Role of Prion Protein (PrP^C) in the Mammalian Dentition

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Die hier vorgelegte Dissertation habe ich eigenständig und ohne unerlaubte Hilfe angefertigt. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keine erfolglosen Promotionsversuche unternommen.

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(Kurt Schneider)

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Contents

Abbreviations

BSE	Bovine spongiform encephalopathy
CWD	Chronic wasting disease
Dpl	Doppel
PRND	gene locus of Dpl
EUE	Exotic ungulate encephalopathy
fCJD	Familial Creutzfeldt-Jakob disease
FFI	Fatal familial insomnia
FSE	Feline spongiform encephalopathy
GSS	Gerstmann-Sträussler-Scheinker syndrome
iCJD	iatrogenic/infectious Creutzfeldt-Jakob disease
PRNP	gene locus of PrP
PRNT	gene locus of a presumed pseudogene of Dpl
PrP	prion protein
PrP^{C}	cellular (physiological) PrP
PrP^{Sc}	scrapie-associated (pathological) PrP
sCJD	Sporadic Creutzfeldt-Jakob disease
sFI	Sporadic fatal insomnia
TME	Transmissible mink encephalopathy
vCJD	(new) Variant Creutzfeldt-Jakob disease

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Von denen mancherley Kranckheiten des Schaafviehes, und was vor Curen damit vorgenommen werden.

Der Trab ist auch eine Kranckheit der Schaafe, und ist ansteckend.

Es bekommen auch manche Schaafe den Trab, welches eine Kranckheit ist, die daran zu erkennen, wenn sich das Stücke, das solchen bekommt, niederleget, und beisset mit dem Maule an den Füssen und um die Beine, und reiben sich mit dem Creuze an denen Stangen, verlieren das Gedeyen, fressen auch nicht recht, und verlahmen endlich; sie schleppen sich lange, verzehren sich nach und nach, und zuletzt müssen sie sterben. Welches Vieh diese Staupe bekommt, wird nicht besser. Daher denn das allerbeste ist, daß ein Schäfer, welcher ein Stücke von dem Trabe befallen, gewahr wird, es balde wegschafft, und vors Herrschafftliche Gesinde schlachtet. Es muß ein Schäfer ein solches Stücke Vieh also gleich von dem gesunden Vieh absondern, denn es stecket an, und kan vielen Schaden unter der Heerde verursachen.

J. G. Leopoldt (1750): Nützliche und auf die Erfahrung gegründete Einleitung zu der Land-Wirthschafft [182]

Summary

Transmissible Spongiform Encephalopathies (TSEs) are a group of disorders affecting members of several orders of mammals and inevitably leading to death within short periods of time. These ailments stand out in that the causative agent is a malformed protein referred to as 'prion'.

The raw material for the generation of prions is a cellular protein (cellular prion protein, PrP^{C}), the physiological function of which is still obscure. In the course of the disease, PrP keeps its amino acid sequence while adopting an alternative folding, resulting in scrapie associated prion protein (PrP^{Sc}).

Although PrP^{C} is expressed nearly ubiquitously, the central nervous system is the organ mostly affected by defects developing during pathogenesis. Nevertheless, there are pointers to a link between PrP^{C} and the mammalian dentition. Not only is PrP^{C} expressed in dental tissues during embryogenesis, but teeth grinding is also an important diagnostic marker for TSEs.

Prompted by the descriptions of others about the detection of PrP mRNA in rodent embryos, a search for PrP^{C} in dental tissues was conducted.

By comparing teeth of wild-type, PrP-knockout and PrP overexpressing mice, prominent differences in the dentin structure were found. While wild-type mice have a mean variability of the number and size of dentin tubules, these structures are more variable, greater in size and less in number in PrP knockout mice. Teeth of PrP overexpressing mice, on the other hand, have more and smaller tubuli, the variability of which is less.

Cultured human dental cells were used to extract RNA, which was reverse transcribed and amplified by PCR. Pulpal cells, cells from the periodontal ligament and ectomesenchymal cells were found to express PrP, thus extending the findings in rodent embryos to adult human dental tissues.

Extracted human teeth were used to detect PrP^{C} at the protein level. As expected, nerve fibers were PrP positive. Strikingly, the only other dental cells which turned out to be PrP positive were those engaged in biomineralization: odontoblasts, cementoblasts and epithelial rests of Malassez (ERM).

It has long been known that PrP^C has copper and manganese binding capability. Therefore, molars of wild-type, PrP knockout and PrP overexpressing mice were subject to a determination of their copper and manganese contents. While there were no differences in manganese concentration, molars of PrP knockout mice had one third less copper content than teeth of wild-type mice. Teeth of PrP overexpressing mice had a slightly elevated copper content compared with teeth of wild-type mice.

The findings of this study suggest (but cannot prove) a link between PrP's copper binding capability and the activity of copper binding enzymes in crosslinking collagen. As collagen is an important constituent of predentin as well as of desmodontal fibers, an alteration of its processing can be expected to have influence upon its structure and durability. The lowered contents of copper in teeth of PrP knockout mice could lead to a decreased stability of dentin, thus explaining its crowded structure that could be observed in SEM figures.

Our findings are hard to classify because never before has PrP^C been associated functionally with dentinogenesis. This problem notwithstanding, in days to come PrP^C may possibly turn out to be an important constituent of a cocktail that enables dental practitioners to evoke a third generation of teeth to grow, erupt and serve as a tool for mastication just as deciduous and permanent teeth are so far.

Zusammenfassung

Transmissible Spongiforme Enzephalopathien (TSEs) sind eine Gruppe von Erkrankungen, die Mitglieder einiger Säugerordnungen befallen und unweigerlich innerhalb kurzer Zeit zum Tode führen. Das Besondere an diesen Krankheiten ist die Tatsache, daß der Erreger ein fehlgefaltetes Protein ist, das als 'Prion' bezeichnet wird.

Prionen entstehen aus einem zellulären Protein (zelluläres Prion Protein, PrP^{C}), dessen physiologische Funktion noch immer unklar ist. Im Laufe der Erkrankung ändert PrP seine Faltung unter Beibehaltung seiner Aminosäuresequenz. Dadurch entsteht das scrapie-assoziierte Prion Protein (PrP^{Sc}).

Obwohl PrP^{C} nahezu in jedem Gewebe exprimiert wird, machen sich Ausfälle, die im Laufe der Erkrankung auftreten, besonders im Zentralnervensystem bemerkbar. Dennoch gibt es auch Hinweise darauf, daß ein Zusammenhang zwischen PrP^{C} und dem Gebiß von Säugetieren besteht. Zum einen wird PrP^{C} im Laufe der Embryogenese in dentalem Gewebe exprimiert. Zum anderen ist Zähneknirschen ein wichtiger Marker bei der Diagnose von TSEs.

Die Beschreibung der Entdeckung von PrP mRNA in Nagerembryonen war der Anlaß für eine Suche nach PrP^{C} in dentalen Geweben.

Beim Vergleich der Zähne von Wildtyp, PrP-knockout und PrP überexprimierenden Mäusen wurden auffällige Unterschiede in der Dentinstruktur gefunden. Während Wildtypmäuse eine mittlere Variabilität in der Anzahl und Größe ihrer Dentintubuli zeigen, sind diese Strukturen bei PrP-knockout Mäusen variabler, größer und in geringerer Zahl vorhanden. PrP-überexprimierende Mäuse andererseits haben mehr und kleinere Tubuli, derer Variabilität geringer ist.

Aus Zellen dentalen Gewebes menschlichen Ursprungs wurde RNA extrahiert, die revers transkribiert und mittels PCR amplifiziert wurde. Bei Pulpazellen, Zellen des Periodontalligaments und Ektomesenchymzellen wurde eine Expression von PrP nachgewiesen. Diese Befunde erweitern die Beschreibungen in Nagerembryos auf adulte humane Gewebe dentalen Ursprungs.

Extrahierte menschliche Zähne wurden für den Nachweis von PrP^C auf Proteinebene verwendet. Erwartungsgemäß fand sich PrP Expression in Nervenfasern. Interessanterweise waren die einzigen anderen dentalen Zellen, die PrP positiv waren, solche, die für die Biomineralisation verantwortlich sind: Odontoblasten, Cementoblasten und die epithelialen Reste von Malassez (ERM).

Es war seit langem bekannt, daß PrP^C die Fähigkeit hat, Kupfer- und Manganionen zu binden. Infolgedessen wurden Molaren von Wildtyp, PrP-knockout und PrP überexprimierenden Mäusen auf Ihren Kupfer- und Mangangehalt hin untersucht. Während sich die Zähne nicht im Mangangehalt unterschieden, zeigten die Molaren der PrP-knockout Mäuse einen um ein Drittel geringeren Kupfergehalt verglichem mit Zähnen von Wildtyp Mäusen. Die Zähne der PrP überexprimierenden Mäuse hatten einen vergleichsweise leicht erhöhten Kupfergehalt.

Die Befunde dieser Studie legen, ohne es beweisen zu können, einen Zusammenhang zwischen der Kupferbindungsfähigkeit von PrP und der Aktivität kupferbindender collagenvernetzender Enzyme nahe. Da Collagen ein wichtiger Bestandteil des Prädentins und der desmodontalen Fasern ist, kann man von einer veränderten Collagenprozessierung erwarten, daß sie Einfluß auf dessen Struktur und Widerstandsfähigkeit hat. Der verringerte Kupfergehalt in Zähnen von PrP-knockout Mäusen könnte zu einer verringerten Stabilität des Dentins führen, was die Unregelmäßigkeiten erklären könnte, die in den rasterelektronenmikroskopischen Aufnahmen zu erkennen waren.

Da PrP^C noch nie in funktionellen Zusammenhang mit der Zahnentstehung gebracht wurde, sind unsere Befunde schwierig einzuordnen. Trotz dieses Hindernisses könnte sich PrP^C eines Tages als wichtiger Bestandteil eines Cocktails erweisen, der Zahnärzten ermöglicht, eine dritte Generation von Zähnen wachsen zu lassen, die — genau wie Milch- und bleibende Zähne bisher — durchbrechen und als Kauwerkzeug dienen können.

Part I

Introduction

1 PrP is found in different isoforms

Prion protein (PrP) is a developmentally regulated [202] glycoprotein which is found in all mammals. Sequences related to PrP have been reported in other vertebrates [336] (see chapter 1.2).

According to structures found in PrP's gene promoter reminding of housekeeping genes [24], PrP^{C} is ubiquitously detectable. Bendheim *et al.* [29] found it in nearly all hamster tissues. While highest levels of PrP are found in the CNS, it can also be detected in the peripheral and enteric nervous system and non-neural tissues [136].

PrP was discovered during the search for pathogens eliciting inevitably fatal diseases of the nervous system [212] (see below). It can be found in different isoforms, at least one of which is pathogenic:

- the native, or physiological, isoform of PrP (designated PrP^{C}) is found in many cells within all vertebrates (see chapter 1.2)
- the pathogenic isoform, PrP^{Sc} (Sc for scrapie), has the same primary, but different secondary – and probably tertiary and quarternary – structure as PrP^{C}, but is characterized by some features that enable it to be readily differentiated from PrP^{C}

The process of refolding from PrP^{C} to PrP^{Sc} is believed to be a key event in the pathogenesis of disorders collectively referred to as "transmissible spongiform encephalopathies" (TSEs / "prion diseases"). Stanley B. Prusiner [240] proposed the term "prion" to denote *pro*teinaceous *in*fectious [217] (or, alternatively, *pr*oteinaceous *i*nfectious *on*ly [3]) particles. Rumour has it that the original term "proin" was exchanged for prion for better sound [184] and pronouncableness. Table 1 lists known TSEs in man and mammals.

The propensity of proteins to acquire different higher structures has also been found in proteins of yeast [117, 192, 243, 304, 316, 322, 323, 324, 325, 326, 339] and fungi [77].

While the vertebrate PrP can, in some isoform(s), be pathogenic for mammals, prions in yeast are not generally pathogenic, but instead, act as elements of inheritance consisting solely of proteins [170]. This finding led to the notion of a prion in a wider sense, that is "a protein that has undergone a change such that it no longer carries out its normal function but has acquired the ability to convert the normal form of the protein into the same form as itself, the prion form" [325].

Thus, the idea of a protein conformation predicted a priori [9] by amino acid sequence must be extended for mammalian, yeast and fungal prion proteins alike. Obviously, for this group of polypeptides, there is more than one stable conformation that can be adopted. However, maybe conditions for refolding of non-prion proteins are only incompatible with conditions for life on earth, thereby preventing them from changing conformation. As soon as transitional intermediates are sufficiently energetically unfavourable, refolding may never be observed under non-denaturing conditions.

The goal of the present investigation was to elucidate the role of PrP in the mammalian dentition. All experiments conducted and results presented in this work concern exclusively the physiological isoform (PrP^C) of the prion protein.

1.1 Features of the Prion Protein and its Gene

There are several remarkable features in the prion protein and its gene:

- the complete sequence coding for the protein, the open reading frame (ORF), is located in the last and largest exon [24, 97, 191, 235, 309] (exon 2 in hamster, exon 3 in mouse, sheep and man).
- the primary translation product is a 253 residue precursor protein [309].

- several posttranslational modifications are reported for PrP [97, 306] (figure 1 on page 24):
  - a signal sequence is found spanning the N-terminal 22 amino acids [168, 191, 309], which is clipped after translation [311].
  - a carboxy-terminal signal sequence is cleaved upon attachment of a glycosylphosphatidylinositol (GPI) anchor [191, 282, 309] to Ser_{231} [311].
  - up to two Asn-linked oligosaccharides of the high-mannose type are attached in the endoplasmic reticulum [124] at position 181 and 197 [3, 234, 235]. Upon processing in the Golgi, sialic acid is incorporated into these oligosaccharide chains [124]. During this maturation, the oligosaccharide chains acquire resistance against digestion by endoglycosidase H, which is delayed in mutant PrP compared with wild-type PrP [124]. While four different modes of glycosylation are possible (unglycosylated, monoglycosylated at the first or at the second Asn, as well as diglycosylated), only three different states of glycosylation are electrophoretically discernible: unglycosylated, monoglycosylated and diglycosylated [52].
  - two Cys residues at position 179 and 214 are joined by an intramolecular disulfide bond [234, 235, 305, 306].
  - upon amino acid sequencing, Arg residues at position 3 and 15 (of the mature protein) eventually are not detectable, suggesting a posttranslational modification [306].
- after posttranslational modification, PrP^{C} extends from amino acid 23 to 231, relative to the primary transcript (figure 1).
- PrP generated in wheat germ cell-free protein synthesizing systems was demonstrated to generate two different forms: a soluble form that is secreted and a form that spans the membrane bilayer at least twice, with both, the amino- and the carboxy-terminal regions pointing to the extracytoplasmic space [128].

- most PrP^C synthesized by cells is lacking a transmembrane domain, as it is released by treatment with phosphatidylinositol-specific phospholipase C (PIPLC) [283].

- PrP expression is developmentally regulated [242]. Early reports detected only little PrP mRNA in brains of neonatal and newborn hamsters using Northern blot, but levels increased within the first days of life [205]. Thereupon, PrP mRNA levels stayed constant [202], or decreased [205]. In contrast, using *in situ* hybridization, Salès *et al.* [261] found high levels of PrP mRNA in the embryonal hamster brain at E14.5.

- while PrP^C has a high content in α-helix and a low content in β-sheet (42% and 3%, respectively), PrP^{Sc} has a low content in α-helix and a high content in β-sheet (30% and 43%, respectively) [288].

- PrP^C contains three α-helices, extending from amino acid residue 144 to 154, 172 to 193, and 200 to 227, and two short β-strand regions, extending from amino acid residue 129 to 131 and 161 to 163 [91, 288, 341].

- PrP^C has an electrophoretic mobility in the range of 33 to 35 kDa [139, 191, 212].

- PrP^C has a fast turnover rate with a half-life which is reported to lie between one hour or less [52] and 5.2 hours [35].

- an amino acid octarepeat (in mammals) or a hexarepeat (in birds) is found at the N-terminal half of the protein.

- PrP^C is believed to be converted to a pathological isoform (PrP^{Sc}) by a posttranslational change of conformation in the course of TSE disease. The pathogenic isoform PrP^{Sc} is considered to be capable to initiate the posttranslational conversion of native PrP^C to a likeness of itself [4, 73, 74, 135, 277, 289]. Upon conversion, one important step of which seems to be PrP^C/PrP^{Sc}-heterodimerization, the α-helix content decreases, whereas the β-sheet content increases [139, 191].

- while PrP^{C} is completely digested upon treatment with proteinase K [95], PrP^{Sc} generates different fragments that are resistant to digestion and can be used to characterize the type of TSE.

- PrP^{C} and PrP^{Sc} are thought to be anchored to the cell membrane [203] by a GPI anchor [52, 191, 282, 315].

- by inoculation with tissue of deceased individuals into healthy ones of the same species, the ready transmission of the disease with a remarkably constant incubation period is found [53, 54, 66, 92, 135]. Transmissibility of TSEs was first demonstrated for scrapie by Cuillé and Chelle [80, 81, 82, 83].

- some kilo bases downstream (16 kb in the mouse [206] and 20 kb in man [189]), there is another gene designated Prnd [27], encoding a protein referred to as Dpl or Doppel (German for double) [27, 206], with 25% similarity to Prnp [99], the gene encoding PrP. Different from PrP, Dpl normally is not expressed in the CNS [206]. The knockout of Dpl leads to male sterility [28, 218].

- another gene, designated Prnt, located 3 kb 3' of Prnd is found in the human genome [189] which might be a pseudogene originating from duplication of Prnd [232].

Based on PrP's frequency of amino acid pairs and its propensity to bind copper [43, 233] , Rode *et al.* [253] considered the prion protein to date back to the early days of chemical evolution, long before the first nucleic acids formed. While the octarepeat region, which is most important in binding copper, is missing in amphibian PrPs [287], thus implying that it appeared only in the course of vertebrate evolution beyond the amphibians, there are still other locations in PrP beyond the octarepeat region where copper can be bound [127, 309].

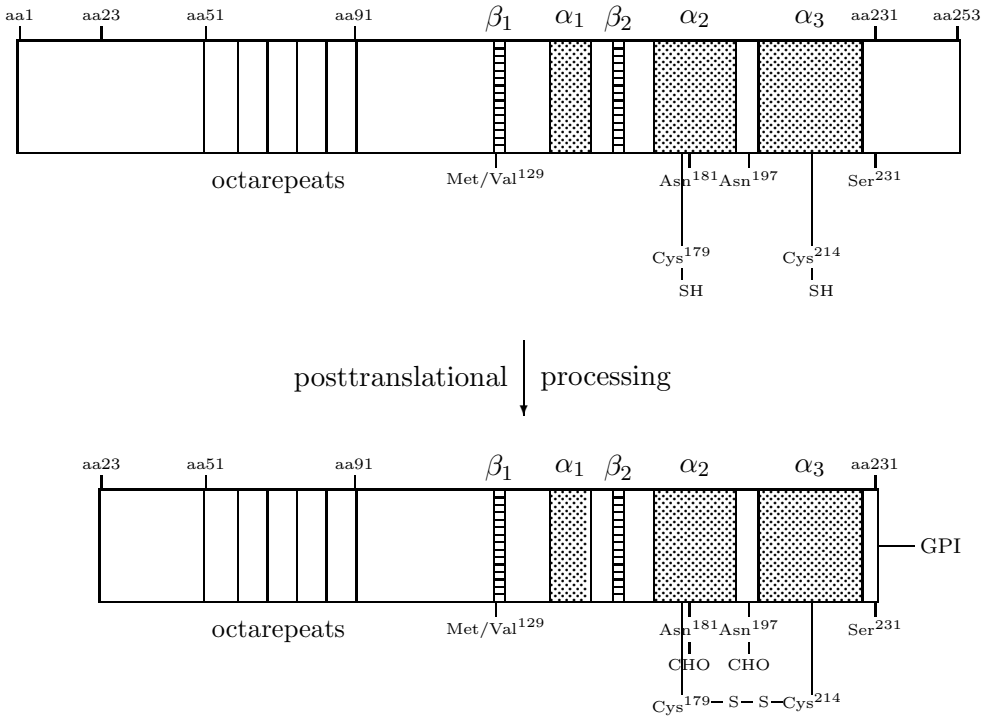

Figure 1: Posttranslational processing of PrP^C
(according to information in [91, 288])

1.2 Prion Proteins in Vertebrates

The prion protein is coded by a cellular single-copy [282] gene [212] located on the short arm of the human chromosome 20 (20p12-pter) [111, 209, 241, 279], the homologous region of chromosome 2 in the mouse [234, 237, 241, 279] and on chromosome 13 in sheep [302]. It is found in all vertebrates examined so far, preferentially expressed in neurons [85, 241], but also found in other tissues. Prion protein sequences have been detected in mammals, marsupials (brush-tailed possum, *Tricosurus vulpecular*) [334], chicken [125], turtle [272], *Xenopus laevis* [287], *Fugu rubripes* (puffer fish) [213, 252], *Salmo salar* [109, 213] and others [336]. Thus, the origins of the prion protein reach back at least to the common ancestor of all vertebrates some 550 million years ago [252]. Some papers date the origins of this protein back even more by citing its detection in evertebrates, as, for example, *Drosophila melanogaster* [109].

Considering this long evolutionary history and the observable conservation of PrP, it came as a great surprise to find PrP knock-out mice ($PrP^{0/0}$) to be viable, reproductive, and obviously devoid of any deficits [59]. Many potential binding partners for PrP have been described (e. g., the tyrosine kinase fyn, suggesting an involvement of PrP in signal transduction [207], and copper ions, suggesting a role in cellular copper uptake [41], modulation of neuronal copper content [45], and copper homeostasis [310]). Nevertheless, PrP's physiological role is still poorly understood.

1.3 PrP^C and the Mammalian Dentition

There are three different interrelations between PrP and the mammalian dentition that can be found in the literature:

- Teeth grinding
- Threat of disease transmission during dental treatment
- Histologic studies of PrP expression

Each of them will be presented in turn.

1.3.1 Teeth Grinding

One of the most constant behavioural peculiarities of individuals affected with TSEs described in the literature is teeth grinding. This behaviour is documented for:

- sheep affected with scrapie [1, 19, 32, 68, 87, 104, 121, 122, 130, 132, 141, 150, 198, 201, 229, 250, 266, 267, 281, 285, 286, 296, 300, 308, 312, 313, 344, 345, 346]
- goats affected with scrapie [64, 266, 300, 301]
- cattle affected with BSE [19, 20, 39, 86, 120, 130, 178, 264, 266]

- elk affected with CWD [13, 112, 119, 262, 278, 332, 333]
- mule deer affected with CWD [13, 107, 112, 262, 331, 332]
- humans affected with CJD [146, 147]

Among 285 different observations (e. g., hyperaesthesia, pruritus, ataxia) used to supply an artificial intelligence algorithm with information for diagnosing scrapie in sheep, teeth grinding actually turned out to be the 11^{th} most important symptom with a sensitivity of 99.5% and a specificity of 99.4% [172]. Likewise, for diagnosing BSE in cattle, teeth grinding proved to be one of the ten most relevant signs [258]. However, teeth grinding is also observed in other conditions, e. g., listeriosis [258], meningitis and/or encephalitis [258], acute copper intoxication [215] or plain hunger (personal observation).

1.3.2 Threat of Disease Transmission during Dental Treatment

Due to the extraordinary resistance of prions against inactivation and decontamination [6, 7, 8, 50, 51, 179, 180], there are serious concerns about a transmission of disease from affected to hitherto unaffected persons.

Person to person transmission does not seem to occur [152]. Albeit, this should be considered a statement only valid in everyday intercourse. While cases of iatrogenic CJD [31, 49, 74, 78, 94, 102, 111, 152, 245, 289, 321] demonstrate that this does not hold true as soon as direct contact to tissues is involved, the causal connection between BSE and vCJD may be considered proven [53, 134].

Moreover, several case reports of patients analyzed the risk of infection based on occupation. Groups of special interest comprised health care workers [47, 193], persons employed in agriculture and meat production [193], as well as cooks and housewives [193]. Nevertheless, all attempts to find a causal connection have been unrewarding. Especially the potentially high levels of infectivity that health care workers are exposed to make a direct link unlikely [30].

As vCJD seems to have another pathogenesis and another distribution of contagiosity than the other human TSEs [17, 321], there is serious concern about

the risk of transmission to humans. Especially in biopharmaceutical production based on natural products (as blood, serum or tissue) [151] and where medical instruments are in routine use [103], as in dentistry, physicians are discussing how to protect their patients, their staff and themselves from getting infected with TSEs [21, 26, 33, 67, 89, 111, 129, 152, 230, 231, 273, 274, 275, 276, 318, 320, 321].

While filling cavities is not considered to be a process that could transmit TSE infectivity as it leaves the pulp chamber intact, root canal instrumentation, with its direct contact to the terminals of the trigeminal nerve, might very well pose a hazard [339, p. 197–199].

The dental pulp is the most important tissue in the mouth for infection with spongiform encephalopathies as it can harbour infectivity in the nerve endings. Scrapie infectivity can be detected in the dental pulp, in gingival tissues and in the tongue [298]. vCJD PrP is found in trigeminal ganglia and lymphoid tissue including tonsils. In the trigeminal ganglion of cattle, infectivity can be demonstrated even during preclinical phases of BSE [274]. Mice were reported to express PrP^{C} in their trigeminal ganglion [101], as were sheep [257].

Guiroy *et al.* [118] found degenerative changes in the ganglion cells of the trigeminal ganglion of three CJD patients. The authors concluded an involvement of the trigeminal ganglion in CJD. Shankar *et al.* [268] went one step further and considered the trigeminal ganglion a traffic junction for CJD infectivity, channeling CJD infectivity from the periphery to the CNS as well as from the CNS to the periphery.

Transmission of infectivity through lesions in oral mucous membranes in mice [65], through the dental pulp in hamsters [143] and through the tongue in hamsters [23, 208] have been demonstrated. All these findings notwithstanding, the UK Department of Health considers dentistry a low risk activity for transmitting TSEs [152].

Hill *et al.* [135], using mice challenged with a strain of hamster prions that is believed not to be pathogenic for mice (strain Sc237), demonstrated that infectivity is nevertheless propagated in these mice. Upon inoculation of ham-

sters with the brains of these inoculated but asymptomatic mice, the hamsters promptly fell ill. Mice with the longest postinoculation period turned out to supply the heaviest load of infectivity, thus arguing for a propagation instead of a mere preservation of infectivity from the original inoculum.

Cases of geographically (non-familial) clustered CJD [199], none of which could ever reach statistical significance because of the little number of cases, raised the question whether infections in the course of dental procedures, which are expected to shine up in geographical vicinity, could be responsible for the disease. Two confirmed and two possible cases of CJD in one family (one of the confirmed cases lacking consanguinity with the other three) [330] leave other explanations than dental procedures (e.g., close social contact). Three other cases only 8 km apart in Japan, obviously having nothing in common, succumbed to CJD in the same year [16]. Two of them attended the same dentist, which at least leaves room for questions.

Adams *et al.* [2] infected mice with ME7 strain scrapie. 15 weeks later, these mice were sacrificed and their gingiva was injured with a rotating dental burr. These burrs in turn were, without cleaning, used on healthy mice to injure their gingivae. These recipient mice were killed after 15 months. None of them showed astrocytosis which is considered characteristic for scrapie. Astrocytosis did, however, develop if 5 mg of infected gingival tissue was injected intraperitoneally. Used burrs were contaminated with between 50 and 100 μg tissue. Thus – the authors concluded – there is a 1 in 50 chance for transmitting the disease through burrs.

Smith *et al.* [273] analyzed the surfaces of endodontic files after root canal instrumentation. They collected files having been used, cleaned, sterilized and decleared ready for re-use from seven dental practices and one dental hospital. Examination was done with a dissecting microscope. Random tests were performed with a scanning electron microscope (SEM). None of the participating institutions delivered exclusively instruments that were without debris. While the files from the dental hospital were devoid of debris in 86% of the cases, one of them was heavily contaminated on more than 75% of its length. On the

other hand, one of the practices delivered exclusively files (5 of 5) that were contaminated on more than 75% of their length. These data are to be interpreted in view of the fact that more than one million endodontic operations are performed annually in England and Wales, that 80-88% of the general dental practitioners re-use their endodontic files [273, 318], and that the scrapie agent was found to adhere to stainless steel surfaces despite washing and formaldehyde treatment, which reduced infectivity only 30-fold but did not succeed to abolish it [347].

Whittaker *et al.* [318] verified the results of Smith *et al.* when analyzing ready-to-reuse endodontic files with SEM. They found visible contaminations with a size of more than 50 μm, probably consisting of residual protein and dentin debris, on 33% (5 of 15) files. The remaining files were found to be contaminated with smaller particles. In succeeding analyses for organic matter using X-ray analysis, files were subject to a treatment with low pressure oxygen plasma at 30°C. Contamination still remaining after this treatment was described as consisting of residual dentin. The authors admitted to have obtained a reduction, but not a complete removal, of organic / protein contamination. Nevertheless, plasma cleaning proved to be a promising improvement for routine cleaning of surgical instruments.

Flechsig *et al.* [98] inserted steel and gold wires for five minutes into brains of infected but still asymptomatic animals. Symptoms were expected only one month after culling. Nevertheless, the wires transmitted disease to all indicator mice, obviously without losing any infectivity during exposure to recipient brain.

Current suggestions for the treatment of all patients suffering from TSEs is to use disposable instruments [49, 277, 291, 292, 327] or, where this is not possible (e. g., handpieces of dental practitioners), to treat the instruments as if they were disposable, that is, disposing them of by incineration after use [21, 26, 152, 231]. Instruments used with patients at risk of being carriers of TSEs (that is, patients with a family history of TSEs) should be quarantined until the status of the patient is confirmed [111, 152, 277, 321]. If there was

contact between the instrument and the central nervous system of the patient, the instrument should be incinerated in any case [88, 111].

Not only instruments used to treat affected patients, but all instruments within the operative field are to be considered, as they might have been exposed to aerosolic contamination [26].

At least with persons currently incubating vCJD asymptomatically [277], even this strategy may fail as the physician as well as the patient are completely unaware of the risk. Nevertheless, there might be infectivity in the tissues of the patient [321].

At the University clinics of Göttingen a pool of instruments was set up from where German clinics can borrow the equipment to treat patients at risk [12, 290]. This idea could be extended to handpieces for dental practitioners, too.

1.3.3 Histologic Studies of PrP Expression

Two studies analyzing the expression levels of PrP in embryos gave a hint that this protein might be involved in the development of the mammalian dentition:

- Manson *et al.* [190] analysed the expression of PrP in mouse embryos on the mRNA level by detecting transcripts through *in situ* hybridization. The authors wrote about neuronal tissue:

  > "Transcripts were detected in cranial and sympathetic ganglia including the trigeminal ganglion (fifth cranial nerve), the superior cervical sympathetic ganglion and sympathetic trunk and ganglia" [190].

  and about non-neuronal tissue:

  > "The PrP gene is expressed in non-neuronal cells. At 13.5 days PrP gene expression was detected in early stages of differentiation of dental lamina. By 16.5 days, hybridisation in tooth buds was higher than in any other area of the embryo and was

present in both inner and outer epithelial layers, which give rise to the enamel-forming cells" [190].

- Using hamster embryos and *in situ* hybridization, Salès et al. [261] confirmed these findings:

  "Hybridization of E14.5 whole body sections with digoxigenin labelled cRNA complementary to hamster PrP^C mRNA showed a strong signal in the brain, tooth bud and lung" [261].

Other studies found PrP in tissues accompanying or innervating the mammalian dentition. Horiuchi *et al.* [136] detected it in the parotid gland in sheep by immunoprecipitation. Ironside *et al.* [144] reported negative PrP immunocytochemistry in salivary glands of victims of the new variant of Creutzfeldt-Jakob disease (vCJD), but found it in the trigeminal ganglion.

Part II

Aims of this Study

In this investigation, the following problems should be approached:

1. Do animals lacking one ($PrP^{+/0}$, heterozygotes) or both ($PrP^{0/0}$, knockouts) allels of the prion protein have any peculiarities in their dentition?
2. Is there any influence upon the copper and/or manganese contents in the dentition of knockouts or PrP overexpressing animals?
3. Can the findings of Manson *et al.* [190] and Salès *et al.* [261] be reproduced in man by amplification (using PCR) of mRNA extracted from cultivated human dental cells?
4. Where exactly in teeth and in the periodontium is PrP expressed?

Problem 1 and 2 were solved with animal teeth, problem 3 and 4 were tackled with human teeth.

Part III

Materials and Methods

2 Mouse Teeth

Prion protein knockout mice of the Zürich I type ($PrP^{0/0}$) [59], heterozygotes ($PrP^{+/0}$), tga20 mice (overexpressing the prion protein 10-fold) [96], and C57BL wild-type mice were analyzed to find differences between the four genotypes.

The experimental design was approved and controlled by the Animal Welfare Officer of the Heinrich-Heine-University of Düsseldorf.

2.1 Genotyping of mice

The genetics of the mice was verified by genotyping.

2.1.1 Tissue preparation

Tail tips of a length of 1 cm were harvested and digested overnight in 300 μl of a digestion mix at 55°C. This digestion mix consisted of 50 ml 1M KCl, 10 ml 1M Tris pH 8.3, 2,5 ml 0.4% gelatine, 1 ml NP40, 1 ml Tween 20, and 1 ml Proteinase K (50 mg/ml). On the following day, the mix was inactivated by heating to 95°C for 5 min.

2.1.2 DNA Isolation and PCR

1 μl of the solution resulting from the digestion was subject to a PCR using Taq polymerase (Qiagen, Hilden, Germany). The three primers used were as described by [59]:

Primer	Sequence
P3	ATT CGC AGC GCA TCG CCT TCT ATC GCC
P4	CCT GGG AAT GAA CAA AGG TTT GCT TTC AAC
P10	GTA CCC ATA ATC AGT GGA ACA AGC CCA GC

The P3/P4 primer pair results in a product of 850 bp length, whereas the P4/P10 primer pair gives rise to a product of 1.1 kb length.

2.2 Determination of Copper and Manganese Contents

8 $PrP^{0/0}$ mice, 9 C57-129Sv wild-type littermates aged between 52 and 174 days and 10 tga20 mice aged between 620 and 676 days were sacrificed by cervical dislocation.

2.2.1 Tissue preparation

The sculls were frozen at -25°C until use. Jaws were dissected free, and molars were extracted. For first molars, the crestal alveolar bone was removed partly. Thereafter, a probe was inserted between the mesial and distal parts of the root and the teeth were gently pulled out of the alveolar cavity. Third molars were completely broken free of the crestal alveolar bone with a curette. For second molars, either way of extraction proved viable. Residual alveolar bone trapped between the roots of extracted teeth was removed with a probe. As mouse molars were too small to separate the pulp from the hard tissue, the metal content of intact molars was determined.

2.2.2 Cu and Mn Determination

The determination of metal content was done by a service provider (Hygiene-Institut des Ruhrgebiets, Gelsenkirchen, Germany). For this determination, teeth of tga20, knockout and wild-type mice, respectively, were split into two

equally sized aliquots each, dried at 40°C and dissolved in 65% nitric acid. After dilution with distilled water to lower the acid concentration, the contents of the teeth of copper and manganese were determined by electrothermal atomic absorption spectrometry (ET-AAS).

2.3 Scanning Electron Microscopy

Analyses with the SEM were employed to find structural differences between $PrP^{0/0}$, $PrP^{+/0}$, tga20 mice, and C57BL wild-type mice.

2.3.1 Tissue preparation

Prion protein knockout mice of the Zürich I type ($PrP^{0/0}$) ($n = 10$), heterozygotes ($PrP^{+/0}$) ($n = 3$), tga20 mice (overexpressing the prion protein 10-fold) ($n = 3$), and C57BL wild-type mice ($n = 9$) were sacrificed, jaws were dissected out and immediately fixed in 2% glutaraldehyde at 4°C for at least 24 hours. The jaw halves were mounted on Palavit G (Heraeus Kulzer, Hanau, Germany) blocks. A transverse section of the molars was ground using a cylindrical burr under visual control until the pulp chamber was cut open (figure 2).

To remove residual pulp tissue, the blocks were treated with 1.5% NaOCl and 0.1% trypsin according to table 2.

After being mounted onto aluminum stubs (Plano, Wetzlar, Germany), the jaws were sputtered with gold in an Edwards Sputter Coater S150B for 90 seconds (figure 3).

2.3.2 Scanning EM

The specimens were observed under a DSM950 scanning electron microscope (Zeiss, Oberkochen, Germany) at 20 kV accelerating voltage. Aspects of the specimens which showed the dentin surface as perpendicular to the line of vision as possible, were selected.

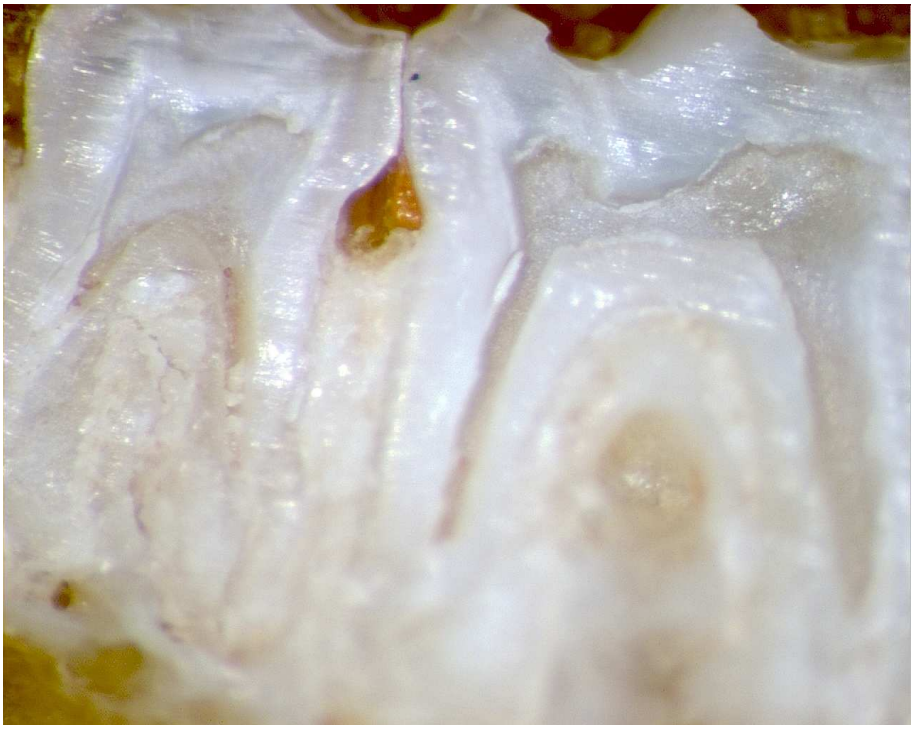

Figure 2: Teeth (M_2, M_1) cut open, ready for sputtering

2.3.3 Quantification of data

Photographs of dentin samples were analyzed morphometrically using ImageJ software (V1.35p, National Institute of Health). The mean size and number of dentin tubules were evaluated in 25 SEM photographs of each group (comprising an area of 10^4 μm^2).

2.3.4 Statistical analysis

Results were statistically analyzed using one-way ANOVA and post-hoc test (Scheffé procedure, SPSS V8.0, SPSS Inc., Chicago, Ill.). A statistically significant level was assumed for $p < 0.01$.

2.4 Histology and Immunohistochemistry

Investigations were carried out with PrP knockout mice and wild-type mice. Generation of the knockout mice was described previously [59].

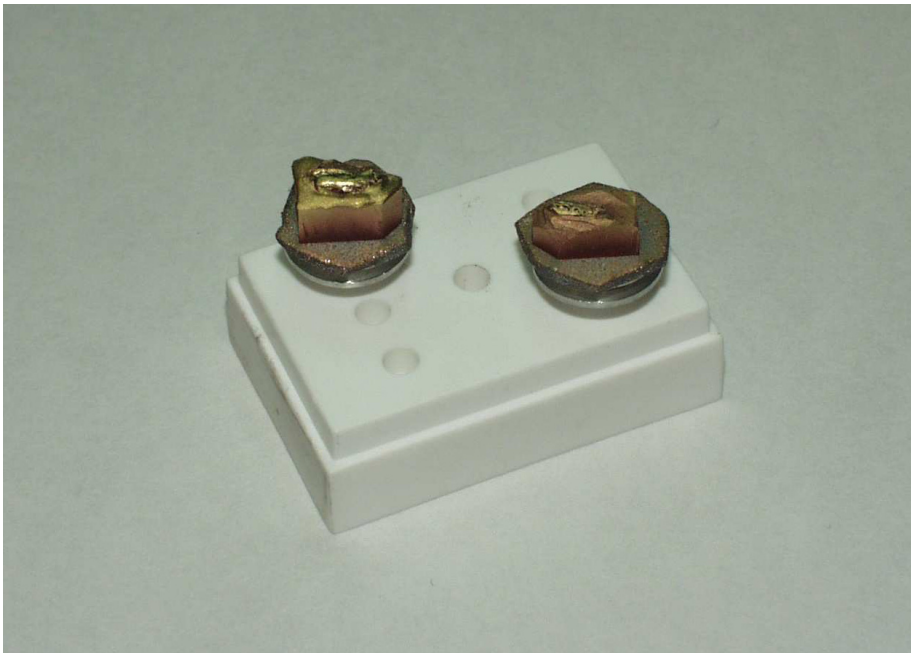

Figure 3: Murine half jaws embedded in Palavit G, mounted on aluminum stubs and sputtered with gold, ready for SEM analysis

Animals were killed by cervical dislocation. The jaws with molar teeth were prepared and immersion-fixed using a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer salt (PBS), pH 7.4 for 24 h at 4°C. Thereupon, samples were demineralized for 14 days in 4 M formic acid. After washing in 0.1 M PBS, pH 7.4 for 24 h at 4°C, specimens were cryoprotected with 30% saccharose in 0.1 M PBS, pH 7.4 for 48 h at 4°C. Specimens were embedded in Tissue Tek and snap-frozen in fluid N_2. Specimens were cryosectioned with a thickness of 30 μm in consecutive sections.

2.4.1 H&E Staining

H&E staining was performed in order to compare histological features and find differences between wild-type and knockout mouse calcified tissues. The dentin structures and alveolar bone structures were characterized. The sections were then stained by H&E staining as described in 3.2.3.

2.4.2 Immunohistochemistry

To block unspecific binding of mouse-derived antibodies in mouse tissue, blocking solutions were used: Fab Fragment of unconjugated anti-mouse IgG [unconjugated Affini Pure Fab Fragment Goat Anti-Mouse IgG (H+L), Jackson Immuno Research Labs] in combination with the use of a biotin-conjugated Fab Fragment Anti-Mouse IgG [Biotin-SP-conjugated Affini Pure Fab Fragment Goat Anti-Mouse IgG (H+L), Jackson Immuno Research Labs] was used. The sections were incubated in avidin-biotin-peroxidase complex and following in DAB+$NiSO_4$ as described in 3.2.4.

2.4.3 Immunohistochemical Controls

In combination with blocking solutions, the immunohistochemical controls were performed by omission of the primary and secondary antibodies in tissues of wild-type and knockout mice, respectively.

3 Human Teeth

Human teeth and tooth germs were collected from healthy patients undergoing surgical and/or orthodontic treatment. The study protocol was approved by the Ethics Committee of the University of Düsseldorf and informed consent was granted by each patient.

3.1 Cell Culture

Permanent human molar teeth and tooth germs were used for cell culture.

3.1.1 Collection of Samples

Immediately after extraction, teeth were repeatedly rinsed with PBS and the crowns were separated from the roots using a sterile diamond burr (Meissinger,

Düsseldorf, Germany). Pulpal tissue from the crown and root canals was sampled for cell culture (pulpal cells). The adherent periodontal ligament (periodontal ligament cells) was scraped off using a sterile curette (Hu-Friedy, Leimen, Germany). Ectomesenchymal tissue was harvested from tooth buds (ectomesenchymal cells).

3.1.2 Isolation of Cells

All tissue samples were minced (≤ 1 mm^3), sequentially digested with a mixture of 1 mg/ml collagenase (type IV, Sigma, Deisenhofen, Germany) and 4.5 U/ml elastase (type IV, Sigma, Deisenhofen, Germany) in serum-free medium (2 h at 37°C). The released cells were washed with serum-containing medium to inhibit collagenase and transferred to 10 cm^2 cell culture flasks (Greiner GmbH, Frickenhausen, Germany). Cells were grown in a medium consisting of DMEM F-12, 11% FBS, 5 μg/ml insulin-transferrin-sodium selenite, penicillin-streptomycin and 2 mM glutamine (all from Sigma, Deisenhofen, Germany).

3.1.3 Cultivation of Cells

The cultures were incubated in humidified atmosphere (5% CO_2, 37°C). Primary cells were passaged at the time of confluency (3–4 weeks) and the first subculture cells were used for RNA isolation.

3.1.4 RNA Isolation and PCR

For harvesting, cells were trypsinized, centrifuged, resuspended in Qiazol (Qiagen, Hilden, Germany) and snapfrozen in liquid N_2. After storage at -80°C, cells were thawed and passed through disposable syringe needles of 0.9 mm (20 gauge) and 0.6 mm (23 gauge) diameter, respectively. RNA was precipitated by the phenol-chloroform procedure. Genomic DNA was digested with DNase I (Qiagen, Hilden, Germany). RNA was purified with RNeasy

MinElute columns (Qiagen, Hilden, Germany) and reverse transcribed with Revert-Aid (Fermentas, Vilnius, Lithuania) using random hexamer primers according to the manufacturer's instructions, followed by amplification with PCR using Taq polymerase (Qiagen, Hilden, Germany). Sequences for custom made primers were determined using the program Primer3. The PrP primer sequences were 5'-CCG AGT AAG CCA AAA ACC AA-3' (forward primer) and 5'-TCC CTC AAG CTG GAA AAA GA-3' (reverse primer) (Operon, Cologne, Germany). The forward primer thus corresponded to the amino acid sequence Pro_{102}-Ser_{103}-Lys_{104}-Pro_{105}-Lys_{106}-Thr_{107}-Asn_{108} of the human prion protein. The reverse primer bound 511 bp downstream on the complementary strand of the nucleotide sequence (corresponding to roughly 170 amino acids, which is beyond the coding region of the mature protein). The GAPDH primer sequences were 5'-GTC AGT GGT GGA CCT GAC CT-3' (forward primer) and 5'-CCC TGT TGC TGT AGC CAA AT-3' (reverse primer) (Operon), resulting in a PCR product of 251 bp length. Identity of PCR products was verified by sequencing (GATC Biotech, Konstanz, Germany).

3.1.5 Western-Blot

Since PrP is membrane bound, we performed a preparation specific for membrane proteins (homogenization using an Ultra Turrax for tissue or repeated extrusion from disposable syringes with 26 gauge needles for cultured cells, sedimentation of nuclei at 125 g for 30 min, precipitation of proteins at 1,400 g for 30 min, twice washing of the pellet, followed by centrifugation at 30,000 g for 30 min each, everything at 4°C). These protein preparations were used throughout the Western-Blots.

For the demonstration that the 6H4 (Prionics AG, Schlieren, Switzerland) antibody binds to a protein of the expected size, a titration of the membrane proteins derived from a C57BL/6 wild-type mouse brain was performed. 10 to 100 μg of protein were applied to the lanes and resulted in the expected banding pattern.

3.1.6 Induction and Detection of Calcifying Potential

Cells were plated at $8 * 10^4$ cells/well in a volume of 3 ml in 6-well plates (Corning/Costar, Wiesbaden, Germany) and cultured at 37°C and 5% CO_2 in DMEM medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. After overnight culture, ascorbic acid (50 μg/ml; Sigma, Taufkirchen, Germany), β-glycerol-phosphate (10 mM; Sigma) and dexamethason (0.1 μM; Sigma) were added to induce the cells [162]. Control cultures were continued without these supplements. Medium including differentiation factors was exchanged every 3 to 4 days. At various time points, calcification was detected by Alizarin-red staining: medium was removed; cells were fixed for 10 min in 70% cold ethanol, washed with bi-distilled water and incubated in 2% Alizarin-red solution (Abcam, Frankfurt, Germany) in bi-distilled water for 10 min at room temperature. After 5 washing steps with bi-distilled water, PBS was added and wells were evaluated microscopically.

3.2 Histology and Immunohistochemistry

3.2.1 Collection of Samples

Twelve human teeth were included in the study. The sound third molar germs ($n = 11$) in different developmental stages from 16- to 22-year-old patients were extracted for orthodontic reasons. One sound premolar from a 28-year-old patient was extracted after a frontal trauma. All patients agreed to have the teeth examined for research purposes.

3.2.2 Tissue Preparation

Immediately after extraction, teeth were immersion-fixed for 48 h in a fixative containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer saline (PBS), pH 7.4. The samples were demineralized for 14 days in

4 M formic acid. Then, tissues were cryoprotected with 30% sucrose solution in 0.1 M PBS, pH 7.4, for 48 h, embedded in Tissue Freezing medium (Leica Microsystem Nussloch GmbH, Nussloch, Germany), frozen in liquid N_2, stored at -80°C and sectioned on a cryostat at 40 μm.

3.2.3 H&E Staining

The free floating human and mouse sections (see section 2.4.1) were dried onto slides. The sections were immersed in Mayer's haemalaun for 6 min. Then, sections were washed in running tap water for 2–3 min or until the sections turned blue. The sections were treated with eosin for 30 s under agitation. Thereafter, sections were washed and differentiated in running tap water for about 5 min. The sections were dehydrated in 50%, 70%, 95% and two changes of 100% ethanol (3 min each). The sections were cleared in xylene and covered in Entellan (Merck, Darmstadt, Germany).

3.2.4 Immunohistochemistry

Free-floating sections were incubated with 0.3% H_2O_2 in 0.05 M Tris-buffered saline (TBS) for 20 min to inhibit endogenous peroxidase. Then sections were washed. The nonspecific immunoglobulin binding sites were blocked by incubation in 10% normal horse serum (Vector, Burlingame, CA, USA) and 2% bovine serum albumin (Sigma, St. Louis, MO, USA). Then, sections were incubated for 48 h with monoclonal antibody 6H4 (1:3000; Prionics), monoclonal antibody 8H4 (1:3000; Alicon AG, Zürich, Switzerland) and monoclonal antibody 3F4 (1:800). After washing, the sections were incubated with biotinylated horse anti-mouse IgG for 1 h, washed and then incubated with avidin-biotin-peroxidase complex (1:100) (Vector). The reaction product was developed with 0.05% 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in 0.05 M Tris-HCl buffer, pH 7.6 containing 0.01% H_2O_2 and 0.01% $NiSO_4$.

3.2.5 Immunohistochemical Controls

3.2.5.1 Immunohistochemical Controls by Omission of Antibodies In immunohistochemical controls, sections were first incubated in the absence of the primary antisera. In the second immunohistochemical controls, sections were incubated in the absence of the secondary antisera.

3.2.5.2 Immunohistochemical Controls by Preabsorption Technique In the present study, preabsorption control experiments were performed. The specificity of antisera was tested by preabsorption with the respective peptide concentration in a 1-, 5- and 10-fold excess (in working dilutions of 6H4, 1:3000) for monoclonal anti-PrP antibody 6H4 peptides (Genaxxon Bioscience, Biberach, Germany).

3.2.5.3 Immunohistochemical Controls using Non-Relevant Antibodies The sections were incubated as described in immunohistochemistry (3.2.4). The sections were incubated with rabbit polyclonal soluble guanylate cyclase (sGC) α_2-subunit (1:800) and sGC β_1-subunit (1:800) (S. Behrends, University of Carolo-Wilhelmina at Braunschweig, Germany), rabbit polyclonal calcitonin gene-related peptide (CGRP) (1:2000) (Peninsula, San Carlos, USA) and with rabbit polyclonal TrkA (1:800) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 48 h. Then, sections were incubated with biotinylated anti-rabbit IgG (1:500) (Vector). The sections were then treated as described above in 3.2.4.

3.2.5.4 Immunohistochemical Controls by Immunoblot It is known that the antibody specificity is best determined by immunoblot techniques [62]. The specificity of 6H4 (Prionics) antibody used in the present study was confirmed by our immunoblot analysis on human teeth cell line.

3.2.6 Densitometry

The densitometrical staining intensities of the 6H4 in odontoblasts, nerve fibers, cementoblasts and in the ERM were measured by grey values of immunostaining. The background grey value was measured from four selected regions at a cell free area. Odontoblasts, nerve fibers, cementoblasts and the ERM grey values were measured from four selected areas of the stained cell areas at 50×. Immunostaining intensity was presented as the mean of measured cell (odontoblasts, cementoblasts, nerve fibers, and the ERM) grey value minus mean of measured background grey value. For staining intensity detection a Zeiss Axiophot microscope coupled to a 3-chip CCD-camera was used and the analysis was performed using the Optimas 6.00 image analysis program (Imaging Technology Inc., San Diego, CA, USA).

3.2.7 Statistical Analysis

Differences among groups were compared with student's t-test for unpaired observations. The data shown are means ± SEM. The level of significance was set to $p < 0.05$. All p-values reported were based on two-sided tests.

Part IV

Results

4 Mouse Teeth

We compared teeth of wild-type, $PrP^{0/0}$ knockout and tga20 mice (which overexpress PrP) with each other.

4.1 Characterization of $PrP^{0/0}$ Mice by Genotyping and Western-Blot

4.1.1 Genotyping of $PrP^{0/0}$ Mice

The genotype of the mice was verified by PCR. The PCR products had the expected sizes of 850 bp (knockout mice) and 1,100 bp (wild-type mice), respectively (figure 4).

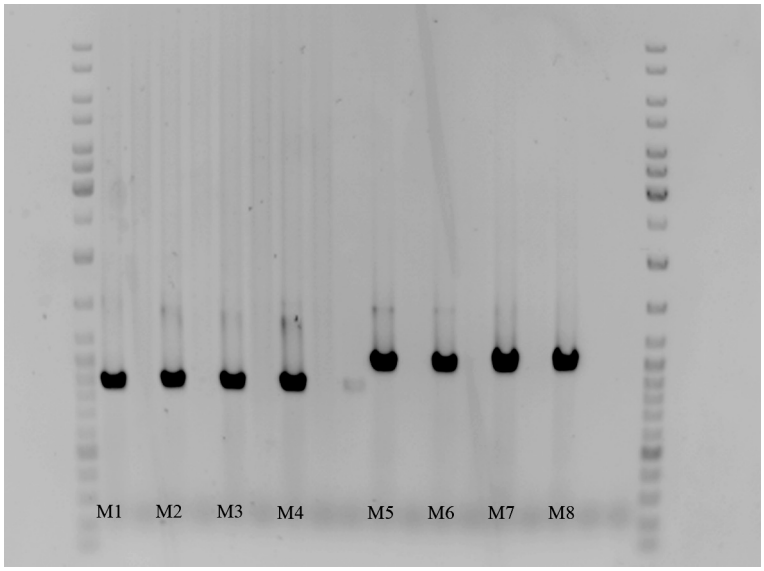

Figure 4: Genotyping PCR of $PrP^{0/0}$ (lanes 2, 4, 6, 8 (M1 – M4); uneven lanes are controls without template) and wild-type (lanes 11, 13, 15, 17 (M5 – M8); even lanes are controls) mice. Knockout and wild-type mice can be discerned by the size of their PCR products (850 bp and 1,100 bp, respectively). Size standard (lane 1 and 20) is multiples of 100 bp.

4.1.2 Western-Blot Characterization of $PrP^{0/0}$ Mice

To verify the absence of PrP in knockout and the presence of PrP in wild-type mice, we performed a Western-Blot, the results of which were presented in figure 13 in chapter 5.1.2. As expected, no PrP was detectable in cells originating from knockout mice, while wild-type mice clearly expressed PrP.

4.2 Determination of Cu and Mn contents

Reports about the propensity of PrP^{C} to bind copper, and to a lesser extent manganese, ions prompted an analysis of the copper and manganese contents of molars originating from wild-type, knockout and PrP^{C} overexpression mice.

4.2.1 Cu and Mn Determination

Teeth of $PrP^{0/0}$ mice contained one third less copper than teeth of wild-type mice. On the other hand, molars of tga20 mice contained one sixth more copper than wild-type mice. Therefore, tga20 mice, which were found in the SEM to have the smallest tubuli diameters and the highest number of tubuli had also the highest PrP expression level *and* the highest copper content. Mice with the greatest tubuli and the smallest number of tubuli found in the SEM had a PrP expression level of zero (PrP knockout mice) *and* the lowest copper level (table 3 and figure 5, see chapter 4.3).

Yet, these findings do not necessitate a causal correlation between copper content and the irregular structure of dentin observed in the SEM.

4.2.2 Quantification of data

Limits of detection for copper and manganese by ET-AAS were 0.4 mg/kg and 0.5 mg/kg, respectively.

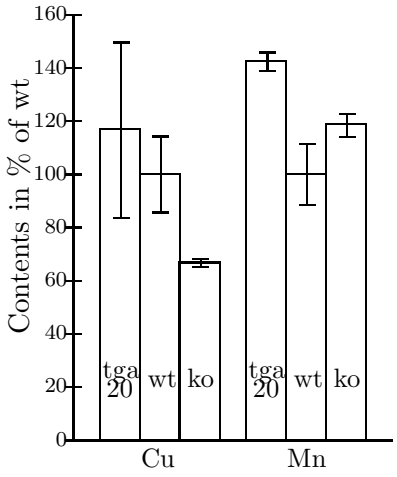

Figure 5: Mean contents of copper and manganese in molars of tga20 (n=10), C57-129Sv (n=9) and $PrP^{0/0}$ (n=8) mice (mean ± S.E.M.). Ordinate is in relative units with the values of wild-type mice at 100. While no correlation could be found between manganese contents and PrP^{C} expression (right), there is a clear correlation between PrP^{C} expression and copper content (left).

4.3 Scanning Electron Microscopy

We asked if PrP^{C} is required for normal teeth development. Therefore, we analyzed teeth from wild-type, knockout and PrP^{C} overexpressing mice. We determined the impact of the expression level of PrP^{C} on size and diameter of dentin tubuli. For this, molars of the three groups of mice were extracted, cut in the mesio-distal plane, and analyzed in a scanning electron microscope.

4.3.1 Scanning EM

Profound differences were found in dentin structure between wild-type, $PrP^{0/0}$ and tga20 mice. While dentin tubules of wild-type mice have a certain variability, the variability as well as the diameter of the tubuli orifices found in $PrP^{0/0}$ was greater, while the variability and tubuli orifice diameter found in tga20 mice was smaller (figure 6).

We analyzed dentin samples derived from different animals within each of four groups: $PrP^{0/0}$, $PrP^{+/0}$, C57BL/6 wild-type mice, and tga20 transgene mice. 25 SEM photographs of dentin samples per group were analyzed morphometrically using the software package ImageJ V1.35p (National Institute of Health).

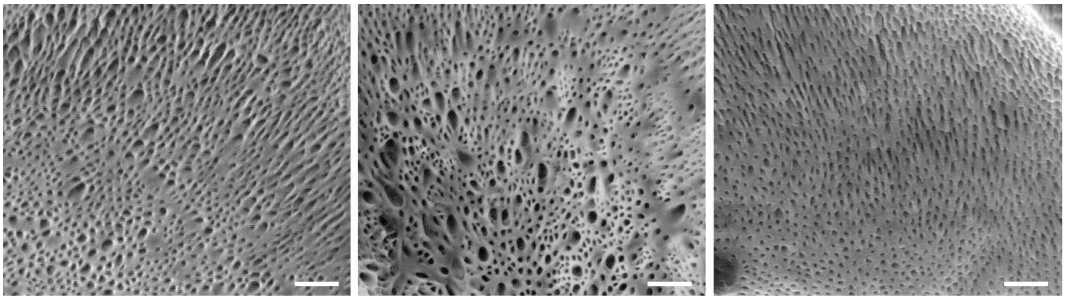

Figure 6: Pulpal aspects of murine dentin (×500). Wild-type dentin is regularly structured. Orifices of dentin tubules show little variability in diameter (left). Tubuli of $PrP^{0/0}$ dentin are bigger and show great variability in diameter (middle). Tubuli of tga20 dentin are smaller and show little variability in diameter (right). Bar is 20 μm

The SEM images were processed for making them comparable. For standardization, the figures' grey values were equalized. Minimum circularity of evaluated tubuli was assumed to be at least 0.25 (ImageJ) in order to exclude artefacts from being analyzed (figure 7). Mean size and number of dentin tubuli were evaluated in 25 SEM photographs of each group.

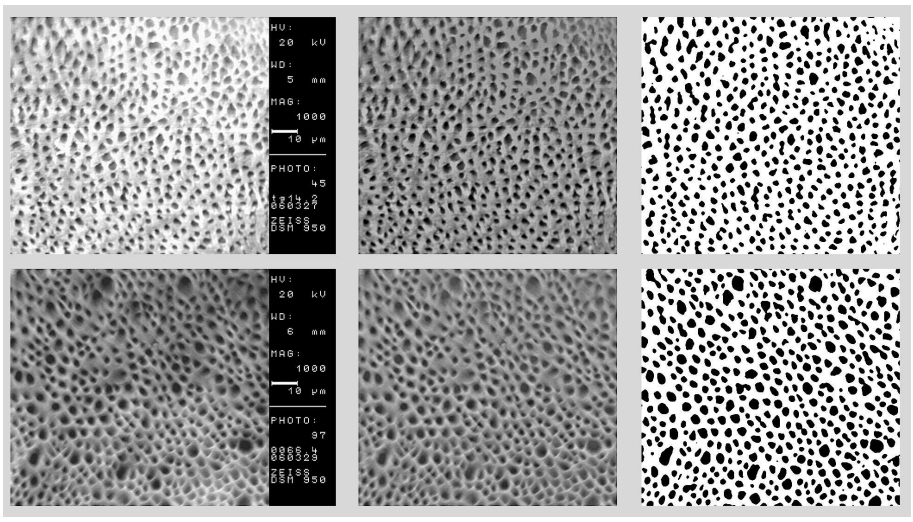

Figure 7: Digital processing of SEM figures. While the original photographs (left) had different levels of brightness, correction of grey values (middle) was used to make the photographs comparable for binarization (right)

The analyzed $PrP^{0/0}$ knockout animals were derived from different sources (Düsseldorf, Berlin and Munich), in order to eliminate location specific factors,

such as climate or feeding.

Comparisons by scanning electron microscopy between molars of wild-type, $PrP^{0/0}$ knockout, $PrP^{+/0}$ and tga20 mice revealed a functional influence of PrP upon dentin structure (figure 6 and table 4). The genotypes (except heterozygotes) demonstrated statistically significant differences in mean area and number of tubules.

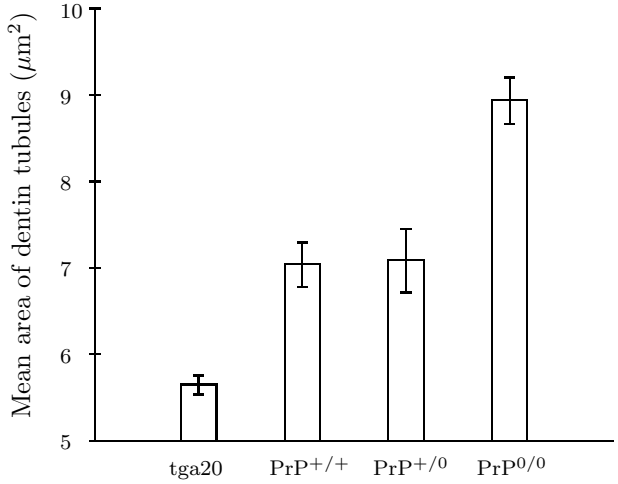

Figure 8: Mean area of dentin tubules (mean ± S.E.M.). While wild-type and heterocygote mice are indistinguishable in the mean area of their dentin tubules, PrP overexpressing mice have much smaller areas and knock-out mice have much greater areas of their dentin tubules, respectively.

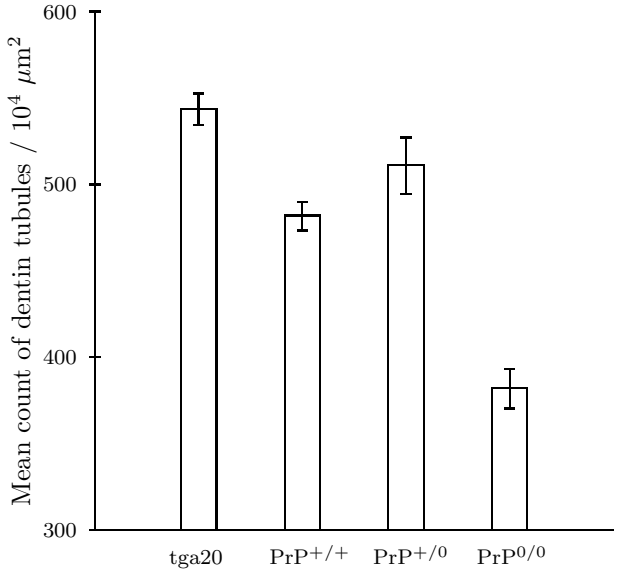

Figure 9: Mean count of dentin tubules (mean ± S.E.M.). While wild-type and heterocygote mice have comparable numbers of tubuli per unit area, tga20 mice have more, while knock-out mice have much less.

Occasionally, thinning of intertubular dentin walls in $PrP^{0/0}$ mice seemed to have led to a breakdown of the entire wall, thereby uniting several formerly independent smaller tubuli to a single bigger one (figure 10).

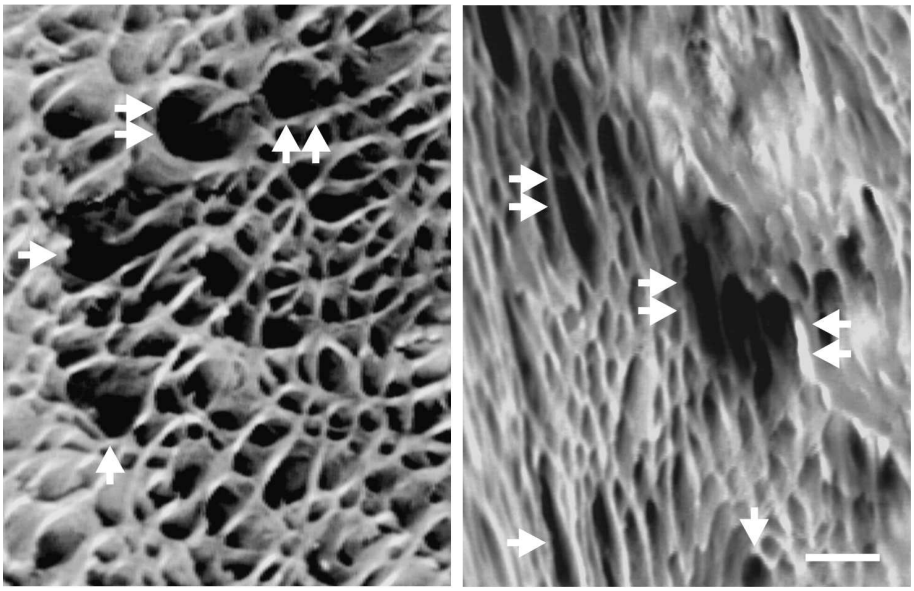

Figure 10: Intertubular walls seemed to have broken down in a first molar of a $PrP^{0/0}$ mouse, thereby uniting two (?, single arrow) or several (double arrows) previously independent smaller tubuli into one big tubulus (right). Tubuli developing from this breakdown of intertubular walls were found in shapes still revealing the original tubuli (single arrows) or as tubuli having attained a circular circumference (double arrows) in a second molar of a $PrP^{0/0}$ mouse (left). Comparisons of small and big tubuli demonstrate the variability of tubulus diameter in $PrP^{0/0}$ mice. Bar is 10 μm

4.4 Histology and Immunohistochemistry

4.4.1 Immunohistochemical Localization of PrP in Mouse Teeth

In this study mouse monoclonal antibodies against PrP were used. Detection of PrP in mouse tissue with mouse antibodies is complicated due to unspecific bindings of secondary mouse antibodies to mouse Igs in mouse tissues. Blocking of these unspecific bindings was associated with reduction of unspecific bindings but the complete elimination of these unspecific bindings was not possible. Furthermore, the specific immunohistochemical staining was very weak (epitope recognition was strongly reduced). Therefore, a weak localization of PrP was detectable in odontoblasts (data not shown).

The characterization of PrP protein in $PrP^{0/0}$ mouse teeth cells will be investigated using a rabbit polyclonal antibody against PrP in our next experiments. Therefore, in the present work the structures of dentin as well as bone of wild-type mice and $PrP^{0/0}$ mice were characterized and compared by H&E staining.

4.4.2 Characterization of Dentin and Bone Structures in Wild-Type and in $PrP^{0/0}$ Mice by H&E Staining

H&E staining was performed in order to compare histological features and differences between wild-type and knockout mouse calcified tissues such as dentin and alveolar bone (figure 11). Important differences were detected in dentin as well as in alveolar bone structures between wild-type mice and $PrP^{0/0}$ mice dentin and alveolar bone (figure 11). There was a regular organization in dentin structures with regular dentin tubuli and homogenous dentin areas within intertubular regions in wild-type mice (figure 11B). Similarly, alveolar bone was regularly organized with blood vessels, bone marrow, compacta as well as with spongious bone structures of the wild-type mice tissues (figure 11C). In comparison, $PrP^{0/0}$ mice revealed irregular organization in dentin structures and in dentin tubuli (figure 11E). Alveolar bone of $PrP^{0/0}$ mice was characterized by great areas and increased amount of bone marrow and irregular alveolar bone structures (figure 11F).

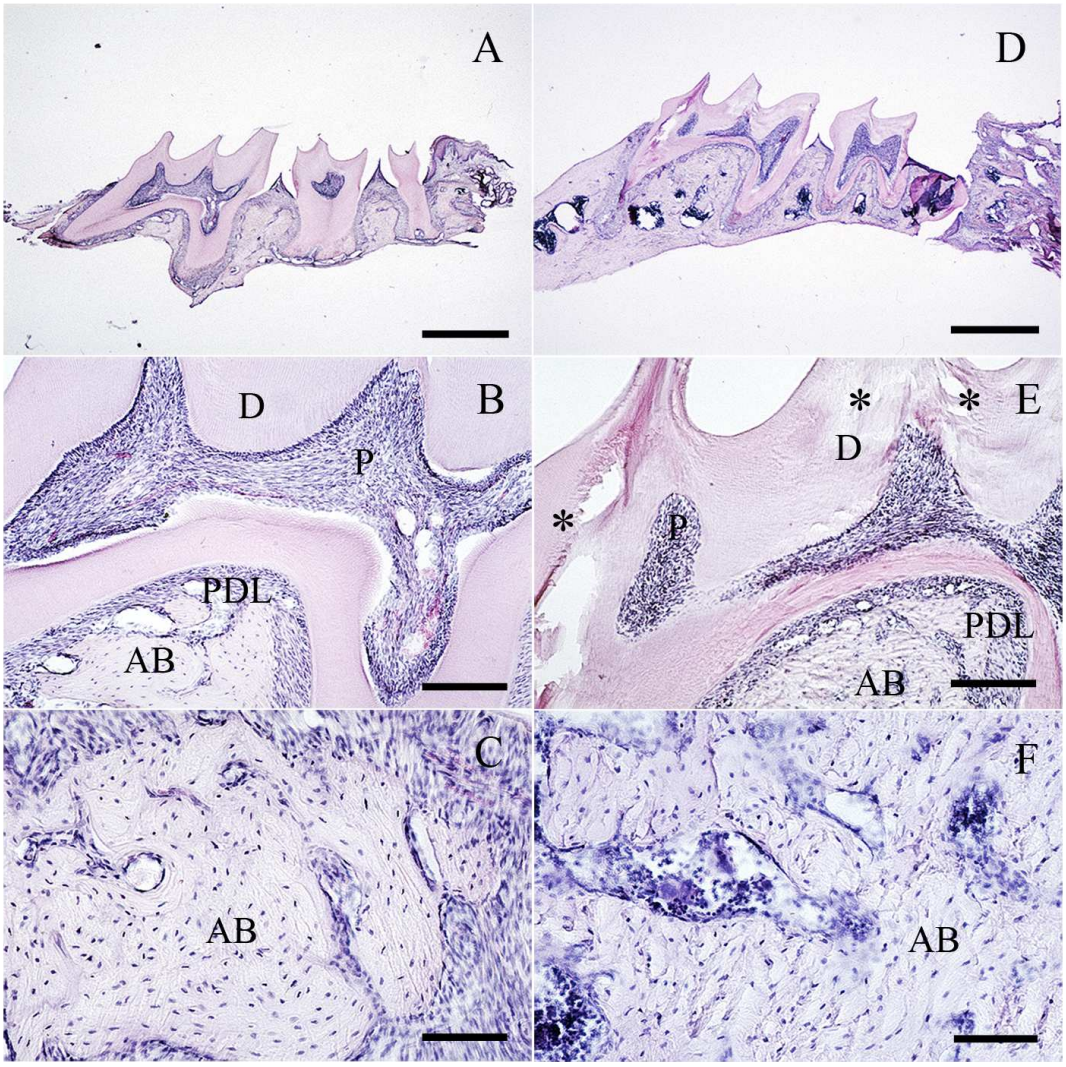

Figure 11: Comparison of dentin and bone structures in wild-type and $PrP^{0/0}$ mice by H&E staining. The differences in dentin and alveolar bone structures between wild-type (A, B, C) and $PrP^{0/0}$ mice (D, E, F) were demonstrated by H&E staining. There was a regular organization in dentin structures (B) and alveolar bone (C) of wild-type mice, while knockout mice typically showed irregular dentin tubules (E; asterisks) and alveolar bone (F) structures. In comparison to the wild-type mice (A, C), there was an increase in the area and amount of bone marrow of the $PrP^{0/0}$ mouse (D, F) alveolar bone. D = dentin, P = dental pulp, PDL = periodontal ligament, AB = alveolar bone. Bars: A, D: 900 μm, B, C, E, F: 100 μm.

5 Human Teeth

5.1 Expression and Detection of PrP in Different Types of Human Cells

To determine the expression level of PrP on the transcriptional level, RT-PCR of human dental cells was performed. This analysis demonstrated a clear expression of PrP on the mRNA level.

5.1.1 Detection of PrP at the mRNA Level in Different Types of Human Cell Lines

Cultured human cementoblasts, pulp cells, and ectomesenchyme were analysed using PCR. All three PCR setups (lane 3–5 in figure 12) where cDNA was used as template showed a strong signal after PCR with PrP primers at the expected size of 511 bp. Lane 6–8, where the corresponding amount of RNA (without the step of reverse transcription) served as template, were negative controls. As expected, no signal was seen, thus ruling out the possible amplification of genomic DNA. Lanes 9–11, with GAPDH primers, served as loading control, while lane 12+13 proved the absence of contaminating nucleic acids in the water.

By using PCR to amplify PrP cDNA, it was possible to verify the results presented by Manson *et al.* [190] (in mouse) and Salès *et al.* [261] (in hamsters) in man. Human cementoblasts, pulp, and ectomesenchyme were found to express PrP. Thus, PrP was demonstrated to be not only transcribed during rodent embryogenesis, but also in adult human dental tissues.

As the presented findings are only snapshots in time, they don't permit any statements about the time course of PrP's expression. It would be interesting to see whether there is a temporal correlation between PrP^{C} expression and dentinogenesis. This, however, must be left to further studies (see chapter 10.2).

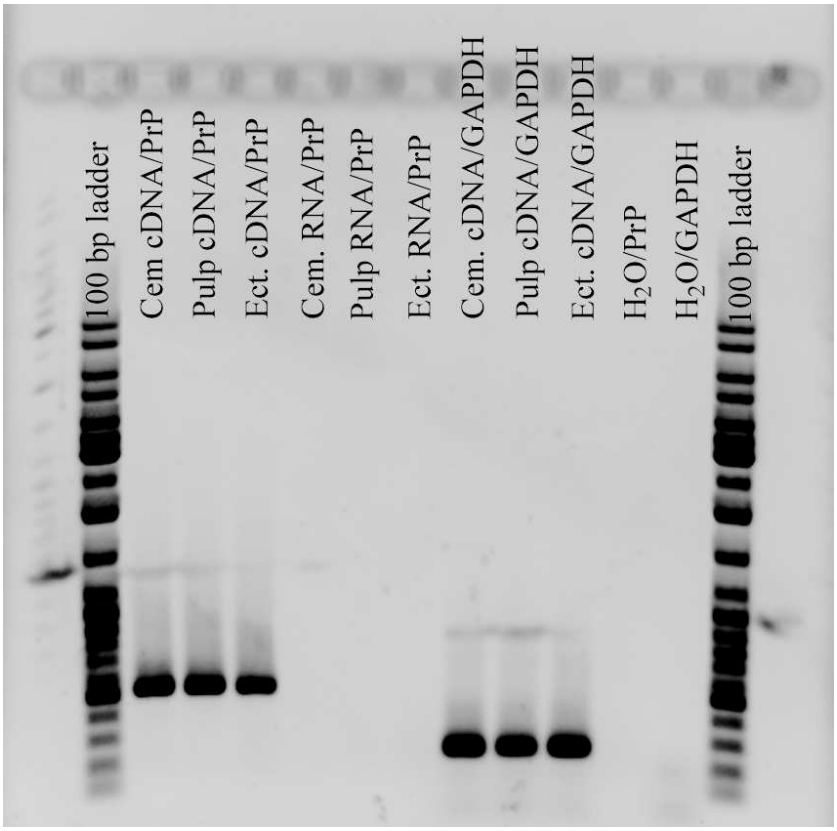

Figure 12: RT-PCR of cultured human dental cells. Lanes 3–5: cementoblast, pulp, and ectomesenchyme cDNA (primer: PrP). Controls: Lanes 6–8: cementoblast, pulp, and ectomesenchyme RNA (primer: PrP), Lanes 9–11: cementoblast, pulp, and ectomesenchyme cDNA (primer: GAPDH), Lane 12: H_2O (primer: PrP), Lane 13: H_2O (primer: GAPDH), Lanes 2+14: 100bp ladder (500bp is darker). cDNA from cementoblasts, pulp and ectomesenchyme are suitable templates for amplification (lanes 3–5), while RNA extracted from these tissues is not (lanes 6–8). This demonstrates that there is no contamination by genomic DNA. Loading control (lane 9–11) demonstrates equal loading

5.1.2 Western-Blot Detection of PrP in Different Types of Human Cell Lines

Brain cells of $PrP^{0/0}$ knockout and wild-type mice and cultivated human cells were subject to Western-Blot. As expected, $PrP^{0/0}$ knockout mouse cells, which served as negative control, revealed no signal. Cells from wild-type mouse brain cells and human pulp cells and ectomesenchyme were clearly detectable, while cementoblasts revealed a prominent signal on Western-Blot, thus verifying the specificity of the used antibody (6H4) (figure 13).

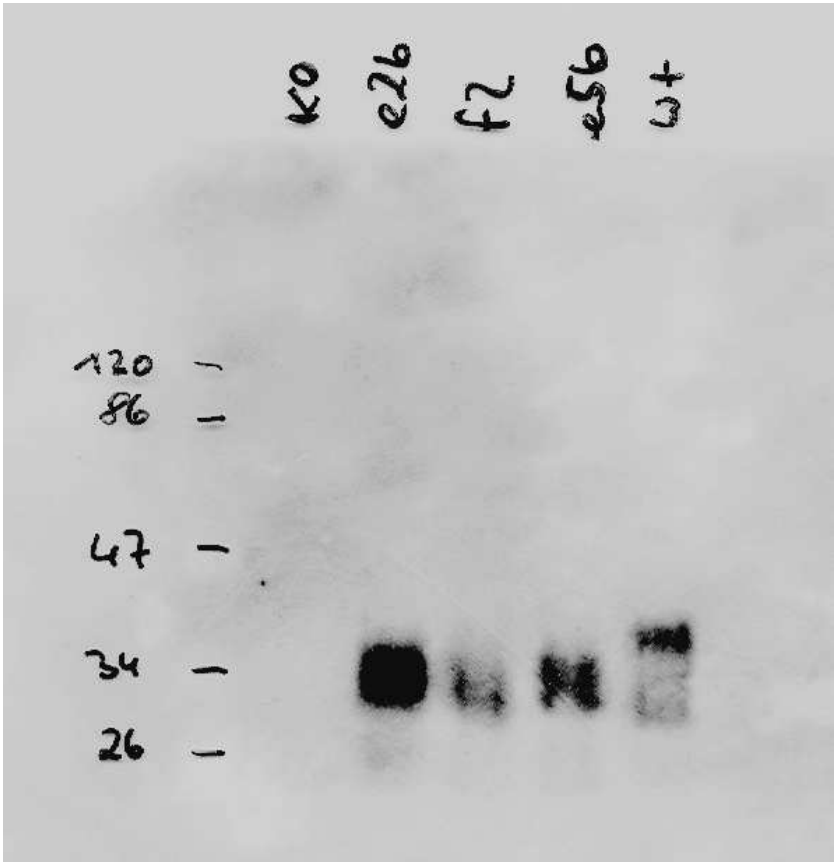

Figure 13: Western-Blot of mouse and cultured human cells. Controls: lane 1: brain of $PrP^{0/0}$ knockout mouse (negative control), Lane 5: brain of wild-type mouse (positive control). Human cell lines: lane 2: cementoblasts, lane 3: pulp cells, lane 4: ectomesenchyme. There is no darkening in the negative control lane, medium darkening in the lanes loaded with pulp cells, ectomesenchyme and wild-type mouse brain, and strong darkening in the cementoblasts lane. The different sizes of the proteins point to different glycosylation states in human dental tissues compared to mouse brain cells. Antibody: 6H4.

5.2 Immunohistochemical Detection of PrP in Different Cell Types of Human Teeth

5.2.1 Dentin-Pulp Complex

In the dentin-pulp complex, PrP was detected in odontoblasts and in nerve fibers. Blood vessels and stroma cells of the dental pulp were negative for staining of PrP.

5.2.1.1 Odontoblasts In odontoblasts, PrP was found with a strong staining intensity in cell bodies. The odontoblasts' processes were negative for PrP. In the odontoblast layer, numerous odontoblasts revealed PrP staining. The odontoblast cell bodies were PrP positive with different staining intensities. Immunohistochemical localization of 6H4 was also confirmed with another PrP antibody (8H4) against a different epitope (figure 14).

5.2.1.2 Nerve Fibers In the root pulp, PrP was detected in thick nerve fiber bundles and in fine nerve fibers. Numerous free and blood-vessels associated with nerve fibers are distributed in the coronal pulp. PrP was identified in numerous thick and thin nerve fibers distributed perivascularly in the subodontoblastic plexus. The nerve fibers showing immunoreactivity to PrP penetrated also into the odontoblast layer (figure 15).

5.2.2 Periodontal Ligament

In the periodontal ligament (PDL), PrP was identified with different staining intensities in cementoblasts, ERM and in nerve fibers of the tooth germ. Stromal cells of the PDL were negative for staining of PrP.

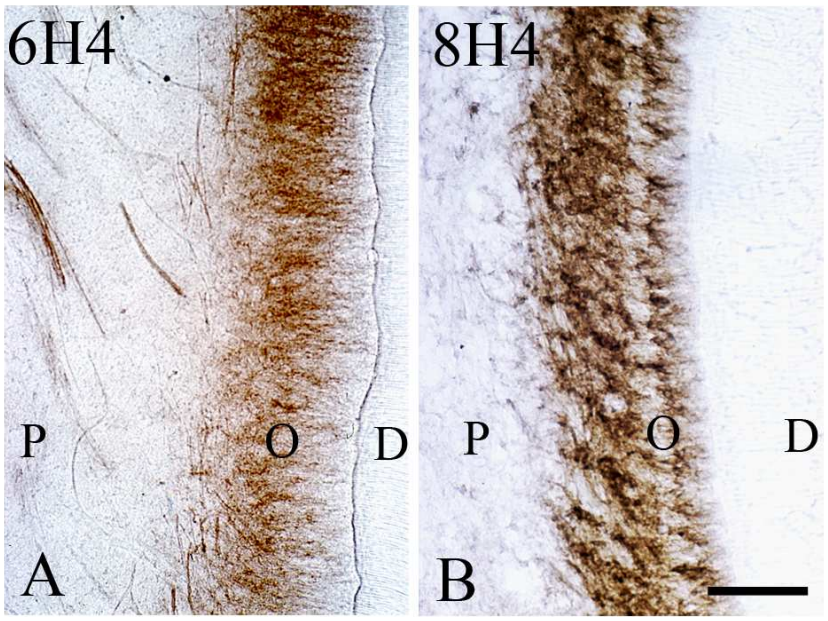

Figure 14: PrP in human molar odontoblasts. In the odontoblasts' cell layer 6H4 was detected in numerous cell bodies of odontoblasts (A; O). This localization pattern of 6H4 was confirmed with 8H4 antibody that recognized another epitope (B; O). In comparison, the staining intensity of 6H4 (A) was weaker than that of 8H4 in the odontoblast layer. P = dental pulp, O = odontoblasts, D = dentin. Bar: A and B = 190 μm.

5.2.2.1 Cementoblasts In fully as well as not yet fully developed teeth, expression of PrP was more pronounced in apically located cementoblasts than in cervical ones (figure 16).

5.2.2.2 Epithelial Rests of Malassez The epithelial rests of Malassez distributed near the cementoblasts in the PDL of a tooth germ revealed strong PrP immunoreactivity. Staining intensities of PrP in the ERM were homogenous. PrP immunoreactivity was detected with the same staining intensities in the cervical PDL as in the apical half of the PDL. In adult PDL, by immunohistochemical results, it was found that ERM are distributed in higher number in the cervical areas, while in the apical areas the number of ERM decreased.

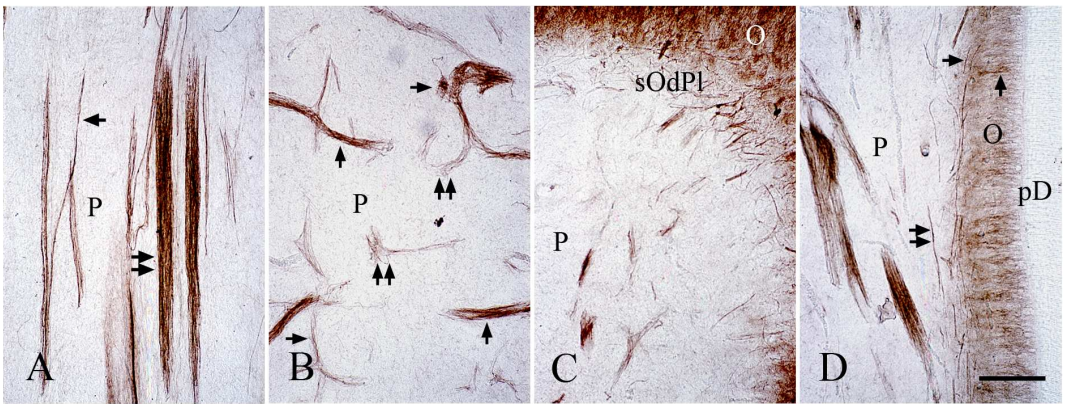

Figure 15: Localization of PrP in nerve fibers of the dentin-pulp complex. The PrP positive nerve fibers pass through the radicular pulp (A) with little branching, but form numerous branches in the coronal pulp (B and C). The distribution of PrP was detected in the perivascular (B; double arrows) as well as in free nerve fibers (B; arrows) of the dental pulp. The PrP immunoreactive nerve fibers were distributed in the subodontoblastic plexus (sOdPl), penetrated the odontoblast layer (D; double arrows) and reached predentin (D; arrows). P = dental pulp, sOdPl = subodontoblastic plexus, O = the odontoblast layer, pD = predentin; Bars: A-D = 190 μm.

5.2.2.3 Nerve Fibers In the PDL, thick nerve fiber bundles were strongly positive for PrP. These nerve fibers were distributed throughout the PDL. There were also numerous thin nerve fibers which revealed a strong immunoreactivity for PrP. These nerve fibers were free nerve fibers as well as nerve fibers around blood vessels (figure 17).

5.3 Increased Expression of PrP beneath a Dentin-Cavity Filling

The expression pattern of PrP was characterized during secondary dentin formation under a filling using 6H4 and 8H4 antibodies.

5.3.1 Characterization of Dentin-Pulp Complex Cells by H&E

In a tooth with a filling cavity, cells of the dentin-pulp complex were characterized by H&E staining (figure 18A). Throughout the coronary and root odontoblast layer, there was a thick layer of secondary dentin formation (fig-

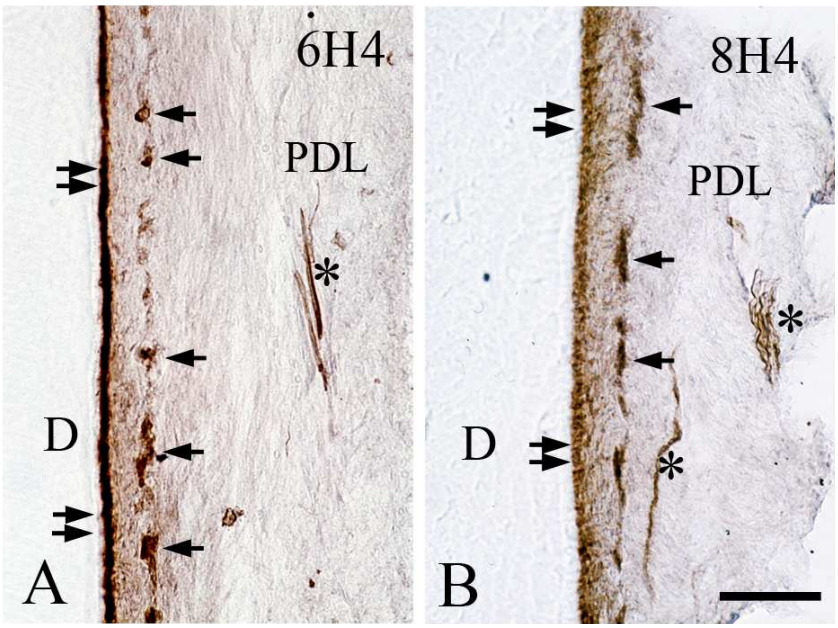

Figure 16: **PrP in cementoblasts and ERM.** In cementoblasts at the apical half, PrP was detected (A, B; double arrows). PrP was also detected in the ERM (A, B; arrows) and in nerve fibers (A, B; asterisks) of the PDL. Detection of PrP with 6H4 (A) was confirmed by 8H4 PrP-antibody in the same cells (B). D = dentin, PDL = periodontal ligament. Bar: A and B = 190 μm.

ure 18D). In comparison to the other secondary dentin areas around the dental pulp, the layer of the secondary dentin area beneath the filling was found to be thicker (figure 18D). Odontoblasts, blood vessels, nerve fibers and pulp stroma cells were detected in a healthy arrangement (figure 18G, H).

5.3.2 Expression of PrP in Pulp Stroma Cells and Odontoblasts Beneath a Dentin-Cavity

The staining intensity of odontoblasts was found to be greater beneath fillings compared to other odontoblasts (figure 18B). Localization of 6H4 was detected with higher staining intensities in odontoblasts as well as pulpal stroma cell populations with a close association to the secondary dentin areas in all coronal odontoblasts (figure 18E). Odontoblasts near the secondary dentin area beneath a filling cavity revealed high staining intensities for 6H4 (figure 18E).

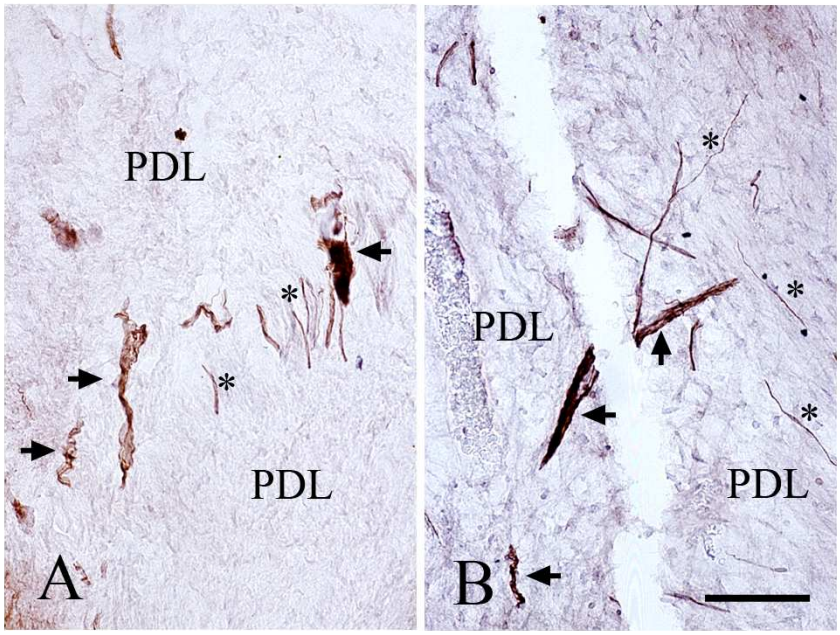

Figure 17: PrP in nerve fiber bundles and in thin nerve fibers of the PDL. In thick nerve fibers of the PDL PrP was detected with a higher staining intensity (A and B; arrows). The thin nerve fibers were strongly positive for the PrP immunoreactivity in the PDL (A and B; asterisks). PDL = periodontal ligament. Bar: A and B = 190 μm.

This staining pattern was confirmed by another PrP monoclonal antibody (8H4) against another epitope with different amino acid sequences (aa 175-185) in the human PrP under a filling cavity (figure 18C) and underlying the pulpal coronal odontoblast layers (figure 18F).

5.4 Immunohistochemical Controls

Incubations without the primary or secondary antisera and preabsorption were carried out as negative controls.

5.4.1 Immunohistochemical Controls by Omission of Antibodies

Sections were incubated in the absence of the primary [mouse monoclonal IgG1 (6H4)] or secondary (biotinylated horse anti-mouse IgG) antisera. In sections

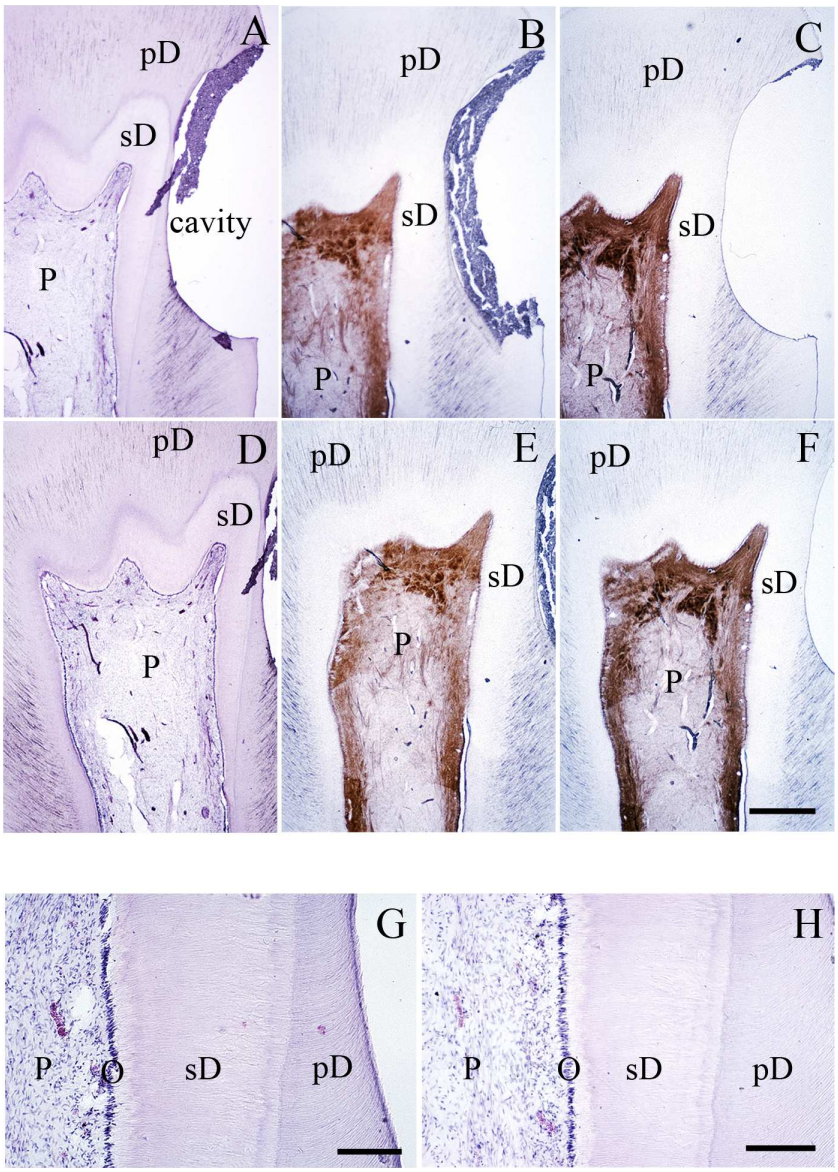

Figure 18: Expression of PrP in odontoblasts beneath the secondary dentin areas and beneath a filling. In H&E staining, secondary dentin was identified (A, D). Odontoblasts revealed a staining for 6H4 under secondary dentin areas (B, E) and beneath a filling with a higher intensity (B, E). In consecutive sections, 8H4 was detected in odontoblasts under secondary dentin regions as well as beneath a filling cavity (C, F). H&E staining revealed healthy cell components with a secondary dentin area (G, H).
pD = primary dentin, sD = secondary dentin, D = dentin, P = dental pulp, O = odontoblasts. Bar: A-F = 900 μm; G and H = 190 μm.

incubated without primary antisera no immunoreactivity was detectable in odontoblasts (figure 19A), in nerve fibers and cementoblasts (data not shown),

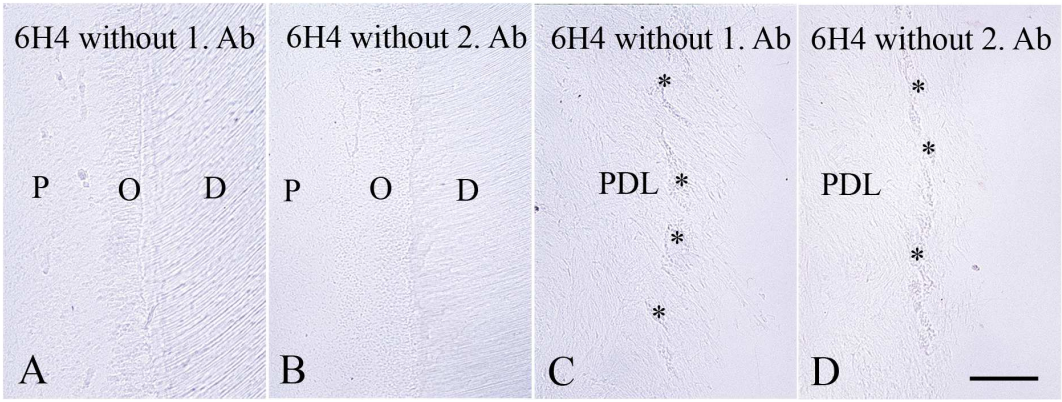

Figure 19: IHC-control incubations without primary and secondary antibodies in human molar sections. In the sections incubated without primary antisera [mouse monoclonal IgG1 (6H4)] no immunoreactivity was detected in odontoblasts (A), in nerve fibers and cementoblasts (data not shown) or in the ERM (C). ERM in the human PDL was negative (C and D; asterisks). After omission of secondary antibody (biotinylated horse anti-mouse IgG), immunoreactivity was absent in odontoblasts (B), in nerve fibers, cementoblasts (data not shown) and in the ERM (D). P = dental pulp, O = odontoblast layer, D = dentin, PDL = periodontal ligament. Bars: A–D = 190 μm.

and in the ERM (figure 19C). Immunoreactivity was also absent in odontoblasts (figure 19B), in nerve fibers and cementoblasts (data not shown) and in the ERM (figure 19D) when the secondary antibody was omitted.

5.4.2 Immunohistochemical Controls by Preabsorption Technique

The specificity of antisera was tested by preabsorption with the respective peptide concentration in 1-, 5- and 10-fold excess (in working dilutions of 6H4, 1:3000) for monoclonal anti-PrP antibody 6H4 peptides (Genaxxon Bioscience, Biberach, Germany). A very weak (in 1-, figure 20A) or no immunostaining [in 5- (figure 20B) and 10-fold (figure 20C) excess] was detectable after preabsorption of the antisera with the respective active peptide.

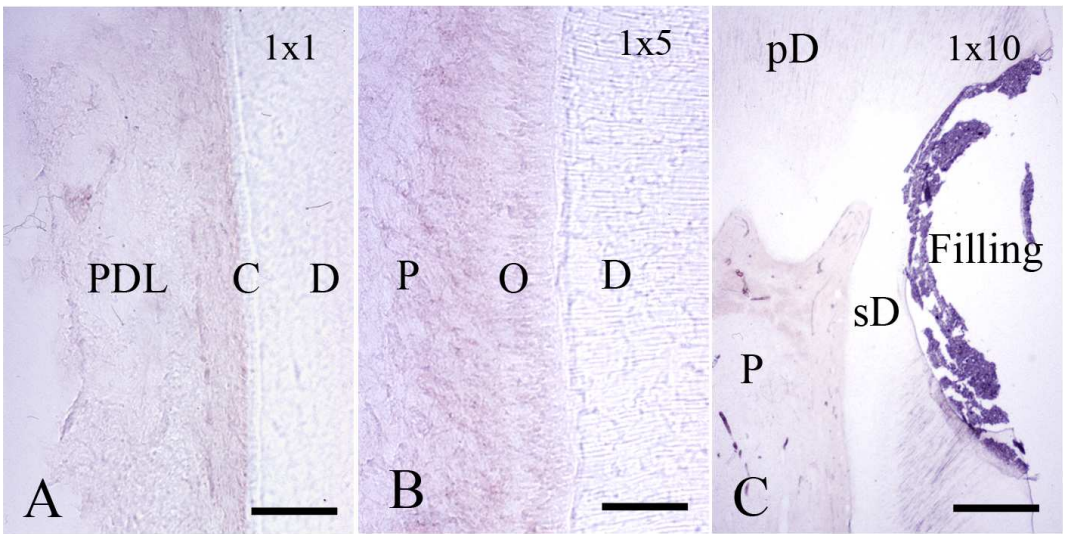

Figure 20: Preabsorption with the respective peptide concentration in 1-, 5- and 10-fold excess (6H4, 1:3000) for monoclonal anti-PrP antibody 6H4 peptides. A weak (in 1-, A) or no immunostaining [in 5- (B) and 10-fold (C) excess] was detectable after preabsorption of the antisera with the peptides. PDL = periodontal ligament, C = cementum, D = dentin, P = dental pulp, O = odontoblast layer, sD = secondary dentin, pD = primary dentin. Bars: A-B = 190 μm, C = 900 μm.

5.4.3 Positive Immunohistochemical Controls Using Non-Relevant Antibodies

The cellular localization patterns of 6H4 (figure 21A) in odontoblasts were confirmed with localization of soluble guanylate cyclase (sGC) α_2-subunit (figure 21B) and sGC β_1-subunit (figure 21C), in odontoblasts [164]. 6H4 localization in nerve fibers bundles and in thin nerve fibers (figure 21D) was confirmed with the localization pattern of calcitonin gene-related peptide (CGRP) positive nerve fiber bundles (figure 21E) and in thin nerve fibers (figure 21F) [271] in the dental pulp. 6H4 in the ERM (figure 21G) and in nerve fibers of PDL was also confirmed with localization of TrkA (figure 21H) and CGRP in the ERM and CGRP in the nerve fibers (figure 21I) of the PDL [271].

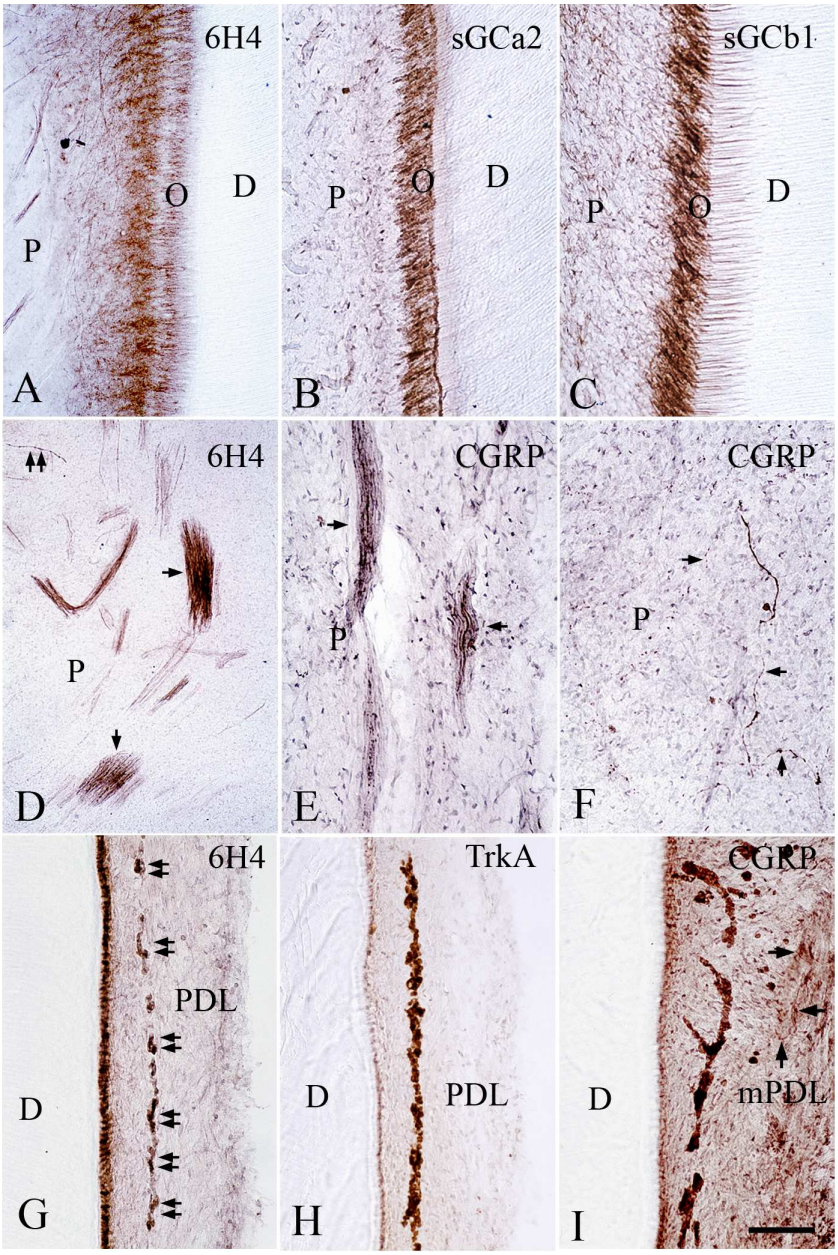

Figure 21: Positive controls of the immunohistochemical localizations in the human dentin-pulp complex and PDL. The localization of 6H4 (A) in odontoblasts was confirmed with localization of sGC α_2-subunit (B) and sGC β_1-subunit (C), in odontoblasts. In the human dental pulp, localization of 6H4 in nerve fiber bundles and in thin nerve fibers (arrows; D) is comparable with the existence of CGRP in nerve fiber bundles (arrows; E) and in thin nerve fibers (arrows; F) in the human dental pulp. Localization of 6H4 in the ERM was confirmed with localization of TrkA (H) and CGRP in the ERM and CGRP in the nerve fibers (I) of the PDL. P = dental pulp, O = odontoblast layer, D = dentin, PDL = periodontal ligament, mPDL = middle PDL. Bar: A–I = 190 μm.

5.5 Densitometry and Statistical Analysis

We have analyzed 6 different states of development of human teeth, characterized by the length of their roots. We classified root development according to figure 22.

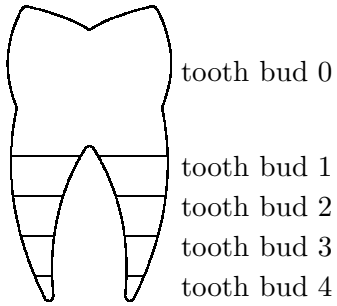

Figure 22: States of root development

By using densitometry of the immunostains, we quantified the amount of PrP in different layers of the teeth. The results of this densitometry are presented in figure 23.

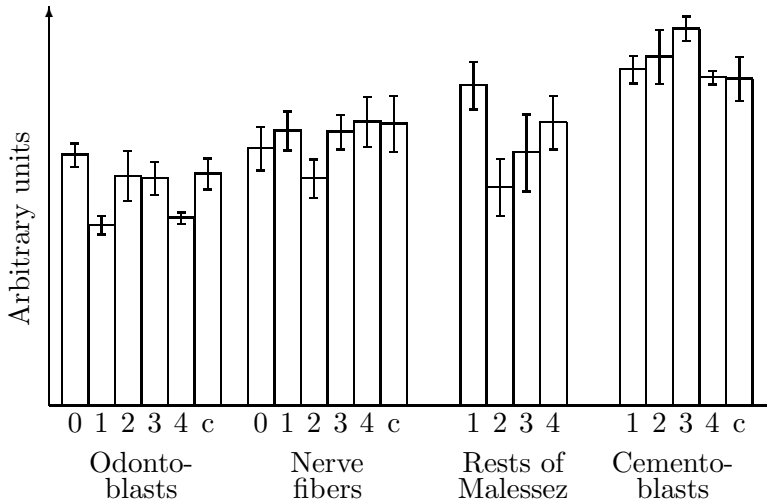

Figure 23: Densitometric analysis of immunostains. There were no significant differences in PrP expression between the different states of root development. 0: Tooth bud 0, 1: Tooth bud 1, 2: Tooth bud 2, 3: Tooth bud 3, 4: Tooth bud 4, c: Fully developed, caries free tooth. Ordinate values: arbitrary units ± S.E.M.

Rests of Malassez and cementoblasts are not present as long as root development has not set in. Comparing different stages of root development, there was

no clear trend in the amount of PrP in the teeth. No statistically significant developmental dependence of PrP expression could be detected.

5.6 Characterization of Cultivated Human Dental Cells by Alizarin-Red Staining

By staining cultivated human dental cells with Alizarin-red, their propensity for calcification could be demonstrated after 6, 9, 10, and 14 days of incubation (figure 24).

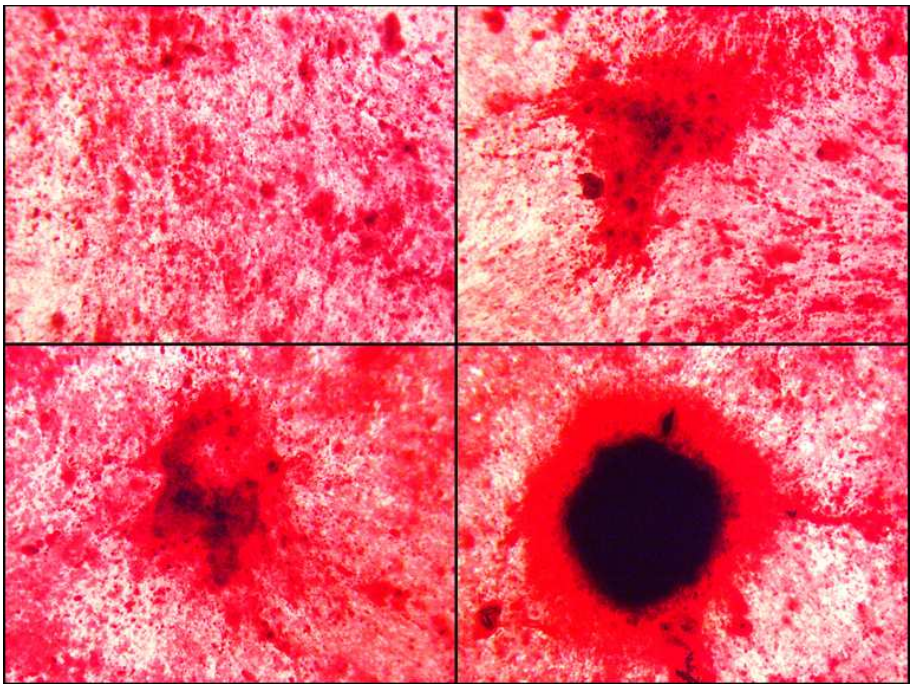

Figure 24: Progress of calcification of extracellular matrix *in vitro* after incubation with β-glycerophosphate, ascorbic acid, and dexamethasone is shown by alizarin red staining. Upper left: after 6 days, upper right: after 9 days, lower left: after 10 days, lower right: after 14 days of incubation.

Part V

Discussion

This study was designed to detect the existence and distribution of PrP in cells of the dentin-pulp complex and in cells of the PDL. The experiments focused on the localization and expression of PrP in different types of cells in normal developmental stages of human teeth. The cell specific localization and expression of PrP was found to be compatible with a role of PrP in matrix formation (in formation of dentin and cementum) and matrix biomineralization (in biomineralization of dentin and cementum) as well as in neurotransmission in the dentin-pulp complex and in the PDL during development and in adult human teeth.

Mice expressing PrP at different levels (knockout, wild-type and overexpressing) turned out to have markedly different dentin structures. These findings may or may not be causally associated with the different levels of copper found in the teeth of these mice.

6 Methodical Considerations

6.1 Scanning Electron Microscopy

For examining the differences between wild-type, heterozygous, knockout and PrP overexpressing animals, only mice could be used during the examination. Knockout cattle [249] were only reported about after the examination had been performed. Furthermore, it might have been close to impossible to come by teeth of these cattle for examination. Cattle teeth, however, would have had the advantage of a greater size which would have made the SEM examination as well as the determination of the metal contents easier.

We have compared $PrP^{0/0}$ (knockout), $PrP^{+/0}$ (heterozygotes), $PrP^{+/+}$ (wild-type) and tga20 (PrP overexpressing) mice. $PrP^{0/0}$ of the Zürich I type, as

they were used in this study, have a mixed genetic background of 129/Sv and C57BL/6J [58]. Our wild-type mice, which were used for comparisons in the SEM study, were C57BL/6J only, whereas for the determination of the copper content, wild-type littermates of the knockout mice were available. While it can not be completely ruled out that the genetic background has some influence upon dentinogenesis, our SEM findings in wild-type mice turned out to be in the middle between the findings in knockout and overexpressing mice, respectively. This is exactly what would be expected in case that PrP^{C} is causally influencing dentinogenesis. This does not, however, give any glue or even proof as to how this influence of PrP^{C} upon the dentin structure is brought about.

Due to personal restrictions in the animal breeding facility of the University Düsseldorf, and also to eliminate location specific factors, mice were obtained from three different suppliers (University Düsseldorf, Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin, Berlin, and Center for Neuropathology and Prion Research, University Munich). The described anomalies of $PrP^{0/0}$, $PrP^{+/0}$ and tga20 mice seen in the SEM were found in mice from Düsseldorf and Berlin. Only later the mice from Munich were received which surprisingly did not have the same peculiarities, although they were reported to be of the same genotype (Zrch I [59]). Unfortunately, no direct contact with the caretakers of any of the employed mice was establishable or the caretakers did not have the requested information anymore (What did the mice eat and drink? Did they have any infections? Have they been immunized against anything?).

One possible explanation for the different findings in SEM figures between mice from Düsseldorf and Berlin on the one hand and mice from Munich on the other hand could possibly be a different diet. Especially different levels of copper supplementation might explain the differences (see chapter 7.1.3). However, this is no longer reproducible. Any other suspicion about the cause of these differences would be pure speculation.

6.2 Determination of Copper and Manganese Contents in Mouse Teeth

Due to the small size and thus small weight of mouse molars, the determination of copper and manganese content could only be performed once. To detect systematic errors during the determination, the teeth were split in two groups and analyzed independently. No statistical evaluation was possible.

6.3 Detection of PrP Expression by PCR

Using separate amplification of cDNA from cementoblasts, pulp and ectomesenchyme, it was possible to demonstrate the transcription of PrP in human dental tissues. However, this method has several limitations:

- As a bulk of cells was subject to isolation of RNA for subsequent reverse transcription and PCR, no differentiation between the transcription level of individual cells was possible.
- PCR is not a quantitative method. As soon as the amplification goes into saturation due to an exhaustion of primer, nucleotides or polymerase, the amplification slows down. Therefore, an estimation of the level of transcription and thus of the amount of mRNA was not possible.

6.4 Comparison of Knockout-, Wild-Type Mouse Brain and Cell Culture Cells

To demonstrate that the antibody does not bind to protein derived from $PrP^{0/0}$ mice, and to demonstrate the expression of PrP in our cell culture cells, we applied the membrane proteins of our cultivated cells, as well as $PrP^{0/0}$ brains (negative control) and $PrP^{+/+}$ brains (positive control) to an SDS-PAGE gele.

Since care was taken to use cultured cells of the same passage as those used for isolation of RNA, only little material was available. Therefore, and to ensure

equal loading of the lanes, only 8 μg protein could be loaded. Ponceau S staining was used to verify equal loading of the lanes. The $PrP^{0/0}$ lane is clearly devoid of any signal, as was expected, after 2 minutes as well as after 6 minutes of exposition. The lanes of the cultured cells and the wild-type mouse brain show the expected lanes at 33 to 37 kDa.

While in wild-type brain the most prominent band is the diglycosylated, the proteins in the cell culture lanes showed a higher electrophoretic mobility. There are different possible explanations for this finding. First, cell culture cells might have a higher proportion of un- and monoglycosylated PrPs. Second, although the three glycoforms are synthesized in equal quantities, the unglycosylated isoform accounts for only 5% of cell-surface PrP^{C} [63]. Thus, another possible explanation for the different mobility in the cell culture lanes compared with the wild-type brain might result from the small amount of protein that was available for preparation. While during brain preparation a protein pellet was clearly visible, no pellet was recognizable during preparation of cell culture cells. Thus, the greater proportion of proteins with a higher mobility might be explainable by residual buffer containing cytoplasmic PrP molecules.

6.5 Involvement of PrP in the Regulation of Different Cells in the Dentin-Pulp Complex and Periodontal Ligament

In the present study the localization of 6H4 was investigated in cells under physiological conditions during tooth development. Healthy teeth showed a staining for 6H4 in odontoblasts, cementoblasts, ERM and in nerve fibers of the dental pulp and periodontal ligament with different intensities during development. These results from teeth with different developmental stages support an involvement of PrP in the regulation of these cells during human tooth development.

6.5.1 Immunohistochemistry

The specificity of the PrP immunostaining was tested by showing the immunolocalization of PrP with non-relevant antibodies, with preabsorption technique and with immunohistochemical incubations of sections with different antibodies directed to different epitopes of PrP. For immunohistochemical experiments with monoclonal PrP antibodies in mouse tissue sections a blocking solution was used due to the binding of secondary anti-mouse antibody to endogenous mouse tissue Igs and other antigenic mouse components. For an optimal immunohistochemical localization the primary and secondary antibody must be developed from other species (e.g., rabbit). Even by employing blocking solutions, the unspecific staining was not avoidable with mouse antibodies in mouse tissue sections. Therefore, in the present study, these results were not presented in $PrP^{0/0}$ mouse tissue sections. In comparison, the localization of PrP with mouse antibodies in human tissue is associated with higher specificity. However, these localization patterns were also tested with non-relevant antibodies, with the preabsorption method, with antibodies directed against different regions of PrP and with positive and negative controls.

6.5.1.1 Immunohistochemical Controls with Non-Relevant Antibodies

The cellular localization patterns of 6H4 in different cells of the dentin pulp complex and PDL were confirmed by the use of non-relevant antibodies with known localization of these antibodies in odontoblasts [soluble guanylate cyclase (sGC) α_2-subunit, sGCβ_1-subunit, pulp and periodontal ligament nerve fibers [calcitonin gene-related peptide (CGRP)], and epithelial rests of Malassez, [TrkA, sGCα_2, sGCβ_1 and CGRP]. The known localization patterns of these different antibodies in rat tissues [164, 165, 271, 340] were verified in human tooth sections and demonstrated the same immunohistochemical localization patterns of 6H4.

6.5.1.2 Immunohistochemical Controls by Preabsorption of PrP Antibodies The specificity of antisera was tested by preabsorption with the respective peptide concentration in a 1-, 5- and 10-fold excess (in working dilutions of 6H4, 1:3000) for monoclonal anti-PrP antibody 6H4 peptides. A very weak (in 1-fold) or no immunostaining [in 5- and 10-fold excess] was detectable after preabsorption of the antisera with the respective active peptide.

6.5.1.3 Immunohistochemical Controls by Staining with Antibodies Directed Against Different Epitopes of PrP The mouse monoclonal antibody 6H4 recognizes the amino acid sequence 144-152 of human PrP. The localization of 6H4 was detected in nerve fibers of the PDL and dental pulp, in odontoblasts, cementoblasts and in ERM. In order to fortify the data, additional immunohistochemical experiments were performed with another mouse PrP monoclonal antibody, 8H4, against another epitope with a different amino acid sequence (175-185) in human PrP. The localization patterns of 8H4 confirmed in all cases (in odontoblasts, in nerve fibers of the dental pulp and in PDL, in cementoblasts and in ERM) the localization patterns of 6H4. The 3F4 antibody detects amino acid sequence 109-112 of human PrP. The results (localization of PrP in nerve fibers, odontoblasts and cementoblasts) were also confirmed with 3F4 antibody (data not shown).

6.5.1.4 Positive and Negative Immunohistochemical Controls In the immunohistochemical localization of 6H4, sections were incubated in the absence of the primary or secondary antisera. In the human sections incubated without primary antisera no immunoreactivity was detectable in odontoblasts, in nerve fibers and cementoblasts (data not shown) and in the ERM. Immunoreactivity was also absent in odontoblasts, in nerve fibers and cementoblasts (data not shown) and in the ERM when the secondary antibody was omitted.

6.5.1.5 Immunohistochemical Incubations and H&E Staining Comparing Wild-Type with Knockout Mouse Teeth 6H4 antibody is a mouse IgG1 subtype antibody. It is known that antigen detection with mouse primary antibody on mouse tissues is complicated by high levels of background staining due to the binding of secondary anti-mouse antibody to endogenous mouse tissue Igs and other components [186]. Blocking this binding by preincubation with Fab Fragment of unconjugated anti-mouse IgG [in wild-type and knockout mouse immunohistochemical protocols unconjugated Affini Pure Fab Fragment Goat Anti-Mouse IgG (H+L) was used, Jackson Immuno Research Labs] in combination with the use of a biotin-conjugated Fab Fragment Anti-Mouse IgG [Biotin-SP-conjugated Affini Pure Fab Fragment Goat Anti-Mouse IgG (H+L), Jackson Immuno Research Labs] led to elimination of most of the unspecific background staining and achieved satisfactory but not optimal result (due to reduced epitope recognition). Therefore, 6H4 was detected weakly in odontoblasts (data not shown).

For this reason, H&E staining was performed in order to compare histological features and differences between wild-type and knockout-mouse calcified tissues. Important differences in dentin and alveolar bone structures were detected. There was a regular organization in dentin structures and alveolar bone of wild-type mice, while knockout mice showed typical irregular dentin tubules and alveolar bone structures. The results of the present work revealed the importance of a histological staining for a characterization and comparison of the cells and organ structures between any wild-type and knockout mouse tissues.

6.5.1.6 Demonstration of the Specificity of the 6H4 Antibody by Western-Blot Four different states of glycosylation are possible for PrP (un-, mono- (at the first and at the second location), and diglycosylated), three of which can be discriminated in the Western-Blot, resulting in bands between 33 and 37 kDa. These three bands are clearly visible in our Western-Blot in lanes from wild-type mice, with the diglycosylated being the most prominent one, as reported before by others [228].

7 Involvement of PrP in Matrix Formation, Matrix Biomineralization and in Neurotransmission in Cells of the Dentin-Pulp Complex and in Cells of the Desmodontal (Cement, Periodontal Ligament and Alveolar Bone) Unit

7.1 Copper and Manganese Contents in Mouse Teeth

There are several publications establishing a relationship between copper as well as manganese on the one hand and PrP on the other:

Copper:

- PrP is known to bind copper [43, 166, 169, 284] at its N-terminal octarepeats [44, 139, 140, 145, 167, 311, 319], around His_{96} and His_{111} [145] as well as at its C-terminal end [70].
- PrP is involved in cellular copper uptake [41], modulates neuronal copper content [45], and is involved in copper homeostasis [310].
- PrP is endocytosed from the cell surface in the presence of copper [225, 227], a mechanism which is abolished upon mutation of the octarepeat region [227].
- Cu^{2+} hampers conversion of PrP into amyloid fibrils [34] and modulates the aggregation properties of the PrP fragment PrP106–126 [149].
- One of the cases described by Creutzfeldt [79] turned out later to be Wilson's disease [42], a disease linked to mutations in copper transporters.
- Cuprizone (biscyclohexanoneoxaldihydrazone), a copper chelator, was reported to produce histopathological similarities to scrapie [153, 155, 223, 224], although these similarities were characterized as superficial [154].

Manganese:

- Kuru symptoms have been reported to resemble manganese poisoning [10, 11, 343].
- Manganese was reported to be the only other cation, besides copper, capable to be bound by PrP [46].
- Brain tissues of sCJD subjects were found to have decreased copper levels (by up to 50%) and up to 10-fold elevated manganese levels [335].

In order to elucidate the molecular mechanism(s) underlying the anomalies found in the hard tissues of mice with different PrP expression levels, we performed a determination of copper and manganese contents in $PrP^{0/0}$, tga20 and wild-type mice.

While there seems to be no correlation between the expression level of PrP and the concentration of manganese in mouse molars, we found a positive correlation between the expression level of PrP and copper. Therefore, the subsequent discussion will focus on copper alone.

There are two different groups of values concerning copper contents that can be found in the literature: comparisons of copper contents of soft tissues harvested from $PrP^{0/0}$ and wild-type mice on the one hand, and contents of copper in teeth of mammals (including humans), but not $PrP^{0/0}$ mice, on the other hand. Since our numbers represent the copper content of whole teeth [tooth hard tissue (enamel, dentin and cementum) as well as the dental pulp], differences between our observations and those found in the literature were to be expected.

7.1.1 Copper Contents in Soft Tissue

As PrP is most concentrated in nervous tissue, the brain's copper content is most important. However, grey matter has a higher copper content than white matter [263], which hampers comparisons between different species. Moreover,

the copper content of the (human) brain is dependent on age of the individual [263].

Brown *et al.* [43] found a copper content of 1.3 ± 0.05 μg/g wet weight in membrane-rich extracts from $PrP^{0/0}$ mouse brains, while the same extracts from wild-type mice had a copper content of 20.1 ± 1.4 μg/g wet weight, which is a decrease by 93%. Remarkably, 6 years later, the same author stated that he never found reliable data to suggest these differences between $PrP^{0/0}$ and wild-type mice [45]. Waggoner *et al.* [314] reported that brain extracts of $PrP^{0/0}$, wild-type and tga20 mice (which overexpress PrP) did not differ significantly in their copper content, a finding that is in line with the assumption that another protein, Ctr1, is the primary copper transporter in mammalian cells [173]. Thus, the different copper contents of $PrP^{0/0}$ and wild-type mouse teeth might be caused by different copper contents in the tooth hard tissue. Herms *et al.* [133] found no difference in copper content of whole brains between wild-type, $PrP^{0/0}$ and tga20 mice. However, copper contents of synaptosomal fractions was reported to be reduced by about 50% in $PrP^{0/0}$ mice compared with both, preparations from wild-type and tga20 mice [133].

7.1.2 Copper Contents in Teeth

A huge variability of copper content in teeth can be found in the literature (table 5 and 6 in section 12).

Reference [161], whose values are far from average, has only been included for the sake of completeness. These values exceed those found in molars of bank voles living in contaminated areas near heavy metal mills [15] by 62-fold (!). Unfortunately, the authors of [161] did not state whether the teeth were from humans, nor whether these individuals inhabitated a heavily contaminated area. The authors of [269] reported to have found no manganese in their teeth.

Our results are at the lower end of the reported numbers. Especially in human teeth, the copper content is heavily influenced by restorations. An obvious

source contributing to elevated levels of copper in human tissues is the use of tools, e. g., cutlery. Another factor influencing the relative contents of copper in teeth might be the way of drying the teeth. Many authors reported to have treated their teeth with some hundreds of °C before analysis, while our specimens were subject to the application of only 40°C for drying until weight constancy was achieved. Residual fluid trapped within the dentinal tubules would reduce the relative contents of any trace element in any tissue.

7.1.3 Suggested Role(s) of PrP^C by Copper Binding in the Desmodontal Connective Tissue Formation and Tissue Maintenance as well as in the Dentin- and Bone-Matrix Formation and Matrix Biomineralization

Collagen is a main component of predentin and desmodontal fibers [265, 293]. There are several copper containing molecules that were described to bind to (SOD3 [14]) and cross-link (protein-lysine 6-oxidase (lysyl oxidase) [254, 255, 256]) collagen. Lysyl oxidase deficiency, on the other hand, was described to result in diseases manifesting in connective tissue disorders, such as Ehlers-Danlos and Menkes syndrome [171]. Copper deficiency was reported to lead to immature collagen in fetal membranes, in turn inducing preterm births [183]. Knockdown of lysyl oxidase in zebrafish demonstrated an involvement of this enzyme in muscle and cartilage development [248]. In *in vitro* experiments, incubation of collagen with copper ions led to an increased resistance against pepsin digestion and reduction of solubility in sodium dodecyl sulfate mercaptoethanol, compared with incubation without copper ions [259]. Another *in vitro* study presented copper concentration dependent collagen synthesis by oral fibroblasts, supporting the hypothesis that areca nut (which has a high content of copper) chewing is involved in the pathogenesis of oral submucous fibrosis [303]. Also *in vitro*, Cu^{2+} has been shown to upregulate collagen anabolism in human articular chondrocytes [131]. An *in vivo* study described bones of chickens which had received a diet deprived of copper as less tolerant to deformation and torsional force and more prone to fracture than the bones

of control animals. Even lowest levels of supplementation of copper, however, were sufficient to prevent these deficiencies [214].

Crowded odontoblasts and dissimilar sizes of tubules were reported in mice fed exclusively with raw beef muscle, which is supposed to contain low levels of copper [263] and to have a low Ca/P ratio [307]. Although the author analyzed the effect of copper and manganese supplementation and observed the partial alleviation of other deficiency syndromes, such as osteoporosis, upon copper supplementation, he did not differentiate between mice that were fed a diet supplemented or not supplemented with copper while describing the structure of dentin. Possibly, the applied copper supplementation was sufficient to partly cure anomalies in bone, but insufficient to avoid the disturbance in dentinogenesis.

Copper deficiency in sheep, detected by determination of liver copper content, was reported to be associated with tooth problems as excessive wear, periodontal disease, maleruption and mandibular osteopathy, all of which could be prevented in flock fellows that were reared on another farm during the period of permanent tooth formation. This observation is in line with the influence of the degree of crosslinking upon the biomechanical properties of collagen-rich tissues [255]. Unfortunately, no copper content of teeth and no dentin structure were reported in these animals [56]. Nevertheless, as lysyl oxidase, a copper containing protein, is involved in collagen (which is an important constituent of dentin) formation [56] and cross-linking [255, 256], an association between copper content of teeth and dentin structure is conceivable. However, as Ctr1, the above-mentioned main copper transporter, is also found in developing tooth buds of mice by day E18.5 [173], the effects of PrP deficiency on dentinogenesis are not expected to be prominent.

Thus, while the findings presented here do not prove a direct link between PrP^C expression, copper content and the laydown of hard tissues containing a collagen matrix, it is neverthelesss noteworthy that especially cells engaged in biomineralisation proved to be PrP positive.

Dentin wall thickness correlated positively with PrP^C expression levels. Occa-

sionally, in SEM figures, inter-tubular dentin walls of $PrP^{0/0}$ mice seemed to be thinned far enough to have broken down, resulting in confluence of two or more tubuli, thereby unifying several smaller tubuli to one big tubulus, an observation that could be explained by insufficient collagen matrix cross-linking.

According to the reasoning presented above, disturbances in dentinogenesis might be caused by an unspecific lack of copper due to the loss of PrP^{C} as copper carrier and/or storage pool.

There are many other ways how PrP^{C} could influence dentinogenesis. However, no evidence for any of these alternative ways could be obtained with the results of this study.

7.2 Cellular Regulation of Odontoblasts and Nerve Fibers by PrP

7.2.1 PrP may be Involved in the Formation and Biomineralisation of Dentin Matrix

Immunocytochemical localization of PrP in different odontoblast cell bodies within the odontoblast layer is compatible with an involvement of PrP in the regulation of cell bodies' activity of odontoblasts. The absence of PrP in the odontoblast processes indicates a role of PrP only in cell bodies of odontoblast. The matrix formation occurs in the cell bodies while biomineralization of this matrix is regulated at the predentin. This fact is compatible with a role of PrP only in the matrix formation in odontoblast cell bodies and in matrix biomineralization.

In experiments of the present study the role of PrP was further confirmed in the matrix biomineralization during secondary dentin formation beneath a filling. In immunohistochemical results, 6H4 was found with strong staining intensities in odontoblasts as well as pulpal stroma cell populations with a close association to the secondary dentin areas in all coronal odontoblasts and especially with a greater area beneath a filling. In the odontoblast layer of all dental pulp areas, odontoblasts located near the secondary dentin area revealed high staining intensities for 6H4 compared to the central pulp cells.

Especially, this staining was increased beneath filling cavities. These results of 6H4 were confirmed with another PrP monoclonal antibody (8H4) against another epitope (compared with 6H4) with a different amino acid sequence (175–185) in human PrP. These results indicate that PrP may be involved in the matrix formation and matrix biomineralization of dentin wounds and thereafter in the organization of the secondary dentin.

In the $PrP^{0/0}$ mouse dentin tissue, the structural defects and disorganization in the dentin tubuli were detected by comparison of the dentin structures of wild-type mice and $PrP^{0/0}$ mice. The dentin tubules were distributed not in the same order as it was found in wild-type mouse dentin. There were defects in dentin which were not observed in the wild-type mice. These findings indicate that PrP is involved in the formation of dentin. Disorganization and defects detected in alveolar bone of $PrP^{0/0}$ mice support an involvement of PrP in matrix formation and in matrix biomineralization in calcified tissues such as dentin and bone.

To our knowledge, there are no references about the involvement of PrP in dentin matrix formation, in dentin wound healing or in formation of secondary dentin. In the present results, it was indicated for the first time that PrP may be expressed in the odontoblast during tooth development as well as in the case of dentin wound healing and secondary dentin formation beneath a dentin cavity filling. Thus, PrP may be involved in dentin matrix formation and dentin biomineralization under physiological as well as under pathophysiological conditions. In our current study, the mechanism of PrP in the formation and biomineralization of the dentin matrix was investigated with physiological experiments.

7.2.2 PrP may be Involved in the Regulation of Nerve Fiber Formation and/or Neurotransmission in the Dentin-Pulp Complex During Human Tooth Development

In the present study, localization of PrP was increased in numerous nerve fibers in the dentin-pulp complex in the last stages of tooth development. In

comparison to the first stage of tooth development the staining intensity of PrP in nerve fibers was increased. These results revealed that PrP is involved in the mechanisms of nerve fiber formation dependent on the developmental stages of the tooth. PrP may regulate the nerve fiber organization during tooth development under physiological conditions. To our knowledge, there is no information about a role of PrP in peripheral nerve fibers formation in human tissue (wisdom tooth). It is possible that PrP may be involved in the neurotransmission in nerve fibers during tooth development. We have also designed new experiments to investigate the involvement of PrP in the formation of peripheral nerve fibers under pathological conditions, such as inflammation of the dentin-pulp complex.

7.3 PrP in Cells of the Periodontal Ligament

7.3.1 Formation of Cementum may be Regulated by PrP during Human Tooth Development

In the tooth germ of the second, third and fourth developmental stage of the tooth root, there were strong staining intensities of PrP in cementoblasts. These staining intensities were higher especially at the apical areas, compared to cervical areas. In the apical areas at the cellular cementum, cementoblasts were active and formed cementum with higher cellular activity. The higher staining of PrP in cementoblasts at the cellular cementum areas indicates that PrP may be involved in the formation of matrix and biomineralization of cementum. The absence or very weak PrP staining in acellular cementum areas at the cervical zone is compatible with a decrease in matrix formation in the acellular cementum area. In comparison, the cementoblasts in the apical regions at the levels of the cellular cementum of the fully developed teeth indicate that PrP is also involved in the cementum remodelling in the cellular cementum regions not only in the tooth germ but also in fully developed teeth. These results lead to an important role of PrP in cementum remodelling and a possible use in the treatment of the cementum associated diseases in the PDL.

7.3.2 PrP Localization in the ERM is Compatible with an Involvement of PrP in the Epithelial Differentiation to Development of Enamel or Epithelial Transformation to Cysts and Ameloblastoma

The inner and outer enamel epithelial tissue fuse by mitotic activity to produce a bilayered epithelial sheath, termed the Hertwig's epithelial root sheath (HERS). The inner surface of the HERS (inner dental epithelium) induces odontoblast differentiation in the adjacent mesenchymal cells which begin to form root dentin [36]. Then, after root dentin has been formed [185, 297], the adjacent epithelial cells of the root separate from their surface and lose continuity [188]. There are some epithelial cells from the fragmented root sheath surrounded by a basement membrane, referred to as epithelial rests of Malassez (ERM) that persist in the mature periodontal ligament [36, 188].

The restoration of the diseased cementum to its original form and function is a major goal of periodontal therapy that requires regeneration of the diseased cementum through formation of new cementum [116, 216]. The mechanisms by which PrP may be involved in the regulation of cell differentiation in adult ERM in a close association to the acellular cementum is important to the understanding of the physiological remodeling or repair of acellular cementum. In the present study, PrP was detected with higher staining intensity in the ERM during root development as well as in the ERM of the adult teeth. It is known that HERS cells undergo epithelial mesenchymal transformation into cementoblasts producing cementum [297, 342]. The localization of PrP in the ERM of the adult PDL may point to its involvement in the cell differentiation of the ERM to the formation and/or remodelling cementum. This possible role of PrP is further investigated using ERM cell culture.

The ERM have the potential to differentiate into cementoblasts [36, 297], in squamous metaplasia forming cysts [69] and in ameloblasts-like cells [270] forming odontogenic tumors [57]. These known transformations of the ERM and the developmental expression of PrP in the ERM of human teeth renders the suggestion possible that PrP under pathological conditions might be involved in the pathological transformation of the ERM to odontogenic cysts and/or

odontogenic tumors. However, the involvement mechanisms of PrP in the transformation of the ERM into odontogenic cysts and odontogenic tumors remains to be elucidated.

7.3.3 PrP may be Involved in the Formation Mechanisms of Nerve Fibers and/or Neurotransmission in the Periodontal Ligament during Human Tooth Development

PrP positive nerve fibers in the PDL were distributed in the PDL as thick nerve fibers as well as as thin nerve fibers. The PDL is richly innervated by sensory innervation including numerous nociceptors and mechanoreceptors. It is possible that thin nerve fibers, which stained positive for PrP, can be nociceptive in nature, while thick nerve fiber bundles may be considered to be mechanosensory nerve fibers. Similar to the results in the dentin-pulp complex, the nerve fibers which were positive for PrP occur in dependence of PDL development. In association with PDL development, PrP positive nerve fibers revealed higher density in the adult tooth PDL. The developmental stages dependent density of the PrP positive nerve fibers indicate that PrP may be involved in the mechanisms of the formation of nerve fibers in the PDL. In our further studies, the role of PrP will be investigated in these mechanisms under pathological conditions.

7.3.4 Alveolar Bone Matrix Formation and Matrix Biomineralisation may be Regulated by PrP

In comparison to the alveolar bone structures from wild-type mice, there were important differences in $PrP^{0/0}$ mice which were demonstrated by H&E staining. In wild-type mice, alveolar bone cells were detected in their regular arrangement. We found a regular alveolar bone with osteoblasts, osteoclasts, bone marrow, blood vessels and calcified matrix structures. In $PrP^{0/0}$ mice, however, there were tissue defects, irregular bone structures and disorganization of the bone cells. The defects detected in dentin structures of $PrP^{0/0}$

support a role for PrP in matrix formation and matrix biomineralization in the alveolar bone. In addition, PDL thickness of $PrP^{0/0}$ mice was small in comparison to wild-type mice. In alveolar bone of $PrP^{0/0}$ mice, there was an increase in the number of bone marrow areas. In our upcoming experiments, this important result will be further investigated.

8 Considerations Concerning the Transmission of TSEs

Compared with previous knowledge, the findings presented in this study show that PrP is more readily accessible from the environment than expected. Due to the fact that only morphological, histological and biochemical evidence has been presented, no predictions to other topics can be given. Some epidemiological considerations are nevertheless appropriate.

8.1 Risks by and for Animals

While the ways of transmission of scrapie among sheep have been elucidated [221, 222, 246], the ways of transmission of chronic wasting disease (CWD) in free ranging deer are still obscure.

8.1.1 Shedding of Teeth

The finding that PrP is expressed in a tissue that is regularly shed during growing-up raises the question as to whether this could be a possible way of transmission. Ruminants have 20 deciduous teeth, as humans have. However, they have a different tooth formula (figure 25).

–	–	–	–	p1	p2	p3
i1	i2	i3	c1	p1	p2	p3

Figure 25: Tooth formula of the deciduous dentition of ruminants

Before deciduous teeth are shed, the root is resorbed, thereby loosening the tooth from the alveolar cavity. Thus, cementoblasts are scarcely to be expected in shed teeth. Odontoblasts, however, are trapped in the pulp chamber and thus arrive in the environment.

The situation is different if permanent teeth are lost due to old age or due to diseases like "broken mouth" in sheep [200, 280], which leads to premature loss of permanent incisors, probably caused by gingivitis. Here, teeth including their roots are released into the environment.

PrP^{Sc} has been demonstrated to be extremely resistant against inactivation (e. g., thermal [50, 51] or by using UV-radiation [6, 7, 8, 179, 180]) and to outlast long periods of time [48]. Both, deciduous and permanent teeth, however, are the most enduring tissues of a mammalian body. It takes centuries to decompose teeth, so that the amount of PrP freed in a given period of time may seem negligible. Of course, no studies exist that would give any hint to the longevity of PrP^{Sc} over such long periods.

8.1.2 Wear of Teeth

Due to the composition of the foodstuff, molars of ruminants are affected by heavy attrition, leading to loss of enamel and dentin. The deeper the attrition, the wider are the lumina of the dentin tubules. While we have found no PrP in the odontoblast processes, these most distal parts of odontoblasts are nevertheless in direct contact with the rest of the cells. Therefore, there is at least a (however improbable) morphological evidence for a possible route of infection. Nobody knows yet whether this absorbed infectivity (if any) could be passaged from odontoblasts to the nervous system.

8.2 Risk by and for Humans

Up to now, a risk of infection was considered possible only for root canal instrumentation, during which endodontic files come into close contact with nervous tissue.

8.2.1 Preparation of Cavities

The described expression of PrP in odontoblasts, cementoblasts and ERM relocates the frontier of PrP positive tissue to a more distal position than before. Even burrs coming into contact with odontoblast processes can come into contact with PrP expressing cells.

8.2.2 Subgingival Scaling

Treatment of periodontal disease comprises subgingival scaling, which serves to remove subgingival calculus from the root cementum surface. During this procedure, the instruments (scaler tips and curettes) come in close contact with the cementoblasts, which were demonstrated in this study to be PrP positive.

8.2.3 Validation of Risk of Transmission

As for CWD, no one knows if transmission of TSEs through odontoblasts, cementoblasts or ERM is a real menace for patients (during treatment), dental practitioners and/or their staff (during handling and cleansing of the instruments). However, considering the number of cavity fillings and subgingival scalings performed each year, even a tiny chance for entrance (through these cells) or translocation (through the used instruments) of infective material should not be neglected.

9 Conclusions

RT-PCR experiments and electron microscopical as well as immunohistochemical findings suggest an important role of PrP during dentinogenesis. Human pulpal cells, cells from the periodontal ligament and ectomesenchymal cells were demonstrated to contain PrP mRNA. Subsequent immunohistochemical analyses could identify the specific cells expressing PrP. We could not only

demonstrate that nerve fibers of the dentin-pulp complex and the periodontium contain PrP, but also cells engaged in biomineralization. Bodies (but not processes) of odontoblasts, cementoblasts and epithelial rests of Malassez express PrP. While this might suggest a cytoprotective task for PrP, the absence of this protein leads, as expected, to obvious defects during dentinogenesis.

Part VI

Perspective

There are certainly many paths on which PrP's role in the mammalian dentition could further be elucidated. Three of them, which directly result from the findings in this thesis, are presented below.

10 Future Studies

10.1 Search for PrP's Interaction Partners Using Bioinformatics

Possible interaction partners of PrP could be searched for by applying methods from bioinformatics. During the verification of PrP's expression in dental tissues using PCR, we also analyzed the mRNA levels of a hundred cytokines which were suspected to be involved in dentinogenesis [175]. Each of them is a potential interaction partner of PrP. By using docking programs like ftdock[1], Hex [251][2], PTools [260] or any other protein-protein docking tool, it might be possible to detect one or more interaction partners. The problem, however, would be the validation of the results. If PrP and any other protein turn out to free up a great amount of energy during docking *in silico*, this is still no proof that they are interaction partners *in vivo*. Verification of the bioinformatics results would be left to *in vitro* experiments.

10.2 Determination of PrP's Temporal Expression Pattern

Expression of PrP^C in permanent human teeth has been demonstrated in this study. It would be interesting to learn when expression of PrP is turned on during embryogenesis. To this end, animal experiments could be used to establish a time scale for PrP^C expression. As dentinogenesis starts long

[1] Homepage at `http://www.bmm.icnet.uk/docking/`

[2] Homepage at `http://www.csd.abdn.ac.uk/hex/`

before teeth start to erupt, a first study to narrow down the most promising time span could focus on the perinatal period. While rodents turned out to be poor candidates due to cross-reactivity of the antibodies, first experiments with cats were promising.

10.3 Personal and Environmental Factors Influencing PrP's Expression

It is unknown whether PrP expression is influenced by individual circumstances (age, gender) or any factors involving dental health. Therefore, human teeth extracted for orthodontic or other reasons could be used to establish possible correlations between the expression of PrP and habits, circumstances and situations. Factors which could be compared in this study include, besides others:

- deciduous vs. permanent teeth
- teeth of young vs. old people
- teeth of male vs. female persons
- incisors vs. premolars vs. molars
- periodontitis vs. no periodontitis
- sound vs. carious teeth
- teeth with vs. without restorations

11 Concluding Remarks

None of these studies is sure to deliver the desired answers to the question what physiological function PrP^C has. Maybe no interaction partner of PrP^C is identified, maybe PrP^C expression turns out not to be associated with any

events known to be important in dentinogenesis and no correlation between PrP^C expression and the factors mentioned above is found.

But after all, mother nature teaches us that she is parsimonious. Thus, we might well expect this protein not to be expressed in dental tissues if there were no function for PrP^C in teeth.

Due to the fact that nobody has detected a vital role for PrP^C in the body, let alone in teeth, the range of upcoming elucidations might reach from "still no finding (yet)" to another contribution to the technique of how to make a third generation of teeth grow during the lifespan of a mammal [142].

Part VII

Appendix

12 Tables

Disease	Affected	Predominant distribution	First citation	Important citation(s)
Scrapie	sheep, goats	worldwide	1750	[75, 182, 204, 220]
fCJD	man	worldwide	1920/21	[79, 146]
sCJD	man	worldwide	1920/21	[79, 146]
GSS	man	worldwide	1936	[108]
Kuru	man	New Guinea	1957	[105, 343]
TME	mink	USA	1965	[60, 126]
iCJD	man	worldwide	1974	[31, 94, 193]
CWD	cervids	USA	1980	[331, 333]
FFI	man	worldwide	1986	[187]
BSE	cattle	United Kingdom	1987	[317, 328]
EUE	kudu, nyala, oryx, eland	United Kingdom	1988	[100, 148, 156, 157, 158]
FSE	felidae	United Kingdom	1990	[181, 226, 337, 338]
vCJD	man	United Kingdom	1995/96	[25, 40, 71, 329]
sFI	man	worldwide?	1997	[5, 194, 195, 219, 237]

Table 1: Spongiform encephalopathies of mammals and man. Citations in part according to [137].

Treatment	Temperature	Duration
1.5 % NaOCl with ultrasonic	room temp.	10 min
1.5 % NaOCl without ultrasonic	room temp.	1 hour
0.1 % trypsin without ultrasonic	37°C	24 hours

Table 2: Solutions for treatment of SEM specimens

	wet wt mg	**dry wt mg**	**copper mg/kg dry wt**	**manganese mg/kg dry wt**
tga20, Sample A	47,0	44,7	1,34	33,1
tga20, Sample B	51,5	49,7	2,01	32,0
C57-129Sv, Sample A	67,3	63,1	1,58	21,0
C57-129Sv, Sample B	63,2	58,1	1,29	24,7
PrP$^{0/0}$, Sample A	57,8	53,1	0,94	26,4
PrP$^{0/0}$, Sample B	55,2	51,2	0,97	27,8

Table 3: Copper and Manganese contents in murine molars. Limits of detection: Copper: 0,4 mg/kg, Manganese: 0,5 mg/kg

	tga20	**PrP$^{+/+}$**	**PrP$^{+/0}$**	**PrP$^{0/0}$**
mean area of dentin tubules in μm^2	5.64 ± 0.224	7.03 ± 0.518	7.08 ± 0.738	8.93 ± 0.541
mean count of dentin tubules per 10^4 μm^2	543.46 ± 18.06	481.63 ± 16.7	510.87 ± 32.74	381.71 ± 22.78

Table 4: Mean area and mean count of dentin tubules in mice of different expression levels of PrP. While the area of the tubuli correlated inversely with the expression level of PrP, the number of tubuli correlated positively with the level of PrP

Tissue	**Cu content in ppm**	**Citation(s)**
human deciduous teeth without filling	0.20 – 0.64	[18]
human deciduous teeth without filling	1.08 ± 0.81	[61]
carious human deciduous teeth's crowns	1.56 ± 0.21	[110]
carious human deciduous teeth's roots	2.01 ± 0.41	[110]
1 (!) human deciduous tooth	≈20	[269]
human permanent teeth without filling	0.20 – 4.40	[18]
human permanent teeth without filling	6.14 – 22.7	[176]
carious human permanent teeth's crowns	3.2 ± 0.31	[110]
carious human permanent teeth's roots	2.56 ± 0.32	[110]
human permanent teeth	0.1 – 6.0	[72]
6 human permanent teeth	<20	[269]
human cariesless teeth	3.76 – 11.69	[93]
human carious teeth	2.67 – 9.93	[93]
human teeth (age $\leq$ 25 yr)	3.76 – 9.51	[93]
human permanent teeth (age > 50 yr)	4.01 – 11.69	[93]
human teeth without filling	0.7 ± 0.3	[196]
human teeth with amalgam filling	24.8 ± 6.8	[196]
human (?) teeth	10 – 100	[299]

Table 5: Values for the copper content of teeth in the literature

Tissue	Cu content in ppm	Citation(s)
human enamel	5 – 30	[55]
human enamel	0.4 – 3.9	[177]
human (?) enamel	970 – 4820	[161]
outer human enamel	9.5 ± 7.8	[211]
inner human enamel	11.3 ± 9.0	[211]
human enamel (aged 10–12 years)	11.5 ± 2.0	[90]
human enamel (aged 13–16 years)	14.4 ± 1.8	[90]
human enamel (aged 17–24 years)	10.1 ± 1.9	[90]
human enamel (aged 25 and over)	11.7 ± 1.9	[90]
human dentin	0.5 – 10.9	[177]
human (?) dentin	1120 – 1840	[161]
human dentin (aged 10–12 years)	7.5 ± 1.3	[90]
human dentin (aged 13–16 years)	7.8 ± 1.2	[90]
human dentin (aged 17–24 years)	5.8 ± 1.2	[90]
human dentin (aged 25 and over)	7.4 ± 1.2	[90]
rat teeth	1.16	[210]
rat teeth	8.75 ± 0.45	[247]
cattle tooth	12	[299]
bank vole molars (min. value)	3.79 ± 0.34	[15]
bank vole molars (max. value)	15.5 ± 3.8	[15]
C57-129Sv mouse molars	1.435 ± 0.21	this study
human supragingival calculus	4	[84]
miniature pigs' calculus	10	[84]
ovine dental deposits	17	[84]

Table 6: Values for the copper content of teeth in the literature (cont.)

13 Software

Operating System: S.u.S.E. Linux 11.1 (S.u.S.E. Linux, Nuremberg, Germany[3])

Text Processing: The text was prepared with the TEX typesetting system [159, 160][4] using the LATEX extension [113, 163, 174]

- the text on page 14 was typeset with the oldgerm and dropcaps packages[5]
- Photographs (like gele photographs) were included as Encapsulated PostScript files [114]
- Literature management was done with the BIBTEX package[6]

Literature, Genome and Nucleotide Searches: these searches were done using the PubMed site[7]

Primers: PCR primers were picked using the program Primer3[8]

Chromatograms: Output of sequencing machines was interpreted using the program FinchTV for Linux (Geospiza [106])

Image Processing: Images were processed with ImageJ[9]

Statistics: Statistical analyses were performed with the R-project Software for Statistical Computing [295], and with SPSS V8.0

[3] `http://www.novell.com/linux/suse`
[4] `http://www.ctan.org`, `http://www.tug.org`
[5] `http://www.ctan.org`, `http://www.tug.org`
[6] `http://www.ctan.org`, `http://www.tug.org`, `http://www.literateprogramming.com/btxdoc.pdf`
[7] `http://www.pubmed.org`
[8] `http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi`
[9] `http://rsbweb.nih.gov/ij` and `http://rsb.info.nih.gov/ij`

Part VIII

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