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Comparing placental phenotype similarities between Neuropathy Target Esterase and the Canonical Wnt Signaling Pathway

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GENERAL INTRODUCTION

A mass spectrometry analysis of Wnt components revealed that LGR with high probability to a protein called Neuropathy Target Esterase (NTE). This was interesting as both NTE and certain Wnt components were known to have placental phenotypes in mice. Therefore, the hypothesis arose that NTE could have a similar role or a potential interaction with the Wnt pathway. This thesis explores this possibility by first giving a detailed review of what is known to date about the Wnt signaling pathway and the NTE enzyme. This is followed by a description of normal placental development in mice and then a section on how this development is defective in both NTE knockout mice and mice with certain Wnt component mutations. Subsequently, an analysis comparing the phenotypes presented will be conducted and from this a discussion will be started.

Following this extended literature review a scientific report will explore the protein R-Spondin and its function as an agonist for the canonical Wnt pathway as well as showing to which receptor it needs to bind for this role. This scientific report therefore underlines an important regulation component of the canonical Wnt pathway which is shown to play an important role in placental development.

This thesis will conclude with a reflection describing the struggles and triumphs of the writing process as well as my time at Hubrecht Institute within the group of Dr. Hans Clevers under the guidance of Dr. Wim de Lau.

LITERATURE REVIEW

Comparing placental phenotypes of Neuropathy Target Esterase and the Wnt signaling pathway

Amy van der List [1,2]

The Wnt Signaling pathway has been studied intensively since the late 1900s. This review explores what has been discovered up to now as well as a potential link between the Wnt pathway and a relatively unstudied enzyme, Neuropathy Target Esterase (NTE). Despite radically different functions within the cell, the Wnt pathway and NTE are shown to have similar placental phenotypes in knockout mice. with defective labyrinth development. Both NTE knockout mice and mice with deficiency in the Wnt components, Frizzled5, R-Spondin3, and Wnt2 ,show defective labyrinth development. This review analyzes this phenotype to try and elucidate whether the cause for the labyrinth defects for Wnt and NTE knockouts are the same. Through this review potential links between Neuropathy Target Esterase and the Wnt pathway are explored and light is shed on their role in placental development.

The Wnt Signaling Pathway

In 1982, Nusse and Varmus, identified the proto-oncogene *integration-1* (int-1) in mice as a mammalian homolog of the gene responsible for segment polarity in Drosophila, *wingless* (wg) [1]. This gene was then renamed Wnt1, a fusion of the terms 'wg' and 'int' [2]. The Wnt1 gene was discovered to have a role in tumor formation when it was overexpressed by a proviral insertion at the *Wnt1* locus [3]. This study resulted in spontaneous mammary hyperplasia and tumours in mice, suggesting a causative role for WNT1 in mammary tumorogenesis [4]. It is now known that the Wnt signaling pathway is a signal transduction cascade that takes on many pivotal roles within the cell. This pathway is determinant in embryonic development, cell growth and differentiation, cell motility, polarity, primary axis formation and most recently, it has been implicated in stem cell renewal [5]. There are three main established Wnt signaling pathways: 1) the canonical pathway, 2) the planar cell polarity (PCP) pathway, and 3) the Wnt-Ca2+ pathway [6]. The latter two pathways, the so-called 'non-canonical' Wnt pathways, play an important role in embryonic development. The PCP pathway governs the orientation of structures according to the polarity in the epithelia. For example, the PCP pathway controls the orientation of hair follicles, sensory bristles and the hexagonal array of ommatidia in the eye [6]. The second branch of the non-canonical pathway, the Wnt-Ca2+ pathway is crucial in 'the negative regulation of dorsal axis formation, promotion of ventral cell fate, regulation of tissue separation and convergent extension movements during gastrulation, and later in heart formation' [6].

In contrast to the PCP and the Wnt-Ca2+ pathway, the canonical pathway is unique in that it is dependent on the protein β-catenin, encoded by the gene CTNNB1. Past studies discovered the influence of Wnt1 on the protein β-catenin and the crucial role for hyperactivated β-catenin/Wnt signaling in colorectal cancer [7]. It is now known that canonical Wnt signaling is heavily involved in development and in the maintenance of adult tissue homeostasis through the regulation of cell proliferation, differentiation, migration, genetic stability and apoptosis [8]. Due to its role in a multitude of important functions, it is not surprising that aberrant regulation of this pathway causes a variety of diseases, including cancer, fibrosis and neurodegeneration [8].

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It is likely that Wnts can signal through both the "canonical" and the "noncanonical" pathways. Which pathway is chosen is determined by the context, and the specific combination of receptors. While the term "noncanonical" is widely used in literature, its definition is quite loose. Canonical refers to the pathway components that lead to stabilization of beta-catenin in response to certain Wnt ligands. Any other biological outcomes of Wnt signaling are termed non-canonical. Although the molecular basis of β-catenin–independent signaling pathways is still poorly understood, there is a general consensus that β-catenin–independent signaling antagonizes the β-catenin pathway [9]. Crosstalk between the different Wnt signaling cascades is possible, and it is also possible that all Frizzled receptors may couple to β-catenin–dependent and –independent pathways. This thesis will focus on the canonical Wnt or β-catenin pathway as it is the most well-known.

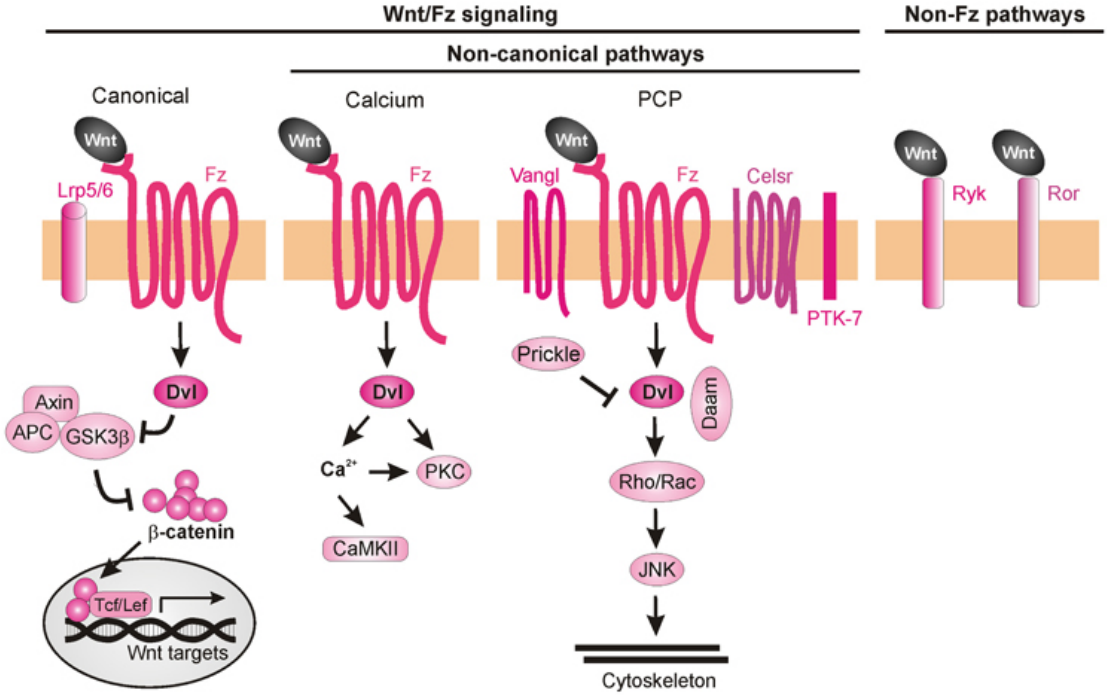


Figure 1: Wnt signaling pathways. Wnt ligands can activate at least three differed Frizzled-dependent (Fzd) pathways: one canonical pathway which is dependent on Beta-catenin and two non-canonical pathways, the calcium pathway and the PCP pathways. All Fzd-dependent pathways Dishevelled (Dvl). The canonical pathway requires the co-receptors Lrp5/6 to recruit Dvl and inhibit the "destruction complex" composed of Adenomatous polyposis coli (APC), Axin, and Glycogen synthase 3β (GSK3β). Wnt binding leads to the phosphorylation of GSK3β in this complex and, as a consequence, to the accumulation of unphosphorylated β-catenin, which can enter the nucleus and together with the transcription factors Tcf/Lef induce the expression of target genes. Activation of the calcium pathway results in an increase of cytosolic calcium (Ca2+) and the subsequent activation of calcium-dependent kinases (CaMKII). In the PCP pathway, Rho GTUPases and JNK are activated by the recruitment of the intracellular proteins Prickle and Daam by transmembrane proteins Vangl, Celsr, and PTK7. This PCP pathway promotes cytoskeleton remodeling. Fzd-independent Wnt signaling is also possible where Wnt ligands bind directly to receptors such as Ryk or Ror. However, the intracellular signaling cascades in these pathways is poorly understood [10].

The Canonical Wnt / β-catenin Pathway

Wnt signaling is required for the accumulation of β-catenin in the cytoplasm. β-catenin is a protein known for its engagement in cadherin-based adherens junctions, an important feature in the support of tissue architecture and morphogenesis [11]. In the Wnt pathway, β-catenin accumulates in the cytoplasm and is translocated into the nucleus [12]. Once in the nucleus, β-catenin acts as a transcriptional co-activator along with others such as cAMP response element-binding protein (CBP), as well as other components of the basal transcription machinery such as Bcl-9 and Pygopus (Pygo) [13]. Together these co-activators activate transcription factors that belong to the lymphoid enhancer-binding factor/T-cell factor (LEF/TCF) family to modulate expression of context-specific target genes [7]. For example, the

activation of these transcriptional factors by β-catenin transcribes multiple downstream target oncogenes, such as c-myc, cyclin-D1, and Axin2, thus enhancing cellular proliferation [14].

When the canonical Wnt pathway is inactive (no Wnt is present), the LEF/TCF transcription factors associate in a repressive complex with transducing-like enhancer protein, TLE or Groucho, co-repressor proteins [4]. These proteins promote the recruitment of histone deacetylases (HDACs) to repress β-catenin target genes [4]. Without a Wnt ligand present, β-catenin is continuously degraded by a destruction complex, preventing its accumulation in the cytoplasm. The destruction complex includes the following proteins: Axin, adenomatosis polyposis coli (APC), glycogen synthase kinase 3 (GSK3) and casein kinase 1α (CK1α). APC and Axin act as scaffolding proteins that bind β-Catenin. The CK1α kinase phosphorylates a set of highly conserved Ser and Thr residues of β-catenin on its N-terminal and GSK3 targets it for ubiquitination by the F-box protein, β-TrCP (β-transducin repeat containing protein), which leads to the subsequent destruction of β-Catenin by the proteasome [6,7].

The function of the destruction complex is disrupted upon Wnt activation by its binding to a receptor complex composed of Frizzled (Fzd) and Low Density Lipoprotein Receptor-related Proteins (LRP) 5 and 6 [6]. The destruction complex is translocated to the plasma membrane where its negative Wnt regulator, Axin, binds to the cytoplasmic tail of LRP-5/6. Axin becomes de-phosphorylated and its stability and levels are decreased. The Dishevelled (DVL) protein then becomes activated via phosphorylation and its DIX and PDZ domains inhibit the GSK3 activity of the destruction complex [4]. Upon LRP binding, the destruction complex can still capture and phosphorylate β -catenin but ubiquitination by β-TrCP is blocked [15]. This allows β-catenin to accumulate, translocate to the nucleus and subsequently associate with TCF/LEF family transcription factors to induce the expression of Wnt target genes.

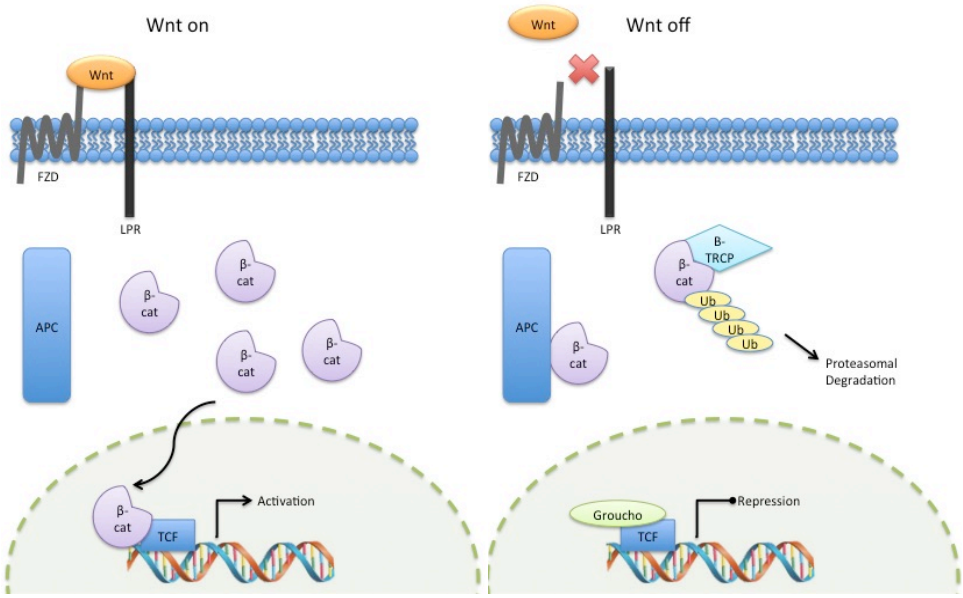


Figure 2: Diagram of the Canonical Wnt/ β-Catenin pathway. The image on the left shows the pathway in the presence of Wnt (Wnt is activated) and the image on the left shows the pathway in the absence of Wnt or with impaired Wnt binding to the receptor complex (Fzd and LPR).

Receptors of the Canonical Wnt pathway

In order for the Wnt pathway to be activated and Beta-catenin to begin accumulating, the Wnt protein needs to bind to its receptors on the surface of the cell. When interacting with target cells, Wnt proteins bind a heterodimeric receptor complex, consisting of a Frizzled (Fzd) and an LRP5/6 protein. A single Wnt can bind multiple Fzd receptors and vice-versa and it has been suggested that LRP6 protein contains separate binding sites for different classes of Wnt proteins [16]. This section will go into more

detail into the structure and function of the proteins which make up this complex.

Wnt Protein. For most mammalian genomes, including the human genome, the WNTs are a family of 19 secreted glycoproteins genes (Papkoff et al. 1987) which fall into 12 conserved Wnt subfamilies (Clevers & Nusse 2012). Interestingly, Wnt is highly conserved with 11 of its 12 subfamilies also occurring in the genome of Cnidaria (the sea anemone Nematostella vectensis) (Clevers & Nusse 2012). Furthermore, the importance of the Wnt proteins in proper cellular function have been emphasized by the variety of hereditary conditions identified due to mutations of six Wnt genes (Nusse & Lim 2015).

Wnt glycoproteins are produce in the Endoplasmic Reticulum and are then released outside of the of the cell where they can interact with Frizzled receptors (Fzd) on the cell membrane [17]. Crystal structure analysis has revealed that Fzd is gripped between two slender digit-like b-hairpin loops, the so-called thumb and finger of Wnt. The thumb has a lapidates Ser187 at its tip and protrudes from the N-terminal domain which bears evolutionarily distant hallmarks of a saposin-like fold. The finger is the C-terminal domain shares the topology of cysteine-knot cytokines such as platelet derived growth factor [18].

Frizzled and LRP5/6 Receptors. Members of the Frizzled family are seven transmembrane proteins which function as receptors for Wnt. 10 Frizzled receptors have been identified in mammals. The extracellular cysteine-rich domain (CRD) at the N-terminal of Frizzled is what binds Wnt [19]. The CRD possesses 10 conserved cysteines within a domain of 120±125 amino acids [19].

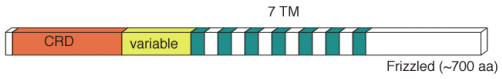


Figure 3: Schematic structure of Frizzled proteins (Fzd). The Fzd receptor is a seven transmembrane protein. Its N-terminal end of consists of the extracellular cysteine-rich domain (CRD), containing 10 conserved cysteines (Nusse & Lim, 2015).

Current knowledge on the structure of Frizzled receptors is limited to that of Fzd8 in complex with *Xenopus* Wnt8 (Figure 4).

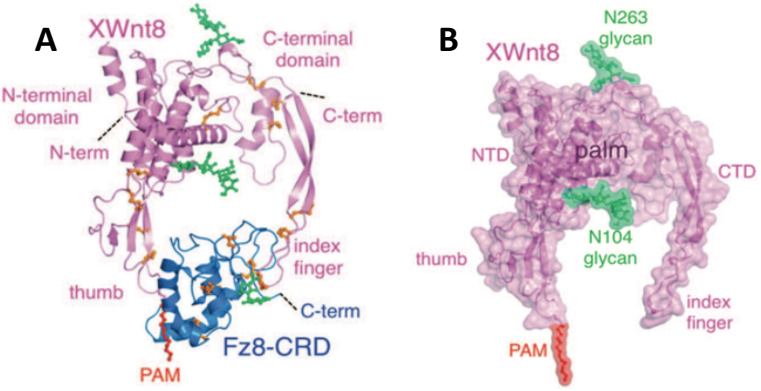


Figure 4: The structure of *Xenopus* Wnt8 (XWnt8) in complex with the Cysteine Rich domain of Frizzled 8 (Fz8-CRD). (A) Ribbon models of XWnt8 (pink) and Fz8- CRD (blue) as viewed "face on" (B) Surface representation of XWnt8 without Fz8-CRD in the complex structure. Figure adapted from [73]

LRP proteins function as co-receptors with Frizzled for Wnt proteins [19]. LRPs are long single-pass transmembrane proteins. There are two LRPs that can act in Wnt signaling in vertebrates, LRP5 and LRP6. LRP5 and LRP6 each contain an extracellular domain with EGF (epidermal growth factor) repeats and LDLR (low-density lipoprotein receptor) repeats, followed by a transmembrane region and a cytoplasmic domain lacking recognizable catalytic motif [20]. The cytoplasmic domain of LRP performs an important function of interacting with Axin through a phosphorylated domain [20]. For Axin binding to the tail of LRP to occur it is phosphorylated by two protein kinases: GSK3 and CK1gamma [21]. In addition to disrupting Axin function, the Fz-LRP receptor complex likely accomplishes the task

of Wnt signaling by activating Dishevelled (DVL) [22].

Wnt/β-Catenin Regulation.

The Wnt pathway is regulated by naturally secreted antagonists. Two antagonists, the Secreted-Frizzled-related proteins (sFRPs) and Wnt inhibitory protein (WIF), work to inhibit Wnt and Wnt receptor interaction by binding directly to Wnt. Other Wnt inhibitors work by binding LRP5/6, including proteins of the Dickkopf (DKK) and the WISE/SOST families. APCDD1 is a membrane-bound glycoprotein that inhibits Wnt signaling by binding both Wnt and LRP [15].
In addition to these naturally secreted antagonists, the protein R-Spondin acts as a Wnt agonist by binding both LGR protein and ZNRF3/RNF43 E3 ligases. Once bound to LGR and ZNRF3/RNF43 E3 ligases, the Frizzled receptors within the cell membrane are no longer removed and degraded by the E3 ligases. Therefore, through a yet unclear mechanism, R-Spondin prevents the E3 ligases from being able to remove Fzd from the surface of the cell. Therefore, the overall outcome of having more Fzd receptors is an increase in Wnt signaling.

The following section will explore the three proteins present in this complex in more detail, LGR, RNF43/ZNRF3, and R-Spondin.

Lgr5 (homologs Lgr4, 6). Lgr5 and its homologs Lgr4 and Lgr6 are important seven-transmembrane (7TM) receptors for the ligand R-Spondin (RSPO). Lgr5 has been demonstrated to be a marker of multiple Wnt-driven adult stem cell types, including those of the adult intestinal crypts. A lack of Lgr5 has also been linked to colon cancer.

LGRs belong to a subgroup of eight LGRs within the super- family of Rhodopsin G-Protein Coupled receptors (GPCRs) [23]. LGR stands for 'Leucine-rich repeat-containing G protein-coupled receptor' and, true to its name, each LGR consists of a large extracellular domain (ECD) composed of a string of leucine-rich repeat (LRR) units flanked by N-terminal and C-terminal cap modules. The eight LGRs can be further subdivided into class A, B, and C receptors [23]. Type A receptors contain seven to nine LRRs and have a long Hinge region which connects this LRR region to the 7TM domain. Type A receptors consist of Lgr1, the follicle-stimulating hormone receptor (FSHR); Lgr2, the luteinizing hormone receptor (LHR); and Lgr3, the thyroid-stimulating hormone receptor (TSHR). Type C receptors consist of Lgr7 and Lgr8 and closely resemble type A receptors besides having a shorter Hinge region. Type C receptors bind insulin-like peptide hormones and are characterized by the presence of a LDLa motif. Type B receptors include the remaining Lgr4, Lgr5, and Lgr6. Their ECD consists of 17 LRRs and their Hinge domain is of intermediate length.

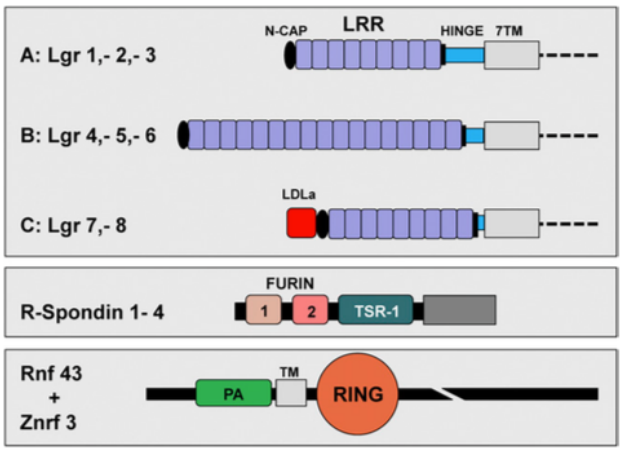


Figure 5: Basic structure of the LGR family, R-Spondin ligand, and the E3 ligases, Rnf43 and Znrf3. The LGR receptors are seven transmembrane proteins which consists of a leucine-rich repeat (LRR) flanked by N-terminal and C-terminal cap modules. Subclass A of LGR contain a long hinge region. Subclass B consists of 17 LRRs and an intermediate length Hinge domain. Subclass C is characterized by an LDLa motif. R-Spondin has four homologs each with two Furin domain and a TSR-1 domain. E3 ligases, Rnf43 and Znrf3, are single pass transmembrane proteins characterized by their RING domain and a PA domain necessary for binding with R-Spondin (de Lau et al., 2014).

Rnf43 & Znrf3. RING finger 43 (Rnf43) and Zing and RING finger 3 (Znrf3) are homologous RING-type E3 ligases which have been identified as the most potent negative feedback regulators in the Wnt pathway [24]. These single-pass transmembrane E3 ligases remove the 7TM Wnt receptor, Frizzled, from the cell surface through ubiquitin ligase activity, leading to their degradation. This was demonstrated through a study by Hao et al. in 2012 where all the Lysine residues in FZD4 were mutated to Alanine residues, preventing the ubiquitin molecules from attaching [25]. The mutated FZD4 was shown to no longer be ubiquitinated and a decrease in the concentration of ZNRF3 lead to the number of wild-type FZD4 to increase while the number of Ala-FZD4 did not change. This interaction between Fzd receptors and the E3 ligases was further supported when it was shown that co-expressed HA-tagged ZNRF3 and Myc- tagged FZD8 could be co-immunoprecipitated. In addition, HA-tagged ZNRF3 can be co-immunoprecipitated with LRP6 or FZD6 [25]. This shows that the E3 ligases are indeed, directly or indirectly, responsible for the ubiquitination of Fzd receptors and lead to the reduction of Frizzled receptors able to interact with Wnt proteins, thereby reducing Wnt signaling response [25]. This function is evolutionarily conserved with the *C.elegans* ortholog of ZNRF3 and RNF43 (PLR-1) being recently reported [26].

Interestingly, Rnf43 and Znrf3 are also encoded by Wnt target genes making them actors in a negative feedback loop. The structural basis for how Rnf43 and Znrf3 identify Frizzled receptors as their specific substrates are currently not exactly known [23]. What is known is that Rnf43 and Znrf3 are structurally related to Grail which belongs to the Goliath family of RING domain E3 ligases. They have an extracellular PA domain and a cytoplasmic RING domain.

R-Spondin. R-Spondins are secreted ligands which act as agonists of Wnt-initiated signaling, enhancing its signal. There are four R-Spondin homologs, R-Spondin 1-4, each of which is present in all vertebrates and some primitive chordates [27]. The R-spondins are members of a much larger family of proteins characterized by the presence of thrombospondin repeats (TSRs). Apart from this TSR domain, R-Spondins contain two N-terminal Furin repeats, both required for Wnt signal enhancement [23]. In fact, the Furin repeats are referred to as the 'business end' of the protein as the presence of both is necessary and sufficient to exert the Wnt-enhancing activity [23]. The Furin-1 domain interacts with RNF43/ZNRF3 E3 ligases and the Furin-2 domain recruits and binds the type-B LGRs, Lgr4-6.

The prefix R in the R-spondin members refers to the observed expression of murine R-spondin1 in the embryonic (R)oof plate neural epithelial tissue [28]. The role of R-Spondins as agonists of Wnt-initated signaling was first revealed through an expression screen in early frog embryos [23]. Depletion of R-spondin2 in single blastomeres at the eight-cell stage in early frog embryos or depletion at the gastrula stage resulted in a failure to transcriptionally activate the myoD and myf5 genes, leading to impaired muscle development. Similar phenotype effects occurred in chicks and mammals whose basal Wnt activity was manipulated in the developmental stage. This suggested a functional link between R-Spondin and Wnt which was confirmed through Wnt reporter assays in HEK293T cells [23].

Identification of R-spondin as ligands of the type-B receptors (Lgr4, Lgr5 and Lgr6). Several studies found evidence for specific binding of R-spondin to the B type LGR receptors (Lgr4, Lgr5, and Lgr6). One study revealed high-affinity interactions between Lgr4 or Lgr5 and R-spondins through tests with Fc-tagged versions of R-spondin [29]. Wim de Lau et al. obtained similar results through the use of a tagged R-spondin-1 as a bait molecule for surface receptors on HEK293T cells. Mass spectrometry identified the captured proteins as Lgr4 [30]. Knock-down studies of Lgr4 in the same cells resulted in a drop in R-spondin sensitivity which could only be rescued by the Lgr4, Lgr5 and Lgr6 but not by other homologs. In conclusion, all four R-spondins were found to bind with high affinity to all three Lgrs of the B-type [29,30].

Identification of Rnf43 and Znrf3 as substrates of R-spondin. The two E3 ligases were implicated with

R-spondin after a series of biochemical experiments indicated that the addition of R-spondin reversed the membrane clearance of Wnt receptors mediated by Rnf43 and Znrf3 [25]. This lead to the creation of a model which stated that both B-type Lgr and Rnf43/Znrf3 binding to the Furin domains of R-spondin would lead to the membrane clearance of Rnf43/Znrf3. Thus, R-spondin–Lgr complexes would neutralize Rnf43/Znrf3, allowing for the Frizzled receptors to remain on the cell surface where they can continue to increase Wnt signal strength [23]. Two studies confirmed this model by presenting the structural data derived from R-spondin-1/Znrf3 [31] and R-spondin-2/Rnf43–Znrf3 [32], leaving no doubt of the interaction of R-Spondin with Rnf43 and Znrf3.

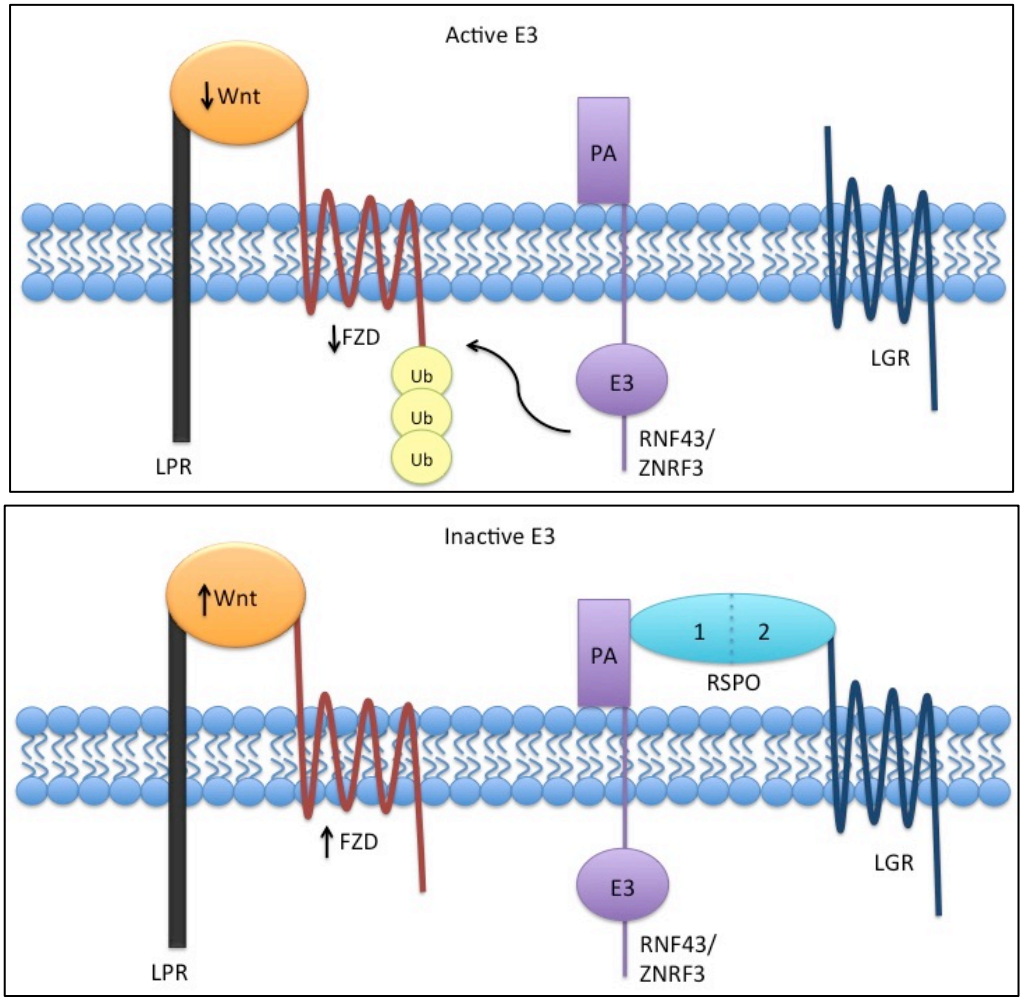


Figure 6: The R-Spondin, E3 ligase (Rnf43/Znrf3), LGR complex and it's influence on Frizzled receptor expression on the surface of cell membranes. E3 ligases are active in the absence of R-Spondin and lead to the ubiquitination and subsequent degradation of Fzd, thus decreasing Wnt signaling. E3 ligases are inactive when bound by their PA domain to R-Spondin, resulting in an increase in Fzd receptor expression and Wnt signaling.

Neuropathy Target Esterase

Neuropathy Target Esterase, otherwise known as SPG39, is the 6th member of a 9-protein family called patatin-like phospholipase domain-containing proteins, PNPLA1-9 [33]. Although the biological role of NTE remains unknown, recent evidence indicates that it is vital for normal development, because NTE knockout mice are not viable beyond embryonic day 11 [34]. There is a general distribution of NTE in different adult tissues including testes, kidney, liver, intestine, placenta and lymphocytes [35]. The highest specific activities of NTE are found in brain tissue, with substantially less in spinal cord and peripheral nerve [36]. The gene encoding NTE, PNPLA6, was mutated in the second most common Motor neuron disease (MND), Hereditary spastic paraplegia (HSP) characterized by distal wasting in all four limbs [33]. This highlights NTE's important function in maintaining viable neuron function.

NTE discovery and related disorders

Neuropathy Target Esterase was discovered in the late 1960s by M.K. Johnson when he investigated the mysterious occurrence of arm and leg weaknesses after drinking a "medicinal" alcohol substitute during the Prohibition Era in the US. The drink was found to contain an organophosphorous ester (OP) called trio-*o*-cresyl phosphate (TOCP) which was identified as the toxic agent responsible. It was found that this toxin lead to paralysis due to inhibition of the protein, Neuropathy Target Esterase (NTE). The condition induced by NTE inhibition through OPs was termed Organophosphate-induced delayed neuropathy (OPIDIN) and is characterized by the degeneration of nerve axons [37,38]. Thus, Organophosphorous compounds have been found detrimental to human heath as they cause irreversible deactivation of NTE through phosphorylation of its esterase active center, leading to damage in neurons of the central and peripheral nervous systems where it is found. Unfortunately, OPs have been also been widely used as pesticides for insect control and due to the similarity of neurochemical processes, these compounds are likely to be neurotoxic to the human brain as well as to insects. Indeed, there is evidence that pesticides reduce motor activity, latency, and caused visuospatial deficits after high exposure [38].

Unfortunately, OPs are not the only cause of NTE impairment and today it is known that a mutation in Neuropathy target esterase is associated with a spectrum of neurodevelopmental and neurodegenerative disorders such as spastic paraplegia 39, Gordon-Holmes syndrome, Boucher-Neuhauser syndrome, Oliver-McFarlane syndrome and Laurence-Moon syndrome [39].

NTE structure

While the tertiary structure of NTE is currently unknown, it has been determined that NTE is a multi-domain consisting of an N-terminal transmembrane domain (TMD), three cyclic nucleotide binding sites (CNP1-3) in the R-domain, and a catalytic domain (C-domain) that has 30% homology to plant protein patatin [40].

Elucidation of NTE's structure began in 1998 after the full-length NTE cDNA clone was isolated by Lush et al. [41]. The NTE cDNA clone D16 encoded a polypeptide of 1327 amino acids. This isolation was made possible through affinity purification of NTE after the discovery of a novel reagent, S9B [1-(saligen cyclic phosphoro)-9-biotinyldiamino-nonane]. S9B was able to react rapidly and specifically with NTE in brain microsomes, resulting in covalent attachment of a biotin molecule via a long alkyl spacer to the active serine residue of NTE [40].

Based on its primary sequence and its requirement for detergent for solubilization and predictions, NTE was demonstrated to be an integral membrane protein [40]. This was also demonstrated through its phase partitioning characteristics. Liposomes containing the C-terminal catalytic domain of NTE

termed NEST (NTE –esterase domain) were extracted with a nonionic detergent and extracts were then warmed to allow separation into aqueous and detergent-rich phases, the great majority of NEST was found in the detergent-rich phase, a characteristic of integral membrane proteins [42]. Due to it being an integral membrane protein, the crystal structure of NTE has been difficult to obtain and the tertiary structure of NTE is still unknown and various models have been proposed.

At first it was predicted that the NEST domain contained four transmembrane segments [36]. At the center of TM4, NTE was predicted to have an active site serine residue, S966, which labels NTE as a serine hydrolase.

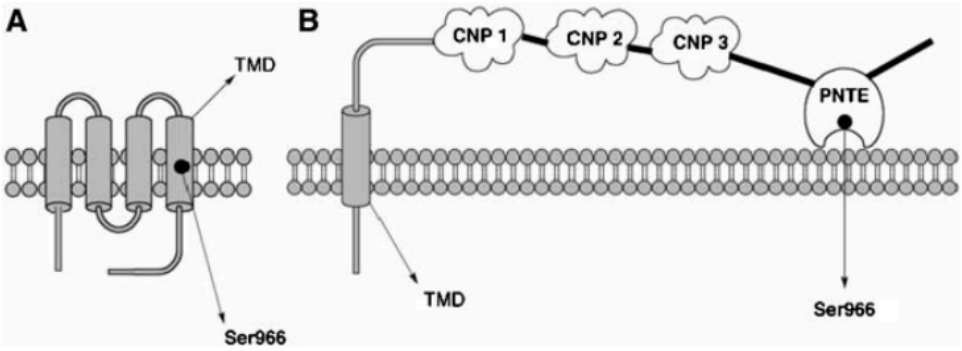


Figure 7: Models for NTE. (A) Model proposed previously by Atkins and Glynn (2000) showing four transmembrane domains with the active site Ser966 residue located within the TMD4 (B) Model proposed by Wijeyesakere et al. (2007) depicting a single transmembrane domain (TMD: residues 9-31), the PNTE domain (residues 933–1099) containing the NTE active-site dyad and three putative cyclic nucleotide-binding (CNP) binding domains (CNP domains; residues 163–262, 480–573 and 597–689) [43].

However, analysis of the catalytic domain of NTE from residues 933 to 1099 (termed the PNTE domain) by Wijeyesakere et al. revealed NTE to have a single transmembrane domain. This discovery was made after it was noted that NTE was homologous to the plant protein patatin whose crystal structure has been solved. This information was used to develop a homology model for PNTE which suggested the presence of a catalytic center consisting of a Ser966-Asp1086 dyad (see figure x) [40]. A Ser-Asp dyad is common to all members of the PNPLA family [44]. NTE contains a single N-terminal TMD (residues 9–31) that is anchored in the ER, and the catalytic site of NTE is not contained within a membrane-spanning domain [43]. Instead the catalytic site is likely located in the cytosol. The hydrophobic face of PNTE, which contains the NTE active site, is associated with the membrane. Currently unknown are the details of the relative spatial arrangement of the TMD, putative cyclic nucleotide binding domains, and the catalytic domains [43]. What is known is that its R- and C-domains interact with the cytoplasmic face of the ER. The NTE protein is probably anchored in the ER membrane via its amino-terminal TM with residues 33-1327 exposed to the cytoplasm [45]. Various studies have demonstrated evidence for this including that NTE has been found to be enriched in microsomal membrane fractions [40]. Moreover, NTE colocalized with calnexin, an ER marker [46] and NTE immunostaining filled neuronal cell bodies (except the nucleus) and sometimes extended into the proximal axon [40]. This suggests that NTE is associated with (at least) the endoplasmic reticulum and possibly with other sub-cellular organelles [40].

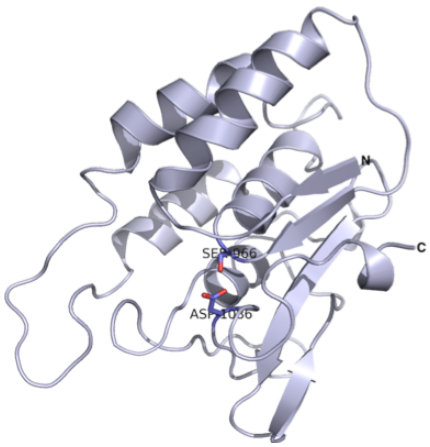


Figure 8: Homology model PNTE depicting the Ser966-Asp1086 dyad catalytic center (Richardson et al. 2013).

Characteristics of NTE: relation to function

The physiological role of NTE has not been identified conclusively. In 2008, it was discovered that NTE belongs to the patatin-like phospholipase domain containing protein (PNPLA) family [33]. Patatins are a group of plant storage glycoproteins that show lipid acyl hydrolase activity, using the enzymatic activity of phospholipase to catalyze the cleavage of fatty acids from membrane lipids. NTE may be a member of the enzyme class, Phospholipase B, a family of hydrolases which specifically act on carboxylic ester bonds. This is because it has been shown that it can hydrolyze phosphatidylcholine (PtdCho), a major component of cell membranes, into one glycerolphosphocholine molecule and two free fatty acids, which are likely important for membrane fluidity, especially in neurons (Bohdanowicz & Grinstein, 2013; Wijeyesakere & Richardson, 2010). Moreover, the typical B-esterase activity of NTE is demonstrated by its inhibition by OP compounds [40]. More specifically, NTE has serine esterase activity and efficiently catalyzes the hydrolysis of phenyl valerate (PV) in vitro, but its physiological substrate is unknown [37]. Due to its activity on carboxylic ester bonds, NTE's physiological substrate is likely to be a metabolite of a carboxylic acid, including free acids, esters of monohydroxy alcohols, esters of glycerol and amides [36]. Therefore, the phospholipase B activity of NTE suggests that NTE could be partially responsible for membrane lipid homeostasis and fluidity [48].

The C-terminal effector domain of NTE, containing its esterase activity, is highly conserved in bacteria, yeast, nematodes, and insects [37]. NTE has a putative homologue in *Saccharomyces cerevisiae,* suggesting it may be involved in a fundamental process common to cells from yeast to neurons [45]. Human NTE is 41% identical to a Drosophila neuronal protein called Swiss cheese (SWS) and a region of 200 amino acids near the C-terminal of human NTE sharing 29% with its homolog in *E.coli*. There is also high similarity between an N-terminal domain of SWS (also present in NTE), and the cycle A-binding regulatory subunit of protein kinase A [40]. As Protein Kinase A plays an important role in the regulation of lipid metabolism, this similarity hints to NTE's potential function in the same.

Placental phenotypes of NTE and Wnt component knockout mice

Many studies have knocked out genes of the Wnt pathway and multiple have resulted in embryonic lethality in mice. Knockout of the PNPLA6 gene also leads to embryonic lethality. Interestingly, both knockout mice models of the gene PNPLA6 and various genes present in the Wnt signaling pathway, share the same phenotypes during embryogenesis. Both result in mice with impaired placentas due to impaired labyrinth development and a lack of vasculature. It is interesting to compare the phenotypes of these mutants to deduce whether NTE and the Wnt signaling pathway could have a similar role in embryogenesis. Even more interesting is the potential NTE has for taking part in the Wnt pathway or vice versa. The hypothesis that they may have a related function is especially promising as the Clevers group in Hubrecht Institute under direction of Wim de Lau performed a mass spec in which Isoform 1 or 3 of Neuropathy Target Esterase was found bound to LGR4 and Fzd5 with a probability above 95% (unpublished data). NTE was identified with 39 distinct peptides with LGR4 and with 3 distinct peptides with Fzd5. To discover whether NTE and Wnt indeed have a function in common, I will first give an overview of normal placental growth and then cover the main embryonic phenotypes of NTE and Wnt knock out mutants respectively, followed by a comparison of the two.

Placental Development

The placenta is essential for proper development of an embryo as it serves the fundamental purpose of exchanging oxygen, nutrients and waste between mother and embryo. This is made possible through

the placenta establishing a utero-placental circulation system in which the circulation systems of the mother and of the embryo are brought closer together, thus allowing the exchange of gases and metabolites via diffusion. In humans and mice, the fully developed placenta is composed of three main layers: the inner layer composed of highly branched villi designed for efficient exchange, the middle region which attaches the fetal placenta to the uterus and contains trophoblast cells that invade the uterine wall and maternal vessels, and the outermost layer, the maternal layer that includes the decidual cells of the uterus as well as the maternal vasculature.

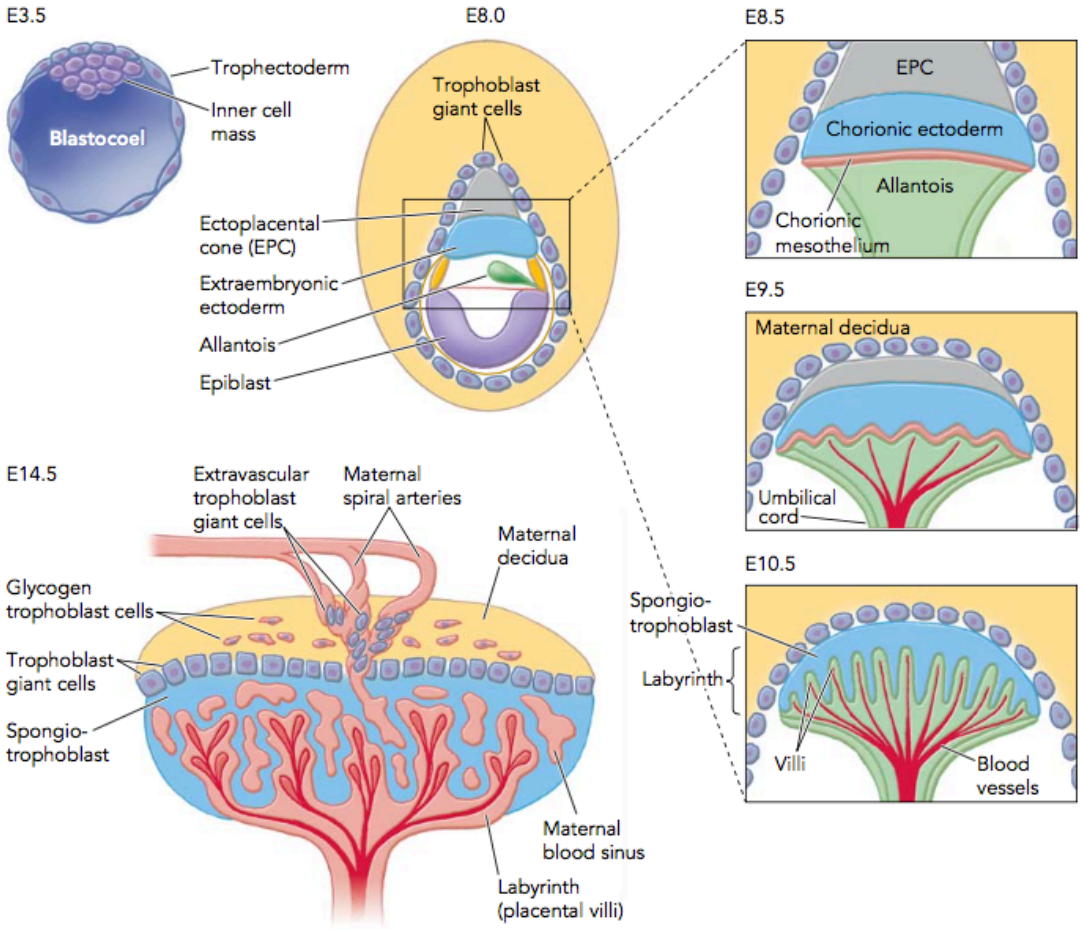


Figure 9: Placental development of the mouse. E3.5 marks the formation of the blastocyst with an outer trophectodermal epithelium and an inner cell mass. At E8.0 chorioallantoic fusion takes place, followed by branching morphogenesis from the chorionic mesothelium into the chorionic ectoderm, forming villi. Blood vessels than grow from the allantois into these villi, forming the labyrinth necessary for nutrient exchange. The mature placenta (E14.5) consists of three layers: The labyrinth, the spongiotrophoblast and the maternal decidua. [49]

For mammalian embryos, the trophoblast layer of the placenta arises from the differentiation of the outer trophectoderm of the blastocyst. During embryonic day 3.5 (E3.5) of mice, the outer trophectodermal epithelium and the inner cell mass (ICM) are developed. The outer trophectodermal epithelium will differentiate to become the fetal part of the placenta while the ICM develops into the embryo proper. On E4.5 implantation of the blastocyst occurs in the uterine wall. During implantation, the outer trophectoderm differentiates into the different trophoblast cell types that occur. One part of the outer trophectoderm, the polar trophectoderm, is found nearest to the ICM and differentiates to form the extraembryonic endoderm and the ectoplacental cone. The second type of trophoblast cells arise from the trophectoderm cells furthest away from the ICM, termed mural trophoblasts. The mural trophoblasts stop dividing but continue to endoreduplicate their DNA to form trophoblast giant cells, a process which occurs in rodents but not in human development.

At E6.0 the cells of the ICM have developed into the epiblast which begins to differentiate at E6.5 through the process of gastrulation. Gastrulation involves the formation of three primary germ layers: the ectoderm, mesoderm and endoderm. Each is fully formed on E8.0 when the outer trophectoderm layer of the blastocyst differentiates into the trophoblast layer of the placenta. At E8 the mesoderm cells from the embryo migrate to the inner surface of the visceral endoderm to from vascular cells in the yolk sac. The mesoderm cells also migrate to form the allantois. At E8.5 chorioallantoic fusion takes place. This process involves the allantois arising from the mesoderm at the posterior end of the embryo and making contact with the ectoplacental cone of the chorion. Hours later, folds appear in the chorion that mark the sites where the chorionic trophoblast cells will undergo extensive villous branching to create a densely packed structure called the labyrinth. Morphogenesis begins with selection of trophoblast cells in the basal chorions that express *Gcm1* (Glial Cells Missing Homolog 1). These cells have left the cell cycle but later begin to thin and elongate, creating a primary villous into which under-lying fetal blood vessels grow (Cross, 2006). As these chorionic trophoblast clusters elongate, Gcm1 remains expressed on the distal tip of their branches.

As the trophoblast cells which make up these branches elongate, they differentiate into two cell types. The trophoblast cells form a mononuclear trophoblast cell type which lines the maternal blood sinuses or fuse to form multinucleated syncytiotrophoblast cells which surround the fetal endothelium of the capillaries. Syncytiotrophoblast cells are multinucleated cells that form as a result of cell-cell fusion of trophoblasts and function as the major transport surface for nutrient and gas exchange between the maternal and fetal circulation in the villous part of the placenta [50]. Lastly, the fetal component of the placental vascular network is derived from the extraembryonic mesoderm (allantois) which will invade the trophoblastic cells, forming the blood vessels of the labyrinth.

On day 12.5 the chorioallantoic placenta is fully developed and has three distinct layers: the maternal decidua, the junctional zone with trophoblast giant cells nearest the maternal side, and the labyrinth zone. The junctional zone in mice is made up of two types of cells, the spongiotrophoblasts and the trophoblastic glycogen cells. While the labyrinth is developing it is supported structurally by the spongiotrophoblast, which forms a compact layer of cells between the labyrinth and the outer giant cells. Evidence from histological studies and the continuity of marker gene expression suggest that the spongiotrophoblast is derived from the cells of the ectoplacental cone [50].

NTE & Wnt placental phenotypes

Many knockout genes are known to lead to defective placental development. The three main stages in placental development which are impaired in mutants are chorioallantoic fusion, branching morphogenesis of the labyrinth, and vascularization of the labyrinth. Mutation studies have revealed that these three stages of placental development depend on a number of intercellular signaling pathways. These include Fgf, Egf, Notch, Lif, Pdgfb, and Wnt [49]. Both NTE and the Wnt components, Wnt2, Wnt7b, Fzd5, and R-Spondin3 have demonstrated placental phenotypes in knockout mice models. At first glance, the phenotypes of NTE appear similar to those of the Wnt components. However, a closer look is needed to determine whether their phenotypes are indeed similar, suggesting a potential interaction or common pathway.

NTE: related placental phenotypes

Two studies, one by Winrow et al. (2003) and one by Moser et al. (2004), discovered that disrupting the gene expressing NTE leads to embryonic lethality around embryonic day 10. Of the 231 live-born progeny, none were homozygous for mutant NTE. The study by Moser discovered that embryonic lethality from homozygosity for the NTE mutation was likely due to impaired placental development.

Analysis of the embryos during early growth stages revealed that NTE-null mice had severe growth retardation with NTE-null mice being half the size and having developed only 2 to 6 somites compared to 8 to 12 in the wild-type or heterozygous littermates at E8.5. In addition, the cranial neural plate had not yet fused and embryos had not completed their process of turning.

Although the process of gastrulation had proceeded normally and all three germ layers were formed by E7.5, the condensed packed cells of the ectoplacental cone were almost absent. Indeed, the ectoplacental cone was smaller than normal and the extraembryonic ectodermal sheet of the chorion itself seemed to be thinner. Overall, the chorionic plate an the ectoplacental cone were less densely packed in NTE mutants. This indicates that there was a reduction in trophoblast differentiation from the polar trophectoderm, the area on the blastocyst from which the extraembryonic endoderm and the ectoplacental cone arise. This is supported by the finding that NTE is expressed in the chorion trophoblast and the ectoplacental cone of the placenta. Thus, these phenotypes indicate that NTE is necessary for trophoblast differentiation into the chorion trophoblast and the ectoplacental cone. In contrast, the mural trophoblasts seem to proliferate normally, since at 7.5 the morphology, number and distribution of trophoblastic giant cells in NTE-deficient embryos was the same as in the control. However, at E8.5 also the number of trophoblast giant cells was decreased. This was demonstrated with the marker Mash-2, used to label the ectoplacental cone, the chorion, and their derivatives, the syncytiotrophoblast cells of the labyrinth. Mash2 was detected in the chorionic plate at E8, the reduced ectoplacental cone at E8.5, but, interestingly, almost no Mash-2 signal was detected at E9.5. This indicates a differentiation failure into trophoblast giant cells and the syncytiotrophoblast cells of the labyrinth. Interestingly, the ectoplacental cone cells of E9.5 had succeeded in differentiating spongiotrophoblasts. This was indicated by the marker 4311 which is specific to a group of cells of the ectoplacental cone. 4311 marks the cells that will form the densely packed spongiotrophoblast later, but not the trophoblast giant cells of the labyrinth layer of the placenta [51]. Therefore, NTE-deficient placentas likely have problems with efficient nutrient exchange as trophoblast differentiation into syncytiotrophoblast cells of the labyrinth has failed.

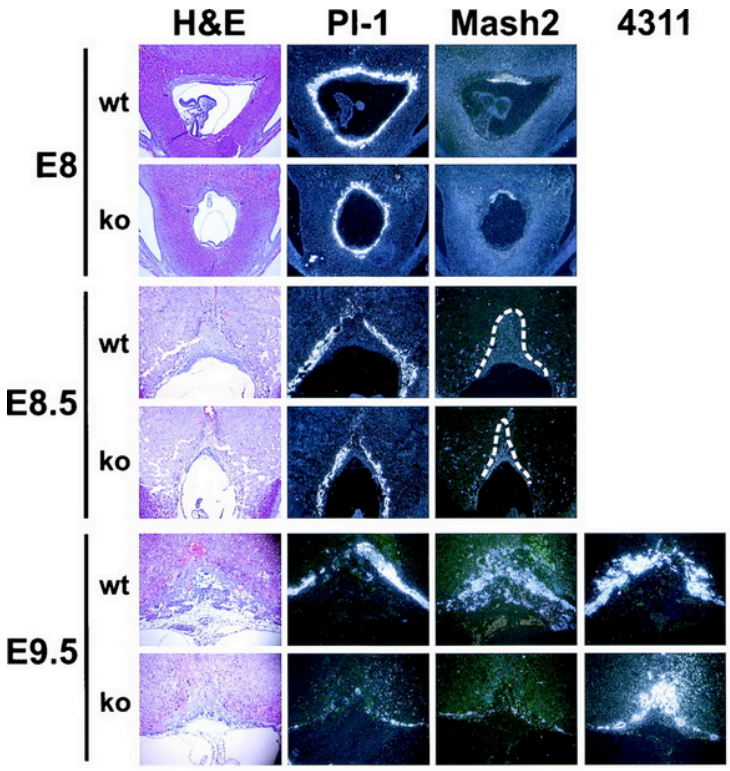


Figure 10: Trophoblast marker analysis of NTE knockout mutants at E8.0, E8.5 and E9.5. Serial sections of wild-type (wt) and NTE knockout mutants (ko) conceptuses are shown probed with antisense riboprobes for Pl-1, Mash2 and 4311. Pl-1 is expressed in trophoblast giant cells; Mash2 in the chorionic plate at E8.0, the ectoplacental cone at E8.5, and the labyrinth and spongiotrophoblast cells at E9.5; 4311 is expressed in the spongiotrophoblast layer of E9.5 placentas. Note that at E9.5 almost no giant cells are labeled and no labyrinth has formed in NTE mutants, whereas spongiotrophoblast cells are present (4311). Strikingly NTE mutants are negative for Mash2 while wild-type placentas are noe. All pictures were taken with the same magnification. Dotted lines indicate the size and shape of the ectoplacental cone. H&E, hematoxylin and eosin [52].

In NTE-null mutants, the labyrinth had failed to form. Importantly, chorioallantoic fusion took place as normal though slightly delayed, occurring at E8.5 in NTE-null mice instead of at E8.0 as in control mice. The chorionic trophoblast differentiation and morphogenesis had begun as normal through interdigitation of the allantoic mesoderm. Moreover, underlying fetal vessels with chorionic plate trophoblast cells begun to form branches and at E9.5 syncytiotrophoblast precursor cells formed around the developing fetal vessels and invaded toward the maternal blood sinuses. However, these trophoblast cell branches failed to elongate and bifurcate and collapsed completely at E10.

Yolk sac circulation is initially well established but is blocked when blood vessels are formed. The yolk sac is made up of mesodermal and visceral endodermal cell layers. The mesodermal cells proliferate and differentiate into blood islands which then fuse by vasculogenesis to form a primitive vascular network. The NTE mutants had normal yolk development in the early phases (E8.0 and E8.5) with normal blood island formation and newly formed blood vessels lined by endothelial cells. However, at E9.5, the mesodermal and endodermal cell layers had collapsed and no vascular network had been established [52]. A cessation of blood flow occurs within the yolk sac plexus which results in growth retardation, a pale coloring to the yolk sac, and swelling of the pericardium.
Vasculogenesis occurs within the embryo at E9.5 with a functional vitelloembryonic circulation being formed. Although the vessels formed in the embryo were lined with endothelial cells and filled with nucleated blood cells, many vessels were dilated and severe hemorrhages were discovered in the head. Due to these alterations in vessel formation of both the yolk sac and in the embryo proper, it is likely that NTE plays a role in vasculogenesis.

Table 1 | Placental phenotypes of mice with homozygous deletion of Neuropathy Target Esterase

Mouse Embryonic Day	Normal Phenotypes	Abnormal Phenotypes
E7.5	• Gastrulation (all three germ layers formed, normal extraembryonic membranes). • Number and distribution of trophoblastic giant cells.	• Condensed packed cells of the ectoplacental cone almost absent. • Ectoplacental cone smaller than normal • Extraembryonic ectodermal sheet of the chorion itself seemed to be thinner
E8.0		• Embryonic cavity of the decidual swelling smaller • Chorioallantoic fusion slightly delayed • Proliferation of placental cells restricted to the chorionic plate
E8.5	• Chorioallantoic fusion occurs	• Growth retardation. • Decrease in number of trophoblast giant cells
E9	• Chorioallantoic branching is initiated with the formation of simple villi	
E9.5	• Syncytiotrophoblast precursor cells form around the developing fetal vessels and invade toward the maternal blood sinuses • Functional chorioallantoic fusion with vessels containing embryonic blood cells has been formed	• Syncytiotrophoblast precursor cells failed to elongate and bifurcate and collapsed completely at E10 • Proliferation of placental cells restricted to the chorionic trophoblast stem cells • Strong developmental delay • Cranial neural plate not yet fused • Pale color of embryo proper • Enlarged pericardium • Incomplete process of turning

All in all, the study by Moser et al. (2004) revealed that NTE is required both for placenta formation and for vasculogenesis within the extraembryonic yolk sac and the embryo itself. Moreover, NTE is essential for the differentiation of cells of the extraembryonic ectoderm of the chorion, the ectoplacental cone, and trophoblast giant cells. Without NTE the labyrinth does not form, the ectoplacental cone is reduced and there is a giant cell defect [52].

Wnt components: related placental phenotypes

The Wnt pathway is essential for many cellular processes, also during development. The Wnt pathway is known to play an important role throughout embryogenesis in regulating cell proliferation, survival, differentiation, polarization, and migration, as well as being critical for blastocyst activation, adhesion and implantation [50,53]. Knock out studies have revealed that certain aspects of the Wnt pathway are essential for proper embryo development, including development of the placenta. Firstly, knock-outs of all nineteen Wnt ligands have been done and it has been found that Wnt 2 and Wnt7b mouse mutants have impaired placental phenotypes, die at midgestation stages and have reduced embryonic or adult vasculature [54]. In placental development specifically, Wnt7b and the transcription factors of the canonical Wnt signaling pathway, Tcf/Lef1, are required for normal chorioallantoic fusion [55,56]. Wnt2 null mice display impaired placental angiogenesis, failed labyrinth development, reduced birth weight, and a higher rate of mortality [57]. Moreover, a role for β -catenin, the key mediator of WNT signaling, has also been implicated in trophoblast adhesion, survival, and differentiation [58]. Embryos bearing homozygous deletion of β-catenin develop to the blastocyst stage but are affected upon gastrulation. The three germ layers and the allantois fail to form [59] and β -catenin is required for embryonic ectoderm development past E7.5 [60]. Therefore, the canonical Wnt signaling pathway clearly plays a role in proper development of the placenta. Table 2 summarizes the homozygous knockouts of Wnt components which lead to placental phenotypes.

Table 2 | Placental phenotypes of mice with homozygous deletion of Wnt signaling components

Gene knock-out	Expression in placenta	Placental Phenotype	Reference
Wnt 2	Allantoic mesoderm, fetal blood vessels in the labyrinth, chorionic plate	• Chorioallantoic fusion not impaired • Failed labyrinth development • Oedema • Reduction in the size and proportion of the labyrinthine zone and the chorioallantoic plate region • Reduced fetal vasculature • No difference in the number or distribution of the trophoblast giant cells or the spongiotrophoblast cells. • Fibrinoid deposition	50 61 57
Wnt 7b	Chorionic trophoblast	• Impaired chorioallantoic fusion • Reduced vasculature • Morphologically abnormal and disorganized diploid trophoblast cells in the chorionic plate	55 56
Tcf/Lef1	Placenta trophoblast (unpublished data)	• Impaired chorioallantoic fusion	55
Fzd5	Labyrinth trophoblast, yolk sac	• Chorioallantoic fusion not impaired • Severely retarded fetal growth • Excessive proliferation of trophoblast cells lining the branchpoint sites in the chorionic plate • Pale yolk sacs devoid of blood vessels • Failed labyrinth development • Reduced penetration of vessels into the trophoblast-specific chorion • Reduced Gcm1 expression • Initiation and progression of branching morphogenesis from the chorion failed • Embryonic blood vessels were completely separated from maternal blood flow. • Similar numbers of trophoblastic cells • No vascular defects in embryo proper	58 61 62

R-Spondin 3	allantoic component of the labyrinth; in early embryogenesis in brain, neural tube, tail, heart, somites and limbs	• Chorioallantoic fusion not impaired • Failed labyrinth development • Reduced vasculature in yolk sac, embryo proper and placenta • Impaired penetration of vessels into chorion • Primary capillary plexus was formed and vessels appeared normal at E9.5 • Vessels from chorion failed to undergo proliferation • Impaired penetration of vessels into labyrinth layer • Reduced Gcm1 expression • Decrease in proliferating endothelial cells	[63]

Fzd5-null mice. *Fzd5* knockout mice (-/-) displayed severely retarded fetal growth, defects in vascularization, and defective labyrinth layer formation [62]. In a study by Ishikawa et al. the Fzd5 mutant yolk sac had poorly developed large vitelline vessels and the capillary plexus was disorganized compared with those of the wild-type. Moreover, homozygous yolk sacs contained fewer blood cells inside the vasculature. This observation was supported by a more recent study where the yolk sacs were found to be pale and devoid of blood vessels at E10.5. This indicates that Fzd5 may play an essential role in yolk sac angiogenesis [58].

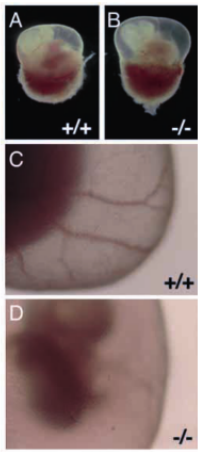
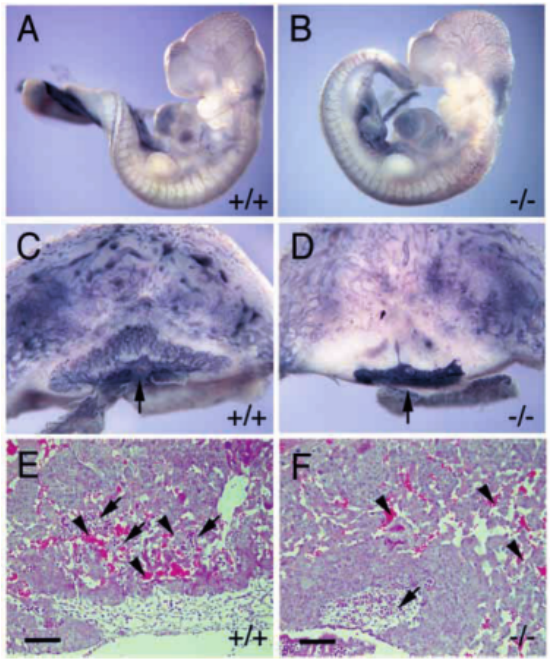


Figure 11: Morphology of wild-type (+/+) and Fzd5 homozygous mutant (-/-) embryos at E10.75. (A, B) Embryos isolated together with their yolk sacs, (C,D) Yolk sacs of embryos shown in A, B at a higher magnification. Note that the vessels visible in the wild-type (+/+) are missing in the homozygous mutant (-/-) yolk sac (Ishikawa et al. 2001)

Moreover, the endothelial cells that lined the inside of the yolk sac were poorly developed and peeled off the expanding yolk sac. It was hypothesized that Fzd5-dependent signals such as Wnt could support endothelial cell growth but were not essential for their initial differentiation. Consistent with this interpretation, the BrdU labeling experiments by Ishikawa et al. (2001) showed a marked decrease in proliferating endothelial cells in the mutants by E10.25, 12 hours before the morphological defects became apparent.



Fzd5 knockout mice display normal attachment of the chorion and allantois at E8.5. However, at E9.5 initiation and progression of branching morphogenesis in the chorion failed to occur

Figure 12: Angiogenesis in embryo proper and placenta at E10.75. (A, B) The Fzd5-homozygous and wild-type embryos proper stained with anti-Flk antibody. Note the normal vascular pattern of the homozygote (B), indistinguishable from the wild-type control embryo. (C, D) The placental vasculature visualized by anti-Flk immunostaining. Note that the embryo-derived blood vessel penetrate deeper in the wild-type placenta (C) than in the homozygote (D). (E, F) Sections of the placenta shown in C, D, respectively and stained with Hemotoxylin and Eosin. Note the extensive intermingling of the maternal (arrowheads) and embryonic blood vessels (arrows) in the wild-type placenta (E). Note the lack of blood vessel penetration into the labyrinth layer in the homozygous mutant placenta (F). Scale bars: 100 μm in E, F (Ishikawa et al. 2001).

leaving the chorion flat and devoid of primary villous branches [58]. This may be due to reduction in *Gcm1* expression which takes place in the chorionic plate at E8.5-10.5 in Fzd -/- mutants. Without *Gcm1* expression, branching initiation does not take place. In general, trophoblast proliferation did occur although the trophoblastic cells within the chorion continued proliferation while the control proliferation had ceased. This highlights the importance of Fzd5 in maintaining normal trophoblast proliferation rates. Moreover, fetal blood vessels failed to penetrate into the Trophoblast-specific chorion.

Few studies have addressed which Wnt–Fzd pairs are expressed in the extra-embryonic tissues around the time of early labyrinth development. In regards to Fzd5, it has been demonstrated to interact with Wnt2, Wnt5a and Wnt10b by Ishikawa et al. (2001). In the same study, they found Fzd5 to be expressed in the labyrinth trophoblast and in the yolk sac [62]. Two of the Wnt ligands, Wnt5a and Wnt10b, were also expressed in the yolk sac. Therefore, it is possible that Wnt5a or Wnt10b are physiological ligands of Fzd5. However, mutating either Wnt5a or Wnt10b does not lead to abnormal yolk sac phenotypes. In comparison, Wnt2 is expressed in allantoic mesoderm and its homozygous mutation leads to placental defects similar to that of Fzd5. These include failed labyrinth development and decreased numbers of fetal capillaries suggesting that Wnt2 may play a crucial role as a ligand for Fzd5 during later placental development. However, despite this similarity the phenotype of the Fzd5 homozygous mutant embryo is still distinct from that of the Wnt2 knockout (Monkley et al., 1996). The lethal stage of the Fzd5 homozygous mutant embryo is earlier than that in the Wnt2-deficient embryos. Therefore, although Wnt2 may be a physiological ligand of Wnt2 it is unlikely to be the sole ligand. However, all that is known is that after chorioallantoic fusion, Wnt2 and Fzd5 are both essential for labyrinth development.

RSPO3-null mice. In a study by Aoki et al. (2007), a mutation resulting in RSPO3-null mice was shown to lead to embryonic lethality with development arresting at E10.5. Interestingly, the phenotypes which resulted are nearly identical to those in Fzd5 knockout mice. This is logical due to the role of R-Spondin in reducing Fzd5 clearance from the cell surface, and therefore also being essential for normal canonical Wnt signaling. The knockout RSPO3 mice had severe defects in the development of the labyrinth, and a failure of branchpoint initiation in the chorionic plate [63]. Although, chorioallantoic fusion occurred normally, there was no chorioallantoic branching and no penetration of the fetal blood vessels into the chorion [63]. Moreover, *Gmc1* expression in the chorionic trophoblast cells was significantly reduced at E9.5 and E10.5 [63].

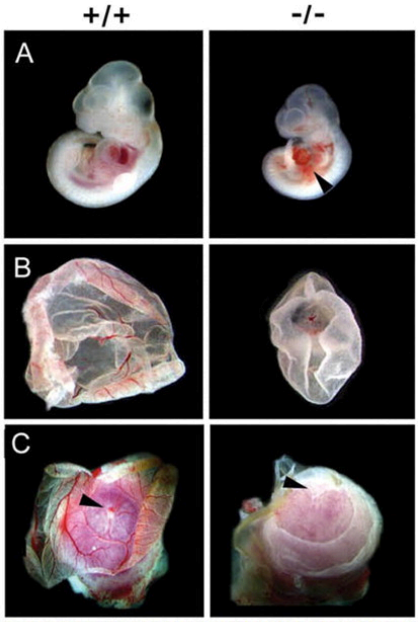
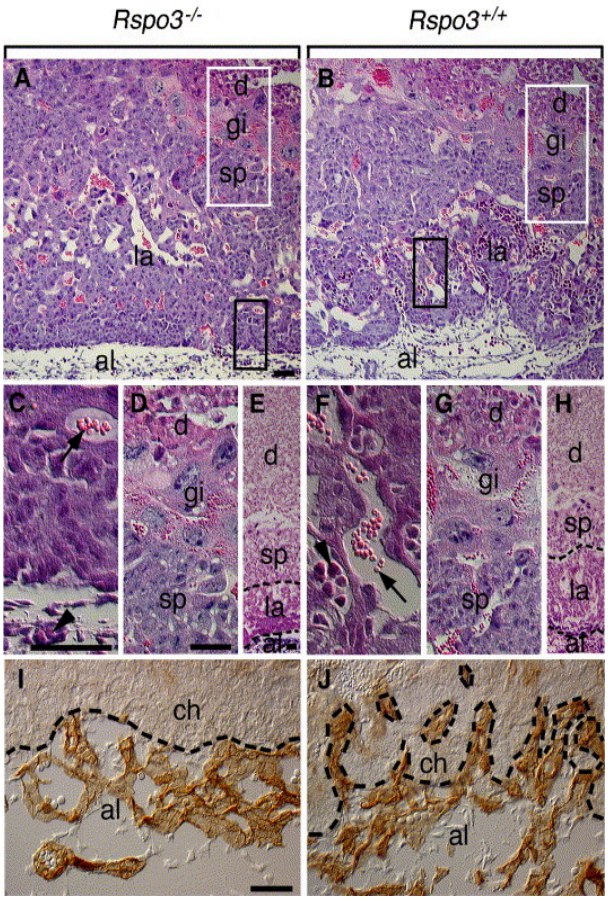


Figure 13: Rspo3 is required for mouse vascular development. Representative samples from 20 wild-type and 20 knockout embryos are shown. (A-C) External morphology of wild-type and Rspo3-/- embryos (A), yolk sacs (B) and placentas (C) (E10.5). Enlarged pericardial sac with hemorrhage is marked by arrowhead in A. Note pale Rspo3-/- yolk sac in B and pale Rspo3-/- chorionic plate in C (arrowheads) [54].

Another study by Kazanskaya et al. (2008) showed similar results in Rspo3 mutant mice which had arrested development around E10 because of placental defects. The nature of these defects seem to be defective angiogenesis in yolk sac and placenta. The mouse mutants displayed ventral edema and vascular defects with lower vessel development in the yolk sac and placenta. This suggests that Rspo3 plays an essential role in vertebrate vasculogenesis and angiogenesis [54]. This argument is supported by the observation that Rspo3 is prominently expressed in sites of vasculogenesis and angiogenesis. Already in E8.0 embryos, Rspo3 is expressed in the posterior primitive streak and the



allantois, which are prominent sources of hematopoietic cells. Rspo3 expression persists in the allantois at later stages and also becomes expressed in the chorionic plate of the placenta at E9.0 and in heart and umbilical cord starting from E9.5. Rspo3 is also expressed in the blood vessels of the embryo proper.

Figure 14: Effect of Rspo3 mutation on placenta. (A-H) Hematoxylin/eosin-stained sections of the homozygous mutant (-/-) (A, C, D, E) and wild-type (+/+) (B, F, G, H) placentas at E9.5 (A-D, F, G) and E10.5 (E, H). The regions outlined in panel A by the black and white boxed areas are shown at higher magnification in panels C and D, respectively. Likewise, the black and white boxed areas in panel B are shown in panels F ang G. Arrows indicate maternal blood cells and arrowheads indicate fetal blood cells. The dotted lines indicate the borders of the labyrinth layer with the allantois and the spongiotrophoblasts. (I, J) PECAM-1 staining for endothelial cells in the homozygous mutant (I) and wild-type (J) placentas at E9.5. Note the flat chorionic plate indicated by the dotted line in panel I, compared to the penetration of fetal blood vessels in the chorion in panel J. allantois (al); chorion (ch); decidua (d); trophoblast giant cells (gi); labyrinth layer (la); spongiotrophoblast layer (sp). Scale bars: 50 μm [63]

BrdU labeling experiments showed a marked decrease in proliferating endothelial cells in the mutants by E9.5. This is also consistent with the phenotypes identified in Fzd5 knockout mice where BrdU labeling experiments showed a marked decrease in proliferating endothelial cells in the mutants by E10.25 [62]. The only difference between the two is that the reduction in proliferation occurs slightly later in Fzd5 mutants and the Fzd5 mutants were shown to have excessive trophoblast cell proliferation while this is not present in RSPO3. That bot Fzd5 and RSPO3 lead to reduced endothelial cell proliferation could mean they are required to promote endothelial cell growth and remodeling. This argument is supported by the fact that the *Vegf* (Vascular endothelial growth factor) gene had reduced expression when R-Spondin was removed. Thus, R-Spondin can act directly on endothelial cells to promote angiogenesis by promoting canonical Wnt signaling to activate *Vegf* expression. Therefore, a compromised VEGF expression, which is under the regulation by RSPO3-mediated WNT/β-catenin signaling, is likely the major cause of the angiogenesis defect [54].

Fzd5 and RSPO3 knockout mice also have the same phenotype in regards to branching morphogenesis of trophoblast cells from the chorionic plate and the penetration of blood vessels into these villi. Like in Fzd5, Gcm1 expression is reduced and villi formation never initiates. Furthermore, although the primary capillary plexus was formed in the placenta and vessels appeared as normal at E9.5, these vessels failed to undergo proliferation and remodeling embryonic blood vessels failed to invade the labyrinth layer [54]. RSPO3 expression was mainly detected in the edge of the chorioallantoic fusion interface. The expression then expanded into the center of the allantois at E9.5. At E10.5, the expression expanded further, and was detected in the allantois penetrating into the chorion. No expression was detected in the yolk sac or in trophoblast lineages, such as chorionic trophoblast cells. This suggests that RSPO3 is needed for vessel invasion into the chorion but is not needed for chorionic trophoblast differentiation [63]. This indicates that the lack of branching initiation from the chorionic plate is likely due to the reduction of *Gcm1* expression.

LGR-null mice. Lgr4-6 receptors take part in the canonical Wnt pathway through interacting with R-Spondin. As the R-Spondin3 mutant phenocopies Fzd5, it seems that R-Spondin3 works to rescue Fzd5 receptors within placental development. However, in order for R-Spondin3 to be able to do this, it would need to bind to an LGR receptor. It has been demonstrated by our lab that R-Spondin3 binds to Lgr4 which are expressed on HEK293T cells [23]. As illustrated in the scientific report section of this thesis, a TOP/FOP luciferase assay demonstrated that the addition of R-Spondin3 to HEK293T cells conditioned with Wnt leads to an increase in Wnt activity. This was followed up by a cell fluorescence assay which showed that this increase in Wnt activity was due to R-Spondin3 binding to Lgr4 receptors expressed on HEK293T cells. Surprisingly, however, there is apparently no necessity for the Lgr receptors in the placenta as their knockout results in no placental phenotype. Instead, homozygous deletion of LGR5 leads to neonatal death associated with ankyloglossia, gastrointestinal distension, cyanosis and respiratory failure [64]. LGR4 null mice showed similar symptoms and weight reductions as newborns of humans, rodents and pigs with the syndrome of IUGR (Intrateurine Growth Restriction) (Mazerbourg, 2004) as well as showing perinatal death, open eyelids, and abnormal renal development [65].

The lack of a placental phenotype could be explained by redundancy between Lgr4 and Lgr5 meaning that the removal of one protein would lead to no effect as the other can compensate and take over its function. However, a study by Mustata et al. (2011) has published findings on a Lgr4/Lgr5 double knockout which surprisingly resulted in mice who survived the neonatal period, albeit with noticeable growth retardation. It was only after a month that the mice died, likely due to severe kidney lesions, phenocopying the Lgr4 knockout mice [66]. However, since only one study was done on a double knockout for Lgr4/Lgr5, it would be beneficial for another study to confirm these results. A remaining possibility for this phenomena could be that Lgr6 takes over for Lgr4 and Lgr5. Therefore, it would be interesting to make a triple knockout to test this. However, as both Lgr4 and Lgr5 are highly expressed in the placenta while Lgr6 is expressed in very low amounts, it is unlikely Lgr6 has sufficient expression to compensate for Lgr4/Lgr5 knockout and thus knocking out Lgr4-6 receptors is unlikely to induce a placental defect.

NTE & Wnt: Similarities

Both NTE and the Wnt components, Wnt2, Wnt7b, Fzd5, and R-Spondin3 have demonstrated placental phenotypes in knockout mice models. At first glance, the phenotypes of NTE appear similar to those of the Wnt components. However, a few differences should be noted. Firstly, while chorioallantoic fusion occurs normally in NTE knockout mice, this process does not occur in Wnt7b and Tcf/Lef1 (-/-) homozygous mutant mice. This makes analyzing the similarities between NTE and Wnt7b knockout mutant phenotypes difficult as the latter never reaches the labyrinth formation stage. However, Wnt2, Fzd5 and R-Spondin3 knockouts did have normal chorioallantoic fusion and thus their phenotypes will be compared with that of NTE. The two main stages of labyrinth development in the placenta will be covered, namely, the penetration of trophoblast cells into the chorionic ectoderm to form branches of the labyrinth and second, the invasion of fetal vessels into these branches. Trophoblast cells in the chorionic plate that express *Gcm1* thin and elongate to create a primary villous made up of trophoblasts into which the under-lying fetal blood vessels grow. The allantois then invades the trophoblast cells, forming the blood vessels that compose the fetal components of the placenta vascular network. This vascular network is needed in order to establish the close connection between the embryonic blood vessels and the maternal blood flow in the placenta for efficient exchange.

Trophoblast differentiation and proliferation into villi of the labyrinth. The main difference between the NTE phenotype and that of Fzd5 and R-Spondin3 is that in NTE the branching of villi from the chorion was still initiated. It is possible that Fzd5 and R-Spondin3 cannot initiate branching from the chorion since sufficient *Gcm1* expression is lacking. This decrease in *Gcm1* expression is likely due to a reduction in canonical Wnt signaling as it has been demonstrated that β-catenin is

involved in directly targeting the GCM1 pathway [67]. Interestingly, NTE may also have a link with *Gcm1*. Similar to the phenotype of Gcm1-null mice, further differentiation of syncytiotrophoblast cells failed in NTE mutants, resulting in the complete absence of the labyrinth. The observed phenotype correlates with the expression of NTE in the extraembryonic ectoderm of the chorion, the ectoplacental cone, and trophoblast giant cells [52].

Invasion of vessels into trophoblast branches of labyrinth. In NTE-null mutants, initiation of underlying fetal vessels in the chorion had initiated formation into branches and at E9.5 syncytiotrophoblast precursor cells formed around the developing fetal vessels and invaded toward the maternal blood sinuses. However, these trophoblast cell branches failed to elongate and bifurcate and collapsed completely at E10. In the embryo proper vasculogenesis also initiated successfully but developed vessels were often dilated or contained severe hemorrhages. Moreover, vessel formation in the yolk sac was initiated but circulation is not maintained. Therefore, effective cardiovascular circulation was not developed fully without the presence of NTE in neither the placenta, embryo proper, nor the yolk sac. This phenotype is repeated by Fzd5 and Rspo3 where the yolk sac is under vascularized and reduced penetration of vessels into the trophoblast-specific chorion occurs and embryonic blood vessels in the mutant placenta remain completely separated from the maternal blood flow [62]. Therefore, both NTE and Fzd5 and R-Spondin3 are clearly needed for proper maintenance of vessels but not for vasculogenesis initiation. However, a main difference between NTE and Fzd5/Rspo3 vasculogenesis of the placenta is that Fzd5 and Rspodin3 the vessels do not invade the trophoblast branches of the labyrinth while in NTE this process is still initiated. This impairment in vessel invasion may have occurred since the trophoblast braches or villi from the chorion to invade are never formed. However, since limited vessel growth also occurs in the yolk sac it is more likely that the vessels themselves were unable to elongate or could not be maintained than that the invasion did not occur simply due to lack of chorion branched villi from the chorion to the labyrinth layer.

Many different defect combinations of the two stages just described (trophoblast branching and vessel invasion) are known (Figure 8). For example, in *Esx1* mutants the villi appear to undergo normal branching morphogenesis, yet the fetal vasculature does not develop normally. The *Esx1* gene encodes a homeobox transcription factor that is expressed solely in trophoblast cells of the labyrinth. This indicates that trophoblast cells are actively involved in the vascularization of the labyrinth [49]. In contrast, *Rb* mutant labyrinths show defects in trophoblast proliferation and differentiation into trophoblast villi. The *Rb*-deficient placenta attempts to compensate for reduced nutrient transport by increasing their fetal capillary density. The phenotype which NTE covers is that of the "Small-labyrinth" mutant where the villi initiates but is reduced and there is an apparent reduction in vascularization. This reduction in vascularization is likely due to the vessels not being able to extend into the underdeveloped trophoblast villi.

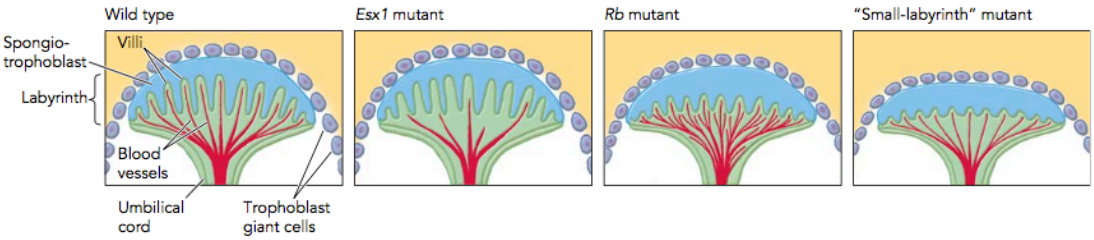


Figure 15: Fetoplacental vascularization defects in various mutant placentas. The two stages of labyrinth formation occur normally in wild-type placentas with trophoblast cells proliferating and differentiating into trophoblast villi in which blood vessels invade. Esx1 mutants differ by lack of vasculogenesis and Rb mutant by lack of villi elongation and increased vasculogenesis. The "Small-labyrinth mutant" has reduced branching morphogenesis of the villi and apparent reduction in vascularization [49].

In the case of Fzd5 and R-Spondin3, the branching morphogenesis never initiates and this could block the blood vessels from elongating. In other words, the fact that the villi fail to form may be the reason vasculogenesis doesn't occur. As all small labyrinth phenotypes with fewer villi would also be

described as having fewer overall vessels, it is important to more accurately discover whether there is an impairment in vasculogenesis. This could be done by comparing vascular density with the density of the differentiated villi to test it the reduction in fetal blood vessels is simply proportional to reduction in villi [49].

Discussion

Canonical Wnt involved in placental growth. Strong evidence suggests that canonical Wnt signaling is involved in placenta development as Wnt2, Fzd5, and Rspo3 mutant mice have nearly identical placental defects at the level of the labyrinthine zone marked by a reduced amount of fetal blood vessels [57]. *Gcm1* and *Vegf* are also known to be regulated by the canonical Wnt pathway and both are down regulated in Fzd5 and Rspo3 mutant mice. Especially as Wnt2 is the suggested ligand for Fzd5 in the placenta, it is indicated that Rspo3 functions in association with them [68]. Surprisingly, LGR receptors don't seem involved, even when taking account of redundancy. Since no other LGR receptors are expressed as high as Lgr4 and Lgr5 in the placenta, it is unlikely that a non-canonical pathway is involved. A plausible explanation is that that R-Spondin circumvents the need for an LGR receptor. For example, R-Spondin3 may associate with another receptor other than an Lgr, resulting in activation of Wnt signaling. However, there is no clear explanation for this phenomena and further research is needed to verify whether Lgr4-6 are involved with Fzd5 and Rspo3 in the placenta.

Wnt transcription factors involved in labyrinth formation. Other than lack of *Gcm1* expression, it is possible that a downregulation of the vascular endothelial growth factor (VEGF) is involved in the lack of vessel invasion into the labyrinth. VEGF is known to play an important role in vasculogenesis and it is conceivable for VEGF to act as an attractive force, attracting and facilitating the invasion of fetal vessels from the allantois into the space created by chorionic branching. VEGF can function as a growth factor to promote vessel growth as well as a chemoattractant and is strongly up-regulated by Wnt signaling during tumorigenesis [58]. Vegf164 mRNA was shown to be expressed in chorion trophoblast cells at E9.0 in control placentas [58]. Interestingly, Vefg164 mRNA is expressed at extremely low levels in Fzd5 mutants, suggesting that fetal vessel infiltration is impaired by Fzd5 mutation [58]. Moreover, Kazanskaya et al. (2008) identified Vegf induction in the placenta as the cause of hampered angiogenesis in mice and Xenopus where RSPO3 was removed by morpholino and mutation respectively. Therefore, the trophoblast cells of the villi may themselves regulate vascularization of the labyrinth. Conversely, fetal vasculature may be important for initiation of branching from the chorioallantoic interface [49]. This is because mutations in various genes appear to block chorioallantoic branching that are only expressed with the allantoic mesoderm/blood vessels.

Both NTE and the canonical Wnt pathway are essential for proper angiogenesis. This is even seen in heterozygous mutations of NTE which lead to many neurological syndromes in humans. For example, Boucher-Neuhauser and Gordon Holmes syndromes are allelic diseases, both caused by recessive PNPLA6 mutations [69]. These two syndromes are characterized by chorioretinal dystrophy which affects the choroid or vacular layer of the eye. It provides oxygen and nourishment to the outer layer of the retina, strikingly similar in structure and function to the placenta. Similarly, Oliver–McFarlane syndrome and Laurence–Moon syndrome, are characterized by severe chorioretinal atrophy. Laurence-Moon syndrome also includes clinical presentation of chorioretinopathy, where leakage is caused due to impaired choroidal neovascularization (CNV). CNV involves the growth of new blood vessels that originate from the choroid [70]. These clinical presentations seem extremely similar to those found in the placenta when NTE is removed. Therefore, it would seem NTE indeed has an important role in maintaining blood vessels and it would be interesting to do more in depth analysis into its potential molecular mechanism in this area.

Interestingly, research has shown that the Wnt pathway is also involved in vasculogenesis with point mutations and deletions in the coreceptor Frizzled 4 (Fzd4) and LRP5 leading to diseases of the retina.

One such disease is autosomal dominant familial exudative vitreoretinopathy (FEVR), a blinding disease characterized by a disruption of the retinal vascular system. Moreover, a second disease, called Norrie disease, has a retinal vascularization phenotype similar to FEVR [71]. Therefore, NTE and Wnt components are clearly involved in similar functions with essential roles of vasculogenesis maintenance in similar developmental regions. However, with the failure of labyrinth formation in NTE and Wnt component mutants initiating at such different points, it is unlikely they take part in the same molecular mechanism.

Although, at first glance, the NTE and Wnt components knockout placental phenotypes seemed similar, it is now clear that they are in fact separate. Moreover, despite the mass spectra analysis performed at Hubrecht Institute having indicated that LGR5 was found bound to NTE, analysis of the placental phenotypes suggest that NTE is not involved in the canonical Wnt pathway. However, it is still possible that LGR and NTE are involved in a separate pathway and do not necessarily activate the Wnt pathway. Further research, however, will need to be conducted to elucidate which other pathway this might be.

Research into these placental phenotypes has elucidated the important function of Neuropathy Target Esterase and the canonical Wnt pathway in maintaining proper vessel growth in mice. Although the gross architecture of the human and mouse placentas differ somewhat, their overall structures and the molecular mechanisms underlying placental development are thought to be quite similar [72]. Therefore, elucidation into the mechanisms behind vessel growth in the mouse placenta could give us insight into potential therapies for pregnancy disorders such as pre-eclampsia, characterized by the abnormal formation of blood vessels in the placenta.

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SCIENTIFIC REPORT

Antibody blocking of R-Spondin and LGR4/5 binding

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Canonical Wnt signaling is enhanced by the ligand, R-Spondin. R-Spondin homologs 1-4 bind to LGR receptors 4-6 with high affinity as well as to the E3 ligases, Znrf3 and Rnf43. The binding of R-Spondin to LGR and the E3 ligase has been shown to save Frizzled receptors on the cell surface from being degraded. This research report performs a TOP/FOP luciferase assay to illustrate that R-Spondin3 increases Wnt activity. Moreover, a cell fluorescence assay demonstrates that R-Spondin3 binds to Lgr4 through the use of antibodies against R-Spondin. This experiment also illustrates the potential for antibodies against R-Spondin in blocking R-Spondin binding to Lgr4, therefore reducing aberrant Wnt activity which could lead to many diseases and cancers.

Introduction

The Wnt signaling pathway serves and important function in cell fate determination, cell migration, cell polarity, neural patterning and organogenesis during embryonic development. It is activated by Wnt glycoproteins which bind to Frizzled receptors on the cell surface and can be enhanced or inhibited by many secreted ligands. One such ligand is called R-Spondin. R-Spondin has four homologs, R-Spondin 1-4, each of which is present in all vertebrates and some primitive chordates [1]. The R-Spondins are members of a much larger family of proteins characterized by the presence of thrombospondin repeats (TSRs) [2]. R-Spondin works as an agonist for Wnt-initiated signaling by preventing the cell surface clearance of the Frizzled receptor (Fzd) by two transmembrane E3 ubiquitin ligases, Zinc and RING finger 3 (ZNRF3) and its homolog, RING finger 43 (RNF43). Znrf3 and Rnf43 are single pass transmembrane proteins characterized by their RING domain and a PA domain (de Lau et al., 2014). They work to ubiquitinate the Fzd receptor, targeting it for removal from the membrane and subsequent degradation by a proteasome. As the Fzd receptors are necessary to bind Wnt ligands and initiate the Wnt signaling pathway, removal of these receptors causes a decline in Wnt activity. R-Spondin counteracts this activity by binding to both the E3 ligase and Leucine-rich repeat-containing G protein-coupled receptor (LGR) through two N-terminal Furin repeats [2]. These Furin repeats are referred to as the 'business end' of the protein as the presence of both is necessary and sufficient to exert the Wnt-enhancing activity (de Lau, 2014). The Furin-1 domain interacts with the E3 ligases, ZNRF3 and RNF43 [3,4]. The Furin-2 domain of R-Spondin recruits and binds the type-B Leucine-rich repeat-containing G protein-coupled receptors 4–6 (Lgr4-6) [5,6].

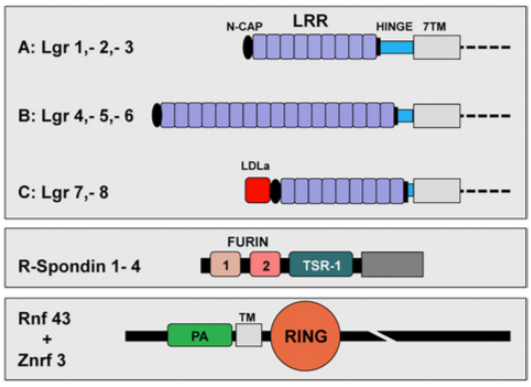

Figure 2: Schematic representation of the domain composition of the 8 LGR receptors, R-Spondin 1-4 homologs, and Rnf43 and Znrf3 E3 ubiquitination ligases. The LGR receptors are characterized by a 7 transmembrane domain (7TM), connected by a Hinge domain to an LRR domain. The R-Spondin secreted ligand is characterized by its TSR-1 domain and two Furin domains. The Furin-1 domain is used to interact with Rnf43 and Znrf3 while the Furin-2 domain binds to the Lgr4-6. RNF43 and ZNRF3 are RING-type E3 ligases which are structurally related to Grail. They consist of a single-pass transmembrane domain, a PA domain, and a cytoplasmic RING domain. The E3 ubiquitin ligase activity exerted by the RING domains of Rnf43 and Znrf3 targets lysine residues in the 7TM loops of Frizzled receptors [2].

LGRs are seven- transmembrane (7TM) receptors which belong to a subgroup of eight LGRs within the super- family of Rhodopsin G-Protein Coupled receptors (GPCRs) [2]. LGR receptors consists of a large extracellular domain (ECD) composed of a string of leucine-rich repeat (LRR) units flanked by N-terminal and C-terminal cap modules.

The Lgr4-5 homologs have an ECD which consists of 17 LRRs and a Hinge domain of intermediate length. The function of these seven-transmembrane (7TM) receptors for the ligand R-Spondin (RSPO) is unknown but it's presence is necessary for RSPO function in blocking Frizzled downregulation as a result of E3 ubiquitination. This was demonstrated through several studies where LGR receptors bind R-Spondins with high affinity and are essential for signal enhancement of low-dose Wnt [5,6].

In summary, if R-Spondin is present it binds to the PA domain of the E3 ligases via its Furin-1 domain while its Furin-2 domain recruits the LGR receptors. Once bound to R-Spondin, the E3 ligases are inactivated and no longer ubiquitinated the Fzd receptors. Therefore, there is no more clearance of the Fzd receptors from the cell surface and the Wnt-initiated signaling increases (Figure 2). Thus, R-Spondin takes part in a control mechanism for the Wnt pathway. If the Wnt pathway is very active, the nucleus produces more ZNRF3 and RNF43 E3 ligases which work to reduce Wnt pathway activity and as a control the nucleus also produces R-Spondin in order to keep the E3 ligases in check, thus, regulating Wnt signaling which is essential for proper cell functioning. Another method of controlling Wnt signaling is through the use of antibodies.

Antibody targeting as therapy

The Canonical Wnt pathway is implicated in many disorders such as osteoporosis and many forms of cancer. Being able to influence the expression of Wnt is therefore a very promising therapy. To date, many inhibitors have been found which target a variety of components of the Wnt pathway such as β-catenin, Dvl, Porcupine, WIF1, SFRPs and Wnt proteins [7]. These inhibitors include small molecules, existing drugs such as aspirin, RNA interference and antibodies [8]. Antibodies developed can target the Wnt pathway either at the extracellular level, when

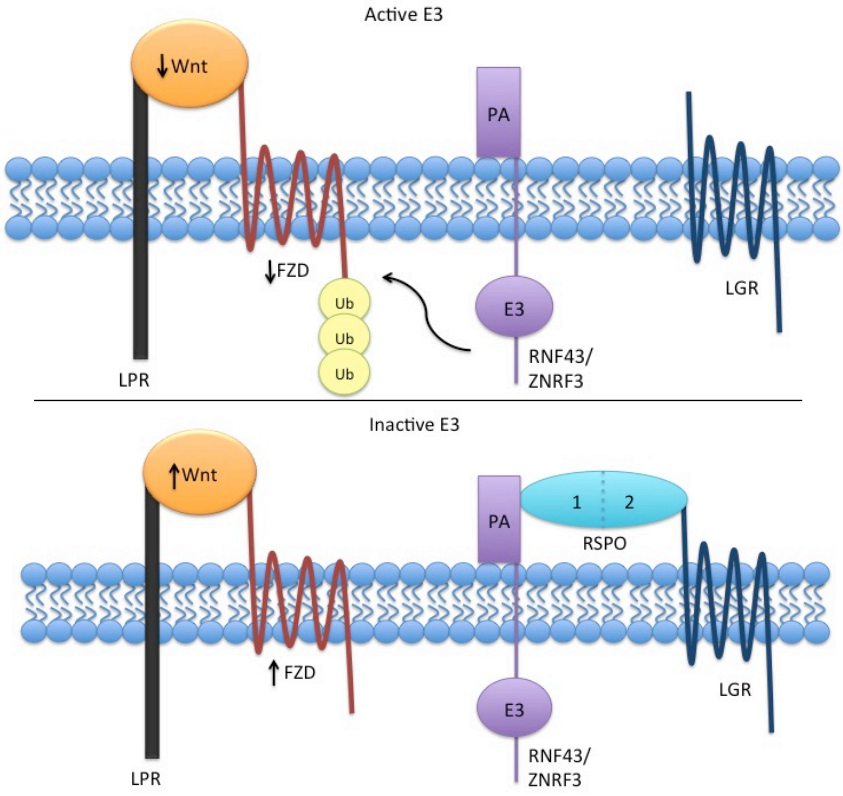

Figure 3: The R-Spondin, E3 ligase, LGR complex and it's influence on Frizzled receptor expression on the surface of cell membranes. E3 ligases are active in the absence of R-Spondin and lead to the ubiquitination and subsequent degradation of Fzd, thus decreasing Wnt signaling. E3 ligases are inactive when bound to R-Spondin, resulting in an increase in Fzd receptor expression and Wnt signaling.

Wnt binds to the surface receptors, or at the cytoplasmic level where β-catenin resides. Targeting the Wnt pathway at the extracellular level has lead to a repertoire of antibody targets including Wnt ligands themselves, Wnt antagonists such as sclerostin or DKK-1, and Wnt receptors such as LGR and FZD.

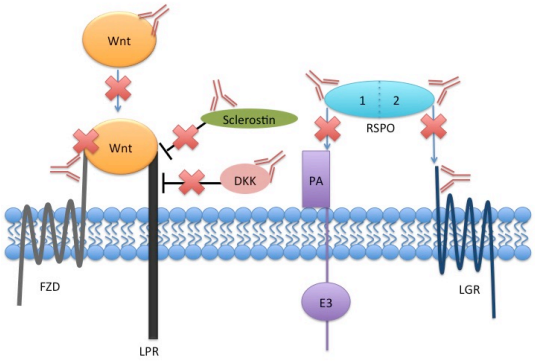

Figure 4: Diagram of the various antibody targets for the Wnt pathway including its receptors as well as the Wnt ligand, R-Spondin protein, and the Wnt antagonists Sclerostin and Dishevelled (DKK).

Wnt ligands themselves can be targeted. Two studies have successfully developed therapeutic monoclonal antibodies against Wnt-1 and Wnt-2 which inhibit Wnt signaling and suppress tumor growth in hepatocellular carcinoma and melanoma respectively [9,10]. The study conducted by Wei et al in 2009, demonstrates that antibodies can be developed which block Wnt-1, offering a potential pathway-specific therapeutic strategy for the treatment of a subgroup of Hepatocellular carcinoma (HCC) cells that over-expresses Wnt-1. The study revealed that an anti-Wnt-1 antibody leads to decreased viability and proliferation of human HCC cell lines. Huh7 and Hep40 cells overexpressing Wnt-1 whilst leaving normal hepatocytes with undetectable Wnt-1 unaffected [9]. Similarly, You et al. have shown that an anti-Wnt-2 monoclonal antibody induced apoptosis and inhibited tumor growth in malignant melanomas [10].

Wnt antagonists can be targeted. Another approach that promises potential is restoring the expression of secreted Wnt antagonists, including WIF-1, Dkk, and sFRPs, in antagonist-deficient tumors [11]. Current preclinical and clinical data suggest that blocking the Wnt antagonists, sclerostin or Dkk-1, using neutralizing antibodies can treat patients with skeletal disorders. Downregulating these two antagonists has been associated with an increase in bone mass density, trabecular bone volume, osteoblast number and bone formation in rodents [12]. Pozzi et al. (2013) have developed an anti-Dkk1 neutralizing monoclonal antibody which has shown promise in counteracting multiple myeloma bone disease [13]. Furthermore, a monoclonal antibody named AMG785 has been developed against sclerostin by the biotech giant Amgen. Phase II results released by Amgen strongly suggest that this inhibitor holds promise for osteoanabolic therapy of osteoporosis [14].

Wnt receptors can be targeted. Receptors of the Wnt proteins on the surface of cells can also be antibody targets for therapy. The most advanced clinical program so far is that of the antibody against Frizzled receptors developed by OncoMed Pharmaceuticals, having entered into Phase 1b clinical trials in combination with standard-of-care chemotherapy in distinct solid tumor indications in 2013 (Gurney et al. 2012). The monoclonal antibody, named OMP-18R5, was initially identified by binding to Frizzled 7 and interacts with five Fzd receptors (FZD1, FZD2, FZD5, FZD7, and FZD8) through a conserved epitope within the Extracellular Cysteine Rich Domain (ECD) (Gurney et al. 2012). By binding to Frizzled receptors, the antibody blocks canonical Wnt signaling induced by multiple Wnt family members and has demonstrated remarkable anti-tumor efficacy in many preclinical patient derived xenograft models. It's ability to suppress Wnt signaling is demonstrated by a luciferase reporter assay performed by Gurney et al. in 2012 in which it was shown that all forms of Wnt activity were decreased significantly upon addition of 18R5. Oncomed has also developed a Frizzled-8 Fc fusion protein and an antibody against R-spondin/Lgr5 (Gurney et al. 2012).

Wnt agonists can be targeted. R-Spondins ability to enhance Wnt signaling is an interesting feature with the potential of being used in therapy. Thus far, developing an antibody against components of the Wnt pathway. This scientific report will demonstrate the agonistic effect of R-Spondin on Wnt through a TOP/FOP assay, its binding to LGR4 through an immunofluorescence assay and the potential for antibody inhibition of R-Spondin through an ELISA.

MATERIALS & METHODS

Cell culture and transfection. HEK293T cells are used as they naturally express the Lgr4 receptor. HEK293T cells were maintained in Dulbecco's modified Eagle medium (DMEM, Invitrogen) supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, and 10% fetal bovine serum (FBS).

For transfection, cell culture medium removed from the plate with HEK293T cells and replaced with PBS0 for a washing step. Subsequently, Trypsin Replacement Enzyme is added followed by transfection medium (DMEM (1x) + GlutaMax) to stop the trypsin process. All cells are resuspended and then a sample is taken from the cell suspension for experimental use.

Cells are Polyethylenimine/PEI-transfected (Polysciences) with a mix of plasmids encoding a Wnt reporter (TOP-luciferase), Wnt independent Renilla-luciferase, and a blank plasmid (pEF myc/His c). The absolute concentrations of these plasmids are 20ng, 2ng, and 178ng/well, respectively. The mix of plasmids (DNA) and PEI are combined in a 1:5 ratio (DNA to PEI) and left to rest at room temperature for at least 20 minutes. The DNA and PEI mixtures are then added to the cell suspension and replated on a flat-bottomed 96 well plate. Cells are maintained in an incubator at 37 degrees Celsius with 5% CO_2 for 24 hours.

Preparation of mRSPO3 conditioned medium. Transfection of HEK293T cells with *Rspo3* and harvest of conditioned medium were as described. Anti-FLAG M2-Agarose beads (Sigma) were incubated overnight with *Rspo3*-conditioned medium or control medium from untransfected HEK 293T cells. After washing, Rspo3FLAG recombinant protein was eluted from beads using FLAG peptide (Sigma) A filtering step was taken to remove any remaining cell debris.

Sequencing. Mouse R-Spondin 3 homolog (mRSPO3) was sequenced by the company Macrogen © and subsequently analyzed using the program, Seqman Pro. For sequencing by Macrogen ©, 5 microliter of the mRSPO3 plasmid of concentration 100ng/microliter was mixed with 5 microliter of 5 micromolar forward or reverse primer. The primers used for sequencing are listed in the table below.

Table 1. mRSPO3 Primers used in this study

Primer name	Sequences (5'→ 3')
mRSPO3-F1	ATGCACTTGCGACTGATTTC
mRSPO3-F2	CAACAATCATACTATGGAATG
mRSPO3-F3	CGGTATCAGTCAGCACTGTAC
mRSPO3-R1	ATTTCTGTTGCTTGTCTCGG
mRSPO3-R2	AACCCTTCTGGGCAACTGTC

TOP/FOP Luciferase assay. Cells are PEI-transfected (Polysciences) with a mix of plasmids encoding a Wnt reporter (TOP-luciferase), Wnt independent Renilla-luciferase, and a blank plasmid (pEF myc/His c) as described.

For the TOP/FOP assay, the third homolog of R-Spondin, RSPO3, is used. After 24 hours of transfection the culture medium is replaced for fresh culture medium. To selected wells, Wnt3a-conditioned culture medium or control conditioned medium were added. To selected wells containing Wnt3a-conditioned medium, add 100 microliters of mouse RSPO3 concentrated medium at final concentrations of 1, 1/2, 1/4, 1/8, 1/16, 1/32 and 1/64 of the initial concentration. Both Wnt3a-conditioned medium and mRSPO3-conditioned medium were supplemented with 10% FCS. Cells were stored in an incubator at 37 degrees Celsius with 5% CO_2 for 24 hours.

After 24 hours, the culture supernatant was removed and the cells are processed according to the instructions of the Dual luciferase kit (Promega), namely, passive lysis buffer was added to each well and the supernatant was re-plated onto a 96-well F-bottom Lumitrac 200

white microplate from Greiner bio-one (REF 655075) for reading. TOP luciferase counts were measured using the *Mikrowin 2000* software and normalized for transfection efficiency and variation in cell numbers by dividing the Wnt reporter-luciferase counts of individual wells by the Renilla luciferase counts of the same well. A correction factor for this ratio was applied to achieve the value of 1 for the average of CM wells. The same adjustment was used for all wells.

Immunofluorescence Assay. HEK293T cells were transfected with Lgr4 similar to described previously. Medium was removed from transfected cells and 4% Paraformaldehyde (PFA) added. 4% PFA fixates cells to plate. Not kept on longer than 5 minutes. Removed and then added PBS w/BSA (except from controls). Concentrated mouse R-Spondin 3 medium (m-RSPO3-huFc1) was added to wells (except for controls) as well as anti-rat antibody with fluorescent marker 488 Alexa (antihuIgG-Alexa488) (diluted by a factor of 200). In designated wells, MAB41201 antibody (R&D systems) which binds to Human/Mouse R-Spondin 3 was also added. After 30 minutes a washing step took place to remove the concentration mRSPO3 medium and thus any unbound R-Spondin. The plate is covered with aluminum foil to protect against light and measured using a fluorescent microscope.

Dot Blot. Used a PVDF membrane from *Immobilon* and activated it in methanol. Once methanol was removed, added Methanol (MeOH) transfer buffer (TB). Placed membrane on filter paper and added dilutions of the supernatants (Lgr4/Lgr5/mRSPO3). Membrane was returned to MeOH TB solution and washed several times with PBS. 5% milk powder in PBS solution was added and soaked the membrane for approximately 30 minutes to block. This milk powder solution was then replaced with the same but containing a Monoclonal Anti-Flag HRP and left for 30 minutes on a shaker. Subsequently, the membrane was washed with PBS and ECL prime substrate was added (solution A and solution B are mixed in a 1:1 ratio). The membrane was then transferred to transparent plastic, the ECL prime mix added and developed in a dark room (or in a Chemiluminescence Tray). The positive control used was recombinant RSPO-Fc and the negative control was F12 medium without mRSPO3.

Maxi-prep. A maxi-prep procedure was performed for Top10, Fop10, Renilla (RL), and empty vector plasmid for use in transfection procedure. AMP (Ampicillin) plates are taken from an incubator. Needed bacteria are mixed with in a 50:1 ratio with desired maxi and undergoes a heat shock procedure followed by the addition of LB. This mix was then centrifuged, spinning the bacteria to a pellet at the bottom. Most of the medium is removed and then the pellet is resuspended and 100 microliters of it is placed on AMP plate which is left overnight.

The following day the contents of the AMP plate are transferred to a LB flask and placed on a shaker table. The contents of the LB flask are centrifuged. The DNA is then purified using the instructions from the PureLink® HiPure Plasmid Filter Maxiprep Kit (solutions R3 w/RNase, L7 and N3). The resulting mixture is then centrifuged and then loaded onto an equilibrated column and allowed to drain by gravity flow. The column is then washed with 60 ml wash buffer (W8) and the flow through is discarded. After this washing step a sterile centrifuge tube is placed under the column. 15 ml elution buffer (E4) is added to the column to elute the DNA. The elution tube now contains the purified DNA so the column is discarded and isopropanol is added to the elution tube and mixed well. The elution tube is then centrifuged at and the resulting supernatant is discarded. The pellet is subsequently resuspended in 1 ml 70% ethanol, centrifuged, and the resulting pellet is air-dried for 10 min and then finally resuspended in 200 microliter TE buffer.

RESULTS

TOP/FOP Luciferase Assay

A TOP/FOP luciferase assay was conducted (as described in methods) to determine whether the addition of R-Spondin to HEK293T cells conditioned with Wnt would lead to an increase in Wnt activity. For the TOP/FOP luciferase assay conditioned medium of mouse R-Spondin3 with a human Fc tag (mRSPO3-huFc1) was used. Mouse R-Spondin is very similar to human R-Spondin, only differing by 10% according to the program SeqMan Pro. Most of the differences present between the used mRSPO3 construct and its cDNA are in the Basic amino acid rich domain, a domain that is not essential for R-Spondin activity (Figure 5).

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10         20         30         40         50
MHLRLISCFF IILNFMEYIG SQNASRGRRQ RRMHPNVSQG CQGGCATCSD
        60         70         80         90        100
YNGCLSCKPR LFFVLERIGM KQIGVCLSSC PSGYYGTRYP DINKCTKCKV
       110        120        130        140        150
DCDTCFNKNF CTKCKSGFYL HLGKCLDSCP EGLEANNHTM ECVSIVHCEA
       160        170        180        190        200
SEWSPWSPCM KKGKTCGFKR GTETRVRDIL QHPSAKGNLC PPTSETRTCI
       210        220        230        240        250
VQRKKCSKGE RGKKGRERKR KKLNKEERKE TSSSSDSKGL ESSIETPDQQ
       260        270
ENKERQQQQK RRARDKQQKS VSVSTVH
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Figure 5: Mouse RSPO3 Sequence. Amino acids in red are different from those of hRSPO3. Amino acid 45-85: Cysteine Furin 1, 86-134: Cysteine Furin 2, 134-214: Thrombospondin Type 1 domain.

A dot blot was performed as described in Methods to test for concentration of mRSPO3 in the mRSPO3-conditioned medium. Subtracting the negative control stains from that of mRSPO3-Fc, there is still very strong staining. Thus, we can conclude there are high concentrations of mRSPO3 (Figure 5B).

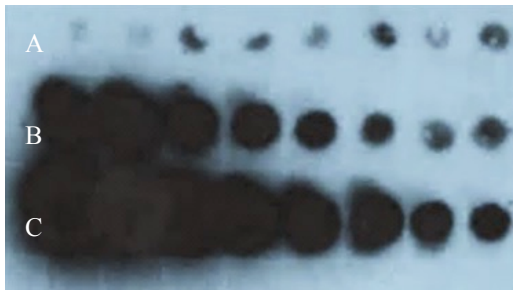
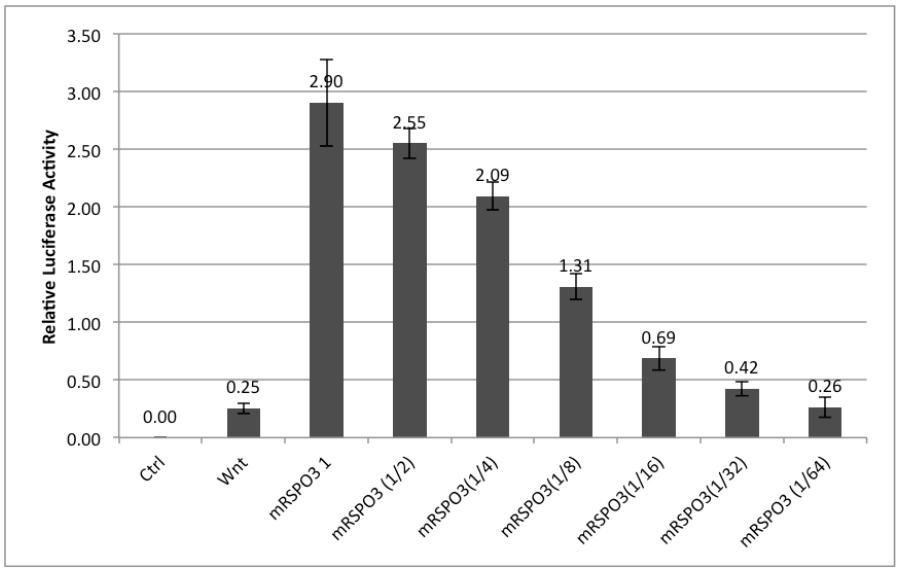


Figure 6: Dot blot for mRSPO3 concentrated medium. (A) Negative Control (F12 medium), (B) mRSPO3-Fc, (C) positive Control (RSPO-Fc). Used anti Fc-HRP antibody.

The results of the TOP/FOP are illustrated in Graph 1. It is clearly shown that the addition of mRSPO3 to the cells with Wnt leads to a 10 fold increase in Wnt activity, with a change in the average ratio of Luc/Renilla from 0.25 to 2.9. As the mRSPO3 concentration is decreased by half, the Wnt activity also steadily decreases until the



Graph 1: Relative Luciferase Activity. HEK293T cells were transfected with a Wnt pathway-specific (TOP) luciferase reporter in combination with a TK driven Renilla reporter. The result illustrated is the ratio of Luciferase to Renilla reporter activity. Each condition was tested five times and the average of the five is indicated. Control wells (Ctrl) were incubated in normal DMEM while the Wnt control (Wnt) had Wnt3a-conditioned culture medium added. In the wells with mRSPO3 condition, both Wnt3a-conditioned culture medium and mRSPO3-conditioned medium were added. mRSPO3 conditioned medium added at final concentrations of 1, 1/2, 1/4, 1/8, 1/16, 1/32 and 1/64 of the initial concentration. Bars indicate standard deviation from mean.

original Wnt activity is regained.

This result demonstrates that R-Spondin3 is indeed responsible for an increase in Wnt activity. As described earlier, this increase is due to R-Spondins function in rescuing Frizzled receptors from the surface of cells by binding to the E3 ligases, Znrf3/Rnf43 and an LGR4-6 receptor, thus blocking E3 ligase activity.

Immunofluorescence Assay

To test whether R-Spondin3 binds to Lgr4 receptors transfected in HEK293T cells, a cell fluorescence assay was performed, as illustrated in figure 8. The fluorescent tag used was attached to an antibody, antihuIgG-Alexa488, which binds to the mRSPO3-huFc1 within the concentrated medium. Therefore, at 500 ng of transfected Lgr4 and no mRSPO3-huFc1 is added, no fluorescence is visible. The m-RSPO3-huFc1 only remains on the cells if it has an Lgr receptor to bind to. Therefore, when the concentration of Lgr4 receptors transfected decreased, the fluorescence, or the level of mRSPO3, also decreases. This shows that mRSPO3 binds to Lgr4.

The addition of an rat antibody against RSPO3 (MAB 41201) lead to a decrease in fluorescence signaling for all concentration of transfected Lgr4, with fluorescence completely disappearing at 8 ng of Lgr4. This indicates that the anti-RSPO3 antibody used inhibits the RSPO3 from being able to bind to the Lgr4 receptor. Indeed, the MAB 41201 antibody has an epitope for amino acid bases Ser21 to Gly209. Therefore, the Furin-2 domain known to bind Lgr4, is blocked. This illustrates the successful inhibition of RSPO3 and LGR receptor binding through the use of antibodies against RSPO3. This antibody application could be a potential therapy for stopping R-Spondin from inhibiting E3 ligase activity in the cells, thereby decreasing excessive Wnt-initiated signaling.

DISCUSSION

There is high correlation between many cancers such as sporadic colon cancer and breast cancer, with aberrant Wnt signaling activity. This report has demonstrated that R-Spondin plays an important role in maintaining Wnt signaling activity levels through counteracting E3 ligase activity. The TOP/FOP has illustrated that RSPO3 works to enhance Wnt signaling and the cell fluorescence assay demonstrated that RSPO3 binds to Lgr4 on the surface of cells. The cell fluorescence assay also showed the potential of antibodies against R-Spondin in effectively blocking the R-Spondin3 binding to Lgr4. If unable to bind to Lgr4, the R-Spondin loses its function and can no longer prevent the E3 ligases from ubiquitinating Frizzled receptors, leading to their degradation and a subsequent decrease in Wnt activity. Therefore, antibodies against R-Spondin have a high potential as therapies in many types of cancers where Wnt signaling is abnormally high. However, although these antibodies have shown promise as therapies against certain cancers and disease, it is important to consider that drugs targeting at the extracellular/membrane level would not be effective to treat those tumors containing mutations downstream in the pathway. Therefore, a vast repertoire of antibodies against many Wnt components should be investigated and developed. Overall, it is clear that R-Spondin has a strong influence on Wnt signaling activity and is dependent on Lgr receptor binding for its function.

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REFLECTION

I am extremely grateful to have had the opportunity of working as an intern at the Hubrecht Institute. I started in late February and worked three to four times a week in the lab until end May. During this time I learnt an incredible amount from how to use new techniques to interacting with co-workers in a lab. I learnt how to perform a dot blot, a maxiprep, and the proper protocol behind working in a fume hood and a sterile environment. I undertook cell fluorescence imaging experiments and TOP/FOP luciferase assays and I have noticed that if I take the time to stop and ask questions, both to myself and to my supervisors, that I perform much better.

Throughout my internship I tested antibodies for companies which targeted multiple components of the Wnt pathway, such as the E3 ligases, Frizzled and LGR receptors. Testing these antibodies allowed me to use many different techniques and also gave insight into the relationship between a company and a research institute. After undertaking these tests I chose to investigate an aspect of the Wnt pathway, the regulation of Frizzled receptors on the cell surface by E3 ligases. This I hoped to do by determining whether the PA domain on the E3 ligases was necessary for their function. However, circumstances and new discoveries lead to a need for a change in topic, as will be discussed below.

Replacing the PA Domain of E3 Ligases

Just when you think you have an interesting research question for your thesis, a paper is published which completely derails your plans. I was hoping to look into RNF43 and ZNRF3 interaction with Frizzled (Fzd) through their PA domain. If it turned out that this PA domain was essential for Fzd removal from the cell surface I planned to introduce point mutations on the PA domain of Znrf3 and Rnf43 to determine which amino acids were essential. However, while proposing this to my supervisor he mentioned that a paper had just been published indicating that the E3 ligases don't interact with Fzd through their PA domain. In fact, the paper claims that an adaptor molecule in the E3 ligase complex is responsible for Fzd interaction. Furthermore, Stefan Ziesche in our lab had discovered just the day before that replacing the PA domain of the E3 ligases with a different PA domain does not influence activity of Fzd. This hints that the PA domain isn't needed for Fzd interaction. Therefore, in just one day, my plans of discovering an interaction between E3 ligases and Fzd receptors were no longer valid.

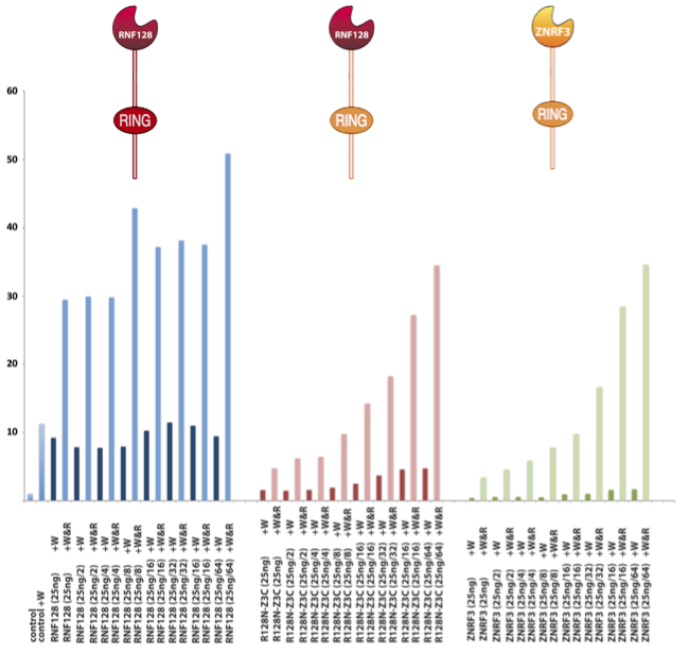


Figure 7: TOP/FOP Luciferase assay comparing the Wnt activity of a PA domain of ZNRF3 with a like-PA domain from RNF128. Note that the Wnt activity of normal ZNRF3 (far left) is nearly identical to that of the ZNRF3 mutant with PA domain replaced by that of RNF128 (R128N-23C) (middle). Source: Stefan Ziesche

From, this experience I learnt how important it is to be flexible and ready to adapt in science. New discoveries are made every day and some of these could mean your plans or approaches have to

change. Although this may be frustrating at times it is also exciting, as each day at Hubrecht showed, you never know before hand what you will be up to in the lab.

ELISA Optimization

In the second month of my time as an intern at the Hubrecht institute, I started optimizing an ELISA experiment. The ELISA was designed to measure whether an antibody against mouse and human R-Spondin would block the Lgr4 and Lgr5 receptors from binding. There were many other experiments available which could test this for us such as a FACs analysis or a cell fluorescence assay. However, the ELISA seemed advantageous as it was a quick and convenient way to get results. An overview of the ELISA design is shown in Figure 1.

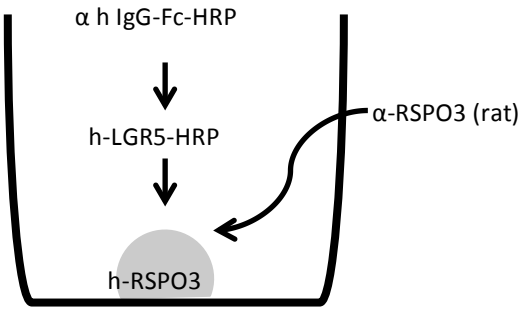
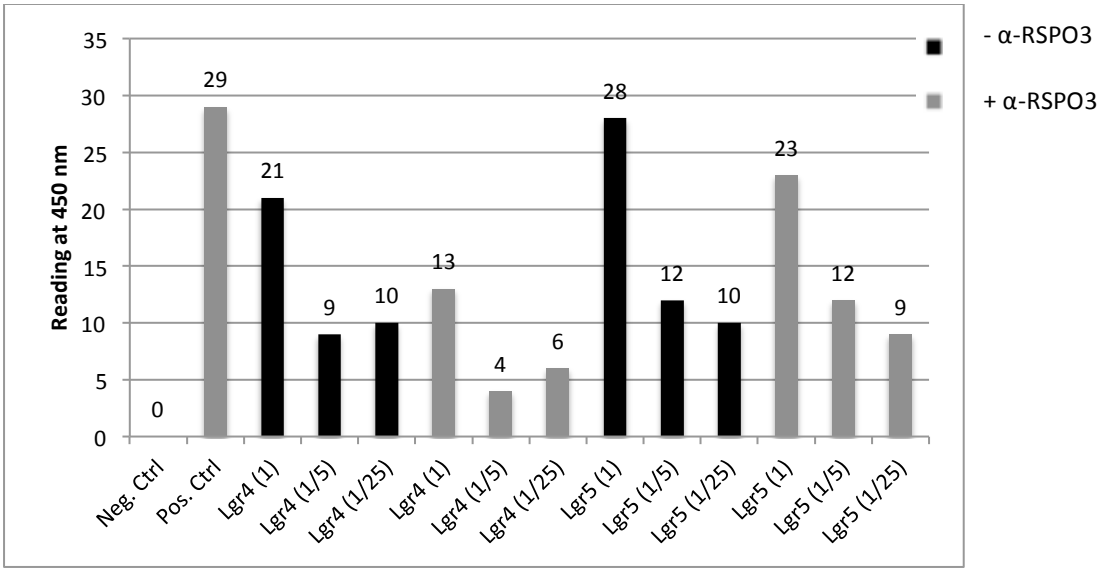


Figure 8: Schematic representation of the ELISA set-up for one well. A carrier free human R-Spondin 3 (hRSPO3) was coated on the plate and then incubated in Lgr5-HRP conditioned medium. An antibody against human IgG-Fc with an HRP tag was then added to bind to the Lgr5-HRP. However, in certain wells we added the same anti-RSPO3 antibody used in the immunofluorescence assay, to see whether this would block the h-Lgr5-Fc-HRP from binding to the h-RSPO3 and thus not being detected by the anti-h-IgG-Fc-HRP antibody.

Unfortunately, after two months of working on optimizing the ELISA, none of the results obtained were useful. Therefore, I learnt how time-consuming and difficult it can be to perfect a research technique. Especially an ELISA, which consists of multiple steps where many factors can play a role, it was difficult to tell where the experiment went wrong or what part could be improved further. The ELISA brought many challenges. For example, our first results seemed promising, as they showed the trend we were expecting. However, on further analysis it became clear that the change in absorbance measured was not significant enough to be able to draw definitive conclusions. This is shown in our results in Figure 2 where the positive control had a low value of 29 when correspondence with other labs indicated it should be near a value of 100. It turned out that this low value was due to the fact that we were using R-Spondin conditioned medium instead of carrier-free R-Spondin.

Figure 2: ELISA results of LGR4/5 Binding Blocking Genentech α-RSPO3 Antibody (403)



After switching to carrier-free RSPP3, however, still many controls had to be performed to optimize the ELISA. For example, we tested RSPO1 and RSPO3 to see which protein would be more effectively blocked. We also tested Lgr4 and Lgr5 respectively and at different concentrations. We also had to optimize for the concentration of R-Spondin we coated the wells with as well as for the amount of antibody we added. Too much R-Spondin on the plate or too little antibody would lead to inefficient blocking of the antibody to the Lgr receptors, giving minimal varying in the absorbance reading. Therefore, what started out as a simple experiment turned out to be quite complex.

Some of the challenges we faced were in gaining consistent readings. Even though we continued to used the same Lgr5 conditioned medium, the readings for Lgr5 within an ELISA of equal concentration would still vary too much (from 0.237 to 0.377). Moreover, we soon discovered that an agent (NaN3) we added to dispel bacterial growth was actually too acidic and affected the ELISA substrate enzymes responsible for coloring the wells.

Over time, the company we were optimizing the ELISA for decided they would prefer a FACs analysis being done over an ELISA and the optimization experiment was stopped. This was disappointing as I felt I was finally getting the results I was looking for. However, from my time working on the ELISA I have learnt an incredible amount.

Overall, I found my time at Hubrecht Institute very enjoyable. Everyone was kind and helpful and I felt I was learning more each and every day. I think my biggest take away from my time at Hubrecht Institute was the understanding of how important it is to constantly ask yourself why you are doing a particular step and what you hope it may teach you. By being aware of every step I made and thinking through my actions before making them, I found I made much fewer mistakes. I plan to take this mindset into the next internship I take and look forward to gaining even more