ORIGINAL RESEARCH ARTICLES

ES CELLS-DERIVED ECTOMESENCHYMAL CELLS FOR TOOTH ENGINEERING.

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Running title
Differentiation of pluripotent cells in ectomesenchyme

Key words
Tissue engineering, Pluripotent cells, Ectomesenchyme, Neural crest, Differentiation.

Abstract
Recent progresses in stem cell biology and tissue engineering allow considering the possible development of new therapies for compensating the dental tissue losses associated with traumas, pathologies or ageing. The possibility of generating a tooth by mimicking development through reassociations between dental epithelial cells and ectomesenchymal cells derived from the neural crest (NC) has been demonstrated in the mouse. In the search of cell sources to be used for a human transfer, pluripotent stem cells could represent a good alternative. Our study thus focuses on obtaining, ectomesenchymal cells from pluripotent ES cells, capable of promoting tooth histomorphogenesis, when reassociated with a competent dental epithelium. To this end, two ES differentiation protocols, using cyclopamine or a combination of FGF2 and BMP4, have been developed and tested for their capacity to generate such cells. The differentiated ES cells were characterized by quantitative RT-PCR. Both protocols led the cells to acquire in 10 days a mesenchymal-like cell morphology. Rapidly after induction, the cells loose their expression of pluripotent genes while sequentially activating typical NC specifiers. However, the kinetics of gene activation differed between the 2 protocols. Interestingly, Twist, a gene whose expression in the NC is associated with a commitment towards an ectomesenchymal fate, is only activated under the influence of FGF2 and BMP4. Reassociation experiments with a competent epithelium will allow testing the odontogenic potential of the differentiated ES cells. These experiments performed in the mouse system should allow defining a strategy for obtaining odontogenic competent human cells.

Résumé
Les progrès en matière de biologie de cellules souches et d'ingénierie tissulaire permettent d'envisager le développement de nouvelles thérapies pour pallier les pertes de tissus dentaires consécutives à des traumatismes, des situations pathologiques ou au vieillissement. La possibilité de générer une dent en mimant le développement, par réassociations entre cellules dentaires épithéliales et mésenchymateuses dérivées des crêtes neurales (CN), a été démontrée chez la souris. Dans la recherche de ressources cellulaires utilisables pour un transfert chez l'homme, les cellules souches pluripotentes pourraient constituer une alternative. Notre but est d'obtenir à partir de ces dernières, des cellules ectomésenchymateuses capables d'interagir avec un épithélium dentaire pour promouvoir l'histomorphogenèse d'une dent. Pour cela, deux protocoles de différenciation de cellules ES, utilisant la cyclopamine ou une combinaison de FGF2/BMP4, ont été mis au point.

Les cellules induites ont été caractérisées par PCR quantitative. Les deux protocoles de différenciation amènent les cellules à acquérir en 10 jours, une morphologie de type mésenchymateux. Après induction, l'expression des gènes de pluripotence chute de façon drastique alors que celle des gènes spécificateurs de CN est activée. Toutefois, la cinétique varie selon le protocole. Le gène Twist, dont l'expression dans les CN est associée à un engagement vers l'ectomésenchyme, n'est activé significativement que sous l'action de FGF2/BMP4. Des expériences de réassociations avec un épithélium dentaire sont en cours pour évaluer le potentiel odontogène des cellules ES différenciées. A terme, ces approches menées chez la souris devraient permettre de définir une stratégie pour l'obtention de cellules compétentes humaines.

Introduction

Research on tissue engineering has been very active during the last decade. Highlighting the presence of stem cells in most adult organs and the development of protocols to generate pluripotent cells from adult somatic cells, the induced pluripotent stem cells (iPSCs) (1), has raised new possibilities for reparative and regenerative therapies. Odontology is at the forefront in this field with the identification of stem cells in various tissues constituting the dental organ (2). This discovery not only stimulates research on the development of innovative therapies involving these cells to treat different types of lesions of the tooth or its periodontium (artificial pulp, implant cellularization, stimulation of intrinsic stem cells ..) but also more fundamental research on the molecular mechanisms involved in the recruitment and differentiation of these cells.

Tooth development is regulated by sequential and reciprocal inductive interactions between ectomesenchymal cells, derived from the neural crest (NC), participating in the formation of the dental follicle and papilla, and the dental epithelium, leading to the formation of the enamel organ (3-5). Several groups have demonstrated the possibility of generating a mouse tooth through the re-association of dental embryonic cells (6-10). This process involves two steps: 1) an in vitro culture of reassociations between dental "competent" epithelial cells and ecto-mesenchymal cells, isolated most often from tooth germs at embryonic day (ED) 14. This step allows crown histomorphogenesis and the initiation of odontoblast differentiation (2, 5, 8), 2) an ectopic in vivo implantation of these cultured reassociations (subcutaneous or renal capsule), which allows to reach ameloblast functional differentiation as well as root and periodontal tissues formation (2, 8, 11).

It has recently been shown that tooth buds formed from such re-associations, were able to develop into a functional tooth, after implantation in a cavity left by a molar extraction (12). Although this study was debated on certain points, it suggests the possibility of reforming a functional tooth capable of de novo integration into the surrounding tissues. If the "proof of concept" of the possibility of tooth engineering seems established in the mouse, the nature and source of cells to be used for a transfer to human remain to be identified. Analyzing the epithelial / mesenchymal interactions that may take place in vitro is still the only integrated way to test the odontogenic potential of a given cell population after it has been reassociated with a competent epithelium. For this reason, the search for alternative source of cells to form a human tooth must first be investigated using the murine model. Regarding the mesenchymal compartment of the tooth, two types of stem cells can be considered, at the present time, for a therapeutic purpose: the adult mesenchymal stem cells (MSCs), with in particular those present in the dental pulp, and the embryonic stem cells (ES) or their equivalent derived from adult cells, the iPSCs.

We have previously shown that dental pulp stem cells, clonal cell lines or dental pulp stem cell population enriched on their expression of stromal cell markers were not able to establish a dialogue with the dental epithelium from ED14 to initiate tooth histogenesis (13; Acuna Mendoza et al., unpublished observations). Two non-exclusive hypotheses can explain these results: 1) the loss, upon culture, of the proper cell microenvironment and therefore of the signals allowing maintenance of the intrinsic stem cells properties (13) or 2) the loss, in these adult cells during the course of their maturation, of the ability to dialogue with the dental epithelium, a property likely associated with an immature state. Until now, most published works have indeed been focused on the ability of dental pulp stem cells to participate in the repair of injured adult tissue (for review14) and few studies have questionned their capacity to behave in an embryonic environment. Embryonic stem cells or iPSCs (15)

could provide an alternative cell source for tooth engineering. This would require to specifically induce these pluripotent naive stem cells towards a "dental mesenchyme" identity. This process would likely involve an initial induction towards an ectomesenchymal/neural crest (NC) cells phenotype and maybe a second more specific specification towards a "dental mesenchyme" potential. The potential of these cells to induce a dental histogenesis would then be evaluated in the reassociation system for each induction step.
Several protocols to derive NC cells from mouse ES cells have been described, starting from embryoid bodies, co-cultures or complex environments (16-19). To try to obtain cells with a NC phenotype capable of recruitment towards the ectomesenchymal fate, through direct differentiation of ES cells in monolayer culture, two induction protocols have been designed and tested.

Materials and Methods.
ES Cell Culture
The mouse ES cell line E14TG2a (20) was performed on irradiated MEF in a humidified atmosphere of 5% CO2 at 37°C in medium GMEM supplemented with L-glutamine (2 mM), sodium pyruvate (1 mM), non-essential amino acids (1X), fetal calf serum (15%), β-mercaptoethanol (0,1mM) and LIF 10^6 units/ml. This medium is referred as "ES medium" (ES). The medium was renewed every day. To induce differentiation, the cells were transferred to 0.1% gelatin-coated plates.

Differentiation protocols towards the Neural Crest Phenotype.
Two protocols of differentiation were evaluated after modifications. In both cases, the basic differentiation medium was: DMEM/F12 Glutamax, N2 supplement (1X), 0.1 mM solution of nonessential amino acids, 1 mM sodium pyruvate, 500 mg/ml serum albumin bovine, 0.1 mM β-mercaptoethanol and 50 U/ml penicillin/streptomycin. This medium is referred as "differentiation medium" (DM). In the first protocol (Protocol A) cells were inoculated at a density of 5 x 10^3 cells/cm2 on gelatin-coated plates in ES medium. Two days later, the medium was changed to DM. This day was considered as day zero of differentiation (D0). Two days later (D2) the culture medium was renewed in the presence of cyclopamine (1 μM) (see Figure 1A). For the second protocol (Protocol B), cells were inoculated at a density of 3.5 x 10^3 cells/cm2 on gelatin-coated plates in ES medium. After two days, the medium was changed to DM and supplemented with FGF-2 (10 ng/ml) and a solution of insulin, transferrin, selenium (ITS 1X). This was considered as D0 of differentiation. At differentiation day 4 (D4), BMP4 (10 ng/ml) was added in combination with FGF2 and ITS in DM (see Figure 1B). In both differentiation protocols, the medium was changed every two days.

RT-PCR and Quanitative Real-time PCR.
Briefly, total RNA was extracted from the cultured cells using a total RNA extraction kit (Qiagen, Hilden, Germany) and reverse-transcribed using a Quantitect RT kit (Qiagen, Hilden, Germany). Quantitative real-time PCR was performed using SYBR Green PCR Master Mix (Roche Diagnostics GmbH Mannheim, Germany) according to the supplier's directions in a LightCycler thermocycler 480R. Expression analysis of a series of characteristic pluripotent genes (Oct4, Nanog) and neural crest genes (Ap2-α, Snail, Sox9, Sox10, FoxD3, Twist) was performed. Values were calculated with the LightCycler software 1.5.0 (Roche). For each gene, values were normalized to β-actin for the cyclopamine protocol (protocol A) and GAPDH and Tata Binding Protein (TBP) for the FGF2/BMP4 protocol (Protocol B). Results were analyzed by the method of Δ Δ Ct. The primers were obtained from Qiagen (Qiagen, Hilden, Germany). The mRNA levels in differentiated cells were expressed relative to naïve ES cells or to cells from ED14 dental mesenchyme, which were taken as 1.

Immunofluorescence Analysis.
The cells were cultured on glass coverslips coated with 0.1% gelatin. Briefly, cells were fixed in 4% paraformaldehyde, permeabilized with PBS 0.5% Triton- X100 during 5 min at room temperature. The blocking step was carried out in 2% fetal calf serum (FCS)/PBS. The cells were then reacted with the primary antibodies: anti-Oct4 and anti-nanog (both from Genetex, Euromedex, France). Primary antibody binding was visualized with an Alexafluor 488-conjugated second anti-rabbit IgG (Invitrogen, Carlsbad, CA).

Results
Induction of ES cells to the CNC cell phenotype.

Two protocols have been developed and evaluated for their ability to generate cells with a phenotype of cranial neural crest (CNC) cells (Figure 1). The first protocol (A, Figure 1A) uses as differentiation inducer, cyclopamine, a SHH pathway inhibitor. This molecule has

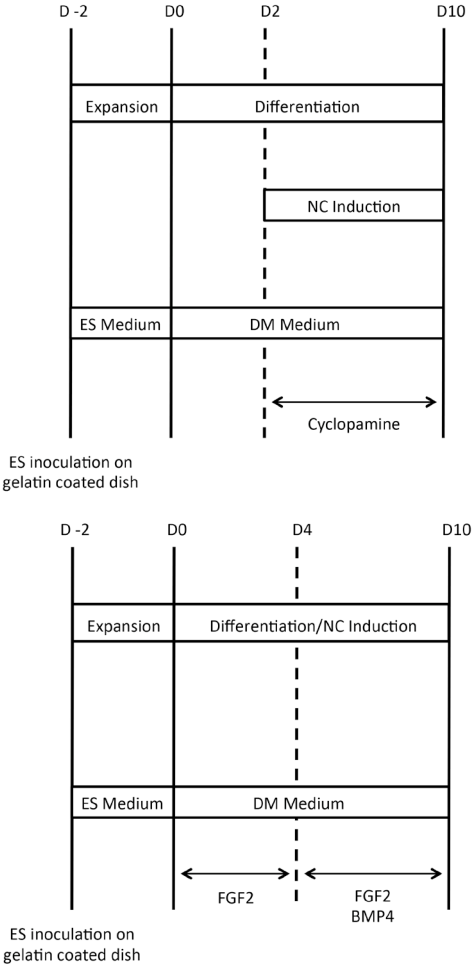

Figure 1
Timetable of ES cell differentiation towards the NC phenotype.
Periods of induction and culture conditions are indicated.
ES cells were seeded in monolayer and expanded in ES medium onto gelatin-coated dishes. Two days later, ES medium was changed for DM. This day marks the initiation of differentiation and was considered as differentiation day 0 (D0).
(A) Cyclopamine Protocol (Protocol A)
(B) FGF2/BMP4 Protocol (Protocol B)

previously been used for generating cortical neurons (21). The second protocol (B, Figure 1B) uses a combination of FGF2 and BMP4, as molecules involved in the recruitment towards an ecto-mesenchymal phenotype (22).

Differentiation of ES cells is highly dependent on the presence of extracellular matrix components and therefore, the culture medium composition together with an adequate pretreatment of the plastic dishes for the culture are essential for the survival and differentiation of ES cells towards a specific pathway. As a first step, the effect of coating the plastic culture dishes with either gelatin or laminin on the cell differentiation was thus investigated. Plastic coating with laminin did not sustain the attachment and spreading of the cells expected to permit differentiation (not shown) whereas gelatin did (Figure 2). All subsequent experiments were thus performed exclusively on gelatinized plasic dishes.
In both protocols, the switch of naïve ES cells (Figure 2A) to differentiation medium (DM) was accompanied by cell death during the first 24 hours. This cell death was, however, significantly more important when the cells were induced in protocol A conditions. Subsequently, in both protocols, cells initially spread on the coated plastic (Figure 2D) and then

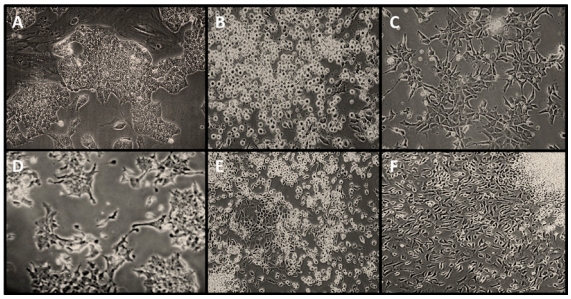

Figure 2
Cell morphology during NC differentiation processes.
In both protocols, the cells successively evolved from a flat to a round/refringent and finally to a mesenchymal-like cell morphology. (A) Undifferentiated ES cells in ES culture condition. (B, C) ES cells differentiated in cyclopamine culture conditions at D4 and 10 respectively. (D) ES cells after 48 h in DM. (E, F) ES cells differentiated in FGF2/ BMP4 culture conditions at D4 and 10 respectively.

acquired a “round refringent cell” morphology (Figure 2B and E). The cells then progressively spread again, assuming a mesenchymal-like phenotype, which concerns their great majority at differentiation day 10, for both protocols (Figure 2C and F). The kinetics of appearance of these phenotypes slightly differed between the 2 protocols, the phenotype “round refringent cell” appearing two days later in protocol A that in protocol B.
Phenotypic analysis.
A quantitative RT-PCR analysis of the expres-

sion kinetics of the pluripotency genes (Oct4 and Nanog) as well as of a series of "markers" genes of the neural crest (AP2α, Snail, FoxD3, Sox 9, Sox10 and Twist) (23) was performed at different time points of the differentiation protocols A and B (Figures 3 and 4). Soon after induction of ES cell differentiation, a drastic decline in the expression of Oct4 and Nanog was observed in the two protocols. This classically signs the commitment of the cells into differentiation (Figure 3A and B). This drop in transcript number was reflected at the protein level, as seen by immunofluorescence analysis (Figure 3C for protocol B).

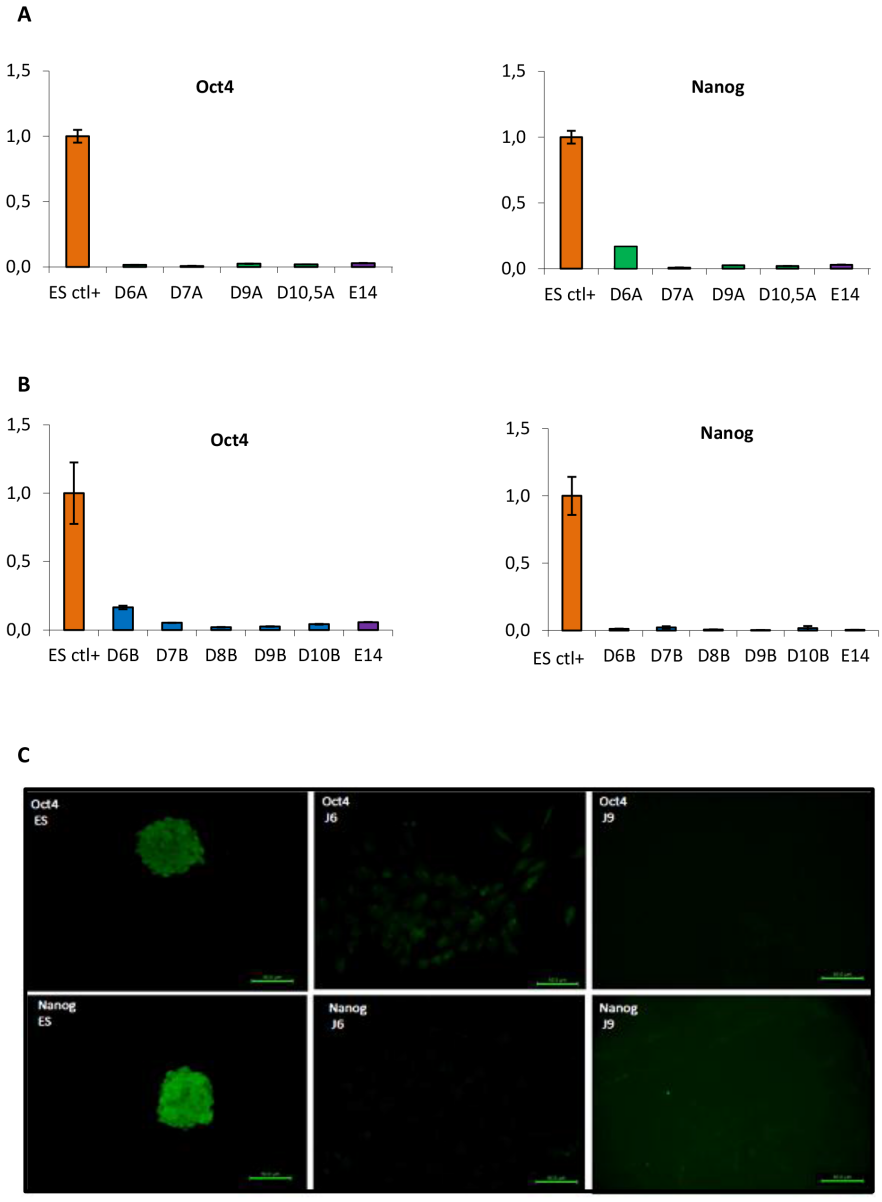

Figure 3: Mouse ES cells cultured in the presence of cyclopamine or FGF2/BMP4 loose their expression of the pluripotent genes Oct4 and Nanog.
Real-time PCR analysis of the Oct4 and Nanog expression levels in undifferentiated and cyclopMINW (A) or FGF2/BMP4 (B) differentiated ES cells at different time points of the differentiation process. The expression levels were normalized to α-actin (for the cyclopamine protocol), GAPDH and TBP mRNAs (for the FGF2/BMP4 protocol). The mRNA levels in differentiated cells were expressed relative to naïve ES cells (ES ctrl+), which was taken as 1.
(C) Immunofluorescence analysis of Oct4 (a-c) and Nanog (d-f) protein expression in ES cells undifferentiated (a, d) or differentiated with protocol B at day 6 (b, e) and day 9 (c, f).

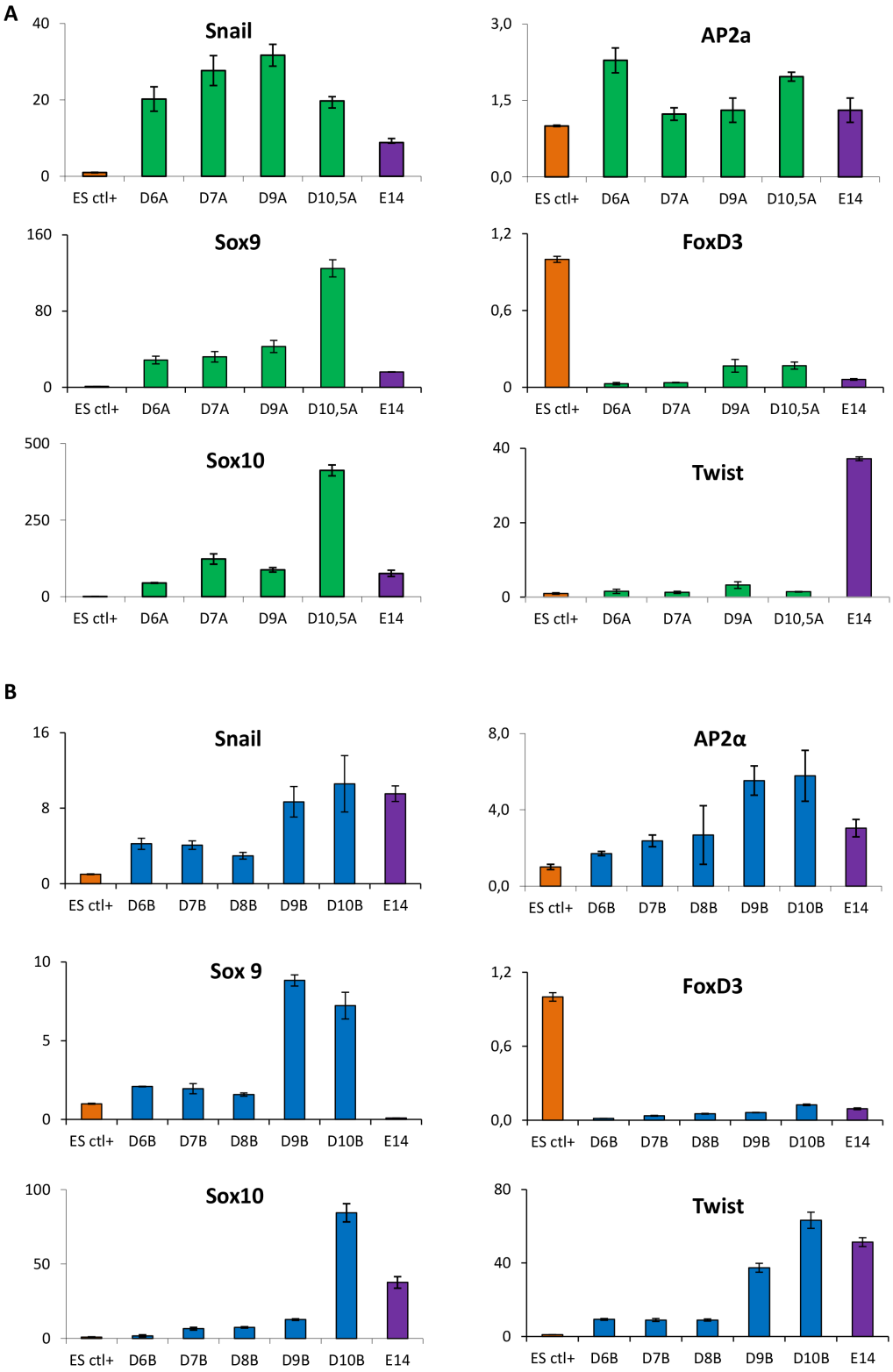

Figure 4: Kinetics of NC cell marker expression in induced ES cells.
Mouse ES cells were induced to differentiate with cyclopamine (A) or FGF2/BMP4 (B) in DM during 10 days and the mRNA expression levels of the neural specifier genes (Snail, AP2a, Sox9, FoxD3, Sox10 and Twist) were determined by real-time PCR. Expressions levels were normalized to α-actin (for the cyclopamine protocol) or GAPDH and TBP mRNAs (for the FGF2/BMP4 protocol). The mRNA levels in differentiated cells were expressed relative to naïve ES cells (ES ctrl+), which were taken as1.

Indeed, while an intense staining for Oct4 and Nanog proteins was observed in the ES cells (Figure 3Ca and d), these proteins were only barely detectable at differentiation day 6 and totally undetectable at day 9 of differentiation (Figure 3Cb, c, e and f).
In parallel, as compared to ES cells, the expression of AP2α, FoxD3, Snail, Sox9 and Sox10, NC specifier genes, which reflects the acquisition of an NC identity, started to be activated as soon as differentiation day 6, with both types of induction. However, the kinetics and extent of gene activation differed between the protocols. Upon cyclopamine induction, activation of the NC specifiers appeared to take place in two steps, with Snail and AP2α expression first activated at day 6 followed by a FoxD3, Sox9 and 10 activation at day 9 (Figure 4A). Upon FGF2/BMP4 induction, an activation of the expression of all NC genes was already detected at day 6 and their level then gradually increased to a maximum at day 10 (Figure 4B). Significantly, while the expression of the Twist gene remained close to the baseline in protocol A, it was strongly activated from day 9, in protocol B, concomitantly with the peak of expression of all other CN specifier genes. This differential expression of Twist gene thus distinguished the NC phenotype generated by the two types of induction.

Discussion

In searching for a new potential source of ectomesenchymal cells for tooth tissue engineering, attemps were made to induce mouse ES pluripotent cells, towards a potential similar to that of a dental mesenchyme. This potential will then be tested by reassociation experiments with a dental competent epithelium at the same stage (2). If these cells do indeed display such an odontogenic potential, their molecular characterization should enable obtaining their human counterparts, since the signaling pathways involved in ES comittment towards a given differentiation program are largely conserved between human and mouse. Several teams have recently sought to generate neural crest-like cells from murine ES cells or iPS cultured under conditions of embryoid bodies, co-cultures or complex medium (16-19). These studies were mostly designed to obtain neural derivatives. We are seeking to specifically derive, directly and at high frequency on adherent cultures, cells with a cranial NC/ectomesenchyme phenotype, competent to engage in tooth histomorphogenesis.
For that purpose, two differentiation protocols for ES cells were tested to recruit the cells towards the NC phenotype. Both differentiation were performed in a defined medium devoid of serum. The first protocol involves induction of the ES cells with cyclopamine, an inhibitor of Sonic Hedgehog (21, 24) while the second uses the sequential addition of FGF2 and BMP4. Differentiation induction was accompanied by an important cell death, that appears less pronounced in the second protocol presumably thanks to the mitogenic properties of FGF2 (25). Subsequently, in both protocols, ES cells undergo similar successive phenotypic changes: first, a rapid cell spreading, characteristic of a commitment to differentiation, then, a transiently “refringent and round” phenotype, before acquisition of a mesenchymal-like morphology. The morphology displayed by the differentiated ES cells appears similar to that observed for NC cells derived from ES by Aihara et al., (22), or recently from iPS cells by Otsu et al., (26), despite different differentiation strategies. However, the kinetics of acquisition of this morphology is different in both protocols, the conversion towards the “refringent round cell” phenotype taking place more rapidly in protocol B. These variations are most likely related to the inducing molecules used. Indeed, FGF2 allowed a better survival of ES cells upon switch into the differentiation medium as already mentionned earlier.
The understanding of the gene network and hierarchy involved in the specification of neural crest cells has made significant progress in recent years (23, 27). It is now established that NC cells express a set of genes called “NC specifiers “ that include c-Myc, Id 3, AP2α, Snail, FoxD3, Sox 9 and 10. Among these, some are “early” specifiers, like c-Myc, Snail or Ap2α and others, “late” specifiers, like FoxD3, Sox9 and Sox10 (23). Expression of these specifiers often selectively persists in more differentiated derivatives where they play essential roles in differentiation. For example, Sox9 is expressed in ectomesenchymal derivatives of the NC, where it will for instance control cartilage differentiation, while Sox 10 expression will persist in cells giving rise to cranial glia and in melanocyte precursors (27). The Twist gene is part of the NC specifier family, but its expression pattern appears essentially restricted to the cranial regions of the embryo. This suggests that this protein may act by providing the NC precursors with a potential of generating mesodermal derivatives (27). At the molecular level, the cells

that we have generated by the two protocols express, from differentiation day 4, the Msx1 and Pax3 genes (results not shown) These two genes are neural specifiers involved in initial recruitment towards the NC identity. Subsequently, the cells sequentially express early NC specifier genes (AP2α and Snail), and late specifiers (FoxD3, Sox9 and Sox10), as observed during development (23). The differentiated ES cells therefore present the molecular signature classically associated with NC progenitors (23). The kinetics of activation of these genes and their activation level vary depending on the protocol (A or B). At this stage of our analysis, we can only assume that these variations may reflect distinct "commitment states" of the NC cells. Such an hypothesis is supported by the observation that Twist, the NC specifier gene associated with the ability to commit NC cells towards mesenchymal derivatives, is only strongly activated in protocol B.

Our results also raise the question of the proportion of cells having acquired the NC identity among the ES cultures. Reassociation experiments require a large number of cells and it is therefore important to have at disposal, an efficient differentiation protocol. The NC gene activation pattern, as detected by PCR, may reflect either a low activation in most cells or a strong activation in a small cell subpopulation. Preliminary results obtained by AP2α immunofluorescence analysis on protocol B differentiated cells at day 6 and 9, suggest an homogeneity of differentiation together with a progressive activation of expression in cells over time.

Reassociation of competent dental epithelium with these ES-NC cells at different time points of induction are now under progress. They only will determine whether the induced cells have acquired an odontogenic potential or if a further, more specific, induction towards dental mesenchyme is requested. Our first results showed that NC-ES cells at differentiation day 6 do not permit an initiation of dental histomorphogenesis. This may be related, to the observed lack of "adhesive" properties of the cells at this stage, which are fundamental for the establishment of the correct dialogue with the epithelium. Such a lack of adequate "adhesive" properties had also been reported for bone marrow cells, when tested reassociation assays (28). Yet at this early stage, ES-NC cells do not yet express the complete set of neural crest specifiers. Experiments are underway to determine if later, in particular at differentiation day 10, when Twist expression is maximum, these cells may become able to initiate dental histomorphogenesis.

Acknowledgements

This study was funded by grants from the Paris Descartes University and the Institut Français pour la Recherche Odontologique (IFRO). SAM was supported by a fellowship from the Chilian government (Becas Chile).

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