



RESEARCH NOTE

REVISED Assessing the bipotency of *in vitro*-derived neuromesodermal progenitors [version 2; referees: 2 approved, 1 approved with reservations]

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v2 First published: 28 Apr 2015, 4:100 (doi: [10.12688/f1000research.6345.1](https://doi.org/10.12688/f1000research.6345.1))
 Latest published: 31 Jul 2015, 4:100 (doi: [10.12688/f1000research.6345.2](https://doi.org/10.12688/f1000research.6345.2))

Abstract

Retrospective clonal analysis in the mouse has demonstrated that the posterior spinal cord neurectoderm and paraxial mesoderm share a common bipotent progenitor. These neuromesodermal progenitors (NMPs) are the source of new axial structures during embryonic rostrocaudal axis elongation and are marked by the simultaneous co-expression of the transcription factors T(Brachyury) (T(Bra)) and Sox2. NMP-like cells have recently been derived from pluripotent stem cells *in vitro* following combined stimulation of Wnt and fibroblast growth factor (FGF) signaling. Under these conditions the majority of cultures consist of T(Bra)/Sox2 co-expressing cells after 48-72 hours of differentiation. Although the capacity of these cells to generate posterior neural and paraxial mesoderm derivatives has been demonstrated at the population level, it is unknown whether a single *in vitro*-derived NMP can give rise to both neural and mesodermal cells. Here we demonstrate that T(Bra) positive cells obtained from mouse epiblast stem cells (EpiSCs) after culture in NMP-inducing conditions can generate both neural and mesodermal clones. This finding suggests that, similar to their embryonic counterparts, *in vitro*-derived NMPs are truly bipotent and can thus be exploited as a model for studying the molecular basis of developmental cell fate decisions.

Open Peer Review

Referee Status:

	Invited Referees		
	1	2	3
version 2 published 31 Jul 2015	 report		
version 1 published 28 Apr 2015	 report	 report	 report

- Patrick P. L. Tam**, Children's Medical Research Institute Australia, **Pierre Osteil**, Children's Medical Research Institute Australia
- Jacqueline Deschamps**, Hubrecht Institute Netherlands
- Kate G. Storey**, University of Dundee UK, **Laure Verrier**, University of Dundee UK

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How to cite this article: Tsakiridis A and Wilson V. **Assessing the bipotency of in vitro-derived neuromesodermal progenitors [version 2; referees: 2 approved, 1 approved with reservations]** *F1000Research* 2015, 4:100 (doi: [10.12688/f1000research.6345.2](https://doi.org/10.12688/f1000research.6345.2))

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Grant information: This study was supported by the Medical Research Council (MR/K011200).

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: No competing interests were disclosed.

First published: 28 Apr 2015, 4:100 (doi: [10.12688/f1000research.6345.1](https://doi.org/10.12688/f1000research.6345.1))

REVISED Amendments from Version 1

The new manuscript version has incorporated some of the reviewers' comments regarding our observations, conclusions and quality of figures. We have also noted some errors in the clone scoring in Figure 3; some of the clones were either misplaced or were missing from the dataset presented. The updated graphs are now correct to the best of our knowledge. We wish to apologise for these errors.

See referee reports

Introduction

Axis elongation in vertebrate embryos proceeds in a rostral-to-caudal sequence and involves the coordinated production of spinal cord neuroectoderm and paraxial mesoderm/somites from a population of neuromesodermal progenitors (NMPs) (for a review see 1). The bipotent status of these axial stem cells was demonstrated in the mouse by retrospective clonal analysis². NM-potent cells are located in the node-streak border and the adjacent caudal lateral epiblast of early somite stage embryos and in the chordoneural hinge (CNH) region of the tail bud of later stage embryos^{3–5} i.e. in areas exhibiting high levels of Wnt and FGF signaling¹. The main hallmark of these cells is the co-expression of the mesodermal transcription factor T (Bra) together with the neural marker Sox2^{6–9}. NMPs are not only an excellent model for deciphering the mechanisms controlling cell fate choice (neuroectoderm vs mesoderm), but also comprise an attractive source for generating trunk spinal cord neuroectoderm cells and skeletal muscle *in vitro*.

We and others have recently shown that mouse and human pluripotent stem cells cultured for 48–72 hours in the presence of FGF2 and the Wnt signaling agonist CHIRON99021 (CHIR) yield a high percentage of T(Bra)⁺Sox2⁺ double-positive cells that transcriptionally resemble embryonic NMPs^{10,11}. These NMP-like cells were also shown to efficiently differentiate exclusively into paraxial mesoderm and posterior neuroectoderm both *in vitro* and *in vivo* upon grafting into cultured mouse and chick embryos¹⁰ suggesting an NM bipotent character. However, these studies were carried out at the population level and it would thus be important to test the NM potency of single cells. Here we address this issue by showing, through the clonal plating of T(Bra)⁺ cells generated after culture of epiblast stem cells (EpiSCs)^{12,13} in NMP-inducing conditions, that individual *in vitro*-derived NMPs are truly bipotent as they give rise to colonies consisting of both neural and mesodermal cells.

Methods

Cell culture, differentiation and flow cytometry

T(Bra)-green fluorescent protein (GFP) reporter (TGFP) EpiSCs were derived from TGFP embryonic stem (ES) cells (sourced from 14) and cultured routinely in fibronectin-treated plates in N2B27 medium containing 10 ng/ml FGF2 (R&D Systems) and 20 ng/ml Activin A (Peprotech) as previously described in 15. For NMP differentiation TGFP EpiSCs were plated at a density of approximately 1500–2000/cm² in N2B27 medium containing 20 ng/ml FGF2 and 3 μM CHIRON99021 (Stemgent) on fibronectin for 48–72 hrs¹⁰. For clonal plating experiments *in vitro*-derived NMPs were pre-treated

with 10 μM ROCK inhibitor Y-27632 (Calbiochem) for 1 hr prior to fluorescence-activated cell sorting (FACS). After this they were re-plated at a density of 4,000 cells/well in 12-well plates in medium containing either FGF2, or FGF2/CHIR alongside Y-27632 for the first 8 hours. We have previously found that when 1:1 mixtures of GFP⁺ and GFP⁻ EpiSCs are plated at a total of 5,000 cells/well in 12-well plates (or 10,000 cells/well in 6-well plates) then 95% of the resulting colonies between 2–8 cells are of monoclonal origin. Here we also included for scoring colonies of up to 10 cells since we employ a smaller initial plating density (4,000 cells/well)⁶. For non-clonal plating of *in vitro*-derived NMPs, approximately 40,000 cells/well (12-well plate) were used. Cell sorting was performed using a FACSAria (BD Biosciences).

Immunocytochemistry and microscopy

For immunocytochemistry cells were fixed with 4% paraformaldehyde, washed with PBS/0.1% Triton X-100 (PBST), treated with 0.5 M Glycine and blocked in PBST/3% donkey serum/7.5% bovine serum albumin (BSA). Primary antibody incubations were performed overnight at 4°C, followed by PBST washes the following day, incubation with secondary donkey Alexafluor antibodies (Life Technologies) for 2–3 hrs at room temperature and further washes in PBST. The primary antibodies used were: donkey polyclonal anti-T(Bra), 1 μg/ml (RRID: R&D Systems Cat# AF2085 RRID:AB_2200235), rabbit monoclonal anti-Sox2, 0.5 μg/ml (RRID: Abcam Cat# ab92494 RRID:AB_10585428) and goat polyclonal anti-Tbx6, 0.5 μg/ml (RRID: R&D Systems Cat# AF4744 RRID:AB_2200834). Fluorescent images were captured using an Olympus IX51 inverted microscope (Olympus) using a x20 objective and the Volocity software (PerkinElmer). Nuclear segmentation followed by single cell fluorescence quantification was performed as described previously¹⁶. T(Bra) and Sox2 protein positivity scoring of individual clones was carried out manually.

Results

To track the emergence of NMPs *in vitro* we employed a T(Bra) reporter EpiSC line (TGFP) generated from ES cells carrying a GFP transgene knocked into the T(Bra) locus¹⁴. This reporter line has been shown to faithfully recapitulate endogenous T(Bra) expression. In line with our previous findings¹⁰, culture of TGFP EpiSCs in the presence of FGF2/CHIR for 48 or 72 hours gave rise to a significant number of TGFP⁺ cells, many of which were also positive for Sox2 expression (55% of the total TGFP⁺ population at 48 hours and 65% at 72 hours) as revealed by antibody staining and image analysis (Figure 1). Interestingly, TGFP⁺Sox2⁺ cells appeared in “patches” and not in a “salt and pepper” manner, possibly reflecting our previous findings on the mutually exclusive emergence of distinct mesodermal precursors from a heterogeneous starting EpiSC population (6) or non-synchronous generation of NMP-like cells *in vitro*. In summary, these results indicate that at least half of the TGFP⁺ cells emerging in the presence of FGF2/CHIR are NMP-like and thus we used TGFP expression under these conditions to enrich for cells with NMP identity.

We have previously found that prolonged (i.e. more than 72 hours) culture in FGF2/CHIR mediates further differentiation of NMPs into mutually exclusive paraxial mesoderm and neuroectoderm cells¹⁰. Therefore apart from promoting an NMP state these

conditions simultaneously provide an environment for the production of the natural differentiation products of NMPs. We thus utilized culture in FGF2/CHIR in order to test the NM potency of TGFP⁺ NMPs at the population level. TGFP EpiSCs were cultured in NMP-promoting conditions for 48 hours and the resulting GFP⁺ cells were sorted by flow cytometry and re-plated at high density for a further 48–72 hours in the presence of FGF2/CHIR (Figure 2A). We have previously shown that under these conditions hardly any pluripotent cells persist in the differentiating cultures as evidenced by analysis of Nanog/Oct4 expression and grafting into the pluripotency-permissive environment of cultured E7.5 embryos¹⁰. Immunofluorescence analysis of the final cultures showed that sorted TGFP⁺ cells generated predominantly mutually exclusive single T(Bra) positive mesoderm and single Sox2⁺ neuroectoderm

(Figure 2B). The cultures also contained clusters of Tbx6⁺ cells which were distinct from the T(Bra)⁺ and Sox2⁺ domains (Figure 2B) and, since this gene uniquely marks emergent paraxial mesoderm, these cells probably arose from the T(Bra)-expressing population. Together these data confirm that the TGFP-expressing cells produced in NMP inducing conditions possess the ability to generate both neural and mesodermal cells upon further differentiation.

We next examined the behaviour of TGFP⁺ NMPs at the single cell level. TGFP⁺ cells induced after 48 or 72 hrs of FGF2/CHIR treatment were flow sorted (purity >99%) and re-plated at clonal density in FGF2/CHIR-containing medium (Figure 3A,B). After 48 hours the resulting colonies were analysed by immunofluorescence and categorized based on their composition (Figure 3C).

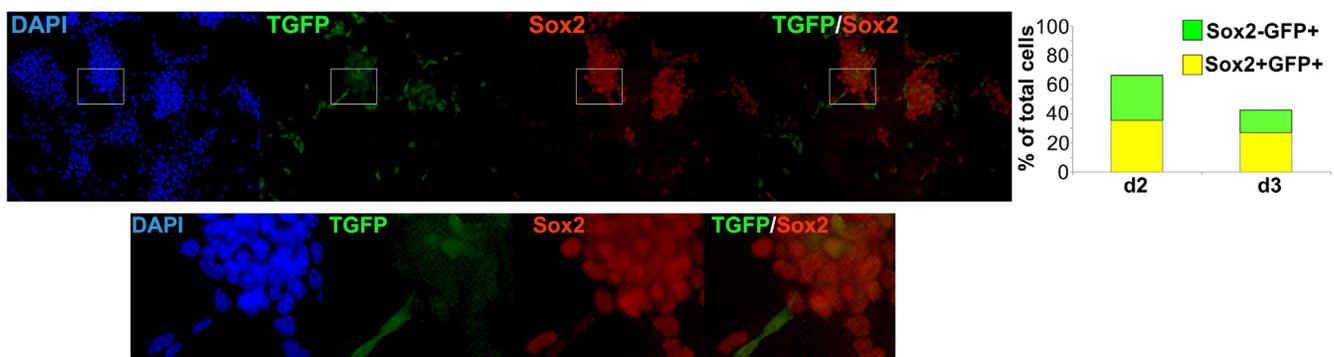


Figure 1. Left: Fluorescence analysis of TGFP and Sox2 expression in TGFP EpiSCs cultured for 48 hours in FGF2/CHIR following antibody staining against Sox2. Right: Quantification of TGFP⁺Sox2⁺ and TGFP⁺Sox2⁻ expressing cells in TGFP EpiSCs differentiated in NMP-inducing conditions after 2 (d2) or 3 (d3) days following immunocytochemistry and image analysis. Magnified versions of the areas marked by a white box are shown in the bottom panel.

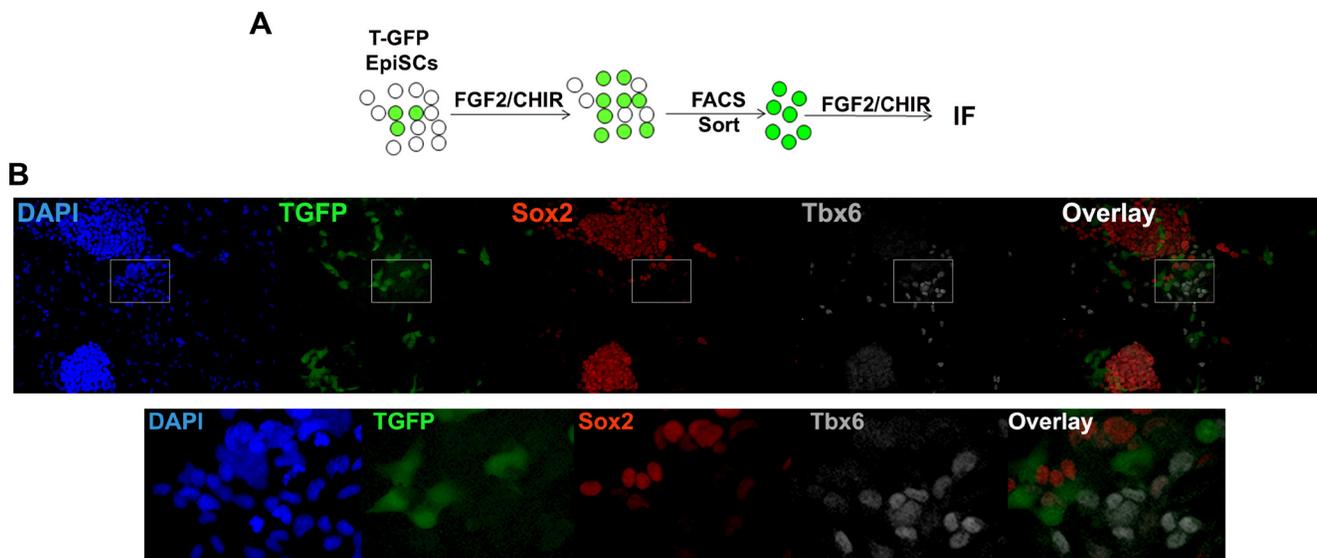


Figure 2. (A) Scheme depicting the differentiation and re-plating of *in vitro* induced NMPs at high density after flow sorting. (B) Fluorescence analysis and immunocytochemistry of TGFP, Sox2 and Tbx6 expression of *in vitro*-derived NMPs sorted at day 2 of differentiation and re-plated at high density in the presence of FGF2/CHIR for 2 days. In all cases cell nuclei were visualized using DAPI. IF: immunofluorescence. Magnified versions of the areas marked by a white box are shown in the bottom panel.

Strikingly, most (55–60% of total) clones obtained from both day 2 and day 3 FGF2/CHIR-induced TGFP⁺ cells were composed exclusively of single Sox2⁺ neuroectodermal cells indicating a strong neurogenic capacity (Figure 3D,E). The proportion of single Sox2⁺ colonies was significantly enhanced to 76% (p value < 0.05 based on a two-tailed z test) with a concomitant decrease in the proportion of T(Bra)⁺ cells when isolated single TGFP⁺ cells produced after 2 days in FGF2/CHIR medium were

re-plated in the presence of FGF2 alone for 48 hrs prior to clone scoring (Figure 3D) confirming the pro-mesodermal effect of Wnt activity on NMPs^{8,10}. We also observed purely mesodermal clones consisting of T(Bra)⁺ cells which were particularly prominent in the case of sorted day two TGFP⁺ NMPs (Figure 3D,E). These data suggest that many *in vitro*-derived NMP cells are biased by the signaling environment towards unilinear differentiation into either neuroectoderm or mesoderm. However, we did observe clones which

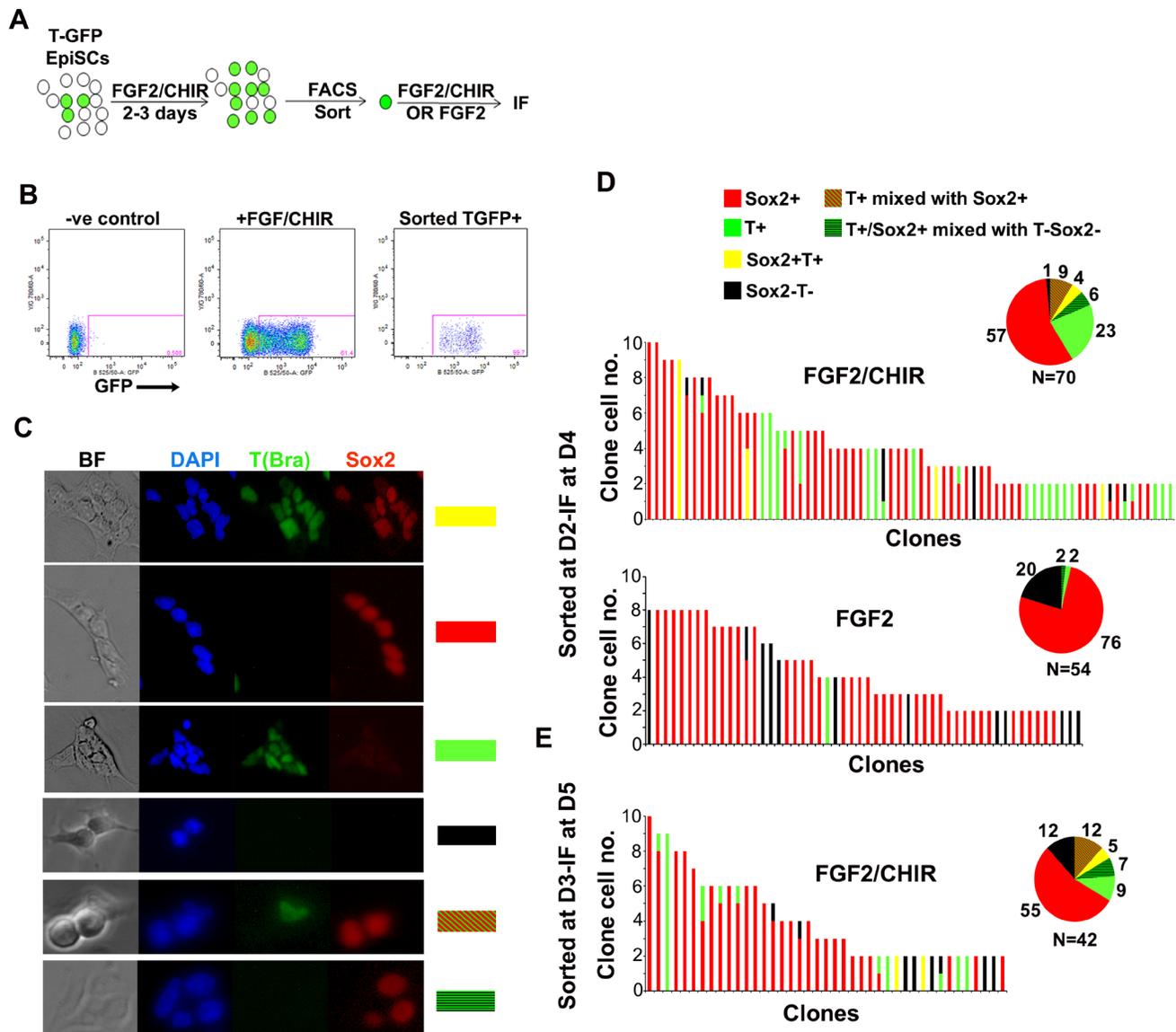


Figure 3. (A) Scheme depicting the differentiation and re-plating of *in vitro* induced NMPs at clonal density after flow sorting. (B) FACS plots depicting analysis of TGFP expression in day 3 FGF2/CHIR-treated TGFP EpiSCs (middle). The purity of the GFP⁺ sorted population and a negative control (wild-type EpiSCs) are also shown. (C) Representative examples of the clones obtained after culture of single sorted TGFP⁺ NMPs in FGF2/CHIR medium following immunofluorescence analysis of T(Bra) and Sox2 expression. The colour-coded bars on the right correspond to the scoring groups shown at the top of panel 3D. (D–E) Composition of colonies obtained after clonal plating of TGFP⁺ NMPs sorted at day 2 (D) or day 3 (E) for a further 48 hrs in FGF2/CHIR or FGF2 only. The cell number of each clone (in x axis) is shown in the y axis. Colour codes: Sox2+: single Sox2+ cells; T+: single T+ cells; Sox2+T+: Double positive cells; Sox2-T-cells: double negative cells; T+ mixed with Sox2+: clones consisting of single T(Bra)+ cells mixed with single Sox2+ cells; T+/Sox2+ mixed with T-Sox2-: clones consisting of single T(Bra)+ or single Sox2+ cells mixed with double negative cells. Pie charts: percentages of different classes of clones representing groups of the colour-coded phenotypes described above. Total numbers of clones scored are shown below each pie chart.

comprised combinations of single positive T(Bra)⁺ and Sox2⁺ cells (9% for day 2 and 12% for day 3 TGFP⁺ NMPs) and were thus indicative of neuromesodermal potency. A few clones were found to contain only T(Bra)⁺Sox2⁺ double positive cells (Figure 3D,E) possibly reflecting NMP self-renewal. Finally, a small number of colonies were composed only of T(Bra)⁺Sox2⁻ negative cells (Figure 3D,E) which may represent more differentiated NMP derivatives such as spinal cord cells or mesodermal lineages other than paraxial (e.g. lateral/ventral) derived from sorted TGFP⁺Sox2⁻ cells. Interestingly, we detected no Tbx6⁺ cells present in the clones (Representative, raw images shown in Dataset 5) despite their presence in cultures derived from sorted day 2 FGF2/CHIR-induced TGFP⁺ cells plated at high density under the same conditions. This suggests that the presumed maturation of T(Bra)⁺ cells into Tbx6-positive paraxial mesoderm depends on paracrine (e.g. FGF)¹⁷ or juxtacrine (e.g. Notch)¹⁸ signaling effects which are absent from the low density, clonally-derived cultures.

Supplementary data for 'Assessing the bipotency of *in vitro*-derived neuromesodermal progenitors'

5 Data Files

<http://dx.doi.org/10.6084/m9.figshare.1371001>

Discussion

The production of axial tissues during embryonic elongation is driven by posteriorly-located progenitors emerging round the end of gastrulation. A long-standing question in the field has been whether this cell population represents a mixture of separate unipotent neural and mesoderm-committed precursors or consists of bipotent progenitors. Genetic marking of single cells and their derivatives using the LaacZ system in mouse embryos shed light on this problem by revealing that spinal cord neurectoderm and paraxial mesoderm originate from bipotent neuromesodermal progenitors². These NMPs have also recently been captured *in vitro* through the culture of pluripotent stem cells in Wnt and FGF signaling agonists^{10,11}. However, the bipotent status of these cells had not been previously demonstrated at the clonal level. Here we show that single *in vitro*-derived NMPs can give rise to mixed clones containing both neural (Sox2⁺T(Bra)⁻) and mesodermal (Sox2⁻T(Bra)⁺) cells, a finding which indicates that FGF2/CHIR-induced cultures contain *bona fide* NM bipotent cells.

Interestingly, a considerable fraction of individual sorted NMPs produced exclusively neurectodermal or mesodermal clones suggesting that a proportion of the Sox2⁺T(Bra)⁺ cells induced from EpiSCs after 2–3 days in the presence of FGF2/CHIR may already be biased towards adopting a neural or mesodermal fate under conditions promoting both lineages. This may be a reflection of heterogeneity in the relative levels of Sox2 and T(Bra) protein/transcript within the *in vitro*-derived Sox2⁺T(Bra)⁺ population with double-positive cells exhibiting higher levels of Sox2 showing a pro-neural bias while T(Bra)^{high} cells are predisposed to mesoderm differentiation. Indeed such heterogeneity in Sox2 and T(Bra) levels (as well as other mesodermal and neural transcripts) has been shown by single cell transcriptomic analysis of mouse ES cell-derived cultures resembling our *in vitro*-generated NMPs¹¹. Nevertheless, the clonal-based assay we employed here establishes bipotency of *in vitro*-derived NMPs and reveals the responsiveness of individual cells to environmental signals.

Data availability

Figshare: Supplementary data for 'Assessing the bipotency of *in vitro*-derived neuromesodermal progenitors' doi: [10.6084/m9.figshare.1371001](https://doi.org/10.6084/m9.figshare.1371001)¹⁹

Author contributions

AT designed and performed experiments. AT, VW prepared the manuscript. All authors have agreed to the final content of the manuscript.

Competing interests

No competing interests were disclosed.

Grant information

This study was supported by the Medical Research Council (MR/K011200).

I confirm that the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Acknowledgments

We wish to thank Fiona Rossi and Clair Cryer (MRC Centre for Regenerative Medicine FACS facility) for their assistance with flow cytometry.

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Current Referee Status:



Version 2

Referee Report 03 August 2015

doi:10.5256/f1000research.7383.r9743



Patrick P. L. Tam

Embryology Unit, Children's Medical Research Institute, Sydney, NSW, Australia

The responses and revisions have addressed the concerns adequately, except for:

3. (d) This is an after-thought argument, as one may not know before the experiments that these markers are not co-expressed in the cells of interest. While it turned out the experimental strategy has not produced any major confounding outcome, it would be prudent to perform the expression analysis using different fluorescent reporters initially.

3. (g) Data of the missing experiment would have enhanced the merit of this study on the differences in clonal types..

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Version 1

Referee Report 12 May 2015

doi:10.5256/f1000research.6803.r8552



Kate G. Storey, Laure Verrier

Division of Cell and Developmental Biology, College of Life Sciences, University of Dundee, Dundee, UK

Following a study on the differentiation of dual-fated Neuromesodermal Progenitors (NMPs) at the population level, Tsakiridis and Wilson describe here the ability of a single NMP cell to generate both neural and mesodermal derivatives. Using T(Bra)-GFP expressing EpiSCs, the authors performed a clonal analysis of NMP differentiation driven by a FGF2/CHIR regime. They show that single T(Bra)-GFP expressing cells can generate both mesodermal T(Bra)-GFP and neural Sox2 expressing cells in the same clone. This main finding demonstrates the neuromesodermal bipotency of in vitro derived NMP at the single cell level, recapitulating the behaviour of NMPs as identified by retrospective clonal analysis in the mouse embryo in Tzouanacou *et al.* 2009.

Overall, the experiments presented are well designed and the results are carefully analyzed. However, the paper would benefit from improvement of specific points.

Main comments:

Methods

1. The authors should comment on the use of the ROCK inhibitor Y-27632 in their protocol for cell sorting and during subsequent plating at low density.

Experimental approaches

1. Figure 2 describes the co-appearance of distinct T(Bra)⁺ and Sox2⁺ cells from an NMP population treated by FGF2/CHIR as a way to induce simultaneously neural and mesodermal lineages. Why did the authors not perform neural and mesodermal differentiation in parallel using two distinct protocols?
2. In this study, we are dependent on the previous finding that cells plated at low density give rise mainly (95%) to monoclonal colonies in the time frame of the experiment. It would be reassuring here if the authors had demonstrated that sorted individual T-GFP +ve cells were also Sox2 co-expressing at the time of plating – even if this required immuno-cytochemistry, it would at least establish the proportion of bra/sox2 co-expressing cells in the starting conditions.
3. Does the negative control in Fig3B represent WT EpiSCs treated with the FGF2/CHIR regime, or are they just undifferentiated cells? A good negative control would be to analyse T(Bra)-GFP undifferentiated EpiSCs as they will have the same genetic background as the NMPs analyzed later but won't express T(Bra). This control should be included as well.
4. Figure 3C shows clones of various sizes in terms of cell number. Can the size of the clone have an impact on cell lineage identity? It would be good to standardize the analysis by looking at the different expression patterns in colonies with comparable cell numbers.
5. Figure 3D and E, the authors comment on the appearance of T(Bra)/Sox2 double negative colonies, and suggest that they are likely to represent further differentiated derivatives. The authors should address this by looking at other neural and mesodermal markers, such as Pax6 or Sox1 and Paraxis. It would be very informative to know what those cells become. Indeed, the suggestion that a higher density of cells is needed for paraxial mesoderm differentiation might indicate that those negative clones are not mesodermal derivatives.

Minor comments:

In figure 1, the authors describe the establishment of an NMP population. Using a FGF2/CHIR differentiation protocol, they only obtain around 38% of T(Bra)-GFP/Sox2 coexpression, with maximum 60% of T(Bra)+ cells in the whole population. However, other studies show up to 80% of coexpression in the same conditions. The authors should comment on that.

Figure 2B: the figure legend should indicate “all nuclei” instead of “ell nuclei”.

The legend for figure 3 (D-E) is confusing and should be clarified.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Author Response 23 Jul 2015

Anestis Tsakiridis, University of Edinburgh (Subscriptions), UK

We would like to thank all three reviewers both for their positive comments and constructive suggestions which improved significantly the quality of our manuscript. Our responses below are shown in italics while reviewer comments are in bold.

Main comments:

Methods

- 1. The authors should comment on the use of the ROCK inhibitor Y-27632 in their protocol for cell sorting and during subsequent plating at low density.**

The ROCK inhibitor Y-27632 is a well-established reagent in human embryonic (hES) and epiblast stem cell (EpiSC) culture used to enhance survival of single cells (Watanabe et al., 2007) and thus we routinely employ it in FACS sorting experiments as a means of counteracting dissociation-induced apoptosis.

Experimental approaches

- 1. Figure 2 describes the co-appearance of distinct T(Bra)⁺ and Sox2⁺ cells from an NMP population treated by FGF2/CHIR as a way to induce simultaneously neural and mesodermal lineages. Why did the authors not perform neural and mesodermal differentiation in parallel using two distinct protocols?**

Clonal analysis is required because pluripotent stem cell differentiation is never 100% efficient at the population level. We thus aimed to assess the ability of single NMP cells to generate both neural and mesodermal derivatives at the same time using conditions promoting the simultaneous emergence of both lineages (i.e. FGF/CHIR treatment).

- 2. In this study, we are dependent on the previous finding that cells plated at low density give rise mainly (95%) to monoclonal colonies in the time frame of the experiment. It would be reassuring here if the authors had demonstrated that sorted individual T-GFP +ve cells were also Sox2 co-expressing at the time of plating – even if this required immuno-cytochemistry, it would at least establish the proportion of bra/sox2 co-expressing cells in the starting conditions.**

We agree that ideally the extent of T(Bra)/Sox2 co-expression should be assessed at the time of low density plating. However, in our opinion, its determination can only be achieved using a T/Sox2 double reporter cell line. The alternative option suggested by the reviewers involving the use of immunocytochemistry would be technically challenging given that freshly sorted and plated TGFP+ cells require a few hours to attach properly thus precluding antibody staining at the very start of the experiment.

3. **Does the negative control in Fig3B represent WT EpiSCs treated with the FGF2/CHIR regime, or are they just undifferentiated cells? A good negative control would be to analyse T(Bra)-GFP undifferentiated EpiSCs as they will have the same genetic background as the NMPs analyzed later but won't express T(Bra). This control should be included as well.**

The control used in Fig. 3B was indeed differentiated E14tg2a EpiSCs. This is the same genetic background as the TGFP+ ES cells (E14.1, 129/Ola; Fehling et al., 2003) we used to derive the EpiSC line employed in this study. It is not possible to utilize undifferentiated TGFP EpiSCs as a negative control for FACS as they also express significant levels of both T(BRA) protein and the TGFP reporter under self-renewing conditions (i.e. in FGF2 and Activin A) in line with previous reports (Tsakiridis et al., 2014; Kurek et al., 2015).

4. **Figure 3C shows clones of various sizes in terms of cell number. Can the size of the clone have an impact on cell lineage identity? It would be good to standardize the analysis by looking at the different expression patterns in colonies with comparable cell numbers.**

Splitting the data in Fig. 3C-D based on clone size would be a good way to decipher a link between colony cell number and lineage identity acquisition, a possibility which is indeed very interesting. However, this type of representation would be more suitable for a larger dataset. Our clone numbers are too small to support any solid conclusions on this issue and we believe that the representation we opted for is the best way to illustrate graphically both parameters (i.e. clone size and lineage composition). We will be happy to incorporate any specific suggestions and or/consider alternative ways of depicting the results.

5. **Figure 3D and E, the authors comment on the appearance of T(Bra)/Sox2 double negative colonies, and suggest that they are likely to represent further differentiated derivatives. The authors should address this by looking at other neural and mesodermal markers, such as Pax6 or Sox1 and Paraxis. It would be very informative to know what those cells become. Indeed, the suggestion that a higher density of cells is needed for paraxial mesoderm differentiation might indicate that those negative clones are not mesodermal derivatives.**

This is a good point which deserves further investigation. Our preliminary data indicate that T(Bra)⁻Sox2⁻ colonies are also negative for Sox1. However, a thorough analysis of these clones will require significant effort and is beyond the scope of this short research note. We have added a sentence in the last paragraph of the results section raising the possibility that double negative clones may also comprise differentiated derivatives of single sorted TGFP⁺ Sox2⁻ cells which are probably precursors of mesodermal cell types other than paraxial, also emerging upon culture in FGF2/CHIR.

Minor comments:

In figure 1, the authors describe the establishment of an NMP population. Using a FGF2/CHIR differentiation protocol, they only obtain around 38% of T(Bra)-GFP/Sox2 coexpression, with maximum 60% of T(Bra)+ cells in the whole population. However, other studies show up to 80% of coexpression in the same conditions. The authors should comment on that.

Line-to-line variation in terms of differentiation potential is a common phenomenon in pluripotent stem cell cultures (e.g. see Osafune et al., 2008) and in our hands we also observe some variation between different EpiSC lines both in terms of the extent of induction of NMP-like cells upon culture in FGF2/CHIR as well as the timing of their emergence. The lower numbers of T(Bra)⁺Sox2⁺ double positive cells we observed in this study when compared to the high efficiency of induction exhibited by the in vivo derived EpiSC line R04-GFP (Gouti et al., 2014) is an example of such variation. One interesting possibility is raised by the fact that the T(Bra) reporter line we employ here contains a GFP cassette knocked into the first exon of the T(Bra) gene (Fehliing et al., 2003). The resulting heterozygosity may lead to lower efficiency of T(Bra)⁺Sox2⁺ cell generation.

Figure 2B: the figure legend should indicate “all nuclei” instead of “ell nuclei”.

We cannot locate the phrase the reviewers are referring to. The exact figure legend wording in Fig. 2B is “In all cases cell nuclei were visualized using DAPI”.

The legend for figure 3 (D-E) is confusing and should be clarified.

We have addressed this point in the new version.

Competing Interests: No competing interests were disclosed.

Referee Report 12 May 2015

doi:10.5256/f1000research.6803.r8488



Jacqueline Deschamps

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Tsakiridis and Wilson present the evidence that single cells from a neuromesodermal axial progenitor (NMP) population produced *in vitro* from mouse epiblast stem cells (EpiSCs) can give rise to both neural and mesodermal cell descendants, and are therefore truly bipotent.

In earlier work they had demonstrated the existence of NMPs in the posterior aspect of the developing early somite embryos, and characterized these NMPs as being T Brachyury/Sox2 double positive. In more recent experiments they and others succeeded in defining high Wnt and high Fgf signaling conditions to culture EpiSCs into a cell population wherein more than half the cells are NMP-like. However, it remained to be proven that an individual NMP-like cell expressing both T Brachyury and Sox2 is able to generate neural derivatives (expressing exclusively Sox2), and mesodermal derivatives (expressing exclusively T Brachyury). It is what the authors achieved in this report, by elegantly making use of EpiSCs derived from T Bra Gfp transgenic embryos. By fluorescence activated cell sorting applied to these EpiSCs cultured in NMP-promoting conditions, they could purify the T Bra positive NMPs and analyze their clonal descendants. They clearly obtained neural and mesodermal descendants from single NMP clones, and could demonstrate that the environmental culture conditions influence the bias of individual NMPs to differentiate into cells with a neural versus mesodermal fate.

The experiments are well designed and executed. The methods are well described, and the results are clear, thoroughly analyzed and discussed appropriately. The data in this manuscript convey a clear message that represents an advance in the field.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 07 May 2015

doi:10.5256/f1000research.6803.r8486



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This study on the differentiation of single NMP progenitors generated by FGF2/CHIR treatment of EpiSC is a sequel to the previous study on lineage differentiation of these cells at the population level. Findings of this study are consistent with the assertion that individual T⁺/Sox2⁺ cells are likely to possess dual potential for differentiation into mesoderm (T⁺ only) and neuroectodermal (Sox2⁺ only) cells. This provides the requisite experimental evidence that some cells *in vitro* may have acquire the attributes of the bipotent NMPs, which are presumed to exist in the node-streak interface and the chordoneural hinges *in vivo*.

Issues to be clarified:

1. Inconsistency of experimental data

Data shown in Fig 3D indicates that T⁺ clones contain 2 and 4 cells (the majority) with a few having up to 6 cells. The example of T⁺ve clone shown in Fig 3C contains at least 9 cells. This result is not included the dataset of Fig 3D (Day 2 sorted) or E (Day 3 sorted). Were all or only subsets of clones scored for these clonal culture experiments?

2. Quality of the image data

a. It is difficult to discern the co-expression, or otherwise, of T-GFP and Sox2 in individual cells at the resolution of Fig 1 and 2.

b. It appears that cells with mixed gene expression are only found in some colonies (Fig 1), rather than in a salt and pepper manner in every colony. This may require an explanation in the context of clonal development.

c. It can be confusing when different colour schemes were used to show the fluorescence results, e.g. T-GFP signals are shown variously in green (Fig 1), white (Fig 2B) and red (Fig 3C), and Sox2 is shown in red (Fig 1) and green (Fig 2B, 3C), rather than red (which is for Tbx6, Fig 2B). Given that the FGF2/CHIR treated cells were sorted based on GFP activity, T-GFP signal should consistently be displayed- in green for all figures.

3. Additional data / information may help:

a. While it is plausible that the Tbx6⁺ cells might be descendents of the mesoderm progenitor, the results do not unequivocally show that they are derived from the T⁺ve cells.

- b. What is the evidence for that T⁻/Sox2⁻ cells (which also did not expressing Tbx6) were “more differentiated” NMP derivatives?
- c. Absence of Tbx6 cells in low density culture is an intriguing result. Is there any precedence that the differentiation of Tbx6-expressing cells is dependent on any “paracrine” signals?
- d. Were the Sox2 and Tbx6 immunofluorescence signals captured in emission channel other than for green fluorescence? If not, would the IF results for these two markers be confounded by the T-GFP background?
- e. Which are the examples of two types of mixed clones in the legend (“T⁺ mixed with Sox2⁺” and “T⁺/Sox⁺ with T⁻/Sox2⁻”) in the FGF2/CHIR and FGF experiments (Fig. 3D)?
- f. The bottom panel of Fig 3C: The “T⁺/Sox2⁺ mixed with T⁻/Sox2⁻” clone showed no T⁺/Sox2⁺ cells among the four cells in this figure.
- g. Is there any difference in the clonal types between “Sorted at D2-IF at D4” and “Sorted at D3-IF at Day 5 FGF2/CHIR” groups? What is the rationale for testing the effect of an extended culture to Day 3 before sorting, and was there a parallel culture of “FGF2 only” to Day 3?

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Author Response 23 Jul 2015

Anestis Tsakiridis, University of Edinburgh (Subscriptions), UK

Our responses below are shown in italics while reviewer comments are in bold.

Issues to be clarified:

1. Inconsistency of experimental data

Data shown in Fig 3D indicates that T⁺ clones contain 2 and 4 cells (the majority) with a few having up to 6 cells. The example of T⁺ve clone shown in Fig 3C contains at least 9 cells. This result is not included the dataset of Fig 3D (Day 2 sorted) or E (Day 3 sorted). Were all or only subsets of clones scored for these clonal culture experiments?

We wish to thank the reviewers for spotting the mistake. All clone examples shown in Fig. 3C are taken from the culture experiments described in this study and are included in the scoring graphs in Fig. 3D-E. The T(Bra)⁺Sox2⁻ clone the reviewers are referring to was mistakenly shown in Fig. 3E as consisting of 7 instead of 9 cells. We have rectified the mistake in the new version (3rd clone from the left, depicted in green). We also re-examined all data and found two further errors:

(1) a T(Bra)⁺Sox2⁺ double positive clone (also shown as an example in Fig. 3C, top row) was accidentally omitted from the top graph in Fig. 3D

(2) a 3-cell clone in the same graph was wrongly depicted as containing two Sox2+ cells and a double negative instead of two Sox2+ cells and a T(Bra)+ cell.

These changes have now been incorporated into the new figure version and the data is now correct to the best of our knowledge. We would like to apologise for these errors.

2. Quality of the image data

a. It is difficult to discern the co-expression, or otherwise, of T-GFP and Sox2 in individual cells at the resolution of Fig 1 and 2.

We have now included higher magnification images in figures 1 and 2 to address this issue.

b. It appears that cells with mixed gene expression are only found in some colonies (Fig 1), rather than in a salt and pepper manner in every colony. This may require an explanation in the context of clonal development.

This is an intriguing observation supporting the idea that NMPs segregate separately from other mesodermal/neural precursors emerging simultaneously in culture conditions promoting a late primitive streak-like environment. It is likely to reflect the fact that the starting EpiSC population is heterogeneous in self-renewing conditions, consisting of cells with differential capacity to generate separate distinct lineages. For example we have previously reported that T(Bra)+Sox2+ and T(Bra)+Foxa2+ mesodermal precursors emerge in a mutually exclusive manner after Wnt signaling stimulation of EpiSCs (Tsakiridis et al., 2014). It may also be true that the generation of NMP-like, T(Bra)+Sox2+ cells is non-synchronous. We have added a sentence in the first paragraph of the results section to comment on this.

c. It can be confusing when different colour schemes were used to show the fluorescence results, e.g. T-GFP signals are shown variously in green (Fig 1), white (Fig 2B) and red (Fig 3C), and Sox2 is shown in red (Fig 1) and green (Fig 2B, 3C), rather than red (which is for Tbx6, Fig 2B). Given that the FGF2/CHIR treated cells were sorted based on GFP activity, T-GFP signal should consistently be displayed in green for all figures.

We have adopted the reviewers' recommendation and in the new figure versions T(Bra) expression appears in green while Sox2 is depicted in the red.

3. Additional data / information may help:

a. While it is plausible that the Tbx6+ cells might be descendants of the mesoderm progenitor, the results do not unequivocally show that they are derived from the T+ve cells.

We agree that the data presented here do not show directly that Tbx6+ cells are derived from T(Bra)+ cells. To indicate this we added the word "presumed" before the sentence: "maturation of T(Bra)+ cells into Tbx6-positive paraxial mesoderm" at the end of the Results section. However, we believe that our hypothesis is reasonable since it is based on

published studies demonstrating that a considerable fraction of paraxial mesoderm arises from T(Bra)+ late primitive streak precursors (e.g. [Anderson et al. 2013](#); [Cambray and Wilson, 2007](#)).

b. What is the evidence for that T-/Sox2- cells (which also did not expressing Tbx6) were “more differentiated” NMP derivatives?

We are interested in characterizing further the T-/Sox2- clones (see also our response to Referees 1 above). We speculate that these are differentiated NMP-derivatives based on the observation that EpiSCs generating NMPs do not (as far as we can tell) lose Sox2 expression during that process and thus its subsequent loss is likely to indicate further differentiation to downstream NMP products. However, in the absence of any solid data on this point we decided to tone down our statements and expand our hypotheses:

1) We modified our statement related to the double negative clones (last paragraph of results section) by replacing the phrase “are likely to represent” with “may represent”

2) We raised the alternative possibility that these clones may arise from sorted single T(Bra)+ mesodermal precursors.

c. Absence of Tbx6 cells in low density culture is an intriguing result. Is there any precedence that the differentiation of Tbx6-expressing cells is dependent on any “paracrine” signals?

Emergence of Tbx6+ cells is likely to depend on FGF signaling ([Ciruna and Rossant, 2001](#)) -we have now incorporated this possibility into the text together with the speculation that Notch-based regulation of Tbx6 transcription may also be critical for cell density effects ([White et al., 2005](#)). We have also shown previously that paracrine Wnt signaling promotes pioneer T(Bra)+ mesodermal precursors in EpiSCs cultured in self-renewal conditions ([Tsakiridis et al., 2014](#)).

d. Were the Sox2 and Tbx6 immunofluorescence signals captured in emission channel other than for green fluorescence? If not, would the IF results for these two markers be confounded by the T-GFP background?

We find this possibility unlikely especially since the Sox2+, Tbx6+ and TGFP+ expression domains shown in Fig. 2 were in most cases mutually exclusive from each other and very few or no double or triple positive cells (which could potentially represent a background artefact) were observed.

e. Which are the examples of two types of mixed clones in the legend (“T+ mixed with Sox2+” and “T+/Sox+ with T-/Sox2-“) in the FGF2/CHIR and FGF experiments (Fig. 3D)?

The class of clones defined as “T+ mixed with Sox2+” refers to colonies containing T(Bra)+ single positive cells mixed with Sox2+ single positive cells (example shown in the 5th row in Fig. 3C) while the group defined as “T+/Sox+ mixed with T-/Sox2-“ describes clones containing T(Bra)+ OR Sox2+ single positive cells mixed with double negatives (example shown in bottom row in Fig. 3C). We would like to apologise for the lack of clarity. We tried

to address this issue by making the legend of Fig. 3D-E more detailed.

f. The bottom panel of Fig 3C: The “T+/Sox2+ mixed with T-/Sox2-” clone showed no T+/Sox2+ cells among the four cells in this figure.

We use the slash (“/”) to indicate “OR” (see our response directly above). Again we apologise for the confusion.

g. Is there any difference in the clonal types between “Sorted at D2-IF at D4” and “Sorted at D3-IF at Day 5 FGF2/CHIR” groups? What is the rationale for testing the effect of an extended culture to Day 3 before sorting, and was there a parallel culture of “FGF2 only” to Day 3?

We observed a significant number of T(Bra)+Sox2+ cells both at 48 and 72 hrs of culture in FGF2/CHIR and thus we wished to examine whether these two populations are equivalent in their capacity to clonally generate both neural and mesodermal cells. Since the goal was to compare these two populations, we did not carry out an experiment involving a 3 day culture FGF2 prior to sorting and re-plating.

Competing Interests: No competing interests were disclosed.
