

Proliferation and pluripotency potential of ectomesenchymal cells derived from first branchial arch

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Abstract. Cranial neural crest-derived ectomesenchymal cells are multipotential progenitors that contribute to various tissue types during embryogenesis. Their potential to be expanded in culture as a monolayer and to be induced into different cell lineages *in vitro* has not been previously reported in detail. In this study, the ectomesenchymal cells in the first branchial arch were enzymatically isolated from the mandibular processes of BALB/c mice and were maintained in an intact state in a medium containing leukaemia inhibitory factor. Here, we first evaluated the proliferative activity of the cells after the third passage, using bromodeoxyuridine labelling and *in situ* hybridization of telomerase mRNA. Positive staining for expression of HNK-1, S-100 and vimentin confirmed that the population of stem cells originated from the ectomesenchyme, which did not express cytokeratin. Then we investigated the molecular and cellular characteristics of the ectomesenchymal cells during their differentiation towards neurogenic, endothelial, myogenic and odontogenic lineages. Expression of multiple lineage-specific genes and proteins was detected by utilizing a range of molecular and biochemical approaches when the cells were transferred to inductive medium. Histological and immunohistochemical analysis of the induced cells at various intervals indicated obvious phenotypic alteration and presence of specific proteins for the differentiated lineages, for example nestin, factor VIII, α -SMA and dentin sialophosphoprotein (DSPP), respectively. Correlatively, results of reverse transcription-PCR corroborated at mRNA level the expression of the characteristic molecules during differentiation. Therefore, it is suggested that the ectomesenchymal cells derived from the first branchial arch may represent a novel source of multipotential stem cells capable of undergoing expansion and variant differentiation *in vitro*.

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INTRODUCTION

In the early embryo, cranial neural crest cells (CNCC) migrate from forebrain, midbrain and hindbrain regions to populate the fronto-nasal, maxillary and mandibular processes. Except for the enamel of the tooth crown, cranial neural crest-derived ectomesenchymal cells form the remaining structures of the tooth, including the dentine, pulp and periodontal tissues (Romer 1972; Tan & Morriss-Kay 1986; Bronner-Fraser 1993; Chai *et al.* 2000). Recent studies have well advanced our understanding of this important pluripotent cell population, and how genetic and epigenetic mechanisms mediate their subsequent lineage segregation, proliferation, differentiation and final contribution to a particular cell type (Chai *et al.* 2000; Tian *et al.* 2004; Yan *et al.* 2004; Jiang *et al.* 2005; Xie *et al.* 2005). During craniofacial development, the proliferative activity of these cells produces discrete swellings that demarcate each branchial arch. Following epithelial–mesenchymal interactions, post-migratory ectomesenchymal cells differentiate into multiple phenotypes and contribute to the formation of various head and neck structures. Growth and transcription factors have been implicated during the specification and determination of neural crest cells (Yan *et al.* 2004; Jiang *et al.* 2005; Xie *et al.* 2005). It is clearly important to identify this novel source of mesenchymal stem cells, which may represent a promising pool of candidate cells for the engineered repair of tissues and organ systems.

Stem cells possess self-renewal, long-term cell viability and multilineage differentiation potential, all of which are taken as their significant characteristics. Bone mesenchymal stem cells have excellent capacity to proliferate in culture and to differentiate into a wide variety of cell types in response to an appropriate culture system (Bianco & Gehron Robey 2000; Gronthos *et al.* 2003). In this study, cranial neural crest-derived ectomesenchymal cells were passaged *in vitro* to evaluate their proliferative potential using bromodeoxyuridine (BrdUrd) labelling. Meanwhile, expression of telomerase was measured by *in situ* hybridization, as active telomerase can prevent cellular senescence and extends the lifespan of cultured cells (Bodnar *et al.* 1998; Vaziri & Benchimol 1998). A key function of telomerase is to add telomere repeats to the ends of chromosomes during cell division. Telomerase activity remains high in stem cells and precursor cells (Tang *et al.* 2001; Xu *et al.* 2001), which are capable of infinite self-replication and differentiation into a variety of cell types (Morrison *et al.* 1996; Weissman 2000).

According to previous reports, each brachial arch develops according to a unique program of spatial and temporal patterning. As the tooth germ develops from the bud to cap stage, ectomesenchymal cells are concentrated at the interface with the dental epithelium, suggesting critical function of these cells in epithelial–mesenchymal interactions during tooth germ development. At the cap stage, the dental epithelium forms an aggregated cell mass (the enamel knot), which serves as a signalling centre to guide cusp formation. As tooth development advances from the cap to the bell stage, the cranial neural crest-derived dental mesenchyme and the inner dental epithelium begin to terminally differentiate into the pre-odontoblast and pre-ameloblast, respectively (Chai *et al.* 2000; Jernval & Thesleff 2000; Yan *et al.* 2004).

In view of the fact that the ectomesenchymal cells are regulated by growth factors and downstream transcription factors before they become committed to a number of different cell types (Noden 1983; Lumsden 1988; Le Douarin *et al.* 1993; Imai *et al.* 1996), the cultured undifferentiated cells were transferred to a specific medium with appropriate ingredients and were then induced to develop into a variety of lineages, including endothelial cells and neuroblasts. These findings on how the fate of the ectomesenchymal cells was determined were important for us to elucidate the mechanism of normal and abnormal orofacial development. Based on results of this study, we also could improve tissue-engineering approaches to repair orofacial structures

(Alsberg *et al.* 2001) and promote the molecular research on the signals that trigger periodontal (Ripamonti & Reddi 1997) or dental regeneration (Smith & Lesot 2001).

MATERIALS AND METHODS

Isolation and culture of ectomesenchymal cells

Eight-week-old BALB/c mice were used in line with the International Guiding Principles for Animal Research (1985). Noon of the day on which vaginal plugs were detected was considered as E0.5. The first branchial arches were dissected from embryos aged E9.5 (Sadler 2000), were minced and then incubated in the solution with 0.125% trypsin and 1 mM EDTA at 37 °C for 5 min. After neutralization of the trypsin, cells released from mandibular arch specimens were collected by centrifugation at 1000 g for 5 min. The cell pellet was resuspended and seeded in medium containing DMEM/F12 (1 : 1, Gibco), 10% foetal bovine serum (FBS, Gibco), 10⁶ U/l leukaemia inhibition factor (LIF, Chemicon International, Temecula, CA) (Wright *et al.* 2003; Deng *et al.* 2004; Tian *et al.* 2004; Jiang *et al.* 2005), 100 U/ml penicillin and 100 g/ml streptomycin. After incubation in plastic flasks for 20 min, most of the ectomesenchymal cells were attached and epithelial cells were still suspended in medium. The supernatant with these unattached cells was removed and was replaced with fresh medium. Cultures were maintained in a humidified atmosphere of 5% CO₂ at 37 °C and cells were passaged three times prior to differentiation or measurement. When 80% confluent, monolayers were trypsinized (0.25% trypsin, 5 min) and the ectomesenchymal cells were further selected (because of their longer time detached from the flask compared to epithelial cells).

BrdUrd labelling for cell proliferation analysis

Cells at passage 3 were resuspended and were seeded into 6-well plates with glass slides, at a density of 1 × 10⁵/well. BrdUrd labelling was performed in 1.0 ml of medium containing 5 μM BrdUrd for 48 h. The slides with labelled cells were washed with PBS, fixed in 4% buffered paraformaldehyde for 20 min and were incubated in 1 M HCl for 30 min. Then cells were washed with PBS again, before being blocked in 1% bovine serum albumin and 1.5% normal goat serum at room temperature for 30 min. Slides were then incubated at 37 °C with monoclonal antibody against BrdUrd (Dako), and secondary antibody labelled with FITC to detect BrdUrd presence in nuclei of the cells.

In situ hybridization for telomerase mRNA

Slides covered with passage 3 cells were pre-treated with 4% buffered paraformaldehyde and 0.1% (v/v) DEPC for 1 h. Hybridization was carried out according to the protocol of the kit (Santa Cruz, CA). Briefly, cells were digested with pepsin to expose the appropriate mRNA fragment and were fixed in 4% buffered paraformaldehyde again. First, slides were pre-hybridized and then hybridized with the telomerase probe, labelled with digoxin overnight in a humidified chamber at 40 °C. Sequentially, they were incubated with secondary biotinylated antibody and horseradish peroxidase conjugated streptavidin (Santa Cruz, CA). The peroxidase reaction was developed using 3,3'-diaminobenzidine tetrahydrochloride (DAB) as chromogen.

TRAP analysis

To statistically assess telomerase activity in the cultured ectomesenchymal cells, we used a TRAP ELISA kit (Roche Molecular Biochemicals, Indianapolis, IN, USA); we performed the TRAP

assay according to the manufacturer's instructions. Primary bone marrow stromal stem cells (BMSSCs) were taken as positive control. In our preliminary experiments, we found that for ectomesenchymal cells or BMSSCs, the amount of PCR-amplified telomerase product detected with the TRAP ELISA kit increased linearly with the total cell number from 1×10^5 to 5×10^6 . So 1×10^6 cells from each cell line were lysed and the extracted protein was used to compare the telomerase activity.

Identification of cultured cells

At passage 3, few epithelial cells remained; most of the culture was made up of mandibular arch ectomesenchymal cells. To verify that the majority were undifferentiated ectomesenchymal cells, cultures were stained with monoclonal antibody to HNK-1 at 1 : 100 dilution, monoclonal antibody to vimentin at 1 : 30 dilution, monoclonal antibody to S-100 at 1 : 100 dilution and monoclonal antibody to cytokeratin at 1 : 100 dilution. (All antibodies were obtained from Dako Cytomation (Carpinteria, CA).)

The proportion of positive staining cells was quantitatively analysed with a colour image analysis system (Media Cybernetics: Image-Pro Plus 5.0). At least 10 000 cells in 100 different regions were counted per slide. These regions were equally distributed throughout the slides. The positive ratio, defined as the number of cells with positive staining divided by the total number of cells counted and expressed as a percentage, was calculated.

Multilineage differentiation of ectomesenchymal cells

The specific medium contained the following components listed in Table 1 to induce multilineage differentiation of ectomesenchymal cells. The medium was replaced every 3–4 days, and this process was repeated till differentiated cells were confluent and harvested for studies.

Histological and immunohistochemical analysis

Cellular morphological features of induced cells, in monolayers, were assessed with standard haematoxylin and eosin staining. Differentiated phenotypes of variant cells were viewed and compared by phase-contrast microscopy. For immunohistochemical analysis, monolayers of cells were prepared on slides fixed in 4% buffered paraformaldehyde. Fixed cells on glass slides were incubated with 3% hydrogen peroxide in methanol for 30 min to inhibit endogenous peroxidase activity. After washing with PBS, they were blocked in 1% bovine serum albumin and 1.5% normal goat serum at room temperature for 30 min. Cells on slides were then incubated overnight at 4 °C with antibodies against nestin, factor VIII, α -SMA (Dako Cytomation (Carpinteria, CA)) and DSPP (Fourth Military Medical University, Xi'an, China). Then, sections were incubated

Table 1. Lineage-specific differentiation induced by medium supplementation

Lineage	Medium	Serum (%)	Supplementation
Neurogenesis	DMEM	None	25 M forskolin, 1% antibiotic/antimycotic
Endothelial cells	DMEM	20% FBS	10 ng/ml VEGF, 1 ng/ml bFGF and 2 ng/ml IGF-I, 1% antibiotic/antimycotic
Myogenesis	DMEM	10% FBS, 5% HS	50 M hydrocortisone, 1% antibiotic/antimycotic
Odontogenesis	DMEM	10% FBS	12 ng/ml TGF- β , 300 μ g/ml BMP-2, 200 μ g/ml Dental Matrix non-collagen proteins (DMNCPs) ^a , 1% antibiotic/antimycotic
Control	DMEM	10% FBS	None

^aThe preparation of DMNCPs was according to Smith & Leaver 1979.

Table 2. Specific primers for PCR amplification with expected fragments size and optimal annealing temperature

Gene	Primers	Annealing temperature (°C)	Fragment (bp)	GenBank No.
β -actin	5'-ACTCTTCCAGCCTTCCTTCC-3' 5'-ACTCGTCATACTCCTGCTTGC-3'	55	313	BC013835
Nestin	5'CCCTCACCCTCTATTTTA-3' 5'-ACTATCTAAACCTTTAGGAGAA-3'	47	439	NM_016701
GFAP	5'ACAGAGGAGTGGTATCGGTC-3' 5'-GAAAACCGCATCACCATT-3'	53	381	NM_010277
vWF	5'GCCTGCGGACATTTTCTC-3' 5'-GGTGGGAAATGAGGGTTG-3'	58	591	NM_011708
α -SMA	5'-CAGGGAGTAATGGTTGGA-3' 5'-CTACTGCCGAGCGTGAGA-3'	54	502	NM_007392
DSPP	5'-GAATAGCACCAACCATGAGG-3' 5'-TCTAACGGAAGTGACGAAAG-3'	63	313	NM_010080
DMP-1	5'-GTGGAGATGACACCTTTGGCGATGA-3' 5'-TATGAGGTCGGAAGAATCTAAAGG-3'	58	546	NM_016779

with secondary biotinylated antibodies, horseradish peroxidase conjugated streptavidin, and were developed using DAB. After rinsing in distilled water, slides were dehydrated in ascending ethanol concentrations, were cleared in xylene and were covered with coverslips for microscopy.

RNA isolation and reverse transcription-PCR

Total RNA was extracted from all the specimens using the TRIzol reagent (Life Technologies, Rockville, MD) according to the manufacturer's protocol. About 1 g of total RNA was reversed transcribed by murine leukemia virus reverse transcriptase (TaKaRa, Jap) and PCR amplification of target message RNA was performed with a TaKaRa PCR kit (TaKaRa, Jap). PCR oligonucleotide primers and annealing temperature are listed in Table 2. The products were electrophoresed on 1.5% agarose gels, stained with ethidium bromide and visualized with QUANTITY ONE software (Bio-Rad).

RESULTS

Proliferative potential of ectomesenchymal cells

Most of the primary cells harvested from the first branchial arch tissues (Fig. 1a) showed fibroblast-like morphology with an elongated spindle-shape (Fig. 1b). Cultured in 10% FBS-containing medium, the undifferentiated ectomesenchymal cells had a striking proliferative potential with a high level of BrdUrd labelling (Fig. 1c). This suggested that the ectomesenchymal cells cultured as monolayers *in vitro* still maintained active renewal potential. *In situ* hybridization for telomerase mRNA provided the reason of why so few of the ectomesenchymal cells reached senescence or apoptosis after several passages (Fig. 1d). Expression of telomerase in isolated cells was at such a high level that it is only otherwise to be seen in cancer cells or in other types of stem cell (Smith & Leaver 1979; Drummond *et al.* 2005; Serakinci *et al.* 2004). Furthermore, telomerase activity in primary ectomesenchymal cells was maintained for a long period until passage 3 was reached (Fig. 1e).

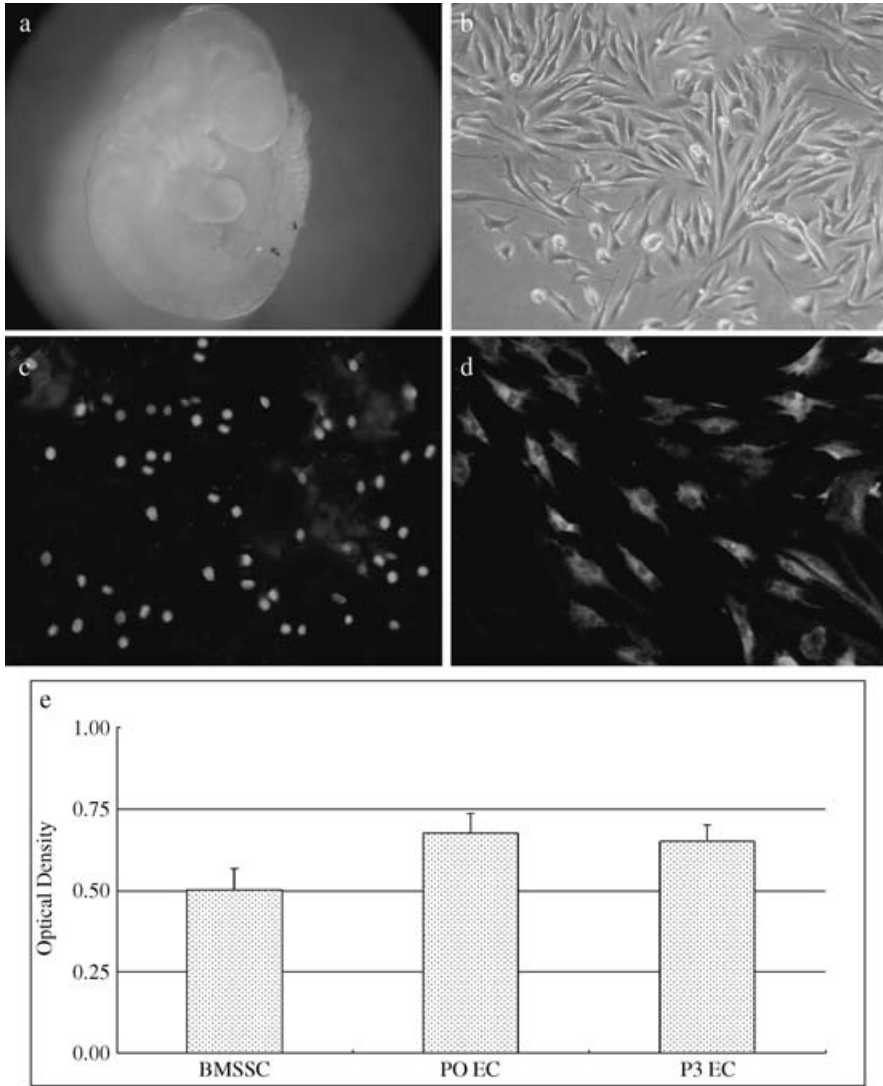


Figure 1. Proliferative potential of ectomesenchymal cells. The first branchial arch primordia (a); most of the primary cells harvested from the first branchial arch tissues showed fibroblast-like morphology with an elongated spindle-shape (b). High levels of BrdUrd labelling indicate a striking proliferative potential of the undifferentiated ectomesenchymal cells (c). The ectomesenchymal cells express high levels of telomerase mRNA (d) and indicate correspondingly high levels of telomerase activity (e).

Characteristics of ectomesenchymal cells

Ectomesenchymal cells attached to the culture flask more rapidly than epithelial cells and remained as monolayers for longer time periods during trypsinization for subsequent passaging. This led to further purification of the ectomesenchymal cell population during this natural time period. By the third passage, cultured cells were positively stained with monoclonal antibody to HNK-1 (Fig. 2a, more than 95% ectomesenchymal cells positive), vimentin (Fig. 2b, more than 90% ectomesenchymal cells positive) and S-100 (Fig. 2c, ~30% ectomesenchymal cells positive), all

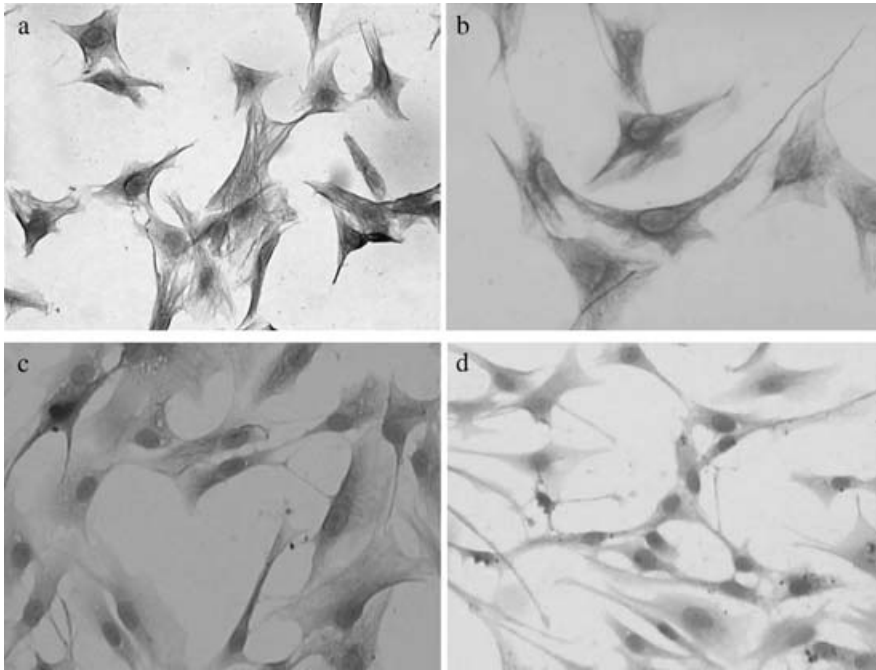


Figure 2. Characters of ectomesenchymal cells. Ectomesenchymal cells positively stained with monoclonal antibodies to HNK-1, vimentin, and S-100, respectively (a,b,c), but almost nothing with the antibody to cytokeratin (d).

being markers of undifferentiated ectomesenchymal and neural crest cells, but most cells were not stained with the antibody to cytokeratin (Fig. 2d, most ectomesenchymal cells negative).

Neurogenesis

Cultured cells were induced to palisade-like parallel arrangement after 2 days in the medium containing 25 nM forskolin. Then they became bipolar and larger. Treated after 4 days, morphological changes in the cells were distinguished; they became long and asymmetrical with time (Fig. 3a). Results of the immunohistochemical assays showed that most induced cells expressed nestin (Fig. 3b, ~60% ectomesenchymal cells positive). Meanwhile, reverse transcription (RT)-PCR also confirmed neurogenesis in the cell cultures with the expression of nestin and GFAP at mRNAs (Fig. 5).

Induction of the endothelial phenotype

Morphology of induced cells indicated that they developed the endothelial phenotype in conditioned medium with 20% FBS, 10 ng/ml VEGF, 1 ng/ml bFGF and 2 ng/ml IGF-I after 14 days culture. Cells shortened and rounded like paving stones during the differentiative process (Fig. 3c) and were strongly positively stained with the antibody to factor VIII protein (Fig. 3d, ~90% ectomesenchymal cells positive). Intensities of vWF bands in RT-PCR analysis further verified the expression of endothelial cell marker genes (Fig. 5), whereas non-induced negative controls were stable with no sign of phenotypic alteration.

Myogenesis

After being in the myogenic medium for 3 days, the ectomesenchymal cells exhibited changes in their morphological features. They became larger and flatter like myofibroblasts. With increasing culture

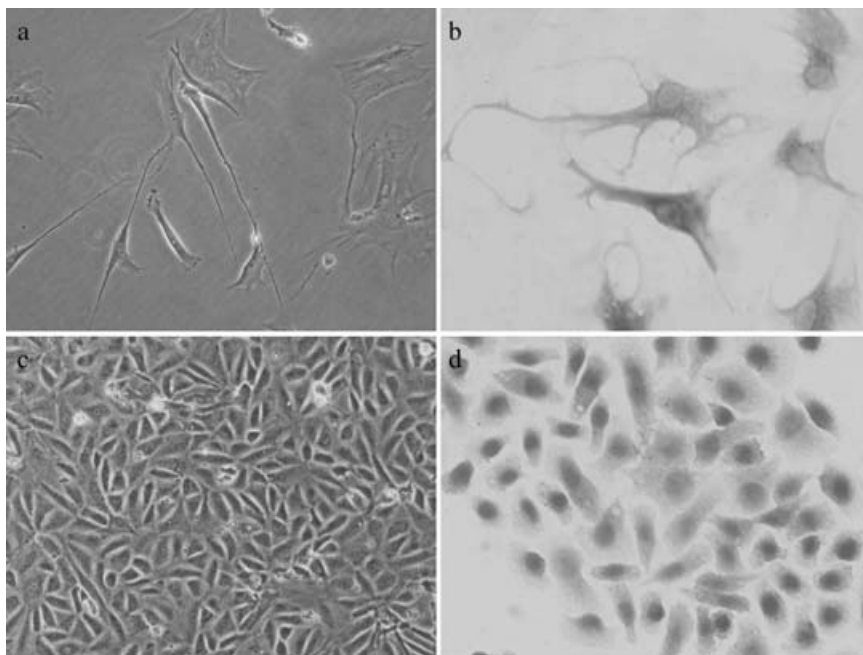


Figure 3. Neurogenic and endothelial potential of ectomesenchymal cells. After 4 days of neurogenic culture, morphological changes of cells were distinct; they became long and asymmetric with increasing time (a). Immunohistochemical assay showed that a high proportion of the induced cells expressed nestin (b). After culture in endothelial medium for 14 days, ectomesenchymal cells became shortened and rounded like paving stones, during differentiation (c), and became stained with the antibody to factor VIII protein (d).

time, this trend became more obvious, finally with almost all cells taking on a flattened morphology at 7 days (Fig. 4a). The majority of the flattened cells expressed α -SMA, a well-characterized smooth muscle marker (Fig. 4b, ~70% ectomesenchymal cells positive). Prolonging the culture period with induction medium, to 2 weeks, could increase the proportion of α -SMA positive cells. In contrast, the cells remaining without myogenic supplements did not stain with the antibody to α -SMA. RT-PCR analysis of mRNA using α -SMA specific primers showed that the induced ectomesenchymal cells had a high level expression, but no expression was observed in the control group (Fig. 5).

Odontogenesis

After 2 weeks of induction, ectomesenchymal cells had significantly switched to a bipolar morphology when compared to intact controls (Fig. 4c). Accompanied by the changes in phenotype, DSPP expression at mRNA and protein levels both increased dramatically (Fig. 4d, ~65% ectomesenchymal cells was positive). Measurement of mRNA encoding DMP-1 and DSPP indicated that there was high expression in odontogenic cells (Fig. 5). These genes were not observed expressed in the cells incubated in control medium.

DISCUSSION

In mammals, CNCC migrate ventrolaterally as they populate the branchial arches during craniofacial development. The ectomesenchymal cells of the first branchial arch originate from the rostral

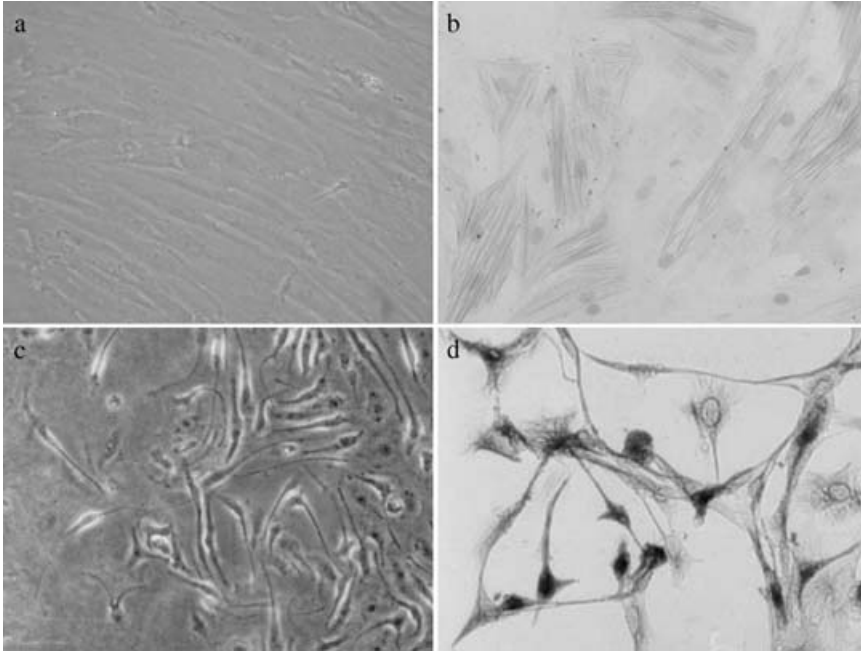


Figure 4. Myogenic and odontogenic potential of ectomesenchymal cells. After being placed in myogenic medium for 7 days, ectomesenchymal cells became larger and flatter resembling myofibroblasts (a). The majority of the flattened cells expressed α -SMA, a well-characterized smooth muscle cell marker (b). After 14 days of odontogenetic inductive culture, ectomesenchymal cells became polygonal in shape and were characterized by long cytoplasmic processes (c). Immunocytochemical staining demonstrated that DSPP was expressed (d).

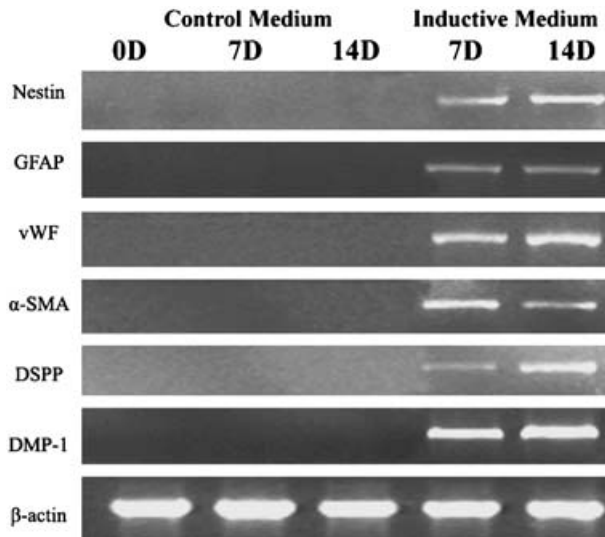


Figure 5. Expression of specific genes was analysed by RT-PCR in inductive and control groups. Each group was assayed at different time points including 0, 7 and 14 days. Results in each group were reproducible.

hindbrain or caudal midbrain (Osumi-Yamashita *et al.* 1994; Lee *et al.* 1995; Imai *et al.* 1996) and develop into a diverse array of head and neck structures during embryogenesis. The present study has focused on proliferative potential and multilineage capacity of these ectomesenchymal cells.

Normal mouse adult cells undergo a finite number of cell divisions and ultimately enter into an arrested state called senescence. But the ectomesenchymal cells here had a high proliferative potential in monolayer culture conditions *in vitro*. Following several cell expansions, they still labelled extensively with BrdUrd, in the same manner as the primary cells. *In situ* hybridization indicated that their expression of telomerase mRNA was stable and high during culture time in the undifferentiated state. It has been proposed that telomere shortening is the molecular clock that triggers cellular senescence, so viability of these ectomesenchymal cells may have been correlated with the activity of telomerase. Previous studies have reported that telomerase is present in proliferative stem cells of renewal tissues and it is required for the maintenance of the stem cell state for cell proliferation and for differentiation. It has been suggested possibly as a common marker for stem cells (Shi *et al.* 2002; Simonsen *et al.* 2002; Lin *et al.* 2004). LIF is a member of the interleukin (IL)-6 family, a multifunctional cytokine that acts on a wide range of cell types. LIF has also been identified as an inhibitor of differentiation of mouse embryonic stem (ES) cells. Mouse ES cells can be maintained without feeder layers in the presence of mouse LIF and fetal bovine serum. In previous studies, our group has isolated undifferentiated ectomesenchymal cells from the first branchial arch, which express HNK-1, in accordance with characteristic properties of migrating neural crest cells. These cells were able to renew while grown in the presence of LIF and retained the potential to differentiate into different phenotypic progeny, thereby displaying the characteristics of stem cells (Tian *et al.* 2004; Yan *et al.* 2004; Jiang *et al.* 2005; Xie *et al.* 2005). However, without the existence of LIF, the cells showed a tendency to spontaneously differentiate. These differentiated cells expressed α -SMA or type I collagen, but not GFAP or NF in the absence of LIF, which suggested that those cells would have differentiated along the smooth muscle cell or osteoblast (although not glial or neural) lineages (Shellard *et al.* 1996; Wright *et al.* 2003; Deng *et al.* 2004).

Stem cells are considered to possess not only self-replicating potential but also the ability to differentiate terminally to multiple lineages (Hall & Watt 1989). Until now, the embryonic stem cell has been the gold standard, which is capable to be induced into cells from all three embryonic germ layers (Shamblott *et al.* 1998; Park & Han 2000). Our findings have suggested that, like embryonic stem cells, ectomesenchymal cells isolated from the first branchial arch had a *trans*-germ potential to differentiate into endothelial, odontogenic and neurogenic lineages. Traditionally, it was believed that the differentiation of adult stem cells progressed in a limited manner and their fate was restricted within a germ lineage. Recent reports, however, have shown the neurogenic potential of MSCs (Mizuno & Hyakusoku 2003), the induction of HSCs into hepatocytes (Legasse *et al.* 2000) and the conversion of neurogenic precursors into muscle and blood (Bjornson *et al.* 1999; Galli *et al.* 2000). In view of the plasticity observed in adult stem cells, we thought it of great value to study the differentiative potential and optimized culture conditions of ectomesenchymal cells from the mandibular arch.

This present study has significantly advanced the understanding of how this important population of pluripotent cells was initially established and the mechanisms that mediated their subsequent lineage proliferation, differentiation and final contribution to a particular cell type. Both intrinsic and extrinsic regulatory signals are critical for the proper migration and proliferation of this CNCC population (LaBonne & Bronner-Fraser 1999). Growth and transcription factors, such as BMP, TGF- β , FGF, Hox, Msx and Pax, have been implicated during the fate determination of neural crest cells (Garcia-Castro & Bronner-Fraser 1999; Christiansen *et al.*

2000). In early embryogenesis, ectomesenchymal cells of the mandibular arch have been described as rostral odontogenic or caudal skeletogenic cells, respectively. Teeth develop from the rostrally located ectomesenchymal cells *via* interactions with the overlying oral epithelial cells. It is accepted that signals from the epithelium are necessary for tooth development. More caudal ectomesenchymal cells do not form teeth *in vivo* but give rise to the skeletal tissues of the mandible (Tucker *et al.* 1999). Fibroblast growth factor-8 (FGF-8) has been identified as one of the key signalling molecules from oral epithelium capable of inducing and/or maintaining expression of several different mesenchymal genes in the mandibular arch (Neubüser *et al.* 1997; Ferguson *et al.* 1998; Kettunen & Thesleff 1998). In this study, the ectomesenchymal cells were seeded in medium containing dental matrix non-collagenous protein and growth factors such as TGF- β 1, BMP-2 and FGF-8 during odontogenesis (Tian *et al.* 2004; Yan *et al.* 2004; Jiang *et al.* 2005; Xie *et al.* 2005). In our previous studies, we tried to investigate the effects of FGF-8 on CNCC differentiating into ectomesenchymal cells of the first branchial arch and to determine the appropriate dose and stage of CNCC exposure to FGF-8. Cranial neural crest explants were cultured in serum-free medium containing modified DMEM/F12 and were supplied with varying doses of FGF-8. The differentiation types of CNCC were determined by *in situ* hybridization for *Hoxa2* and immunocytochemistry was for vimentin expression (Yan *et al.* 2004). These studies proved that the pre-emigrating CNCC did not express *Hoxa2* but did express vimentin after treatment with 100 μ g of FGF-8. Both the post-emigration CNCC group and the control group expressed *Hoxa2* and vimentin. Thus, we concluded at an early stage of CNCC emigration that the first branchial arch phenotype of CNCC could be induced by treatment with FGF-8 and that this experiment could provide an *in vitro* model for study of the mechanism of tooth-jaw regeneration (Tian *et al.* 2004; Yan *et al.* 2004; Jiang *et al.* 2005; Xie *et al.* 2005).

Differentiation into various lineages was assessed using immunochemical staining and molecular analysis of specific genes or proteins. Accumulation of type I collagen and mineralization demonstrated the onset of overt odontogenesis, coincident with DSPP and DMP secreted by induced cells. Dentin is a mineralized tissue with contents similar to those of bone. The dentin extracellular matrix is composed of approximately 47% (v/v) mineral, 30% (v/v) protein and 21% (v/v) water (Legeros 1991). In dentin, type I collagen accounts for about 90% of the protein fraction and most of the dentin non-collagenous proteins are also expressed in bone. However, some proteins appear to be more specifically expressed in dentin even though they can be found, albeit weaker, in other tissues. For instance, odontoblasts express DMP-1 and DSPP, the latter turns into dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) after proteolytic cleavage (Ritchie & Wang 1996; MacDougall *et al.* 1997; Gu *et al.* 1998). DPP is involved in matrix mineralization with a high affinity for calcium ions (Marsh 1989) and hydroxyapatite crystals (Fujisawa & Kuboki 1991).

In addition to odontogenesis, ectomesenchymal cells can be induced to differentiate into further mesodermal lineages such as to endothelial cells. Phenotypic alteration in the cells occurred when seeded into conditioned medium, paralleling with expression of factor 8 and vWF, which are essential molecules of the haemostatic mechanism (Escolar *et al.* 1998) and markers of endothelial differentiation (Chung-Welch *et al.* 1989). Simultaneously, high expression of nestin after specific induction, rapidly verified the procedure of early neurogenic differentiation in these ectomesenchymal cells. Nestin is an intermediate filament protein expressed at high levels in neural stem cells (Sanchez-Ramos *et al.* 2000). Its expression has also been observed in myogenic cells, endothelial cells and hepatic cells, indicating that it cannot be used as a marker for putative neurogenic potential. However, neurogenic induction of cells has also resulted in the assumption of neurogenic morphology and the increased expression of GFAP, the two of which bind together to form the main intermediate filament found in specialized brain cells (astrocytes). Astrocytes are star-shaped cells that support the functions of nerve cells in the brain and spinal cord. In

summary, the expression of several lineage-specific genes and proteins revealed that these ectomesenchymal cells had both mesodermal and ectodermal capacity.

The biological function of these cranial neural crest-derived cells has been studied in a variety of animal models. At first, it was proposed that they carried certain pre-acquired molecular signals as they migrated into the branchial arch. Recent studies have shown that they acquire positional identity at the time they reach their final destination and contribute to the formation of various craniofacial structures. Certain growth and transcription factors have been implicated as important regulators for the critical epithelial–mesenchymal interactions through which these multipotent neural crest-derived cells become progressively restricted to form neural crest derivatives and eventually develop into individual cell types (Anderson 1997; Thesleff & Sharpe 1997; Tucker & Sharp 1999). In agreement with these findings, we induced multiple germ layer differentiation of ectomesenchymal cells in appropriate conditions.

Thus, it is suggested that ectomesenchymal cells derived from first branchial arch might represent a novel source of multipotential stem cells capable of undergoing expansion and variant differentiation *in vitro*. Based on the results, we have obtained valuable information concerning the fate of the ectomesenchymal cells. It is important that we have elucidated a mechanism in which normal or abnormal orofacial development occurs. Also, we have made it clear which growth factors or cytokines play key roles during isolated cells differentiating into many kinds of committed lineages. With optimization of inductive conditions and manipulation of pivotal genes by vectors, we could improve the tissue-engineering approaches to repair of the orofacial structures (Alsberg *et al.* 2001) and promote molecular research into signals that trigger periodontal (Ripamonti & Reddi 1997) or dental regeneration (Smith & Lesot 2001) in the future.

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