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Wnt/PCP signaling in early brain development and disease

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Dedicated to my family

ABSTRACT

Wnt/Planar Cell Polarity (PCP) signaling is involved in many cellular processes throughout the development of the embryo. These include control of proliferation, differentiation, migration and cell fate decisions. Deregulation of Wnt/PCP signaling can lead to many developmental abnormalities and cancer. This thesis investigates the effects of altered Wnt/PCP signaling during neurulation in mouse embryos, its importance in cell-cell adhesion and its role in pediatric cancer.

The developing central nervous system (CNS) comprises different patterning centers consisting of groups of cells with organizer-like properties. These produce signals that influence the fate, histogenic organisation and growth of adjacent tissues, resulting in spatial patterning. Members of the Wnt gene family are among few secreted proteins expressed in patterning centers of the developing CNS, such as the pallial-subpallial boundary (PSB) and the cortical hem. This suggests that they might have a role in demarcating and specifying regions in the developing brain.

We decided to investigate the influence of Wnt/PCP signaling on forebrain development and regionalization. The method we chose was transgenic overexpression of Wnt7a, Wnt7b and later the PCP signaling component Vangl2, in neural progenitor cells. To our surprise we found drastic changes in early embryonic stages with a decreased body size, smaller forebrain structures and even neurulation defects starting from the midbrain region and further rostral. Analyses of Wnt7a transgenic embryos (paper I) showed that the neural tube adherens junctions, i.e. specialized tight junctions, were affected. This was manifested by decreased expression and impaired distribution of actin microfilaments, N-Cadherin and beta-catenin – the latter both a component of the cytoskeleton and a mediator of canonical Wnt signaling. Since PCP signaling acts on actin and the cytoskeleton, we investigated the downstream signaling components of this pathway and found an increased expression of Vangl2 and a misdistribution of Scribble1.

In our following study (paper IV) we confirmed and further described the neurulation defects, the deregulation of adherens junctions and the altered distribution of cytoskeletal components, by analyzing transgenic mouse embryos overexpressing Vangl2 and Vangl2 mutant (loop-tail) mouse embryos. We could demonstrate that Vangl2 targets the Rho family small G-protein Rac1 to sites of actin polymerization at the cell's adherens junctions.

It is known that Wnt signaling can affect the proliferation and differentiation of neural progenitor cells, and that beta-catenin signaling affects target genes that control these cellular processes. Analyses of the neuronal population of transgenic Wnt7a and Wnt7b embryos showed decreased numbers of newly born neurons (beta-tubulin III+ cells) and an aberrant positioning along their migrational axis (paper II), which we concluded to be due to a delay

in neuronal differentiation. This mechanism was corroborated and further investigated in Wnt7b transgenic embryos, which also displayed a decreased proportion of proneural transcription factors Tbr1 and Tbr2. However, the proportion of pax6 expression was similar to the wild type, suggesting an unaffected neural progenitor cell pool (paper III).

Finally, we set out to investigate the influence of Wnt/PCP signaling on pediatric tumor cell characteristics and viability (paper V). For long, the PCP signaling pathway has been considered to counteract parts of the canonical Wnt signaling pathway, acting in part as a tumor suppressor. In our study we characterized several medulloblastoma and neuroblastoma cell lines for their native expression of Wnt/PCP components. We found that an increase in PCP protein expression correlated with decreased levels of phosphorylated beta-catenin. In neuroblastoma cells, PCP gene knockdown increased, while PCP gene over-expression decreased tumor cell viability in an in vitro MTT assay. These results correlated well with clinical data and survival estimates from open access databases, including gene array data from several neuroblastoma patients.

In conclusion, the results presented in this thesis increase our knowledge on Wnt/PCP signaling and how it can affect the correct closure of the neural tube. Also, we discuss its influence on neural stem/progenitor cell behavior and differentiation, where imbalances on Wnt/PCP components have great implications on the characteristics and viability of pediatric tumors.

LIST OF SCIENTIFIC PAPERS

I. Shariatmadari M., Peyronnet J., **Papachristou P.**, Horn Z., Sousa KM., Arenas E., Ringstedt T. *Increased Wnt levels in the neural tube impair the function of adherens junctions during neurulation.* (Mol Cell Neurosci. 2005 Nov; 30(3):437-51)

II. Horn Z., **Papachristou P.,** Shariatmadari M., Peyronnet J., Eriksson B., Ringstedt T. *Wnt7a overexpression delays beta-tubulin III expression in transgenic mouse embryos.* (Brain Res. 2007 Jan 26;1130(1):67-72)

III. ***Papachristou P.**, *Dyberg C., Lindqvist M., Horn Z. and Ringstedt T. *Transgenic increase of Wnt7b in neural progenitor cells decreases expression of T-domain transcription factors and impairs neuronal differentiation* (Brain Res. 2014 Aug 12;1576:27-34)

IV. *Lindqvist M., *Horn Z., Bryja V., Shulte G., **Papachristou P.**, Ajima R., Dyberg C., Arenas E., Yamaguchi T., Lagercrantz H., Ringstedt T. *Vang-like protein 2 and Rac1 interact to regulate adherens junctions*. (J. Cell Sci. 2010 Feb 1;123(Pt 3):472-83)

V. *Dyberg C., ***Papachristou P.**, Thomas Ringstedt, Per Kogner, Hugo Lagercrantz, John-Inge Johnsen and Malin Wickström. *Planar cell polarity gene expression correlates with tumor cell viability and prognostic outcome in neuroblastoma* (Manuscript)

**denotes equal contribution*

CONTENTS

LIST OF ABBREVIATIONS

BrdU	bromo-deoxyuridine
ca	constitutive active
CNS	central nervous system
Dkk	dickkopf
dn	dominant negtive
DNA	deoxyribonucleic acid
E	embryonic day
Emx2	empty spiracles homeobox 2
eGFP	enhanced green fluorescent protein
FGF	fibroblast growth factor
Foxg1	forkhead box protein G1
Frz	frizzled
GABA	gamma-aminobutyric acid
GOF	gain-of-function
GTP	guanosine-5'-triphosphate
HEK	human embryonic kidney
IRES	internal ribosomal entry site
LOF	loss-of-function
MDCK	Madin-Darby canine kidney
mRNA	messenger RNA
NCS	neural stem cell
Ngn	neurogenin
ORF	open reading frame
Pax6	paired box 6
PCP	planar cell polarity
PCR	polymerase chain reaction
PFA	paraformaldehyde
PSB	pallial-subpallial boundary
RA	retinoic acid
Rac1	ras-related C3 botulinum toxin substrate 1

RhoA	ras homolog family member A
RNA	ribonucleic acid
RNAi	RNA interference
SFRP	secreted frizzled related protein
Shh	sonic hedgehog
Tbr1	T-box brain 1
Tbr2	T-box brain 2
UTP	uridine-5'-triphosphate
Vangl2	van-gogh like 2
VZ	ventricular zone
SVZ	subventricular zone
WIF-1	wnt-inhibitory factor-1
Wg	wingless
Wnt	wingless integration factor

SUMMARY OF THESIS - *SWEDISH*

Ökad kunskap kring de molekylärbiologiska mekanismer som styr utvecklingen av det centrala nervsystemet (CNS), däribland hur hjärnans olika regioner bildas, är viktig för att förstå uppkomsten av utvecklingsbiologiska och neuropsykiatriska störningar. Flera proteiner som styr planläggning av framtida regioner i fostrets hjärna är konserverade i ryggradsdjur - däribland medlemmar ur Wnt/PCP proteinfamiljen. I denna avhandling används så kallade transgentekniker som ändrar aktiviteten av dessa proteiner. Vi kan därmed studera hur de påverkar beteenden hos nervsystemets stamceller, som celldelning, cellmigration och celldifferentiering.

Forskning kring de proteiner som styr celldelning, utmognad och organisation av nervceller under hjärnans planläggning är viktig för att förstå uppkomsten av och förbättra framtida behandling av sjukdomar relaterade till hjärnans och nervsystemets utveckling.

Utvecklingen av CNS styrs via en komplex kommunikation mellan olika cellpopulationer. Specifika regioner i CNS utmärker sig med en unik uppsättning av genuttryck. Uttrycket hos vissa Wnt gener, t.ex. Wnt7b, är lokaliserat till specifika centra i den tidiga utvecklingen, vilka senare definierar och organiserar viktiga strukturer och framtida regioner i fostrets hjärna.

Det finns 19 olika Wnt gener hos ryggradsdjur, där flera förekommer i närliggande former (exempelvis Wnt5a/Wnt5b och Wnt7a/Wnt7b). De är först beskrivna som cancergener. Produkten av dessa Wnt gener - Wnt proteinerna - har en intercellulär kommunikationsväg, vilket påverkar celler i den närmsta omgivningen. Resultatet av Wnt signalen är *koncentrationsberoende*, och påverkas således av omkringliggande cellers avstånd till ursprungssignalen. Störningar i Wnt proteinernas signalvägar har bland annat visat sig påverka anläggningen av kroppsorgan (som njurar, lungor, extremiteter och delar av hjärnan), utmognaden av nervsystemet, samt uppkomsten av cancer.

Wnt proteinerna är så kallade *extra*cellulära ligander och för att kommunicera sin verkan till andra, närliggande, celler behöver de binda till en receptor - i det här fallet receptorer ur Frizzled (frz) familjen. Wnt liganderna binder till frz receptorer med varierande specificitet. Först efter en korrekt interaktion mellan ligand och receptor kan den *intra*cellulära signalvägen aktiveras, vilket ofta sker i flera steg och skapar på så sätt en slags förstärkning eller amplifiering av den ursprungliga signalen.

Den först beskrivna och bäst utredda intracellulära signalvägen för en Wnt-frz interaktion benämns den kanoniska vägen eller ”*canonical signaling pathway*” och går via stabilisering av proteinet beta-catenin. Inne i cellen ansvarar ett större proteinkomplex för den kontinuerliga nedbrytningen av fritt beta-catenin, vilket förhindrar vidare signalering. När en

frz receptor aktiveras av en Wnt ligand skickar den en signal som hämmar proteinkomplexets aktivitet. Därmed kan det uppstå ett överskott av fritt beta-catenin inne i cellen, som då får möjlighet att ta sig vidare in till cellkärnan. Väl där inne kan det, via specialiserade proteiner som reglerar genuttryck (transkriptionsfaktorer ur Tcf/LEF familjen), utöva sin verkan och aktivera uttryck (transkription) av målgener. Denna kanoniska signalväg har visat sig vara överaktiv i flera cancersjukdomar. Det finns även ytterligare en eller två signalvägar (det är osäkert om de utgör en och samma eller två skilda signalvägar). Dessa så kallade icke-kanoniska eller ”*non-canonical signaling pathways*” går via proteiner som Jun-kinase och/ eller Ca^{2+}-joner och reglerar inte främst genuttryck utan istället cellens cytoskelett, vilket påverkar cellmotilitet och cellmigration. Gemensamt för samtliga signalvägar är proteinet Dishevelled (dsh), som likt ett växelspår skiftar mellan de olika signalvägarna beroende på kombinationen Wnt ligand – frz receptor. Andra proteiner än Wnt som påverkar ”non-canonical” signalering är PCP (Planar Cell Polarity) proteinerna. Dessa ansvarar för att upprätthålla polaritet och asymmetri i cellerna genom interaktion med cytoskelettet under embryots och fostrets organutveckling.

Studier av genfunktion hos möss med s.k. knock-out teknik har lett till intressanta resultat. Bland annat utvecklar Wnt1-defekta möss ingen mellanhjärna, medan de som saknar Wnt7b dör tidigt under fosterutvecklingen eftersom placentan och lungorna utvecklas felaktigt. Olika Wnt gener såsom Wnt3, Wnt7b och Wnt8b uppvisar specifika uttrycksmönster under hjärnans tidiga utveckling. Det ligger därför nära till hands att anta att de har betydelse för hjärnans regionalisering.

I syfte att öka kunskapen om Wnt och PCP proteinernas betydelse för hjärnans tidiga utveckling, har vi studerat transgena musembryon. De benämns transgena då vi genom molekylärbiologiska gentekniker tillfört extra genetiskt material som tillåter ett högre genuttryck av Wnt eller PCP proteiner i neuronala stam-/progenitorceller under den tidiga hjärnutvecklingen.

Analys av transgena Wnt7a och Wnt7b embryon vid 10,5 dagars embryonal ålder (E10.5) visar på en störd utmognad av nya nervceller utan att påverka celldelning (proliferation) eller programmerad celldöd (apoptos). De transgena embryona har ett utseende (fenotyp) med förkrympta hjärnstrukturer. I de allra flesta fall har deras neuralrör inte slutit sig som det ska. I våra experiment har vi kunnat visa att slutningsdefekten beror på en störd reglering av cellernas förmåga att binda till varandra. Denna styrs av en specialiserad del av cytoskelettet som benämns ”*adherens junctions*”, och har stor betydelse för korrekt slutning av neuralröret.

Vi har dragit slutsatsen att ökat genuttryck av Wnt7a i neuronala progenitorer leder till en försenad nybildning av nervceller och en störd funktion hos neuralrörets cytoskelett, vilket leder till en slutningsdefekt av neuralröret (*artikel I-II*). Vidare har vi kunnat bekräfta en försening av nybildningen av nervceller i Wnt7b transgena embryon. Den beror delvis på ett minskat uttryck av nervcellsspecifika transkriptionsfaktorer, vilka ska ansvara för att öka produktionen av nervceller vid en viss korrekt tidpunkt under den normala utvecklingen (*artikel III*).

Förändringar i PCP signalering leder ofta till felaktig reglering av cytoskelettets funktion, vilket påverkar celladhesion och cellmigration. Hos transgena Wnt7a embryon kunde vi även visa på en ökad aktivitet hos Vangl2 genen samt felaktig lokalisering av proteinet Scribble1 i neuralröret (bägge två proteiner av PCP typ). Genom studier av ökat eller minskat Vangl2 uttryck i transgena möss samt liknande förändringar i cellkultur, kunde vi bekräfta tidigare resultat med Wnt7a samt föreslå en mekanism (se Fig. 3) där Vangl2 proteinet påverkar integriteten av cytoskelettet, celladhesion och migration. Detta kunde vidare kopplas till en protein-protein interaktion och samlokalisering med proteinet Rac1, som har en viktig roll för styrningen av cytoskelettets funktion (*artikel IV*).

Utveckling av cancer utgår från förändringar i normala stamceller. Det är känt att Wnt/PCP signalering har en betydande roll vid cancerutveckling. PCP och ”*non-canonical*” signalering är även känd som en motpol till Wnt och ”*canonical*” signalering – där komponenter ur PCP signaleringsvägen visat sig ha cancermotverkande egenskaper. En ökad förståelse av hur cancerceller avviker från den normala utvecklingen ger stora möjligheter för en bättre sjukdomsprognos och forskning inom Wnt/PCP signalering kan lyfta fram nya angreppssätt i jakten på en bättre behandling till flera cancersjukdomar.

I det sista delarbetet valde vi att analysera uttrycksmönster för olika PCP proteiner i två av de vanligaste barncancerformerna, med ursprung i neuronal vävnad – medulloblastom och neuroblastom. Vi studerade flera cellinjer av medullo- och neuroblastom och kunde visa att en ökad PCP aktivitet korrelerar med minskad överlevnad av tumörceller i cellkultur. Likaså var en minskat PCP aktivitet korrelerat till ökad överlevnad av tumörceller i cellkultur (*artikel V*). Våra resultat överensstämmer med statistiska överlevnadsdata hämtade ur öppna forskningsdatabaser, där neuroblastomceller från olika patienter analyserats med *gene array expression* (analysmetod för tusentals olika gener). Statistiskt validerad data visade på en fördelaktigare prognos och en ökad överlevnad 5 år efter diagnos hos de neuroblastompatienter med ett högre uttryck av PCP gener.

Sammanfattningsvis kommer forskningen kring Wnt/PCP signalvägar utgöra en viktig grund för framtida diagnostik och behandling av flera sjukdomar, framför allt genom en ökad förståelse av hur dessa proteiner påverkar stamceller – både vid normal organbildning och vid cancerutveckling.

SUMMARY OF THESIS - *GREEK*

Η γνώση των μοριακών μηχανισμών που ελέγχουν την ανάπτυξη του εμβρύου, χρησιμεύει στην κατανόηση των διαταραχών και των νόσων που θα εκδηλωθούν στο μελλοντικό κεντρικό νευρικό σύστημα (ΚΝΣ). Υπάρχουν πολλά σηματοδοτικά μοριακά μονοπάτια εμπλεκόμενα στο σχεδιασμό των περιοχών του εγκεφάλου του εμβρύου. Μέσα σε αυτά ανήκει και η ομάδα Wnt/PCP. Στην πάρουσα διδακτορική διατριβή μελετήσαμε με τη χρήση διαγονιδιακών τεχνικών, τις αλλαγές στην έκφραση των γονιδίων Wnt/PCP και το πως αυτές επηρεάζουν τη συμπεριφορά των βλαστικών κυττάρων και την εξέλειξη του ΚΝΣ. Επιπλέον, εξετάσαμε την κυτταρική διαίρεση, την διαφοροποίηση και την μετανάστευση των βλαστικών κυττάρων, που βοηθούν στην κατανόηση της αιτιολογίας και της μελλοντικής θεραπείας ασθενειών που σχετίζονται και με την εξέλιξη του εγκεφάλου και μορφών καρκίνων.

Η ανάπτυξη του ΚΝΣ ελέγχεται από μία πολύπλοκη επικοινωνία διαφορετικών κυτταρικών πληθυσμών. Συγκεκριμένες περιοχές του ΚΝΣ χαρακτηρίζονται από ένα μοναδικό μοτίβο γονιδιακής έκφρασης της ομάδας Wnt. Οι πρωτεΐνες αυτές είναι εντοπισμένες σε συγκεκριμένα κέντρα του εμβρύου στην πρώιμη ανάπτυξη, τα οποία καθορίζουν και οργανώνουν σημαντικές δομές και περιοχές του αναπτυσόμενου εγκεφάλου.

Μεχρι στιγμής είναι γνωστά 19 διαφορετικά γονίδια *Wnt* (στα σπονδυλωτά) πολλά άπο τα οποία έχουν παρόμοιες μορφές (για παράδειγμα, *Wnt5a/Wnt5b* ή *Wnt7a/Wnt7b*). Τα *Wnt* έχουν περιγραφεί αρχικά ως γονίδια σχετιζόμενα με τον καρκίνο. Η μεταγραφή και μετάφραση των γονιδίων *Wnt* έχει ως αποτέλεσμα τα μόρια ή πρωτεΐνες Wnt. Η δράση των πρωτεϊνών Wnt γίνεται μέσω διακυτταρικής επικοινωνίας και επηρεάζει τα κύτταρα του αμέσου περιβάλλοντος. Διαταραχές στη δράση μοριακής σηματοδότησης των Wnt επηρεάζουν την ανάπτυξη των διάφορων οργάνων του σώματος (όπως τα νεφρά, τους πνεύμονες, τα άκρα και διάφορα τμήματα του εγκεφάλου). Επίσης επηρεάζουν την ωρίμανση του νευραλογικού συστήματος και την ανάπτυξη και εξέλιξη καρκίνων.

Οι πρωτεΐνες Wnt είναι *εξω*κυτταρικά μόρια ή δεσμευτές και πρέπει να δεσμευτούν σε ένα συγκεκριμένο υποδοχέα για να επικοινωνήσουν αποτελεσματικά με τα γειτονικά κύτταρα. Η συγκεκριμένη ομάδα υποδοχέων ονομάζεται *Frizzled* (frz) και αποτελείται από δέκα μέλη. Τα διάφορα Wnt προσδένονται με συγκεκριμένους frz υποδοχείς με διαφορετικά αποτελέσματα. Μετά από μια σωστή αλληλεπίδραση μεταξύ Wnt και frz, μπορεί να ενεργοποιηθούν οι *ενδο*κυτταρικές σηματοδοτήσεις.

Η πρώτη και καλύτερα διερευνημένη ενδοκυτταρική σηματοδότηση της αλληλεπίδρασης Wnt-frz ονομάζεται «κανονική» (*canonical Wnt signaling pathway*). Καταλήγει στην σταθεροποίηση της πρωτεΐνης β-κατενίνης. Ένα σύμπλεγμα πρωτεΐνων διασπά συνεχώς την ελεύθερη κυτταροπλασματική β-κατενίνη. Όταν ενεργοποιηθεί σωστά ο κατάλληλος

υποδοχέας frz, τότε στέλνεται ένα σήμα που αναστέλλει την δραστηριότητα του συμπλέγματος με αποτέλεσμα να αυξάνονται τα επίπεδα της β-κατενίνης η οποία στη συνέχεια μπορεί να εισέλθει στον πυρήνα του κυττάρου. Εκεί, μέσω των μεταγραφικών παραγόντων Tcf/LEF, μπορεί να ενεργοποιηθεί η μεταγραφή γονιδίων στόχων. Η «κανονική» σηματοδότηση έχει αποδειχθεί ότι υπερλειτουργεί σε διάφορες μορφές καρκίνου.

Υπάρχει επίσης τουλάχιστον μια πρόσθετη μορφή σηματοδότησης που ονομάζεται «μη κανονική» (*non-canonical Wnt signaling pathway*). Λειτουργεί μέσω αλληλεπιδράσεων με διάφορες πρωτεΐνες όπως την Rac1 και την Jun-kinase ή και μέσω αλλαγών της συγκέντρωσης των ενδοκυτταρικών ιόντων ασβεστίου (Ca^{2+}).

Κοινό στοιχείο για όλους τους τρόπους σηματοδότησης Wnt είναι η πρωτεΐνη Dishevelled (dsh), η οποία λειτουργεί ως διακόπτης και μεταφέρει, ανάλογα με την αλληλεπίδραση Wnt-frz, την κάθετη ενδοκυτταρική σηματοδότηση. Μια άλλη ομάδα πρωτεΐνων που εμπλέκονται και επηρεάζονται από την «μη κανονική» σηματοδότηση, ονομάζεται PCP (Planar Cell Polarity proteins). Αυτές οι πρωτεΐνες είναι υπεύθυνες για την διατήρηση της κυτταρικής πολικότητας και ασυμμετρίας μέσα από την αλληλεπίδρασή τους με τον κυτταροσκελετό.

Πολλές μελέτες για το ρόλο των Wnt έχουν γίνει με τη χρήση διαγονιδιακών ζώων με εξαλείψεις συγκεκριμένων γονιδίων *Wnt* (νοκ-άουτ). Γιά παράδειγμα, δεν αναπτύσεται ο μεσεγκέφαλος (*midbrain*) στα ποντίκια με εξάλειψη Wnt1, ενώ στα ποντίκια με εξάλειψη Wnt7b υπάρχει πρόωρος θάνατος κατά τη διάρκεια της εμβρυογένεσης λόγω λανθασμένης ανάπτυξης του πλακούντα και του πνεύμονα. Διάφορα γονίδια *Wnt* όπως τα *Wnt3*, *Wnt7b* και *Wnt8b* παρουσιάζουν μοναδικές μορφές έκφρασης κατά την πρώιμη ανάπτυξη του εγκεφάλου. Ως εκ τούτου, μπορούμε να υποθέσουμε ότι έχουν σημασία στην περιφερειακή ανάπτυξη του εγκεφάλου.

Για να αποκτήσουμε περισσότερες γνώσεις σχετικά με τη λειτουργία και τη σημασία των πρωτεΐνων Wnt και PCP στην πρώιμη ανάπτυξη του εγκεφάλου, μελετήσαμε διαγονιδιακά έμβρυα ποντικιού. Ονομάζονται διαγονιδιακά διότι με τεχνικές μοριακής γενετικής έχουμε προσθέσει επιπλέον γενετικό υλικό το οποίο επιτρέπει αυξημένη έκφραση των γονιδίων *Wnt* (*Wnt7a* και *Wnt7b*) ή *PCP* (*Vangl2*) μέσα στα νευρικά βλαστικά κύτταρα.

Στην πρώτη φάση της μελέτης η ανάλυση διαγονιδιακών εμβρύων Wnt7a και Wnt7b, ηλικίας 10,5 ημερών, παρουσίασε μια διαταραγμένη ωρίμανση νέων νευρώνων (*beta-tubulin-III*$^{+}$) χωρίς καμία επίδραση στην διαίρεση των κυττάρων ή στον προγραμματισμένο κυτταρικό θάνατο (απόπτωση). Στην εμφάνισή τους, τα διαγονιδιακά εμβρύα είχαν υποανάπτυκτες εγκεφαλικές δομές και στις περισσότερες περιπτώσεις μία ελαττωματική ολοκλήρωση του νευρικού σωλήνα. Αποδείξαμε ότι η ακεραιότητα του νευρικού σωλήνα εξαρτάται από τη διαταραγμένη ρύθμιση της κυτταρικής προσκόλλησης σε ένα εξειδικευμένο τμήμα του

κυτταροσκελετού που ονομάζονται ζώνες προσκόλλησης (*adherens junctions*), οι οποίες συνεισφέρουν στη σωστή ολοκλήρωση του νευρικού σωλήνα.

Είδαμε ότι η αυξημένη έκφραση της Wnt7a στα νευρικά βλαστικά κύτταρα οδηγεί σε καθυστέρηση της νευρογένεσης και στην διαταραγμένη λειτουργία του κυτταροσκελετού, κάτι που επηρεάζει την ολοκλήρωση του νευρικού σωλήνα (άρθρο I και II). Επιπλέον, έχουμε περιγράψει πως η καθυστέρηση του σχηματισμού νέων νευρικών κυττάρων στα διαγονιδιακά έμβρυα Wnt7b οφείλεται εν μέρει στην γενική μείωση των μεταγραφικών παραγόντων *Tbr-1* και *Tbr-2*, οι οποίοι είναι υπεύθυνοι για την αύξηση της μεταγραφής συγκεκριμένων γονιδίων που προάγουν την παραγωγή νέων νευρώνων (άρθρο III).

Οι αλλαγές στην σηματοδότηση μέσα από τα PCP οδηγούν συχνά στην απορρύθμιση της λειτουργίας του κυτταροσκελετού, η οποία επηρεάζει την κυτταρική προσκόλληση και μετακίνηση-μετανάστευση. Στα διαγονιδιακά έμβρυα Wnt7a εντοπίσαμε μία αυξημένη έκφραση της Vangl2 και έναν ανακριβή εντοπισμό της Scribble1 (και οι δύο είναι πρωτεΐνες τύπου PCP). Μέσω μελετών αυξημένης ή μειωμένης έκφρασης της Vangl2 σε διαγονιδιακά ποντίκια, καθώς επίσης μέσα από κυτταρικές καλλιέργειες, καταλήξαμε στο συμπέρασμα ότι η πρωτεΐνη Vangl2, όπως και η Wnt7a, επηρεάζει την ακεραιότητα του κυτταροσκελετού, την κυτταρική προσκόλληση, την μετανάστευση και την ολοκλήρωση του νευρικού σωλήνα. Εντοπίσαμε ένα κοινό στόχο της Vangl2, την πρωτεΐνη Rac1. Στην εικόνα 3 περιγράφεται ένα μοντέλο δράσης που εξηγεί την σωστή λειτουργία μεταξύ των δύο πρωτεΐνων, του κυτταροσκελετού και την διαρύθμιση των ζωνών προσκόλλησης (άρθρο IV).

Η ανάπτυξη και εξέλιξη του καρκίνου βασίζεται στις γονιδιακές μεταλλάξεις σε φυσιολογικά βλαστικά κύτταρα τα οποία εντοπίζονται σε όργανα του σώματος με εγγενή αναγέννηση. Είναι γνωστό ότι η «κανονική» σηματοδότηση Wnt/PCP παίζει σημαντικό ρόλο στην ανάπτυξη του καρκίνου. Οι πρωτεΐνες PCP και η «μη κανονική» σηματοδότηση μπορούν να δράσουν αντίθετα με την «κανονική» σηματοδότηση και ως εκ τούτου διαθέτουν αντικαρκινικές ιδιότητες. Η κατανόηση για το πώς τα καρκινικά κύτταρα διαφέρουν από την κανονική ανάπτυξη, μας δίνει την ευκαιρία για μία καλύτερη πρόγνωση του καρκίνου. Η έρευνα στον τομέα σηματοδότησης Wnt/PCP μπορεί να προσφέρει νέες προσεγγίσεις στην ανάπτυξη αποτελεσματικής θεραπείας ενάντι πολλών μορφών καρκίνου.

Στην τελευταία μελέτη μας, επιλέξαμε να εξερευνήσουμε την πρότυπη έκφρασης μορίων PCP στις δύο πιο κοινές μορφές του νευρολογικού καρκίνου της παιδικής ηλικίας, τα μυελοβλαστώματα και τα νευροβλαστώματα. Μελετήσαμε διαφορετικές κυτταρικές σειρές (από διάφορους ασθενείς) με μυελοβλαστώματα και νευροβλαστώματα μερικά από τα οποία είχαν αυξημένη έκφραση πρωτεΐνων PCP, η οποία συσχετίστηκε με μειωμένη επιβίωση των καρκινικών κυττάρων μέσα σε κυτταρική καλλιέργεια (άρθρο V). Τα αποτελέσματά μας ήταν σύμφωνα με τα στατιστικά δεδομένα μία ανοιχτής επιστημονικής βάσης δεδομένων (*R2 database*). Αυτή περιέχει διάφορες κυτταρικές σειρές, συμπεριλαμβανόμενων και των

νευροβλαστωμάτων, οι οποίες έχουν μελετηθεί με την μέθοδο ανάλυσης έκφρασης γονιδίων σε συστοιχίες (*gene array*). Τα νευροβλαστώματα με αυξημένη έκφραση γονιδίων PCP είχαν καλύτερη και πιο ευνοϊκή πρόγνωση επιβίωσης πέντε χρόνια μετά την διάγνωση.

Ολοκληρώνοντας, η ομαλή σηματοδότηση Wnt/PCP οδηγεί στην φυσιολογική ανάπτυξη του ΚΝΣ, ενώ διαταραχές στη σηματοδότηση αυτή επηρεάζουν αρνητικά την ανάπτυξη του εγκεφάλου. Η διαταραγμένη σηματοδότηση ενδέχεται να είναι συνυπεύθυνη για την αρνητική εξέλιξη νευρολογικών νόσων ή και στην νοητική υστέρηση στα παιδιά. Επίσης δεν πρέπει να μας διαφεύγει ότι τα *Wnt* είναι γνωστά ως ογκογονίδια οπότε η κατανόηση των διαφόρων τρόπων σηματοδότησης Wnt/PCP έχει μεγάλη σημασία για την κατανόηση της καρκινογένεσης, αλλά και αποτελεί μια σημαντική βάση για την συνεχή έρευνα πάνω στα βλαστικά κύτταρα.

BACKGROUND

DEVELOPMENT OF THE CENTRAL NERVOUS SYSTEM

Gastrulation and neurulation

Development of a mammalian embryo is dependent on the highly coordinated organization of groups of cells and their internal communication. After implantation of the blastocyst embryo, epiblast cells derived from the inner cell mass undergo dramatic morphological changes in response to intrinsic and extrinsic (from the trophoblast cells) signals. During this gastrulation process, three different germ layers (endoderm, mesoderm and ectoderm) are formed (Tam and Loebel, 2007). The induction of the ectoderm by underlying signals from mesoderm and rostral endoderm will produce a neural plate, a thickened, pseudostratified region with a unique set of molecular markers. Proliferation and cellular movements will elongate and narrow the embryo in a process termed convergent extension. Cellular changes in the midline and the lateral parts of the neural plate will cause the bending of the plate at the midline and the elevation of the lateral edges, which will eventually meet and fuse in a process termed neurulation to produce the neural tube (Vieira et al., 2010; Copp et al., 2003; Smith and Schoenwolf, 1997).

Induction and regionalization

The rostral part of the neural tube will give rise to the forebrain, midbrain and hindbrain whereas the caudal part will give rise to the spinal cord. Patterning along the rostro-caudal and dorso-ventral axes is accomplished by the restricted expression of transcription factors and secreted molecules in the developing CNS, which outline the different subdivisions of the future brain. *Fibroblast growth factor* (FGF), *retinoic acid* (RA) and *Wnt* signals are responsible for inducing dorsal neural character whereas *Shh* is responsible for the ventral neural character (Guérout et al., 2014; Vieira et al., 2010).

Corticogenesis

All higher mammalian functions reside in the cerebral cortex, the most superficial part of the telencephalon where the projection neurons and interneurons have their soma. The cerebral cortex develops in an inside-out fashion into a six-layered structure where early born neurons reside in the deep layers (V & VI; and also layer I) and later ones born in more superficial layers (II & III).

In mice, the first cortical neurons generate the preplate structure during midgestation. The preplate will then split and give rise to the marginal zone (layer I) and the subplate. Specialized cells of the marginal zone, called Cajal-Retzius cells, will secrete Reelin protein which is necessary for the subsequent inside-out development of the cortical layers. The two main classes of cortical neurons are the GABA-ergic interneurons and the Glutamatergic projection neurons. The vast majority of cortical interneurons in rodents migrate tangentially from the ventral telencephalon to populate the developing cortex. The projection neurons are exclusively derived from the dorsal telencephalon and migrate radially into the cortical plate.

The position of each neuron and its function are regulated by complex intrinsic mechanisms, which in turn regulate the location in the germinal zone and the timing of cell cycle exit of all cortical progenitor cells. Expression of different intrinsic programs are controlled by combinatorial expression of factors like Foxg1 (Hanashima, 2004), Ngn1 & 2 (Schuurmans, 2004), Tbr1 (Hevner, 2001), Tbr2 (Sessa, 2008) and pax6 (Englund, 2005) which are required for proper corticogenesis (reviewed in Campell, 2005; Marin and Rubenstein, 2001).

THE CYTOSKELETON

The cytoskeleton is an intracellular scaffold that not only provides cell shape, stability and rigidity, but is also a dynamic structure responsible for cell motility, cell polarity and cellular differentiation (Stiess and Bradke, 2011). It is subdivided into three major components - microtubules, intermediate filaments and microfilaments - responsible for separate functions within the cell. The microtubules consist of hollow cylinders and have important cellular functions within intracellular transportation of molecules and the arrangement of the mitotic spindle at cell division (Poulain and Sobel, 2010). The intermediate filaments are rope-like structures that provide mechanical strength to the cells (Yuan et al., 2012). The microfilaments consist of actin filaments, concentrated at the cells periphery or cortex, and confer changes in cell shape and motility (Luo, 2002). Microtubules and microfilaments are composed of repeating numbers of smaller subunits and can rapidly undergo rearrangements in response to the cell's intrinsic and extrinsic environment. The dynamics of the cytoskeletal system is maintained by accessory proteins that control the assembly of the cytoskeletal protein subunits and their transportation to an active site of cytoskeletal polymerization.

Adherens junctions

The cytoskeleton makes contact with the extracellular environment via anchoring proteins within the plasma membrane. The cadherins belong to the transmembrane adhesion protein family that mechanically attaches individual cells to each other or to the extracellular matrix.

Adherens junctions are specialized adhesive structures that connect cadherins with the actin microfilaments via alpha-catenin and beta-catenin, thereby connecting the cytoskeleton of neighboring cells (Harris and Tepass, 2010). In the neural tube, adherens junctions are present as ring-like structures surrounding the apical end of the cells, which face the lumen of the neural tube. During nervous system development, defects in the integrity of adherens junctions lead to defects in cranial neural tube closure (Ybot-Gonzalez and Copp, 1999).

WNT SIGNALING

The Wnt gene family encodes secreted lipid-modified glycoproteins and constitute important intercellular signaling molecules involved in embryonic development. In humans, 19 Wnt genes have been identified and many of these appear in homologous forms like Wnt5a/5b and Wnt7a/7b (Miller, 2002). The Wnt genes are closely related to the Drosophila Wingless gene (Wg). Wnt1 is the most studied member of the Wnt family. It was originally isolated as a proto-oncogene in mouse (Nusse and Varmus, 1982). During development, Wnts have diverse roles in governing cell fate (Castelo-Branco et al., 2003), proliferation (Rajagopal et al., 2008), differentiation (Rosso et al., 2005) and axonal migration (Zaghetto et al., 2007). Knocking out Wnt genes in mice have resulted in early morphogenetic effects. Wnt1 knockout mice (Wnt1 -/-) do not develop a midbrain and cerebellar structures, Wnt4a -/- mice lack kidneys and Wnt7a -/- mice have ventralized limbs (Miller, 2002).

The Wnt ligands bind to cell-surface receptors of the Frizzled (Frz) family together with LDL-receptor-related protein (LRP5/6) and Ryk co-receptors (Huelsken and Behrens, 2002). The Frz receptors comprise a large family of transmembrane receptors (10 members are known to date), where the general structure resembles that of G-protein-coupled receptors (Fanto and McNeill, 2004). Wnt signaling is modulated in the extracellular space by diverse secreted proteins which either bind directly to Wnts or prevent their interaction with the Frz receptors. These Wnt inhibitors include Secreted frizzled related proteins (SFRP), Wnt-inhibitory factor-1 (WIF-1), Cerberus and Dickkopf (Dkk) (Fanto and McNeill, 2004; Miller, 2002; Cadigan and Nusse, 1997).

The Wnts act through distinct signaling pathways. The most studied pathway is often referred to as the canonical pathway. All other pathways are referred to as non-canonical pathways (Fanto and McNeill, 2004; Veeman et al., 2003; Cong et al., 2003; Miller, 2002; Cadigan and Nusse, 1997).

Signaling through the canonical Wnt pathway depends on the level of beta-catenin in the cell. In the absence of Wnt, beta-catenin is kept low because of phosphorylation-dependent ubiquitination/proteasome degradation. Wnt signaling prevents the phosphorylation of beta-catenin leading to its accumulation in the cytoplasm. The accumulated beta-catenin translocates to the nucleus where it interacts with members of the Tcf/LEF family of transcription factors, thereby activating target genes (Seto and Bellen, 2004). Dishevelled (dsh) is an essential intracellular protein in the Wnt signaling cascade. It is a key player as it responds to different Wnt and Frz signals by shuttling from intracellular vesicle compartments to the cell membrane, acting like an on/off switch between the canonical and non-canonical pathway (Pandur et al., 2002). The Wnt/Ca^{2+} and the Wnt/planar cell polarity (PCP) pathway are two of the non- canonical Wnt signaling pathways (Cong et al., 2003; Montcouqiuol et al., 2003; Pandur et al., 2002; Kühl, 2002) . In the Wnt /Ca^{2+} pathway dsh stimulates calcium flux and activation of the Ca^{2+}-sensitive enzymes such as protein kinase C (PKC), calmodulin-dependent kinase II (CamKII) and calcineurin (Cong et al., 2003; Kühl, 2002). The Wnt/PCP pathway act on the cytoskeleton and reorganize it, thereby affecting convergence and extension movements. Activation of Jun-N-terminal kinase (JNK) and asymmetrical expression of cell membrane bound proteins like Vangl2 are parts of the mechanism (Bastock et al., 2003; Cong et al., 2003; Montcouqiuol et al., 2003; Veeman et al., 2003).

Planar cell polarity signaling

The Planar Cell Polarity (PCP) proteins establish polarity within a tissue plane by asymmetric expression of core proteins such as Vangl2, Prickle1 and Celsr1. They are involved in early cell movements in gastrulation and neurulation and hence have a regulatory effect on the cytoskeleton. Mutations in PCP genes result in disturbed convergent-extension movements and neurulation defects. Mutants also lack the proper localization of the other wild type PCP core proteins, reflecting an interdependence of asymmetrical localization of all PCP components within the cell (Gray et al., 2011; Goodrich, 2008). This is evident in the naturally occurring Vangl2 mutant mouse (*Looptail* mouse) that does not only display neurulation defects but also an aberrant distribution of PCP components in the polarized mammalian inner ear (Montcouquiol et al., 2003).

Wnt signaling in the developing CNS

The Wnt genes 1, 3, 3a, 5, 5a, 7a, 7b and 8b are expressed in unique patterns in the developing brain. Wnt genes 3, 3a, 7b and 8b exhibit sharp boundaries of expression in the forebrain that may predict subdivisions of the region later in development (Parr et al., 1993). In the spinal cord there is also a difference in Wnt expression patterns between dorsal and ventral regions (Garda et al., 2002; Lee et al., 2000).

The developing CNS comprises different patterning centers consisting of groups of cells with organizer-like properties that produce signals which influence the fate, histogenic organization and growth of adjacent tissues, resulting in spatial patterning. Wnt7b, Wnt8b and Wnt3 are expressed in such patterning centers, including the pallial-subpallial boundary (PSB) and the cortical hem suggesting a possible role in demarcating and specifying regions in the developing brain (Garda et al., 2002; Kim et al., 2001; Lee et al., 2000).

Wnt7a is first expressed in the CNS at E9.5, when expression in the ventral brain and spinal cord begins (Parr et al., 1993). Wnt7a expression has also been observed in the telencephalon (Grove et al., 1998). Wnt7a has been demonstrated to activate both canonical (Hirabayashi et al., 2004; Caricasole et al., 2003; Lucas and Salinas, 1997) and non-canonical (PCP) (Shariatmadari et al., 2005; Dabdoub et al., 2003; Kengaku et al., 1998) signaling. In E10.5 mouse embryos, Wnt7a has been shown to suppress cell cycle exit (Viti et al., 2003). However, in E11.5 embryos, Wnt7a can direct the neuronal differentiation of cortical precursors (Hirabayashi et al., 2004). Wnt7a mutant mice do not display any gross brain abnormalities, but the formation of synaptic contacts between mossy fibers and granule cells in the cerebellum is delayed (Hall et al., 2000).

During vertebrate embryonic development Wnt7b is expressed in the kidneys, lungs and the CNS (Shu et al., 2002). Wnt7b is also expressed in the chorion where it is required for fusion of the chorion and allantois during placental development. Knock-out mice lacking the third exon of Wnt7b die in midgestation (before embryonic day 10.5; E10.5) due to placental abnormalities (Parr et al., 2001). Another homozygous Wnt7b mutant, with the first exon replaced by lacZ, dies at birth due to respiratory failure (Shu et al., 2002). In conditional Wnt7b knock-out mice (Nes-Cre/Sox2-Cre), it was shown that Wnt7b together with Wnt7a regulate angiogenesis in the CNS (Stenman, 2008).

Previous studies have revealed important roles for Wnt7b in patterning and specification of cells in the CNS. Pax-6 mutant mice have defects forming the pallial-subpallial boundary (PSB). These mice lack expression of secreted frizzled related protein-2 (SFRP-2) and Wnt7b at the PSB and lose SFRP-2 expression in the diencephalon (Kim et al., 2001). Wnt7b is also expressed in specific subsets of mediolateral (M-L) clusters of the cerebellar cortex. It has been suggested that expression of Engrailed-2 (En-2) or Wnt7b in the newly

born Purkinje cells (PC) defines a specific subset of M-L clusters (Millen et al., 1995; Hashimoto and Mikoshiba, 2003). Other experiments have demonstrated that Wnt7b affects proliferation of CNS progenitor cells and that FGF2 or Sonic hedgehog (Shh) mediates the effect of Wnt molecules on progenitor cell maturation from the ventricular zone (VZ) to subventricular zone (SVZ) progenitors (Viti et al., 2003). It is also possible that Wnt7b regulates the expression of Emx2, a homeobox gene with restricted spatial expression throughout the dorsal telencephalon (Theil et al., 2002). It has also been shown that Wnt7b can regulate neuronal branching and increase dendritic development by activating the non-canonical signaling pathway (Rosso et al., 2005).

Wnt/PCP signaling in pediatric tumors

Increased Wnt/beta-catenin signaling is found in many forms of cancer (Katoh, 2005), including childhood cancers medullo- and neuroblastoma, where it is associated with upregulation of proliferative genes. Medulloblastoma is the most frequent malignant brain tumor in childhood and 10-15% of medulloblastomas show strong nuclear beta-catenin staining (Fattet et al., 2009; Eberhart et al., 2000). In about 22% of these, mutations have been found in the beta-catenin signaling pathway (Taylor et a., 2012; Giles et al., 2003; Koesters and von Knebel Doeberitz, 2003). Neuroblastoma is a frequently lethal childhood tumor and a subset of neuroblastomas display amplification of the MYCN proto-oncogene, which accounts for a more aggressive phenotype. However, high-risk neuroblastomas without MYCN amplification display increased levels of nuclear beta-catenin. This correlated with an increased expression of MYC and other proto-oncogenes, that are targets of the canonical Wnt signaling pathway (Liu et al., 2008).

AIM

The main objective of this thesis was to characterize the function of Wnt/PCP signaling in early CNS development utilizing transgenic overexpression of Wnt and PCP genes. Also, we wanted to investigate how Wnt/PCP signaling interacted and interfered with tumor cell properties.

Specific aims of the studies were:

- To investigate the effects of Wnt signaling on neuronal progenitor cell differentiation in early CNS development.

- To investigate the mechanisms whereby Wnt/PCP signaling interact with the cytoskeleton and regulate cell adhesion, the formation of adherens junctions and neural tube closure.

- To investigate PCP signaling in pediatric tumor cell lines and its effect on tumor cell viability.

METHODOLOGY

In this section, a brief summary and aspects of the methodology will be highlighted. Detailed information about each specific methods used can be found in the papers as listed below.

• Generation of DNA expression constructs	*Papers I - V*
• Generation of transgenic and mutant embryos	*Papers I - V*
• Phenotyping and genotyping	*Papers I - V*
• Immunohistochemistry	*Papers I - V*
• In situ hybridization	*Paper I - IV*
• Cell culturing and transfections	*Papers IV & V*
• Immunocytochemistry	*Papers IV & V*
• Aggregation and wound assays	*Paper IV*
• Immunoprecipitation and GTPase activity assay	*Papers IV & V*
• Western blotting	*Papers IV & V*
• Fluorescence and confocal microscopy	*Papers I - IV*
• Viability (MTT) assay	*Paper V*
• Luciferase assay	*Paper V*
• quantitative *real-time* PCR	*Paper V*
• Bioinformatics	*Paper V*

IN VIVO AND IN VITRO MODELS

Transgenic mouse embryos

The Wnt genes seem to have important roles as determinants of cell proliferation and differentiation during the development of CNS. Several studies have used the knockout technique to describe different gene functions. But there is a limitation to this technique. Many genes involved in animal embryogenesis are used over and over again during development. If these genes are deleted, the early effects on pattern formation, proliferation or cell survival are likely to overshadow any functions these genes might have later in development. A way to overcome this is the use of conditional knock out techniques, which confine the ablation of a specific gene to a specific developmental time window and cell population.

A complementary approach in developmental gene studies is ectopic expression (or transgenic overexpression) of the gene confined to a specific cell population during a certain developmental time interval. This can be achieved by choosing an appropriate promoter/ enhancer. The resulting phenotype can then be ascribed to a specific function of the protein, and functional or structural interactions with other proteins, in a given time frame and cell type. A disadvantage is that the integration of the transgene into the genome is random and could generate an insertional mutation that interferes with the function of an existing gene. In the production of transgenic mice the gene construct is introduced in one-cell mouse embryos by means of pronuclear injection. The injected DNA recombines in a random fashion and integrates in the mouse genome.

Nestin is an intermediary filament and considered a marker of neural stem cells (NSCs). Constructs with the nestin gene result in strong overexpression in NSCs between embryonic days 7.5 (E7.5) and E16.5 (Johansson et al., 2002, Lothian et al., 1999; Lothian and Lendahl, 1997). Expression continues in areas that develop later, such as the cerebellum. Tissue specificity of nestin expression is governed by enhancer elements located in its first and second intron. The promoter is not relevant. The first intron directs expression to developing muscle tissue and the second to neural stem cells. The vector (nes1852tk/lacZ) used in this study includes only the second intron of the *human* nestin gene and has a documented function (Lothian and Lendahl, 1997). The lacZ gene has been excised to create the nes1852tk vector, which will be referred to as the *hnes* vector. It has further been altered by the addition of an eGFP reporter gene separated by an IRES sequence, in order to label and follow the Nestin+ progenitor cell pool.

For this thesis, transgenic mouse embryos overexpressing Wnt7a, Wnt7b and Vangl2 were generated by Karolinska Center for Transgene Technologies (KCTT), using DNA constructs made by us. Briefly, a linearized DNA construct is microinjected into 1-cell stage embryos, into the male pronucleus (slightly larger than the oocytes nucleus). These fertilized oocytes are reimplanted into the oviducts of pseudopregnant dams (mated with vasectomised males) and let to develop. The day of the vaginal plug was set to embryonic day 0.5 (E0.5).

Pregnant dams with embryos of the desired gestational age were sacrificed by spinal dislocation, and the embryos (<E14.5) were rapidly dissected out and immersed in ice cold 4% paraformaldehyde (PFA). Embryos older than E14.5 were rapidly dissected out, decapitated and subsequently immersed in ice cold 4% PFA. The transgenic embryos were identified by PCR, using yolk sac DNA as a template (100 ng). A sense primer complementary to human nestin second intron was combined with an antisense primer complementary to the gene of interest ORF. Embryos identified as transgenic by PCR were further investigated by in situ hybridization. Only PCR positive embryos unambiguously overexpressing the gene of interest were included in the transgenic group.

Mutant mouse embryos

The Looptail mouse provides a model for the most severe neural tube defect known as craniorachischisis and was found to have a natural mutation in the Vangl2 gene (Kibar et al., 2001). In paper IV we describe that altered distribution of cytoskeletal components correlates with Vangl2 function.

All animals were treated according to the ethical approval from the Northern committee in Stockholm and to European Communities Council guidelines (directive 86/609/EEC).

Neural, epithelial and tumor cell lines

Different cell lines were employed for this thesis depending on the specific purpose of the study. The mouse neural stem cell line C17.2 was used to assess cytoskeletal interactions and migration of neural cells, whereas the epithelial cell lines HEK293 and MDCK were used to study adherens junctions and cell adhesion. Medullo- and neuroblastoma cells were assessed for their native Wnt/PCP protein expression and used in the viability (MTT) assay.

CELL AND TISSUE PROTEIN LABELING

Immunohistochemistry/immunocytochemistry

Cultured cells and mouse embryo tissue sections were processed according to the specific protocols (see respective article), in order to label and study the distribution of a specific protein within the cell and the specific structures of the developing brain.

All antibodies used in the experiments, have been previously used and validated for the specific labeling of the desired epitope/protein, therefore experiments included only a negative control where the primary antibody was omitted in order to subtract for the eventual background staining of the secondary antibodies. However, one must be aware of the pitfalls using unvalidated antibodies, or antibodies validated in other experimental setups. In such cases, there is a risk of unspecific labeling, giving a false positive result. It is then recommended to test each antibody used with a positive control (cells expressing the desired protein), and negative controls, such as preincubation with the binding epitope peptide fragment and/or immunostaining against knock out cells or tissue (lacking expression of the desired epitope/protein).

GENE AND PROTEIN EXPRESSION STUDIES

In situ hybridization

Histological gene expression studies employ the technique of *in situ* hybridization. Here one can study the spatial and temporal distribution of a particular gene in both whole embryos and organs or in tissue sections and cell cultures. An antisense probe, i.e. a short (100-300 base pairs) ribonucleotide sequence, is designed and let to hybridize with the sense strand of the mRNA sequence encoded by the gene of interest. The *in situ* probe can be labeled in different ways for subsequent detection and relative quantification. Traditionally probes are labeled with a radioisotope (e.g. ^{35}S), which enables for development on x-ray film and make it possible to process the images for relative quantification. The other labeling method uses an epitope tag, usually digoxigenin labeled UTP, during the synthesis of the antisense probe. This enables both chemical and immunofluorescent labeling of the gene transcript and the possibility to use several differently labeled probes, or to process the tissue for protein expression analysis with immunohistochemical methods. In our studies we have used both ^{35}S (*paper I and II*) and digoxigenin (*paper III and IV*) labeled probes, as well as doublestaining with digoxigenin labeled probe and subsequent immonofluorescense (*data not shown*).

quantitative *real-time* PCR

Total RNA was prepared from cells with the RNeasy kit (Qiagen, Hilden, Germany) followed by cDNA synthesis with the High capacity RNA-to-cDNA kit (Applied Biosystems). PCR was performed with Power SYBR Green (Applied Biosystems, Life Technologies, Thermo Fisher Scientific Inc., Waltham, MA USA) on a 7500 Real-Time PCR system (Applied Biosystems) with primers (Applied Biosystems). Relative expression was calculated with the $2^{-\Delta\Delta Ct}$ method.

Western blotting

For the analysis of protein expression from cell and tissue extracts, we performed protein immunoblot staining on PVDF nitrocellulose membranes, where immobilized sample proteins where transferred to after separation through SDS-polyacrylamide gel electrophoresis. Immunoblots were exposed to x-ray films and further processed after computer scanning with ImageJ (National Institutes of Health; http:// rsbweb.nih.gov/ij/)for pixel density analysis and relative quantification of protein expression.

CELL ADHESION AND MIGRATION

Aggregation assay

One of many assays to study cell adhesion is through an aggregation assay. For the experiments the use of a cell line that forms tight connections, such as MDCK cells, is a prerequisite. Using different expression constructs, affecting the integrity of adherens/tight junction formation, we could calculate an aggregation score, rating adhesive properties of the different cytoskeletal and Wnt/PCP signaling components assayed.

Wound assay

An easy, fast and economical way to study cell migration *in vitro* is by using a wound healing assay. This method is based on the properties of cells to migrate into a formerly colonized area and replace the lost cells. For this experiment, described further in paper IV, we used C17.2 cells which are suitable due to their high migration capacity (Ourednik *et al.*, 2002). The results were quantified both by a calculation of the recolonized area percentage based on processed binary images in ImageJ, and manually by counting the proportion of cells in the wound area.

CHEMILUMINESCENT REPORTER ASSAYS

Viability assay (MTT)

The effects of increased (RNAi) or decreased (over-expression) PCP gene expression on neuroblastoma cell line viability was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoleum (MTT) assay (Sigma-Aldrich). Briefly, cells were seeded in 96-well plates, allowed to attach overnight, and transfected with cDNA or RNAi constructs of PCP genes Vangl2, Prickle1 and Scribble1. The medium was then replaced by 50 µl serum-free medium containing 5 mg/ml MTT. Cells were incubated for 3 h, and 150 µl isopropanol was then added to dissolve crystals. The plates were then shaken overnight at 4°C, and absorbance was measured using a microplate reader at 570 nm.

Luciferase assay

Cells were seeded in 24-well plates, left to attach and transfected with a TCF reporter plasmid (Super 8x TOPFlash; 400 ng) together with a Renilla-Luc plasmid (40 ng) using Lipofectamine 2000 (Invitrogen). Twentyfour hours after transfection cells were drug treated with either 25 µM or 50 µM Rho-associated coiled-coil kinase (ROCK) inhibitor Fasudil (HA-1077, LC laboratories, Boston USA). A Dual Luciferase Assay Kit (Promega, Fitchburg, Wisconsin USA) and a luminometer (Perkin Elmer, Waltham Massachusetts USA) were used to measure luminescence. The values were normalized to the Renilla reporter before calculating relative levels.

PROTEIN ACTIVITY AND INTERACTION

Immunoprecipitation

Protein-protein interactions can be determined by the immunoprecipitation method, whereby a cell lysate is incubated together with an antibody for the protein of interest and then pulled down by sepharose beads with high affinity for the antibody. We employed this method to study interactions of different variants of Vangl2 and Rac1 in *paper IV* and different variants of Vangl2 with Prickle1 in *paper V*. Control experiments were done both by transfection of a control plasmid and by precipitation with an unspecific IgG antibody. As described in the respective paper, this method is suitable for investigating if a specific protein is part of the same interacting complex as another protein. It is also possible to use mutated or truncated protein forms to identify necessary binding/interacting regions.

Rac and Rho activation assays

In order to study downstream signaling in the Wnt/PCP pathway we used a modified immunoprecipitation protocol were sepharose beads coated with recombinant proteins with high affinity for the active, GTPbound state of Rac1 or RhoA were used to pull down the target protein. The immunoprecipitate was processed by Western blotting. Compared to a standard Western blot, this is a far more delicate procedure, since all previous experimental steps needs to be done on ice in order to preserve the GTP-bound state.

STATISTICAL METHODS

In all five studies, statistical differences between groups were determined using Student's *t*-test for equal variances or a variant known as Welch's unpaired *t*-test for unequal variances. Differences were considered to be statistical significant at $P < 0.05$*, $P < 0.01$ **, and $P < 0.001$ *** (two-sided). Data are presented as mean ± SD. For the transgenic and mutant embryos, only littermates at a specific age were compared between groups. Kaplan-Meier survival estimates and gene correlation graphs were extracted from the R2 database (R2: microarray analysis and visualization platform (http://r2.amc.nl)).

RESULTS AND DISCUSSION

WNT/PCP SIGNALING DISTURBS NEURULATION BY AFFECTING THE CYTOSKELETON

In our first studies we investigated the aberrant phenotype of Wnt7a and later Wnt7b transgenic embryos (*papers I-III*). As Wnt genes are expressed in different regions of the developing brain, we expected that transgenic overexpression could interfere with the early CNS regionalization. To our surprise transgenic embryos showed underdeveloped forebrain structures and neurulation defects, further characterized as defects in the neural tube adherens junctions – an essential cytoskeletal component in both neurulation and neural tissue rigidity. Transgenic embryos showed decreased levels of the adherens junction components N-cadherin and beta-catenin, the latter also a mediator of canonical Wnt signaling. The cytoskeletal components N-Cadherin and beta-catenin were found to be mislocalized within the neuroepithelium. Relative quantification of *in situ* hybridizations indicated an increase of N-Cadherin expression throughout the neural tube. Since focal presence of the N-cadherin protein at the adherens junctions was clearly lowered, our results suggest that N-cadherin levels were increased in the neural tube, but not in the adherens junctions. We conclude that a mislocalization of N-cadherin protein but not a reduction in N-cadherin mRNA expression was the cause of the absence of N-cadherin in the adherens junctions (*paper I*).

Transgenic overexpression of the Wnt7a homologue Wnt7b produced an embryo phenotype with no neurulation defects, but still the embryos were small in size with underdeveloped forebrain structures. Embryos analyzed at later stages had normal morphology and the neural expression pattern of different histological markers were similar between littermates. This suggested either lethality or inactivity of the Wnt7b transgene at later developmental stages. However, one transgenic Wnt7a embryo that was analysed at E12.5 displayed a considerably larger brain than its wild type littermates. As for the E8.5-E10.5 Wnt7a transgenic embryos, we could describe the phenotype of the E12.5 transgenic embryo with a similar disturbance in the adherens junctions and an impaired neurulation. We presented a hypothetic model of brain alteration in the transgenic Wnt7a embryos described in the following schematic image (Fig. 1).

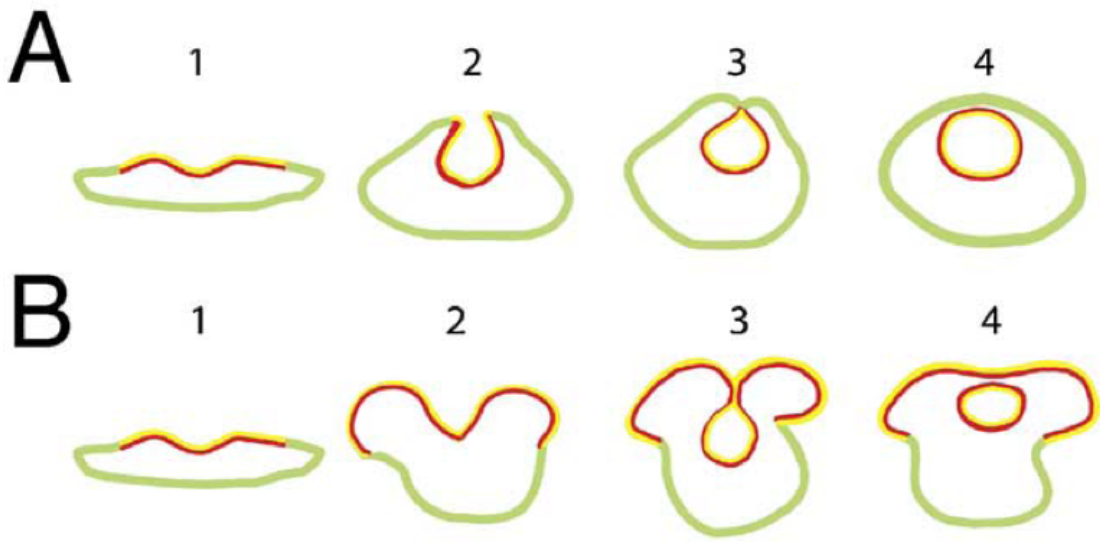

Figure 1. Model of the alterations in the brain development of Wnt7a transgenic mice.

(A) A schematic representation of neurulation in wild type embryos. The neural plate/tube is drawn in yellow and red, where yellow indicates apical and red basal. (B) A hypothetic depiction of cranial neurulation in the E12.75 nestin-Wnt7a embryo. The neural folds fail to elevate properly (2) as observed in E8.75–E9.75 nestin-Wnt7a embryos. Without the rigidity imposed by the adherens junctions, the neural tube walls splay outwards. If they meet and fuse mediolaterally (3), a neural ‘‘cap’’ with a reversed apico-basal polarity is created. This process will also lead the medial part of the neural tube to bud off (3 and 4). This secondary neural tube will have a normal apico-basal polarity (4) and might continue to develop structures resembling those arising during normal brain development (see Figs. 7G and 7R in Shariatmadari et. al., 2005). An impaired cranial neurulation leads to the aberrant brain morphology. Increased proliferation contributes to the phenotype.

INCREASED WNT SIGNALING IN NEURAL PROGENITOR CELLS DELAYS NEUROGENESIS

It has been shown that Wnt signaling can affect the proliferation and differentiation of neural progenitor cells and that beta-catenin signaling affects target genes that control these cellular processes. Transgenic overexpression of beta-catenin (under control of the nestin enhancer) in neural progenitor cells increases their proliferation, and in particular favours symmetrical cell divisions. This, expands the progenitor cell pool which results in an enlarged cortex (Chenn and Walsh, 2002, 2003).

Transgenic Wnt7a and Wnt7b embryos analyzed at E10.5 for proliferative markers such as BrdU (*paper I*) or phospho-Histone 3 (*paper II & III*) did not show any differences in proliferative rates compared to their wild type littermates, which suggested indirectly that Wnt7a/Wnt7b did not act through increased stabilisation of beta-catenin before E10.5.

However, neural differentiation was impaired in both Wnt7a and Wnt7b transgenic embryos, indicated by a decreased number of beta-tubulin III positive cells in the transgenic forebrains compared to wild type litter-mates (*paper II & III*). Also, the transgenic embryos were smaller in size than their wild type littermates. Since the proliferative and also apoptotic rates of neural progenitor cells were unaffected, one can speculate whether the proportion of symmetric versus asymmetric progenitor cell divisions was shifted so that the impaired neural differentiation was a consequence of premature cell cycle exit.

Several transcription factors and morphogens have important functions during the initial expansion of the neural progenitor cell pool. Disturbances in their expression may cause premature cell cycle exit and therefore a decreased proportion of symmetrical divisions, depleting the progenitor cell pool and decreasing the size of the developing embryo (Hatakeyama et al., 2004). Tbr1 and Tbr2 are forebrain-specific transcription factors which drive the production of both cortical plate neurons and intermediate progenitor cells (IPCs) respectively. In the transgenic Wnt7b embryos, expression of Tbr1 and Tbr2 was grossly reduced (42.7% and 19% of wild type expression respectively (*paper III*)). However, the transgenic embryos displayed a proportion of pax6 expressing cells similar to wild type littermates, implying an unaffected neural progenitor cell pool (Fig. 2). Taken together, the results (*paper II & III*) indicate that the impaired neuronal differentiation might be due to a delay in neurogenesis, neither owing to an increased progenitor symmetrical divisions nor depletion of the progenitor cell pool by premature cell cycle exit.

In order to confirm our hypothesis we could have performed double immunolabelling against BrdU and Ki67 in our transgenic embryos. We had previously delivered BrdU to all of the pregnant dams either 4 or 24 hours prior to sacrifice, and at the desired embryonal day of investigation. Preliminary results for two Wnt7b transgenic embryos and wild type littermates analysed at E10.5 showed a similar proportion of $BrdU^{+}/Ki67^{+}$ double labelled progenitor cell nuclei and thus no indication for increased cell cycle reentry. The numbers of $BrdU^{+}/Ki67^{-}$ nuclei were also similar to wild type which indicated that there was not an obvious increase of premature neural progenitor cell cycle exit (*data not shown*).

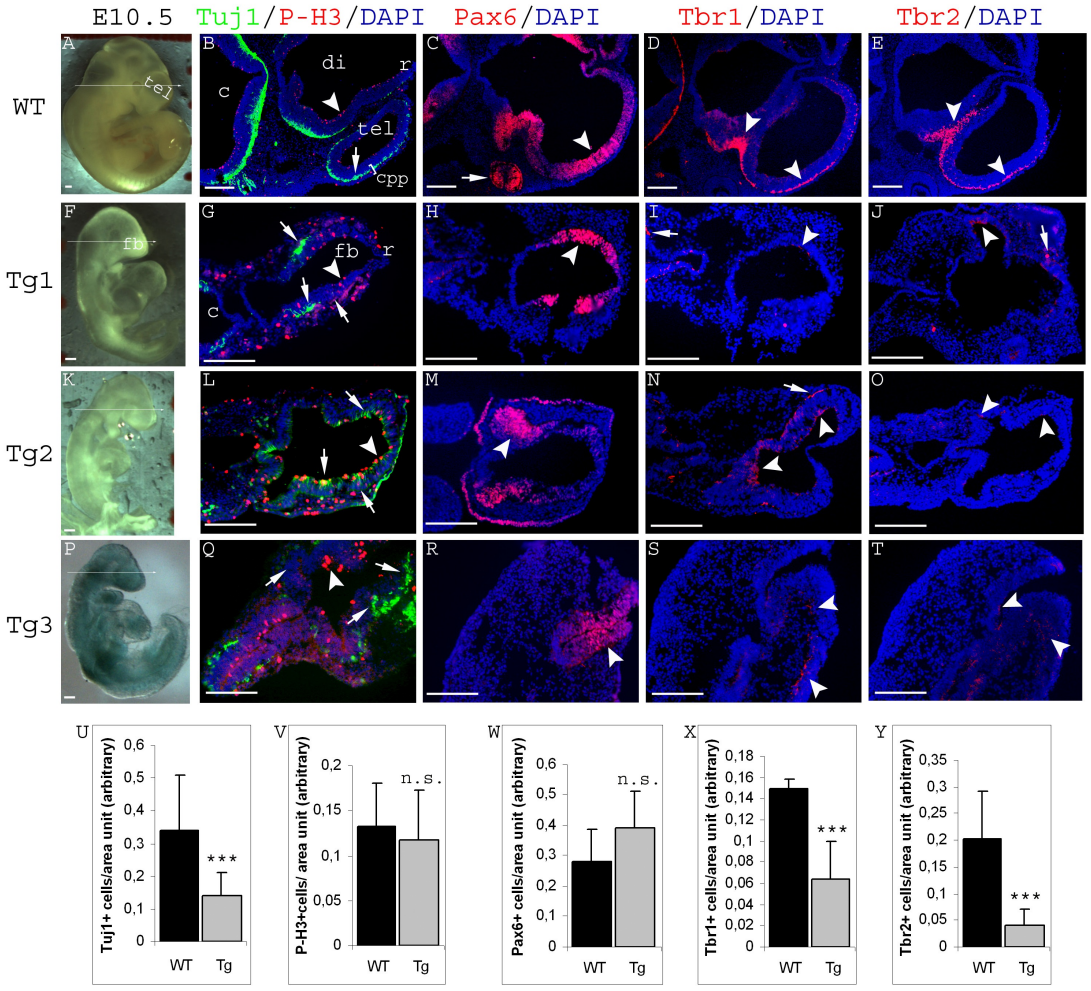

Figure 2. Wnt7b transgenic embryos show decreased labeling against beta-tubulin-III, Tbr1 and Tbr2.

Wild-type (A) and transgenic (F, K and P) E10.5 embryos; micrographs of 10-12 µm transversal tissue sections labeled with antibodies against beta-tubulin-III (Tuj1), phospho-Histone-3 (P-H3), pax6, Tbr1 and Tbr2 and visualized with fluorescent conjugated secondary antibodies respectively. DAPI nuclear counterstain was used for tissue visualization. Wild-type E10.5 mouse embryos showed an even distribution of Tuj1+ neurons populating the preplate (arrow in B) as opposed to the Wnt7b transgenic embryos where Tuj1+ neurons are sparse and scattered (arrows in G, L and Q). The wild-type expression of Tbr1 and Tbr2 is visible in the emerging forebrain/cortical structures (arrow heads in D and E). In Wnt7b transgenic embryos the expression of Tbr1 and Tbr2 was decreased and in some parts completely absent (arrows and arrow heads in I-J, N-O and S-T respectively). P-H3 and pax6 expression was similar in both wild-type and transgenic embryos (arrows in B, G, L and Q; arrowheads in C, H, M and R respectively; arrow in C indicates pax6 expression in developing eye). U-Y; Quantification of cellular marker expression, $P < 0.001$ (***). Abbreviations; WT – wild-type, Tg – transgenic; tel – telencephalon; di – diencephalon; cpp – cortical preplate; fb – forebrain; r – rostral; c – caudal; Scale bars are 200 µm.

INTERACTION BETWEEN VANGL2 - RAC1 AND THE ORGANIZATION OF ADHERENS JUNCTIONS

Vangl2 is an essential component of the Wnt/PCP pathway, which acts via activation of the Rho family of small GTPases RhoA and Rac1 (Habas et al., 2003). In our study (*paper IV*), both decrease and increase of Vangl2 levels resulted in aberrant cellular distribution of actin microfilaments, Rac1 and other cytoskeletal components. We could demonstrate that Vangl2 was present in a protein complex with Rac1 and that the PDZ-binding domain of Vangl2 was essential for this interaction. However, the overall expression level of Rac1 and Rac1 activation were not affected by Vangl2. On the basis of these observations, we propose a model in which Vangl2 does not affect Rac1 activity directly, but recruits Rac1 to locally increase its concentration at the membrane or other cellular compartments. We suggest that Vangl2, which is located at adherens junctions, is required for the proper recruitment of Rac1 to the adherens junction (Fig. 3).

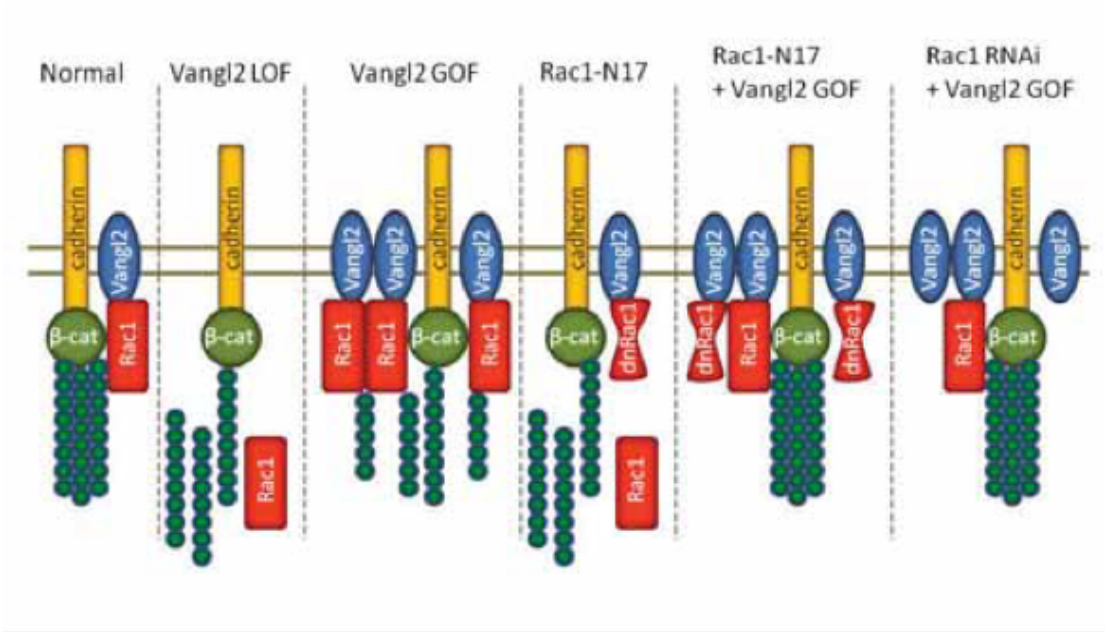

Figure 3. Model of interaction between Vangl2 and Rac1.

The model shows six different conditions of Vangl2 and Rac1 expression with our proposed mechanism of interaction. Actin microfilaments are displayed as strings of pearls (green). In the normal condition, Vangl2 targets Rac1 to the plasma membrane and promotes the formation of adherens junctions, in which microfilaments link to cadherin via catenins. In Vangl2 loss-of-function (LOF), Rac1 lacks a membrane targeting signal and the adherens junctions fail to form. In Vangl2 gain-of-function (GOF), excess Vangl2 and Rac1 at the membrane results in disordered microfilaments. When Rac-N17 is expressed, the dominant negative Rac1 (dnRac1) competes with endogenous Rac1 for Vangl2's binding site and inhibits the formation of adherens junctions. Vangl2 GOF rescues Rac1 blockade (Rac-N17 or Rac1 RNAi) by recruiting remaining endogenous Rac1 to the adherens junctions.

Active Rac1 is known to promote actin polymerization at the cell periphery (Hall and Nobes, 2000). The crucial function of Rac1 in the actin cytoskeleton and cell adherence has been demonstrated previously (Jou and Nelson, 1998), and is also confirmed in our experiments with RNAi knockdown of endogenous Rac1 or dominant-negative Rac1. Importantly, previous studies have demonstrated that the actin cytoskeleton is disrupted both by increased and decreased Rac1 signaling (Jou and Nelson, 1998).

Cell adhesion and cell migration are biological processes that are hijacked in cancer cells in order to promote tumor characteristics, such as invasiveness and metastasis. Deregulation of Wnt/PCP signaling has been shown to contribute to cancer development (Katoh, 2005). In our study (*paper IV*) we showed that proper Vangl2 expression was important for cell adhesion and cell migration, assessed through aggregation and wound healing assays. Our results suggested that Vangl2 gain-of-function (GOF) affected cell adherence in a Rac1-dependent manner, and that the PDZ-binding domain was essential for this. Also, Vangl2 overexpression increased cell migration and this effect could be antagonized by coexpression of dominant negative Rac1-N17 (dnRac1; Fig. 4).

Loss of Vangl2 function in human cancer cell lines increased the cell migration and promoted matrix metalloproteinase-dependent extracellular membrane invasion (Cantrell and Jessen, 2010). Also, components of the PCP pathway, including Vangl2, were upregulated in B lymphocytes of patients with chronic lymphocytic leukemia (CLL) and this feature was required for the migration and transendothelial invasion of CLL cells (Kaucká et al., 2013). Increasing evidence has been gathered for Vangl2 and Scribble1 expression suggesting that deregulation of these polarity genes plays a role in tumor cell motility, metastasis and clinical prognosis (Feigin et al, 2014; Godde et al., 2014; Hatakeyama et al., 2014).

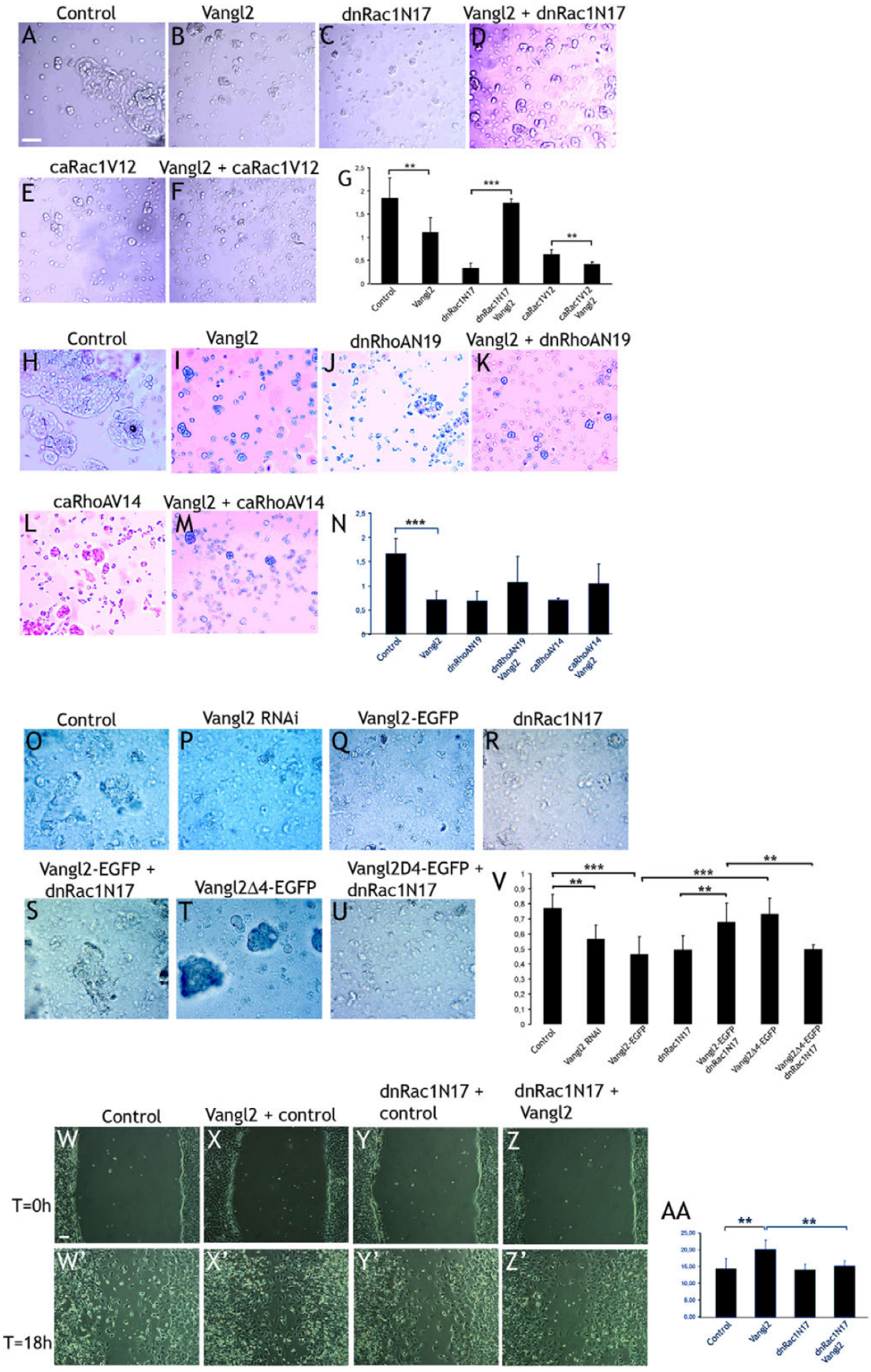
Control
Vangl2
dnRac1N17
Vangl2 + dnRac1N17
A
B
C
D
caRac1V12
Vangl2 + caRac1V12
E
F
G
Control
Vangl2
dnRac1N17
dnRac1N17 Vangl2
caRac1V12
caRac1V12 Vangl2
Control
Vangl2
dnRhoAN19
Vangl2 + dnRhoAN19
H
I
J
K
caRhoAV14
Vangl2 + caRhoAV14
L
M
N
Control
Vangl2
dnRhoAN19
dnRhoAN19 Vangl2
caRhoAV14
caRhoAV14 Vangl2
Control
Vangl2 RNAi
Vangl2-EGFP
dnRac1N17
O
P
Q
R
Vangl2-EGFP + dnRac1N17
Vangl2Δ4-EGFP
Vangl2D4-EGFP + dnRac1N17
S
T
U
V
Control
Vangl2 RNAi
Vangl2-EGFP
dnRac1N17
Vangl2-EGFP dnRac1N17
Vangl2Δ4-EGFP
Vangl2Δ4-EGFP dnRac1N17
Control
Vangl2 + control
dnRac1N17 + control
dnRac1N17 + Vangl2
T=0h
T=18h
W
X
Y
Z
W'
X'
Y'
Z'
AA
Control
Vangl2
dnRac1N17
dnRac1N17 Vangl2

Figure 4. Vangl2 affects cell adhesion and migration in interaction with Rac1

Aggregation assays with MDCK cells. (A-D) Control cultures display a high proportion of aggregated cells (A), whereas Vangl2 (B) or dominant negative (dn)Rac1N17 (C) significantly reduced cell aggregation. (D) Vangl2 combined with dnRac1N17 resulted in a close-to-normal level of cell aggregation. (E,F) constitutively active (ca)Rac1V12 reduced cell aggregation (E), which was further enhanced by Vangl2 (F). (G) Graph with the respective aggregation indexes. (H-M) Vangl2 (I), dnRhoAN19 (J) or caRhoAV14 (L) reduces cell aggregation compared to control transfected cells (H). Addition of Vangl2 does not modify the effects of dnRhoAN19 (K) or caRhoAV14 (M). (N) Quantification of the results. (O,P) Vangl2 RNAi reduces cell aggregation compared to control transfected cells. (Q-S) Vangl2-EGFP (Q) and dnRac1N17 (R) alone reduces aggregation, whereas the combination of Vangl2-EGFP and dnRac1N17 results in close to normal aggregation levels (S)(T) Vangl2Δ4-EGFP results in normal aggregation (95% of control). (U) However, Vangl2Δ4-EGFP is not able to counter the effects of dnRac1N17. (V) Quantification of the results. (W-Z) Vangl2 increases cell migration in a wound assay. Pictures of the same area were taken at the start of the experiment (T=0, W-Z), and after 18 hours (T=18 hours, W'-Z'). Vangl2-transfected cells (X') colonize the inflicted wound to a higher extent than the control transfected cells (W'). Dominant negative Rac1 blocked the effects of Vangl2 in cell migration (Z'), but did not by itself affect cell migration (Y'). (AA) Quantification of the results. Scale bars: 100 μm. Error bars in G, N, V and AA represent std **P<0.03-0.05, ***P<0.01.

WNT/PCP GENE EXPRESSION IN PEDIATRIC TUMOR CELL LINES AND TUMOR CELL VIABILITY

Understanding the mechanisms that regulate and promote tumor development is of great importance for establishing future prognostic markers and therapeutic interventions in childhood cancers. Increased Wnt/beta-catenin signaling is found in many forms of cancer (Katoh, 2005), including the childhood cancers medullo- and neuroblastoma where it is associated with upregulation of proliferative genes such as MYCN. Analysis of medulloblastoma and neuroblastoma cell lines for their native active beta-catenin and core PCP protein expression revealed an inverse correlation between Prickle1, Vangl2 and beta-catenin expression levels (*paper V*). Further, we showed that *in vitro* manipulation of PCP gene expression affected tumor cell viability in a MTT assay. Prickle1, Vangl2 or Scribble1 RNAi knockdown in neuroblastoma cells increased the relative absorbance and indirectly cell viability by 31 to 42 %, compared to control cells treated with a scrambled RNAi sequence (Fig. 3; $P<0.0001$). Overexpressing hPrickle1, hVangl2 or hScribble1 with cDNA constructs, decreased the relative absorbance and indirectly the cell viability by 39 to 68 %, compared to cells treated with vehicle (Fig. 3; $P_{Prickle1}=0.0044$; $P_{Vangl2}<0.0001$; $P_{Scribble1}=0.0003$).

Loss of polarity gene function has been associated with induction of dysplasia, increased tumor cell motility and metastasis (Feigin et al, 2014; Godde et al., 2014; Hatekayama et al., 2014).

Analysis of Wnt/PCP genes in open access gene array databases (e.g. R2: microarray analysis and visualization platform (http://r2.amc.nl)) show that high expression of PCP genes in neuroblastoma patient material are associated with a better clinical prognosis (5-year survival) compared to neuroblastoma patients with lower expression.

Many PCP genes interact with each other and have interdependence both for their asymmetric localization within the cell, and for their capacity to establish cell and tissue polarity. In our study, we could by means of immunoprecipitation show that Prickle1 was found in the same protein complex as Vangl2, a protein known to interact with the cytoskeleton (Lindqvist et al., 2010). A different study with hepatocellular cancer cells showed that Prickle1 was a negative regulator of beta-catenin stabilization, mediated through its interaction with Dishevelled (dvl3; Chan et al., 2006).

Further experiments are needed in order to investigate whether Prickle1 mediates its action through cytoskeletal interaction with Vangl2 or via Dishevelled and beta-catenin in neuroblastoma cells. We are also interested in studying the genetic profile of aggressive and highly metastatic medullo- and neuroblastoma cells versus their counterparts that rarely metastasize. This can be done by gene expression array analysis.

CONCLUSIONS

This thesis describes the role of Wnt/PCP signaling in neurulation, cell adhesion,

neural differentiation, neural cell migration and pediatric tumor cell line characteristics and viability. The main findings can be summarized as follows:

- Transgenic overexpression of Wnt7a and Vangl2 resulted in neurulation defects due to impaired adherens junctions and neural tube rigidity.
- Transgenic overexpression of Wnt7b resulted in a similar, but milder, phenotype compared to the Wnt7a transgenic embryos. Neurogenesis was impaired and the expression of neural specific transcription factors was decreased.
- Studies of Vangl2 gain- and loss of function, both *in vivo* and *in vitro*, described a Vangl2-dependent interaction and redistribution of Rac1 within the cell to active sites of actin microfilament rearrangement at the cellular cortex.
- Neuroblastoma and medulloblastoma cells showed differential expression of beta-catenin and PCP components. Manipulation of PCP gene expression in neuroblastoma cells correlated with tumor cell viability and predicted clinical prognosis (open access gene array data).

RELEVANCE AND FUTURE PERSPECTIVES

The results presented in this thesis aim to increase our understanding of the mechanisms governing Wnt signaling and patterning in the developing brain. Studies of Wnt/PCP signaling pathways may increase our knowledge of the etiology and treatment of diseases related to the brain and its development, e.g. neural tube defects, plus neurological and psychiatric diseases.

When utilizing transgenic overexpression techniques, one can in a fast and fairly economic way study an ectopic or increased gene expression *in vivo* depending on the spatial and temporal characteristics of the promoter/enhancer that is used. The drawback of a non-inducible transgenic technique is the lack of temporal control, with a rather early onset and offset of the transgene product. Also, the injected transgene DNA recombines in a random fashion and integrates in the mouse genome at different chromosomal locations when studying embryos from primary injections. This could result in phenotypical differences, as the transgene could integrate in a DNA region containing other silencer or enhancer elements, which can affect the overall expression of the transgene product.

This shortcoming can be overcome by analyzing milder transgenics postnatally in order to establish a transgenic mouse line which one can produce transgenic embryos from (Hamasaki et al., 2004). An even better way is to choose a transgenic system which allows more control over the temporal and spatial expression of the gene. These inducible expression systems, such as the tet transactivator system, or Cre/loxP system have great advantages but can still be expensive and time consuming, with an extensive breeding in order to produce a stable transgenic mouse background.

Wnt/PCP signaling has been implicated in both embryogenesis and carcinogenesis. Disturbances in Wnt/PCP signaling have been shown in many cancer forms, such as mammary gland tumors (Zhan et al., 2008), colorectal cancer (Piazzi et al., 2013), prostate cancer (Pearson et al., 2011), neuroblastoma (Liu et al., 2008) and medulloblastoma (Giles et al., 2003). The latter represent the most common form of malignant brain tumor in children (Koesters and von Knebel Doeberitz, 2003). Genetic profiling of different tumor cells can be of great value in terms of understanding tumor cell characteristics, which may lead to better prognostic predictions and development of tailored therapeutic strategies.

In summary, the results described in this thesis will increase our knowledge on how proliferation and differentiation of neural progenitor cells is controlled, which will be of relevance for stem cell research. It will also further our understanding of the mechanisms that control cell adhesion and motility, features that are often deregulated in carcinogenesis and metastasis.

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