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## **Androgen Receptor Involvement in Rat Amelogenesis: An Additional Way for Endocrine-Disrupting Chemicals to Affect Enamel Synthesis**

Katia Jedeon, Sophia Loiodice, Khaled Salhi, Manon Le Normand, Sophia Houari, Jessica Chaloyard, Ariane Berdal, Sylvie Babajko

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# Endocrinology

## Androgen receptor involvement in rat amelogenesis: an additional way for endocrine disrupting chemicals to affect enamel synthesis

--Manuscript Draft--

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<p>SPECIAL REQUESTS:</p> <p>Enter specific comments or requests to the editors here. Enter "none" if there aren't any.</p>	<p>Dear Dr. Andrea C. Gore, Editor-in Chief of Endocrinology, Dear Dr. Gail Prins, Associated Editor of Endocrinology,</p> <p>Thank you for favorably considering our paper. We are grateful for the reviewers feedback and have carefully considered all their remarks and comments which have helped to improve the paper.</p> <p>Please find attached the revised form of our paper EN-16-1342R1 entitled Androgen receptor involvement in rat amelogenesis: an additional way for endocrine disrupting chemicals to affect enamel synthesis, by K Jedeon, S Liodice, et al. All the modifications brought to the revised version of the paper were highlighted in yellow. The paragraphs that we added to this new version (requested by Reviewer 2) required 7 additional references (the total number of references is now 48 in the revised version of the manuscript). In addition, we made a minor correction to the figure 1C and to the corresponding legend, with the stratum intermedium (SI) for the secretion stage and the papillary layer (PL) for the maturation stage (Figure 1 new).</p> <p>We replied to each point raised by the reviewers (Point-by Point Rebuttal). The IRB approval for animal studies (requested by Reviewer 1) was also included at the end of the downloading process.</p> <p>All authors have read the manuscript, agree that the work is ready for submission, and accept responsibility for the manuscript. The authors declare no competing financial interests, nor any conflict of interest.</p> <p>We look forward to hearing from you in due course. Sincerely, Sylvie Babajko, Ph.D</p>
<p>EDITOR-IN-CHIEF SELECTION:</p>	<p>Dr. Andrea Gore</p>

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**Requested Editor:**

We are grateful for the reviewers' feedback and have carefully considered all their remarks and comments which have helped to improve the paper.

Please find below our detailed response to each point raised by the reviewers, and modifications made in light of the referees' comments.

#### Replies to the Reviewer 1:

We thank the referee for his comments and we address the issue raised below:

Reviewer #1: Jadeon et al investigates the role of EDCs and androgens in the regulation of amelogenesis through targeting the androgen receptor. The study build upon previous research in this area while still clearer extending upon this work in novel ways. The combination of animal models with cell lines to dissect effects of both androgens and several key EDCs (vinclozolin and BPA) is a strength. The studies are well controlled and comprehensive in testing the role of AR (localization, mRNA and protein expression, activation, use of flutamide) in regulation of enamel genes (KLK4) . Overall this paper provides insight into mechanisms of EDC targeting AR function in a unique tissue. This raises an important insight into potential impact of EDCs and androgens on enamel mineralization that has potential relevance to other species

Institutional IRB approval for animal studies needs to be confirmed.

Institutional IRB approval for animal studies is attached, and the current number (A75-06-12) of agreement inserted in the revised version of the manuscript.

#### Replies to the Reviewer 2:

We thank the referee for his useful comments which have helped to improve the paper. We address each issue (in the same order as the reviewer) raised below (pages were annotated according the first version and all corrections made in the revised version are highlighted in yellow).

Reviewer #2: EN-16-1342Androgen receptor involvement in rat amelogenesis: an additional way for endocrine disrupting chemicals to affect enamel synthesis.

This manuscript convincingly describes a role for androgen receptors in regulating enamel synthesis in dental epithelium during development in male rats. The study will be of interest to readers of Endocrinology in that it provides a plausible mechanistic explanation for observed sex differences in tooth development and related pathologies in humans; suggests that exposure to EDCs in the natural environment could explain individual and population differences in abnormal dentition (specifically, Molar Incisor Hypomineralization); and could in future provide a novel biomarker of early developmental exposure to EDCs in humans and animals.

For the most part, the study is methodologically sound and the manuscript is concise and clear. However, considering that endocrinologists, for the most part, are not familiar with dental development and differentiation, the paper would benefit from a brief basic description of the

processes of dental development and, specifically, amelogenesis and enamel mineralization. As they are using rats, but cite human studies and allude to human health implications and sex differences, it is necessary to compare and contrast the developmental time course of ameloblast differentiation in rats and humans and what is known about male-female differences in both groups. This information could be added to a reorganization of the introduction (and discussion) and used to segue into their earlier research findings, unanswered scientific questions addressed in the present study, and their main findings here.

A detailed paragraph on amelogenesis and enamel mineralization was added to the introduction part of the revised version of the manuscript, as well as a comparison between rodent and human amelogenesis and susceptibility to EDCs. The introduction part of the paper has been reorganized: the same ideas as those of the first version are developed with more details and in a different sequence.

The differences between male and female amelogenesis haven't been really investigated until now considering that amelogenesis occurs before pubertal increase of estrogens and testosterone (omitting the perinatal high level of testosterone (in males) because neither the expression of sex steroid receptors nor the responsiveness to steroids by the ameloblasts (or any dental epithelial cells) have ever been reported).

Additionally, in Methods, it is important for the authors to describe, briefly at least, the procedure used for microdissection of the oral epithelium and how the procedure was validated...presumably in their earlier studies. Validation is important for interpreting their earlier and present results. It should not be necessary for the reader to search out the earlier paper to obtain critical methodological information.

The procedure used for microdissection of the oral epithelium and its validation was added to the corresponding paragraph in the Materials and Methods.

Specific questions/comments:

1. p. 4. "MIH essentially affects the teeth that mineralize from the third trimester of fetal life to four-five years after birth, which exactly corresponds to the window of the highest susceptibility to EDCs" Reference this statement. Do rats display MIH? Is the critical period known? Surely, this information is needed to justify use of rats as animal model for the condition.

Our previous study (Jedeon et al., 2013 = reference 12) showed that the first mineralizing incisor of rats at 30 days of age was affected when rats were exposed to low-dose BPA from the beginning of the fetal life whereas the rats at 100 days of age didn't present any enamel defects clinically observable. This situation in rats is comparable to human MIH which also affects the first mineralizing teeth (essentially first permanent molars and permanent incisors). Interestingly, both series of teeth mineralize during the period of time of the highest sensitivity to xenobiotics:

In rats, glucuronidate-BPA conjugates produced in the maternal circulation can cross the placenta and can be deconjugated back to active BPA in the fetus (Nishikawa et al., 2010 = ref 15). The fact that this, together with the fact that UGT2B1 glucuronidase activity against xenoestrogens is undetectable in rat fetus and in the neonate, and only reaches adult levels by day 21 (Matsumoto et al., 2002 = ref 16) indicate that the risk of BPA exposure is greatest in the fetus and neonate.

In the case of humans, the teeth mostly affected by MIH begin to mineralize between the last trimester of fetal life and 5 months of age, which identifies the window of susceptibility for human MIH. In addition, human newborns exhibit plasma BPA concentration 11 times greater than that found in adults (Edginton & Ritter, 2009 = ref 17). This finding agrees well with the hypothesis that

exposure to BPA during the first months of life affect the development of the teeth that are developing during that window time, thus giving rise to MIH.

A paragraph has been added in the new version of the introduction to compare the rat experimental model of enamel hypomineralization with human MIH.

2. p. 4 "more selectively expressed"....more than what?  
"more" has been suppressed

3. p. 5 "only the androgen receptor (AR) was specifically expressed in late stage ameloblasts".  
Preface this sentence by "The present study shows....". Replace "specifically" with "preferentially" or "most highly".

The recommendations of the Reviewer have been followed.

4. Methods, p. 5. Why 5 week old rats? Why 10 mg/kg testosterone? Explain these choices.  
We chose to work on young adult rats of 5 weeks of age with a completed growth of the first incisor and with adult levels of testosterone (when non-castrated).

The dose of 10 mg/kg testosterone per injection is a common procedure to study the short-term effects of testosterone (see Engi SA et al., Int J Dev Neurosci. 2015 as an example of a recent reference).

5. Methods. p. 7. Here add microdissection procedure in brief and replace heading to read "Microdissection, RNA extraction....etc."

The recommendations of the Reviewer have been followed.

6. Results. Para 1. Heading is not accurate. Replace heading with "Steroid receptor expression in dental epithelium and localization in ameloblasts".

The recommendation of the Reviewer has been followed.

7. Same para. Second sentence. Preface with "As measured by qPCR in microdissected components of the dental epithelium

The recommendation of the Reviewer has been followed.

8. Results. P. 10. Modify heading to read "Anti-androgenic effects of EDCs in rat ameloblasts in vivo and in cell lines"

The recommendation of the Reviewer has been followed.

9. Discussion. Much of the first paragraph beginning with sentence 2 belongs in the Introduction to provide necessary rationale for the present study. Begin Discussion with present findings not past findings and rationale.

The recommendation of the Reviewer has been followed.

10. p. 12. Discussion. In conclusion....."we demonstrate, for the first time, that dental epithelial cells are androgen responsive and thus provide an explanation of the sexual dimorphism of enamel quality and the preferential impact of EDCs on the enamel of males". The logic of this statement is unclear, perhaps because the authors have not given the reader necessary background and references on male-female differences (see above). Or, do the authors assume that only males have androgens in early development? Or, do they think that only males have androgen receptors? Clarify

in the context of male-female differences in circulating androgens and estrogens during early development in rats which is well-defined in the literature.

We showed that AR is similarly expressed in male and female dental epithelial cells (figure 1). The difference of amelogenesis may thus result from the level of testosterone. We agree with the Reviewer that the testicular activity during the weeks following the birth with an increase of testosterone concentrations is known since a long time (Forest MG et al., 1973 = ref 46). The peak of testosterone (observed in species including humans and rodents) is concomitant with amelogenesis and may explain the male-female difference of amelogenesis due to specific enamel target gene expression modulations. Despite androgens may be the source of estrogens during development, it is also known that testosterone may act *per se*, in brain for example. In addition, estrogens are probably involved in early amelogenesis as suggested by the ER $\alpha$  expression in pre-ameloblasts (enamel quantity) whereas androgens are involved in late stage amelogenesis, thus in enamel quality, as illustrated by AR expression in maturation stage ameloblasts.

In accordance with our hypothesis of androgens involved in enamel quality, a study published in Lancet (Vos T et al., 2012) that shows the higher prevalence of caries in women than in men. Despite the dental decay is a very complex process that involves many factors in addition to enamel quality, these data (and other similar ones) argue for a sexual difference between male and female enamel quality.

Many other papers report the estrogen influence in periodontal disease but it is a matter of alveolar bone (and gingiva), not of amelogenesis.

We added a paragraph in the last part of the discussion to clarify the sexual difference of enamel quality.

**Androgen receptor involvement in rat amelogenesis: an additional way for endocrine  
disrupting chemicals to affect enamel synthesis**

Katia Jedeon<sup>1,2,3\*</sup>, Sophia Loiodice<sup>1,2\*</sup>, Khaled Salhi<sup>1,2#</sup>, Manon Le Normand<sup>1#</sup>, Sophia Houari<sup>1,2</sup>,  
Jessica Chaloyard<sup>1,2</sup>, Ariane Berdal<sup>1,2,3</sup>, Sylvie Babajko<sup>1,2</sup>

<sup>1</sup>Centre de Recherche des Cordeliers, INSERM UMRS 1138, Université Paris-Descartes, Université Pierre et Marie Curie-Paris, Paris Laboratory of Molecular Oral Pathophysiology, Paris, F-75006 France.

<sup>2</sup>Université Paris-Diderot, UFR d'Odontologie, F-75006, Paris, France.

<sup>3</sup>Centre de Référence des maladies rares de la face et de la cavité buccale MAFACE hôpital Rothschild, AP-HP, Paris, France.

\* Katia Jedeon and Sophia Loiodice contributed equally to this work as first authors.

# Khaled Salhi and Manon Le Normand contributed equally to this work as second authors.

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**Corresponding author and person to whom reprint requests should be addressed:**

Sylvie Babajko, PhD

Centre de Recherche des Cordeliers, INSERM, UMRS 1138, Laboratory of Molecular Oral Pathophysiology, 15-21 rue de l'École de Médecine, 75270 Paris cedex 06, France

Tel: +33 (01) 44 27 55 79 / Fax: +33 (01) 44 27 55 91

Email: [sylvie.babajko@crc.jussieu.fr](mailto:sylvie.babajko@crc.jussieu.fr)

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## **Abstract**

Endocrine disrupting chemicals (EDCs) that interfere with the steroid axis can affect amelogenesis, leading to enamel hypomineralization similar to that of Molar Incisor Hypomineralization (MIH), a recently described enamel disease. We investigated the sex steroid receptors that may mediate the effects of EDCs during rat amelogenesis. The expression of androgen, estrogen, and progesterone receptors (AR, ER $\alpha$ , and PGR) was dependent on the stage of ameloblast differentiation, whereas ER $\beta$  remained undetectable. AR was the only receptor selectively expressed in ameloblasts involved in final enamel mineralization. AR nuclear translocation and induction of ARE-containing promoter activity upon testosterone treatment, demonstrated ameloblast responsiveness to androgens. Testosterone regulated the expression of genes involved in enamel mineralization such as KLK4, Amelotin, SLC26A4, and SLC5A8, but not the expression of genes encoding matrix proteins which determine enamel thickness. Vinclozolin and to a lesser extent bisphenol A, two anti-androgenic EDCs that cause enamel defects, counteracted the actions of testosterone.

In conclusion, we show, for the first time, that: 1) ameloblasts express AR; 2) the androgen signaling pathway is involved in the enamel mineralization process; and 3) EDCs with anti-androgenic effects inhibit AR activity and preferentially affect amelogenesis in male rats. Their action, through the AR pathway, may specifically and irreversibly affect enamel, potentially leading to the use of dental defects as a biomarker of exposure to environmental pollutants. These results are consistent with the steroid hormones affecting ameloblasts, raising the issue of the hormonal influence on amelogenesis and possible sexual dimorphism in enamel quality.

## Introduction

The human environment has become increasingly contaminated by various pollutants which may have a role in the modern burst of diseases. Among environmental toxicants, endocrine-disrupting chemicals (EDCs) have been associated over these past 50 years with hormone-dependent cancers, diabetes, obesity, and decreased fertility (1, 2). This is supported by numerous epidemiological surveys and experimental studies. Based on these data, the median health and economic cost that can be reasonably attributed to EDC exposure in the European Union (EU) is €157 billion per year (or \$209 billion, corresponding to 1.23% of the EU's gross domestic product) (3). Among the thousands of EDCs, bisphenol A (BPA) is one of the most active and ubiquitous due to its wide use by the plastic industry. It is also present in dental materials (4). Consequences of exposure to BPA have been studied in detail in the development and pathophysiology of multiple organs, acting on different effectors of the steroid axis. These include the reproductive tract for decreased fertility (5), the pancreas for diabetes (6), the liver for hepatosteatosis (7), adipose tissue for obesity (8), the heart for arrhythmia (9), the brain for neurodevelopmental behavioral disorders (10) or the breast and prostate for increased cancer risk (11). BPA has also recently been shown to induce tooth enamel hypomineralization (12). Chronic perinatal exposure to low-dose BPA, with or without exposure to two other EDCs that disrupt the steroid axis (genistein and vinclozolin), affects dental enamel mineralization **in rats** (13). **Human and rodent** amelogenesis follows a similar well-known sequence of cell proliferation, differentiation, maturation and death characterized by specific gene expression patterns. **It begins with a secretory stage during which a partially mineralized enamel matrix is formed. The enamel matrix is comprised of amelogenins, enamelin and ameloblastin which are subject to extracellular processing by matrix metalloprotease-20 (MMP20). Once the full thickness of the enamel has been deposited, amelogenesis enters the maturation phase during which the ion exchangers (aided by solute carriers SLCs and ion-handling paroteins) allow optimal pH conditions for crystallite formation, and the serine protease kallikrein 4 (KLK4) degrades the enamel matrix proteins. Abnormal retention of proteins or indeed any extraneous proteins such as serum albumin lead to the eruption of hypomineralized enamel.** Finally, ameloblasts disappear during tooth eruption making enamel defects irreparable and irreversible, thus able to retrospectively record

ameloblast pathophysiology. The rodent incisor provides a running record of how a disruption of amelogenesis affects future development as the incisor continues to erupt. Rats at 30 days of age exposed daily to low-dose BPA present asymmetric white opaque spots on their incisors similarly scored as human Molar Incisor Hypomineralization (MIH), a recently described enamel pathology (14), whereas the 100 day old rats didn't present any enamel defects clinically observable (12). Contrary to the incisor observable at day 100, the first incisor (of 30 day old rats) mineralizes during the fetal and neonatal period of time which corresponds to the highest susceptibility to xenobiotics due to the immaturity of the detoxifying system: Glucuronide-BPA conjugates produced in the maternal circulation can cross the placenta and can be deconjugated back to active BPA in the fetus (15). Moreover, the UGT2B1 glucuronidase activity against xenoestrogens is undetectable in rat fetus and in the neonate, and only reaches adult levels by day 21 (16). In the case of humans, the teeth mostly affected by MIH, permanent first molars and permanent incisors, are the first mineralizing teeth. They develop from the third trimester of fetal life to four-five years after birth, which identifies the window of susceptibility for human MIH. Interestingly, human newborns exhibit plasma BPA concentration 11 times greater than that found in adults. This fact indicates that the risk of BPA exposure is greatest in the fetus and neonate (17). The clinical, structural and biochemical incisor abnormalities of rats exposed to low-dose BPA mimic human MIH making them a good experimental model of MIH (12, 18). The prevalence of MIH among six to nine year-old children is 15-18%, and its etiology remains unclear (18-20). However, exposure to EDCs has been associated to MIH. In addition to BPA, two other families of EDCs, polychlorinated biphenyls (PCBs) and dioxins, have also been implicated in enamel hypomineralization (21, 22).

Among enamel genes, we previously identified Enamelin, Amelogenin, KLK4 and SLC26A4 as the BPA targets of which altered expression leads to enamel hypomineralization (12, 23) The mechanisms of action of BPA and EDCs, which have similar effects, are still unclear, particularly in dental cells where the putative receptors are poorly defined, except for the ER $\alpha$  (24). BPA binds to estrogen receptors (ERs) with an equilibrium dissociation constant ( $K_d$ ) of 100 nM (25). The ER $\alpha$ , selectively expressed by undifferentiated ameloblast cells, mediates the increase in proliferation induced by BPA

(24). However, MIH is a hypomineralizing enamel pathology suggesting a disruption of the final stages of enamel synthesis.

The aim of this study was to systematically investigate the steroid receptors which might mediate the effects of BPA and other EDCs during amelogenesis. The present study shows that among them, only the androgen receptor (AR) was preferentially expressed in late stage ameloblasts suggesting its involvement in enamel final mineralization. The exposure of *in vivo* and *in vitro* amelogenesis models to anti-androgenic EDCs or testosterone revealed the importance of AR and androgens in amelogenesis and their implication in enamel defects due to EDC exposure.

## Materials and Methods

**Animals and biological samples.** Castrated and non-castrated young adult Wistar rats of 5 weeks of age were purchased from Janvier France Sarl (Le Genest Saint Isle, France). Animals were maintained in accordance with the French Ministry of Agriculture guidelines for care and use of laboratory animals during the time of the experiments (A75-06-12). All experimentation was conducted in accord with accepted standards of humane animal care.

Eight rats per group (either vehicle injected used as controls, or injected with 10 mg/kg of testosterone, or non-castrated and non-treated rats) were used in this study. Rats were anesthetized by isofurane inhalation, killed and their mandibles immediately dissected. The incisors were extracted and soft dental tissues microdissected for histological and biochemical analysis as previously described (12).

**Cell cultures and treatments.** Rat HAT-7 ameloblast cells (26) were grown in Dulbecco's modified Eagle's medium (DMEM)/F-12 without phenol red (Gibco) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and penicillin-streptomycin (50 IU/ml). Human LNCaP prostate cancer cells (27) were cultured in RPMI without phenol red (Gibco) supplemented with 10% FBS (Invitrogen) and penicillin-streptomycin (50 IU/ml). All culture media were filtered through charcoal to remove any steroids. Cells were cultured without serum for the 24 hours before treatments and during the experiments. Cells were treated with BPA (Sigma-Aldrich), vinclozolin M2 (Cayman chemical, Ann

Arbor, MI) and testosterone (Sigma-Aldrich) solubilized in DMSO (Dimethyl Sulfoxide, Sigma Aldrich); controls were exposed to culture medium containing the same amount of DMSO. For dedicated experiments, flutamide (Sigma-Aldrich), an androgen antagonist (28), was applied to the cells one hour before the beginning of the treatments.

**Immunofluorescence assays.** Dental soft tissues and cell lines were fixed by immersion in a 4% paraformaldehyde solution for 24 hours. After washing in PBS for 4 hours at 4° C, the samples were dehydrated in ethanol, rinsed in Safesolv (Labonord-France) and paraffin-embedded (Paraplast plus, Sigma). Serial frontal 8 µm sections were cut using a microtome (RM 2145, Leica, France). Sections were deparaffinized and rehydrated in decreasing concentrations of ethanol. Slices were microwaved for 20 minutes, endogenous peroxidases were blocked by incubation for 20 minutes in a freshly prepared 3% H<sub>2</sub>O<sub>2</sub> solution in PBS and the tissues permeabilized with 0.5% Triton X-100 for 10 minutes. Sections were then washed in PBS and blocked with 5% milk in PBS for 20 minutes at 4° C before primary antibody incubation. Cells cultured on glass coverslips, were fixed in cold methanol for 10 minutes and permeabilized in 0.1% Triton-X100 for 4 minutes. Both series of samples were incubated for one hour at room temperature with primary rabbit anti-AR polyclonal antibody (N-20:sc-816, Santa Cruz) (1:200). Sections were incubated with secondary goat anti-IgG coupled to Alexa Fluor 594 antibody (A-11072, Life Technologies) (1:500) for one hour in dark. After rinsing with PBS, sections were immersed in DAPI (010M4003-Sigma) (1:100000) for one minute and finally mounted with Fluoromount (Southern Biotech, Clinisciences).

**Cell transfections.** HAT-7 cells were transfected with the luciferase reporter plasmid, ARE-containing MMTV-luc (29), and β-galactosidase vector used for transfection efficiency standardization, according to the manufacturer's instructions (Qiagen, Courtaboeuf, France). After 48 hours of treatment and transfection, 5x10<sup>6</sup> cells were lysed for protein content determination and promoter activity assays. Protein content was determined using a BCA (bicinchoninic acid) protein assay kit (Pierce, France) according to the manufacturer's instructions. Luciferase activity was assayed by mixing 25 µL protein

extract with 75 µL Luciferase Assay Substrate (Promega Corp. Madison WI), and read with a B941 TriStar microplate reader (Berthold, France). The luciferase activity levels in transfected cell extracts are directly dependent on the activity of the promoter being tested. The experiment was independently repeated six times.

Transfection of human and rat siRNAs to a final concentration of 25 nM AR siRNAs (human: 5-CGGGAAGUUUAGAGAGCUAdTdT – rat: 5-CAAGGGAGGUUACGCCAAAdTdT) or scrambled siRNAs (5-UUCUCCGAACGUGUCACGUDTdT) used as controls (Qiagen, France) were performed using lipofectamine according to the manufacturer's procedure (Invitrogen, France) to directly and specifically repress AR expression.

**Microdissection, RNA extraction and gene expression profiling.** As previously described (12), dental epithelial cells from the secretion stage and the maturation stage were separately dissected using the molar reference line for isolation (30). The cervical loop, anatomically distinguishable, was dissected from the apical end of the incisor. The microdissection quality was validated with the specific pattern of enamel gene expression (Enamelin for the secretion stage for example) (23). RNAs were extracted from microdissected cervical loop, secretion and maturation rat dental epithelia using the RNeasy® Protect Mini Kit (Qiagen-France) according to the manufacturer's procedure. Spectrophotometry was used to assess the concentration and purity of RNA by measuring absorbance at 260 nm with a NanoDrop 1000 and RNA Integrity Number (RIN) (threshold > 9.5) with an Agilent Bioanalyzer, respectively. Reverse transcription was carried out with 1 µg total RNA for 45 minutes at 42° C, using a random primer oligodT primer mix, according to the manufacturer's instructions (Superscript II® - Invitrogen). Real-time quantitative PCR was performed using an Opticon Monitor device (Bio-Rad Laboratories, Hercules, CA, United States). Each PCR was independently repeated in triplicate and the results were normalized against those for RS15 and TBP1, two selected reference genes for which the expression did not vary under our experimental conditions. Details of the primers and the corresponding amplicon sizes are presented in (Table 1). The standard curve method was used to calculate the values

corresponding to the relative amounts of test and reference RNAs. Mean ratios of test RNA/standard RNA were calculated for each sample. Similar data were obtained with the  $\Delta\Delta\text{Ct}$  method.

The same RNAs extracted from microdissected dental epithelia at the maturation stage were used for microarray experiments performed with Affymetrix RatGene1.0 ST chip probes at the Genom'IC platform of Cochin Institute (Paris, France) to measure the relative level of each steroid receptor.

**Statistical analysis.** Data resulting from at least three independent experiments are presented as means  $\pm$  S.D. and were analyzed with GraphPad Prism4 software using the non-parametric Mann-Whitney test, two tailed. When  $P < 0.05$  (\*),  $P < 0.01$  (\*\*),  $P < 0.001$  (\*\*\*), the values compared were considered to be significantly different.

## Results

### *Steroid receptor expression in dental epithelium and localization in ameloblasts*

As EDC effects may involve sex steroid receptors, the expression of AR, ERs and PGR was investigated in dental epithelium. Rat dental epithelial cells expressed AR, ER $\alpha$ , and PGR but not ER $\beta$  (Figure 1A). Similar levels of all receptors of interest were measured in males and in females. **As measured by qPCR in microdissected components of the dental epithelium, the** ER $\alpha$  was primarily expressed in early-stage ameloblasts (secretory and pre-ameloblasts), whereas the PGR was expressed all along amelogenesis. The main variations were obtained for AR which expression was 7.3- fold higher in the maturation stage epithelium than in early-stage ameloblasts. PGR and ER expression were much lower than that of the AR regardless of the differentiation stage (Figure 1B). Among the sex steroid receptors, the AR was the most highly expressed in dental epithelium especially in cells involved in enamel terminal mineralization. Similarly, immunofluorescence assays showed the presence of the AR in dental epithelium, exclusively in maturation ameloblasts, not in secretion ameloblast, nor in cells of the papillary layer (Figure 1C). The two other receptors were undetectable in these cells in our experimental conditions.

AR expression was 11.0-fold higher in maturation stage ameloblasts than in the dental mesenchyme containing odontoblasts responsible for dentine synthesis (Figure 1A). The two other steroid receptors were equally expressed in the dental mesenchyme than in the epithelium.

#### ***AR nuclear translocation upon testosterone treatment***

The intracellular localization of the AR upon testosterone treatment was investigated by immunofluorescence assays. An AR signal was detected in the cytoplasm of dental epithelial cells under control conditions and in the nuclei of the same cells one hour following injection with 10 mg/kg testosterone (Figure 2A). Similar data were obtained in facial skin used as a positive control of AR expression and testosterone responsiveness.

*In vitro* experiments also showed AR nuclear translocation one hour after testosterone treatment in rat HAT-7 ameloblast cells and permanent nuclear localization in the prostate cell line LNCaP used as a positive control of AR expression and androgen responsiveness (Figure 2B). *In vitro* HAT-7 model allowed us to characterize the specificity of androgen action using flutamide, an androgen-antagonist, which completely prevented testosterone-induced AR nuclear translocation (data not shown).

#### ***The functional activity of the AR in ameloblasts***

The AR activity in ameloblasts was demonstrated by measuring ARE-containing MMTV promoter activity. Testosterone induced dose-dependent MMTV promoter activity in the HAT-7 ameloblastic cell line with a 3-fold induction of MMTV activity by 1 nM testosterone (Figure 3A). Similarly, testosterone stimulated MMTV activity in the LNCaP cell line (used as positive control because of its high AR expression) in a dose-dependent manner also with a 250-fold induction by 1 nM testosterone (Figure 3B). ERE-containing promoter activity remained unchanged at all testosterone concentrations tested and 1 nM estrogen (E2) was unable to stimulate MMTV activity (data not shown), showing that the effects of testosterone were specific. Testosterone induction of ARE-containing promoter activity was significantly inhibited by the androgen antagonist flutamide in a dose-dependent manner in the HAT-7

ameloblastic cell line as well as in the LNCaP prostate cancer cell line. It was also inhibited by siRNAs directed against the AR in HAT-7 and LNCaP cells by 54% and 73%, respectively.

***Enamel gene expression modulations by androgens and anti-androgens.***

AR expression was up-regulated after 48 hours of testosterone treatment in maturation stage ameloblasts of 40-day old castrated rats (Figure 4A). Testosterone induction of AR expression was also observed *in vitro* in HAT-7 cells. Conversely, AR expression was 1.8-fold lower in castrated rats than in non-castrated ones. *In vitro*, AR expression was reduced by 52% and 58% by flutamide and siRNAs against the AR, respectively. These data showed the specific androgen-dependent autoregulation of the AR expression (Figure 4A).

The KLK4 mRNA level was also significantly up-regulated 1.2-fold in maturation stage ameloblasts of adult rats after 48 hours of testosterone treatment (Figure 4B). It also increased 3.2-fold and 1.2-fold following treatment with 1 nM testosterone in LNCaP and HAT-7 cells respectively. *Klk4* expression was reduced 2.2-fold by siRNAs directed against AR (data not shown).

Testosterone modulations of expression of other previously reported BPA enamel target genes was investigated *in vivo*. The expression of two ion-exchangers, SLC26A4 and SLC5A8, increased 10.8-fold and 4.3-fold, respectively, following injection of 10 mg/kg testosterone (Figure 4B). Conversely, amelotin expression decreased 1.5-fold. We did not observe altered expression of any gene expressed in secretion stage ameloblasts such as amelogenin and enamelin, two major enamel matrix coding genes.

***Anti-androgenic effects of EDCs in rat ameloblasts *in vivo* and in cell lines.***

The anti-androgenic EDCs, vinclozolin and to a lesser extent BPA prevented testosterone-induced AR nuclear translocation (Figure 5A). Both EDCs also prevented testosterone induction of ARE-containing MMTV promoter activity in a dose dependent-manner in HAT-7 ameloblastic cells. Testosterone activation was reduced by 75% and 65% by  $10^{-6}$  M vinclozolin and  $10^{-7}$  M BPA, respectively (Figure 5B). Vinclozolin and BPA significantly reduced both, basal level of KLK4 expression and its testosterone induction (Figures 5C).

## Discussion

EDCs adversely affect different cells, tissues and organs, and their mechanisms of action have begun to be elucidated with the characterization of their putative receptors (25, 31). BPA is able to directly or indirectly act through several receptors including ERs (25), ERR $\gamma$  (32), GPR30 (33), ROR $\gamma$  (34), and AR (35). ERs, and more specifically ER $\alpha$  signalling, are only partially involved in the effects of BPA on enamel, and are affected exclusively during the proliferation stage (24). We focused our study on the AR because of BPA reduction of androgen synthesis (36), and the preferential impact of BPA on male enamel that we have documented (12, 24). The AR is also potentially involved in the mechanisms of action of other EDCs, such as vinclozolin, and PCBs (37, 38) already known to cause enamel hypomineralization (18, 21). The pattern of steroid receptor expression during amelogenesis was still unknown. We showed, for the first time, that ameloblasts express the AR and PGR, in addition to the ER $\alpha$  (24, 39). The presence of steroid hormone receptors suggests steroid control of amelogenesis. Among these receptors, the level of AR expression is much higher during all amelogenesis sequence than the levels of ER $\alpha$  and PR. Moreover, AR expression is the highest in maturation stage ameloblasts involved in terminal enamel mineralization, in contrast to the ER $\alpha$ . The AR is also present in dental pulp (40). Our data show that the AR is more strongly expressed in the epithelium than in the mesenchyme suggesting that the principal effect of testosterone on teeth is the regulation of enamel mineralization. Indeed, our functional studies showed that ameloblasts were highly responsive to androgens as shown by AR nuclear translocation upon testosterone treatment *in vivo*, in young and adult rats, and *in vitro* in ameloblast-like cells. This nuclear translocation was associated with testosterone induction of ARE-containing promoter activities similarly to that in AR positive control prostate LNCaP cells. Our *in vivo* and *in vitro* data reveal the regulation of the expression of androgen-responsive genes, notably the AR, which was up-regulated by testosterone and down-regulated by castration and anti-androgens. These results are in agreement with data on other organs and cells, such as osteoblasts (41), and more recently, Purkinje cerebellar cells (42).

In the current study, enamel genes expressed in maturation ameloblasts directly involved in terminal enamel mineralization, such as SLC5A8, SLC26A4, Amelotin and KLK4, were also regulated by androgens. By contrast, genes expressed earlier such as Enamelin, Ameloblastin and Amelogenin which contribute to enamel matrix deposition, remained unchanged. SLC26A4 (also called pendrin) is an anion exchanger for chloride, bicarbonate, iodine, and formate expressed by maturation ameloblasts which neutralizes acidification of enamel fluid in forming enamel (43). Klk4 and Amelotin are involved in enamel mineralization and hardness. Both processes are controlled by maturation ameloblasts which express the AR, whereas enamel volume and thickness are dependent on secretion ameloblasts which don't express the AR (44). Androgen-regulated enamel genes, such as SLC5A8, SLC26A4, Amelotin and KLK4 are also targeted by anti-androgenic EDCs able to disrupt amelogenesis in male rats, i.e. BPA (12, 23) and vinclozolin (13). *In vivo* data were comforted by *in vitro* experiments. Vinclozolin, and to a lesser extent, BPA inhibited testosterone-induced nuclear translocation, MMTV promoter activity, and KLK4 expression, suggesting that their enamel disrupting effects may be mediated through the AR. In conclusion, androgens appear to influence enamel quality rather than enamel quantity. Such an effect of androgens on overall tooth quality was recently reported in castrated male rhesus macaques (45). As AR is similarly expressed in male and female dental epithelia, the sexual difference of amelogenesis may result from the level of testosterone. The testicular activity during the weeks following the birth with an increase of testosterone concentrations occur in different species including humans and rodents (46) and is concomitant with amelogenesis. Taking into account the ameloblastic expression of AR, androgens may influence amelogenesis per se, by directly inducing AR activity. These findings along with the present data unambiguously showed the influence of androgens on amelogenesis and sexually dependent ameloblast differentiation (47, 48). In the present study, we demonstrate, for the first time, that dental epithelial cells are androgen responsive and that enamel key gene expression may be modulated by testosterone and thus provide an explanation of the sexual dimorphism of enamel quality and the preferential impact of EDCs on the enamel of males. It may be useful to decipher the endocrine-dependent regulation of enamel synthesis to better understand the impact of EDCs which may be considerably different depending on the receptor involved in their action: ER $\alpha$  being involved in early

events and cell proliferation, its disruption may lead to morphogenetic defects observed in MIH, whereas AR being involved in late events by modulating expression of genes involved in terminal enamel mineralization, its disruption may lead to enamel hypomineralization in MIH. Each EDC may specifically and irreversibly affect enamel, depending on their putative receptor activation, generating various defects. Indeed, the clinical phenotype of MIH is variable between individuals. Thus, the careful analysis of enamel may be informative on the causative EDCs, and resulting enamel defects may be used as easy biomarker of exposure to environmental pollutants.

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### **References**

1. De Coster S, van Larebeke N. Endocrine-disrupting chemicals: associated disorders and mechanisms of action. *J Environ Public Health* 2012; 2012:713696.
2. Maqbool F, Mostafalou S, Bahadar H, Abdollahi M. Review of endocrine disorders associated with environmental toxicants and possible involved mechanisms. *Life Sci* 2015; 145:265-273.
3. Trasande L, Zoeller RT, Hass U, Kortenkamp A, Grandjean P, Myers JP, DiGangi J, Bellanger M, Hauser R, Legler J, Skakkebaek NE, Heindel JJ. Estimating burden and disease costs of exposure to endocrine-disrupting chemicals in the European Union. *J Clin Endocrinol Metab* 2015; 100(4):1245-1255.

4. Cooper JE, Kendig EL, Belcher SM. Assessment of bisphenol A released from reusable plastic, aluminium and stainless steel water bottles. *Chemosphere* 2011; 85(6):943-947.
5. Varayoud J, Ramos JG, Munoz-de-Toro M, Luque EH. Long-lasting effects of neonatal bisphenol A exposure on the implantation process. *Vitam Horm* 2014; 94:253-275.
6. Alonso-Magdalena P, Garcia-Arevalo M, Quesada I, Nadal A. Bisphenol-A treatment during pregnancy in mice: a new window of susceptibility for the development of diabetes in mothers later in life. *Endocrinology* 2015; 156(5):1659-1670.
7. Martella A, Silvestri C, Maradonna F, Gioacchini G, Allarà M, Radaelli G, Overby DR, Di Marzo V, Carnevali O. Bisphenol A Induces Fatty Liver by an Endocannabinoid-Mediated Positive Feedback Loop. *Endocrinology* 2016; 157(5):1751-1763.
8. Vom Saal FS, Nagel SC, Coe BL, Angle BM, Taylor JA. The estrogenic endocrine disrupting chemical bisphenol A (BPA) and obesity. *Mol Cell Endocrinol* 2012; 354(1-2):74-84.
9. Gao X, Wang HS. Impact of bisphenol a on the cardiovascular system - epidemiological and experimental evidence and molecular mechanisms. *Int J Environ Res Public Health* 2014; 11(8):8399-8413.
10. Mustieles V, Perez-Lobato R, Olea N, Fernandez MF. Bisphenol A: Human exposure and neurobehavior. *Neurotoxicology* 2015; 49:174-184.
11. Seachrist DD, Bonk KW, Ho SM, Prins GS, Soto AM, Keri RA. A review of the carcinogenic potential of bisphenol A. *Reprod Toxicol* 2016; 59:167-182.
12. Jedeon K, De la Dure-Molla M, Brookes SJ, Loiodice S, Marciano C, Kirkham J, Canivenc-Lavier MC, Boudalia S, Bergès R, Harada H, Berdal A, Babajko S. Enamel Defects Reflect Perinatal Exposure to Bisphenol A. *Am J Pathol* 2013; 83(1):108-118.
13. Jedeon K, Marciano C, Loiodice S, Boudalia S, Canivenc Lavier M-C, Berdal A, Babajko S. Enamel hypomineralization due to endocrine disruptors. *Connect Tiss Res* 2014; 55(Supp1):1-5.
14. Weerheijm KL, Jalevik B, Alaluusua S. Molar-incisor hypomineralisation. *Caries Res* 2001; 35(5):390-391.

15. Nishikawa M, Iwano H, Yanagisawa R, Koike N, Inoue H, Yokota H. Placental transfer of conjugated bisphenol A and subsequent reactivation in the rat fetus. *Environ Health Perspect* 2010; 118:1196-1203.
16. Matsumoto J, Yokota H, Yuasa A. Developmental increases in rat hepatic microsomal UDP-glucuronosyltransferase activities toward xenoestrogens and decreases during pregnancy. *Environ Health Perspect* 2002; 110:193-196.
17. Edginton AN, Ritter L. Predicting plasma concentrations of bisphenol A in children younger than 2 years of age after typical feeding schedules, using a physiologically based toxicokinetic model. *Environ Health Perspect* 2009; 117:645-652.
18. Jedeon K, Berdal A, Babajko S. The tooth, target organ of Bisphenol A, could be used as a biomarker of exposure to this agent. In: Editor YG, editor. *Bisphenol A: Sources, Risks of Environmental Exposure and Human Health Effects*: Nova Science Publishers. 2015:205-225.
19. Jälevik B. Prevalence and Diagnosis of Molar-Incisor- Hypomineralisation (MIH): A systematic review. *Eur Arch Paediatr Dent* 2010; 11(2):59-64.
20. Alaluusua S. Aetiology of Molar-Incisor Hypomineralisation: A systematic review. *Eur Arch Paediatr Dent* 2010; 11(2):53-58.
21. Jan J, Sovcikova E, Kocan A, Wsolova L, Trnovec T. Developmental dental defects in children exposed to PCBs in eastern Slovakia. *Chemosphere* 2007; 67(9):S350-354.
22. Alaluusua S, Calderara P, Gerthoux PM, Lukinmaa PL, Kovero O, Needham L, Patterson DG Jr, Tuomisto J, Mocarelli P. Developmental dental aberrations after the dioxin accident in Seveso. *Environ Health Perspect* 2004; 112(13):1313-1318.
23. Jedeon K, Houari S, Loiodice S, Thuy TT, Le Normand M, Berdal A, Babajko S. Chronic Exposure to Bisphenol A Exacerbates Dental Fluorosis in Growing Rats. *J Bone Miner Res* 2016, in press. doi: 10.1002/jbmr.2879.
24. Jedeon K, Loiodice S, Marciano C, Vinel A, Canivenc Lavier MC, Berdal A, Babajko S. Estrogen and bisphenol A affect male rat enamel formation and promote ameloblast proliferation. *Endocrinology* 2014; 155(9):3365-3375.

25. Delfosse V, Grimaldi M, Pons JL, Boulahtouf A, le Maire A, Cavailles V, Labesse G, Bourguet W, Balaguer P. Structural and mechanistic insights into bisphenols action provide guidelines for risk assessment and discovery of bisphenol A substitutes. *Proc Natl Acad Sci U S A* 2012; 109(37):14930-14935.
26. Kawano S, Morotomi T, Toyono T, Nakamura N, Uchida T, Ohishi M, Toyoshima K, Harada H. Establishment of dental epithelial cell line (HAT-7) and the cell differentiation dependent on Notch signaling pathway. *Connect Tissue Res* 2002; 43(2-3):409-412.
27. Horoszewicz JS, Leong SS, Kawinski E, Karr JP, Rosenthal H, Chu TM, Mirand EA, Murphy GP. LNCaP model of human prostatic carcinoma. *Cancer Res* 1983; 43(4):1809-1818.
28. Liao S, Howell DK, Chang TM. Action of a nonsteroidal antiandrogen, flutamide, on the receptor binding and nuclear retention of 5 alpha-dihydrotestosterone in rat ventral prostate. *Endocrinology* 1974; 94(4):1205-1209.
29. Sun H, Si C, Bian Q, Chen X, Chen L, Wang X. Developing *in vitro* reporter gene assays to assess the hormone receptor activities of chemicals frequently detected in drinking water. *J Appl Toxicol* 2012; 32(8):635-641.
30. Smith CE, Nanci A. A method for sampling stages of amelogenesis on mandibular rat incisors using the molars as a reference for dissection. *Anat Rec* 1989; 225:257-266.
31. Delfosse V, Dendele B, Huet T, Grimaldi M, Boulahtouf A, Gerbal-Chaloin S, Beucher B, Roecklin D, Muller C, Rahmani R, Cavailles V, Daujat-Chavanieu M, Vivat V, Pascussi JM, Balaguer P, Bourguet W. Synergistic activation of human pregnane X receptor by binary cocktails of pharmaceutical and environmental compounds. *Nat Commun* 2015; 6:8089.
32. Matsushima A, Kakuta Y, Teramoto T, Koshihara T, Liu X, Okada H, Tokunaga T, Kawabata S, Kimura M, Shimohigashi Y. Structural evidence for endocrine disruptor bisphenol A binding to human nuclear receptor ERR gamma. *J Biochem* 2007; 142(4):517-524.
33. Sheng ZG, Zhu BZ. Low concentrations of bisphenol A induce mouse spermatogonial cell proliferation by G protein-coupled receptor 30 and estrogen receptor- $\alpha$ . *Environ Health Perspect* 2011; 119(12):1775-1780.

34. Nishigori M, Nose T, Shimohigashi Y. Highly potent binding and inverse agonist activity of bisphenol A derivatives for retinoid-related orphan nuclear receptor ROR $\gamma$ . *Toxicol Lett* 2012; 212:205-211.
35. Lee HJ, Chattopadhyay S, Gong EY, Ahn RS, Lee K. Antiandrogenic effects of bisphenol A and nonylphenol on the function of androgen receptor. *Toxicol Sci* 2003; 75:40-46.
36. Akingbemi BT, Sottas CM, Koulova AI, Klinefelter GR, Hardy MP. Inhibition of testicular steroidogenesis by the xenoestrogen bisphenol A is associated with reduced pituitary luteinizing hormone secretion and decreased steroidogenic enzyme gene expression in rat Leydig cells. *Endocrinology* 2004; 145:592–603.
37. Kojima H, Katsura E, Takeuchi S, Niiyama K, Kobayashi K. Screening for estrogen and androgen receptor activities in 200 pesticides by in vitro reporter gene assays using Chinese hamster ovary cells. *Environ Health Perspect* 2004; 112(5):524-531.
38. Portigal CL, Cowell SP, Fedoruk MN, Butler CM, Rennie PS, Nelson CC. Polychlorinated biphenyls interfere with androgen-induced transcriptional activation and hormone binding. *Toxicol Appl Pharmacol* 2002; 179(3):185-194.
39. Ferrer VL, Maeda T, Kawano Y. Characteristic distribution of immunoreaction for estrogen receptor alpha in rat ameloblasts. *Anat Rec A Discov Mol Cell Evol Biol* 2005; 284(2):529-536.
40. Dale JB, Sarich SL, Bretz TM, Hatton JF, Zachow RJ. Hormonal regulation of androgen receptor messenger ribonucleic acid expression in human tooth pulp. *J Dent Res* 2002; 81(5):360-365.
41. Wiren KM, Zhang X, Chang C, Keenan E, Orwoll E. Transcriptional up-regulation of the human androgen receptor by androgen in bone cells. *Endocrinology* 1997; 138:2291-2300.
42. Perez-Pouchoulen M, Toledo R, Garcia LI, Perez-Estudillo CA, Coria-Avila GA, Hernandez ME, Carrillo P, Manzo J. Androgen receptors in Purkinje neurons are modulated by systemic testosterone and sexual training in a region-specific manner in the male rat. *Physiol Behav* 2016; 156:191-198.

43. Bronckers AL, Guo J, Zandieh-Doulabi B, Bervoets TJ, Lyaruu DM, Li X, Wangemann P, DenBesten P. Developmental expression of solute carrier family 26A member 4 (SLC26A4/pendrin) during amelogenesis in developing rodent teeth. *Eur J Oral Sci* 2011; 119 Suppl 1:185-192.
44. Núñez SM, Chun YP, Ganss B, Hu Y, Richardson AS, Schmitz JE, Fajardo R, Yang J, Hu JC, Simmer JP. Maturation stage enamel malformations in *Amtn* and *Klk4* null mice. *Matrix Biol* 2016; 52-54:219-233.
45. Wang Q, Kessler MJ, Kensler TB, Dechow PC. The mandibles of castrated male rhesus macaques (*Macaca mulatta*): The effects of orchidectomy on bone and teeth. *Am J Phys Anthropol* 2016; 159(1):31-51.
46. Forest MG, Cathiard AM, Bertrand JA. Evidence of testicular activity in early infancy. *J Clin Endocrinol Metab* 1973;148-151.
47. Ribeiro DC, Brook AH, Hughes TE, Sampson WJ, Townsend GC. Intrauterine hormone effects on tooth dimensions. *J Dent Res* 2013; 92(5):425-431.
48. Heikkinen T, Harila V, Tapanainen JS, Alvesalo L. Masculinization of the eruption pattern of permanent mandibular canines in opposite sex twin girls. *Am J Phys Anthropol* 2013; 151(4):566-572.

## Figure Legends

**Figure 1. Expression pattern of sex steroid receptors in rat dental epithelium of mandibular incisors from rats.** A, Sex steroid expression in the cervical loop (L), secretion stage ameloblasts (S), and maturation stage ameloblasts (M) of microdissected rat dental epithelium of males and females. For each gene, the highest level of expression (either in L for ER $\alpha$ , or in M for AR and PGR) was set to 100%. Levels of expression in dental epithelium were compared to those of dental mesenchyme (Mes). B, Relative expression levels of sex steroid receptors in rat maturation ameloblasts were obtained by microarray assays. The corresponding levels of expression in L and S were calculated on the basis of RT-qPCR analysis presented above. C, Immunofluorescence analysis of AR in rat dental epithelium. AR staining was localized in ameloblasts, and higher in maturation stage ameloblasts (involved in enamel terminal mineralization) than in secretion ones. PL, papillary layer, SI, stratum intermedium.

Scale bars, 20  $\mu\text{m}$ . All values are reported as the mean  $\pm$  S.D. (n=4), \*\*,  $P < 0.01$ , \*\*\*,  $P < 0.001$  by Mann-Whitney test.

**Figure 2.** Nuclear translocation of AR in ameloblasts upon testosterone treatment. A, Immunofluorescent (IF) analysis of AR in dental epithelium and facial skin (used as a positive androgen-responsive tissue). AR staining appeared in the nuclei of ameloblasts (upper panel) in rats injected with 10 mg/kg within one hour. Scale bars, 20  $\mu\text{m}$ . B, *In vitro* IF experiments showed the nuclear localization of AR staining in 1 nM testosterone treated rat ameloblastic cell line HAT7 and human AR positive LNCaP cells used as a positive control. Scale bars, 10  $\mu\text{m}$

**Figure 3.** AR-induced transcriptional activation of the ARE-containing MMTV promoter in rat ameloblast-like HAT-7 cells compared to human LNCaP cells. A, Testosterone induced MMTV promoter activity in rat ameloblast-like HAT-7 cells in a dose-dependent manner. Flutamide reduced testosterone induction in a dose dependent manner. Testosterone induced MMTV promoter activity was reduced by siRNAs directed against AR mRNA. B, MMTV promoter activity was measured in human LNCaP prostate cancer cells used as a positive control for androgen responsiveness. Specific inhibition of MMTV promoter activity by AR siRNA and flutamide. All values are reported as mean  $\pm$  S.D. \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , \*\*\*,  $P < 0.001$  by Mann-Whitney test.

**Figure 4.** Regulation of enamel gene expression by testosterone. A, testosterone induction of AR expression *in vivo*, in microdissected dental epithelia of castrated rats and, *in vitro*, in HAT-7 cells. Left panel, the level of AR expression was higher in dental epithelium of testosterone treated rats and in non-castrated rats than in castrated untreated rats (used as control animals). Right panel, *in vitro* induction of AR expression by  $10^{-9}$  M testosterone in HAT-7 cells. Basal level of the AR expression was specifically inhibited by AR siRNAs and by  $10^{-6}$  M flutamide. B, testosterone regulations of genes involved in amelogenesis. SLC26A4, SLC5A8, KLK4 and Amelotin are mainly expressed in maturation stage ameloblasts involved in enamel terminal mineralization. Amelogenin and Enamelin which encode

the two main enamel matrix proteins are expressed by the secretion stage ameloblasts. All values are reported as the mean  $\pm$  S.D. \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , \*\*\*,  $P < 0.001$  by Mann-Whitney test.

**Figure 5.** Anti-androgenic effects of vinclozolin and BPA in dental epithelial cells. A, One  $\mu\text{M}$  vinclozolin and, to a lesser extent, 10 nM BPA inhibited testosterone-induced AR nuclear translocation in rat HAT-7 ameloblastic cells. Scale bars, 10  $\mu\text{m}$ . B, Vinclozolin (V) and BPA reduced testosterone induced MMTV promoter activity in a dose-dependent manner. Neither V nor BPA had an effect on basal MMTV promoter activity. C,  $10^{-6}$  M V and  $10^{-8}$  M BPA reduced basal and testosterone-induced KLK4 expression. All values reported are means  $\pm$  S.D. \*,  $P < 0.05$ , \*\*,  $P < 0.01$ , \*\*\*,  $P < 0.001$  by Mann-Whitney test.

**Table 1.** Primer sequences used for RT-qPCR analyses

<b>Gene</b>	<b>Amplicon size</b>	<b>Primer sequences (5'-3')</b>
RS15	341 pb	GGCTTGTAGGTGATGGAGAA CTTCCGCAAGTTCACCTACC
TBP1	101 pb	CACGAACAACCTGCGTTGATC TTTTCTTGCTGCTAGTCTGGAT
Amelogenin	271 pb	ACACCCTTCAGCCTCATCAC GAGAACAGTGGAGGCAGAGG
Amelotin	379 pb	GCAACAAAACCGACTCCAG CTCCATTCTGCACATCTGG
AR	105 pb	ACCTGACCTGGTTTTCAATGAGTATC GTTATCTGGAGCCATCCAAACTCTT
Enamelin	439 pb	CATGTGGCCTCCGCCAGTCC GTCATCTGGGGGCGGGTCCT
ER $\alpha$	112 pb	CCAGCTACAAACCAATGC GGTCTTTCGTATCCCGCCTTTC
ER $\beta$	274 pb	AACCTGGACGGTGGGCCTGT AATCGCTGCAGACGGGGCAG
KLK4	320 pb	GCATCCGCAGTGGGTGCTGT CACACTGCAGGAGGCTGGGC
PGR	108 pb	CGCCCTACCTCAACTACCTG ATGCTTCATCCCCGCAGATT
SLC26A4	202 pb	CTTAGGCAGCCCACCAGAAA TGTTGGGTCAACAGACGGAG
SLC5A8	92 pb	ACGGTGGAACCTGATAACCCG GAAGCTTCACAAGCGAGTGC

Figure 1

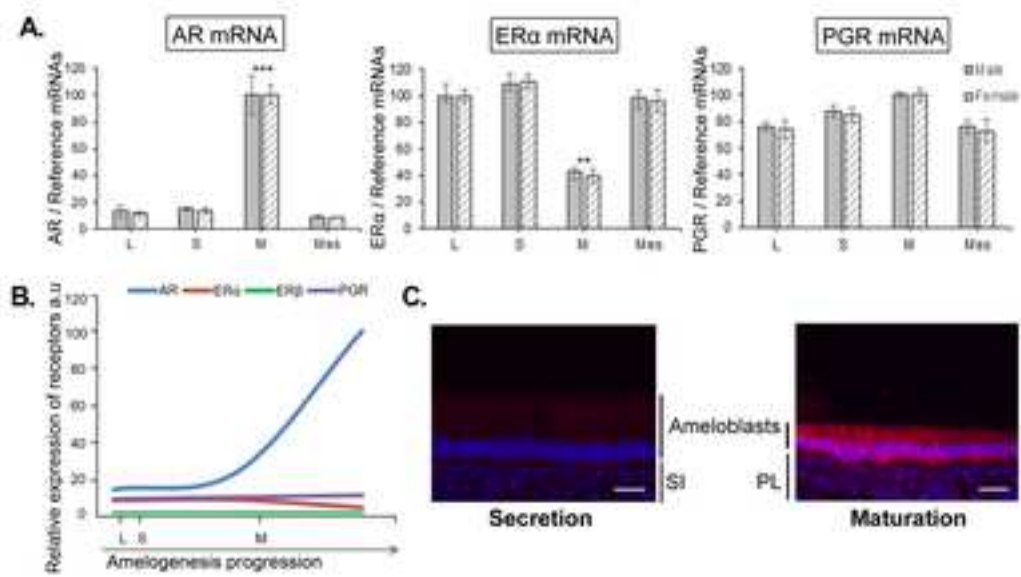


Figure 2

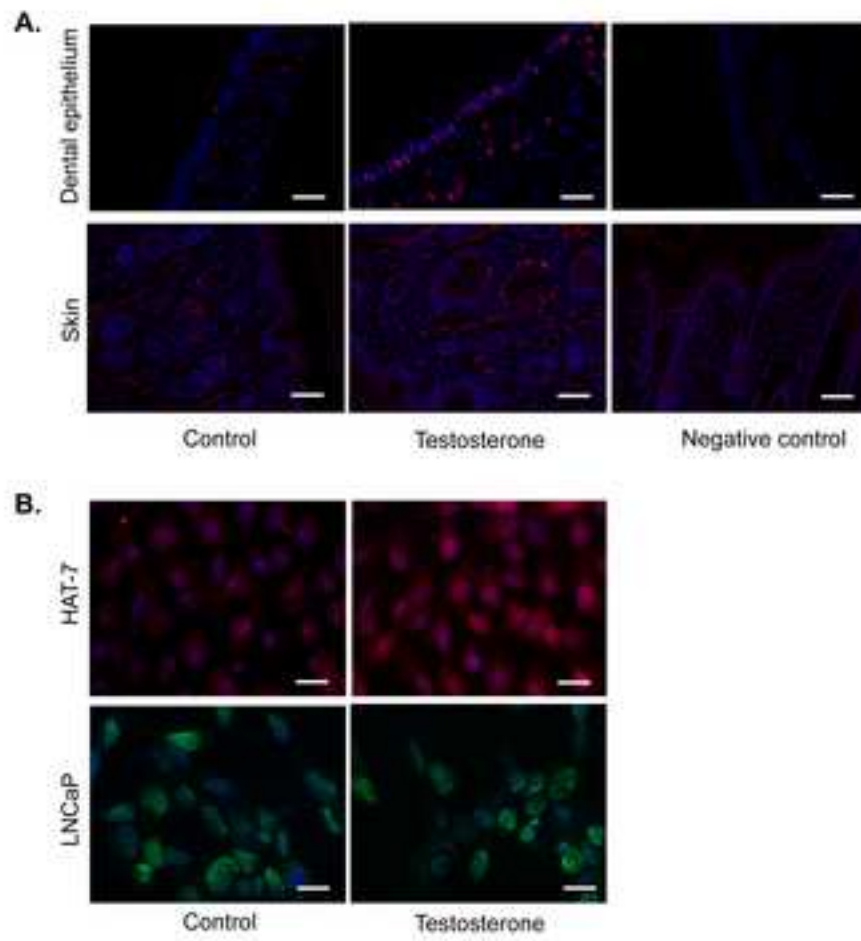


Figure 3

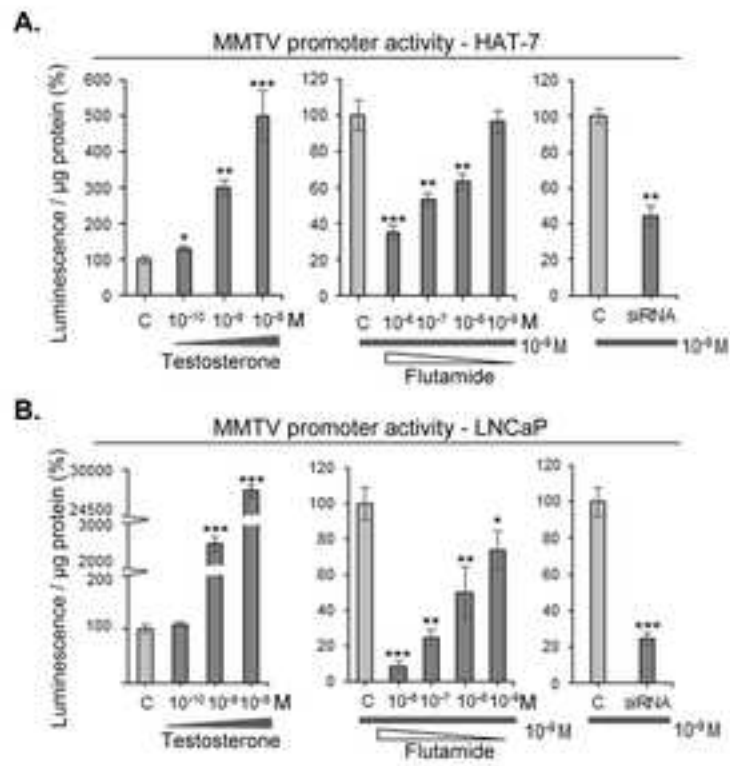


Figure 4

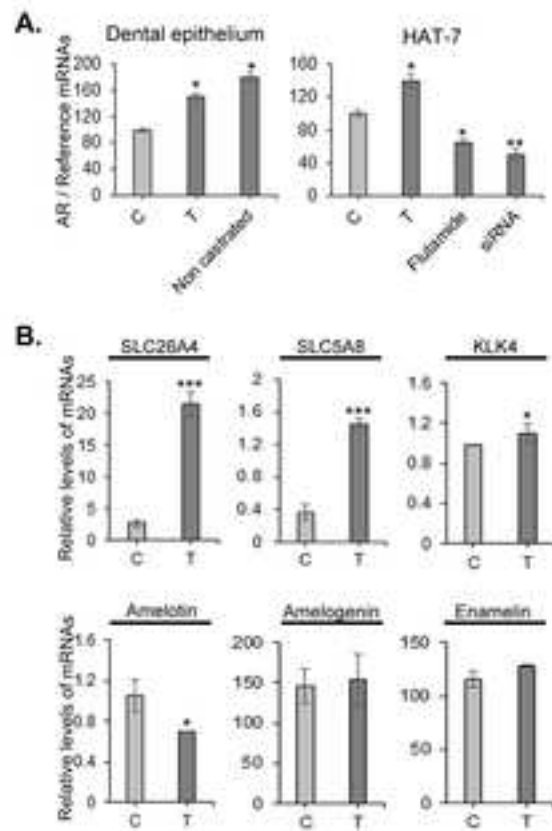
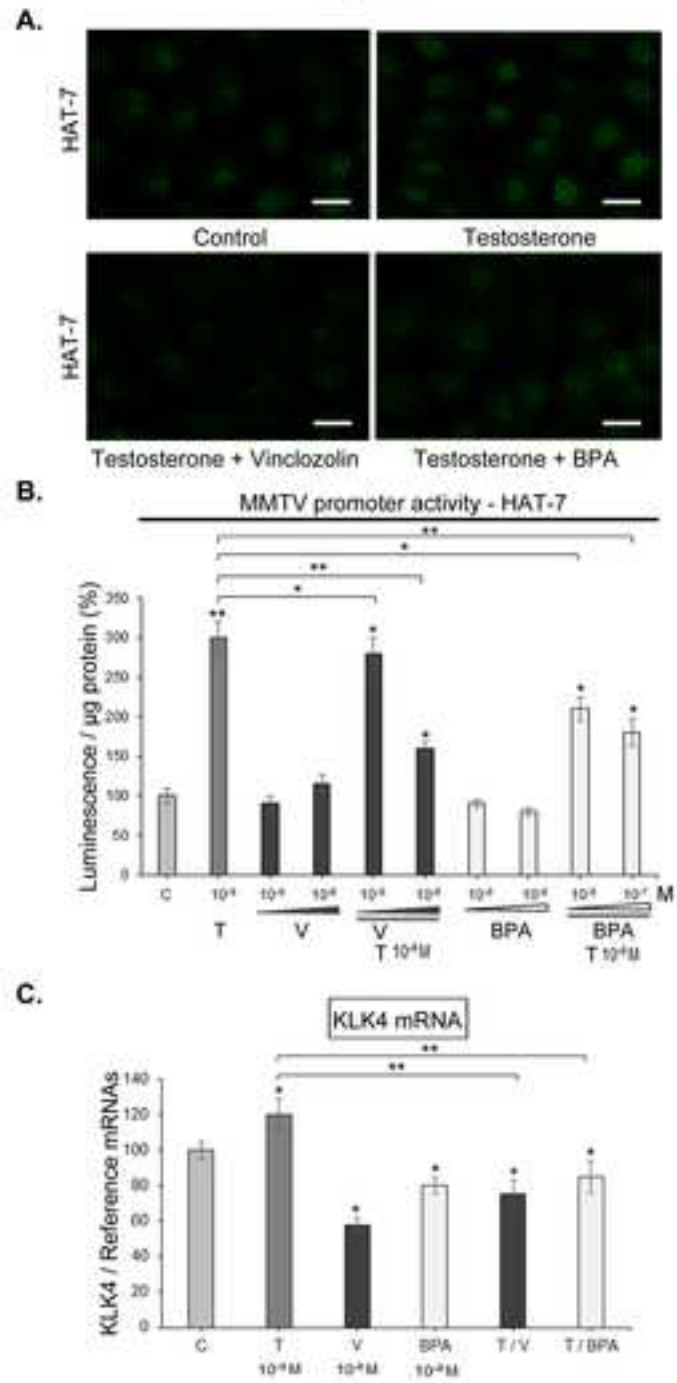


Figure 5

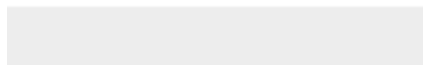




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**Supplemental Material**

**Institutional Approval for animal studies.pdf**



## Antibody table

<b>Antibody</b>	<b>Reference</b>
Primary rabbit anti-AR polyclonal antibody	N-20:sc-816, Santa Cruz
secondary goat anti-IgG coupled to Alexa Fluor 594 antibody	A-11072, Life Technologies

<b>Concentration</b>
1:200
1:500