

***N*-methyl-*N*-nitrosourea-induced changes in epithelial rests of Malassez and the development of odontomas in rats**

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Received February 9, 2012; Accepted April 4, 2012

DOI: 10.3892/etm.2012.559

Abstract. Morphological changes in the epithelial rests of Malassez (ERM) and the development of odontogenic tumors in the molars of female Lewis rats treated at 4 weeks of age with a single intraperitoneal injection of 50 mg/kg of *N*-methyl-*N*-nitrosourea (MNU) were examined at 12, 18 and 30 weeks of age. Following MNU exposure, the total number and average area of ERM in the cervical and furcational regions of the first, second and third molars of the mandible and maxilla were compared with age-matched control animals. The number of ERM at each time point was significantly greater in the MNU-treated group compared to the control group, but there was no time-dependent increase in the number of ERM in either group. The area of ERM was significantly larger in the MNU-treated group compared to the control group at each time point, and it increased in a time-dependent manner in the MNU-treated group. No increases in the number or area of ERM were observed in the control group. At 30 weeks of age, 23% of the MNU-treated rats had developed odontomas (complex type) in the molar region as well as in the incisor region. Immunohistochemically, the expression of tyrosine receptor kinase A (TrkA) and cytokeratin 14 (CK14) decreased, whereas p63 expression remained high during ERM enlargement. In tumors, ameloblast-like cells were positive for amelogenin, TrkA and CK14 but negative for p63, whereas odontoblast-like cells were negative for all antigens examined. In conclusion, a single intraperitoneal injection of MNU

caused the development of odontomas in the molar region; these tumors were possibly derived from ERM.

Introduction

Rodent teeth are sensitive to chemicals and are good models for the evaluation of chemically induced effects in humans. However, their biological features, particularly those of the incisors, are quite different from those of human teeth (1,2). Rats have 16 teeth, comprising an incisor and three molars in each quadrant. Rodent incisors grow, calcify and erupt continuously throughout life. A longitudinal section shows the complete life cycle of tooth development from inception to maturity (3,4). The frequency of odontogenic tumors is low in rodents (5) and humans. The odontogenic epithelium is responsible for tooth development under physiological conditions and can give rise to tumors and cysts in the jaw (6,7). Therefore, tumors derived from the perpetually erupting ameloblasts of the rodent incisor may differ from those in human adults. The chemical compounds that cause morphological changes in rodent teeth include various antitumor or DNA-alkylating agents, such as 5-fluorouracil, doxorubicin, mitomycin C, vinblastine sulphate, docetaxel, irinotecan hydrochloride, cisplatin and nitrosoureas. These compounds cause a variety of dental lesions in the incisors and/or molars (5,8-10). Among these chemicals, *N*-ethyl-*N*-nitrosourea (ENU) and *N*-methyl-*N*-nitrosourea (MNU) have deleterious effects on odontogenic tissues, resulting in tooth deformation and malocclusion and eventually odontogenic tumors in rats (9,11-17) and hamsters (8,15,18-20).

Epithelial rests of Malassez (ERM) were first described by Malassez in 1884. In studies of human teeth, Malassez noted that ERM form a network around the tooth root and that the number of ERM decreases with age (21). ERM are quiescent epithelial remnants of Hertwig's epithelial root sheath that remain in the periodontal ligament throughout life (22-24); the exact function of these structures has not been clarified. ERM subcultured with primary dental pulp cells can differentiate into ameloblast-like cells and generate enamel-like and/or dentin-like tissues (25). ERM have the potential to proliferate

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Key words: amelogenin, cytokeratin 14, epithelial rests of Malassez, *N*-methyl-*N*-nitrosourea, odontogenic tumor, p63, tyrosine receptor kinase A

in response to appropriate extracellular signals such as mitogens, and the proliferation of ERM may lead to the formation of odontogenic tumors or cysts (26,27). However, the changes that occur in ERM during the process of odontogenic tumor development have not been closely examined. The present study focuses on the changes in ERM during the development of odontomas in the molar region and the morphological and immunohistochemical characterization of these lesions induced by a single intraperitoneal injection of MNU into female Lewis rats.

Materials and methods

Animals. A total of 43 3-week-old female Lewis rats [LEW/CrlCrJ] were purchased from Charles River Japan (Atsugi, Japan). All rats were housed 4-5 in a plastic cage with paper bedding (Paper Clean, SLC, Hamamatsu, Japan) in a temperature- (22±2°C) and humidity- (60±10%) controlled animal room under a 12-h light/dark cycle.

Experimental procedures. After an acclimatization period of 1 week, rats were divided into two groups. A total of 25 rats received an intraperitoneal injection of 50 mg/kg MNU (Sigma, St. Louis, MO, USA). MNU was dissolved in physiologic saline containing 0.05% acetic acid immediately prior to injection. The control group consisted of 18 rats that were injected with vehicle only (physiologic saline containing 0.05% acetic acid). All rats were fed a commercial pellet diet (CMF 30 kGy, Oriental Yeast, Chiba, Japan) and had *ad libitum* access to water throughout the experiment. During the experiments, clinical signs were observed once a day, and body weight was measured once a week. At 12 and 18 weeks of age, 5 randomly selected rats in the control group and 6 in the MNU-treated group were sacrificed. The remaining rats in the control group (n=8) and the MNU-treated group (n=13) were sacrificed at 30 weeks of age. Complete necropsies were conducted on all animals. All procedures involving animals were approved by the Animal Experimentation Committee of Kansai Medical University.

Tissue sampling. The rats were anesthetized with isoflurane (Forane®; Abbot Japan, Tokyo, Japan), and the left and right sides of the mandible and maxilla were separately dissected from the surrounding tissues. The collected tissue was immersed in 10% neutral buffered formalin for one week and demineralized in a 10% EDTA solution (pH 7.0-7.3; Osteosoft®, Merk KGaA, Darmstadt, Germany) at room temperature for 4-6 weeks. The samples were then dehydrated with graded ethanol and embedded in paraffin. Sagittal sections were cut in the mesiodistal direction and included the first, second and third molars and the incisors, as previously described (28). Sections were stained with hematoxylin and eosin (H&E) or used for immunohistochemistry. ERM of the first, second, and third molars and the induced tumors were histopathologically evaluated. Histopathological and immunohistochemical evaluations were reviewed by a toxicologic pathologist certified by the Japanese Society of Toxicologic Pathology and/or by the International Academy of Toxicologic Pathology (K.Y. and A.T.), according to the previously defined histopathological terminology and diagnostic criteria (2,29,30).

Immunohistochemistry. The labeled streptavidin biotin (LSAB) technique was performed with an LSAB staining kit (Dako, Carpinteria, CA, USA). The following antibodies were used: rabbit anti-tyrosine receptor kinase A (TrkA) polyclonal antibody (sc-118, 1:50 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a nerve growth factor receptor in periodontal ligament epithelium (31,32); mouse anti-human CK14 monoclonal antibody (clone LL002, 1:20 dilution; Leica, Microsystems, Wetzlar, Germany) as a basal cell keratin marker (25); mouse anti-human p63 monoclonal antibody (clone 4A4, 1:50 dilution; Dako, Glostrup, Denmark) as an epithelial stem cell marker in oral tissue (33-38); and rabbit anti-porcine amelogenin polyclonal antibody (raised by T.U., 1:5000 dilution) (39,40) as an ameloblast marker (41,42). Each primary antiserum or antibody was incubated overnight at 4°C without antigen retrieval. The reaction products were visualized using 3-3'-diaminobenzidine tetrahydrochloride.

Morphometric analysis. H&E-stained sections of the jaw were scanned with a high-resolution digital slide scanner (NanoZoomer 2.2 Digital Pathology, Hamamatsu Photonics, Hamamatsu, Japan) to prepare digital images. The ndpi image files were opened in color mode with NDP.view software (Hamamatsu Photonics), and the area of ERM was measured in both sides of the maxillary and mandibular jaws; the number of ERM was counted in the cervical and furcation regions of the first, second, and third molars of the mandible and maxilla. An experimental dentist (A.K.) performed morphometric analysis using Image J Windows software (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. All discrete values are expressed as mean ± standard error (SE) and were analyzed with the two-tailed independent Student's t-test for unpaired samples after confirming the homogeneity of variance. The statistical analysis was used to examine the significance of differences in the number and the area of ERM between MNU-treated rats and control rats at each time point and between different time points. P-values <0.05 were considered to indicate statistical significance.

Results

General remarks. None of the animals died during the study period. Body weight gain was lower in the MNU-treated rats than in the control rats (data not shown). By the age of 30 weeks, the MNU-treated rats developed mammary tumors (data not shown).

Morphological analysis of proliferative changes in molars. In the MNU-untreated control rats, ERM were observed in the cervical and furcation regions of the first, second, and third mandibular and maxillary molars and were characterized by a high nuclear/cytoplasmic ratio and condensed nuclei (Fig. 1a). Moreover, the size of the ERM remained small, and there were no neoplastic changes in the periodontal ligament at any time point. The ERM in MNU-treated animals at 12, 18, and 30 weeks of age had similar morphology to the ERM in control rats, but the size of the ERM gradually increased after MNU treatment (Fig. 1b). No odontogenic tumors were

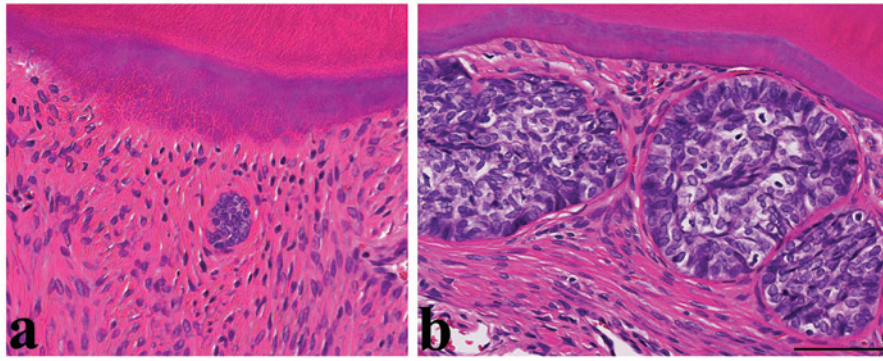


Figure 1. Epithelial rests of Malassez (ERM). (a) Small ERM composed of cells with high nuclear-cytoplasmic ratios are found in a 30-week-old control rat. (b) ERM in a 30-week-old MNU-treated rat are large, however, the morphology is similar to that of ERM in the control rat. H&E staining; bar, 100 μm .

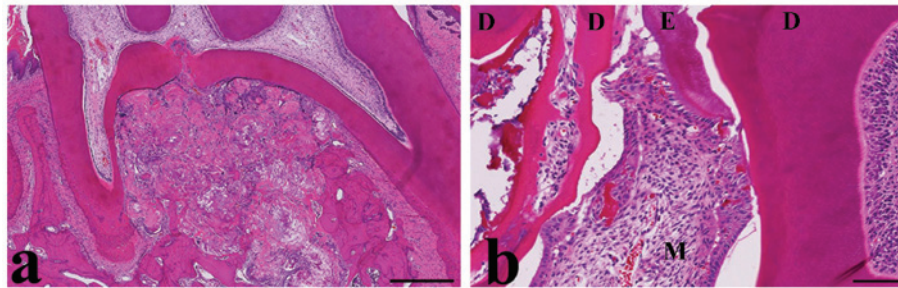


Figure 2. An *N*-methyl-*N*-nitrosourea (MNU)-induced odontogenic tumor. (a) The tumor is located in the furcational region of the second right maxillary molar. H&E staining, bar, 500 μm . (b) The tumor is composed of (D) dentin-like and (E) enamel-like tissues together with (M) dental pulp-like mesenchymal cells, suggestive of a complex odontoma. H&E staining; bar, 100 μm .

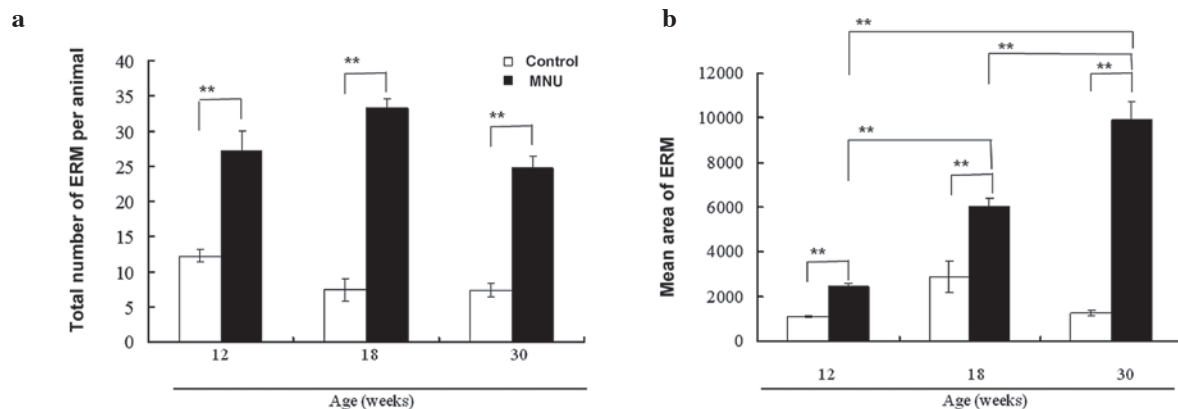


Figure 3. Time-course progression of the number and area of epithelial rests of Malassez (ERM) in the mandibular jaws of control and *N*-methyl-*N*-nitrosourea (MNU)-treated rats. (a) Total number of ERM per rat was significantly higher in the MNU-treated animals than in the control animals at each time point, but the number did not increase in a time-dependent manner. (b) Mean area (μm^2) of ERM was significantly higher in the MNU-treated animals at each time point, and the area increased in a time-dependent manner. Means \pm SE are shown. ** $P < 0.01$.

detected at 12 or 18 weeks of age. At 30 weeks of age, odontogenic tumors were found in three of 13 MNU-treated rats (23% incidence). These tumors were located near the third mandibular molars. Two odontogenic tumors appeared in the cervical and furcation regions, indicating molar origin (Fig. 2a). These tumors contained a mixture of ameloblast-like cells with enamel-like tissue, odontoblast-like cells with multinucleated giant cells and dentin-like tissue, and dental pulp-like mesenchymal cells (Fig. 2b). The third

tumor had similar morphology. It was difficult to determine the origin of this tumor, but it was located at the base of the incisor, indicative of incisor origin. The morphology of the three MNU-induced tumors was indicative of odontoma (complex type).

Morphometrical analysis of epithelial rests of Malassez (ERM). The number of ERM per rat remained low in control rats (12.2, 7.4, and 7.3 at 12, 18, and 30 weeks, respectively), but

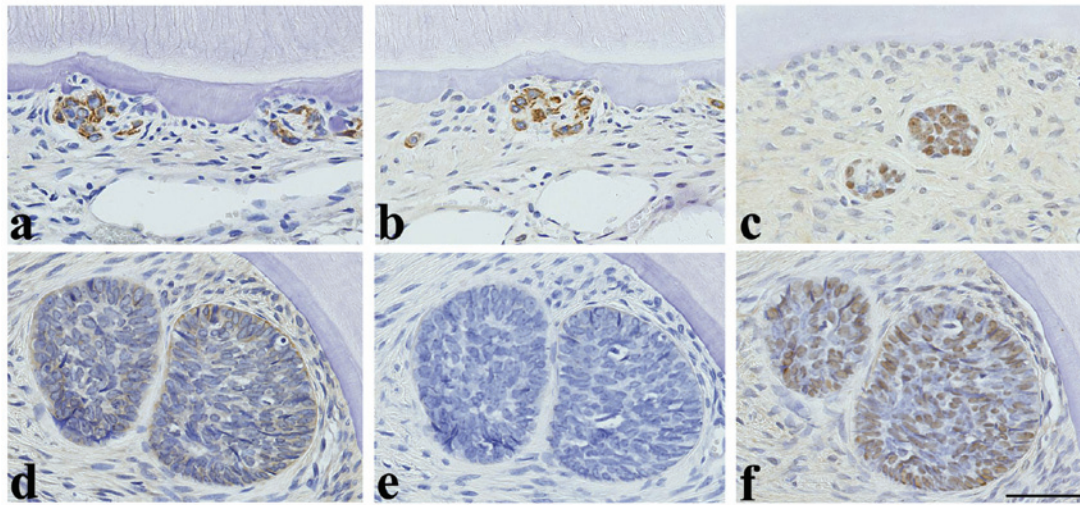


Figure 4. Immunoreactivities of epithelial rests of Malassez (ERM) in 30-week-old rats. (a and d) Tyrosine receptor kinase A (TrkA) immunohistochemistry in the control group and the *N*-methyl-*N*-nitrosourea (MNU)-treated group, respectively. (b and e) Cytokeratin 14 (CK14) immunohistochemistry in the control group and the MNU-treated group, respectively. (c and f) p63 immunohistochemistry in the control group and the MNU-treated group, respectively. Bar, 100 μ m.

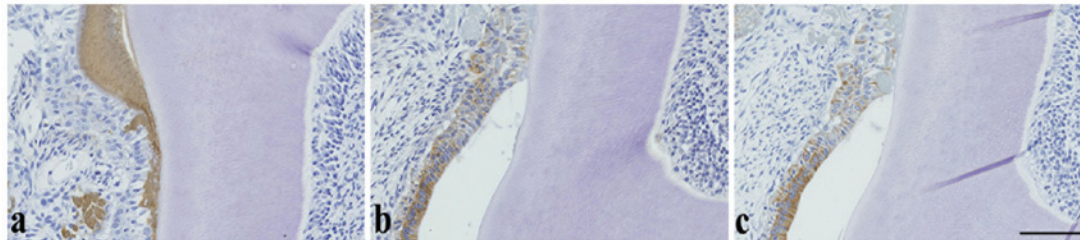


Figure 5. Immunoreactivities of odontogenic tumors induced by *N*-methyl-*N*-nitrosourea (MNU). (a) Amelogenin expression is observed in the cytoplasm of ameloblasts and secretory ameloblasts with tall columnar or cuboidal-shaped cells and in enamel-like tissue. (b) Tyrosine receptor kinase A (TrkA) expression is observed in the cytoplasm of ameloblasts and secretory ameloblasts with tall columnar or cuboidal-shaped cells. (c) Cytokeratin 14 (CK14) expression is observed in the cytoplasm of ameloblasts and secretory ameloblasts with tall columnar or cuboidal-shaped cells. Bar, 100 μ m.

was significantly higher in the MNU-treated rats at each time point (27.2, 33.2, and 24.7 at 12, 18, and 30 weeks, respectively) (Fig. 3a). Although there was no time-dependent increase in the number of ERM in the control or MNU-treated groups, MNU-treated rats had significantly larger ERM as compared with controls, and there was a time-dependent increase in the mean area of ERM (Fig. 3b) (1108, 2878, and 1268 μ m² in control groups vs. 2428, 6061, and 9930 μ m² in MNU-treated groups at 12, 18, and 30 weeks, respectively). This discrepancy between the number and area of ERM may be due to the fusion of enlarged ERM.

Immunohistochemical analysis of proliferative changes in molars. Regardless of MNU treatment, TrkA (Fig. 4a) and CK14 (Fig. 4b) immunoreactivity was observed in the cell cytoplasm and p63 immunoreactivity was observed in the cell nuclei (Fig. 4c) of the normal or relatively small ERM in the cervical and furcation regions of the molars, whereas amelogenin was negative (figure not shown). However, in the larger ERM observed following MNU treatment, TrkA and CK14 expression decreased or disappeared (Fig. 4d and 4e), whereas p63 expression (Fig. 4f) remained constantly high and amelogenin remained negative (figure not shown). In tumors, the expression of amelogenin (Fig. 5a), TrkA (Fig. 5b), and

CK14 (Fig. 5c) was detected in the cytoplasm of ameloblast-like cells and cells with tall columnar or cuboidal-shaped cells (secretory ameloblasts). Amelogenin was also expressed in enamel-like tissue, whereas p63 was negative. None of the antigens examined were expressed in odontoblast-like cells, dentin-like tissue or dental pulp-like mesenchymal cells.

Discussion

The present study examines changes in ERM as a precursor to odontogenic tumor development in the molar region following a single intraperitoneal injection of MNU into female Lewis rats. The number of ERM in MNU-treated rats was significantly higher at all time points as compared to control rats. The area of ERM in the MNU-treated rats was significantly larger than in the control rats, and it increased in a time-dependent manner. Finally, odontomas were observed in the molar and incisor regions in MNU-treated rats at 30 weeks of age.

Herrold was the first to induce odontogenic tumors in animals by exposure to MNU (18). Morphologically, MNU-induced tumors in Syrian hamsters resembled human ameloblastomas. Rats were later found to be susceptible to MNU-induced odontogenic tumors (15). MNU-induced odontogenic tumors in rodents are derived from the ameloblasts of

the continuously erupting incisors, which is located adjacent to the root of the incisors (12,14,17,26). A single injection of 150 mg/kg MNU causes death in approximately one third of rats in three weeks. Younger rats (6 weeks old or younger) were more susceptible to MNU than older rats (8 weeks old), and the susceptibility to the development of odontogenic lesions appears to end at 8 weeks of age (17). Local and multiple injections of ENU, a chemical closely related to MNU, coupled with mechanical injuries (incisor wounding) were found to accelerate the production of odontogenic tumors in the incisor region (9), which indicates that promoter stimuli may be required for a high yield of odontogenic tumors. Odontogenic tumors in the molar region occur in the cervical and furcation regions (but not in the apical region) where ERM always remain as a result of local administration of MNU mixed with alginate impression material to preserve MNU at the treatment site (26). Due to the similarity in locations of ERM and tumors, enlarged ERM may have acquired neoplastic characteristics. In contrast to tumors derived from ameloblasts of incisors, tumors derived from ERM of multi-rooted rat molars are similar to human tumors. However, to the best of our knowledge, it has not been previously reported that a single systemic administration of MNU causes odontogenic tumors in the molar region. In the present study, odontogenic tumors developed in the molar regions of female Lewis rats treated with MNU.

In general, odontogenic tumors are rarely encountered as spontaneous lesions in rodents (1,5,43). In Tg.AC mice, a mouse line created in the FVB/N background by pronuclear injection of v-Ha-*ras* oncogene, the incidences of odontogenic tumors (odontoma) were 16% in a 26-week study and 35% in a 1-year study (5,44). In other transgenic mice, such as Hedgehog transcriptional effector Gli2-overexpressed mice, keratocystic-type odontogenic tumors arise from proliferative ERM (45). In *Smad4*-gene knockout mice (*Smad4*^{Co/Co} OC-*Cre*), a similar type of odontogenic tumor arises from proliferative ERM (46). In the present study, the incidence of odontogenic tumors was 23% at the age of 30 weeks, which is compatible with the incidence in Tg.AC mice.

Several genetic and molecular alterations appear to promote the development and progression of odontogenic tumors via multiple steps associated with tooth development, bone metabolism, and the malignant potential of tumors (6). In an *in vitro* study, ERM in combination with dental pulp cells generate enamel-like or dentin-like tissues in a similar manner to cervical loop epithelial cells (25). ERM have characteristics of stem/progenitor cells (36). Morphological continuity exists between ERM and the induced tumors in rats treated with local administration of MNU (26). TrkA (47), CK14 (25), and p63 (48) are markers for ERM in the periodontal ligament. p63 is a candidate epithelial stem cell marker in oral tissues (33-38). TrkA, CK14 and p63 were constantly expressed in small ERM of control rats, although TrkA and CK14 expression tended to decrease and disappear, respectively, in the larger ERM of MNU-treated rats. p63 was consistently detected in ERM of various sizes. However, ERM of various sizes were negative for amelogenin (25,49). In the present study, the expression of TrkA and CK14 in large ERM was different from that in normal-sized ERM. The decreased levels of expression may corroborate with the change at the early transition or dedifferentiation stage of odontogenic carcinogenesis.

MNU-induced tumors were diagnosed as odontomas (complex type), since they contained dentin-like, enamel-like, and dental pulp-like mesenchymal cells (2,30). Amelogenin is not detected in ERM (49), but it is detected in the cytoplasm of the tall columnar odontogenic epithelium, stellate reticulum-like cells, and their associated extracellular components together with enamel-like tissue in epithelial odontogenic tumors (50). In the present study, amelogenin was detected in the enamel-like matrix, in secretory ameloblasts adjacent to enamel-like matrix, and in cells assumed to be ameloblasts, but not in dentin-like or dental pulp-like mesenchymal cells. Amelogenin-CK14 interactions in ameloblasts occur during enamel formation (51) and cultured ERM express amelogenin and CK14 (25). Epithelial odontogenic tumors express various degrees of amelogenin and CK14 (50,52). Co-expression of amelogenin and CK14 may be a characteristic of epithelial odontogenic tumors, indicating that these tumors have ameloblastic differentiation or odontogenic epithelial properties. In addition, TrkA was expressed in tumor cells with ameloblastic differentiation, although the reasons for this are unclear. p63 is essential for tooth development (53) and is expressed in dental germ cells (35), ameloblasts (37), and various odontogenic tumors including ameloblastomas (48,54). Although p63 was consistently detected in ERM of various sizes, the compound odontomas in the present study were p63-negative. p63 expression correlates to malignancy in odontogenic tumors (48). In conclusion, a single intraperitoneal injection of MNU resulted in the development of odontogenic tumors with mixed enamel and dentin differentiation (complex odontomas) in the molar region, and these tumors may have been derived from ERM. Detailed mechanistic investigations of the molecular basis of odontogenic carcinogenesis are required to further elucidate the relationship between ERM and odontogenic tumors in rodents and humans.

Acknowledgements

The authors thank Ms. T. Akamatsu for her technical assistance and Ms. A. Shudo for manuscript preparation. This work was supported in part by a grant-in-aid for Scientific Research (C) (22591954).

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