

GENETIC APPROACHES FOR THE DIAGNOSIS AND TREATMENT OF
CONGENITAL TOOTH AGENESIS

A Dissertation

by

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ABSTRACT

Congenital tooth agenesis is the most common developmental anomaly in man. More severe forms of tooth agenesis (> 5 missing teeth) demand lengthy and expensive treatment approaches such as bone augmentation surgeries and placement of multiple implants. Tooth agenesis is caused by mutations in genes responsible for early tooth development; and ever since it had been shown that timely injections of functional recombinant gene products can overcome the corresponding, mutation-based developmental disorder, such new therapeutic strategies for the prevention of tooth agenesis should be attempted.

In this research project I have pursued two objectives:

1.) Basic research into the molecular genetics and therapeutics of the tooth agenesis gene PAX9. Since PAX9 is an intra-cellular transcription factor which cannot be replaced directly, suitable downstream targets for therapy have to be identified by comparing wild type and Pax9 deficient tooth organs.

2.) Clinically oriented research into the molecular diagnostics of human tooth agenesis. We use candidate gene sequencing in large numbers of people with tooth agenesis to identify the majority of human tooth agenesis genes and to determine the molecular cause of tooth agenesis in individuals.

In the first study I identify the genes and pathways that are affected by Pax9 deficiency using microarray and q-PCR technology, and find that the Fgf, Shh and Wnt

pathways are more affected than Bmp4 which had previously been considered the main target of Pax9 in tooth development.

The next study shows that it is possible to apply therapeutic approaches to unravel the complexity of molecular signaling within the developing craniofacial complex. Using small molecule Wnt therapies we are able to rescue palatal clefting in Pax9-deficient mice.

Our third study presents a clinical aspect of human molecular genetics where we establish that tooth agenesis does not predispose women to ovarian cancer, as had been previously suggested.

The last study shows that mutations in *WNT10A*, but not in *WNT10B* or *WNT6*, are highly prevalent in populations with tooth agenesis. We also suggest that there must be some kind of heterozygous advantage to retaining mutations in Wnt10a. However, that advantage has not been identified.

DEDICATION

To my loving and beautiful wife and our amazing children.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

OVERVIEW

Teeth, like all other organs of the body, are formed and maintained as the result of temporally and spatially organized expression patterns of genes (Vaahtokari et al., 1996). Disruption of this process results in tooth agenesis, the congenital absence of one or more permanent teeth, which is the most common inherited disorder in humans, affecting up to 10% of the population even when third molars are excluded (Mattheeuws et al., 2004; Shapiro and Farrington, 1983). Third molar agenesis is the most common with an incidence of 20% (JM, 1956; Lavelle et al., 1970); but it is usually disregarded in the missing tooth count and does not require any intervention.

Traditionally, tooth agenesis can be separated into three main categories: a small number (1 to 5) of congenitally missing teeth is commonly referred to as hypodontia and consists typically of missing mandibular second premolars or maxillary lateral incisors with a prevalence of 3-4% and 2%, respectively. Many cases of this form of tooth agenesis may be satisfactorily treated with conventional dentistry. Missing greater than 5 teeth is termed oligodontia and is less common with a prevalence of about 0.1%. A much rarer occurrence of congenitally missing all teeth is known as anodontia. Oligodontia and anodontia patients would greatly benefit from innovative approaches.

Tooth agenesis can arise as an isolated trait and is then also referred to as selective or non-syndromic; or tooth agenesis can present as part of a syndrome. The

syndromic form of tooth agenesis most often affects additional ectodermal appendages such as hair, nails, glands, and the skin itself. Both syndromic and non-syndromic tooth agenesis normally fall within the hypodontia or oligodontia category (Kapadia et al., 2007; Nieminen, 2009). Although tooth agenesis does not present a life-threatening condition, it has a significant and long-term effect on oral health and well-being because it affects mastication, speech, and esthetic appearances for patients. Current treatment protocols for patients with tooth agenesis can impose significant functional, emotional and financial burdens on patients and their families and includes not only orthodontic and conventional procedures, but also expensive therapies such as bone augmentation surgeries and the placement of multiple implants, the latter of which can result in peri-implantitis in 20% of patients or may not integrate into the bone resulting in implant and possible bone loss (Callan, 2007; Mombelli et al., 2012). Restorative features such as fixed partial dentures (bridges), crowns, or removable partial dentures have been shown to be sufficient for long-term use (Bartlett, 2007), however these are not without their negative outcomes as well.

The obvious best approach for treating tooth agenesis patients would be prevention based on knowledge about the genetic origin of the disorder. Understanding the molecular genetics will most likely play an important role in the diagnosis, prognosis, and treatment of dental disorders such as caries, periodontal disease, mineralization defects, and especially tooth agenesis as it already does in the medical field. A thorough understanding on the molecular level would allow researchers to find suitable targets for supplementation with exogenous proteins or for modification by

pharmacologically active small chemicals. Since most tooth agenesis-causing gene mutations lead to haploinsufficiency, a small increase in active gene product can make a big difference in outcome (Das et al., 2002). Also, the permanent dentition is much more frequently affected than the primary dentition, which would probably allow for recombinant protein (or small molecule drug) replacement therapy after birth, perhaps even in the form of a local instead of systemic application. The successful use of recombinant Ectodysplasin-A (EDA) protein to prevent ectodermal dysplasia symptoms including tooth agenesis in affected mice and dogs has proven that such a molecular genetics approach is indeed feasible. The molecular interactions during development are so complex and difficult to dissect that it may be more revealing to make informed guesses and test directly using suspected gene products in experimental animals.

In the following research project I pursued a molecular genetics approach towards the diagnosis of human tooth agenesis and possible treatment options of tooth agenesis caused by PAX9 mutations.

THE MOLECULAR GENETICS OF PAX9 DEFICIENCY

One focus of this research project is on the function of the transcription factor Pax9, specifically the exploration of the molecular mechanisms that lead to tooth developmental arrest resulting from Pax9 deficiency. Pax9 is a member of the Pax (paired box) gene family that contains a paired type homeodomain with DNA binding properties (Neubuser et al., 1995; Wang et al., 2009b). In humans, a severe tooth agenesis phenotype can be caused by the mutation of just one PAX9 allele (Stockton et

al., 2000) and since the disorder is transmitted as an autosomal dominant trait with a 50% chance of transmission, affected members are fairly common in families with a Pax9 mutation (Goldenberg et al., 2000). In mice, both copies of the Pax9 gene have to be disabled to produce a tooth agenesis phenotype and the affected embryos also suffer from cleft palate, polydactyly, lack of thymus and parathyroid glands, and postnatal death.

Pax9 expression during the different stages of tooth development

Embryonic tooth development is a continuous process but is usually divided into several stages, each representing specific attributes and signaling events between the odontogenic epithelial and mesenchymal tissue layers. In mice (and also in humans), Pax9 is expressed very early in the presumptive tooth mesenchyme independent from epithelial signals around embryonic day E11.5, after the formation of the dental lamina around the dental placode stage. Mechanical compaction of mesenchymal cells may be the driving force for the initiation of Pax9 expression (Mammoto and Ingber, 2010).

Pax9 expression becomes stronger during the next stage of tooth development, the bud stage. This stage is characterized by the advancement of the lamina/placode into the underlying mesenchyme of the first branchial arch (Thesleff, 2006) and the shift of odontogenic potential from the dental epithelium to the dental mesenchyme. During this process, mesenchymal cells gather around the tooth bud and form the dental papilla, which later will differentiate into the tooth pulp and dentin-secreting odontoblasts (Kollar and Baird, 1970).

Pax9 continues to be expressed in dental mesenchyme when the bud stage progresses into the cap stage at E14.5 and the enamel knot begins to form (Thesleff et al., 2001). Mesenchymally expressed gene products are involved in the induction of the enamel knot, which acts as a transient signaling center in the tooth epithelium and further drives the morphogenesis of the crown of the tooth (Aberg et al., 1997).

With Pax9 deficiency the advancement from the bud to the cap stage does not occur and the tooth does not form.

Pax9 function during tooth development

Pax9 is a paired box transcription factor and its main task is the regulation of expression of other genes in the dental mesenchyme. Studies in knockout mice (Peters et al., 1998b) have revealed by *in situ* hybridization that Pax9 deficiency causes down-regulation of Msx1, another homeodomain transcription factor, which occasionally can also act as a transcriptional repressor. Also down-regulated are lymphoid enhancer factor 1 (Lef1) and the Tgf β family member bone morphogenic protein 4 (Bmp4). Lef1 is a transcription factor in the Wnt pathway and is important in the dental epithelium, but less so in the mesenchyme. Bmp4 on the other hand has been considered the most important signaling factor for the progression of tooth morphogenesis, therefore the main function of Pax9 was thought to be the transcriptional activation of mesenchymal Bmp4.

In vitro investigations showed that Pax9 can activate a proximal Bmp4 promoter fragment, strengthening this hypothesis. In these experiments it was shown that Msx 1

could potentiate the activity of Pax9 on the Bmp4 promoter, although it could not activate the promoter by itself (Ogawa et al., 2006). The conclusion was that Pax9 and Msx1 form a positive feedback loop for activation of mesenchymal Bmp4. Tooth development arrest in Pax9- and also in Msx1-deficient individuals was caused by the lack of Bmp4.

However, some doubt arose about this theory when it was shown that tooth agenesis-causing Msx1 mutations do not ameliorate the *in vitro* Bmp4 activation (Kong et al., 2011; Wang et al., 2011). Later it was also shown that some tooth types develop without any mesenchymal Bmp4 (Jia et al., 2013).

One goal of this project is to better understand the role of Pax9 during tooth development and to gain more insight into its downstream signaling targets. Because mutations in Pax9 can cause severe agenesis of posterior teeth, they are a desirable target for molecular therapies which can be tested in Pax9-deficient mice. Since Pax9 is an intracellular transcription factor, which cannot be replaced directly, suitable targets for therapy must be sought after among the downstream effector genes of Pax9.

THE CLINICAL GENETICS OF TOOTH AGENESIS

Another goal of my project is to identify the known genes or even find new genes responsible for tooth agenesis in individual patients. There are currently only six genes (MSX1, AXIN2, EDA, EDAR, EDARADD, and WNT10A) other than PAX9 that cause selective (non-syndromic) tooth agenesis in humans. All seven together account for

about half of all cases worldwide. The causative genes for the other half remain to be determined.

Known tooth agenesis genes

Most of these genes have been identified studying large tooth agenesis families with linkage analysis and gene sequencing.

EDA Pathway (EDA, EDAR, EDARADD; X-linked detected in 1996 (Kere et al., 1996); Selective discovered in 2006 (Tao et al., 2006))

Mutations in Ectodysplasin A (EDA) cause X-linked hypohidrotic ectodermal dysplasia (XHED) or selective tooth agenesis (STHAGX1). Males are strongly affected, females are not or only mildly affected. Different sets of mutations cause either the XHED syndrome or the non-syndromic tooth agenesis.

EDA pathway genes Ectodysplasin-A receptor (EDAR) and EDAR-associated death domain (EDARADD) also cause HED, which is often autosomal recessive or they cause selective tooth agenesis, which is present in about 50% of heterozygotes. Primary and permanent dentitions are both affected, with a predilection for incisor agenesis. Perinatal treatment with recombinant EDA protein is curative in mice and dogs with EDA mutations. Clinical trials have started to test the efficacy of this protein in humans with EDA mutations as well. Testing hypodontia patients for EDA mutations may become mandatory in the near future.

MSX1 (detected in 1996 (Vastardis et al., 1996))

Msx1 is a homeodomain transcription factor which can inhibit or activate transcription. It is expressed in tooth bud mesenchyme as well as in the developing heart, limb, and other craniofacial tissue. Inheritance of MSX1-associated phenotypes is autosomal dominant – a mutation in one allele is sufficient to cause tooth agenesis in humans. MSX1 mutations affect only the permanent human dentition. Mice, which have only one dentition, require the loss of both Msx1 alleles to develop a missing tooth phenotype. Humans with mutations in Msx1 normally fail to develop premolars and third molars (Kim et al., 2006) and these mutations are normally located in the homeodomain.

MSX1 mutations can also be found in cleft lip/palate patients and Witkop syndrome.

PAX9 (detected in 2000 (Stockton et al., 2000))

PAX9 encodes a paired box transcription factor expressed in tooth bud mesenchyme, thymus, parathyroid glands, and limb buds. *In vitro*, PAX9 cooperates with MSX1 to induce BMP4, a key signaling factor in tooth development.–The mutation of one PAX9 allele is sufficient to cause tooth agenesis in humans with autosomal dominant inheritance. Most PAX9 mutations affect the permanent dentition while some affect both primary and permanent dentitions. Molars are the predominantly missing tooth group followed by premolars and in rare cases incisors. Functional studies have

shown that loss of DNA-binding is the most common cause for dysfunction of mutant proteins (Wang et al., 2009b).

AXIN2 (detected in 2004 (Lammi et al., 2004)).

AXIN2 is an inhibitor in the Wnt pathway. The colon cancer gene APC (adenomatous polyposis coli) is part of the same pathway. AXIN2-caused hypodontia has been found in 6 independent cases. AXIN2 mutations cause mild to severe tooth agenesis with a mixed distribution pattern. In one family with AXIN2 mutation oligodontia was inherited together with a predisposition for colon cancer, therefore all patients with severe mixed tooth agenesis should be tested for AXIN2 mutations.

WNT10A (detected as selective tooth agenesis gene in 2009 (Bohring et al., 2009) and in 2012 (van den Boogaard et al., 2012)).

WNT10A is in the Wnt family of signaling factors that play major roles in development and oncogenesis. WNT10A mutations have a very high prevalence in human tooth agenesis (van den Boogaard et al., 2012). They can also cause ectodermal dysplasia syndromes and in fact WNT10A was initially discovered as the cause of the rare Odonto-onycho-dermal dysplasia (Adaimy et al., 2007) and Schopf-Schulz-Passarge syndromes (Nagy et al., 2010).

Heterozygous and homozygous mutations cause mild or severe tooth agenesis phenotypes, respectively. The phenotype is similar to the EDA phenotype with incisors being affected in mild forms while a mixed agenesis pattern is seen in severe forms.

Surprisingly, one single mutation (Phe228Ile) is the cause of most cases in European populations.

Suspected tooth agenesis genes

BMP4, MSX2, and PITX2 mutations have been found in single patients with relatively mild tooth agenesis (own observations and (Huang et al., 2013)).

Detection of new tooth agenesis genes

Linkage analysis, the classical method for finding causative genes for genetic disorders, requires large families with many affected individuals. Such large families have become rare in Western societies and we will have to approach gene identification using new means. The latest approach is exome sequencing which is perfectly suited for the detection of the genetic cause of rare, apparently genetic, disorders even when a large family is not available. However, at this point this method is still expensive and not suitable for large-scale screening applications.

In principle, all genes that are expressed during tooth development are candidates for investigation in patients with tooth agenesis. However, some of these genes are more likely candidates than others. For example, genes that also cause tooth agenesis in knockout mice or genes that are downstream of a known tooth agenesis gene, like the downstream genes EDAR and EDARADD in the EDA pathway or genes that cause syndromic tooth agenesis such as EDA and WNT10A, are more likely to be candidate genes for selective tooth agenesis.

For large-scale mutation screening of tooth agenesis samples, a candidate gene approach with direct sequencing of dozens of independent samples is still a useful approach.

Because I am pursuing a DDS/PhD degree, I have engaged in both basic science and clinical research around the common theme of diagnosis and future therapy of tooth agenesis. This project reflects both aspects of scientific research which was tailored to provide me with a more rounded scientific training.

SUMMARY

The two objectives of my research are:

1) Basic research into the molecular genetics and therapeutics of the tooth agenesis gene Pax9.

Mutations in this gene can cause severe agenesis of posterior teeth and are therefore a desirable target for molecular therapies which can be tested in Pax9 deficient mice. Since Pax9 is an intracellular transcription factor which cannot be replaced directly, suitable targets for therapy have to be identified among downstream effector genes of Pax9.

2) Clinical approaches to study and diagnose the genetic causes of tooth agenesis in human populations.

In order to provide as many tooth agenesis patients as possible with the new therapies it is mandatory to find as many human tooth agenesis genes as possible. This can be done by candidate gene sequencing in large numbers of people with tooth agenesis. Candidate gene sequencing is also used for the molecular diagnosis of tooth agenesis in individual families.

Chapter II of this dissertation presents a basic science approach to understanding the downstream effects of Pax9. Using microarray technology and quantitative PCR we identify the genes and pathways affected by Pax9 deficiency.

Chapter III provides evidence that an informed guess about important Pax9 target genes as derived from the results in Chapter II can lead to useful choices for replacement therapies. Using replacement therapies we show that we are able to rescue palatal clefting in Pax9-deficient mice.

Chapter IV presents a clinical aspect of human molecular diagnostics where we establish that tooth agenesis is not likely to predispose women to ovarian cancer, as had been previously suggested.

Chapter V is a study about the prevalence of Wnt10a, Wnt10b and Wnt6 in populations with tooth agenesis.

CHAPTER II

PAX9 DEFICIENCY IN TOOTH DEVELOPMENT AFFECTS MULTIPLE GENETIC PATHWAYS OTHER THAN BMP4

SYNOPSIS

The molecular mechanisms involved in tooth development have been thoroughly studied in recent years, leading to the discovery of all the major signaling pathways that contribute to tooth formation. However, many details are still missing about the exact role of genes that, when mutated, are associated with human tooth agenesis. Deficiency of *PAX9*, a paired domain transcription factor in tooth bud mesenchyme, leads to congenitally missing teeth in both humans and mice which could be partly attributed to the down-regulation of *Bmp4*, a signaling factor required for several critical steps in tooth development. To learn more about the activities of the Pax9 protein, we studied Pax9-dependent gene expression levels in the developing murine tooth using microarray and quantitative PCR. Our findings suggest that *Bmp4* activation may not be the most prevalent activity of Pax9 since *Bmp4* showed only modest down-regulation in Pax9-deficient tooth anlagen when compared to other signaling and transcription factors such as *Fgf*, *Shh*, *Tcfap2*, *Foxf1*, *Egr*, and several others.

INTRODUCTION

Tooth development has been studied since the late 1930's, but only in the last twenty years have many of the underlying molecular mechanisms been discovered,

notably the transcription factors and signaling pathways that are the primary driving forces responsible for this process which involves reciprocal genetic interactions between epithelial and mesenchymal layers of the dental lamina. Other studies (Mammoto and Ingber, 2010) have shown that mechanical forces induced by condensation and cell compaction also play a role in tooth formation by directly activating the expression of genes, such as the paired domain transcription factor Pax9 in tooth bud mesenchyme.

In mice, the Pax9 gene is prominently expressed in the mesenchymal layer of the early tooth anlage. Lack of Pax9 expression leads not only to tooth agenesis, but also to missing thymus and parathyroid glands, cleft secondary palate, and supernumerary digits of the hind limb (Peters et al., 1998b). *In situ* hybridization studies with Pax9 knockout mice revealed a decreased expression of homeodomain transcription factor/transcriptional suppressor Msx1, Tgf β -related signaling factor Bmp4, and Wnt pathway transcription factor Lef1 in tooth bud mesenchyme. Bmp4 had been previously identified as an important signaling factor in tooth development and thus the reduction of Bmp4 expression was considered the most likely cause of the tooth agenesis seen in Pax9-deficient mice and humans. This notion was strengthened by *in vitro* investigations suggesting that Pax9 can activate both Msx1 and Bmp4 promoters and that Msx1 cooperates with Pax9 to induce Bmp4 expression in tooth organ mesenchyme although Msx1 by itself cannot activate the Bmp4 promoter (Ogawa et al., 2006). However, later studies suggested that mesenchymal expression of Bmp4 requires additional factors besides Pax9 and Msx1 and that Pax9 and Msx1 may contribute to the

activation of regulatory networks other than the Bmp4 signaling pathway (Nakatomi et al., 2010; Zhao et al., 2008; Zhou et al., 2011). Therefore, we hypothesized that Pax9 and Msx1 do not regulate Bmp4 to the extent previously thought, and that Bmp4 down-regulation may contribute, but not be the central component that leads to tooth agenesis in Pax9-deficient tooth germs.

Knowledge about the main downstream targets of Pax9 in tooth development is central for the discovery of new therapeutic approaches for the prevention of tooth agenesis in humans, which is caused by haploinsufficiency of Pax9 and presents with severe agenesis of predominantly posterior teeth. Ever since it had been shown that a few, well-timed injections of recombinant ectodysplasin A can rescue hair, gland and tooth formation in animals with ectodermal dysplasia (Gaide and Schneider, 2003), this “missing protein replacement” approach has become a potentially viable alternative to current tooth replacement therapies. Since the intracellular transcription factor Pax9 cannot easily be replaced, substitution of downstream extracellular targets of Pax9 will be necessary to overcome developmental arrest of the tooth germ. Alternatively, small molecule chemicals with the property of pathway activators or inhibitors could be employed once the critical downstream pathways have been discovered.

In this study we evaluated gene expression differences between Pax9-deficient and wild-type (WT) embryonic tooth anlagen of mice using expression microarrays and quantitative PCR to uncover new downstream effector genes of Pax9 in the developing tooth.

MATERIALS AND METHODS

Mouse line

We used a C57/B6 mouse strain in which the first exon and half of the second exon of Pax9 was replaced by an *FRT*-flanked *neo* expression cassette followed by *Myc-Osr2* cDNA segment, referred to as Pax9^{-/-}. This strain was kindly provided by Dr. Rulang Jiang, Cincinnati's Children's Hospital, and was shown to be functionally Pax9-null in homozygous embryos. Since homozygotes (Pax9^{-/-}) die at birth, heterozygous (Pax9^{+/-}) mice were mated and embryos from multiple litters were harvested at either embryonic day E13.5 and E14.5 according to IACUC standards. Immediately after harvesting, mandibular 1st molar tooth anlagen were micro-dissected using a stereo microscope and stored separately in RNAlater (Ambion). After genotyping, RNA extraction was performed on these tissues using an RNeasy Mini Kit (Qiagen). Tooth bud RNA from 15-20 homozygous (Pax9^{-/-}) embryos and from a similar number of wild-type littermates was pooled separately and stored at -80°C.

Data acquisition/analysis

Expression microarrays were performed by the UT Southwestern Medical Center microarray core facility and included Whole Mouse Genome 430 2.0 arrays and a Mouse Exon 1.0 ST array (Affymetrix). For the Whole Genome array the statistical analysis, performed by Ingenuity's iReport, identified 73 significantly differentially expressed genes (DEGs) (I think that there were more in Hannah's 430 and exon arrays and the E13.5 array – Hannah's exon array showed 74, her 430 array showed 176; mine was 179;

this number was obtained using the iReport software, not looking at fold change in particular. We can change to say that we identified DEGs as having a fold change greater than 1.5 instead) out of approximately 39,000 transcripts (representing about 14,000 different genes) with a fold change cutoff value of 1.5 or greater. The Whole Mouse Genome array DEGs were clustered based on molecular function. From all the genes affected by Pax9 deficiency, known mesenchymal transcription regulators and signaling molecules were chosen as the most important candidates for further analysis, followed by epithelially expressed genes with large expression differences. Genes with poorly understood function were also chosen as candidate genes if they showed large expression differences between Pax9^{-/-} and wild-type. The localization of the transcripts of these DEGs were determined using the Eurexpress database: “A Transcriptome Atlas Database for Mouse Embryo” that uses *in situ* hybridization on sagittal sections of E14.5 mouse embryos to depict the expression pattern of most genes down to the single cell level (Diez-Roux et al., 2011).

Real-time quantitative PCR

RNA was quantified with a Nanodrop spectrophotometer (Thermo), and equal amounts of Pax9^{-/-} and WT RNA were used. Two step Reverse Transcriptase quantitative PCR (RT-qPCR) was performed using the GoScript Reverse Transcription System (Promega) with both random and oligo(dT) primers and GoTaq qPCR Master Mix (Promega). cDNA was prepared from total RNA using wild-type versus Pax9^{-/-} mouse tooth organ tissue. RT-qPCR was performed following Minimum Information

for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines.

Technical replicates were performed in triplicate using Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the chosen housekeeping gene. We compared expression levels of genes between the two groups. RT-qPCR and data analysis was performed using a C1000 thermocycler with a CFX96 optical reaction module (Bio-Rad). Primers for the DEGs of interest are as follows: *Adil* forward primer, 5'-CGG AGT GCT CTA TTG GAA GC-3' and reverse primer, 5'-TCC TCC TTG TCC CTG ACA TC-3'; *Bmp4* forward primer, 5'-GGA AGG CAA GAG CGC GAG GC-3', and reverse primer, 5'-GTG CGT CGC TCC GAA TGG CA-3'; *Clqtnf3* forward primer, 5'-CTC CAC AAG CTG GAG GAC TG-3', and reverse primer, 5'-TCC CAT TGT TTC CAT GGT TT-3'; *Daam1* forward primer, 5'-GAA CAC AAG CAT GAG CTG GA-3', and reverse primer, 5'-AAC ACC TCC TCA GAG CCA GA-3'; *Dkk1* forward primer, 5'-TAT GAG GGC GGG AAC AAG TA-3', and reverse primer, 5'-AGG AAA ATG GCT GTG GTC AG-3'; *E2f6* forward primer, 5'-CTG GGG GCA TTC TTG ACT TA-3', and reverse primer, 5'-GAG TTC TGC CTG CAG CTT CT-3'; *Fgf3* forward primer, 5'-GCG CTA CCA AGT ACC ACC TC-3', and reverse primer, 5'-GGC GGG AAG CAT ATG TAT TG-3'; *Foxf1a* forward primer, 5'-GGC CTC CTA CAT CAA GCA AC-3', and reverse primer, 5'-CTG GGC GAC TGT GAG TGA TA-3'; *Gapdh* forward primer, 5'-TTG ATG GCA ACA ATC TCC AC-3', and reverse primer, 5'-CGT CCC GTA GAC AAA ATG GT-3'; *Gstm6* forward primer, 5'-CCG GAC CTG TTC TCA GAC TC-3', and reverse primer, 5'-CCC ATG GCG TAT CTC TTC TC-3'; *Msx1* forward primer, 5'-CTC TCG GCC ATT TCT CAG TC-3', and reverse primer, 5'-TAC TGC

TTC TGG CGG AAC TT-3'; *Odam* forward primer, 5'-AGC CAG ACC TCT CTC AGC AG-3', and reverse primer, 5'-AAA TAG CTG CTG CCC TGT GT-3'; *Osr2* (exon1) forward primer, 5'-CAA CAC GCT CGC TCT TTA CA-3', and reverse primer, 5'-GCA CAG CTT GGA AAG GTC AT-3'; *Osr2* (exons 2-3) forward primer, 5'-AGT TTT GCG GCA GAC ACT TT-3', and reverse primer, 5'-TCC TTT CCC ACA CTC CTG AC-3'; *Pax9* forward primer, 5'-CCA AGG GCA ACA GTC ACC-3', and reverse primer, 5'-GGC GGC TCA GTC TAT CAC TC-3'; *Shh* forward primer, 5'-GCC ATC TCT GTG ATG AAC CA-3', and reverse primer, 5'-CCA CGG AGT TCT CTG CTT TC-3'; *Tfap2b* forward primer, 5'-CCA AGA AGT GGG CTC AGA AG-3', and reverse primer, 5'-TGG CAT CTT CAA CTG ACT GC-3'; *Trp63* forward primer, 5'-TTT GAT GCC CTC TCT CCA TC-3', and reverse primer, 5'-CTT CGC AAT CTG GCA GTA CA-3'.

RESULTS

In this study we investigated the Pax9-dependent gene expression pattern in mouse embryonic day 14.5 (E14.5) tooth organs and their surrounding mesenchymal layer by microarray in order to obtain the overall pattern of gene expression changes around the time of developmental arrest (Table 2-1). We chose to pool tooth bud RNA from 20 to 30 embryos from different litters for each array to avoid sampling bias and the need for cDNA amplifications. Also, although Pax9 is only expressed in the mesenchymal layer of the tooth bud, we chose to isolate whole tooth bud RNA from mandibular first molars for our arrays instead of separating the mesenchymal from the

epithelial layer which could have resulted in greater loss or degradation of material. We adopted this strategy because the spatiotemporal expression pattern of the majority of potential target genes was either well-known or verifiable by consulting the Eurexpress data base and because we were interested in exploring indirect targets of Pax9 as well as direct ones since both are potentially suitable targets for replacement therapies. We confirmed our E14.5 results using RT-qPCR (Figure 2-1) and followed up our analysis with arrays using E13.5 tooth bud RNA, which were added later for the assessment of temporal changes in expression patterns (Figure 2-2).

Our results suggest that Pax9 has a direct or indirect impact on the up- and down-regulation of many diverse genes in the mesenchymal and sub-mesenchymal layer; in addition the differential expression of a large number of transcripts was detected in the epithelial layer and enamel knot, including several that had not been prominently associated with tooth formation. The array data were used mainly as a guide for the more accurate quantitation of expression differences by quantitative PCR.

As expected, Pax9 levels were significantly decreased in Pax9-deficient tooth organ tissue, but surprisingly, the microarray and RT-qPCR data obtained from a comparison of *Pax9*^{-/-} and wild-type tooth organs suggested that *Bmp4* expression was less than 1.5 fold down-regulated by Pax9 deficiency at both E13.5 and E14.5. *Bmp4* did not even once appear as a significantly differentially expressed gene in any of the four arrays even if a cut-off value of 1.3 fold expression difference was chosen; and only rarely was a larger expression difference encountered in RT-qPCR investigations of Pax9-deficient and wild-type cDNAs.

Instead, several other genes showed more impressive differential regulation; among these were a few **signaling factors** such as mesenchymally expressed fibroblast growth factor 3 (*Fgf3*) along with *Fgf4*, *Fgf20*, Lymphotoxin B (*Ltb*) and sonic hedgehog (*Shh*), which are expressed in the epithelial enamel knot signaling center. In fact, *Shh* was the most strongly down-regulated gene in the E14.5 arrays; but it may not be the sole cause of the tooth developmental arrest because *Shh* mutations in humans are not associated with absence of posterior teeth like seen in *Pax9* deficiency; instead they feature a solitary, upper central incisor (Roessler et al., 1996). The exact molecular mechanism of *Shh* expression dependence on *Pax9* is not yet clear, but probably involves Fgf and Wnt pathways. *Fgf3* and *Fgf4*, which are strongly down-regulated in *Pax9*^{-/-} tooth buds at E14.5 have previously been shown to be dependent on epithelial Wnt signaling and their lack of expression in *Lef1*^{-/-} mice was associated with strongly reduced *Shh* expression in the enamel knot (Kratochwil et al., 2002). Notably in humans, *Fgf3* mutations are associated with microtia and microdontia (Alsmadi et al., 2009); furthermore association studies have implied a connection between the *Fgf3* gene and tooth agenesis (Kuchler et al., 2013). Fgf and *Shh* pathway components should be amenable to substitution therapies as a potential future treatment for *PAX9*-associated tooth agenesis.

Also significantly differentially expressed were many **transcription factors** such as early growth response 3 (*Egr3*), transcription factor ap2 (*Tcfap2*), odd skipped related 2 (*Osr2*), distal-less 2 (*Dlx2*), lymphoid enhancer factor 1 (*Lef1*), *Sp6*, forkhead box fl a (*Foxfla*), t-box transcription factor 1 (*Tbx1*), *paired-like homeodomain transcription*

factor 2 (Pitx2) and others. Receptors, signal transduction proteins, structural proteins and pathway inhibitors were affected as well. Among the transcription factors, *Tcfap2b* was found to be one of the more strongly down regulated genes at both E13.5 and E14.5 in *Pax9*^{-/-} mice where it is strongly and exclusively expressed in molar dental mesenchyme. *Tcfap2b* is a member of the AP2 family of transcription factors that are involved in many developmental processes. Mutations in *Tcfap2b* are responsible for Char syndrome (Satoda et al., 2000), which is a condition that affects the development of the face including teeth, heart, and limbs. *Egr3*, which is also strongly and fairly exclusively expressed in tooth bud mesenchyme at E14.5, is substantially down-regulated; but nothing is currently known about its functional significance in tooth development. Several Keratin transcripts, most significantly *Krt17*, were down regulated.

The results for *Osr2* were at first confusing because, contrary to expectation, it was up-regulated in the array obtained with *Pax9*^{-/-} tooth bud RNA. This led to our suspicion that the *Osr2* transgene which was inserted into the *Pax9* locus and can be transcribed but not translated into protein, may have been responsible for this outcome. Indeed, when we performed RT-qPCR using primers to amplify the part of the *Osr2* cDNA not present in the transgene insert, we could show that the expression of endogenous *Osr2* was actually down-regulated by more than 3-fold.

Of the inhibitors that affect the Wnt and Bmp4 pathways, *Sostdc1*, *Dkk4*, *Sfrp4* and *Apcddl* are down-regulated while *Dkk1* and *Chrdll* show modest up-regulation at E14.5 (Ahn et al., 2010; Fedi et al., 1999; Hsieh et al., 1999; Leyns et al., 1997; Sakuta

et al., 2001), suggesting that Pax9 may have a role in fine-tuning *Wnt*-induced activation of the morphogenetic process; possibly to allow for mesenchymal growth before final differentiation. Some of these extracellular pathway inhibitors may also be useful for replacement therapies.

Several of the differentially expressed genes were found in the sub-mesenchymal layer (C1qtnf3, Sfrp4, and others), indicating that the activities of Pax9 are not limited to the tooth bud but may include supporting structures.

DISCUSSION

Human trials for the substitution of recombinant EDA for a curative treatment of hypohidrotic ectodermal dysplasia, including its associated tooth agenesis have started. These trials were born from experiments with EDA deficient mice and dogs which showed that treating affected embryos via maternal injection or the affected newborn mice and dog pups with recombinant Eda could prevent the phenotypic effects of the gene mutation (Gaide and Schneider, 2003). This success inspired the pursuit of similar approaches for the treatment of other genetically linked developmental disorders.

Pax9 deficiency causes tooth developmental arrest at bud stage in both humans and mice, yet the molecular mechanisms that lead to this defective phenotype have remained poorly understood. We pursued this knowledge gap with microarray studies from murine embryonic tooth organs. Only around 200 genes were shown to be differentially expressed greater than 1.5 fold. Using bioinformatics approaches and real-time quantitative PCR we narrowed our results to even fewer genes in which we were

confident were differentially expressed and also located in the developing tooth organ region.

Unexpectedly Bmp4 expression was not significantly altered in our study of Pax9-deficient tooth buds versus wild-type tooth buds. This result requires careful scrutiny since it does not agree with prior investigations. In wild-type mice, Bmp4 had been repeatedly shown by *in situ* hybridization to be prominently expressed in tooth bud mesenchyme at E13.5 and E14.5; at E14.5 Bmp4 was shown to be additionally expressed in the emerging enamel knot signaling center in tooth bud epithelium. In Pax9-deficient mice, however, Bmp4 was reported to be neither detectable in the mesenchymal nor in the epithelial layer by *in situ* hybridization. Therefore we had expected a significant difference of Bmp4 expression in our RNA samples from E14.5 WT and Pax9-deficient whole tooth germs but to our disappointment this was not the case. We did not observe a similar discrepancy between previously reported *in situ* hybridization results and our array or qPCR data with respect to *Msx1*, *Lef1*, *Osr2*, *Shh* and *Fgfs*. At the present it is not clear what caused this disagreement; to our support it should be noted that it has also been shown recently that Bmp4 expression in *Msx1* deficient mice is by far not as extensively down-regulated as had previously been assumed (our unpublished results and (Jia et al., 2013; O'Connell et al., 2012)).

Since Bmp4 expression seems largely unchanged in Pax9^{-/-} deficiency; a slight delay in developmental timing should have allowed for the accumulation of sufficient Bmp4 to proceed with tooth morphogenesis (Miletich et al., 2011). Bmp4 mutations in humans have been described to affect the development of eyes but not teeth; only one

paper (Huang et al., 2013) suggests that a Bmp4 prodomain mutation may be associated with relatively mild premolar agenesis. In summary, our data suggest that BMP4 is not the most important factor in the development of Pax9 related tooth agenesis.

Endogenous Osr2 is significantly down regulated in Pax9^{-/-} tooth bud tissue as expected, since it had been previously described that expression of Osr2 in tooth bud mesenchyme is dependent on Pax9 (Zhou et al., 2011). Osr2 however must be an inhibitor of tooth development because its removal leads to an additional row of teeth or to significant rescue of the normal row of teeth in mice lacking all mesenchymal Bmp4 (Jia et al., 2013). Therefore the down regulation of Osr2 cannot be responsible for the tooth developmental arrest resulting from Pax9 deficiency. In contrast to the situation in Pax9^{-/-} mice, experimental Osr2 down regulation in Msx1^{-/-} tooth germs seems to restore tooth development (Zhang et al., 2009b). This demonstrates quite clearly that the complexity of gene regulatory events during tooth development requires further research.

According to our results Shh and Fgf3 are the most strongly down-regulated genes at E14.5. Also substantially reduced is the expression of two other Fgf genes, Fgf4 and Fgf20, that are well-known for their contribution to odontogenesis. Fgfs and Shh have been previously shown to be integrated into an epithelial-mesenchymal signaling loop which is initiated by epithelial Wnt signaling and mediated by Lef1, a transcription factor in the canonical Wnt pathway (Kratohwil et al., 2002). More specifically, this signaling loop probably starts with Wnt 10 (a or b) around bud stage leading to Lef1 induced Fgf4 expression in the epithelial enamel knot area. Fgf4 then signals to the

dental mesenchyme resulting in mesenchymal Fgf3 activation which in turn is required for subsequent Shh expression in the enamel knot signaling center. This signaling loop is interrupted in *Lef1* deficient mice leading to tooth developmental arrest at bud stage similar to the situation in *Pax9*-deficient mice. In *Lef1* deficiency, tooth development can be completely rescued by the application of Fgf4 (also Fgfs 7, 8a, 9 or 10) but not by application of Bmp4 or Shh. Our results suggest that *Pax9* is somewhere involved in this Wnt-Fgf signaling loop. Additional support for this theory is provided by the finding that constitutional activation of Wnt signaling can override the *Pax9*-associated tooth developmental arrest leading to an abundance of irregular teeth (O'Connell et al., 2012). The importance of the Wnt pathway in odontogenesis is furthermore underscored by the fact that mutation in *WNT10a* appear to be the most common cause of human tooth agenesis (Bohring et al., 2009; Mues et al., 2014; van den Boogaard et al., 2012).

The question now arises at which stage and how *Pax9* becomes involved in this Wnt-Fgf-Shh signaling loop. *Pax9* starts being expressed quite early (before E12) in the condensing mesenchyme and Fgf3 cannot be its most important target because selective Fgf3 deficiency leads only to smaller, not to missing teeth. Similarly, a reduction of Shh cannot be the sole cause of *Pax9*-associated tooth agenesis since human *Shh* mutations produce a much less severe and also different dental phenotype than *Pax9*, featuring only a solitary maxillary central incisor instead of the molar and premolar agenesis encountered in patients with *PAX9* mutations (Das et al., 2002; Roessler et al., 1996).

The modulation of some aspects of Wnt signaling itself may be another factor contributing to the *Pax9*-associated tooth agenesis. This can be inferred from the down-

regulation of the Wnt pathway inhibitors *Sostdc1*, *Dkk4*, *Sfrp4* and *Apcdd1* and the up-regulation of *Dkk1* at E14.5. All these inhibitors show different expression profiles and only *Dkk1* seems to be exclusively located in the dental mesenchyme. *Dkk1* (Dickkopf-related protein 1) is a potent Wnt antagonist and other studies have shown that over-expression of *Dkk1* in a 2.3-kb *Col1a-Dkk1* transgenic mouse leads to malformed second molars and loss of third molars (Han et al., 2011). It would be of great interest to test the effect of these Wnt inhibitors on the craniofacial development of *Pax9*-deficient mouse embryos with or without the addition of *Fgfs* and *Shh*.

Another important role of *Pax9* (and especially *Msx1*) may be the promotion of mesenchymal growth, probably mediated by *Fgfs* like *Fgf3* coupled with a delay of premature differentiation, which could be achieved through temporary global inhibition of epithelial *Wnt* signaling.

Lately it is becoming apparent that mesenchymal *Bmp4* activation is mainly achieved through *Wnt* signaling, but *Pax9* and *Msx1* may contribute slightly through positive regulation of mesenchymal *Lef1* expression (Behrens et al., 1996). On the other hand, the importance of mesenchymal *Bmp4* expression has been diminished by showing that its expression is not required to the extent previously thought (Jia et al., 2013)- possibly a lack of mesenchymal *Bmp4* can be compensated for by epithelial *Bmp4*.

CHAPTER III

A BOOST IN WNT SIGNALING RESCUES PALATE FORMATION

SYNOPSIS

In this study, we found that a few maternal injections of a small molecule Wnt pathway activator prevents cleft palate formation in Pax9^{-/-} mouse embryos without affecting other associated phenotypes such as tooth and thymus agenesis, or hind limb polydactyly. No overt adverse effects of this treatment were detected in mother or normal littermates suggesting that small molecule signaling pathway modulators may be effective for the prevention of developmental abnormalities.

INTRODUCTION

Pax9 is a paired box transcription factor which is required for craniofacial, tooth, and limb development. Mice without a functional Pax9 gene die at birth and have a missing thymus, missing parathyroid glands, cleft secondary palate, tooth agenesis, and supernumerary digits of the hind limb while heterozygous mice are completely normal (Peters et al., 1998b). In humans only heterozygous pathogenic mutations have been found in the *PAX9* gene, all of which cause severe non-syndromic tooth agenesis of mostly posterior teeth (Wang et al., 2009b). Concordantly, a possible association of the *PAX9* gene with orofacial clefting (Ichikawa et al., 2006; Lee et al., 2012; Song et al., 2013) in humans has also been described.

The molecular and pathophysiological mechanisms leading to cleft palate in Pax9-deficient mice have been reported to involve down-regulation of Bmp4, Fgf10, Shh and Osr2 resulting in malformed palatal shelves which fail to elevate, a defect which could be partially rescued by restoring Osr2 expression (Zhou et al., 2013). Pax9 has also been described to play a role in palatal fusion when studied in TGF- β 3 null mice (Sasaki et al., 2007), however, others have shown that Pax9-deficient palates can fuse *in vitro* when placed next to each other suggesting that clefting is primarily due to lack of proper palate morphogenesis and elevation.

In order to gain a better understanding about Pax9 target genes in tooth development, we previously evaluated the gene expression differences between Pax9-deficient (Pax9^{-/-}) and wild-type (Pax9^{+/+}) mouse embryonic tooth bud tissue by expression microarray and qPCR analysis. Unexpectedly, among all the gene expression changes we found several genes involved in the Wnt signaling pathway, which had so far not been described as a Pax9 target although it had been shown that constitutively activated Wnt signaling can overcome the tooth developmental arrest in Pax9 deficient mice (O'Connell et al., 2012).

MATERIALS AND METHODS

To determine if the Wnt pathway does in fact play a role in the pathogenesis of the Pax9^{-/-} phenotype we chose to modulate the Wnt pathway *in vivo* by injecting a small molecule Dickkopf-related protein 1 (Dkk1) inhibitor (Pelletier et al., 2009) (WAY-262611, Enzo Life Sciences) into pregnant Pax9^{+/-} mice which had been mated with

Pax9^{+/-} males. Pax9^{-/-} mice were kindly provided by Rulang Jiang and are previously described (Zhou et al., 2013). Briefly, the Pax9 locus contains a *frt*-flanked *neo* expression cassette followed by an unexpressed *Myc-Osr2A* cDNA cassette which replaces exon 2 of the Pax9 gene. Pax9^{-/-} mice resulting from the mating of heterozygous parents of this strain display phenotypes identical to a previously described Pax9-deficient mouse model (Peters et al., 1998b) such as missing teeth, cleft palate, thymus and parathyroid gland abnormalities, and hind limb polydactyly, all of which are completely penetrant.

The drug used for increasing Wnt signaling, (1-(4-(naphthalen-2-yl)pyrimidin-2-yl)piperidin-4-yl) methanamine, also known as WAY-262611, has been described previously (Pelletier et al., 2009) and was shown to potentiate the Wnt β -catenin cellular signaling pathway through the inhibition of the potent Wnt inhibitor Dkk1. WAY-262611 was dissolved in DMSO and diluted 1:10 with PBS. Three consecutive doses of 12.5 mg/kg were injected in the tail veins of Pax9^{+/-} pregnant mice at embryonic days E12.5, E13.5 and E14.5 (n=15; 6 pregnant females with 15 homozygous recessive embryos total). The injection of vehicle (10% DMSO in PBS) alone at E12.5, E13.5 and E14.5 did not rescue palate formation in the Pax9^{-/-} embryos (n=7; 3 pregnant females with 7 homozygous recessive embryos total). All mouse pups were inspected immediately after birth (P0) and their genotype was determined. The palate phenotype of the Pax9^{-/-} pups was observed both visually with a stereo dissecting microscope and histologically using H&E staining. Wild type and heterozygous Pax9^{+/-} littermates of the Pax9^{-/-} pups as well as their mothers were observed for 12 weeks).

All experiments complied with all relevant institutional and national animal welfare laws, guidelines, and policies.

RESULTS

To determine if the Wnt pathway does in fact play a role in the pathogenesis of the Pax9^{-/-} phenotype we chose to modulate the Wnt pathway *in vivo* by injecting the small molecule Dkk1 inhibitor (WAY-262611, Enzo Life Sciences) into pregnant Pax9^{+/-} mice which had been mated with Pax9^{+/-} males. Since Dkk1 is a potent Wnt inhibitor, expressed coordinately with Pax9 in dental mesenchyme, we expected WAY-262611 to be effective in increasing Wnt signaling activity and reversing any phenotypic symptoms of the Pax9^{-/-} mouse embryos that were caused by down regulation of Wnt activity. We found that WAY-262611 could prevent the cleft palate phenotype of Pax9^{-/-} mouse embryos (Figure 3-1), but not any of the other developmental malformations such as missing teeth, lack of pharyngeal pouch derivatives, or polydactyly. Thirteen out of a total of 15 Pax9^{-/-} pups from 6 litters showed rescued palate fusion. However, the palatal fusion did not prevent postnatal death of the pups, indicating that other Pax9 target organs, such as parathyroid glands or thymus, may be more instrumental in perinatal lethality.

Additionally, the WAY-262611 injections had no negative effects on the wellbeing of the mother or the health of the Pax9^{-/+} or Pax9^{+/+} littermates, both of which were followed for more than twelve weeks. The injection of vehicle (10% DMSO in

PBS) alone at E12.5, E13.5 and E14.5 did not rescue palate formation in Pax9^{-/-} embryos (n=7).

DISCUSSION

In terms of preventing tooth agenesis, it is likely that Pax9 affects additional pathways besides just Wnt and therefore a therapeutic approach that targets several different pathways would probably be needed to be effective. Alternatively, a stronger boost in Wnt signaling or different Wnt pathway activator may be required.

Wnt genes are known to regulate many developmental processes, including craniofacial development, but they are rarely mentioned as contributors to clefting disorders: One report describes a family with homozygous nonsense mutations in WNT3 causing orofacial clefts as part of a Tetra-amelia syndrome (Niemann et al., 2004); and a few association studies also suggest that WNT3 may contribute to an increased risk for cleft lip/palate in humans (Menezes et al., 2010; Mostowska et al., 2012). In mice, investigators have shown that the cleft palate following retinoic acid administration during pregnancy is at least partly caused by inhibition of canonical Wnt signaling (Hu et al., 2013). Other investigators found that cleft lip with cleft palate caused by inactivation of Pbx genes also involves Wnt down-regulation; they succeeded in rescuing cleft lip but not cleft palate formation by ectopic ectodermal Wnt expression (Ferretti et al., 2011).

The implications of our findings are the following: 1) The Wnt pathway seems to have a significant role in orofacial cleft development. 2) The paired domain transcription

factor Pax9 is involved in the regulation of Wnt pathway activity during the development of some craniofacial tissues. 3) Small molecule modulators of major signaling pathways have the potential to become effective drugs for the prevention of developmental malformations. 4) The critical targets of any developmentally active transcription factor are likely to differ from tissue to tissue requiring combination replacement therapy. 5) Temporally restricted administration of these agents may be tolerated without causing severe adverse effects.

Finally, the possibility exists that WAY-262611 or Dkk1 have a wider spectrum of molecular activity besides affecting only Wnt signaling. Further investigation of this drug and the function of Dkk1 is warranted to understand the exact mechanism that is driving their role in palatal growth, morphogenesis, and/or fusion.

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CHAPTER IV

IS THERE A LINK BETWEEN OVARIAN CANCER AND TOOTH AGENESIS?*

SYNOPSIS

An epidemiologic study from the year 2008 found a highly significant increase of congenital tooth agenesis in women with ovarian cancer suggesting that a common genetic etiology may predispose women to both conditions. The finding was reminiscent of a previously described family harboring an *AXIN2* mutation which could be shown to segregate with both the tooth agenesis and the predisposition to colon cancer transmitted in this family. Since tooth agenesis as a marker for susceptibility to ovarian cancer would be of great relevance to both oncologists and women with inborn missing teeth, the relationship between the two disorders requires a thorough assessment. We examined DNA samples from the ovarian cancer patients who participated in the original study, to look for a possible genetic connection between their ovarian malignancies and tooth agenesis. *MSX1*, *PAX9*, *AXIN2*, *EDA*, *WNT10A*, *BARX* and *BRCA1* genes were selected for sequence analysis as they may cause tooth agenesis, are expressed in the female reproductive system, and/or are involved in tumorigenesis in general or specifically in the ovary.

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Our study revealed evidence that one half of the dually affected patients had an independent causation of the two conditions, thus reducing the previously estimated ovarian cancer risk for women with congenital tooth agenesis quite significantly.

INTRODUCTION

In 2008, Chalothorn et al. described an increased prevalence of congenitally missing teeth in women with epithelial ovarian cancer (Chalothorn et al., 2008). Twenty percent of women with neoplastic ovarian disease reported one or two missing teeth, versus three percent in a cancer-free control sample. Surprisingly, ten ovarian cyst patients (unpublished) displayed an even greater prevalence of hypodontia: forty percent. These observations suggested that there may be common genetic factors affecting both tooth development and susceptibility to the formation of epithelial tumors or cysts of the ovary, similar to the sequence variant in the *AXIN2* gene which causes both tooth agenesis and colorectal cancer (Lammi et al., 2004).

Early detection of epithelial ovarian cancer is difficult and as a result, the mortality rate is unacceptably high. If a link were found between tooth agenesis and ovarian cancer, semi-annual screening could become the standard of care for women with tooth agenesis to increase the early detection rate (van Nagell et al., 2007). This would not only provide a new diagnostic tool but also open up new biological insight into epithelial ovarian cancer which, according to latest findings, may actually originate in the fallopian tube epithelium since gene expression patterns in these two tissues resemble each other closely (Kurman and Shih Ie, 2011).

The lifetime risk for neoplastic ovarian disease is only about a fifth of that for tooth agenesis, which occurs in approximately 3% to 9% of the population even when 3rd molars are excluded (Mattheeuws et al., 2004; Shapiro and Farrington, 1983). So far, sequence variants in *WNT10A*, *MSX1*, *PAX9*, *AXIN2* and *EDA* pathway genes have been shown to cause about 50% of selective tooth agenesis in humans (Bergendal et al., 2011; Bohring et al., 2009; Lammi et al., 2004; Stockton et al., 2000; Tao et al., 2006; Vastardis et al., 1996). Several of these genes are also expressed in tumor cells of the female reproductive system, suggesting a possible mechanism for the relationship between ovarian disorders and hypodontia.

The homeobox gene *BARX2* is expressed in maxillary and mandibular arches (Jones et al., 1997) and in the developing tooth and is frequently dysregulated in epithelial ovarian cancer (Sellar et al., 2001; Sellar et al., 2002). *BARX1*, a related homeobox gene, plays an important role in molar morphogenesis (Gould and Walter, 2000).

Overexpression of *Msx1* inhibits ovarian carcinoma cell proliferation by inducing apoptosis through interaction with the tumor suppressor p53 (Park et al., 2005). *MSX1* deficiency has been seen in human ovarian cancer cells (Park et al., 2001) and other malignancies (Peters and Balling, 1999). Sequence variants in *MSX1* have been shown repeatedly to cause oligodontia of premolars, molars and incisors similar to *PAX9*, a paired box transcription factor which is thought to control mesenchymal Bmp4 signaling during odontogenesis (Peters et al., 1998a). *PAX9* expression was also found in five of six epithelial ovarian cancer cell lines examined (Muratovska et al., 2003).

AXIN2 is a member of the Wnt signaling pathway, which is highly conserved in evolution and controls many events during embryogenesis such as morphogenesis, proliferation, motility and cell fate. Individuals with *AXIN2* sequence variants can have both tooth agenesis and a strong predisposition for developing colorectal cancer (Lammi et al., 2004; Mostowska et al., 2006). *WNT10A* is the most commonly altered gene in tooth agenesis (Bohring et al., 2009) with a large number of missing teeth in homozygotes, and a few missing teeth in about 50% of heterozygotes. Sequence variants in *BRCA1* are the best-known causes of breast and ovarian cancer and, although the gene is also expressed in the developing tooth, it has never been implicated in tooth agenesis.

The *EDA* gene is not associated with ovarian cancer or development, however, sequence variants of the *EDA* gene cause the syndrome X-linked Hypohidrotic Ectodermal Dysplasia (Li et al., 2008) and also non-syndromic tooth agenesis. Carrier females may present with one or two missing or malformed teeth. Therefore an *EDA* sequence variant found in any of the ovarian cancer samples would signify coincidental tooth agenesis.

The primary goal of our study was to investigate if the five well-established tooth agenesis genes *WNT10A*, *EDA*, *PAX9*, *MSX1*, and *AXIN2* as well as the *BARX1*, 2 and *BRCA1* genes show any evidence of involvement in the tooth agenesis/ovarian cancer association by doing a thorough sequence analysis of these candidate genes in the original patient sample from the study by Chalothorn.

MATERIALS AND METHODS

Subject recruitment

The original protocol was IRB approved by the University of Kentucky. Fifty subjects with ovarian cancer and another ten with ovarian cystic disease, each with or without tooth agenesis, were recruited from the University of Kentucky Ovarian Screening Clinic. Inclusion criteria were peri- or post-menopausal, ages 45 or older and no obvious signs of a syndrome. Additional unrelated patients with tooth agenesis and no personal or family history of ovarian disease were recruited under a separate IRB approval from Texas A&M University Baylor College of Dentistry. All participants were Caucasians except one who was of Japanese ancestry.

The final patient cohorts consisted of four groups: 1) 10 patients with agenesis of 1 to 2 teeth and ovarian cancer, 2) 40 patients with only ovarian cancer, 3) 35 patients with agenesis of 1-8 teeth without ovarian disorders, and 4) the 10 patients with ovarian cysts of whom 4 had mild tooth agenesis.

The wild-type sequence and allele frequencies of common variants in control populations were obtained from the NCBI SNP database and the NHLBI Exome sequencing project (ESP). Wild-type reference sequences obtained from NCBI are as follows: *MSXI* (NM_002448.3), *PAX9* (NM_006194.3), *AXIN2* (NM_004655.3), *EDA* (NM_001399.4), *WNT10A* (NM_025216.2), *BARX1* (NM_021570.3), *BARX2* (NM_003658.4) and *BRCA1* (NM_007294.3).

Informed consent was obtained and a thorough patient history and dental exam was performed. If a patient was unsure of history, the patient's dentist was consulted to

confirm etiology of any missing teeth. DNA samples were obtained using BuccalAmp swabs and sent to Texas A&M University Baylor College of Dentistry for analysis.

DNA extraction from buccal swabs

Since buccal swabs may not have yielded sufficient material for the analysis of eight to ten genes, the samples were amplified by Whole Genome Amplification (WGA) with the GenomiPhi WGA system (GE Healthcare). Successful genome amplification was verified by gel electrophoresis of amplified samples together with a quantitation marker.

Polymerase chain reaction and sequencing of products

The exons of each gene were PCR amplified with GoTaq reagents (Promega) using a 96-well plate format for the 95 samples and one negative control. Several of the amplicons were very GC-rich and required PCR optimization and the use of 5% DMSO. Quality and quantity of PCR products was confirmed by gel-electrophoresis, followed by treatment with ExoSapIt (Affymetrix) and then addition of specifically designed sequencing primers. Automated dideoxy chain terminator sequencing was done by Seqwright, TX and GenScript, NJ.

Analysis of sequencing results

All sequences were visually inspected for heterozygous base changes and compared to the corresponding wild-type sequences previously mentioned using the

NCBI BLAST program. Once a nucleotide change was found, the SNP (single nucleotide polymorphism) database was consulted to determine if the SNP is a common polymorphism. For common SNPs, the allele frequencies were compared 1) between the different experimental groups and 2) between experimental groups and Caucasian population controls reported in NCBI and NHLBI databases. For *MSX1* polymorphisms, the Caucasian control allele frequencies from a study by Jezewski et al. (supplement) were also employed (Jezewski et al., 2003). Chi-square statistics was used for the determination of statistical significance of allele frequency differences.

RESULTS

Sequence analysis of the *EDA*, *WNT10A* and *BRCA1* genes yielded several interpretable results (Table 4-1):

1) One of the ten patients with combined ovarian cancer/tooth agenesis was a carrier of the known p.Arg69Leu sequence variant in *EDA* (rs132630309), explaining the tooth agenesis, and she also had a frameshift sequence variant in *BRCA1*, p.Gln1096_Ser1097=fs (rs80357686), most probably responsible for the ovarian cancer.

2) Another tooth agenesis/ovarian cancer patient had the *BRCA1* frameshift sequence variant, p.Lys679Ter (rs80357082), which was also present in two other ovarian cancer patients without tooth agenesis, indicating that this patient's ovarian cancer and her tooth agenesis have different roots and also that the investigated population shared common ancestors since the frequency of this sequence variant seems

to be quite high, but no population allele frequencies are available (presumably due to gene patent issues).

3) Three of the remaining eight tooth agenesis/ovarian cancer patients had the *WNT10A* p.Phe228Ile sequence variant (rs121908120), which in heterozygous form is probably the most common sequence variant encountered in mild tooth agenesis while causing severe tooth agenesis when homozygously inherited. There were also three patients with this sequence variant in the “ovarian cancer only” group, however about 50% of people with this sequence variant normally do not present with tooth agenesis.

These results taken together suggest that at least half of the samples from the combined ovarian cancer/tooth agenesis patients identified in the epidemiological study by Chalothorn et al. show evidence for independent causation of the two conditions. In the ten ovarian cyst patients we found sequence variants neither in *BRCA1* nor in the tooth agenesis genes *WNT10A* or *EDA*.

Sequence analysis results from the other investigated genes are presented in Tables 4-2 and 4-3 and are briefly presented below. These results were calculated for the whole group, including ovarian cancer and ovarian cyst groups.

In *BARX1*, the missense sequence variant Ala48Thr was detected in 17 samples, but no significant allele frequency differences between the groups were found. The other five polymorphisms in this gene were also insignificant.

In *BARX2*, we found four heterozygous p.Ser64Pro sequence variants; three of them in individuals with tooth agenesis, with and without ovarian cancer. p.Ser64Pro is fairly conserved among species suggesting its importance in protein function; however

since the control allele frequency reported in the SNP database is similar to the one found in this study, it may not play a role in tooth agenesis. On the other hand, we do not know if the control population used for SNP data was screened for missing teeth. The synonymous p.Pro203= polymorphism in exon 2 occurred in the ovarian cancer cohort in 50% of those with tooth agenesis versus 33% without tooth agenesis; however, the difference was not statistically significant. The remaining polymorphisms found for *BARX2* did not demonstrate allele patterns consistent with an association with a disease phenotype.

A substantial number of polymorphisms were found in the *MSX1* gene including p.Ala40Gly (formerly p.Ala34Gly). The minor allele frequency of this polymorphism was significantly higher in all groups with tooth agenesis (regardless of ovarian disease status) compared to the NHLBI ESP Caucasian control group (n> 2,000). However using the allele frequency values for 154 Caucasian controls which were reported by Jezewsky et al. in their study about *MSX1* sequence variants in cleft lip/palate patients (Jezewski et al., 2003), the differences were not statistically significant. The allele frequencies of the *MSX1* polymorphisms c.*6C>T (rs8670) and c.-18G>A (rs186861426) were also significantly higher in most groups with tooth agenesis using the NHLBI ESP (Table 4-3), but not with the Jezewski control frequencies; we also noticed that these SNPs appear to be syntenic with p.Ala40Gly.

In *AXIN2* we found the missense sequence variant p.Ser762Asn in a single ovarian cancer patient without tooth agenesis. This sequence variant may thus contribute to cancer susceptibility but not to tooth agenesis. We also found the previously reported

c.1994_1995insG sequence variant in one patient with combined ovarian cysts and tooth agenesis; however, we suspect that it is a PCR artifact since its appearance was dependent on PCR conditions and it occurs after a run of seven G nucleotides.

Sequencing of *PAX9* revealed five known and one new polymorphism (Table 4-2). One of these, a C to T transition adjacent to the p.Ala240Pro polymorphism, lead to a synonymous histidine codon which was significantly more frequent in samples from tooth agenesis patients (Table 4-3).

DISCUSSION

A genetic connection between development and cancer is intuitively understandable in that both processes are characterized by rapid cell growth which often involves the same signaling pathways. However it is less intuitively understandable that a developmental deficit like tooth agenesis should be associated with the uncontrolled expansion of cell growth found in cancer. The finding that a nonsense sequence variant in *AXIN2*, which should lead to increased WNT signaling, can cause both severe tooth agenesis and a predisposition for colon cancer in humans (Lammi et al., 2004) is therefore somewhat surprising because increased WNT signaling leads to supernumerary teeth in experimental animal models (Jarvinen et al., 2006) and *Axin2* promotes oncogenicity in colon cancer by the upregulation of *Snail1* (Wu et al., 2012). Thus, only a gain-of function sequence variant, not a nonsense sequence variant, in *Axin2* would be compatible with its double role as suppressor of tooth development and promoter of colon cancer. The double role is easier to fathom in the case of sequence variants in

MSX1, a protein that is able to interact with both DNA and other proteins and can act as a transcriptional suppressor or as an activator depending on cellular context.

Since a causal linkage between tooth agenesis and ovarian cancer would have major ramifications for the large number of women with minor tooth agenesis, the issue requires quite urgent clarification. Therefore, we undertook this investigation and approached the question of common genetic factors in tooth agenesis and ovarian cancer by screening known tooth agenesis causing genes for sequence variants as suggested in the original study by Chalothorn and also added the *BRCA1* gene and the newly attributed tooth agenesis gene *WNT10A*.

We did not find a single candidate gene or sequence variant that could explain the increased co-occurrence of tooth agenesis with ovarian cancer or ovarian cysts with the exception of the controversial c.1994_1995insG sequence variant in *Axin2*. But we did find *BRCA1* sequence variants in two of the ten cases of ovarian cancer/tooth agenesis patients, explaining the ovarian cancer. Additionally, one of these two also had an EDA sequence variant, clearly demonstrating separate origins of ovarian cancer and tooth agenesis. Furthermore, we found well known tooth agenesis-causing *WNT10A* sequence variants in three additional members of the ovarian cancer/tooth agenesis group arguing for independent causation of the tooth agenesis in these patients. Our findings do not prove that the two conditions arose independently from each other but they make it seem quite likely for at least half of the cases.

The observation by Chalothorn et al. that their 10 ovarian cyst patients had an even higher prevalence of hypodontia than the ovarian cancer cohort reinforces this

notion since so far no genetic link has been detected between epithelial ovarian cancer and ovarian cysts making it unlikely that a single gene may be responsible for all 3 disorders.

Additional evidence for independent causation comes from a recent study which did find increased rates of (self-reported) cancer in patients with tooth agenesis (Kuchler et al., 2013). However, ovarian cancer, in contrast to prostate, breast, and nervous system malignancies, was not among the significant results. We also have been enrolling a few hundred subjects in our own “Missing Tooth Study” over the past 10 years and have not noted any case with ovarian cancer or cysts in her/his family’s medical history; however, the reason for this could be the higher prevalence of tooth agenesis and incomplete recall of family health issues.

On the other hand, we know that the investigated genes represent only a minority of all the possible candidates that may be involved in hypodontia and it would certainly be worthwhile to investigate additional genes which are commonly associated with ovarian cancer such as *BRCA2*, BRCA-interacting protein, *ErbB2* and *p53*, for example, which are also quite strongly expressed in the tooth bud of developing mouse embryos (Diez-Roux et al., 2011).

In conclusion we propose that a larger epidemiological study should be conducted to confirm any link between ovarian cancer and tooth agenesis and challenge the currently held notion of a predisposition of developing cancer in tooth agenesis patients. Ideally, a large family with both ovarian cancer and tooth agenesis may be recruited for linkage analysis.

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CHAPTER V

THE WNT10A GENE IN ECTODERMAL DYSPLASIAS AND SELECTIVE TOOTH AGENESIS*

SYNOPSIS

Mutations in the *WNT10A* gene were first detected in the rare syndrome odonto-onycho-dermal dysplasia (OODD, OMIM257980) but have now also been found to cause about 35–50% of selective tooth agenesis (STHAG4, OMIM150400), a common disorder that mostly affects the permanent dentition. In our random sample of tooth agenesis patients, 40% had at least one mutation in the *WNT10A* gene.

The *WNT10A* Phe228Ile variant alone reached an allele frequency of 0.21 in the tooth agenesis cohort, about 10 times higher than the allele frequency reported in large SNP databases for Caucasian populations. Patients with bi-allelic *WNT10A* mutations have severe tooth agenesis while heterozygous individuals are either unaffected or have a mild phenotype. Mutations in the coding areas of the *WNT10B* gene, which is co-expressed with *WNT10A* during odontogenesis, and the *WNT6* gene which is located at the same chromosomal locus as *WNT10A* in humans, do not contribute to the tooth agenesis phenotype.

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INTRODUCTION

Adaimy et al. (2007) performed autozygosity mapping in three consanguineous Lebanese families with the rare Odonto-Onycho-Dermal-Dysplasia syndrome (OODD, MIM 257980) which had been previously characterized phenotypically in the same population (Fadhil et al., 1983). They found that all affected family members were homozygous for the same nonsense mutation in the *WNT10A* gene leading to the phenotypic features of severe hypodontia, onychodysplasia, smooth tongue as well as palmar and plantar hyperhidrosis and hyperkeratosis. The phenotype of heterozygous family members was not recorded. Two years later Bohring et al. (2009) reported that *WNT10A* mutations are not restricted to the rare OODD syndrome but also found in other Ectodermal Dysplasia entities like the Schöpf-Schulz-Passarge syndrome (MIM 224750) which additionally features eyelid cysts and predisposition to adnexal skin tumors. Bohring et al. also described a high prevalence of apparently non-syndromic tooth agenesis among their homozygous patients as well as mild, predominantly dental symptoms in about half of the heterozygous family members. Three further reports about the high prevalence of *WNT10A* mutations in Ectodermal Dysplasia syndromes and in non-syndromic tooth agenesis followed in 2011 (Cluzeau et al.), in 2012 (van den Boogaard et al.), and in 2013 (Plaisancie et al.).

The expression of *Wnt10a* along with *Wnt10b*, *Shh*, *Bmps2* and *4* and other developmentally active gene products during mouse odontogenesis had been investigated as early as 1998 (Dassule and McMahon). They detected *Wnt10a* at around mouse embryonic day 12 (E12) by *in situ* hybridization in the inner epithelial/ enamel

knot area of the tooth bud, where it was co-expressed with Wnt10b. Since Wnt10b expression was recognizable a little earlier and more prominently than Wnt10a expression, further investigations in this study focused on the Wnt10b molecule. At later stages of tooth development (mouse E14 to E18), Wnt10a can also be found in the mesenchymal preodontoblast layer where it contributes to or initiates odontoblast differentiation, possibly through the up-regulation of dentin sialophosphoprotein (Dspp) expression (Yamashiro et al., 2007).

WNT10A, which is located adjacent to *WNT6* at 2q35 in humans, is also active during the development of hair follicles and limbs, and in hematopoiesis. In adult tissues it is expressed in lymph nodes, blood, adrenal gland, prostate, testis, ovary, retina, brain, lung and kidney; and may also play a role in several neoplastic disorders, notably ameloblastomas, keratocystic odontogenic tumors, lymphomas and leukemias but is also found up-regulated in several cancers. Functional studies showed that Wnt10a activates the canonical Wnt pathway and regulates mesenchymal cell fate in that it inhibits adipogenesis and stimulates osteoblastogenesis (Cawthorn et al., 2012).

The general role of canonical Wnt signaling during tooth development has been explored in more detail by stabilization of β -catenin or depletion of Apc, a positive and a negative regulator of canonical Wnt signaling respectively. Both procedures constitutively activate Wnt signaling in tooth bud epithelium leading to the formation of many accessory tooth buds sprouting from the original tooth anlage. The resulting supernumerary teeth are often small but otherwise completely normal (Jarvinen et al., 2006; Wang et al., 2009a). Interestingly, the expression of Pax9 and Msx1, two normally

essential transcription factors in tooth bud mesenchyme, are not required for the formation of these supernumerary teeth. Furthermore, the inactivation of the Wnt secretion facilitator Wntless (Wls) was recently shown to prevent intraepithelial Wnt signaling leading to tooth developmental arrest (Zhu et al., 2013).

When we sequenced the *WNT10A* gene in the random collection of tooth agenesis patients who participate in our “missing tooth” study, we also found a large number of WNT10A mutations in our samples confirming the importance of WNT10A in tooth development.

MATERIALS AND METHODS

Patient recruitment

Tooth agenesis study participants were recruited via website and through collaboration with Drs. Alexandre Vieira (University of Pittsburgh) and Ophir Klein (University of California at San Francisco), following IRB approved protocols. People of all ages with any number of missing teeth except third molars were included, and only patients with overt ectodermal dysplasia symptoms were excluded. The final cohort consisted of 90 unrelated samples; half of them were from Caucasian Americans and the other half from patients from Turkey which are considered to be mostly of Mediterranean- European ancestry. Cheek swab or saliva samples were collected for the isolation of genomic DNA. The wild type sequence and allele frequencies of common variants in control populations were obtained from the NCBI SNP data bases as well as the NHLBI Exome sequencing project (ESP).

DNA extraction from buccal swabs

DNA extraction was performed with the Puregene Buccal Cell Kit (Qiagen). Since buccal swabs do not yield sufficient material for the analysis of multiple genes, the samples were amplified by Whole Genome Amplification (WGA) with the REPLI-g WGA system (Qiagen). Successful genome amplification was verified by gel electrophoresis of amplified samples together with a quantitation marker. DNA samples received from our collaborators were also amplified by WGA.

Polymerase chain reaction and sequencing of products

Exons of the *WNT10a*, *WNT10b* and *WNT6* genes were PCR amplified with GoTaq reagents (Promega) using a 96-well plate format for the 90 samples and the controls. Quality and quantity of PCR products was confirmed by gel-electrophoresis, followed by treatment with ExoSapIt (USB) and addition of the sequencing primers. Automated dideoxy chain terminator sequencing was done by GenScript, Piscataway, NJ.

Analysis of sequencing results

All sequences were visually inspected for heterozygous base changes and compared to the corresponding wild type sequences using the 'BLAST' program. Once a nucleotide change was found, the SNP (single nucleotide polymorphism) data base was consulted to determine if the SNP is a common polymorphism. For appropriate SNPs, the allele frequencies were compared between the experimental groups consisting of

Caucasian and Turkish samples; and between the experimental and the Caucasian population control groups reported in NCBI and NHLBI data bases.

RESULTS

Wnt10A but neither WNT6 nor WNT10B contribute to tooth agenesis

About forty percent of our random group of tooth agenesis patients had at least one missense, nonsense or frameshift mutation in the *WNT10A* gene. The different mutations encountered in our patient samples are shown in Figure 5-1. Most common was the mono- or bi-allelic Phe228Ile mutation with a prevalence of about 31 percent and an allele frequency of 0.21 compared to an allele frequency of about 0.02 in large Caucasian control populations and only 0.007 in African American controls (Table 5-1). The allele frequency of Phe228Ile was also calculated separately for our Caucasian (0.216) and Turkish participants (0.20) to exclude any influence that ethnic background differences could have had on the allele frequency.

Since Phe228Ile is so much more common in Caucasian tooth agenesis patients, it either is the causative factor or is closely linked to the causative mutation. Since *WNT10A* is located only a few kb telomeric of *WNT6* on chromosome 2q35 we included the latter in our analysis but did not find any mutations or polymorphisms that were syntenic with the nucleotide change leading to *WNT10A* Phe228Ile. The common *WNT6* variant Pro155Arg occurred in our tooth agenesis population at a frequency similar to normal control populations and only 1 of the 10 patients who had the *WNT6* Pro155Arg variant also had *WNT10A* Phe228Ile.

Since the WNT10A and WNT10B proteins are co-expressed in the inner dental epithelium of developing teeth and share 62 percent identity, it was conceivable that WNT10B mutations may also cause missing teeth. But sequencing of the whole coding area of the *WNT10B* gene did not reveal any nucleotide changes that could possibly be implicated in the tooth agenesis phenotype.

Phenotypes associated with WNT10A mutations

We did not receive any reports about missing primary teeth although some patients remembered having relatively small deciduous teeth. The number of missing teeth in the permanent dentition depended strongly on whether the affected individual was heterozygous or homozygous/ compound heterozygous for WNT10A mutations. Heterozygous patients were missing up to 6 permanent teeth while homozygous patients were generally missing from 6 to 26, most often 16 teeth.

While all patients with bi-allelic mutations had oligodontia, many heterozygous relatives of study participants were not affected suggesting incomplete penetrance (Figure 5-2). Syndromic ectodermal dysplasia manifestations were not encountered in our study population because they constituted exclusion criteria for participation in the study. One study participant however had a history of benign skin tumors, possibly bearing some resemblance to the Schöpf-Schulz-Passarge syndrome and another one reported mild heat intolerance.

The tooth agenesis pattern in heterozygous patients parallels that of common mild tooth agenesis with a predominant absence of lower second premolars and upper

lateral incisors; but mandibular incisors are also frequently absent, occasionally even a canine or a first premolar. The pattern is similar to EDA pathway associated selective tooth agenesis in that the anterior teeth are more often affected (Mues et al., 2010; Mues et al., 2009; Tarpey et al., 2007). Patients with homozygous *WNT10a* mutations have also posterior tooth agenesis similar to syndromic EDA pathway mutation phenotypes (Lexner et al., 2007).

DISCUSSION

Traditionally we distinguished between syndromic and non-syndromic tooth agenesis. Syndromic tooth agenesis was most often encountered as part of an Ectodermal Dysplasia phenotype while non-syndromic tooth agenesis, also called isolated or selective tooth agenesis/hypodontia (STHAG1-6 and X1 in OMIM), should only affect the dentition without any systemic manifestations. Inherent in the syndromic versus non-syndromic classification was the assumption that the two disorders had fundamentally different genetic causations.

However, the more we learn about the pathogenesis of tooth agenesis the more we realize that there is an extensive overlap between the genetic basis of syndromic and non-syndromic forms of tooth agenesis (Nieminen, 2009) and the phenotypic distinction may not have been helpful for the search of additional tooth agenesis genes. From a genetic point of view, non-syndromic and syndromic tooth agenesis are often caused by the same genes, but the development of some teeth seems to be more sensitive to gene

dosage and thus are the first organs to be affected, while other ectodermal appendices may still form normally (Mues et al., 2010).

The distinction between syndromic and non-syndromic tooth agenesis may also be problematic with respect to the new, biologically based diagnostic and therapeutic approaches for which dental professionals who are traditionally the only health care providers for non-syndromic tooth agenesis patients, may still be little prepared. Classification of the STHAGs as ectodermal dysplasia entities may therefore be desirable from a clinical point of view.

The high prevalence of *WNT10A* mutations is truly astounding, especially since this gene is hardly ever mentioned in the extensive literature about the molecular genetics of tooth development. Even more surprising is the large number of Caucasian tooth agenesis patients with one particular mutation, *WNT10A* Phe228Ile. Pathogenic mutations are usually lost from the gene pool of a population unless they have some kind of survival advantage like for example heterozygous mutations in the hemoglobin genes which are of benefit in areas with high malaria incidence. The survival advantage is often lost in individuals with homozygous mutations like in sickle cell disease, but since homozygously affected individuals are quite rare, the mutation has an overall positive effect on population growth (Fleming et al., 1979). It will certainly be interesting to find a cause for the high prevalence of *WNT10A* Phe228Ile mutations.

Interesting is also that the tooth agenesis pattern of patients with *WNT10A* mutations resembles that of *EDA* pathway associated hypodontia. Both *WNT* and *EDA* pathways are known to operate predominantly in the epithelial layer of the developing

tooth and repeated interactions between the two pathways have been observed during ectodermal appendage formation and *in vitro* (Durmowicz et al., 2002; Laurikkala et al., 2001; Zhang et al., 2009a). It is therefore possible that the new, biological replacement therapies for EDA pathway mutation associated disorders may also be of value in the much more common WNT10A disorder. It would certainly be worth testing this possibility because EDA replacement therapies have shown great efficacy in ameliorating disease symptoms in animals and are currently tested in humans, while the generation of WNT10A specific therapeutics would be quite complicated not only from a chemical engineering point of view but also because of the lack of a one to one correspondence of ligands and receptors in the WNT pathway, potentially leading to severe adverse effects, and even more importantly, a lack of a Wnt10a-deficient, diphyodont animal model for the testing of these therapeutics.

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CHAPTER VI

CONCLUSION

In this project I investigated both the basic science and clinical aspects of the molecular genetics of tooth agenesis in order to:

A) Characterize those target genes of the transcription factor Pax9 which mediate the tooth developmental arrest observed with Pax9 deficiency. These target genes have not only the potential to serve as therapeutics for the rescue of the tooth developmental arrest but may also be tooth agenesis causing genes themselves deserving to be screened for mutations in tooth agenesis patients.

B) Identify the particular mutations in known or suspected tooth agenesis genes that are responsible for the tooth agenesis in each individual of a large group of patients with inborn missing teeth. An accurate molecular diagnosis is required before any molecular therapeutics can be used and the majority of the as yet unknown tooth agenesis genes should be discovered.

The initial project in which I studied the downstream effects of Pax9 revealed that the regulatory mechanisms surrounding tooth agenesis were not as straightforward as previously thought. The most intriguing aspect of this study is that the gene that was presumed to be the central downstream effector of Pax9 on tooth formation, Bmp4, had mRNA levels that were relatively unchanged in our Pax9-deficient model during early tooth formation. This coupled with the fact that many other signaling pathways were much more heavily affected indicates that tooth agenesis cannot be easily attributed to

the loss of a single gene or gene product downstream of the causative gene and may therefore require treatment with multiple replacement factors if the mutated gene product itself is not suitable for replacement.

The signaling pathways that appear to be markedly affected by Pax9 deficiency, Wnt, Fgf and Shh, were previously thought to be relatively minor targets of Pax9 or the result of diminished Bmp4 activity. The latter seems unlikely in light of our finding of barely changed Bmp4 expression.

The impact of Pax9 on Wnt pathway regulators is of special interest because recent clinical studies indicate that mutations in one Wnt pathway member, the WNT10A gene, may be responsible for approximately 40% of all congenital human tooth agenesis. In concord with this clinical finding, many basic research studies have also detected the importance of the Wnt pathway which is gradually being revealed to be the master regulator of tooth formation, much like it is a major player in limb formation. Constitutively activated Wnt signaling has even been shown to overcome the tooth developmental arrest in Pax9 deficient murine tooth buds (O'Connell et al., 2012).

To test the effects of a temporary and physiologically more tolerable increase in Wnt signaling in our Pax9-deficient mouse model we used a small molecule Wnt agonist (an antagonist of the Wnt inhibitor Dkk1 which was upregulated in Pax9^{-/-} mice at E14.5). Injection of small amounts of this molecule into the maternal tail vein at discreet times during embryonic development led to the surprising finding that we could rescue the cleft palate phenotype of the Pax^{-/-} embryos but the tooth phenotype was unchanged.

Nevertheless, another fortunate finding was that there were no apparent deleterious effects of using this molecule on normal and healthy wild-type embryos or the mother.

Originally it was assumed that the cleft palate was to blame for the postnatal death of Pax9-deficient pups. However, pups with rescued palate still died at birth, with the cause now most likely being the lack of development of other missing pharyngeal pouch derivatives. Hind limb polydactyly was also unchanged, presumably because the drug was given after the start of limb and digit formation. It would be interesting to see if this polydactyly would be corrected by earlier application of the drug. Most likely, however, at this earlier stage of development, around E10, the treatment would produce more undesirable side effects. Since we could rescue the cleft palate but no other phenotype manifestations of Pax9 deficiency, we suggest that combination therapy may be needed to totally restore Pax9-deficient mice, for example adding recombinant Fgf3, 4 and/or an Shh agonist like the Smoothened Agonist, SAG.

We also tested the effect of injecting the Bmp4 agonist isoliquiritigenin (not published). This molecule was shown to act as a Bmp4 agonist in cell culture and in zebrafish; however, it had not yet been tested in mammals. Our results showed that there was no effect of this treatment suggesting that either this molecule does not perform well in mammals or that Bmp4 deficiency is not a major cause of the Pax9-deficient phenotype. Based on our microarray and RT-qPCR results it is most likely the latter.

The other aspect of this dissertation focuses on clinical approaches to diagnose the molecular genetic causes of tooth agenesis in individual patients. In order to find new tooth agenesis genes we screened several of our newly discovered Pax9 downstream

target genes (Fgf3, Fgf4, Tcfap2b, Sostdc1, Lef1 and others) for mutations in 90 independent DNA samples from patients with tooth agenesis. None of the chosen genes showed mutations in our tooth agenesis samples except for one rare polymorphism in Lef1 which may have contributed to the tooth agenesis phenotype of the donor of that sample.

Our study also investigated the alleged association of ovarian cancer with tooth agenesis and showed that there was essentially no increased risk for women to acquire ovarian cancer if they had missing teeth because we found through candidate gene sequence analysis that the tooth agenesis and the ovarian cancer most probably arose independently from each other. The importance of our study is obvious since tooth agenesis is the most common developmental abnormality in humans and this high prevalence would lead to unnecessary anxiety in many women who, in reality, are not at an increased risk at all of developing a malignant neoplasm. Studies such as this suggest the need to further investigate issues where clinical associations suggest causality when in fact there is only a correlation.

We also showed through diagnostic gene sequencing in 90 tooth agenesis samples that all known Wnt mutations that affect tooth formation reside within the WNT10A gene and not within other Wnt genes such as WNT6 which is located adjacent to WNT10a on chromosome 2 or Wnt10b which is co-expressed with WNT10A during tooth development. Also of note is that a single mutation, Phe228Ile, is astonishingly common in Caucasian populations and yet there does not seem to be an advantage to this mutation. Usually harmful mutations are lost from the gene pool unless they have

heterozygote advantage such as mutations in hemoglobin in individuals with sickle cell anemia who gain resistance to malaria. It would seem that the great unsolved question is what kind of heterozygote advantage a reduced Wnt10a activity could have.

Our overall conclusion is that it should be possible to rescue genetically caused developmental disorders by substitution of the crucial gene products at the correct developmental time interval. The substitution therapy may consist of recombinant proteins or small molecule genetic pathway regulators and may require only one factor in the case that the dysfunctional gene product can be replaced directly, or it may require the replacement of several factors if downstream targets of the dysfunctional gene product have to be used. In any case the genetic cause of the developmental disorder has to be diagnosed before the appropriate replacement therapy can be determined.

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APPENDIX A

FIGURES

Figure 2-1. RT-qPCR data versus microarray data for E14.5.

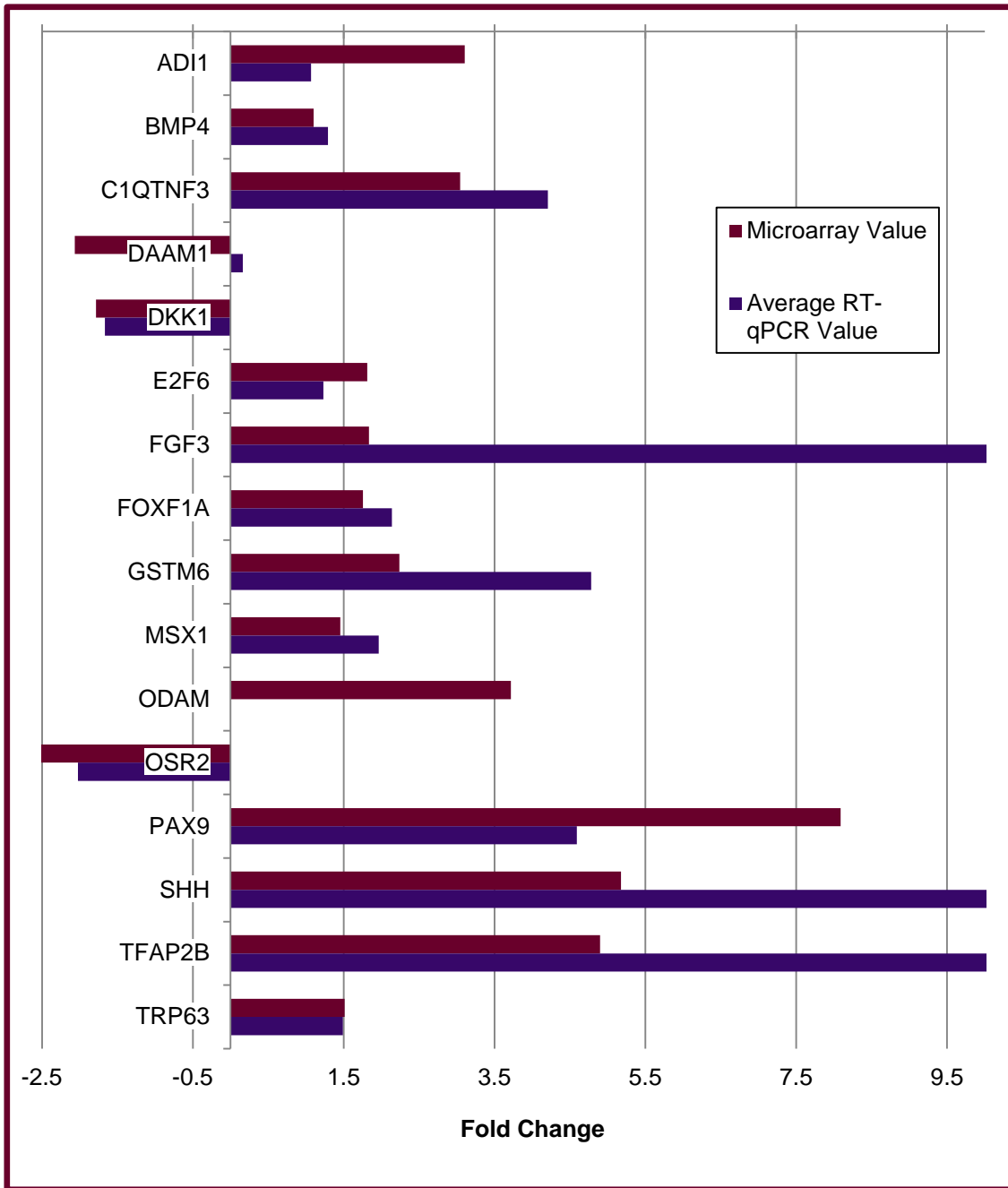
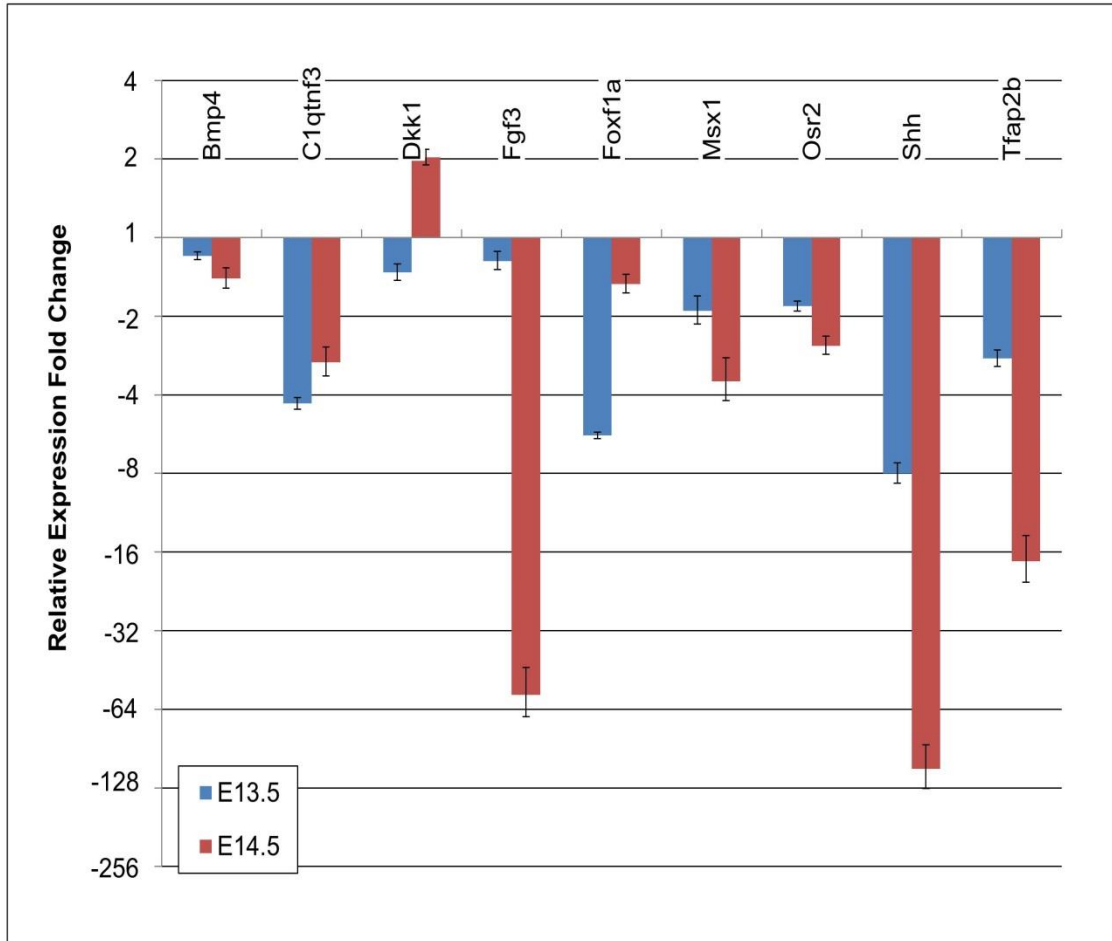


Figure 2-1 Continued.

FGF3, SHH, and TFAP2B all showed a >9.5-fold change (E14.5). Most microarray gene expression values align with their RT-qPCR counterparts except for DAAM1 and ODAM. DAAM1 showed overexpression in the array while RT-qPCR showed very slight under-expression. ODAM's relative fold change was no different from wild-type values when performing RT-qPCR.

Figure 2-2: Relative gene expression pattern in Pax9^{-/-} tooth organs at E13.5 and E14.5



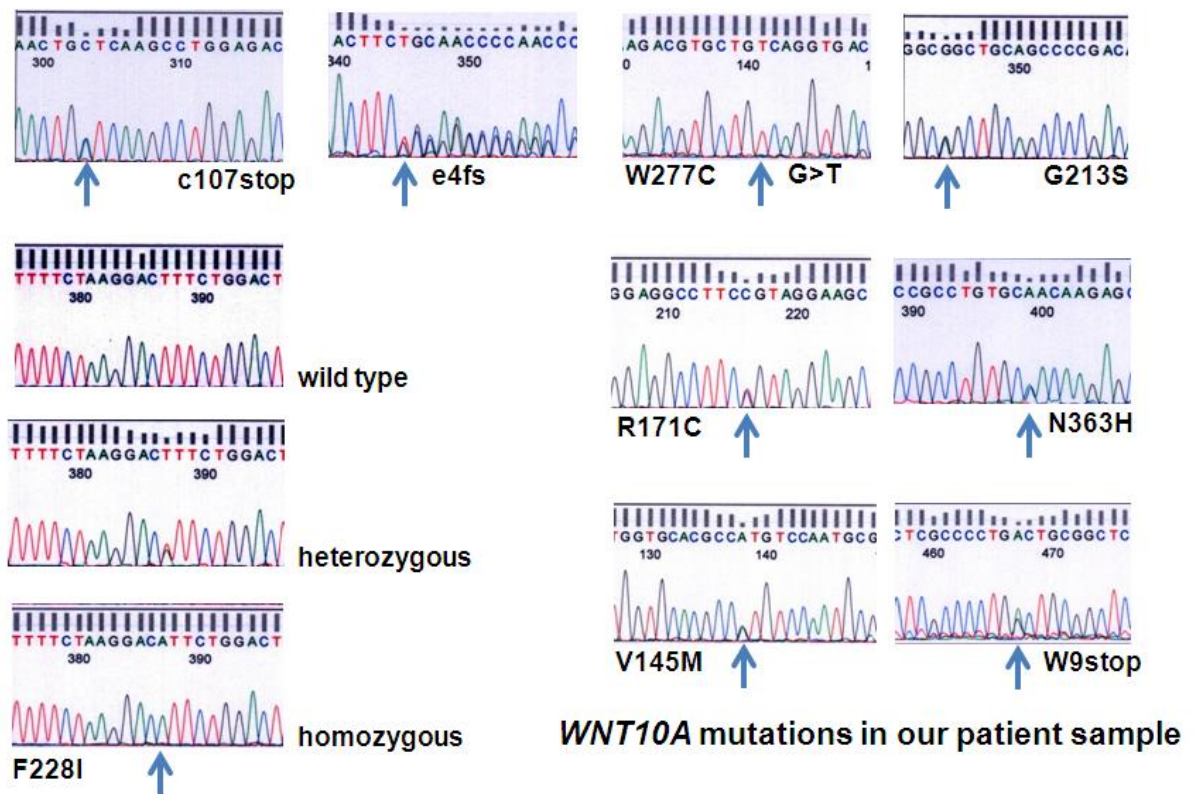
Pax9-deficient gene expression levels in E13.5 and E14.5 relative to wild-type expression. Note that Bmp4 levels are only slightly reduced in the Pax9-deficient tooth organs while Fgf3 and Shh are greatly reduced. Dkk1, a potent Wnt antagonist, has an increased expression level which suggests reduced Wnt activity.

Figure 3-1. Rescue of cleft palate in Pax9^{-/-} E18.5 embryos.



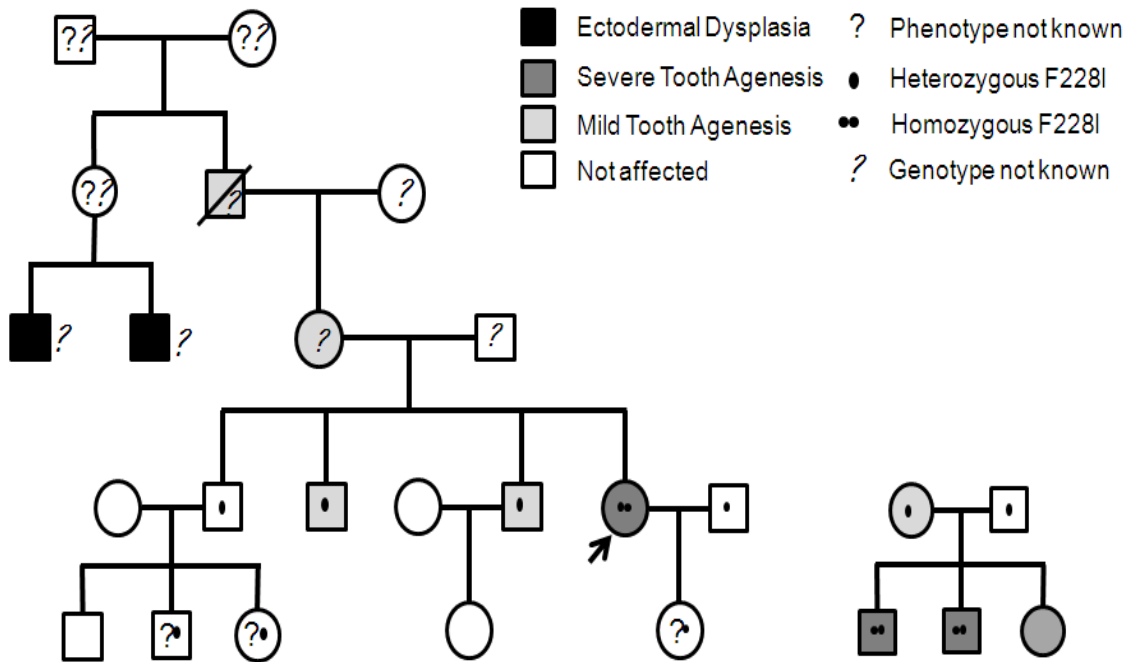
a.) E18.5 Pax9^{-/-} untreated embryo. b.) E18.5 wild-type (Pax9^{+/+}) embryo. Note normal development of palate and tooth organs. c.) E18.5 Pax9^{-/-} treated with 12.5 mg/kg WAY-262611 at E12.5, E13.5, and E14.5. Arrows indicate dental lamina arrested at early bud stage (a and c) and normal tooth organs (b); *** indicates secondary cleft palate; white arrowhead indicates fused palate.

Figure 5-1. *WNT10A* mutations found in our tooth agenesis patient cohort.



e4=exon4 of *WNT10A*, fs=frame shift, F=Phenylalanine, I=Isoleucine, W=Tryptophan, N=Asparagine, R=Arginine, V=Valine, M=Methionine, H=Histidine, C=Cysteine, G=Glycine, S=Serine.

Figure 5-2. Incomplete penetrance and phenotypic variability of WNT10A



Two pedigrees showing incomplete penetrance and variability of the phenotype depending on the number of WNT10A alleles affected. The fifth generation of the pedigree on the left is still too young for phenotype evaluation. An additional feature in this family is that the husband of the homozygous index patient (arrow) also carries the F228I variant, a 1 in 50 chance. The pedigree on the right also shows that two different tooth agenesis genes may contribute to the phenotypes, because the daughter has several missing teeth but no F228I mutation while her two homozygous brothers have severe tooth agenesis. F=Phenylalanine, I=Isoleucine

APPENDIX B

TABLES

Table 2-1. DEGs with a >1.5-fold change between wild-type and *Pax9/Osr2* knock-in mice at E14.5 (microarray data).

Function	Fold Change		
	1.5-2.0	2.0-3.0	3.0+
Transcriptional Regulator	FOXF1A, TRP63, EGR2, MSX1	OSR2, TFAP2C	PAX9, EGR3
Other	DKK1, LEF1	DAAM1, GSTM6, G2E3, HAS3, KRT17, DKK4	ADI1, ODAM, SHH
Unknown	DLX1AS, VWA2	VEPH1	C1QTNF3

Red indicates a down-regulated gene and blue indicates an up-regulated gene. All genes show a positive signal in the E14.5 tooth bud according to the Eurexpress database.

Table 4-1. Sequence variants found in ovarian cancer patients with or without tooth agenesis.

Pt#	<i>BRCA1</i> (NM_007294.3)	<i>EDA</i> (NM_001399.4)	<i>WNT10A</i> (NM_025216.2)	<i>AXIN2</i> (NM_004655.3)	TOOTH AGENESIS PHENOTYPE
2			p.Phe228Ile		
3				p.Ser762Asn	
5	p.Lys679stop				
7	p.Lys894fs				
10	p.Lys527fs				
13	p.Lys894fs				
14					1 upper lateral incisors
16			p.Phe228Ile		1 upper 2nd premolar
19	p.Met1652Ile				
20			p.Phe228Ile		
27					1 lower 2nd premolar
29					2 upper lateral incisors
30			p.Phe228Ile		2 upper lateral incisors
32			p.Phe228Ile		1 upper 2nd premolar
33	p.Gln1096fs	p.R69L			upper lateral peg
34			p.Phe228Ile		
35	p.Lys894fs				2 upper lateral incisors
36					1 upper 2nd molar
43					1 upper lateral incisor
56				<i>c.1994_1995insG</i>	1 upper lateral incisor, 1 upper 1st premolar

Ovarian Cancer patients with/without tooth agenesis who were found to have sequence variants in the breast/ovarian cancer gene *BRCA1* and the tooth agenesis genes *EDA* and *WNT10A*. Bold entries represent patients with combined ovarian cancer/tooth agenesis; the others have ovarian cancer without tooth agenesis. Patient numbers 1-50 are ovarian cancer patients; #56 is an ovarian cyst patient. Phenotypes were previously reported by Chalothorn.

Table 4-2. All sequence variants and their allele frequencies in selected study groups.

SEQUENCE VARIANT			ALLELE FREQUENCY (MINOR ALLELE)					REMARKS
Gene	rs#	Location	Total (n=95)	OV±TA (n=60)	TA±OV (n=49)	OV+TA (n=14)	Control	
PAX9 (NM_006194.3)	rs12881240	p.His239=	0.30 ¹	0.24	0.31 ¹	0.18	0.20	Significant NHLBI
	rs4904210	p.Ala240Pro	0.31	0.34	0.26	0.29	0.36	
	rs61734510	p.K172=	0.011	0.008	0.01		0.008	
	rs17104965	c.*259C>T	0.032	0.033	0.031	0.036	0.02	
	rs17766200	c.*429T>C	0.126	0.075	0.163	0.036	0.093	
		c.*209G>T	0.005		0.01			
MSX1 (NM_002448.3)		c.-243G>A	0.01	0.008	0.01			Promoter region
	rs186861426	c.-18G>A	0.12 ²	0.075 ¹	0.15 ²	0.036	0.025	Significant NHLBI
		c.-7G>T	0.005		0.01			
	rs36059701	p.Ala40Gly	0.22 ²	0.217 ¹	0.255 ²	0.32 ²	0.143	Significant NHLBI
	rs34165410	p.Gly116=	0.026	0.0167	0.04	0.036	0.04	
		p.Thr39=	0.005		0.01			
	rs8670	c.*6C>T	0.29 ²	0.24	0.34 ²	0.286	0.2	Significant NHLBI
	rs1095	c.*68C>T	0.005	0.008		0.036	0.117 ¹	
	rs2229262	c.*70C>T	0.005		0.01		0.019 ¹	
	rs12532	c.*276A>G	0.3	0.31	0.28	0.286	0.24	
rs33929633	c.470-23delT	0.1					See below ²	
AXIN2 (NM_004655.3)	CI002733	c.1994_1995insG	0.005	0.008	0.01	0.036		PCR artifact?
	rs35415678	p.Leu688=	0.016	0.008	0.03	0.036	0.04	
	rs117688560	p.Ser762Asn	0.005	0.008			0.0014	
	rs9906513	c.1907+39T>A		N/D	0.03	0.036	0.04	49TA samples
	rs186861426	c.-18G>A	0.12 ²	0.075 ¹	0.15 ²	0.036	0.025	Significant NHLBI
	rs36059701	p.Ala40Gly	0.22 ²	0.217 ¹	0.255 ²	0.32 ²	0.143	Significant NHLBI
	rs34165410	p.Gly116=	0.026	0.0167	0.04	0.036	0.04	
	rs8670	c.*6C>T	0.29 ²	0.24	0.34 ²	0.286	0.2	Significant NHLBI
	rs1095	c.*68C>T	0.005	0.008		0.036	0.117 ¹	
	rs2229262	c.*70C>T	0.005		0.01		0.019 ¹	
	rs12532	c.*276A>G	0.3	0.31	0.28	0.286	0.24	
	rs33929633	c.470-23delT	0.1					See below ²
BARX1 (NM_021570.3)	rs191789925	p.Ala48Thr	0.096	0.087	0.095	0.036	0.066	
	rs12684081	c.600+30C>T	0.295	0.306	0.274	0.25	0.27	
	rs149299341	c.600+50C>G	0.006	0.01			0.008	
	rs11793856	p.Pro240=	0.26	0.25	0.276		0.267	
	rs2297835	c.*17G>T	0.005	0.008			0.01	
	rs59397123	c.*108G>A	0.06	0.05	0.06		0.092	Low cover
BARX2 (NM_003658.4)	rs117979658	c.187+46G>T		0.017				Ov±TA only
	rs142822705	p.Ser64Pro	0.02	0.017	0.02		0.02	
	rs149011396	p.Ile93=	0.005		0.01		0.0014	
	rs10791010	c.574-5C>T		0.18		0.25	0.2	Ov±TA only ³
	rs10791011	p.Pro203=		0.18		0.25	0.2	Ov±TA only ³
	rs60183112	c.*128_*129insC		0.23		0.286	0.23	Ov±TA only ³
EDA (NM_001399.4)	rs132630309	c.206G>T				0.036	0.011	Ov+TA only
	rs2274469	c.526+27T>A				0.11	0.125	Ov+TA only
	rs11426919	c.527-34_527-33insA				0.5		Artifact
	rs10579679	c.706+12delTinsC T				0.36		Ov+TA only
	rs140058036	c.741+47G>C				0.07	0.06	Ov+TA only
	rs62604271	c.741+125G>T				0.07		Ov+TA only
	rs2296765	c.742-11C>T				0.25	0.37	Ov+TA only
	rs201036606	c.*35C>G				0.036		Ov+TA only

OV±TA: All patients with ovarian disease (cancer and cysts) with or without tooth agenesis. TA±OV: All patients with tooth agenesis regardless of ovarian disease status. OV+TA: All patients with both ovarian disease and tooth agenesis. ¹ethnically unmatched control; ²Homozygotes are not recognizable in the sequencing reaction;

Table 4-2 Continued.

³rs10791010, rs10791011 and rs60183112 appear to be syntenic, the latter also as a separate polymorphism. ' =significant, '' =highly significant difference compared to NHLBI Caucasian control population but not in comparison with the (smaller) control group described by Jezewski, et al. [supplement].

Table 4-3. Detailed analysis of significant allele frequency differences in all study groups.

SEQUENCE VARIANT			ALLELE FREQUENCY (MINOR ALLELE)							REMARKS
Gene	rs#	Location	Total (n=95)	OV no TA (n=46)	OV \pm TA (n=60)	TA \pm OV (n=49)	TAnoOV (n=35)	OV+TA (n=14)	Contr- ol	
<i>PAX9</i>	rs12881240	p.H239=	0.30'	0.25	0.24	0.31'	0.36''	0.18	0.20	Significant
<i>MSX1</i>	rs186861426	c.-18G>A	0.12''	0.087'	0.075'	0.15''	0.2''	0.036	0.025	Significant
	rs36059701	p.Ala40Gly	0.22''	0.185	0.217'	0.255''	0.23'	0.32''	0.143	Significant
	rs8670	c.*6C>T	0.29''	0.23	0.24	0.34''	0.37''	0.286	0.2	Significant

OV no TA: All patients with ovarian disease only. OV \pm TA: All patients with ovarian disease with or without tooth agenesis. TA \pm OV: All patients with tooth agenesis regardless of ovarian disease status. TAnoOV: All patients with tooth agenesis, no ovarian disease. OV+TA: All patients with both ovarian disease and tooth agenesis. '=significant, ''=highly significant when compared to the NHLBI Caucasian control population but not in comparison with the (smaller) control group described in Jezewski et al. [supplement]. Genotype frequency in the NHLBI Caucasian control population displayed Hardy-Weinberg Equilibrium for all *Msx1* SNPs. The *Pax9* SNP CAC>CAT deviated modestly from HWE probably due to its dependence on the adjacent SNP GCG>CCG, creating a potential CpG dinucleotide, which has a tendency to either alter the third position C of the CAC codon to a T or the first position G of the adjacent GCG to a C. rs36059701 and rs8670 are syntenic. NCBI reference sequences for *PAX9* and *MSX1* are NM_006194.3 and NM_002448.3, respectively.

Table 5-1. WNTA10 mutation allele frequencies.

Source	Ethnicity/ study group	Number of detected alleles	F228I allele frequency
Our Tooth Agenesis (TA) study	Caucasians with TA	127 Phe and 33 Ile	0.206
NCBI SNP data base	Caucasians	3348 Phe and 67 Ile	0.02
NHLBI Exome Sequencing Project (ESP)	Caucasians	8389 Phe and 209 Ile	0.024
NHLBI Exome Sequencing Project (ESP)	African Americans	4370 Phe and 32 Ile	0.007
Bohring et al. 2009 Am J Hum Genet 85: 97	Caucasians without TA	396 Phe and 2 Ile	0.005

The WNT10A mutation Phe228Ile is unlikely to be a common polymorphism since its allele frequency is about 10 times higher in the tooth agenesis group than it is in several large control groups.