

Expression of neural crest markers by human embryonic stem cells: an introductory project

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Introduction: Neural crest cells make up a transient migratory population of cells found in all vertebrate embryos. Great advances have been made over the past 20 years in clarifying the molecular basis of neural crest induction and, although much still remains unclear, it appears that it is a process involving several factors acting at different stages of embryogenesis. In the future, an understanding of the precise mechanisms involved in orofacial development, even at the earliest stages, may well be of use to all clinicians interested in the management of these tissues.

Aim: The present study was designed to determine if the early addition of noggin (a bone morphogenetic protein (BMP) antagonist) and/or the late addition of BMP4 would increase the expression of the transcription factors: Msx-1, Snail, Slug and Pax-7.

Method: This involved an assessment of the effects of early addition (Days 0 to 3) of noggin and/or the late addition (Days 4 to 7) of BMP4 on the expression of the neural crest markers by human embryonic stem cells, co-cultured for eight days on a feeder layer of mouse PA6 cells.

Results and conclusions: The expression of the neural crest markers Pax-7, Msx-1, Slug, and Snail by human embryonic stem cells is likely to be affected by the addition of noggin and BMP4. Not all of these effects will necessarily be significant. The late addition of BMP4 is likely to significantly increase the expression of Pax-7 by human embryonic stem cells (hESCs), when compared with the effects of co-culturing with stromal cell-derived inducing activity, alone. The early addition of noggin and the late addition of BMP4 are likely to significantly increase the expression of Msx-1 by hESCs, when compared with the late addition of BMP4, alone. The hESC results support those from animal ESC studies that the late addition of BMP4, especially, may result in the differentiation of neural crest precursors.

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Introduction

Neural crest cells (NCCs) are a transient migratory population of cells found in all vertebrate embryos.¹ They segregate from the dorsal portion of the neural tube and migrate through the embryo to generate a highly pluripotent cell population, giving rise to a diverse range of tissues including bone, cartilage, muscle, connective and peripheral nerve tissues in the head.^{2,3} The differentiation of each particular NCC is dependent on its own innate program as well as signals to which it is subjected from the surrounding

environment. Once NCCs are induced at the border of the neural plate, a genetic cascade of transcription factors is activated. This controls the specification, migration, and final differentiation of the cells.² Great advances have been made in clarifying the molecular basis of neural crest induction, and although much still remains unclear, it appears that it is a process that involves several factors acting at different stages. Some of these factors have been identified in the frog, fish and chick. These factors include bone morphogenetic proteins (BMPs) and BMP antagonists, as well as neural crest transcription factors such as Msx-1, Snail,

Slug and Pax-7.¹ There is growing evidence that, in both the fish and the frog, the neural crest is induced by a gradient of BMPs, which is in part created by the secretion of BMP antagonists, such as noggin. The neural crest has been shown in the frog and the fish to be specified by levels of BMP, intermediate to those required for the specification of the neural plate and epidermis.²

Mizuseki et al.⁴ studied the effects of the addition of BMP4 at different times on the differentiation of mouse and primate embryonic stem cells (ESCs), both of which were co-cultured on a feeder layer of mouse PA6 stromal cells. It was found that the late addition of BMP4, after the fourth day of co-culture, resulted in the differentiation of neural crest precursors, with a higher concentration (5.0 nM) of BMP4 giving rise to neural crest-derived autonomic progenitors and a lower concentration (0.5 nM) giving rise to neural crest-derived sensory progenitors. Early exposure of ESCs to BMP4 (Days 0 to 3) strongly promoted epidermogenesis, rather than neural crest induction. The study by Mizuseki et al.⁴ demonstrated that systemic generation of NCCs from ESCs, *in vitro*, is possible by manipulating the timing of the addition and the concentration of exogenous factors such as BMP4.

Interestingly, Pomp et al.⁵ first reported the expression of neural crest markers in human embryonic stem cells (hESCs), which they co-cultured on a mouse PA6 feeder layer for three weeks. After seven days of co-culture, several molecular markers were detected in association with neural crest development. PA6 cells are mouse stromal cells derived from skull bone marrow.⁶ Kawasaki et al.⁷ discovered that co-culturing mouse ESCs on a mouse stromal PA6 feeder layer promoted neural differentiation of the ESCs. This was termed neural-inducing activity of PA6 cells: SDIA (stromal cell-derived inducing activity). The precise

molecular mechanism of SDIA is still unknown, but it possibly involves a combination of PA6 cell surface-anchored factors and soluble factors secreted by the PA6 cells, themselves.

The hypothesis of the present study was that the early addition of noggin and/or the late addition of BMP4 would increase the expression of the transcription factors: Msx-1, Snail, Slug and Pax-7. It is well known that various orofacial tissues are closely related to overall post-natal growth in the craniofacial region.⁸⁻¹³ It was expected that this work might lead to a greater understanding of the development of the tissues in the craniofacial region, in addition to targeted treatment to replace lost or damaged tissues, mechanisms to increase the amount of available tissue in under-developed areas and methods by which the development of various related tissues and structures might be influenced.

Materials and methods

All experiments were approved by the National Health and Medical Research Council (NHMRC), authorising the use of excess assisted reproductive technology (ART) embryos (Licence number: 309707). They were also performed in accordance with the guidelines from the Standard Operating Procedure (SOP) manual at the Monash Immunology and Stem Cell Laboratory (MISCL). The experimental conditions for all Groups A to E are summarised in Table I.

Human embryonic stem cell culture

Human embryonic stem cells of cell line HES2 (Embryonic Stem-cell International, ESI, Singapore) were co-cultured on a mitotically-inactivated human fibroblast feeder layer in tissue culture dishes and passaged

Table I. Experimental conditions for Groups A to E.

Group	No. wells	Days 0 – 3	Days 4 – 7	No. repeats
A (control) hESCs + Feeder layer	3	-	-	3
B hESCs + Feeder layer	3	-	5 nM BMP4	3
C hESCs + Feeder layer	3	500 ng/mL noggin	5 nM BMP4	3
D hESCs + Feeder layer	3	500 ng/mL noggin	-	3
E (control) Feeder layer only	3	-	-	3

every seven days in hESC medium: 80% KnockOut™ Dulbecco's Modified Eagle Medium (KO-DMEM), 20% KnockOut™ Serum Replacement (KO Serum), 1% Non-Essential Amino Acids (NEAA), 1% Gluta-Max (Gibco™), 1% Pen/Strep, 55mM 2-β mercaptoethanol, 1% Insuline/Transferrin/Selenium (ITS) (Gibco™) and 10 µg/ml fibroblast growth factor (FGF).

Maintenance and preparation of the mouse stromal PA6 cell feeder layer

PA6 cells (Riken Cell Bank, Riken, Japan) were cultured in a T-25 flask (In Vitro, Becton Dickinson, NJ, USA) with Alpha-Minimum Essential Medium (α-MEM) supplemented with 10% foetal bovine serum (FBS) and 1% 50 µg/ml penicillin/streptomycin (Pen/Strep). The medium was changed daily for five days as the average time taken for the cell layer to achieve 90% confluency as observed under the light microscope (×100). The PA6 cells were then mitotically inactivated by the addition of mitomycin-C at the concentration of 50 µl pre-prepared 200 µg/ml mitomycin-C solution per ml of PA6 medium.

Confluent cultures were treated with trypsin/ethylene diaminetetraacetic acid (EDTA) for three minutes to detach them from the base of the flask. The cell suspension was then centrifuged (1,800 rpm for three minutes) to obtain a cell pellet and to allow removal of trypsin/EDTA. The cell pellet was re-suspended in the PA6 medium and transferred to 48-well plates (In Vitro, Becton Dickinson, NJ, USA). Approximately 4.5×10^4 cells were transferred to each well and allowed to attach to the floor of the wells overnight. To pre-equilibrate the PA6 cells prior to co-culturing with human embryonic stem cells, the following day the PA6 medium was replaced with hESC medium.

Co-culture of human embryonic stem cells on the PA6 feeder layer and treatment with noggin and BMP4

Each PA6-containing well was seeded with a $10^{-3} \times 10^{-3} \text{ m}^2$ human embryonic stem cell colony fragment. This was designated as Day 0. The hESC medium was changed daily. The wells were divided into Groups A-E according to which factors were added or omitted from their hESC medium (Table I). Under all conditions, PA6 cells and hESCs were cultured in air containing 5% CO₂ at 37°C. Photographs of

one well, representative of each group, were taken daily using bright field (BF) light microscopy at ×100 magnification (Olympus™ IX81, Japan) to observe any obvious differences in the morphological changes occurring in the various groups.

Ribonucleic acid (RNA) extraction and quantitative polymerase chain reaction analysis

On Day 8, total cellular RNA was extracted from all of the cells (PA6 cells and hESCs) in each well, using the PicoPure™ RNA Isolation Kit (Arcturus, Mountain View, CA, USA). Complimentary deoxyribonucleic acid (cDNA) synthesis was carried out using oligo(dT)₂₀ (Invitrogen) and SuperScript™ III Reverse Transcriptase (Invitrogen). Q-PCR was carried out to analyse the expression of specific messenger RNA (mRNA) (Msx-1, Snail, Slug, and Pax-7) in each well for Groups A to E. Amplification products were designed to cross introns to distinguish cDNA from genomic DNA. All real-time PCR was performed with the Mastercycler ep realplex using the Eppendorf RealMasterMix (Eppendorf AG, Hamburg, Germany). Reactions were set up in 96-well plates (Eppendorf AG, Hamburg, Germany) and contained 8 µL SuperMix, 1 µl of 10 µM forward primer, 1 µl of 10 µM reverse primer, 1 µl of 5 µM hybridisation probe, 9 µl distilled water and 1 µl cDNA. Amplification took place as follows: 95°C for two minutes, then 50 cycles of 95°C for 15 seconds, 52°C for 15 seconds, and 68°C for 20 seconds. A no cDNA control group was also included for each primer pair. The sense (forward) and antisense (reverse) primer sequences are listed in Table II. The probe sequences are listed in Table III.

Table II. Sense and antisense primer sequences.

SNAIL Forward	ACCTATGAGACTGGGTTTTCC
SNAIL Reverse	AAGCCACAGTGATAGTGAAGAG
SLUG Forward	AAAAGTCTCCAAAACCTTCTCC
SLUG Reverse	CGAGTAAACATTGATTGCGTCAC
MSX1 Forward	GCTACAGCATGTACCACCTGAC
MSX1 Reverse	GGAGCAGTGTGAGGGTTAAAGG
PAX7 Forward	CCCCGTCCCCATTCTTCTC
PAX7 Reverse	CTGTCCATCTGGCTGGACTTC
ACTIN Forward	GTGCCAGATAGTGCGGGTATAG
ACTIN Reverse	TTGTTTGCCCTCGTCTTGTC

Table III. Probe sequences.

SNAIL	CCTGGGTGTCCTGTCTGCTGCT
SLUG	ACACAGCAGCCAGATTCCCTCATGTTTGT
MSX1	AGAGGGTCCCAGGTCGCCCCACC
PAX7	TCTGTGAAATGGACATCCCCACCCTGAC
ACTIN	AACCAACCAGATGTGTTCCGTGTCCTCA

Statistical analysis

Fifteen colonies were observed in each experiment and each experiment was conducted three times (including two repeats). Tukey’s Multiple Comparison Test¹⁴ was used to compare the statistical significance of differences between the various groups. *P* values for the statistical significance of differences observed were calculated.

Results

Photography

Photographs taken of representative colonies on Day 0 and Day 8, respectively, are shown in Figure 1. The photographs taken for each of Groups A to D on Day 0 show the initial ‘plate-down’ (seeding) of a sectioned fragment of a human embryonic stem cell colony on the feeder layer of PA6 cells. Each fragment has a relatively well defined border and contains small and homogeneous cells. The photographs taken for each of Groups A to D on Day 8 show the central portions of expanding and differentiating cell colonies. The borders are still reasonably well defined although the cells radiating out from the centre are relatively large in size compared with their appearance on Day 0. They would therefore likely be in the process of differentiation. The photographs taken for Group E (Days 0 and 8) represent control images of only the PA6 feeder layer cells, without the co-culture of the human embryonic stem cells.

On Day 8, the quantitative polymerase chain reaction was used to assess the effects of the early addition of noggin and the late addition of BMP4 on the expression of a small collection of NCC markers: *Msx-1*, *Snail*, *Slug*, and *Pax-7*. The results of the Q-PCR analysis are presented in Figures 2 to 5. It should be noted that the values reported regarding the expression of each particular marker under different experimental conditions are relative rather than quantitative and, as such, the values obtained for the different markers cannot be directly compared with each other.

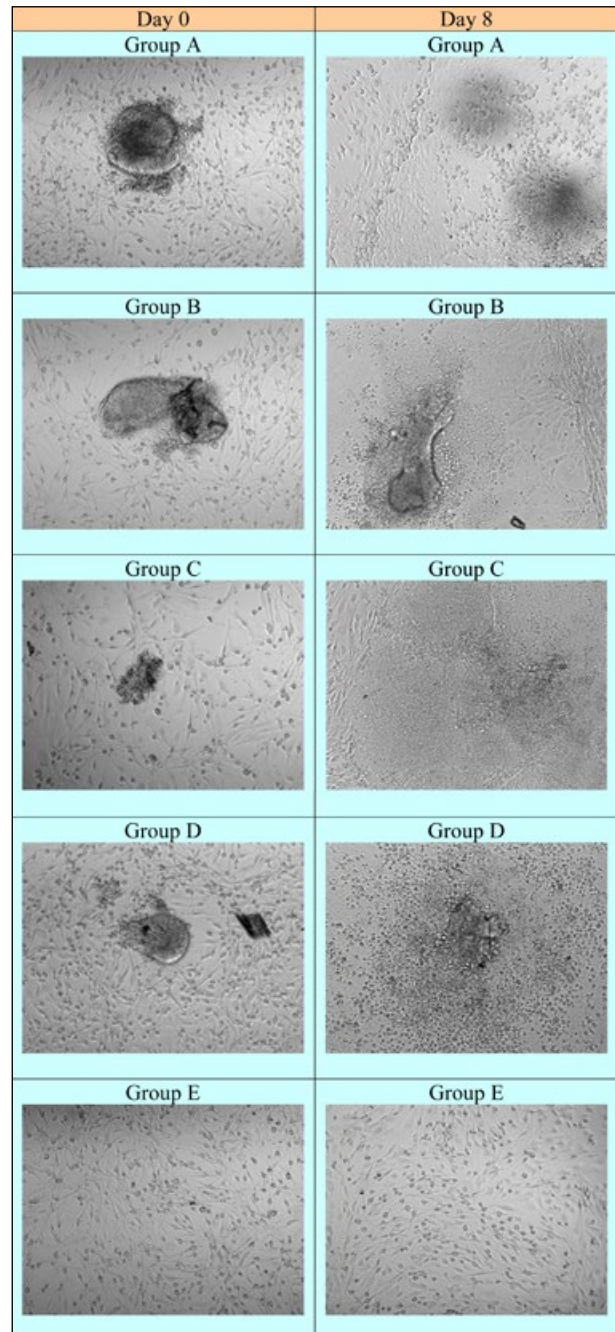


Figure 1. Photographs of human embryonic stem cell colonies using bright field (BF) light microscopy, at x100 magnification (Olympus™ IX81, Japan). Left side Day 0, Right side Day 8.

- Group A: No noggin, no BMP4 added
- Group B: No noggin, BMP4 added
- Group C: Noggin added, BMP4 added
- Group D: Noggin added, no BMP4 added

Pax-7

Q-PCR analysis revealed that the late addition of BMP4 (Group B) significantly* increased the expression of the neural crest cell marker, *Pax-7*, compared with Group A, in which neither noggin nor BMP4 were

added. Differences observed between other groups were not found to be statistically significant ($p > 0.05$) (Figure 2).

Msx-1

The addition of early noggin and late BMP4 (Group C) significantly* increased the expression of the neural crest cell marker, *Msx-1*, compared with Group B,

in which only late BMP4 was added. No statistically significant differences were observed between the other groups ($p > 0.05$) (Figure 3).

Slug and Snail

The apparent differences seen between the groups in the expression of *Slug* (Figure 4) and *Snail* (Figure 5) were found to be insignificant ($p > 0.05$).

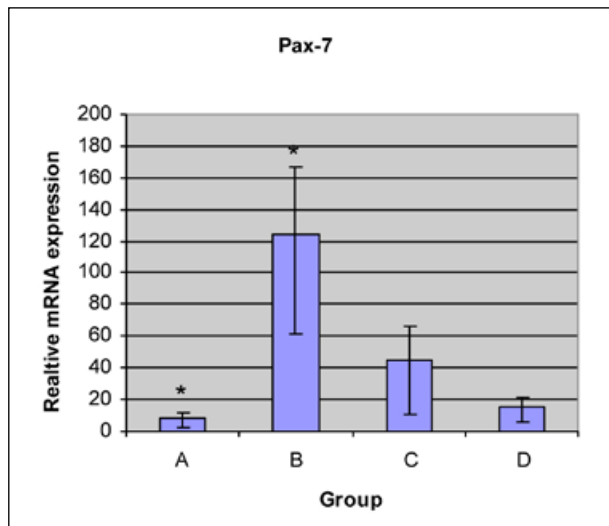


Figure 2. Expression of Pax-7 by human embryonic stem cells – Day 8 (* $p < 0.05$). Late addition of BMP4 (Group B) significantly different to SDIA, alone (group A).

* $p < 0.05$, reflecting a significant difference between the means of the groups.

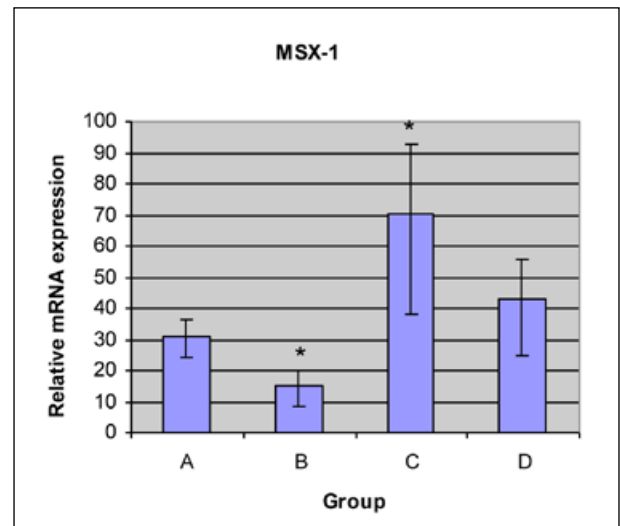


Figure 3. Expression of *Msx-1* by human embryonic stem cells – Day 8 (* $p < 0.05$). Early addition of noggin and the late addition of BMP4 (Group C) significantly different to late addition of BMP4, alone (Group B).

* $p < 0.05$, reflecting a significant difference between the means of the groups.

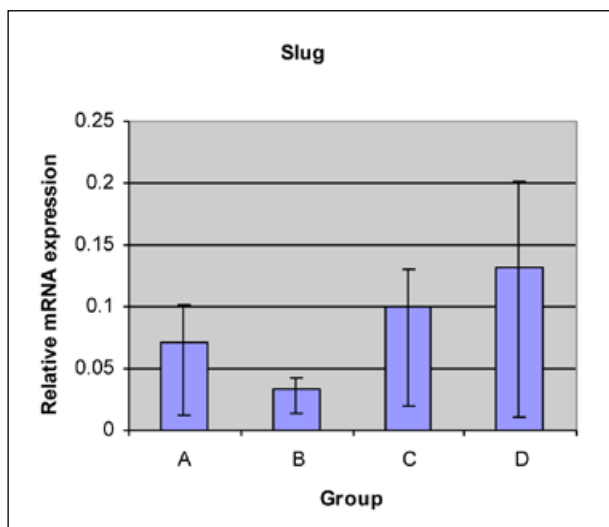


Figure 4. Expression of *Slug* by human embryonic stem cells – Day 8 (NS $p > 0.05$).

* $p < 0.05$, reflecting a significant difference between the means of the groups.

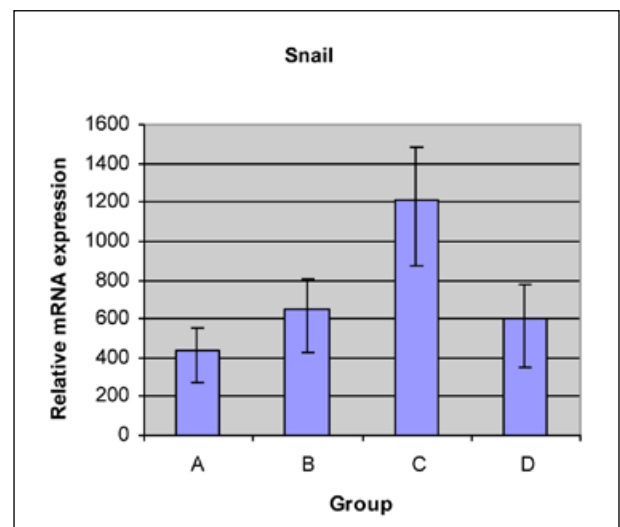


Figure 5. Expression of *Snail* by human embryonic stem cells – Day 8 (NS $p > 0.05$).

* $p < 0.05$, reflecting a significant difference between the means of the groups.

Discussion

The effect of BMP4 concentration in neural crest induction

The results of past animal studies^{4,15} have shown that the addition of exogenous growth factors can modify the expression of target genes of embryonic stem cells but only during a specific window of competence. The factors controlling when and how these windows might be opened and closed remain largely unknown.¹⁶

NCC development, in particular, is known to be susceptible to modulations in BMP signalling.¹⁷ Previously-described models for the formation of NCCs in frogs and fish suggest that a gradient of BMP signalling in the neural fold determines the fate of the local cells. A very high level of BMP signalling has been found to promote epidermogenesis, an intermediate level of BMP induces neural crest formation, whereas a lower level of BMP induces neural plate development.^{2,18,19} Mizuseki et al.⁴ studied the effects of the addition of various concentrations of BMP4 (0.5 nM and 5.0 nM) on the expression of neural crest markers in *primate and mouse* embryonic stem cells. The experiments conducted in the present study examined the effects of the addition of 5.0 nM BMP4 on *human* ESCs, based on the concentration of BMP4 used previously by Mizuseki et al.⁴ It is acknowledged that, for financial reasons, the present study was of a smaller scale compared with that of Mizuseki et al.⁴ and, consequently, the results might not be as conclusive. However, it is conceivable that, even if the larger-scale conditions of the experiment were to be replicated with human ESCs rather than animal ESCs, the concentrations of BMP4 that were found by Mizuseki et al.⁴ to increase the efficiency of differentiation of NCCs from ESCs in animals might not achieve the same results in humans, because of inter-species differences.¹⁷

The effect of the timing of BMP4 exposure in neural crest induction

Kawasaki et al.⁷ previously reported that the stromal cell-derived inducing activity of a PA6 feeder layer promotes neural differentiation of mouse ESCs in co-culture. Mizuseki et al.⁴ expanded on these findings by studying the addition of exogenous BMP4 on ESC differentiation in a ESC/PA6 co-culture. In addition to studying the effects of various concentrations of BMP4 on the efficiency of neural crest cell generation,

the effects of different temporal additions of BMP4 to the ESC/PA6 co-culture were also examined. Mizuseki et al.⁴ found that early exposure (Days 0 to 3) of ESCs to BMP4 drives the ESCs down the ectodermal pathway, rather than promoting neural differentiation.

In the present study, the decision to add BMP4 late in the co-culture on Days 4 to 7 was based on the previously described findings of Mizuseki et al.⁴ One of the primary factors that could explain why the present results differed from those found in mice and primates⁴ is that the temporal expression of BMP4 is species-specific. In fact, such different temporal exposure is believed to contribute to the mechanism by which craniofacial morphogenetic diversity exists amongst the various species.

Stromal cell-derived inducing activity

SDIA is a cocktail of largely unknown factors produced by the PA6 cells, which may potentially include BMP4. In keeping with the previous findings of Kawasaki et al.⁷ that the SDIA of a PA6 feeder layer promotes neural differentiation of mouse ESCs in co-culture, in the present study, neural crest cell markers were observed to be expressed even in Group A in which hESCs were exposed to the SDIA of PA6 cells alone, without the addition of exogenous factors. This experiment was *novel* in that a BMP4 antagonist, noggin, was added in the early phase of co-culture (Days 0 to 3) to counteract the possibility that the SDIA, itself, might contain endogenous BMP4 and therefore reduce the early exposure of hESCs to BMP4, and encourage the differentiation of hESCs along the neural crest pathway, instead of the epidermal pathway.

Noggin

BMP antagonists such as noggin are believed to ensure proper BMP activity levels and to define temporal and spatial boundaries within the early neural plate.¹⁷ Anderson et al. demonstrated that noggin performs a critical function as a BMP antagonist during neural crest development in mice by limiting BMP signaling in and around the presumptive neural crest.¹⁷ In a feeder layer-free culture system, Wang et al.²⁰ found that the addition of 500 ng/ml of noggin to the culture medium maintained hESCs in an undifferentiated state. Based on this finding, the same concentration of noggin was applied in the early phase of the present

experiments to discourage early differentiation along the epidermal pathway. It is acknowledged, however, that the precise concentration and timing of noggin exposure required to modulate the necessary levels of BMP4 for neural crest induction are unknown. Although the results in the present study were found to be inconclusive, conducting this experiment on a larger scale would not necessarily yield results that conform to those found in similar experiments in animals, due to the likely inter-species differences that exist in BMP antagonist expression.^{17,21}

In the present study, the addition of the signalling factors BMP4 and/or noggin had an effect on the expression of the neural crest markers Msx-1, Snail, Slug and Pax-7 by hESCs. However, each marker was affected differently by the various experimental conditions tested and some of the differences were found to be statistically insignificant and possibly related to the relatively small sample size. Future possible avenues for research could involve the investigation of how other signalling factors implicated in neural crest development, such as Wnt protein and FGF, may work in the stromal cell-derived inducing activity system.^{22,23}

Neural crest cell markers

There is no single marker known to be specific for NCCs in animals²⁴⁻²⁷ and so, in previous studies of neural crest cell induction, the expression of a combination of markers has been used as a criterion for NCC identity.^{4,28} During the induction of the neural crest in embryogenesis, neural crest markers are expressed at different levels and at different times in different animals. Snail and Slug are expressed in the pre-migratory and/or the migratory neural crest of the mouse, chick, frog and fish, and have been considered to be amongst the earliest known markers for neural crest presence.²⁹ In frogs, Snail lies upstream of Slug in the genetic cascade required for the specification of the neural crest.¹⁸ By contrast, in chicks, Slug is expressed at a later stage related to the closing of the neural folds, and not at the borders of the open neural plate as in frogs.^{30,31} In mouse embryos, Snail is expressed in a manner similar to that of Slug in the chick.³² In mice, the Slug gene is expressed only in migrating crest cells and not in pre-migratory cells.³³

In an earlier study, Tribulo et al.³⁴ found that, in frogs and fish, Msx-1 is upstream of Snail in the specification of the neural crest. Consequently, it was

proposed that Msx-1 could be the earliest gene to activate the neural crest genetic cascade that, in turn, induced the expression of other specific genes that conferred neural crest identity. Pax-7 is expressed at the neural plate border and later at the dorsal neural tube in the mouse and chick. Pax-7 is also important in neural crest cell migration and, subsequently, also important in the formation of facial structures during embryogenesis. It is considered a 'later' neural crest marker.²⁷

The inter-species differences in the expression of various neural crest markers, described above, highlight the limitation in this human embryonic stem cell experiment of selecting early NCC markers based on the results of previous animal studies. In future experiments, further increasing the number of NCC markers being analysed might increase the validity of findings regarding the effects of exogenous factors on neural crest induction. The expression of only four NCC markers was tested in this study, so an absolute conclusion regarding the effect of BMP4 and noggin on the induction of the neural crest cannot be made. Another potential limitation of the present study was that it only provided a snapshot of the markers expressed on Day 8 of the co-culture period and the expression of markers can fluctuate with time. One improvement in experimental design might be to study the expression of the markers progressively from Day 1 to 8, in a manner similar to that in the animal study of Mizuseki et al.,⁴ in order to gain a more accurate picture of the temporal expression pattern of the neural crest markers under the specified experimental conditions. In the present study, Q-PCR was used to analyse the expression of neural crest markers. Neural crest cells were not morphologically distinguishable from human embryonic stem cells with simple light-microscopy, alone (Figure 1). A further improvement to the experimental design would therefore involve immune-staining the cells for neural crest markers prior to light microscopic examination, which would supplement the Q-PCR results. It must also be pointed out that scientific methods have continued to develop rapidly and various 'in vivo' approaches and iPSC display techniques are also currently and widely used.

Sample size

It is acknowledged that the sample sizes (15 colonies in each of the three experiments conducted) were

relatively small in comparison with those described in previous animal studies. Larger samples would have increased the statistical power of the assessment. Unfortunately, the costs associated with an experiment of this type and size are prohibitive without significant support from major institutional funding bodies.

Conclusions

In keeping with outlined limitations of the present study, the following introductory conclusions may be drawn:

1. The expression of the neural crest markers Pax-7, Msx-1, Slug, and Snail by human embryonic stem cells is likely to be affected by the addition of noggin and BMP4. Not all of these effects will necessarily be significant.
2. The late addition of BMP4 is likely to significantly increase the expression of Pax-7 by hESCs, when compared with the effects of co-culturing with stromal cell-derived inducing activity, alone.
3. The early addition of noggin and the late addition of BMP4 are likely to significantly increase the expression of Msx-1 by hESCs, when compared with the late addition of BMP4, alone.
4. These hESC results support those from animal ESC studies that the late addition of BMP4, especially, may result in the differentiation of neural crest precursors.

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