The oocyte derived growth factors, GDF9 and GDF9B, and their biological activities in *in vitro* cell models

Pro gradu study
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Preface

This work was done in the Biomedicum Helsinki in the Programme for Developmental and Reproductive Biology, in the laboratory of Docent Olli Ritvos, and under supervision of Docent David G. Mottershead.

I would like to thank Olli for the chance & support, and David for all the support.

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Abstract:

The oocyte-derived growth differentiation factors -9 and -9B (GDF9 & GDF9B) are members of the TGF-β superfamily, in which there are also some 40 other homologous proteins affecting cell growth and differentiation.

The role of GDF9 and -9B in ovarian function is particularly interesting since these proteins are strongly expressed in the oocyte and they have been shown to be indispensable for normal folliculogenesis. Naturally occurring sheep mutations for GDF9B have been found, and sheep heterozygous for such a mutation exhibit multiple ovulations, leading to twin and triplet pregnancies. GDF9B is thus the first identified "twinning gene". Sheep immunized with short GDF9 or GDF9B –derived peptides suffered from shutdown of follicular development. Recently the first mutation in human GDF9B was found when two Italian women suffering from hypergonadotropic ovarian failure were found to have a mutation in the pro-region of GDF9B. As the role of GDF9 and -9B in ovarian function is being better understood through basic research there may be a possible future use of these growth factors as fertility drugs.

Previously in the laboratory of Olli Ritvos recombinant GDF9 and -9B proteins from various species have been expressed using stably transfected 293T cell lines. It has been shown previously that mouse GDF9 is active when secreted in conditioned media but human GDF9 and -9B are not showing any activity in *in vitro* cell models using various biological reporters. Therefore studies for investigating the latency of human GDF9 and -9B are of value, one approach being the purification of GDF9 and -9B using epitope tagging. In this study GDF9 and -9B were cloned, expressed and purified as 6-Histidine epitope tagged molecules and characterized both for their biochemical properties and biological activity. Biological activity was followed by monitoring CAGA- and BRE-luciferase reporters for response as a measure of the activation of the downstream target Smads.

With assistance of an international collaborative network we have acquired unique tools for the investigation of the molecular and reproductive biology of GDF9 and -9B, such as unique and specific monoclonal antibodies against these proteins. To verify the biological activity of the GDF9 monoclonal antibodies, their capability to immunoneutralize GDF9 was determined, as well as their specificity and epitopes.

Purification of human GDF9B by immobilized metal affinity chromatography resulted surprisingly in biological activity on human granulosa-luteal cells in both CAGA and BRE -luciferase assays. Ongoing studies on how further downstream processing of human GDF9 and -9B affects the activity of these ligands are being conducted in order to better understand the purified preparations.

Keywords: Transforming growth factor-β (TGFβ), growth differentiation factors (GDF9, GDF9B), immobilized metal affinity chromatography (IMAC), monoclonal antibodies (MAb)

TABLE OF CONTENTS

I SUMMARY IN FINNISH

Munasoluperäiset kasvu- ja erilaistumistekijät -9 ja -9B (GDF9 ja GDF9B, joka tunnetaan myös nimellä BMP-15), ovat kaksi suuren transformoiva kasvutekijä -β (TGFβ) perheen sukulaisjäsentä. Kasvutekijät GDF9 ja GDF9B erittyvät munasolusta ja vaikuttavat kohdesoluihinsa (munasolua ympäröiviin granuloosasoluihin) kasvavan munasolun välittömässä läheisyydessä.

GDF9 ja GDF9B:n on osoitettu vaikuttavan munarakkulan kehitykseen jo sen alkuvaiheissa,. GDF9-poistogeenisillä hiirillä munarakkulan kehitys loppuu ennen kypsymistään sekundaariseksi munarakkulaksi, näin ollen antraalisia munarakkuloita ei löydy ja munasarjojen ulkoasu on hyvin erilainen kuin normaalilla toimivalla munasarjalla. GDF9B ei vaikuta hiiren munarakkuloiden kehitykseen näin dramaattisesti, mutta ihmisellä sen on näytetty olevan elintärkeä rooli munasarjojen normaalin toiminnan kannalta. Onkin epäilty että GDF9:llä ja GDF9B:llä olevan erilaisia rooleja munarakkulan kehityksessä eri lajien välillä. Ihmisellä ja lampaalla, joilla irtoaa 1-2 munasolua per kuukautiskierto, molempien proteiinien on osoitettu säätelevän ovulaatiota, vaikkakin kummankin roolista löydetään jatkuvasti uusia puolia. Hiirellä irtoaa useita, jopa kymmeniä, munasoluja yhden kierron aikana ja kierron kesto on vain noin kolme vuorokautta. Näyttääkin että hiirellä GDF9:n rooli on korostunut ja GDF9B:n rooli on vähäisempi. GDF9:llä on epäilty olevan tekemistä myös ihmisten kaksoisraskauksien kanssa. Kummankin molekyylin epänormaalilla toiminnalla on epäilty olevan tekemistä ennenaikaisen munasarjojen toiminnan loppumisella ihmisillä (POF, PCOS).

Viime aikoina on näytetty että GDF9 ja GDF9B toimivat myös yhteistyössä säädellessään munarakkulan kehitystä mutta tämän vuorovaikutussuhteen perimmäistä biomekaanista taustaa ei tiedetä. Nämä proteiinit käyttävät kuitenkin samantapaisia viestintätapoja säädellessään munarakkulan kasvua, joten on hyvin todennäköistä että nämä hyvin läheiset ja samoihin aikoihin munasolun kehityksessä ilmennetyt sukulaismolekyylit voivat myös fyysisesti muodostaa komplekseja. Tämän ns. parakriinisen kasvun säätelyn ja hormonaalisen, ns. endokriinisen, kasvun säätelyn vuorovaikutussuhteita ei vielä tunneta hyvin. Aiemmin on luultu että munasarjat ovat pääasiallisesti endokriinisesti säädeltyjä,

mutta viimeisten vuosien aikana on käynyt selväksi että munasolun itsensä tuottamat molekyylit ovat elintärkeitä munarakkulan kehitykselle.

GDF9 ja GDF9B käyttävät seriini-treoniini kinaasireseptoriperheen jäseniä välittääkseen signaalinsa kohdesoluihinsa. Seriini-treoniini kinaasiperheen pääasiallinen ja parhaiten tunnettu signaalinvälitysreitti on Smad-perhe. Smad-perheen jäsenet ovat solunsisäisiä seriini-treoniinireseptorien edesauttamana fosforyloitumisella aktivoituvia signaalinvälittäjiä, jotka muodostavat komplekseja ja kulkeutuvat tumaan jossa käynnistävät kohdegeenien ilmentämisen.

Pro gradu – työssäni tutkin miten ilmentää, puhdistaa ja tutkia ihmisen GDF9 ja GDF9B – proteiineja. Ilmentämiseen käytin ihmisen munuaisperäistä solulinjaa (HEK-293T) joihin siirsin tuottovektorin, joka sisälsi haluamani rekombinanttiproteiinin ja antibioottivastustuskyvyn antavan osan jolloin pystyin antibiootin avulla valitsemaan rekombinanttiproteiinia tuottavat solut. Rekombinanttiproteiinit sisälsivät joko villityypin tai 6xHis-leimatun proteiinin, jonka avulla pystyisin paremmin seuraamaan ja puhdistamaan proteiinin. Rekombinanttiproteiinin bioaktiivisuutta tutkin lusiferaasireporttereilla jotka aktivoituvat eri Smad-perheen jäsenten fosforyloitumisen myötä. Näiden avulla pystymme seuraamaan aktivoituuko ns. TGF-β- vai BMP – signalointireitti *in vitro* – solumalleissa. Solumalleina käytin tässä työssä ihmisen primaarista granuloosa-luteal solumallia ja 293T-solumallia. Granuloosa-luteal solut saimme lahjoituksena potilailta jotka olivat käyneet hedelmöityshoidoissa. Lisäksi tutkin työssäni GDF9 ja GDF9B monoklonaalisia peptidivasta-aineita ja niiden kykyä syrjäyttää GDF9:n biologinen aktiivisuus. Tutkin lisäksi mikä on näiden vasta-aineiden epitooppi, jotta saisimme lisätietoa GDF9:n tärkeistä kontaktipinnoista sen kohdereseptorien kanssa. Tulevaisuudessa, sekä kehitetyn puhdistusmenetelmän ansiosta että vasta-aineiden avulla, pystymme jatkamaan tutkimusta siitä miten GDF9 ja GDF9B säätelevät munarakkulan kehitystä granuloosasolujen kanssa keskustelemalla.

II ABBREVIATIONS

aa	Amino acids
ActR	Activin receptor
Ad	Adenovirus
Alk	Activin-receptor like kinase
AMH	Anti-Müllerian hormone
AMHR	AMH receptor
BMP	Bone morphogenetic protein
BMPR	BMP receptor
bp	Base pairs
BRE	BMP responsive element
BSA	Bovine serum albumin
β-Gal	beta-galactosidase
CG	Cumulus granulosa cell
cDNA	Complementary DNA
COC	Cumulus-oocyte complex
Co-Smad	Common partner Smad
C-terminal	Carboxy-terminal
DMEM	Dulbecco's modified Eagle medium
dsRNA	Double stranded RNA
ECL	Enhanced chemiluminescence
FCS	Fetal calf serum
FSH	Follicle stimulating hormone
Fst	Follistatin
GC	Granulosa cell
Ham's F12	Nutrient mixture containing inorganic salts, aa, vitamins etc.
hGDF9	human growth differentiation factor -9
hGDF9B	human growth differentiation factor -9B
hGL	Human granulosa-luteal
HEK	Human embryonic kidney
I-Smad	Inhibitory Smad
IVF	*In vitro* fertilization
kDA	kilodaltons

KL	*c-kit* ligand
LAP	TGF-beta latency associated protein
LH	Luteinizing hormone
LTBP	Latent TGF-beta binding protein
mAb	Monoclonal antibody
MCS	Multiple cloning site
MH	Mad homology
MIS	Müllerian inhibiting substance
mGDF9	Mouse growth differentiation factor-9
mRNA	Messenger RNA
MW	Molecular weight
N-terminal	Amino-terminal
PBS	Phosphate buffered saline
PCOS	Polycystic ovarian syndrome
POF	Premature ovarian failure
PCR	Polymerase chain reaction
rGDF9B	Rat growth differentiation factor-9B
RNA	Ribonucleic acid
RNAi	Ribonucleic acid interference
R-Smad	Receptor regulated Smad
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
Smad	Sma- and Mad-related factor
StAR	Steroidogenic acute regulator protein
TBS-NP40	Tris buffered saline-Nonidet P 40
TβR	TGF-beta receptor
TGFβ	Transforming growth factor-beta
X-Gal	5-bromo-4-chloro-3-indolyl-D- Galactopyranoside

III REVIEW OF THE LITERATURE

3.1 Introduction

Previously the oocyte was considered only a passive recipient of developmental signals from oocyte-associated granulosa cells and from the pituitary produced endocrine factors and hormones, however it is now clear that there is communication between oocytes and granulosa cells which determines the flow of folliculogenesis. Paracrine factors which control the development of the oocyte have recently been the focus of many studies, since it is known that endocrine and local ovarian hormones promote folliculogenesis, but the roles of the factors secreted by the oocyte have been unclear. Paracrine factors act upon the oocyte by regulating granulosa cells, the cells surrounding the oocyte, and by controlling nutrient intake and the growth of the oocyte. It is known that these factors are crucial for normal folliculogenesis and the absence or genetic alterations of these factors have profound effects on fertility /1/. Studies utilizing *in vitro* bioassays using granulosa cells and co-cultures with oocytes have revealed how these paracrine factors signal and modulate folliculogenesis by proliferation, steroidogenesis, the synthesis of inhibin, activin and follistatin /2, 3, 4, 5, 6/, and expression of such key players in ovarian function as the luteinizing hormone receptor /7/ and kit-ligand /8/. These findings have lead to the idea of control over ovarian function using paracrine factors and have applications in endocrine and fertility research /9/.

Growth differentiation factor -9 and -9B (GDF9 and -9B, GDF9B is also known as bone morphogenetic protein 15; BMP15) /10, 11/ have been suggested to play key roles in paracrine modulation of the oocyte, since they are strongly expressed in the oocyte during the early stages of folliculogenesis /12/. They belong based on their structure to the TGF-β superfamily, in which there is also some 40 other homologous proteins affecting cell growth and differentiation /13/. Most notably, the structure of GDF9 and -9B differs from other TGFβ superfamily members due to a missing cysteine residue, which forms a disulfide bond during the dimerization of the other superfamily members /10/.

In mice GDF9 is known to control ovulation, fertility and reproductive processes, and has been shown to be necessary for follicle development /1/, further GDF9 -knockout mice

suffer from a shutdown of follicular development /14/. Also recent work has shown via immunoneutralization of the biological activity of GDF9 that it at least partially accounts for mouse oocyte mitogenic activity /15/.

GDF9B is indispensable in sheep ovarian folliculogenesis as shown by naturally occurring sheep mutations, and sheep heterozygous for such a mutation exhibit multiple ovulations and twin and triplet pregnancies. GDF-9B is thus the first identified "twinning gene" /16/. Indeed a number of mutations have been identified for sheep in both GDF9 and GDF9B /17, 18/. Sheep immunized with short GDF9 or GDF9B –derived peptides suffered from shutdown of follicular development /19/.

Recently the first mutation in human GDF9B was found when two Italian women suffering from hypergonadotropic ovarian failure were found to have a mutation in the pro-region of GDF9B, which is likely to alter the structure of the protein (Y235C) /20/. Variants of the human GDF9 have been implicated in twinning /21,22/ and very recently there have been a number of reports suggesting that variations in either GDF9 or GDF9B may be the cause of abnormal functioning of the ovary in such conditions as Polycystic Ovarian Syndrome (PCOS) and premature ovarian failure (POF) /23, 24, 25, 26/, so it is quite evident that GDF9 and -9B play a critical role in for normal human reproduction.

It has been shown both *in vivo* and *in vitro* that GDF9 and -9B synergize when controlling folliculogenesis and acting upon their target cells /27, 28, 29, 30/, but also species differences in these phenomena have been found based on *in vitro* cell models underlining the requirement for extensive studies on both the ligands and receptors and their species differences.

3.2 Folliculogenesis

3.2.1 General aspects

Female reproductive biology revolves around its cyclical activity, a feature that is also dominating the growth and development of follicles. Normally, the human ovaries produce a single dominant follicle, which results in a single ovulation each menstrual cycle. The dominant follicle is responsible for estradiol production during the follicular phase of the

menstrual cycle. After ovulation, the dominant follicle transforms into the corpus luteum, which secretes large amounts of progesterone during the luteal phase of the menstrual cycle. Estradiol and progesterone then act on the uterus, in preparation for the implantation of the human embryo, thus it is very important to comprehend the menstrual cycle, and its affects on the life cycle of the dominant follicle and female fertility. In the mammalian ovary a single follicle is comprised of the oocyte itself and the granulosa cells surrounding the oocyte. Granulosa cells can be divided into different layers starting with the granulosa cells which are in actual contact with the oocyte called cumulus cells, followed by the mural granulosa cells which are further removed from the oocyte and in turn are covered by a layer of theca cells /31/. When these two populations are separated by a fluid filled compartment called the antrum, the follicle is known as antral follicle. The growing oocyte in different stages is presented in figure 1 /32/.

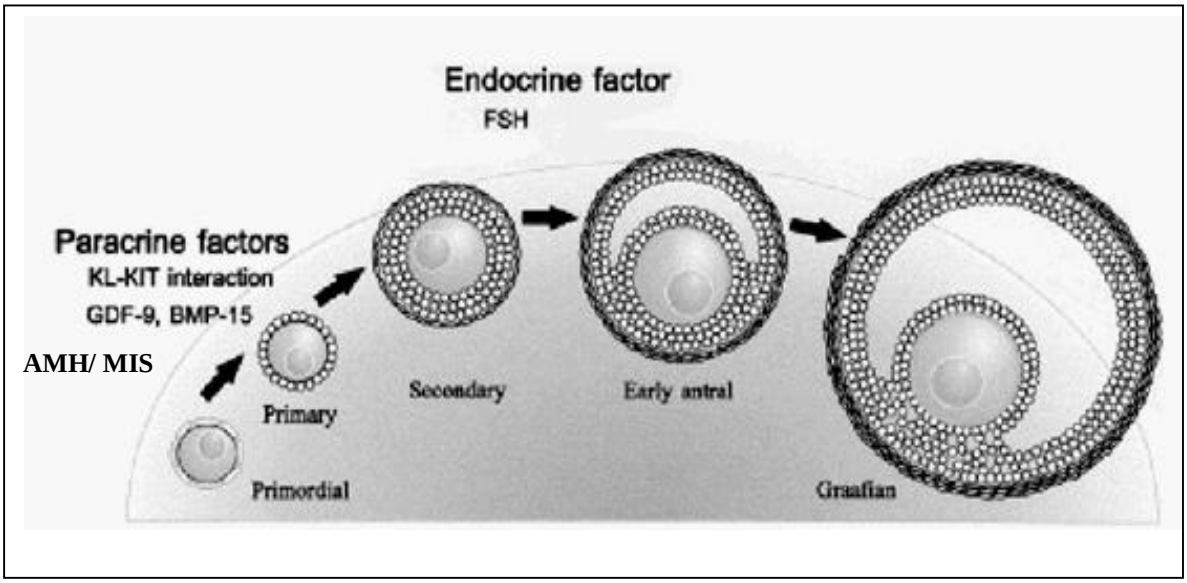

Figure 1. Modified from Senbon et al /32/. Growing follicles are driven to grow by paracrine factors such as GDF-9 and BMP-15 until the early antral stage, where FSH and other hormones join in and start maturing the growing follicle, now known also as Graafian follicle

3.2.2 Flow of folliculogenesis

Folliculogenesis begins with the recruitment of a primordial follicle into the pool of growing follicles and it ends with ovulation or cell death by atresia. Folliculogenesis is a long process, it takes a year for a primordial follicle to grow and develop to the ovulatory stage and it can be divided into two stages. The first, preantral phase is a phase where the oocyte grows and differentiates independently from gonadotrophins, but is driven by growth factors utilizing autocrine and paracrine mechanisms. In the second, antral phase,a so called Graafian follicle grows largely driven by gonadotrophins such as follicle stimulating hormone (FSH) and luteinizing hormone (LH) as well as growth factors. The enlarged and mature Graafian follicle ovulates and the remaining theca and granulosa cells form the corpus luteum /33/.

3.3 TGF-β superfamily

3.3.1 General characteristics

TGF-β superfamily members have various roles in developmental, homeostasis and reproductive biology, in the superfamily there are some 40 factors associated with numerous roles *in vivo*. TGF-β superfamily members are synthesized as prepropeptides, then processed intracellularily with furin /34/ or other proteolytic enzymes to yield an active mature carboxy-terminal dimer. These dimers can be hetero- or homodimers and function as such or they can be bound to a complex and remain latent until released from these complexes by enzymes, or *in vitro* by acid treatment or heat /35/. Although it is well known that the TGF-β superfamily members are usually secreted as latent forms, researchers often overlook this fact or misunderstand latent TGF-β activation. This might due to the unusual biology of TGF-β:

(1) the propeptide of TGF-β remains tightly bound to the mature portion of the protein after the bonds between propeptide and mature TGF-β are cleaved;

(2) the interaction between mature and propeptide renders the growth factor latent;

(3) the TGF-βs are secreted as a complex in which a second gene product is bound to the propeptide either non-covalently or covalently.

A schematic diagram of a large latent complex of TGF-β is presented in figure 2.

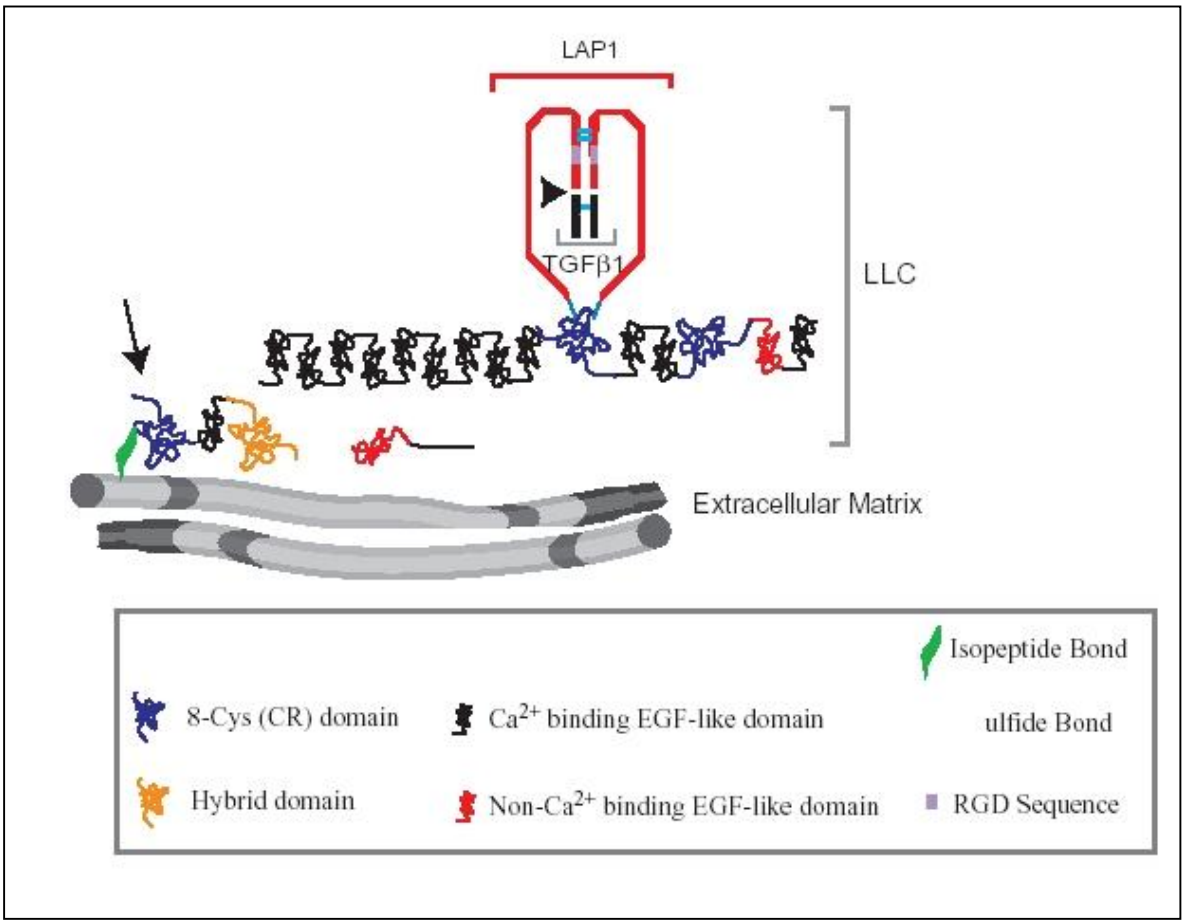

Figure 2. Modified from Annes et al /35/. TGF-β actions are complicated and finely detailed, an inactive TGF-β1 (in black) is bound by latency associated-peptide (LAP), which together with e.g. extracellular matrix (ECM) forms the large latent complex (LLC). For TGF-β1 to be active it has to be released from this complex.

3.3.2 Signalling pathways

The TGF-β superfamily ligands mediate their intracellular messages via specific cell surface receptors which have serine/threonine kinase activity. When the ligand is bound by two type I receptors and two type II receptors, sometimes assisted by accessory receptors such as large cell surface betaglycans, it activates an intracellular signalling cascade by first phosphorylating specific Smad-proteins, presented to intracellular parts of the receptor by proteins like SARA (Smad Anchor for Receptor Activation) /36/. Phosphorylated Smads in turn form complexes with the common Smad4 protein and accumulate in the nucleus where they regulate the transcription of target genes (figure 3). It is known that other signalling pathways may synergize or antagonize the TGF-β pathway. Recent research has shown that

the specificity of the intracellular messages is determined by a combination of receptors bound by the ligand, the selective interactions between Smads /37/ and other non-Smad mediated pathway components /38/. Also a vast number of extracellular antagonists have been shown to suppress or enhance the signalling of these factors /39/.

Although there are less receptors and Smads than there are ligands, versatility is achieved through combinatorial interactions of type I and type II receptors and Smads in oligomeric complexes /40/. These then are complemented by the many transcription factors with which Smads cooperate, resulting in context- or ligand-dependent transcriptional regulation specific for each TGF-β superfamily member. These pathways regulate Smad-mediated responses and also induce Smad-independent responses. Recent progress in understanding the signalling mechanisms of TGF-β-related factors through Smad-dependent and Smad-independent pathways has revealed that these combinatorial interactions in the tetrameric receptor complex allow differential ligand binding or differential signalling in response to the same ligand /38/. The basic Smad-signalling pathway is presented in figure 3 /37/.

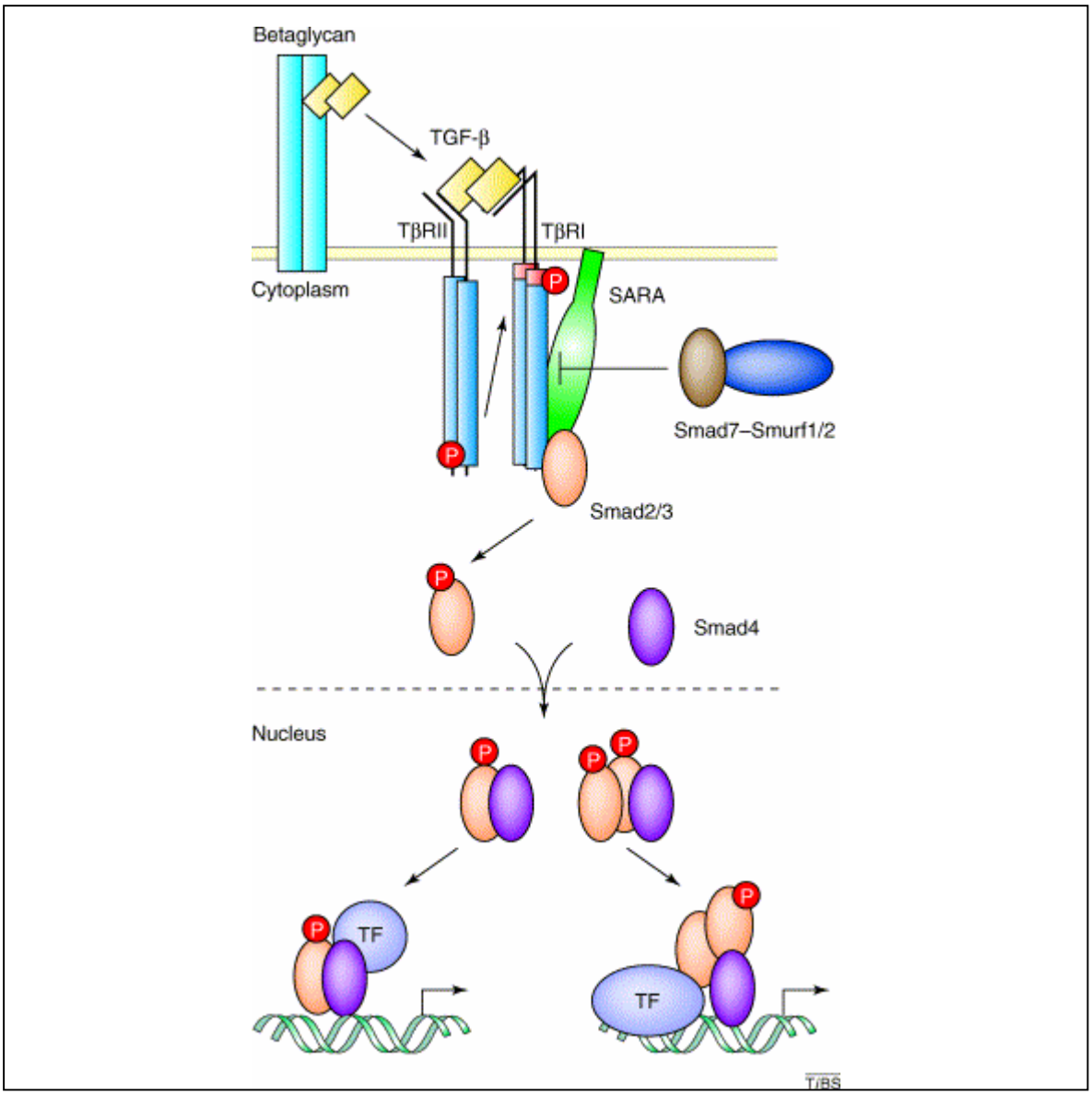

Figure 3. Modified from ten Dijke et al /37/. Basic Smad-signalling pathway, which is regulated by the presence of suitable receptors, accessory receptors (such as betaglycans), SARAs, Smads and of course TGF-β superfamily member ligands .

3.4 Receptors

The serine/threonine kinase receptors which mediate the TGF-β superfamily signalling can be divided into subfamilies based on their functional and sequence characteristics. These proteins are referred to as type I or type II receptors. The type I receptors are more closely related based on their sequence, when comparing kinase domains, than type II receptors. The other way of subdividing the receptors is based on their ability to bind certain TGF-β superfamily members.

Both receptor types are glycoproteins with a molecular weight from 55 to 70 kDa and comprise polypeptides ranging from 500 to 570 amino acids including the N-terminal signalling sequence. The extracellular portion of the protein is only 150 amino acids but is heavily occupied by cysteines which are important in determining the fold of the protein. The extracellular part is also N-glycosylated /13/. A schematic diagram of a general serine/threonine kinase receptor is presented in figure 4.

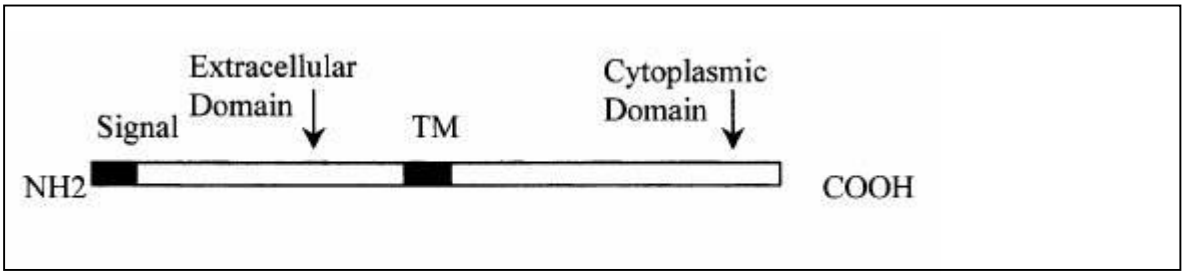

Figure 4. Schematic diagram of typical serine/threonine kinase receptor: receptor-peptide consists of N-terminal signal peptide,extracellular domain, transmembrane domain (TM) and cytoplasmic domain

3.4.1 The type I receptors

In vertebrates, the type I receptors can be divided into three subgroups based on their preference for certain ligands, as said they have similar kinase domains and their intracellular signalling is therefore similar. The first group of the type I receptors contains the TGF-β Receptor type-I (TβR-I), Activin Receptor type-IB (ActR-I) and Activin-like Kinase -7 (ALK7), the second group is dedicated to BMP-binding receptors BMPR-IA and –IB. The third group comprises ALK1 and ALK2. Nomenclature of serine/threonine kinase receptors is complicated due to the fact that they were found around the same time by multiple people and therefore different names were adopted /13/.

3.4.2 The type II receptors

The type II receptors comprise 5 different receptors in vertebrates TβR-II, BMPR-II, AMHR-II, ActR-II and –IIB. The first three, as one might guess, bind TGF-β, BMPs and AMH. ActRIIs bind activins together with activin type I receptors but they can bind BMPs with BMP type I receptors /13/.

It has become apparent that the choice of receptors is crucial for TGF-β superfamily signalling, but one receptor combination often binds different ligands, and patterns of ligand and receptor expression often dictate which receptors are activated. For example, the type II receptors ActRII and ActRIIB can combine with the type I receptor ActRIB/ALK4 and mediate activin signalling, whereas their interactions with BMP-RIA or BMP-RIB allow BMP binding and signalling as well. The BMP type II receptor BMP-RII can combine with three type I receptors, BMP-RIA, BMP-RIB and ActRI/ALK2, to bind several BMPs and mediate BMP signalling /13/. More recently an association of the BMPRII and ALK5 was implicated in GDF9 signalling /41, 42/.

Therefore, a ligand can induce different signalling pathways depending on the composition of the receptor complex. For example, the TβRII interacts not only with the 'classical' type I receptor TβRI/ALK5, which activates Smad2 and Smad3, but also with ALK1, which activates Smad1 and Smad5 /44/.

3.5 Smad-proteins

Smads are structurally related intracellular signalling mediators, which are activated, among others, by serine/threonine kinase receptors to result in a phosphorylated Smad. In total there are eight vertebrate Smads, Smad1 to Smad8, with diverse roles in intracellular signalling and they depend for activation on extracellular ligands which bind to receptor extra-cellular domains /38/. The different Smads are specific for particular receptors as shown in figure 5.

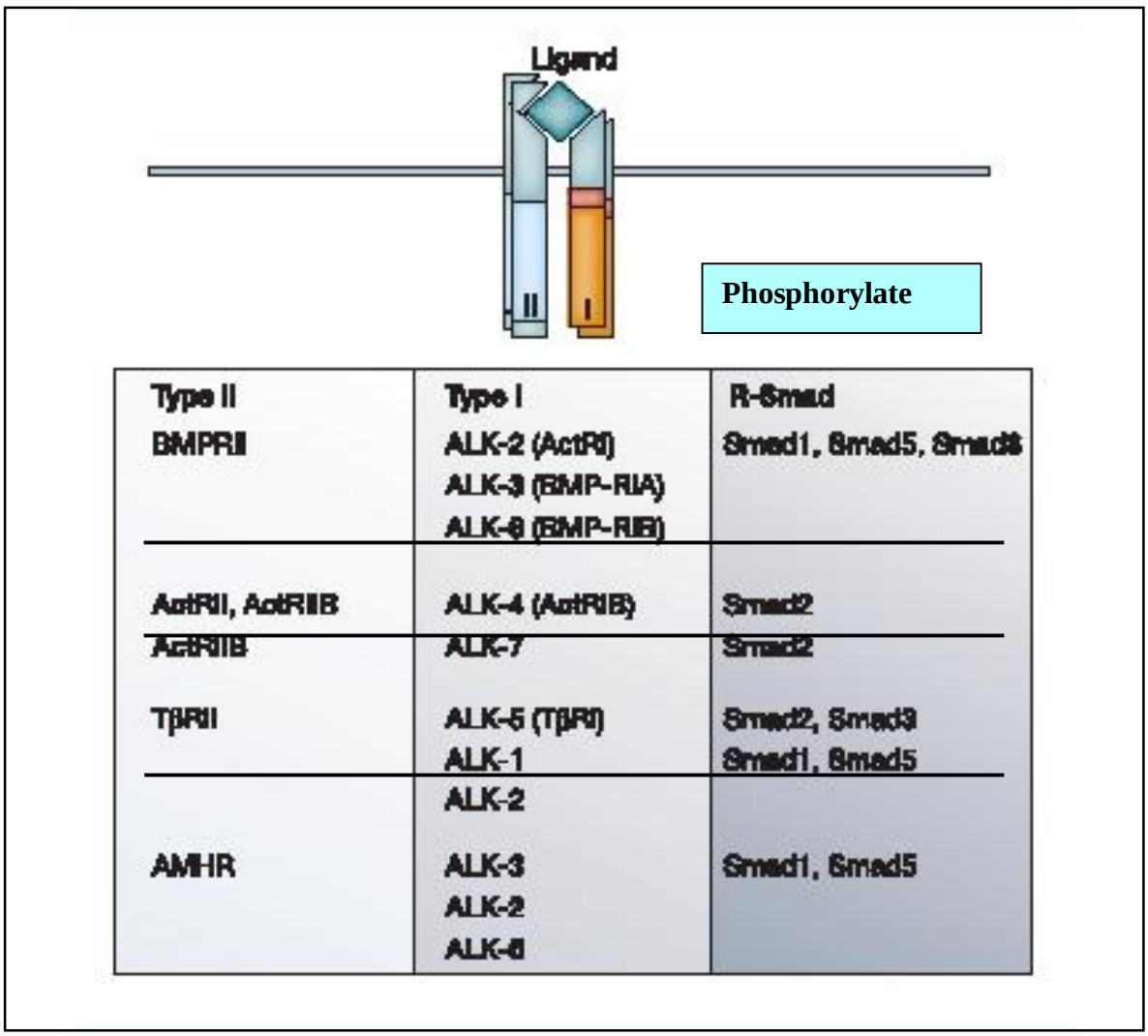

Figure 5. Modified from Derynck et al /38/, Smad – receptor relationships, type-I and type-II receptors form a tetrameric complex which enables intracellular part of the receptors to phosphorylate receptor-Smads.

Smads-2 and -3 are activated through C-terminal phosphorylation by the TβRI and ActR-IB, Smads1, -5 and -8 are activated by ALK-1, ALK2, ALK3 and ALK6 in response to BMPs of the TGF-β superfamily. Therefore it can be said that there are Smads that are more specific for TGF-βs and Smads that are BMP-specific. All in all, these five Smads have the common name R-Smads (receptor-Smads) and when activated they form a trimeric complex with a common Smad, Smad4. The role of Smad4 is not quite clear, it is not required in TGF-β signalling since some TGF-β responses occur in the absence of Smad4 but some Smad4-deficient cell lines have a limited response to TGF-β. Smad6 and Smad7 are structurally divergent and act as inhibitory Smads /43/.

R-Smads and Smad4 contain a conserved N-terminal MH1 (Mad homology domain) and a C-terminal MH2 domain, flanking a divergent middle linker segment (figure 6). Inhibitory Smads however lack this recognizable MH1 domain, but they do have a MH2 domain. The MH2 domain has some similarities with the phosphopeptide-binding domain FHA (forkhead-associated). Both MH domains can interact with selected sequence specific transcription factors, whereas the C-terminus of the R-Smads interacts with and recruits the related coactivators CREB-binding protein (CBP) or p300.

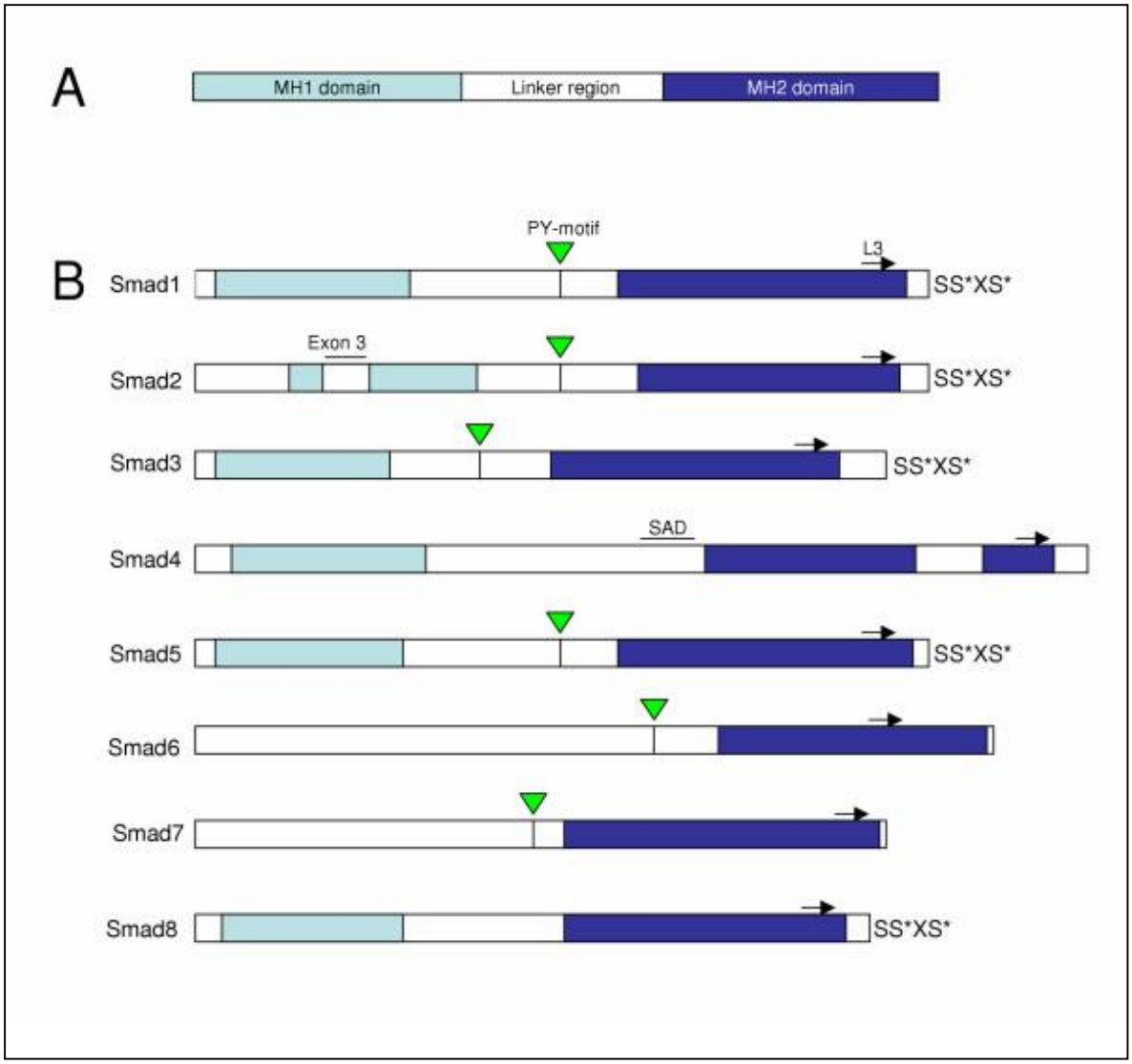

Figure 6. Schematic diagram of the structure of the Smad-protein family members /43/. Smads 1,2,3,5,8 are so called receptor activated R-Smads, Smad4 is a common Smad, and Smads 6 and 7 are inhibitory Smads.

3.6 Growth differentiation factors -9 and -9B

3.6.1 General characteristics

Growth differentiation factor -9 and -9B are oocyte derived factors, which are strongly expressed during folliculogenesis /12/ and have been shown to have an indispensable role in that process. They belong to the TGF-β superfamily and as such are processed as prepropeptides, proteolyzed by a furin-like protease /34/ and secreted as C-terminal dimers after post-translational modifications, such as N-linked glycosylation. A schematic diagram of GDF9 and -9B processing is presented in figure 7 /44/. Members of the TGF-β superfamily have a characteristic structure known as the cysteine knot, which establishes the characteristic fold of the monomer, and is conserved throughout the TGF-β superfamily. A schematic of the cysteine knot is shown in figure 8 /45/.

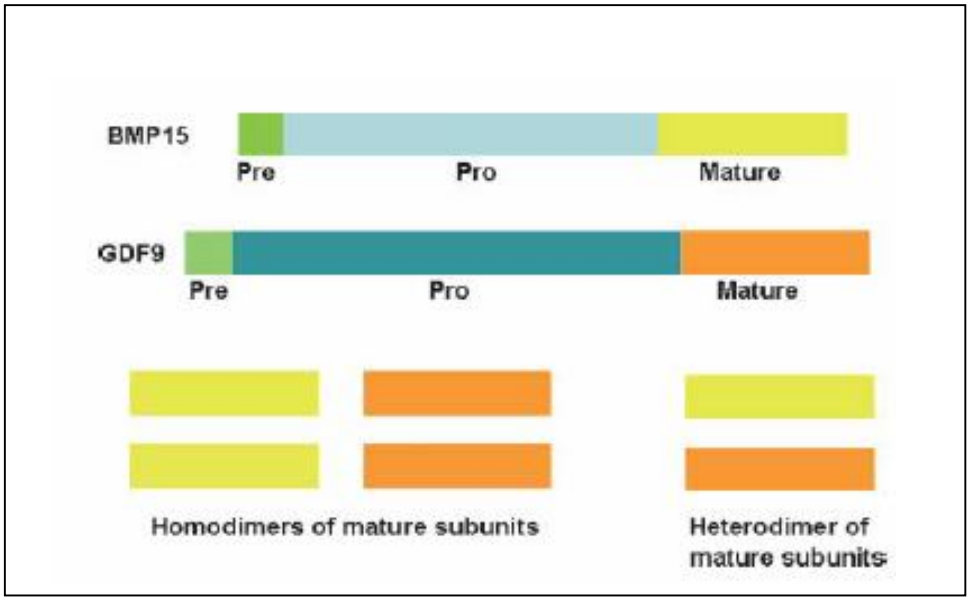

Figure 7. Modified from McNatty et al /44/. Schematic outline of the processing of GDF9 and GDF9B molecules and possible dimerizations of monomers.

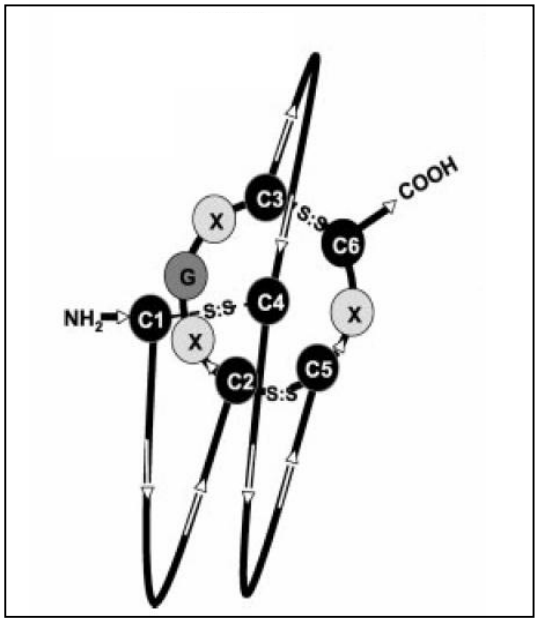

Figure 8. Modified from Avsian-Kretchmer et al /45/. Basic structure of a TGF-β superfamily member monomer with a characteristic cysteine knot to create rigid "palm" and two loops protruding like fingers.

3.6.2 Growth differentiation factor -9

Growth differentiation factor -9 was discovered in 1993 by using degenerate oligonucleotides from conserved regions of TGF-β superfamily members in PCR. With northern blotting -analysis it was shown that GDF9 mRNA was only found in the ovaries of mice /10/. Human in situ –hybridization showed that expression was limited to the ovaries and was expressed during all stages of the folliculogenesis except the primordial phase /46, 47/.

The amino acid sequence of mouse GDF9 consists of 441 amino acids, the NH_2-terminus has a hydrophobic domain, common for secretory proteins. In the peptide there is a proteolytic processing site of four arginines stretching from amino acids 303-306. This is where the mature COOH-terminal dimer is produced by cleavage by a furin-like protease, an enzyme recognizing and proteolyzing within the bibasic sequence of R-X-X-R. The resulting processed protein has molecule weight of 15,6kDa, but in total there are four possible glycosylation sites in the 441-amino acid peptide, one of which is located within the mature protein. Hence the molecular weight of the mature protein is greater than 15,6

kDa due to glycosylation. GDF-9 lacks the conserved cysteine residue that forms the disulfide linkage between subunits in other family members /10/. A schematic diagram of the human GDF9 and GDF9B peptide structures is presented in figure 9.

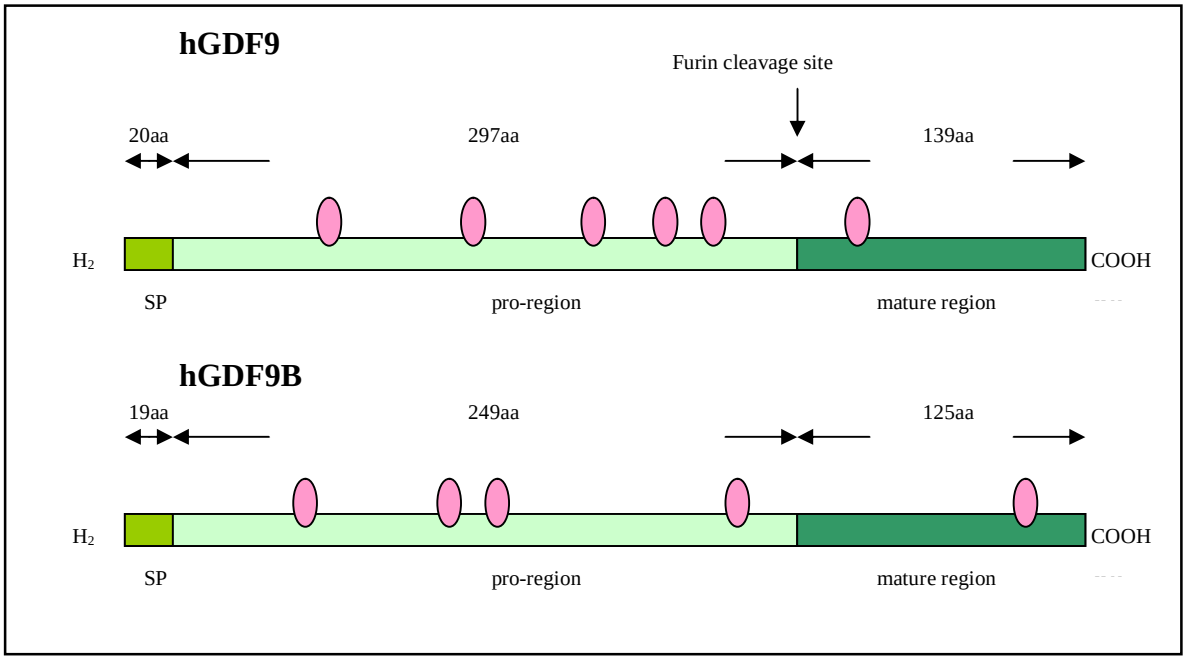

Figure 9. Schematic diagram of human GDF9 and GDF9B peptides. Light green colour for the pro-region and dark green for the mature region, and pink balloons present possible N-linked glycosylations. Furin cleaves hGDF-9 and hGDF9B mature region resulting in 139 amino acid (aa) and 125aa mature proteins, respectively.

The known biological effects of GDF9 are limited to the ovary, where it regulates different functions. To name a few, GDF9 stimulates progesterone production (by increasing steroidogenic acute regulatory gene [StAR] mRNA), cyclooxygenase 2 (COX2) mRNA synthesis, hyaluronan synthase 2 (HAS2) production, cumulus expansion /46, 48, 49/ and inhibin β production /50/, but inhibits urokinase plasminogen activator mRNA expression and FSH-stimulated LH receptor mRNA expression /46/. Recently GDF9 has been shown to interact with hormones when controlling follicle growth /51/ and prevent cumulus cell apoptosis by expressing TGF-β superfamily members /52/, thereby enhancing their developmental competence /53/.

3.6.3 Growth differentiation factor -9B

GDF9B was found by searching the GenBank Expressed Sequence Tag (EST) database with the mouse GDF-9 cDNA sequence. The GDF9B sequence was identified from a mouse 2-cell embryo library, from which an EST cDNA was isolated which encoded a putative member of the TGF-b superfamily, which was named GDF-9B /11/. Using Northern Blot hybridization a 4 kb long mRNA was found in the mouse ovary. With reverse transcription polymerase a 821 bp long complementary DNA was copied from the mouse ovarian RNA. The murine GDF9B C-terminus was found to be 53% homologous to mouse GDF9, further the new GDF9B sequence lacked the cysteine that is shown to link mature dimers of the TGF-β superfamily /11/. With rat GDF9B it was shown that GDF9B has a signal sequence which suggests that it is a secreted protein and it was found that expression pattern is similar to GDF9 /54/. In the human by using RT-PCR it was shown that GDF9B is only expressed in the gonads, further *in situ* –hybridizations showed that GDF9B is expressed at slightly latter stages than GDF9, late primary follicles instead of early primary follicles /12/. At the same time another group cloned also GDF9B and named it BMP15 /55/, hence the two names for GDF9B.

Human GDF9B was found utilizing the mouse GDF9B sequence when screening a human genomic DNA-library, after the sequencing of the PCR-fragment was done it confirmed that this was a GDF9-like new sequence, human GDF9B. Using FISH-analysis the human GDF9B gene was localized in the X-chromosome, Xp11.2. Albeit the similarities in the actual sequence and expression pattern, also exon-intron structure is similar to human GDF9, two exons are split with one intron but a difference can be found in the length of the intron, the human GDF9 intron is 1,6 kb long but GDF9B intron is 4,6 kb long /12/.

3.6.4 GDF9 and GDF9B - signalling pathway and receptors

Recently it was shown GDF9 signalling is mediated by the type I receptor, activin receptor-like kinase 5 (ALK5) /55/ and that its type II receptor is BMP type II receptor (BMPRII) /41/ therefore it mediates signaling by phosphorylated Smad2 and Smad3 /42, 50/. This is an unique combination of type I and type II receptors and the first time when it has been reported that a TGF-β superfamily member utilizes both activin-type receptor (ALK5) and BMP-type receptor (BMPRII) for its signalling.

GDF9B probably utilizes the same type II receptor as GDF9 i.e. the BMPRII, but thetype I receptor has been suggested to be ALK6 /41, 55/, although a lot is not known about GDF9B signalling.

IV AIMS OF THE STUDY

The overall aim of this study was to examine whether we can produce and purify bioactive recombinant human GDF9 and GDF9B. Further, there was a need to characterize a number of monoclonal antibodies raised against GDF9 and GDF9B, and to determine the usefulness of such antibodies in the characterization of the purified proteins.

4.1 Production of wild type human GDF9 and GDF9B

The first aim was to produce human GDF9 and GDF9B, and from previous experience in the lab we knew that we should use HEK-293T cells which are easily transfectable, are easy to grow and should secrete the protein in a processed form. The expression plasmid pEFIRES-p was used, which enables the production of a stable recombinant GDF9 or 9B expressing cell line via puromycin selection /57/.

4.2 Characterization of monoclonal antibodies

In order to study wild type recombinant human GDF9 and GDF9B, monoclonal antibodies were raised against both of these proteins, and their specificity and capability to immunoneutralize GDF9 *in vitro* was determined. These antibodies were epitope mapped which can potentially help when determining which are the hot spots of interaction between the ligands and their receptors. The antibodies discussed in this thesis were produced in collaboration with the laboratory of Prof. Nigel Groome (Oxford-Brookes University, UK) and characterized in the Ritvos laboratory.

4.3 Production of tagged human GDF9 and GDF9B

Previously it has been shown in the Ritvos laboratory that mouse GDF9 is active when secreted into conditioned media, but we found that human GDF9 did not show activity in *in vitro* cell models. Therefore studies for investigating the latency of human GDF9 were of interest and we decided to produce both GDF9 and GDF9B as histidine-tagged forms and purify the proteins to test for bioactivity.

4.4 Purification of human GDF9 and GDF9B

The aim was to purify human GDF9 and GDF9B using immobilized metal affinity chromatography (IMAC). In this study GDF9 and 9B were epitope tagged with a 6-histidine tag and purified using Ni-chelated HiTrap™. Biological activity was followed by monitoring the CAGA-luciferase mediated response for the GDF9 downstream target SMAD3. Two *in vitro* cell models were used to monitor biological activity, either using the HEK-293T cell line or by transducing the reporter construct into primary human granulosa-luteal cells via the use of a CAGA-luciferase adenovirus.

V MATERIAL AND METHODS

5.1 Background on the production of human GDF9 and GDF9B

Previously in the Ritvos laboratory GDF and GDF9B from various species have been cloned from testis RNA and subcloned such that pro-regions from different species have been joined with a mature region from another species to make fast progress in having the mature proteins available for in-house use and for collaborators. Most of the proteins have been cloned such that they are wild type in sequence, therefore having no tagging to enable purification using common known purification methods, based on the presence of an epitope-tag. The reason for first producing wild type proteins was that it was not known whether epitope tagging a TGF-β superfamily member would result in an altered bioactivity. GDF9B has been active when purified via a C-terminal Flag-tag /56/, however for the purpose of scale-up, immunoaffinity purification using the Flag-tag would not be ideal, hence epitope tagging of these factors was carried out utilizing the ability of the 6xHistidine tag to bind metal ions.

Recently it was found that sheep GDF9 and GDF9B wild type proteins were active when applied together but were inactive on their own as non-purified recombinant proteins in crude media /28, 29/. Therefore, in order to study also the interplay between human GDF9 and GDF9B, a construct was made in our laboratory by Mika Laitinen and Marjo Rissanen where human GDF9 and GDF9B are in the same expression plasmid, separated by just by an IRES (11 repeats). This protein was also tested in *in vitro* cell assays in this study.

An expression construct for human GDF9B with a 6-histidine tag in the NH_2-terminus of the mature region (hGDF9B-6H) was previously produced in the lab. This construct had the rat GDF9B pro-region preceding the His6 tagged human mature region. A new expression construct was designed which would generate COOH-terminally 6His-tagged human GDF9. Cell lines were available for the production of wild type recombinant human GDF9. All expression constructs used in this study are presented in figure 10.

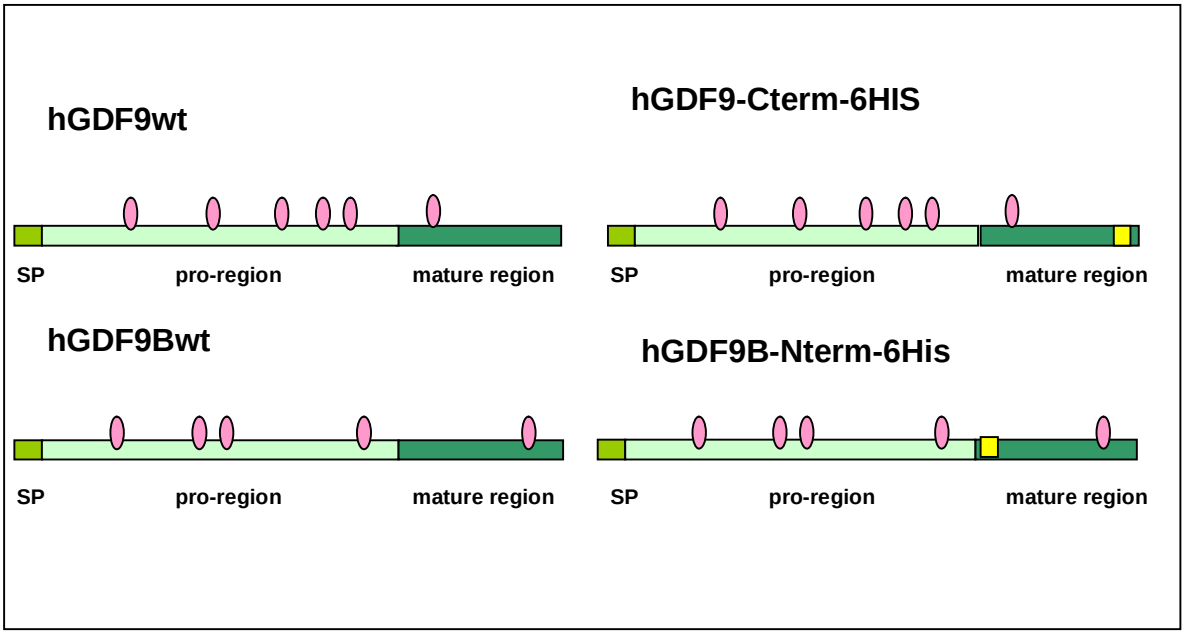

Figure 10. Expression constructs used in this study, yellow bar illustrates the location of the 6-histidine tag on the mature protein, pink balloons represent glycosylations.

For cloning human GDF9 primers were designed such that they would incorporate the 6-histidine tag into the COOH-terminal end of the wild type human mature GDF9, when PCR amplifying from wild type human GDF9 template. Then the PCR-product was ligated into the cloning plasmid pGEM-T Easy, using T-overhangs generated by the PCR-enzyme Dynazyme Ext. The ligated pGEM-T Easy vector was then transformed into DH5-α E.coli strain and plated on ampicillin containing agar plates for selection of the right clones. The selected clones are then grown in 5ml of Luria containing 5ug of ampicillin, in order to produce enough material for a Spin-prep. Spin-prep DNA was sequenced using M13 primers, specially designed for sequencing from pGEM-T Easy. The right clones were this way selected and further cloning done by cutting the insert out with restriction enzymes and ligating it into the expression plasmid, pEFIRES-p.

5.1.2 Expression plasmid and generation of a stable cell line

The expression plasmid used is called pEFIRES-p (figure 11, /57/) which incorporates sequences for the elongation factor 1 alpha (EF-1α) promoter, an internal ribosome entry site (IRES), and a selection cassette for puromycin resistance. The insert from pGEM-T Easy was ligated into pEFIRES such that the orientation was correct. Then a maxi-prep of DNA was made from this construct. With 1-3 μg of pEFIRES-p plasmid containing the wanted insert, a transfection can be made for 293 cells using Fugene6 when cells are at confluency of about 30% in T25-flasks. 293 cells are grown in Dulbecco´s Modified Eagle Medium (D-MEM), supplemented 10% Fetal Calf Serum (FCS) with penicillin streptomycin and L-glutamin, they grow in this media as a adherent monolayer.

After transfection, on the next day, puromycin is applied at 3 μg/ml. At this time cells usually have a confluency of about 50%. The following day the cells were observed and often 30-50% have died and therefore have not incorporated the pEFIRES DNA into the cells. Puromycin selection afterwards is usually done such that cells are grown to a confluency of about 80%, then split (1/3) and puromycin added straight after splitting, puromycin levels are increased 30 - 50% each passage, such that puromycin 160 μg/ml is the final concentration. Usually a transient transfection is made from the wanted expression construct to check whether the recombinant protein is expressed properly, this is done just by simply transfecting cells and leaving the cells to grow for 3-4 days and harvesting the media.

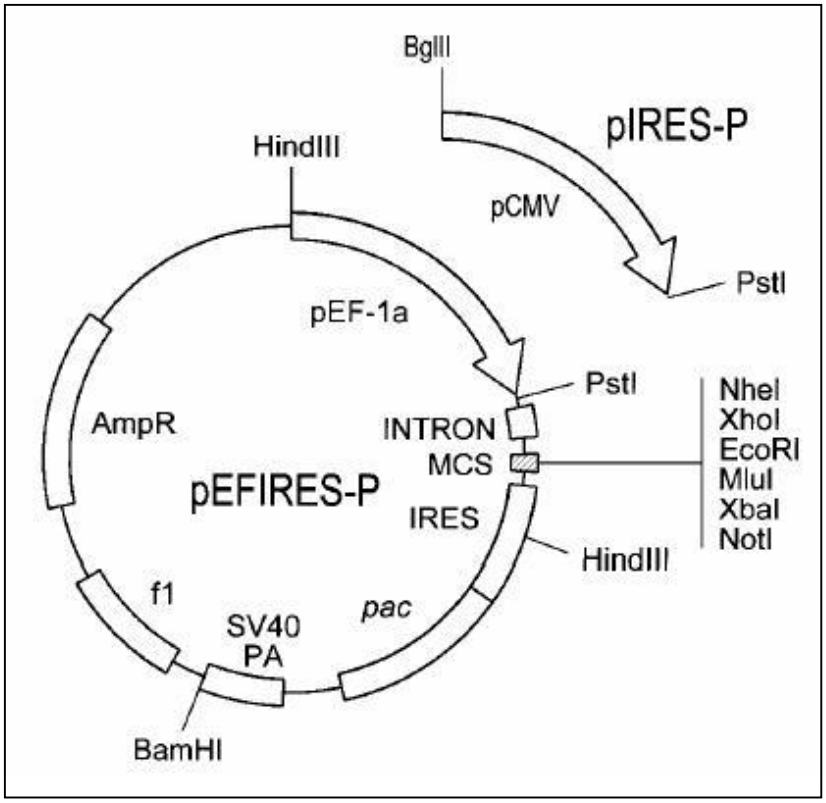

Figure 11. Expression plasmid pEFIRES-P, plasmid contains in brief, internal ribosome entry site (IRES), multiple coding sequence (MCS), antibiotic resistance cassette and Elongation Factor 1-α /12/.

5.2 Reporter construct

Since GDF9 /55/, and possibly also GDF9B, use the Smad2/Smad3 pathway to mediate their response inside the cell it is useful to employ such a reporter that directly corresponds with the activation of this pathway, therefore the TGF-β specific CAGA-luciferase reporter was used in this assay /57/. Since the cells used in this study are mainly human granulosa-luteal cells, and as such are primary cell cultures which cannot be transfected with ordinary methods, an approach utilizing adenovirally mediated gene transfer was adopted based on the Ad-CAGA adenovirus /42/. Briefly, Ad-CAGA comprises the CAGA-response element repeated nine times followed by the luciferase enzyme coding sequence. Cells transduced with this adenovirus and stimulated with TGF-β will produce the luciferase enzyme which can be detected by addition of luciferin substrate to the cell extract and measurement of light emission.

5.3 in vitro cell models

Human granulosa-luteal cells are cells that are left over from patients undergoing IVF (*in vitro* fertilization) -treatments, and with consent from these patients the cells can be used in *in vitro* -studies. Cells were fetched usually twice a week from IVF-clinics in 50ml Falcon-tubes full with follicular fluid, pooled after centrifugation and dispersed from other cells using hyaluronidase. Then they are centrifuged in a Ficoll-Pague gradient where human granulosa cells are left on the surface of the Ficoll and just under the sera. Human GL-cells are then plated on 24-well plates 30 000-40 000 cells per well and are allowed to settle over night under standard conditions, 37 ˚C and 5% CO_2 in 10% FCS DMEM –media supplemented with L-glutamin and antibiotics. The next day 200 µl serum free media containing 0,5 µl of Ad-CAGA was added per well and left for 30 minutes under standard conditions, after which 1 ml 2% FCS DMEM –media is added per well. Cells are then left for one to two days. When the ligands are added fresh 2% FCS DMEM –media is added on to the cells, 500 µl or 600 µl depending on the sample size, is changed and the sample is added.

Also the human embryonic kidney cell line, 293T, was used in some of the assays when hGLs were not available or they were too few. This cell line is also useful for proof-of-princible studies, since these can be grown in reasonable amounts and are easily transfectable, indeed these cells are transfected with the CAGA-luciferase reporter plasmid the same way as transient transfection is done e.g. using Fugene 6 (Roche, following the manufactors instructions).

5.4 Immobilized metal affinity chromatography

Immobilized metal affinity chromatography (IMAC) utilizes the ability of the amino acid histidine to bind certain metal ions. These metal ions are called transfer metal ions and such metals as nickel, copper, zinc and cobalt have the capability to interact with electron donating side chains of amino acids histidine, cysteine and tryptophan. Usually a 6-histidine tag is preferred and nickel as the chelating metal in a column format, especially for large scale applications. It is worth noting that the chelated metal should have a stronger affinity for the immobilizing gel than the protein has for the metal. Usually chelating is

done by the manufacturer and based on the matrix, the most common chelating substance is iminodi-acetic acid (IDA) and matrix often is sepharose, with a long spacer arm these chelated together such that minimal interference is caused for the binding of the tagged protein to the metal ion.

Using IMAC usually consists of three main steps: charging of the gel with a suitable metal ion, binding the protein onto the gel and eluting it out. The binding capacity of these gels is usually very high, in the region of milligrams per millilitre of gel slurry. After binding the protein, the gel is washed with suitable buffer that can contain a small amount of competing substance (e.g. immidazole), so that non-specific binding proteins would elute out with the washes. Elution can be done using various methods: altering the pH, or using competing ligands for the metal ions such as imidazole, ammonium chloride or histamine.

5.5 Monoclonal antibodies

Monoclonal antibodies specific for human GDF-9 were generated by taking an anti-peptide approach /15/. A synthetic peptide corresponding to the annotated amino acids 420–450 of the C-terminal part of the human GDF-9 (GenBank accession no. NP 005251) sequence, VPAKYSPLSVLTIEPDGSIAYKEYEDMIATKC, was made using F-moc chemistry, coupled to tuberculin through the cysteine thiol using a heterobifunctional agent, and used to immunize female BALB/c mice by standard methods. After an initial immunization and two boosts at monthly intervals, the sera of the mice collected by tail bleed were tested against ELISA wells coated directly with the immunizing peptide. High responding mice were boosted *in vivo*, and four days later the spleens were removed and used for fusion to SP2/0 splenocytes by standard methods. Positive hybridomas were tested in Western blots against recombinant GDF-9. Monoclonal antibodies were purified by protein A chromatography using a high salt protocol. The antibody has minimal crossreaction with preparations of recombinant GDF-9B. Also other clones were tested on Western blot and were found to be specific for GDF9.

5.5.1 Western Blotting

Western blotting was done with lab-made SDS-Page 15% gels, transfer was done with Semi-dry transfer and the membrane used was Hybond ECL –membrane from Amersham Biociences. The secondary antibody was a horseradish peroxidase conjugated anti-mouse monoclonal antibody from Jackson Immunoresearch.

5.5.2 Epitope Mapping

Some of the MAbs were found to be able to immunoneutralize the biological activity of mouse GDF9 therefore it was worthwhile determining the epitopes of these MAbs. Epitope mapping was performed by using 14-amino acids (aa) polypeptides attached to cellulose membranes (SPOT peptides) as previously described /58/. By using a 1-aa frame shift, the peptides, each 14-aa long, covered the entire human GDF-9 C-terminal sequence used for immunization, enabling the identification of the minimal peptide sequence of the epitope binding to the various mAb clones /15/. A schematic is presented in Figure 12 to clarify how the protein peptides are synthesized on nitrocellulose membrane

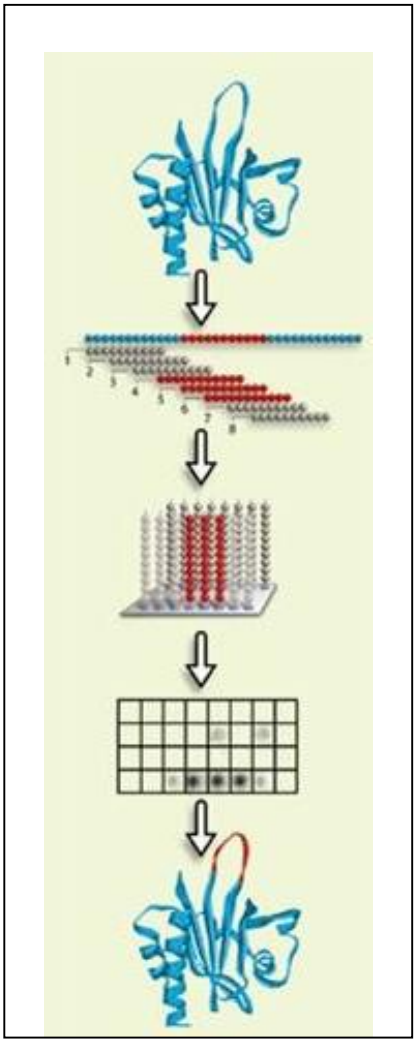

Figure 12. Pep-SPOT schematic diagram /59/. A protein sequence is generated and attached onto a nitrocellulose membrane as short peptides, this membrane is then incubated with possible binding protein and by enhanced chemiluminescence (or other means) interacting peptides are located. Interacting peptides can then be located back to the actual protein sequence, hence minimal epitopes are found.

5.6 Experiment setting and data analysis

Bioassays using 293T or hGLs were always performed in triplicate and from triplicates standard error of means (SEM) were calculated and presented as error bars with results. In some cases β-galactosidase normalization was used to account for variations in the infection and transfection efficacies of the cell lines. However, it was soon noticed that especially adenovirally mediated gene transfer was very consistent through assays and error in assays was probably due to different cell numbers in wells rather than in transduction efficiency between wells.

VI RESULTS

6.1 Production of wild type human GDF9 and GDF9B

The production of wild type human GDF9 and GDF9B recombinant proteins expressing cell lines was achieved by using pEFIRES-p as an expression plasmid /50/, transfected into HEK-293T cell line and selected with an antibiotic puromycin. Processing of GDF9 and GDF9B seems to occur as a clear band is seen around 20kDa, as seen on figure 13.

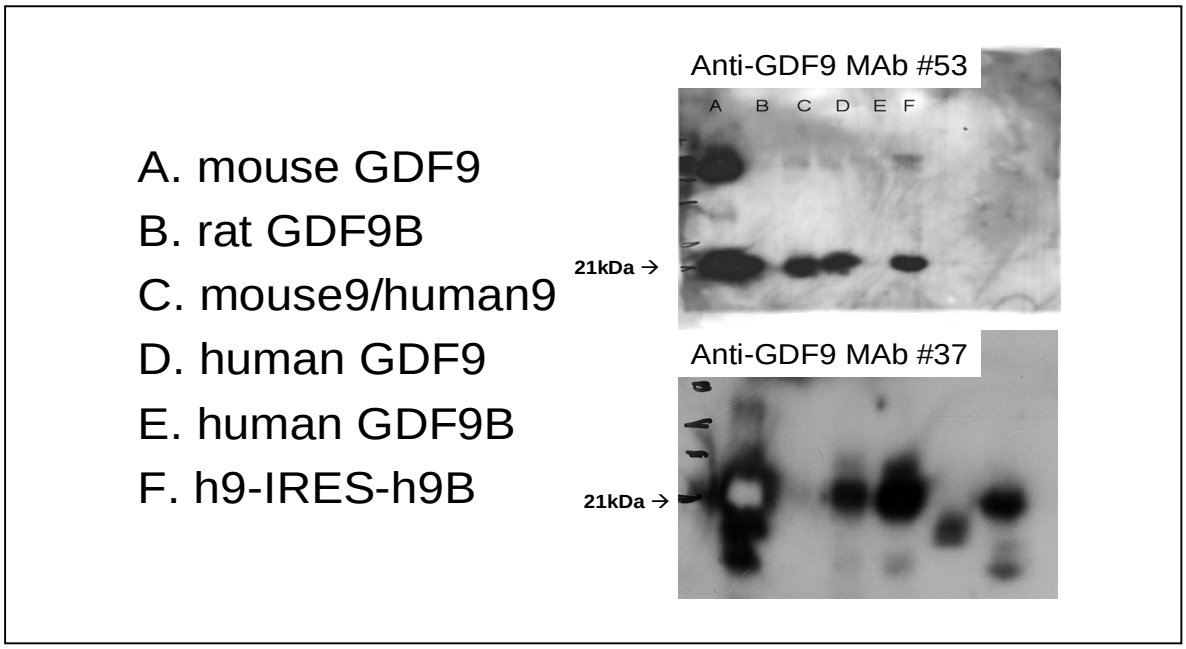

Figure 13. A Western blot analysis was done to determine the whether processing of the GDF9 occurs when expressed as a recombinant protein from HEK-293T cells, a mature portion of the protein runs close to 20kDa as predicted from 15kDa peptide size and allowing for glycosylation.

6.2 Specificity of MAbs

The monoclonal antibodies generated by our collaborator Nigel Groome, Oxford UK were subjected to specificity screening against some of the closely related TGF-β superfamily members (MAbs tested and their specificity presented in table 1). It was found that most of the MAbs did not recognize other TGF-β superfamily members, hence these MAbs would be excellent tools for investigation of GDF9 biology. The specificity of MAb #70 and MAb #53 is presented in Figure 14. Also other MAbs were tested but results are not shown here.

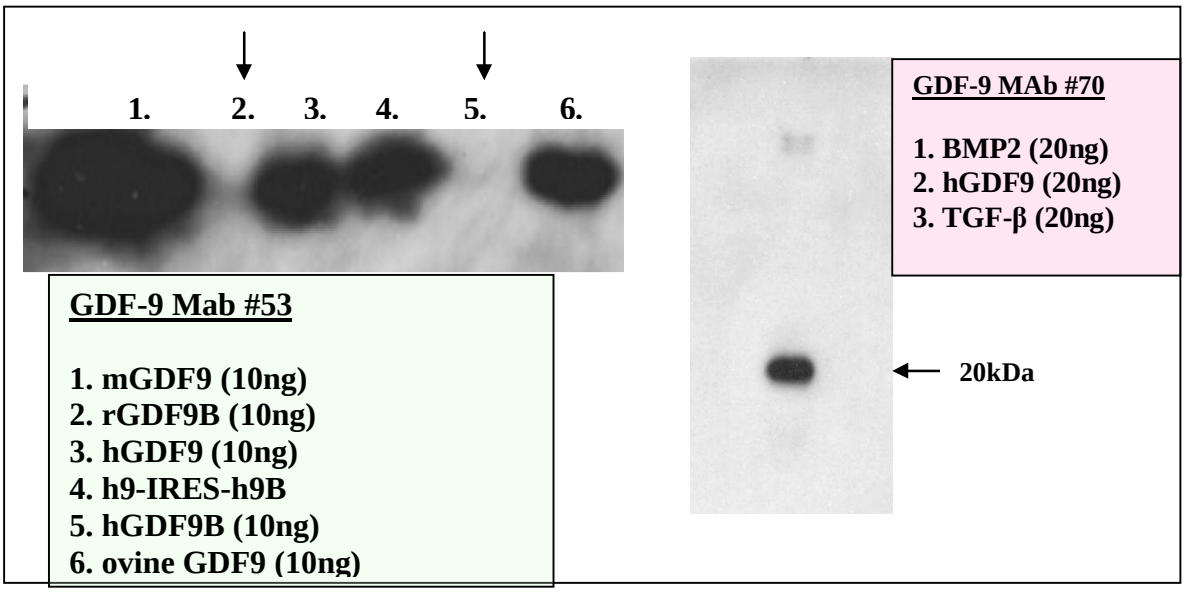

Figure 14. A Western blot analysis was done to determine the specificity of anti-GDF9 MAbs #53 and #70 and they were found to be very specific for GDF9, they did not crossreact even with close homologue GDF9B.

Different MAbs were generated for different GDF9 and -9B recombinant proteins utilizing the same peptide approach and their ability to recognize GDF9 and -9B was tested with Western blotting, MABs and their relative affinities are presented in table 1.

MAb	mouse GDF9	rat GDF9B	human GDF9	human GDF9B	h9-IRES-h9B
anti-human GDF9					
C-terminal **#37**	**XXX**		**XX**	**X**	**XX**
C-terminal **#53**	**XX**		**XX**		**XXX**
C-terminal **#70**	**XX**		**XX**		**XXX**
N-terminal **#72**			**XXX**		**XXX**
anti-human GDF9B					
C-terminal **#1/1**	**X**	**X**	**X**	**X**	**X**
C-terminal **#4/3**		**X**		**XXX**	**XXX**
N-terminal **#28**				**XXX**	**XXX**

Table 1. Affinities of GDF9 and -9B monoclonal antibodies (MAbs) for different species of recombinant GDF9 and -9B, using standard Western blot-protocol and ECL. MAbs were generated either against C-terminal or N-terminal peptides. MAbs generated against the N-terminal peptide of hGDF9 and GDF9B proved to be very specific and effective.

6.3 Epitope mapping of MAbs

In order to identify the epitopes recognised by these MAbs, a PEP-SPOT analysis was carried out /60/ using 14-aa peptides with a frame shift of one amino acid, yielding 19 peptides covering the whole 32-aa immunizing peptide. Epitope mapping was done using a standard Western blot –protocol and chemiluminescence reagents. Epitopes ranging from 4 to 14 amino acids were found, and interestingly the MAb having the shortest epitope (EPDG) was still able to immunoneutralize the biological activity of GDF9 /15/. MAbs and their epitopes are presented in figure 15.

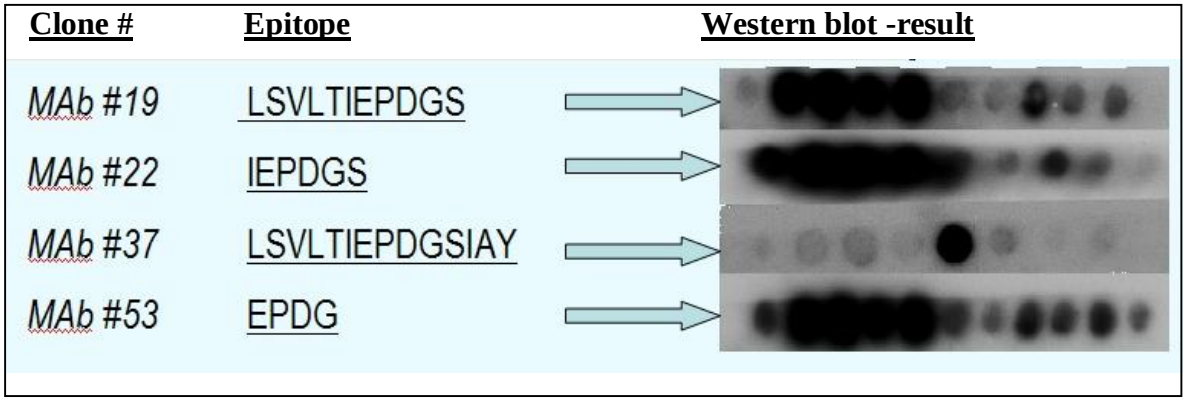

Figure 15. Minimal epitopes of MAbs were determined using the PEP-SPOT method /15/, the more spots that illuminate; the shorter the epitope will be, since more peptides interact with the protein.

These four MAbs were tested in various bioassays to test their capability in immunoneutralizing the biological activity of mouse GDF9 and the most convincing results came when thymidine incorporation assays carried out in collaboration with the laboratory of Dr. Robert Gilchrist (Adelaide University, Australia) were performed using a mouse granulosa cell –model, where MAb #53 was able to neutralize 93% of the biological activity of mouse GDF9 and 40% of oocyte mitogenic activity. Therefore it can be said that mouse GDF9 partially accounts for oocyte mitogenic activity /15/. MAbs and their corresponding capability to immunoneutralize are presented in figure 16.

Anti-hGDF-9 clone[2]	**Epitope**	**% Neutralization**[1] **GDF-9** (80 ng/ml)	**DOs** (12 oocytes/well)	**TGF-β1** (0.5 ng/ml)
control (none)	-	0	0	0
IgG	-	-5	-6	5
mAb-53	EPDG	93	40	-4
mAb-37	LSVLTIEPDGSIAY	67	38	-12
mAb-19	LSVLTIEPDGS	73	17	-4
mAb-22	IEPDGS	75	22	-21

[1] Inhibition of GDF-9-, oocyte- or TGF-β1-stimulated MGC [^{3}H]-thymidine incorporation by various anti-hGDF-9 monoclonal antibodies (or IgG control). Values are mean percent neutralisation expressed relative to the control (mitogen with no antibody). Negative values indicate stimulation of MGC DNA synthesis.
[2] All IgGs at 80 µg/ml

Figure 16. MAbs were tested for ability to immunoneutralize mGDF9 and oocyte mitogenic activity, using MGC thymidine incorporation –assay, surprisingly MAb#53 with the shortest epitope was able to immunoneutralize 93% of recombinant mGDF-9 activity and 40% of oocytes mitogenic activity

Surprisingly MAb #53 epitope (EPDG) was found to be unique amongst the whole TGF-β superfamily for GDF9 and conserved across different species (Figure 17). The closest homolog GDF9B, which is also an oocyte growth factor, had only two out of the four amino acids shared with GDF9. Still MAb #53 is found to be specific for GDF9 and able to neutralize mouse GDF9. Even more, all vertebrate GDF9 proteins have all four amino acids of this epitope in common, therefore making this MAb a versatile and specific tool for analyzing GDF9 function throughout different species.

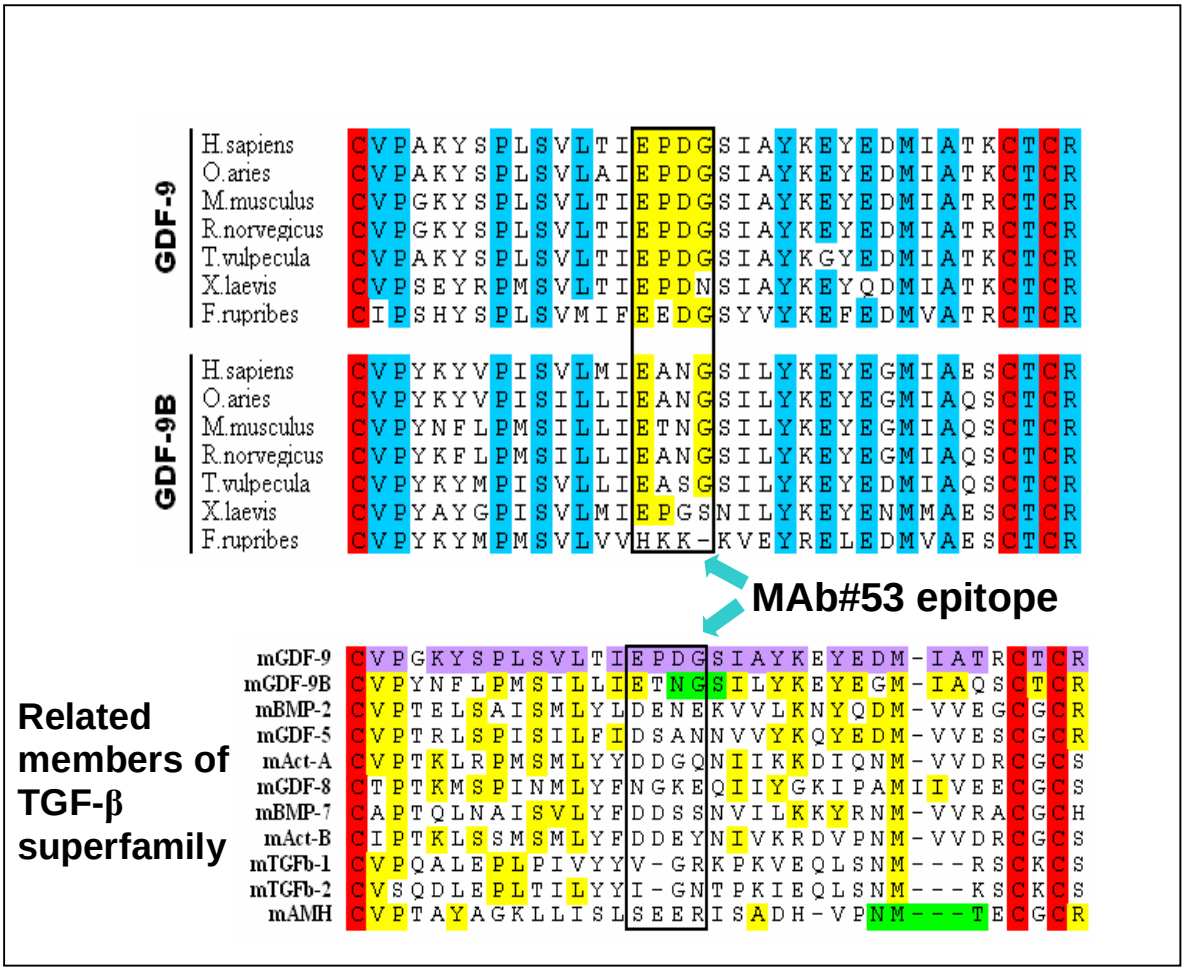

Figure 17. The epitope for MAb #53 and its conservation throughout species and the TGF-β superfamily. The MAb #53 epitope EPDG is well conserved in GDF9, only two amino acids are in common with closest homolog GDF9B. Other TGF-β superfamily members do not share these amino acids with GDF9.

When the epitope of MAb #53 was localized on the molecular model of GDF9 (based on the crystal structure of BMP2) it was found to be located on the second protruding fingertip of GDF9. At this same location GDF9B shares only two amino acids and there is also a potential N-linked glycosylation site. Therefore it was no surprise that MAb#53 was found to be specific for GDF9. In the molecular model (figure 18), which is based on the crystal structure of BMP2, the epitope for MAb #53 is highlighted and corresponding four amino acids of GDF9 and GDF9B are shown as a "spacefilling" magnifications.

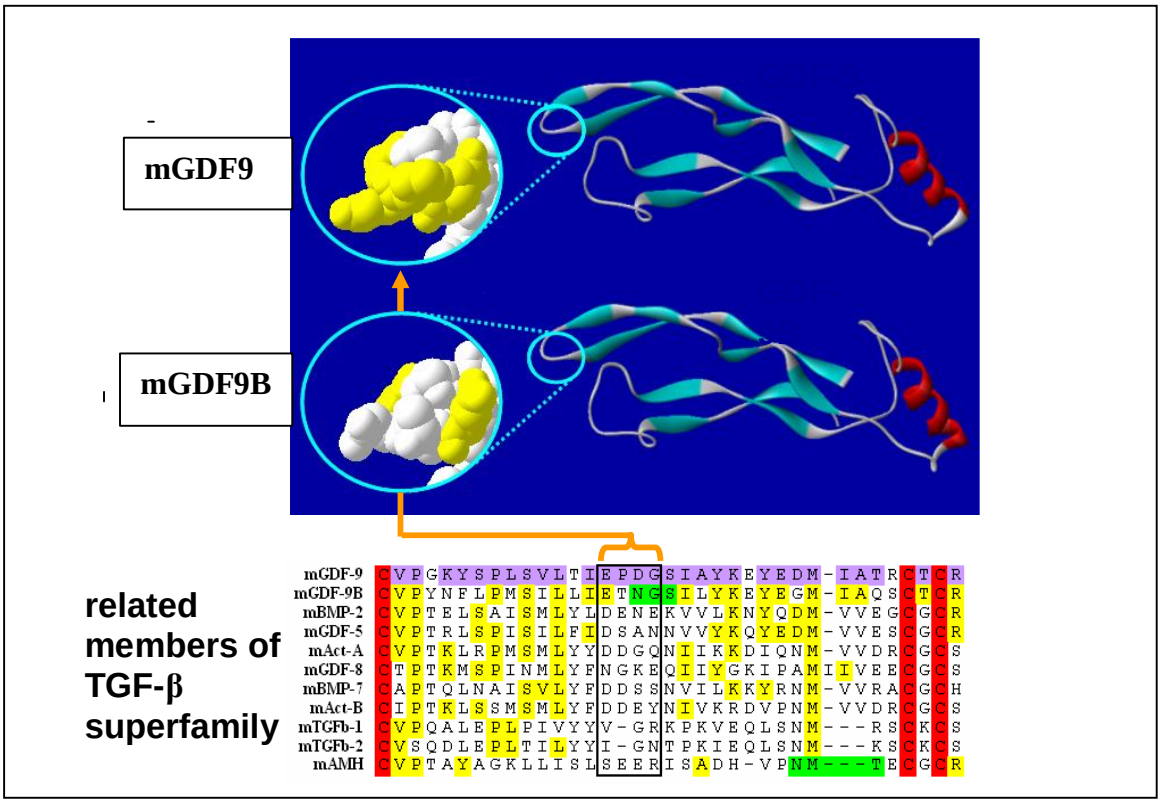

Figure 18. Epitope of MAb #53 localized on a molecular model of mGDF9 and GDF9B (based on crystal structure of BMP2). Epitope can be found on the tip of the protruding finger and when comparing with mGDF9B also a possible glycosylation may contribute for MAb#53´s ability to bind GDF9B.

Using a short synthetic peptide comprising of the amino acids –IEPDGS- the interaction between mouse GDF9 and MAb#53 was dose-dependently blocked (figure 19).

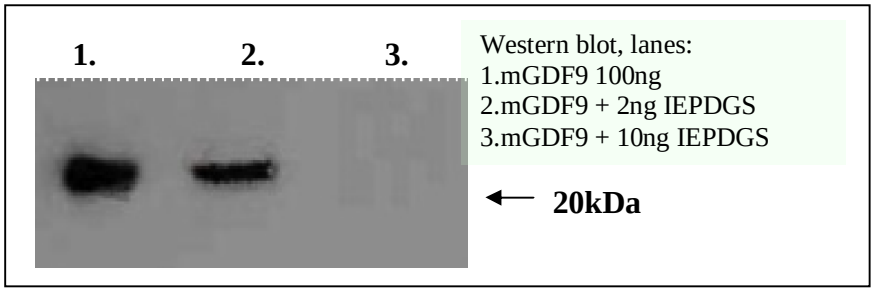

Figure 19 Competing mouse GDF9 interaction with MAb#53 with the IEPDGS-peptide, the peptide had a greater affinity towards the MAb therefore having a similar peptide sequence than the epitope of the MAb is.

6.4 Purifying HIS-tagged human GDF9 and GDF9B

Purification of human GDF9B with a His6 tag at the N-terminus of the mature region was performed with HiTrap Chelating ™- columns by binding $NiSO_4$ onto the matrix and then binding the protein from conditioned media (salt adjusted to 1M NaCl) by circulating 150ml of media through the column overnight in the cold room. After loading of the sample the column was washed first with either low imidazole (10-20mM) containing buffer, followed by 3-7M urea containing buffer, and finally eluted with high imidazole (50-250mM) containing buffer.

The purification result of human GDF9B is shown in figure 20. The elution fraction with 50mM imidazole was chosen to be used in biological activity characterizations.

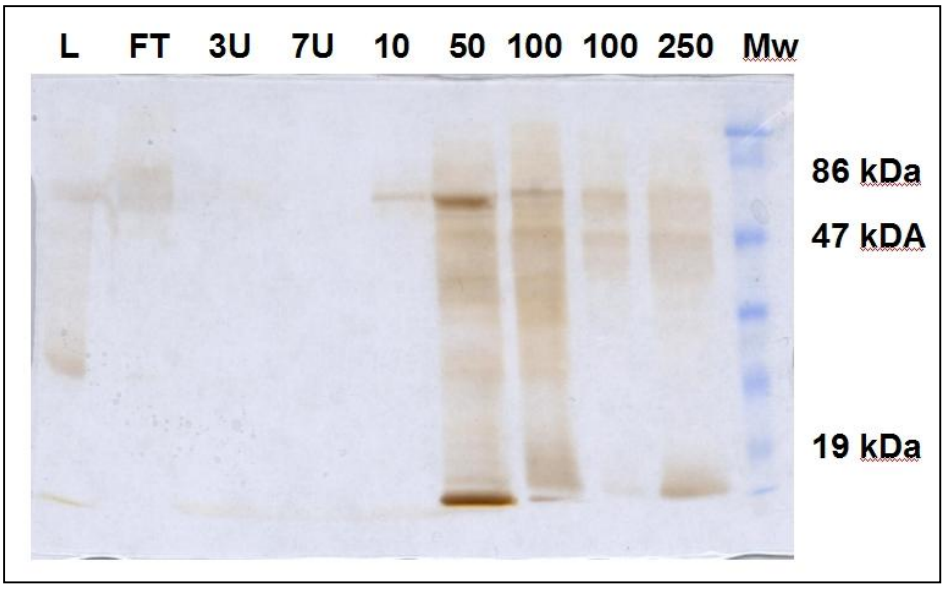

Figure 20. Purification of human GDF9B with urea wash, silver staining.
***Lane 1**: Load-in conditioned media, **lane 2:** flow-through, **lane 3:** 3 M urea wash, **lane 4:** 7 M urea wash, **lane 5:** 10 mM imidazole wash, **lane 6:** 50 mM imidazole elution, **lane 7:** 100 mM imidazole elution, **lane 8:** 100 mM imidazole elution, **lane 9:** 250 mM imidazole elution and **lane 10:** molecular weight marker (Fermentas).*

The purification result of human GDF9B without any urea washes is shown in figure 21. The first elution fraction with 100mM imidazole was chosen to be used in biological activity characterizations. When urea washes are not used, 2 bands of protein migrate in gels around 50-60kDa, these are considered as complexes of the preproprotein of GDF9B, however when urea is used for washing this complex does not appear (figure 20).

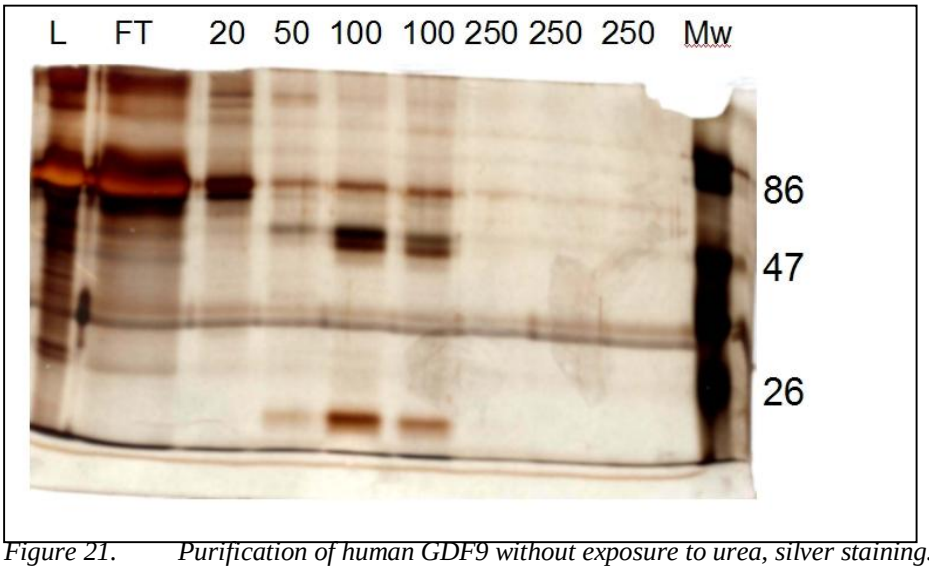

Figure 21. Purification of human GDF9 without exposure to urea, silver staining.
***Lane 1**: Load-in conditioned media, **lane 2:** flow-through, **lane 3:** 20 mM imidazole wash, **lane 4:** 50 mM imidazole wash, **lane 5 & 6:** 100 mM imidazole elution, **lane 7-9:** 250 mM imidazole elutions, and **lane 10:** molecular weight marker (Fermentas).*

6.5 Biological activity of GDF9 and -9B

Previously it had been shown that human GDF9 and -9B were inactive in all tested bioassays formats, when the sample was added as conditioned media from 293T cells. This was in contrast to what was known about mouse GDF9 which wass active in whole untreated conditioned media. As recent knowledge indicated that when ovine GDF9 and GDF9B are mixed together *in vitro* and given to a responsive cell line they result in bioactivity, this approach was also tested on human GDF9 and GDF9B. Also the hypothesis that the interaction of human GDF9 and GDF9B when produced simultaneously from one expression construct would result in bioactive protein was tested.

6.5.1 Biological activity of conditioned media containing GDFs

All produced recombinant human GDF9 and -9B except mouse GDF9 showed no bioactivity either on hGLs or 293Ts when in conditioned media, also a new human GDF9-GDF9B expressing cell line produced inactive protein, as shown in figure 22. This finding lead to the idea that these proteins need to be purified in order to characterize their properties and to reveal whether they are at all bioactive when produced using this system.

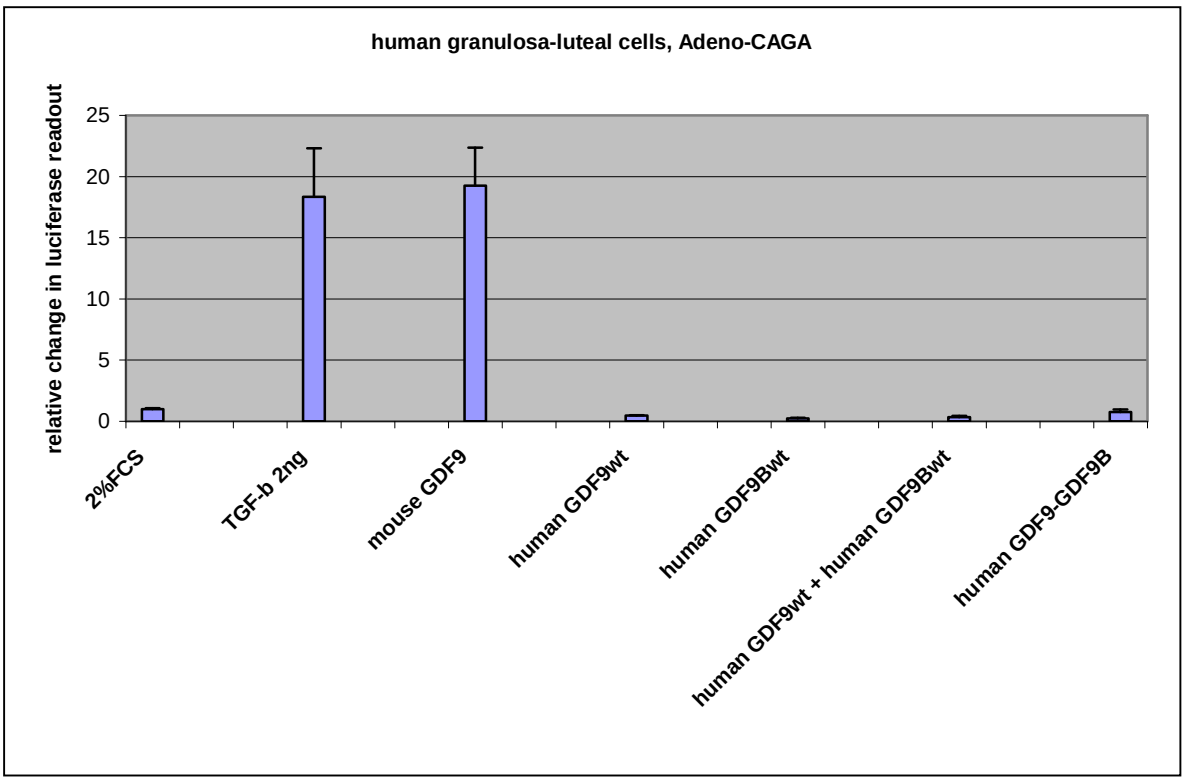

Figure 22. Luciferase readout from human granulosa-luteal adenovirally infected with CAGA-luciferase, all GDF9s and GDF9Bs except mouse GDF9 are inactive when produced into media from 293T cells. TGF-β is a known activator of CAGA-luciferase and used as a positive control. The mixture of human GDF9wt and GDF9Bwt was also tested since it has been shown that these two molecules interact with each other/ 28, 29, 30/

6.5.2 Biological activity of purified preparations of GDFs

Purified human GDF9B-6H showed inconsistent bioactivity when purified without a urea wash –step, ie only after a urea wash step was there consistent bioactivity. Surprisingly in both CAGA- and BRE -assays the purified 9B exhibited bioactivity. (since it has been previously shown to be responsive through BRE) but validation of this purified preparation is under way. Because of these results we hypothesize that the urea wash disrupts a latent complex of GDF9B and renders active the mature protein. In figure 23 human granulosa-luteal cells are used and dose-dependent luciferase readout with purified human GDF9B can be observed. (material from figure 20)

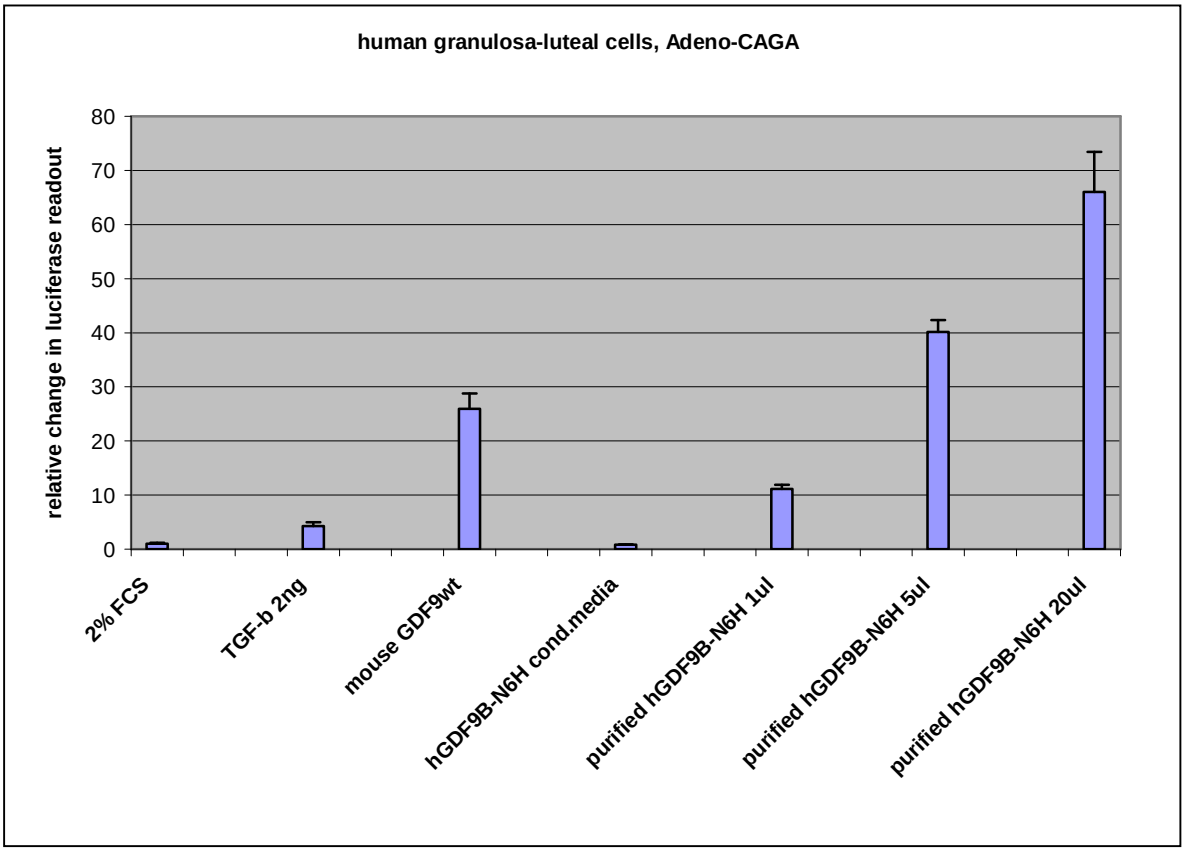

Figure 23. Purified human GDF9B shows dose-depended activity on CAGA-luciferase from human granulosa-luteal cells, surprisingly since previously it has thought to be a possible BRE-luciferase activator.

Surprisingly the same purified human GDF9B also showed activity in the BRE-luciferase assay, which utilizes the same technique, but the responsive element requires the ligand to interact with BMP-responsive Smads, as shown in figure 24.

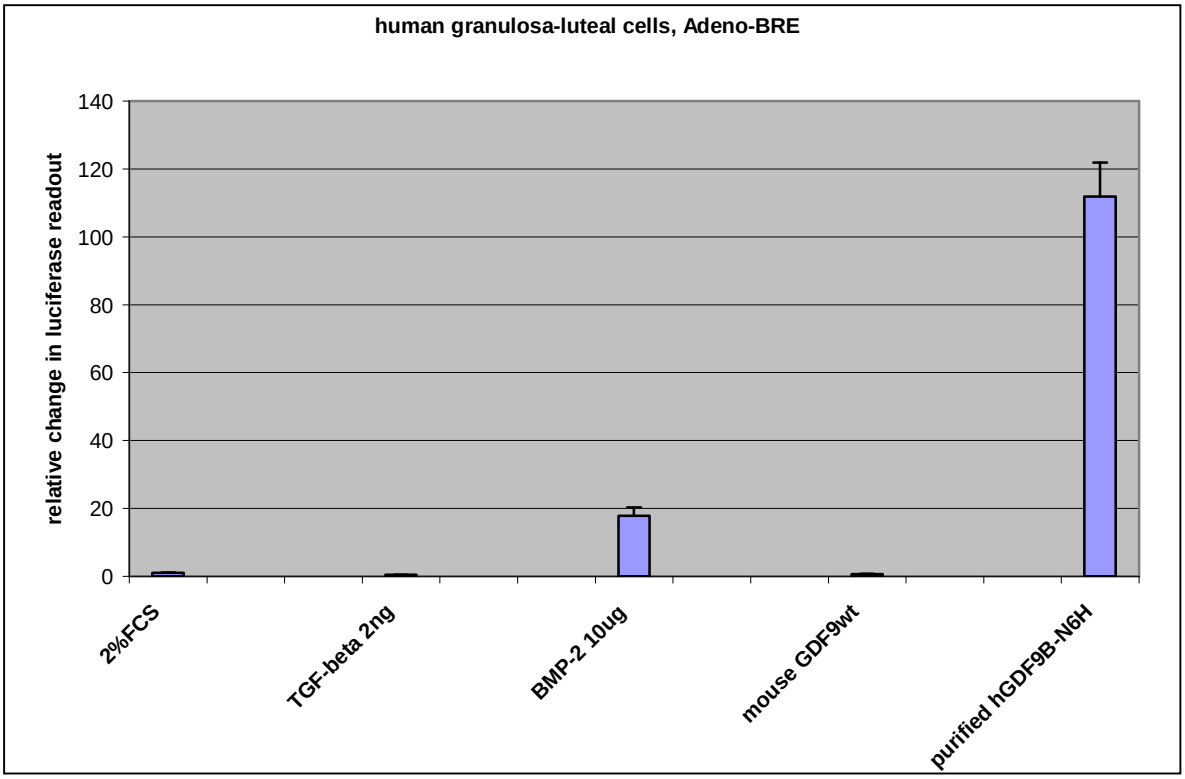

Figure 24. Purified human GDF9B activity on BRE-luciferase from human granulosa-luteal cells, which was surprising since it has already activated CAGA-luciferase pathway. BMP-2 is a known BRE-activator and both TGF-β and mouse GDF9 act as a negative control since they are known CAGA-activators.

This result was very surprising and it has to be validated by neutralizing human GDF9B biological activity both in BRE- and CAGA-luciferase assays, and also further investigating the properties of the purified preparation..

Purified human GDF9 with a C-terminal his6 tag was tested both on CAGA- and BRE – mediated luciferase readout on human granulosa-luteal cells. The results revealed that the protein was in both CAGA- and BRE luciferase assays, as shown in figures 25 and 26.

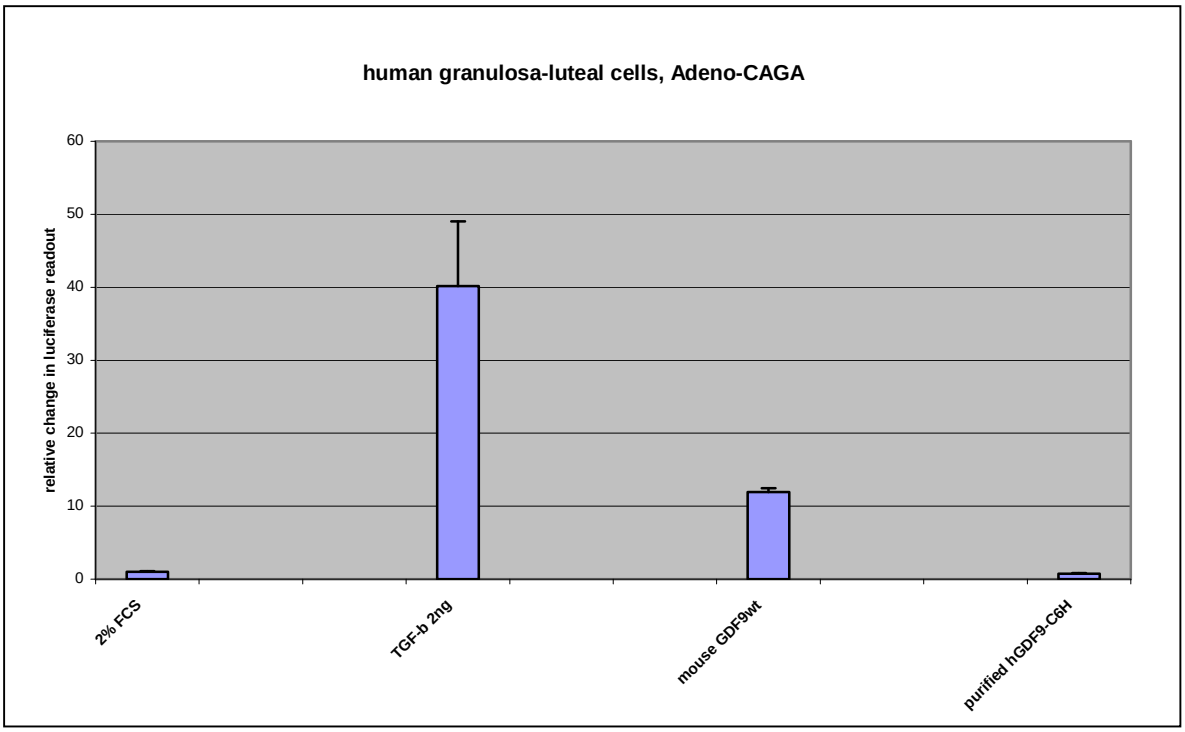

Figure 25. purified human GDF9 tested on human granulosa-luteal cells with adenovirally mediated CAGA-luciferase readout. TGF-β and mouse GDF9 act as positive control, purified hGDF9-C6H exhibits no biological activity.

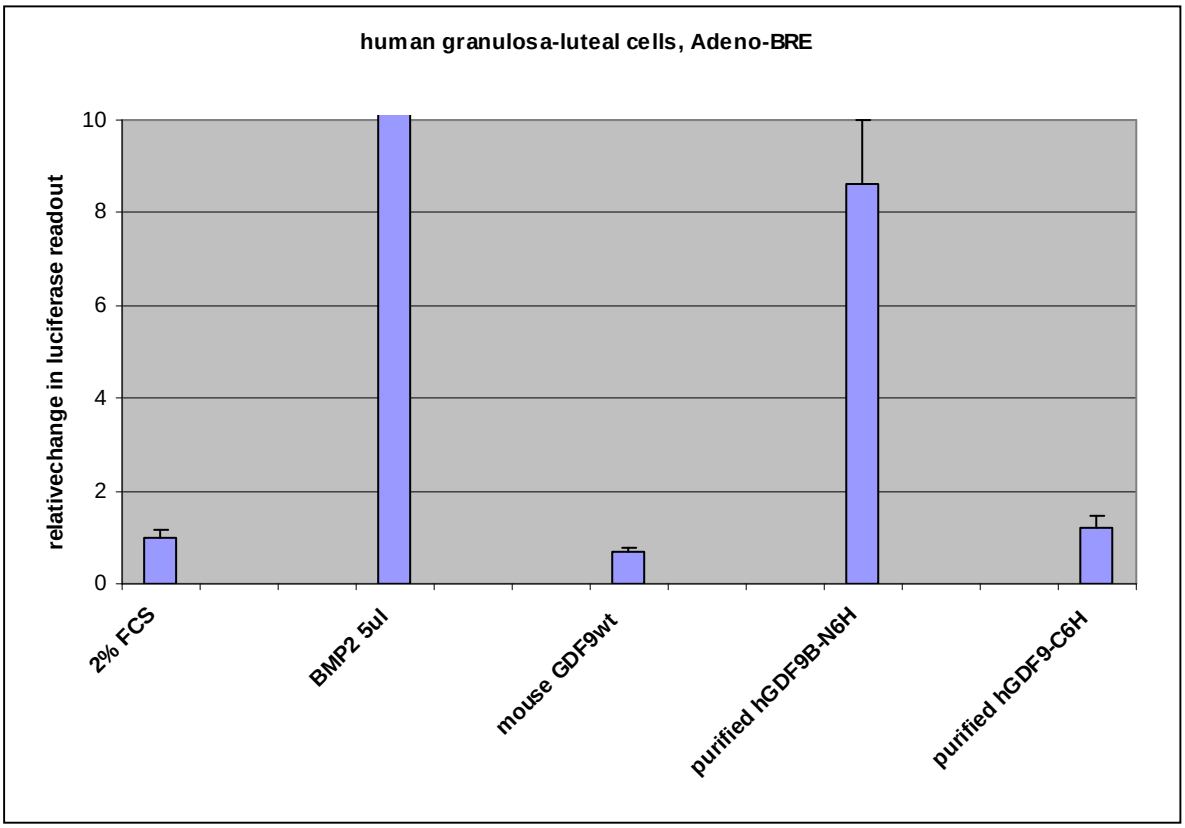

Figure 26. purified human GDF9 tested on human granulosa-luteal cells with adenovirally mediated BRE-luciferase readout. BMP2 acts as a positive control, again purified hGDF9-C6H shows no biological activity, where as purified hGDF9B-N6H exhibits BRE-activity.

As purified human GDF9-C6H did not show any activity after purification, questions of the biological properties of this molecule arose. This led us to believe that the propeptide of the human protein is able to bind the mature protein even more tightly than expected, or that C-terminal His-tagging of human GDF9 would result in an inactive protein, since N-terminal His-tagging resulted in a bioactive molecule in the case human GDF9B-N6H.

VII DISCUSSION

The production of biologically active recombinant GDF9 and GDF9B has been a problem being investingated for some time. When this study was iniated theonly active recombinant GDF9 was mouse GDF9, which was active as a crude supernatant produced from HEK-293T cells, human GDF9 and GDF9B were produced, processed and secreted but they were inactive in their crude supernatant. This opened a question, how do these molecules behave differently and what is the basis of that behaviour? However further studies of these molecules required purified and validated preparations.

The first aim of this study was to produce and purify an active recombinant human GDF9 and GDF9B, for this purpose these proteins were epitope-tagged with 6-histidine to enable purification using immobilized metal affinity columns. The second aim was to characterize a number of GDF9 and GDF9B monoclonal antibodies in regard to their specificity, capability to immunoneutralize recombinant GDF9 *in vitro* and further study the epitopes of these antibodies.

Recombinant human GDF9 and GDF9B were produced as epitope tagged molecules and purified from crude media. To our surprise C-terminally tagged human GDF9 was inactive in our cell models even as a purified preparation, but N-terminally tagged human GDF9B was active, even more it was active in both our reporter constructs which suggests that human GDF9B utilizes two different pathways to mediate its responses to target cells. Although it must be said that purified human GDF9B was not consistent in all assays.

Monoclonal antibodies for GDF9 were found to be specific for GDF9 and immunoneutralize mouse GDF9 *in vitro* to some extent and by this it was found that GDF9 partially accounts for the mitogenic actions of the oocyte. The most effective immunoneutralizing antibody was found to have the shortest epitope of all, and the epitope (4aa, -EPDG-) was conserved through different species of GDF9, but not other TGF-β superfamily members, actually the only homologous amino acids were found from the closest relative GDF9B and even it only shared maximum 2 amino acids of the epitope, also a glycosylation site was located in GDF9B close to this epitope which probably would interfere this antibody from binding GDF9B.

In future, to really understand what are the biological activities of human GDF9 and GDF9B, epitope tagging and its location should be further studied. The latency issue of human GDF9 and GDF9B should be looked at and the possible latency associated molecules should be searched. Since immunoneutralizing antibodies for GDF9 are available the validation of the biological activity in *in vitro* cell models is more reliable, this should bring new answers to questions relating to the signalling of these molecules. Immunoneutralizing antibodies should be tested *in vivo* in mice to see whether they can modulate folliculogenesis by interfering with GDF9 signalling on granulosa cells.

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