Most efficient BMP4 exposure and concentrations of inhibitors for pluripotent stem cell differentiation to trophoblast

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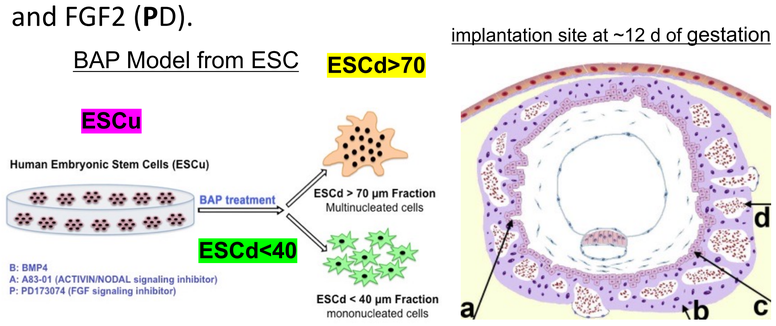
Background and Significance

Trophoblasts first emerge at the blastocyst stage of embryo development and are involved in the process of implantation and most functions of the mature mammalian placenta. Diseases of pregnancy, such as preeclampsia, are believed to be caused by trophoblast dysfunction and a partial failure of the trophoblast to interact properly with the maternal system. However practical and ethical considerations prevent the early stages of pregnancy and hence such diseases to be investigated. Human embryonic stem cells (hESCs) can be coaxed to differentiate into placental trophoblast cells resembling those in early pregnancy by using a protocol (**BAP**) that involves the addition of **B**one Morphogenetic Protein 4 (BMP4), along with inhibitors of **A**ctivin/Nodal (A83) and FGF2 (**P**D).



a primary villous development, **b** primitive STB, **c** primitive CTB, **d** blood filled lacunae.

From *James et al*, 2012

Research Question and Design

Our goal was to determine the minimum time hESCs need exposure to BMP4 and optimal concentrations of A83-01 and PD173074 for complete differentiation to trophoblast, thereby providing additional insight into the process of trophoblast differentiation.

The BAP protocol involves the addition of of **B**one Morphogenetic Protein 4 (BMP4), along with inhibitors of **A**ctivin/Nodal (A83) and FGF2 (**P**D) to hESCs every 24 hours. This procedure has been manipulated to fit our research question. The protocol has been developed by using the findings of Amita et al 2013 PNAS.

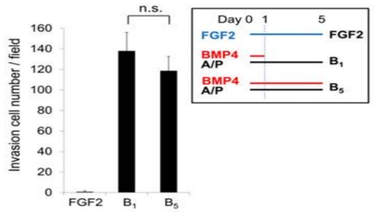
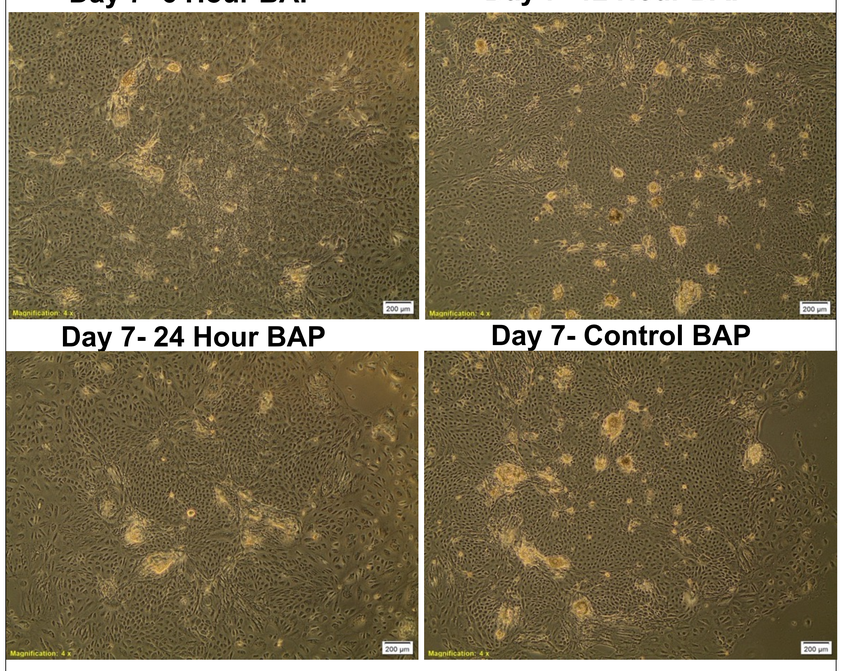


Fig. S7. Acquisition of invasive properties by hESC following short-term exposure to BMP4. The H1 hESC were plated on Matrigel-coated membranes with 8-µm pores inserted into invasion chambers arranged in a six-well format at a density of 5×10^4 cells per chamber. After 5 d culture in MEF-CM supplemented with either FGF2 (blue line in *Inset*), BMP4 plus A/P for 1 d and A/P alone for 4 d (B_1, short red and long black lines in *Inset*), or BMP4 and A/P (B_5, long red and black lines in *Inset*), cells that had migrated through the pores and attached to the undersurface of the membranes were counted. The data represent the combined results of two independent experiments. Five wells were counted for both B_1 and B_5, and two wells were counted for FGF2. Values are means ± SEM. $P = 0.422$.

Preliminary Experiment and Results

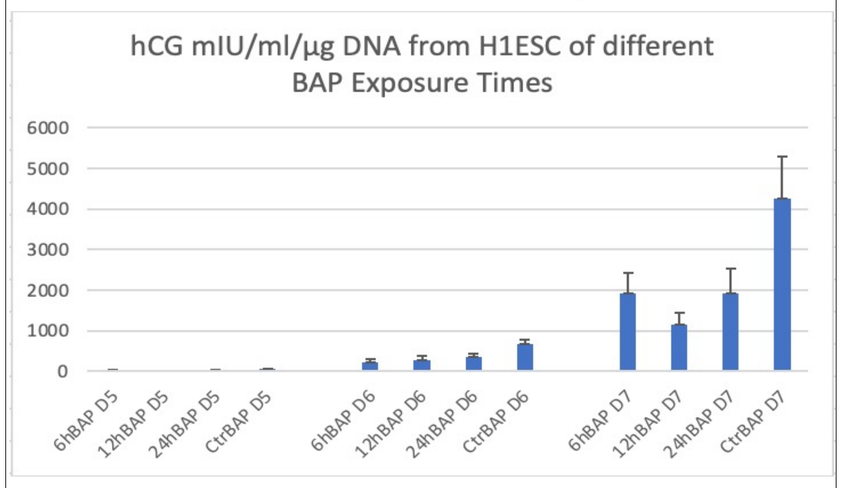
For BMP4 exposure time, BAP medium was replaced with control medium, i.e. minus BMP4, but still containing the inhibitors, at different times during a 7-day culture. The hESC (5×10^5 cells per d35 per well) were cultured under BAP conditions for either 6 h, 12 h, 24 h, or seven days (control). After each set period, the medium was switched to only contain A83-01 and PD173074 (AP), without BMP4 hESC medium. The experiment was run in triplicate, i.e. three culture wells per treatment condition. Medium was collected on days 5, 6, and 7 in order to assess the production of pregnancy hormones. Additionally, images were taken on these days.

H1 ESC Passage 52



The 24h sample shows very similar morphology to the control sample. The 6h and 12h experiments do show syncytialization, but at visibly reduced amounts. Because multinucleated syncytiotrophoblasts secret major amount of pregnancy hormones, such morphological differences are consistent with hCG production amounts (in the graph below).

Human Chorionic Gonadotropin ELISA

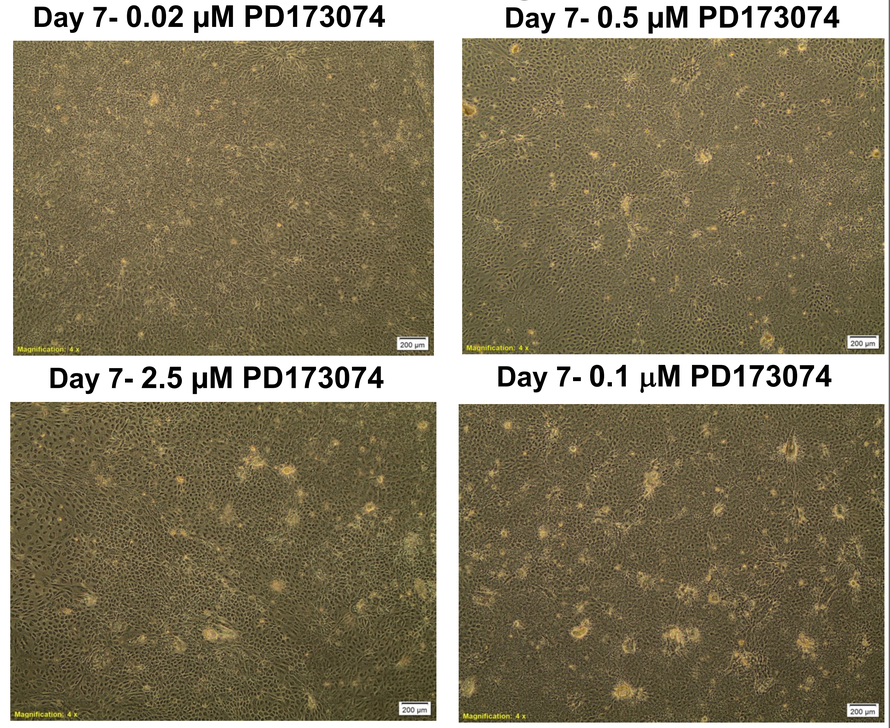


The graph shows the results of the ELISA run on human chorionic gonadotropin in the collected medium of the experiment and normalized with DNA amounts. After running a statistical analysis (ANOVA), we found that there is no difference between the 6h, 12 h, and 24h groups and the control group. This means that as short a time as 6 h exposure to BMP4 may be sufficient to prime the cells for trophoblast differentiation.

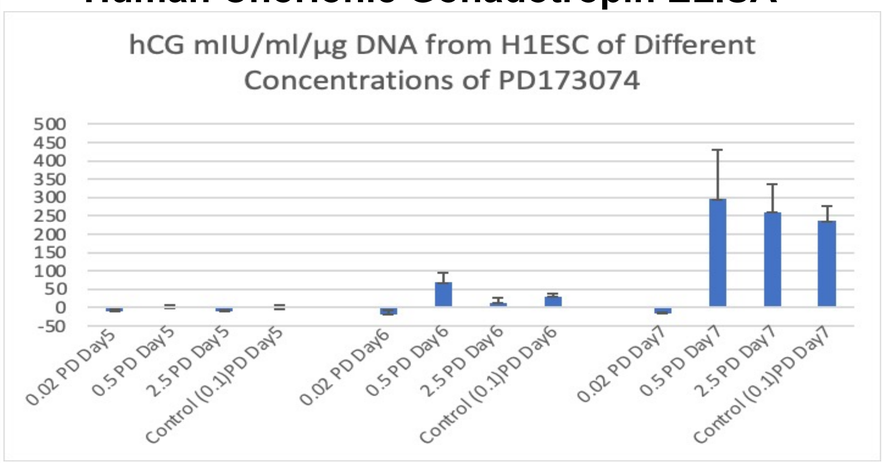
Secondary Experiment and Results

As seen above, the 24h BAP treatment was sufficient to lead to equivalent differentiation as controls as long as inhibitors, A83 and PD were present. Next we attempted to determine an optimal concentrations of PD173074 in presence of a fixed concentration A83-01 for complete differentiation to trophoblast after the hESC had been primed with BMP4 for 24 h. To do this, hESCs were cultured with 10 ng/ml BMP4 for 24 h and continued in culture with 1.0µM of A83-01 and adjusted concentrations of PD173074 (0.02 µM, 0.5 µM, and 2.5 µM with the control at 0.1 µM). After 24 hours, the medium was switched to contain A83 and PD, and no BMP4. The experiment was run in triplicate, i.e. three culture wells per treatment condition. Medium was collected on days 5, 6, and 7 in order to assess the production of pregnancy hormones (progesterone and human chorionic gonadotropin) as the cells differentiated. We also collected photomicrographic images of the cells on these same days. These data were compared with ones obtained from the controls.

H1 ESC Passage 53



Human Chorionic Gonadotropin ELISA



The data above were normalized with DNA amounts. Based on our findings, is seems that 0.5 µM PD173074 may be the optimal concentration. More tests are being run to finalize this finding.

Conclusions and Further Testing

Based on the data from the experiments on BMP4 exposure time, we see that an exposure of just 24h is sufficient to prime trophoblast differentiation. The data from the PD173074 experiment is still being finalized by running additional replicates. These will be used to clarify results from the previous trials.

In the future, we plan to run experiments to find the optimal concentration of the A83-01 inhibitor. To do so, we will vary concentrations of A83-01 (0.2µM, 5µM, and 25µM) with the PD173074 control at 0.1µM. Our control for A83 will be set at 1µM.

Summary

Human embryonic stem cells (hESCs) can be coaxed to differentiate into placental trophoblast cells resembling those in early pregnancy by using a protocol (**BAP**) that involves the addition of **B**one Morphogenetic Protein 4 (BMP4), along with inhibitors of **A**ctivin/Nodal (A83) and FGF2 (**P**D). This protocol was manipulated to allow us to determine the minimum length of time hESCs must be exposed to the different components of the BAP protocol and then optimal concentrations of A83-01 and PD173074 for complete differentiation to trophoblast. By keeping two inhibitors in cultures, cells with BAP for 24 h proves sufficient. Based on preliminary data from the PD173074 experiments, 0.5 µM seems to be optimal, but additional trials are being run to strengthen this finding.

Funding Resources

Pluripotent human stem cells as models for normal and abnormal trophoblast at implantation
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