement with previous work, D53 position mutations may not play a major role in the interaction between wt BMP2 and BMPRIA, but might play a role in interactions with other analogous receptors. However, D53-series mutants may modulate the BMP2-BMPRIA binding affinity due to the lack of steric clashes from smaller side chains or the presence of steric clashes from bulkier residue side chains. Whether such mutants would be biologically active motivates further *in vitro* testing to confirm whether the L51-series of BMP2 mutants are biologically active and to assess the degree of influence of the D53-series substitutions.

5.5 METHODS

5.5.1 *In silico* Analysis of BMP2 Mutants

The crystal structures of BMP2 and BMPRIA PDB ID: 1REW Chains A and B were used as a template for subsequent mutagenesis and minimization using AMBER (AMBER 12, San Francisco, CA, USA) [92]. The BMP2 leucine 51 position residue was mutated to the desired residue using Sybyl (Certara, Princeton, NJ, USA). The AMBER force field used was ff99SB [93] and the AMBER editor xLeap (UCSF, San Francisco, CA, USA) was used for further editing. Disulfide bonds were added manually, the charges on the molecular complex were neutralized by adding the appropriate amount of Na⁺ ions and the complex was solvated in an octagonal box of water molecules (on advice from Dr. Jyothi Kumaran). The BMP2 units from the crystal structure were mutated using Sybyl to the desired amino acid residues. Structures were set to 60 minimization rounds and saved for further evaluation.

Files of the AMBER minimized mutant BMP2 ligand structures were fitted to minimized wild-type BMPRIA and positioned in place of the wild-type ligand using the PyMol superimpose function. [FIGURE 5.3] Measurements were taken of the hydrogen bonding contacts between the main chain binding determinants of the mutated L51 position residue and the Q86 side chain carboxamide group using PyMol measurement functions. Mutants which displayed measurements that corresponded to *bona fide* hydrogen bonds [FIGURE 5.1] were considered for further synthesis in *E. coli* and subsequent CD spectroscopy and alkaline phosphatase activity assays using C2C12 promyoblast cell cultures [79].

5.5.2 Measurement of Mutant BMP2 Dihedral Angles, ϕ and ψ

The dihedral angles ϕ and ψ of the D53-series BMP2 minimized mutated crystal files were measured and tabulated using the PyMol Dyno Plot application after following the scheme outlined in **[FIGURE 5.3]**.

5.2.3 Assessment of Rotameric Conformations and Steric Clashes

D53-series rotameric conformations were evaluated using PyMol's wizard mutagenesis backbone-independent rotamer library for tabulation of number of steric clashes. Ligands were first fitted using the scheme outlined in **[FIGURE 5.3]**, then side chains were rotated through those available with the program library. Steric clashes were denoted by the presence of red discs indicating van der Waals overlap.

6. CIRCULAR DICHROISM SPECTROSCOPY OF THE BMP2 L51-SERIES MUTANTS DERIVED FROM *IN SILICO* SCREENING AND ALKALINE PHOSPHATASE ACTIVITY IN C2C12 CELLS TREATED WITH THE RECOMBINANTLY EXPRESSED MUTANT PROTEINS

6.1 INTRODUCTION

BMP2 is a cytokine growth factor which elicits *de novo* bone formation in adult mammals. BMP2 recruits mesenchymal stem cells to differentiate into osteoblasts, stimulating bone formation. BMP2 is used in surgical applications ranging from spinal fusion procedures to dental implants. A current major drawback is the large quantity of BMP2 needed for efficacy in these procedures. This adds not only to high costs for these procedures but also increases the chances of adverse immune responses, which can result in clearance of implanted BMP2, swelling and pain for the patient and also anti-BMP2 antibody formation [5]. Smaller physical doses may have the potential to reduce these effects. Creating a more potent BMP2 molecule may enable implantation of smaller physical doses while maintaining sufficient potency to achieve successful surgical procedures.

BMP2 belongs to the TGF- β superfamily of growth factor ligands. BMP2 shares sequence homology with the other BMPs to various degrees, as well as a sequence of conserved cysteine residues which enable these molecules to form a cystine-knot motif, as they lack a hydrophobic core [27]. The TGF- β superfamily members are all homodimeric proteins but can also form bioactive heterodimers. The BMP2 homodimer is the biologically active form of BMP2 and binds to its type 1A receptors in a 1:2 BMP2-BMP type 1A receptor complex. Although there are more than 30 TGF- β superfamily ligands and 7 different type 1 receptors and 5 different type II receptors, BMP2 has highest binding affinity for the BMP type 1A receptor [37]. Once BMP2 binds its type IA receptors, the BMP type II receptors are then

able to complete the signalling complex formation and transduce the signal across the plasma membrane to the intracellular compartment.

The binding interface between BMP2 and the BMPRIA is complex [50]. However, one particular binding hotspot is known, that is the interaction between L51 of the BMP2 ligand and Q86 of the BMPRIA. Mutation of the L51 residue to a proline results in a greater than 4000-fold reduction in binding affinity to the BMPRIA [51]. The L51P mutant has also been shown to be biologically inactive as well. The L51 residue is situated in a mobile domain termed the “pre-helix loop” [51]. The BMP2 pre-helix loop is composed of six amino acid residues F49, P50, L51, A52, D53 and H54, which are thought to fluctuate randomly in the free ligand when unbound to BMPRIA [FIGURE 1.4]. Three of the six residues contribute hydrogen bond contacts to the receptor when the BMP2 ligand is bound to the BMPRIA. In the case of L51, it has two main chain hydrogen bond contacts with the side chain of Q86 of the BMPRIA.

Hypothesis: The L51 mutant collection derived from the *in silico* modeling in my previous studies will possess different CD spectra and bioactivity than wt BMP2.

6.2 RESULTS

6.2.1 Sequence Alignment to Show Conserved Homology of BMPs, GDFs and Dpp

The L51 position is conserved amongst the BMPs and GDFs that share significant sequence homology and that bind to BMPRIA as well [25, 27, 48]. Using T-Coffee Espresso multiple

peptide sequence alignment tool, a multiple sequence alignment of the homologous C-terminal human BMPs and GDFs peptide sequences as well as the Drosophila Dpp peptide confirms previous reports that leucine at the analogous BMP2 L51 position in these TGF- β superfamily related molecules is conserved [51, 94] **[FIGURE 6.1]**

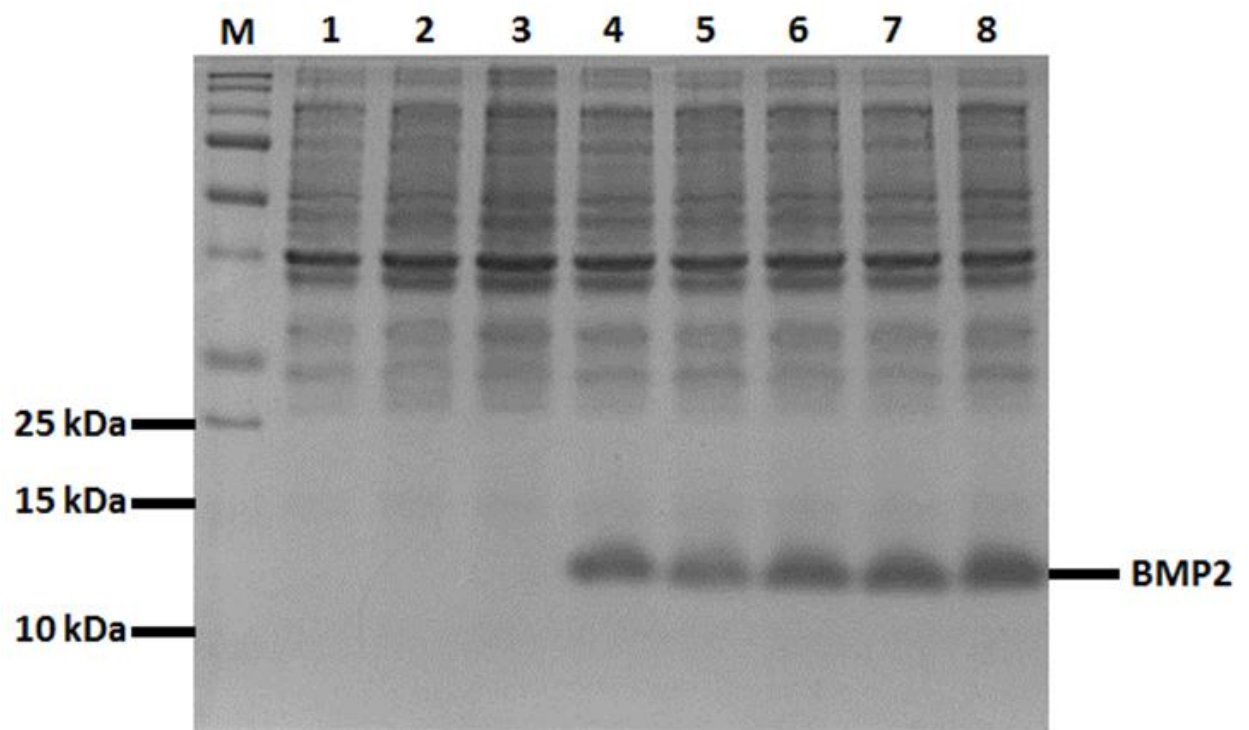
(<http://www.tcoffee.org/Projects/tcoffee/>).

FIGURE 6.1: T-Coffee Alignment of the sequences of BMP2, 4, 7, GDF 5 and *Drosophila Dpp*. The FPLADH domain or analogous domain in these molecules is outlined in a green box (<http://www.tcoffee.org/Projects/tcoffee/>).

6.2.2 Synthesis of mutant BMP2 proteins

BMP2 DNA mutant sequences were synthesized using wt BMP2 ligated in pGEM T-Easy subcloning vector (Promega Corp. Madison WI, www.promega.com). Once mutagenesis was verified by DNA sequencing, mutated sequences were double digested from pGEM and ligated into pET21a(+) using RG cells as the expression host. BMP2 mutant proteins were expressed and refolded as per the method outlined in [78] [FIGURE 6.2]. The complete collection in [TABLE 5.1] was synthesized for further spectroscopic and assay analysis of the mutant proteins.

FIGURE 6.2: SDS-PAGE Analysis of Mutant BMP2 Proteins. *E. coli* expressed, insoluble extract fraction. M-Markers, 1-Rosetta (no plasmid); 2-Rosetta + pET21a(+); no insert; 3-pET21a(+) + Reverse insert; 4-wtBMP2; 5-L51V BMP2; 6-L51A BMP2; 7-L51G BMP2; 8-L51P BMP2. Mutant proteins expressed as per the method outlined in [78].

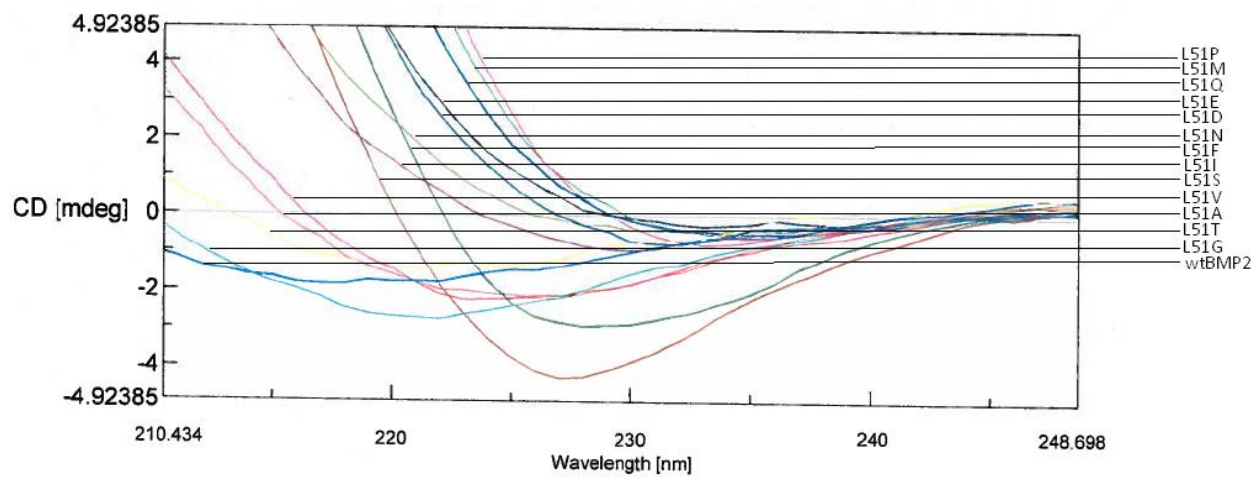


6.2.3 CD Spectrum of BMP2 L51 Mutants

The secondary structure of each of the unbound refolded BMP2 and L51-series of mutant BMP2 proteins was assessed using CD spectroscopy. CD spectral analysis was limited to the range of wavelengths between 200-240nm [95]. A spectrum was obtained for each mutant protein and all spectra differed from the wt BMP2 spectrum [FIGURE 6.3]. Only two mutant proteins, L51G and L51T, had spectra comparable but not identical to the spectrum of wt BMP2. Other mutant proteins displayed spectra that were different from wt BMP2, although residues possessing bulkier side chains seemed to display spectra which resembled that of the BMP2 L51P mutant CD spectrum. The cystine-knot architecture allows for the BMP2 molecule to have a bound structure that has rigidity [96]. The majority of the BMP2 molecular secondary structure is known to be predominantly in β -sheet conformation, however, only a few domains of the molecule possess flexibility and hence may be physically capable of producing some change in secondary structure. The pre-helix loop is known to be mobile in the unbound ligand state and hence the differences in the secondary structure of this domain may be solely responsible for producing the differences in the spectra, as the only difference amongst the proteins is the substituted residue within the pre-helix loop. The spectra of the mutant BMP2 L51-series shown here corresponds to either random coil, loop or hairpin conformation [97] [98]. The minima of all mutant spectra compared to the spectrum of wt BMP2 is right-shifted with the spectra of L51G and L51T being closer to wt BMP2 with the other spectra tending to shift towards the L51P BMP2 spectra [98]. CD spectra have been obtained previously for wt BMP2, however, the buffers used were sodium acetate and guanidinium chloride [99]. All of the present protein samples were prepared in sodium phosphate buffer, pH 3, to obtain the spectra of the wt BMP2

molecule and all other BMP2 mutant spectra, so comparison to the previously published spectra may show differences. The data suggest that substitutions at the L51 site in unbound BMP2 produce differences in secondary structure of the pre-helix loop in solution. However, the secondary structure of the bound ligand-receptor complex was not assessed, only the free homodimeric ligand in solution.

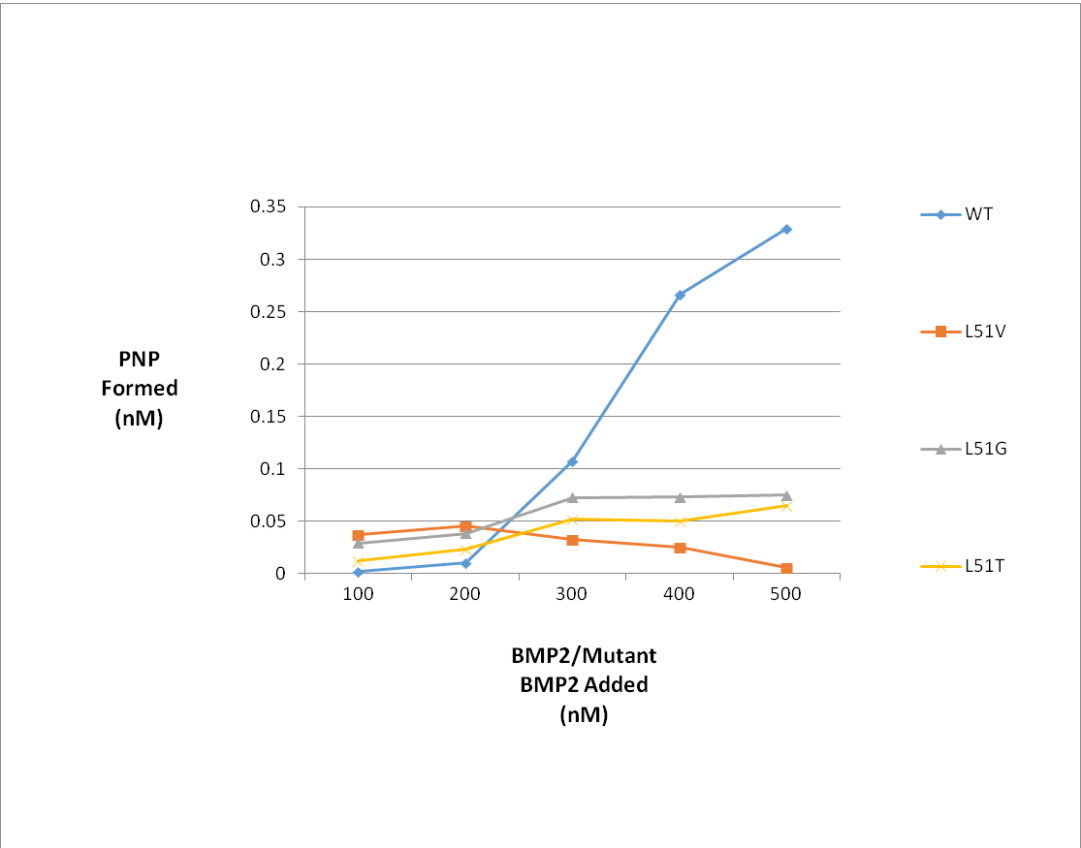
FIGURE 6.3: CD Spectroscopic Analysis of BMP2 Mutant Proteins. The CD spectra of L51-series BMP2 mutant proteins measured between 210-250 nm. CD spectrum of each sample was analyzed using a JASCO J-815 spectropolarimeter (JASCO Analytical Instruments, Easton, MD, USA).



6.2.4 Alkaline Phosphatase Activity of L51-Series Mutants in C2C12 Cells

None of the L51-series BMP2 mutants previously determined via *in silico* had any notable activity in comparison to wt BMP2 concentrations from 300 nM to 500 nM [**FIGURE 6.4**]. Between this dose range, only wt BMP2, produced a dose-dependent response, while no other mutant proteins gave any response over the 3-day incubation with the given L51-series BMP2 mutant proteins (Data shown only for L51V, L51G and L51T). Mutants of other residues of BMP2 have largely been shown to have reduced activity in C2C12 assays, suggesting that the evolutionary design of BMP2 may in fact be highly specific for BMPRIA [79]. Thus, none of the present sterically compatible L51-series mutants [**TABLE 5.1**] were able to yield activity comparable to or above wt BMP2.

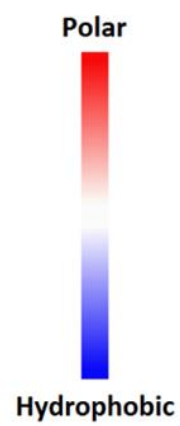
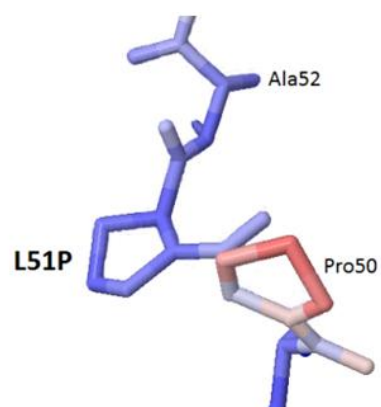
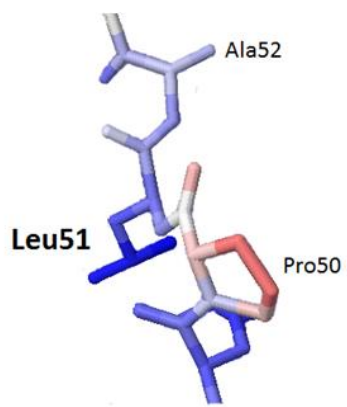
FIGURE 6.4: Alkaline phosphatase induction in C2C12 cells. Cells were incubated in a 96-well microplate in Dulbecco's Modified Eagle Medium (DMEM) with the L51-series of BMP2 mutant proteins (n=3) for three days then were lysed and incubated with 1mg/ml para-nitrophenylphosphate (PNPP) as per the method described by [79]. Shown here are wild type BMP2 (blue line), L51V BMP2 (orange line), L51G BMP2 (gray line) and L51T BMP2 (yellow line).



6.2.5 Atomic Depth Calculation Using Simple Atom Depth Index Calculator (SADIC)

To better appreciate the depth to which the ligand ‘penetrates’ into the receptor binding pocket, each atom’s relative position was estimated by further modeling using a hydrophobic atom depth calculator [100]. Although unbound L51 in the pre-helix loop is thought to be part of the pre-helix loop as a mobile element, and thus could be surrounded with water molecules in solution, upon receptor binding, this motion is largely dissipated. It is speculated that this side-chain may in fact be secured in a hydrophobic pocket when the BMP2 ligand is bound to the BMPRIA. The securing of this side-chain within the hydrophobic region of the protein is thought to provide stability and structure [53]. Submission to SADIC calculates the depths of each atom within a hydrophobic pocket of proteins and maps the degree of surrounding hydrophobic influence as a depth index. SADIC analysis (<http://www.sbl.unisi.it/prococoa>) of the 1REW structure showed that the isopropyl group of the L51 side-chain lies in a predominantly hydrophobic region [**FIGURE 6.5**]. Upon submission of the 1REW PDB file, the BMP2 L51P mutant crystal structure, to the SADIC calculator, the L51P side-chain was observed not buried as deep within a hydrophobic domain compared to the L51 side chain in the wild-type protein. This is in agreement with the previous thinking that the GDF5 L56 side-chain is buried in a hydrophobic core and is analogous to the L51 side chain of BMP2 [53]. Thus the L51 side chain seems to be accommodated by a hydrophobic fitted pocket which structurally is tailored for this side chain only.

FIGURE 6.5: SADIC Analysis of the Wild Type BMP2 and the L51P Mutant. Shown here is the level of hydrophobicity of the atoms within the tripeptide sequence P50-L51-A52 for wild type BMP2 and P50-P51-A52 for the L51P BMP2 mutant. The isopropyl component of the L51 side chain appears to be particularly shaded deep blue and indicates that this portion of the side chain is excluded from external solvent while the side chain of the proline substitution is situated in a much less hydrophobic environment.

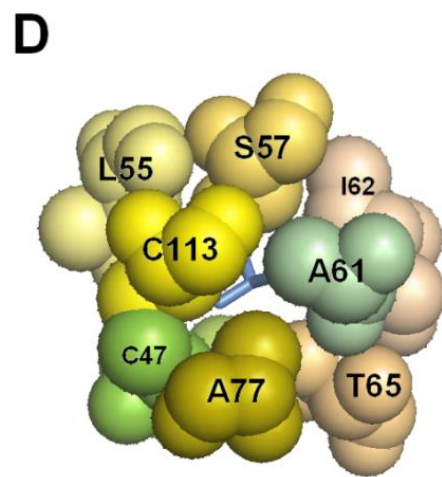
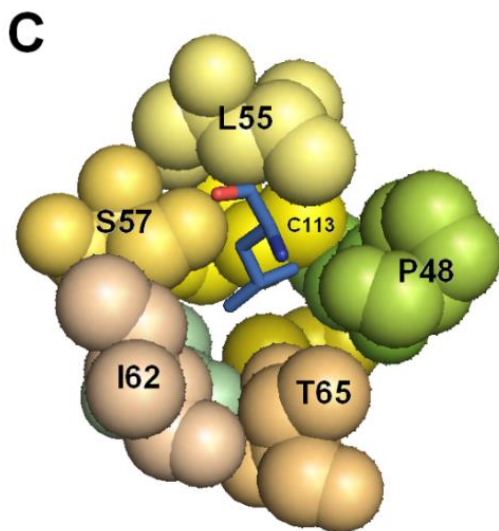
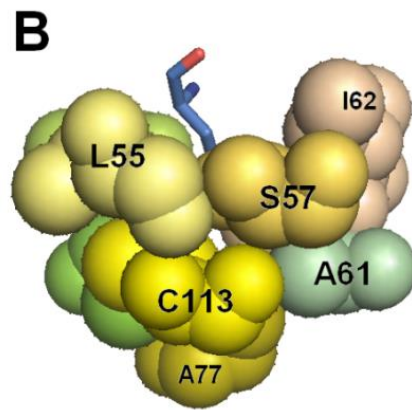
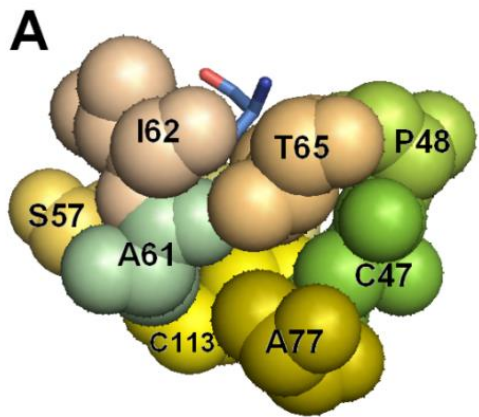


6.3 DISCUSSION

Under the experimental model employed – *E. coli*/C2C12 – wt BMP2 yielded bioactivity readouts comparable to those previously reported for *E. coli* based BMP2. The *in silico* tested sterically compatible mutant collection from the previous chapter did not yield any activity when each of the mutant BMP2 proteins were incubated with C2C12 cells. The absence of bioactivity of the L51-series of mutants tested here might suggest there is another aspect of this residue that contributes to the interaction between BMP2 and BMPRIA. The pre-helix loop is thought to be mobile in the free ligand and hence the conformations of this loop are random and not ordered. The specificity of the L51 side chain was demonstrated in that, despite having an analogous structure to such amino acids as valine or threonine, these analogous side-chains were unable to substitute for the L51 side-chain and produce notable bioactivity.

Upon examining the 1REW crystal structure with the PyMol molecular viewer, the residues that seem to be immediately surrounding the L51 side chain γ -carbon within a 5Å radius are C47, P48, L55, S57, T58, A61, I62, T65, A77 and C113. When bound by the receptor, these residues seem to form a pocket which distinctly appears to accommodate the L51 side chain [FIGURE 6.6]. This might suggest that the L51 side-chain is accommodated by an intramolecular induced-fit mechanism upon binding to the receptor. The GDF5 L60P mutant is known to have greatly decreased bioactivity when assayed [54]. Another residue in BMP2, namely L55, is noted to be present as one of the residues surrounding the BMP2 L51 side chain. Perhaps the reduced bioactivity of GDF5 L60P could be due to the disruption of an analogous pocket in the GDF5 L60P mutant. Two other GDF5 residues, S58T and H59L,

FIGURE 6.6: Intramolecular Pocket Accommodating the Side Chain of L51. Shown here are the amino acid residues of the BMP2 molecule that surround the L51 side chain. The residues shown here are: C47, P48, L55, S57, A61, I62, T65, A77 and C113. The L51 residue is shown as a blue stick for comparison. A) right side view, B) left side view, C) top view and D) bottom view. The BMP2-BMP receptor 1A (PDB ID: 1REW) structure was used for the visualization. The colors provided do not correlate with any physical differences and are only provided for visual distinction for each of the amino acid residues.



were noted to have no bioactivity when tested in vitro [54]. These GDF5 residues are analogous to S57 and T58 in BMP2 which surround the leucine side chain in the crystal structure 1REW. Perhaps these residues may play a vital structural role in forming the appropriate architecture to accommodate the BMP2 L51 (or the L56 side-chain in GDF5) side-chain in particular and mutation of these residues may disrupt the architecture, thus leading to the lack of bioactivity in vitro [54].

The requirement for the L51 side-chain to be aligned for the proposed induced fit mechanism might be a factor, if not an obstacle, in the development of peptide inhibitors that have affinity for the BMPRIA binding interface. Assuming an induced-fit pathway, the side-chain of L51 may have to dock in a certain sequential manner within a hydrophobic cavity to provide the required orientation for the interaction of the main chain hydrogen contacts with the Q86 side chain to produce the final bound complex structure between BMP2 and BMPRIA. Hence, a cyclic peptide inhibitor may be required to accommodate the L51 analog; the analog may need to be designed such that this residue is able to trace an appropriate induced-fit pathway in order to produce a suitable orientation.

It is unknown whether these mutants will have differing interaction activity with the soluble inhibitors such as noggin or chordin. The BMP2 L51P mutant is able to interact with noggin, inhibiting noggin interaction with wt BMP2, thus enhancing osteoactivity [101]. It is also uncertain whether this same effect will carry over with the other L51 variants and remains to be evaluated. It was shown that a mutation in BMP6, K60, (corresponding to BMP2 P36) confers increased noggin resistance and that resistance to noggin can be increased when BMP2 P36 is mutated to lysine [102]. However, this mutation is not located

in the pre-helix loop region and whether the L51 mutants will have differing binding affinities for soluble inhibitors such as noggin is still yet to be determined.

Leucine 51 seems to function as a main binding determinant as it provides two main-chain hydrogen bonding contacts. However, it may also play additional sub-roles during the process of the induced-fit binding mechanism pathway, the side-chain of L51 may have additional intramolecular interactions within the BMP2 ligand itself. The way forward is now clearer to produce improved BMP2 protein in transgenic plant platforms [103].

6.4 CONCLUSION

Substitutions at the L51 site produced differences from wt BMP2 in secondary structure in the unbound pre helix loop region. Leucine 51 is essential for BMP2 activity when tested with alkaline phosphatase assays using C2C12 promyoblasts. This aspect of intramolecular structure needs to be additionally considered for small molecule or peptide mimetics or inhibitors of BMP2 in order for the clinical applications of this protein to be improved.

6.5 METHODS

6.5.1 Site Directed Mutagenesis (SDM) of the BMP2 L51 Position

The BMP2 sequence template was ligated into the pGEM T-Easy subcloning vector and the Stratagene Quick Change SDM kit (Agilent Technologies, Santa Clara, CA, USA) was used to generate mutant DNA sequences. Mutated sequences were excised via double digestion with *NdeI* and *NotI* (New England Biolabs, Ipswich, MA, USA) and ligated into pET21a (+) vectors (EMD Millipore, Billerica, MA, USA) for protein expression in *E. coli*.

6.5.2 Synthesis and Expression of BMP2 Mutant Proteins in E. coli

Recombinant wtBMP2 and mutants were synthesized, purified and refolded as described in the procedure by Long et al. [78].

6.5.3 Circular Dichroism Spectroscopy of wt BMP2 and BMP2 L51-Series Mutant Proteins

Purified and refolded wtBMP2 and thirteen other L51 BMP2 mutant proteins were refolded overnight as described in the procedure by Long et al. [78], resuspended in 0.5M phosphate buffer, pH 3.0 at a concentration of 0.1 mg/mL and the CD spectrum of each sample was analyzed using a JASCO J-815 spectropolarimeter (JASCO Analytical Instruments, Easton, MD, USA).

6.5.4 C2C12 Alkaline Phosphatase Activity Assays

To verify that the recombinant wtBMP2 protein as well as the mutated proteins were bioactive, the recombinant protein samples were incubated with C2C12 murine promyoblast cells in the procedure described by Kirsch et al. [79]. Assays were read on a BioTek PowerWave HT microplate spectrophotometer using Gen5 Data Analysis software (BioTek, Winooski, VT, USA).

7. DISCUSSION

The design and synthesis of the rice codon optimized BMP2 DNA sequence and its verification in *E. coli* by the production of an expected size peptide product enabled verification that this DNA sequence which was otherwise designed for protein expression in rice, was able to produce a bioactive protein product that was able to induce alkaline phosphatase activity when incubated with C2C12 cells with activity similar to that reported in the literature. This DNA sequence still requires further testing to assess whether rice will produce a correctly folded and properly glycosylated bioactive protein product.

Minimizations using AMBER produced a set of twelve mutant L51-series BMP2 that suggested that a subset of residue substitutions would be tolerated at this position, but further examination of the mutant collection showed that these mutants possessed no bioactivity. This result suggested that the *in silico* method used here for the L51 BMP2 mutant series was unable to account for hydrophobic interactions that are crucial to the proper binding of the BMP2 ligand with the BMPRIA if assessed in vitro with C2C12 cells. The stringent requirement for leucine at the L51 position suggests that this side chain is specifically tailored to fit within an intramolecular hydrophobic cavity within the ligand itself [FIGURE 6.6]. From the results obtained in these studies, this residue possesses essential functionality with both its main chain, as shown by Keller et al. [51], and also its side chain which appears to have a key-like fit within the ligand upon binding with BMPRIA. This intramolecular interaction allows the side chain to be used as an “intramolecular rudder” to orient the main chain hydrogen bonding contacts with the Q86 side chain contacts of the BMPRIA.

In the case of the D53-series of mutants, the result that there are less steric clashes with a small subset of mutants with the BMPRIA might suggest that there is less steric disturbance from this residue substitution and this might be responsible for the greater bioactivity shown in the D53A mutant [51]. The rotameric analysis of D53-series of mutants further suggest that that the D53-position of BMP2 may be limited in the number of substitutions that can be tolerated at this position; however, further bioactivity assays are needed to confirm this.

8. OVERALL CONCLUSION OF THESIS

The codon optimized DNA BMP2 sequence designed for rice expression, is able to produce a peptide which, after purification and refolding, is able to produce a bioactive protein product that yields a CD spectrum and also stimulates alkaline phosphatase activity in *in vitro* assays in C2C12 cells in line with results reported from other investigations. Although the method of AMBER minimizations of BMP2 mutants used here show retention of the known hydrogen bonding schemes between the BMP2 ligand and the BMPRIA using the PDB crystal structure 1REW, they fail to account for possible, specific intramolecular hydrophobic interactions within the BMP2 ligand itself.

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CONTRIBUTION OF COLLABORATORS

Dr. Jyothi Kumaran formulated and directed the *in silico* methods used in this study. She also provided guidance with the use of the CD Spectropolarimeter.

Dr. Roger MacKenzie provided access to his lab facilities and equipment for both the CD spectroscopy work as well as the *in silico* work.

Dr. Marc André Langlois provided access to using his culture hoods for the C2C12 assay work.

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APPENDIX

Table 1: Measurements of the L51-series of BMP-2 mutant ligands fitted to 1REW receptors. Receptors are the C and D chains from the crystal structure of 1REW. (Except CS 1REW, which is the original crystal structure without modifications from the PDB file 1REW). Optimal Criteria are given below in the row (H-BOND)*. Mutants and measurements highlighted in red do not meet the selection criteria shown in the (H-BOND)* row. Criteria are taken from [88].

(H-BOND)*	<2.500 Å	<3.900 Å	>90.000°	>90.000°	>90.000°
L51T	r	d	θ	γ	Φ
Thr51A-Gln86					
N-H...O=C	1.365	2.368	169.618	113.129	117.176
O=C...H-N	1.644	2.576	149.649	117.978	128.671
Thr51B-Gln86					
N-H...O=C	1.465	2.452	157.376	108.466	110.186
C=O...H-N	1.831	2.716	143.081	106.475	119.492

L51R	r	d	θ	γ	Φ
Arg51A-Gln86					
N-H...O=C	2.635	3.377	121.797	92.945	106.048
O=C...H-N	2.792	3.556	131.976	114.469	124.660
Arg51B-Gln86					
N-H...O=C	2.363	3.124	130.864	92.440	106.678
C=O...H-N	2.585	3.383	134.917	114.519	125.032

L51K	r	d	θ	γ	Φ
Lys51A-Gln86					
N-H...O=C	1.740	2.439	122.253	77.800	98.004
O=C...H-N	1.885	2.675	131.816	98.020	113.598
Lys51B-Gln86					
N-H...O=C	1.929	2.678	128.086	83.517	100.870
C=O...H-N	2.031	2.859	136.601	105.391	118.987

L51Q	r	d	θ	γ	Φ
Gln51A-Gln86					
N-H...O=C	1.270	2.285	178.388	116.691	116.282
O=C...H-N	1.288	2.245	152.982	121.324	133.175
Gln51B-Gln86					
N-H...O=C	1.575	2.548	158.800	113.674	116.032
C=O...H-N	1.704	2.654	153.143	119.786	129.753

L51W	r	d	θ	γ	Φ
Trp51A-Gln86					
N-H...O=C	2.351	2.959	117.415	71.451	86.408
O=C...H-N	2.842	3.541	126.169	74.750	86.833
Trp51B-Gln86					
N-H...O=C	1.476	2.475	167.546	105.41	106.087
C=O...H-N	1.876	2.637	128.700	91.599	108.395

L51H	r	d	θ	γ	Φ
His51A-Gln86					
N-H...O=C	0.996	1.975	158.585	82.366	93.070
O=C...H-N	1.689	2.318	115.443	79.276	102.659
His51B-Gln86					
N-H...O=C	1.547	2.529	160.842	100.786	103.634
C=O...H-N	1.951	2.772	135.475	99.834	114.514

L51F	r	d	θ	γ	Φ
Phe51A-Gln86					
N-H...O=C	1.551	2.563	173.379	110.036	112.488
O=C...H-N	1.790	2.662	141.126	109.155	122.520
Phe51B-Gln86					
N-H...O=C	1.506	2.480	158.896	107.365	107.837
C=O...H-N	1.876	2.715	137.282	109.619	106.200

L51I	r	d	θ	γ	Φ
Ile51A-Gln86					
N-H...O=C	1.137	2.151	175.762	118.176	116.398
O=C...H-N	1.344	2.287	150.542	114.314	126.954
Ile51B-Gln86					
N-H...O=C	1.366	2.377	172.391	115.043	112.293
C=O...H-N	1.566	2.451	141.853	107.761	122.460

L51P	r	d	θ	γ	Φ
Pro51A-Gln86					
N-H...O=C	N/A	2.516	N/A	N/A	123.780
O=C...H-N	N/A	2.340	N/A	N/A	127.798
Pro51B-Gln86					
N-H...O=C	N/A	2.998	N/A	N/A	122.935
C=O...H-N	N/A	2.811	N/A	N/A	142.194

L51V	r	d	θ	γ	Φ
Val51A-Gln86					
N-H...O=C	1.433	2.443	172.091	119.016	115.736
O=C...H-N	1.654	2.561	145.536	114.355	126.567
Val51B-Gln86					
N-H...O=C	1.839	2.855	176.322	127.273	126.745
C=O...H-N	2.005	2.886	143.037	126.226	136.034

L51G	r	d	θ	γ	Φ
Gly51A-Gln86					
N-H...O=C	0.928	1.934	169.072	101.325	106.616
O=C...H-N	1.590	2.444	137.776	94.587	110.832
Gly51B-Gln86					
N-H...O=C	1.363	2.355	163.472	95.410	101.712
C=O...H-N	1.993	2.741	128.010	97.216	103.890

L51E	r	d	θ	γ	Φ
Glu51A-Gln86					
N-H...O=C	1.834	2.835	167.360	124.592	120.093
O=C...H-N	1.586	2.561	158.038	127.539	136.080
Glu51B-Gln86					
N-H...O=C	1.596	2.596	167.212	126.447	122.563
C=O...H-N	1.342	2.322	158.876	128.778	137.875

L51A	r	d	θ	γ	Φ
Ala51A-Gln86					
N-H...O=C	1.039	1.990	151.184	97.773	100.252
O=C...H-N	2.046	2.895	134.916	77.778	92.263
Ala51B-Gln86					
N-H...O=C	1.406	2.417	174.036	108.376	106.695
C=O...H-N	1.955	2.743	131.839	89.439	105.277

L51S	r	d	θ	γ	Φ
Ser51A-Gln86					
N-H...O=C	1.271	2.278	169.590	113.066	117.627
C=O...H-N	1.622	2.527	145.048	113.937	126.024
Ser51B-Gln86					
N-H...O=C	1.598	2.576	159.843	100.442	103.978
C=O...H-N	2.048	2.842	132.857	94.668	109.918

L51N	r	d	θ	γ	Φ
Asn51A-Gln86					
N-H...O=C	0.809	1.811	166.516	113.886	114.787
C=O...H-N	1.095	1.933	132.121	97.321	119.958
Asn51B-Gln86					
N-H...O=C	1.381	2.353	157.558	103.432	107.101
C=O...H-N	1.821	2.672	138.483	102.548	116.910

L51D	r	d	θ	γ	Φ
Asp51A-Gln86					
N-H...O=C	1.547	2.404	138.471	150.603	137.371
C=O...H-N	1.336	2.339	166.126	140.695	138.257
Asp51B-Gln86					
N-H...O=C	1.215	1.963	123.028	149.056	134.881
C=O...H-N	1.144	1.88	120.579	80.162	105.539

L51Y	r	d	θ	γ	Φ
Tyr51A-Gln86					
N-H...O=C	1.420	2.373	153.350	86.564	97.448
O=C...H-N	2.029	2.752	125.661	89.191	105.977
Tyr51B-Gln86					
N-H...O=C	1.658	2.621	156.679	97.038	103.051
C=O...H-N	2.087	2.881	132.921	97.783	112.360

CS 1REW	r	d	θ	γ	Φ
Leu51A-Gln86					
N-H...O=C	1.781	2.752	157.520	134.521	127.197
C=O...H-N	2.013	3.016	167.223	130.212	132.876
Leu51B-Gln86					
N-H...O=C	1.791	2.758	156.755	133.704	126.917
C=O...H-N	1.964	2.965	166.354	131.204	134.309

CS MIN	r	d	θ	γ	Φ
Leu51A-Gln86					
N-H...O=C	0.988	1.980	161.670	111.401	116.687
C=O...H-N	1.401	2.322	146.648	99.598	113.458
Leu51B-Gln86					
N-H...O=C	1.536	2.540	168.796	124.849	124.612
C=O...H-N	1.643	2.606	155.546	128.414	135.744

TABLE 2: D53-Series Residue Rotamer Steric Clash Analysis. Rotamers for each mutant were assessed using the PyMol rotamer function.

Residue	Side chain molar mass(g/mol)	Number of Rotamers	Number of Steric Clashes	Receptor Residues Involved in Clashes
ASP (min)	59	7	6 out of 7	1/7 - K79, T55; 2/7 - K79; 3/7 - C77, K79; 5/7 - C77, T55; 6/7 - C77; 7/7 - T55
ASN	58	11	11 out of 11	1/11 - M78, K79, T55; 2/11 - K79; 3/11 - M78, K79, C77, T55; 4/11 - K79, 5/11 - K79; 6/11 - T55; 7/11 - T55; 8/11 - K79, M78, C77, T55; 9/11 - C77, T55, K79; 10/11 - T55; 11/11 - T55, M78, K79
GLY	1	1	0 out of 1	N/A
GLN	72	14	13 out of 14	1/14 - T55, I44
TRP	130	7	7 out of 7	1/7 - K79, N56; 2/7 - I54; 3/7 - M78, K79; 4/7 - T55, P44; 5/7 - M78, T55; 6/7 - P34; 7/7 - C77, H58
ARG	100	21	21 out of 21	1/21 - I54; 2/21 - T55; 3/21 - T55, P34; 4/21 - T55; 5/21 - P34; 6/21 - T55; 7/21 - T55; 8/21 - T55; 9/21 - C77, T55; 10/21 - C77, T55; 11/21 - G57, H58; 12/21 - H58, C77; 13/21 - C77, T55; 14/21 - P34, T55; 15/21 - I54, C77; 16/21 - K79; 17/21 - K79; 18/21 - T55, G57; 19/21 - K79;
LYS	72	18	18 out of 18	1/18 - T55, L36; 2/18 - T55; 3/18 - T55, P34; 4/18 - F35, T55; 5/18 - T55; 6/18 - T55; 7/18 - N56; 8/18 - T55, K79; 9/18 - T55, L36; 10/18 - T55, L36, M78; 11/18 - N56, M78; 12/18 - K79; 13/18 - C77; 14/18 - N56; 15/18 - T55; 16/18 - T55; 17/18 - L32;
LEU	58	4	4 out of 4	1/4 - T55, K79; 2/4 - K79, T55; 3/4 - T55; 4/4 - M78, T55
ILE	58	4	4 out of 4	1/4 - M78, C77; 2/4 - M78; 3/4 - P34; 4/4 - K79
TYR	107	4	4 out of 4	1/4 - N56, K79; 2/4 - H58, M78, C77; 3/4 - H58, M78, K79; 4/4 - L33, K79
PRO	41	2	2 out of 2	1/2 - C77, M78; 2/2 - C77
HIS	81	7	7 out of 7	1/7 - N56; 2/7 - N56; 3/7 - K79; 4/7 - M78, H58, C77; 5/7 - H58, M78, C77; 6/7 - C77, M78, K79; 7/7 - C53
VAL	43	2	2 out of 2	1/2 - H58, M78; 2/2 - C44
ALA	3	1	0 out of 1	N/A
GLU	72	13	13 out of 13	1/13 - T55;
THR	45	2	2 out of 2	1/2 M78 2/2 M78 C77
SER	31	3	0 out of 3	N/A
MET	75	13	13 out of 13	1/13 T55, M78, C76; 2/13 T55 M78; 3/13 K79 T55; 4/13 T55; 5/13 K79; 6/13 T55 C77 K79; 7/13 C77 T55; 8/13 K79 T55; 9/13 I54; 10/13 K79; 11/13 K79; 12/13 T55 K79 M78; 13/13 T55 K79 M78

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GRATPERM

Dear George,

RE. Fig. 1. Dong Xu et al. Hydrogen bonds and salt bridges across protein-protein interfaces. Protein Engineering (1997) 10 (9): 999-1012

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George Styles, B.Sc.Hon. (Ottawa 2008)

Ph.D. Biochemistry, University of Ottawa, candidate (current)

Protein Biochemistry / Recombinant Protein Expression and Purification/Design and Analysis of Biopharmaceuticals / Structural Biology / Recombinant Protein Activity Assays / Endosperm Comminution /Milling and Grinding /Amyloplast-Protein Body Separation / Particle Size Characterization / Air Classification/DNA Cloning/ Scientific Schematic and Graphic Design using Modeling and Animation Software/Patent Search and IP Validation

Selected Achievements:

- Design, expression and bioassay screening of mutant proteins.
- Design of novel DNA sequences for optimized protein expression in plants, mammalian cells and bacteria.
- Optimization of plasmid expression of recombinant proteins in plant-based systems.
- Optimization of downstream milling process for the recovery of commercially valuable biopharmaceutical products in plants.
- Start-up, design experience of novel sensing technologies for orthopedic applications.
- Design, modeling of novel fracture-inducing technologies for kernel comminutions.
- Bioactivity assays of recombinant protein products.
- Presentation of scientific concepts and data using Autodesk Maya-based graphics.
- Patent/IP searches for various scientific innovation endeavors.

HIGHLIGHTS

BIOPHARMACEUTIC RESEARCH Altosaar Laboratories, Ottawa ON. 2008-Present.

Multi-disciplinary role and involvement in numerous projects in this diverse laboratory. Reliable work performed in upstream processing involving plant-based protein expression systems as well as downstream processes such as product liberation via column purification, milling and grinding of substrates with associated biopharmaceutical proteins-of-interest.

- Theoretical design of mutant proteins using AMBER structure minimizations and molecular viewers.
- Design and expression of mutant proteins and screening via bioassays.
- Designed experiments aimed at characterizing milling processes for liberation of biopharmaceutical proteins.
- Constructed novel DNA sequences for heterologous protein expression in both plant and bacterial expression platforms.
- Assessed milling-air cyclones for downstream processes.
- Assessed efficacy of systems used in processing.
- Acted as both researcher and technician for all work.

SENSOR DEVELOPMENT for Dr. Hassan Moghadam, Argyle Associates, Ottawa 2008-Present.

Groundwork to develop an intelligent orthopedic drill sensor system. Design of preliminary experiments to verify physical principals involved, as well as in-depth and broad areas. Conducted intellectual property searches at USPTO, WIPO and CIPO.

- Designed a preliminary sensor system for orthopedic applications.
- Consulted a broad range of literature for project assessment.
- Consulted patent databases for existing parallel IP.

RECENT WORK EXPERIENCE

LinkedIn PROFILE PAGE: ca.linkedin.com/pub/george-styles/1a/386/318

MYSPACE: www.myspace.com/g-styles

LEAD GUITARIST/SOUND RECORDING/PRODUCER Musician/Guitarist (1995-2001)

Played lead guitar in a band for 5 years as well as a solo lead guitarist. Also played independently with own compositions. For recordings of former work as well as production and personal music compositions, please see <https://soundcloud.com/georges-guitar-resume>.

ARGENTUM 13 (2007-2008)

Invented and designed solutions for problems in mining applications. Worked on strategies to overcome barriers to precious metal recovery, patent writing and submission for novel technology as well as marketing strategies. Primary contributor to technical drawings and in conceptual design within the project. Work examples can be provided upon request.

Mineralogical sorting, particle size analysis.

Acid digestion and chemical analysis of ore samples.

Physical sample preparation.

Qualitative analysis of samples via sieving and particle size analysis equipment.

Gravimetric analysis as well as post treatment distribution analysis.

LET'S TALK SCIENCE PRESENTER/DEMONSTRATOR Various Schools (2012-2015)

Demonstrated and teach classes of elementary and high school students the techniques used for DNA extraction as well as how to run samples using electrophoresis. Students are also given a brief presentation on how DNA works and its current world applications.

CELEBRATING SCIENCE INSTRUCTOR Biochemistry Workshop, U Ottawa (2007)

Demonstrated and taught a class of high school students the techniques used for extraction of Green Fluorescent Protein (GFP) from transformed *E. coli* cells. Extracted protein was then visualized via acrylamide gel and UV lamp.

LABORATORY INSTRUCTOR UOttawa, BCH 3356 Biochemistry Laboratory (2010, 2011, 2012, 2013)

Instructor for molecular biology and biochemistry techniques to 3rd and 4th year biochemistry students.

TEACHING ASSISTANT UOttawa, BCH4172 Biotechnology (2010, 2011, 2014)

Serve as a teaching assistant for the biotechnology class. Topics include: Bioscience and business, Intellectual property and patenting for the bioscience sector.

LABORATORY INSTRUCTOR UOttawa, BCH2333 Biochemistry Lab Demonstrator (2010, 2011, 2012, 2013, 2014, 2015)

Serve as a demonstrator for the biochemistry laboratories as well as answer online questions in the course forum, marker for mid-terms and final exams. Topics include: Enzymatic activity and assays.

DISCUSSION GROUP INSTRUCTOR UOttawa, BCH2333 Biochemistry (2015)

Explained and went over key concepts and theory related to lectures and assignments from the course BCH2333.

TEACHING ASSISTANT/MARKER UOttawa, BCH4123 Clinical Chemistry (2014)

Marker and proctor of all course assessments for this undergraduate level clinical chemistry course.

ORGANIZATIONS AND COMPETITIONS

UNIVERSITY OF OTTAWA MICROBIOLOGY AND VIROLOGY CLUB (2008)

Members regularly contribute seminars to the group, keep updated on recent topics in microbiology and virology as well as host guest speakers specialized in this area.

SEMI-FINALIST IN THE TVC: TECH VENTURE CHALLENGE (2008)

Planned, organized, and submitted a business proposal for a self-invented product. Major issue surrounding this proposal was the invention of a novel patentable idea. Proposal copy can be sent via email upon request. See webpage: <http://www.techvc.org/>

SEMI-FINALIST IN THE TVC: TECH VENTURE CHALLENGE (2010)

Planned, organized, and submitted a business proposal for a self-invented product. Proposal copy can be sent via email upon request. See webpage: <http://www.techvc.org/>

FINALIST IN THE LEAD TO WIN COMPETITION (2010)

Entrepreneurial competition that promotes start-up businesses to develop a business launching plan that is effective in creating new jobs around the Ottawa area. Was one of three teams that made it to the finals out of >20 teams. <http://www.leadtowin.ca/>

START-UP GARAGE FINALIST (2014)

Selected to be funded in order to startup a bio/cosmeceutical company, Cosmeceutica. Work is in progress to get this startup company up and running by the end of summer 2014. <http://www.startupgarage.ca/>

PUBLICATIONS AND CONFERENCES

GENE TECHNOLOGY IN AGRICULTURE, ENVIRONMENT AND BIOPHARMING:

BEYOND BT-RICE AND BUILDING BETTER BREEDING BUDGETS FOR CROPS (2012)

Zaidi, MA ; El Bilali, J; Koziol, AG; Ward, TL; Styles, G et al. JOURNAL OF PLANT BIOCHEMISTRY AND BIOTECHNOLOGY Vol: 21 Iss: 1 Supplement Pgs: S2-S9 OCT 2012.

30TH ANNUAL MEETING OF THE CANADIAN BIOMATERIALS SOCIETY (2013)

Wednesday, 29 May 2013 to Saturday, 1 June 2013, SITE Building, Ottawa ON. Poster Presentation. Exploiting the key binding determinants of the BMP2/BMPRIA interaction: Using mutagenesis to probe a key binding hotspot of BMP2/BMPRIA.

AMERICAN DIABETES ASSOCIATION 73RD SCIENTIFIC SESSIONS (2013)

June 21 - 25, 2013, McCormick Place Convention Center (West Building), Chicago, IL Observer.

AMERICAN DIABETES ASSOCIATION 75TH SCIENTIFIC SESSIONS (2015)

June 5 - 9, 2015, Boston Convention Center, Boston, MA Observer.

UOTTAWA FIRST NATIONS MINI MED SCHOOL (2014 and 2015) Saturday, January 18, 2014. University of Ottawa, Roger Guindon Campus. Sessions included case-based learning activities, anatomy lab, as well as casting and suturing workshops.

CANADIAN COUNCIL OF DEANS OF SCIENCE (2014)

June 15-17, 2014. Guest speaker for the Science Education and the First Nations Students Panel.

HARROWING, HELLISH HIJINKS ON HOOLIGAN ST. (2015) By: George Styles. A semi-fictional book about my friends and I during our last year together and all the things we did. Currently in submission.

VOLUNTEERING

LET'S TALK SCIENCE (2012-Continuing) Provide in-class science demonstrations, tutorials and experiments for elementary school children. Have done presentations in both the local area as well as in First Nations elementary schools.

PLAN CANADA (2012-Continuing) Child sponsoring by providing monthly financial support as well as bi-yearly correspondence for an adolescent child to participate and attend activities in Plan Canada Program.

GARRY J ARMSTRONG (2014-Continuing) Volunteer at the retirement residence. Main duties to date include assisting with serving at the afternoon tea room as well as joining in afternoon activities with the residents.

MULTIPLE SCLEROSIS SOCIETY OF CANADA (2014-Continuing) Assist and join clients during outings and events that are planned by the society. Also, will be working with clients at their residence and provide support.

GRANTS AND AWARDS

MITACS ACCELERATE (2010) Industrial partnering with university research. Was awarded joint funding between MITACS and Proteins Easy Corporation to develop transgenic rice expressing valuable human growth factors.

MITACS ACCELERATE (2011) Industrial partnering with university research. Was awarded joint funding between MITACS and Hassan G. Moghadam Corporation for the development of more potent human growth factors.

CERTIFICATIONS

SMART SERVE ONTARIO (2014) The Alcohol and Gaming Commission of Ontario, License e140908228998.

EXPERIENCE

BIOCHEMISTRY

EXTENSIVE EXPERIENCE WITH:

- Protein extraction and purification
- DNA/RNA extraction
- Heparin-Sepharose and His-Tag Column Chromatography
- SDS-PAGE and Tricine SDS-PAGE
- C2C12 promyoblast cells
- Acid and Alkaline Phosphatase assays
- DNA polymerase chain assembly (PCA)
- DNA sequencing sample preparation
- DNA sequencing data interpretation
- DNA ligation techniques

- PCR primer design
- Expression vector design and manipulation
- Protein sample dialysis
- Protein refolding
- Molecular viewing software
- Molecular animation software
- PyMOL and Python Molecular Viewer (PMV)
- Site directed mutagenesis primer design
- Various site directed mutagenesis techniques
- Mammalian cell culture
- Pluripotent stem cell culture
- pET expression vector DNA cloning and sub-cloning.
- Protein expression optimization
- Codon optimization of DNA sequences for protein expression
- Cytokine biochemistry
- Stem cell differentiation

EXPERIENCE WITH:

- Circular Dichroism (CD) Spectroscopy
- Totipotent plant cell callus handling and culture
- Transformation of plant callus cells
- Plant tissue GUS staining
- *Agrobacterium* transformation and culture
- Transgenic plant cell lines
- Scripps Autodock
- Bioinformatics
- Binary vector cloning and sub-cloning
- Various colorimetric assays
- AMBER
- SYBYL

INORGANIC AND ANALYTICAL CHEMISTRY

EXPERIENCE WITH:

- UV/Vis and fluorescence spectroscopy
- Circular Dichroism (CD) Spectroscopy
- ¹H and ¹³C NMR spectra
- IR spectroscopy, structure determination by NMR and MS
- HPLC analysis and titration
- Affinity chromatography
- Ion exchange chromatography
- Size exclusion chromatography
- Hydrophobic exchange
- Analysis of samples via acid/base titration
- Redox titration

- Polymeric chemistry synthesis
- Distillation and reagent preparation in analytical chemistry
- Experience in the use and disposal of hazardous reagents
- Solution preparation

ORGANIC CHEMISTRY

EXPERIENCE WITH:

- Organic synthesis
- Multistep organic synthesis
- Crystallization and separation
- Distillation
- Thin-Layer Chromatography
- Limited synthesis experience in medicinal and pharmaceutical synthesis

MICROBIOLOGY

EXTENSIVE EXPERIENCE WITH:

- Plating
- Incubation
- Conjugation
- Bacterial cell transfection
- Microbial genetics
- Microscopy
- Proteomic analysis of bacteria
- Cell culture
- Aseptic technique
- Cell counting, and passage

EXPERIENCE WITH:

- Antimicrobial susceptibility testing
- Identification tests

POWDER TECHNOLOGY

EXTENSIVE EXPERIENCE WITH:

- Milling
- Sieving
- Particle size distribution and analysis
- Laboratory scale Hammer mills
- Fine powder production and handling
- Mill classification and evaluation

- Air classification via air washing
 - Laboratory scale planetary ball mills
-

SKILLS

- Complete computer literacy in Microsoft Appl's, Word, Excel, PowerPoint, Outlook, etc
- Basic training in AutoCAD and AutoDesk Inventor
- Excellent written and oral communication skills
- Excellent proficiency with the English language
- Excellent problem solving skills
- Excellent skills with power tools, hand tools, and general machinery
- Ability to manage multiple and competing priorities in a fast paced environment
- Ability to perform heavy lifting
- Excellent ability in writing technical and scientific reports
- Excellent ability in using and maintaining a scientific notebook
- Able to work in a careful and meticulous manner
- Experience in laboratory work
- Excellent organization and time management skills
- Critical thinker as well as excellent memory
- Able to work comfortably with novel instrumentation and technologies
- Strong technical background and a strong aptitude for conducting research
- Seeking positions that encourage continuous learning
- Able to use Autodesk Maya (Version 2012) for graphics and presentations
- Able to create graphics for scientific presentations and publications
- Familiar with using Molecular Maya add-on for Autodesk Maya
- Familiar with using Adobe Photoshop for graphics and presentations
- Verbal presentation skills for communicating scientific results and ideas
- Reef aquarium fish and coral keeping (>5 yrs experience)
- Freshwater aquarium keeping (>5 yrs experience)