Estimating minimum BMP4 exposure time needed to prime human pluripotent stem cell differentiation to trophoblast

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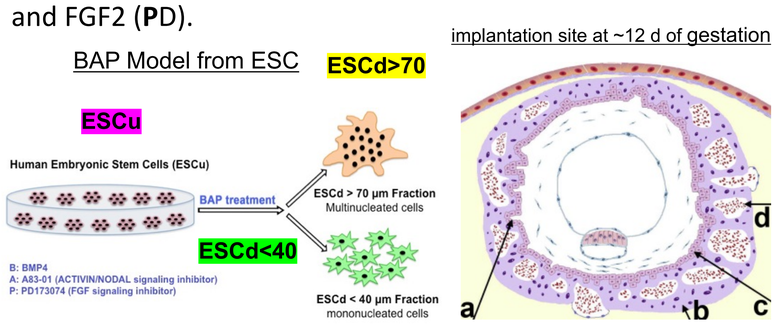
Background and Significance

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

Here, our goal has been to determine the minimum length of time hESCs must be exposed to the BMP4, A83, or PD components of the BAP protocol to provide complete differentiation to functional trophoblast, providing insight into the process of trophoblast differentiation. Previous publication from Roberts lab indicated continuous addition of BMP4 is optional to have trophoblast differentiation, however, a precise minimum time has not been determined yet.
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 using the findings of Amita et al 2013 PNAS.

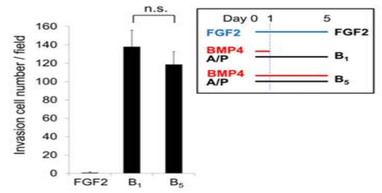
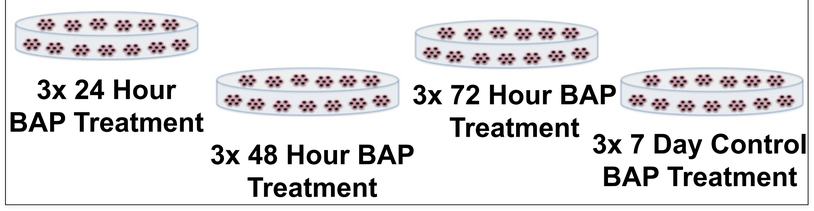


Fig. S7. Acquisition of invasive properties by hESC following short-term exposure to BMP4. The H1 hESC were plated on Matrigel-coated membranes with 8-µm pores inserted into invasion chambers arranged in a six-well format at a density of 5×10^4 cells per chamber. After 5 d culture in MEF-CM supplemented 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. $P = 0.422$.

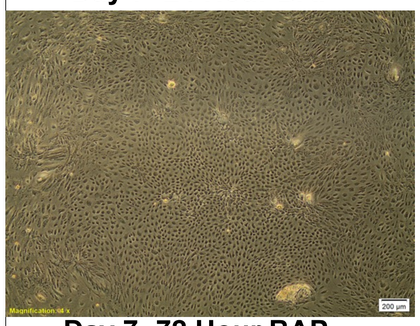
Preliminary Experiment and Results

The BAP medium was replaced with control medium (i.e. minus the BMP4 factor, the A83, and the PD) at different times during a seven-day culture period. The hESC were cultured under BAP conditions for either 24 h, 48 h, 72 h, or for the full seven days (control 1). After each set period, the medium was switched to simply 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.

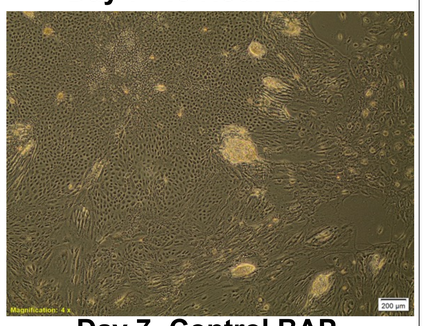


H1 ESC Passage 47

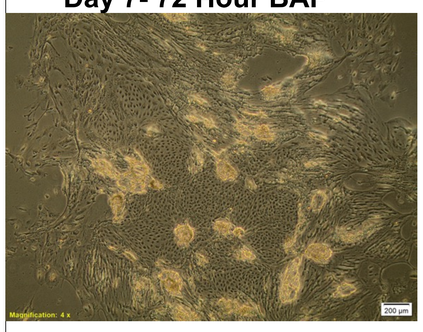
Day 7- 24 Hour BAP



Day 7- 48 Hour BAP



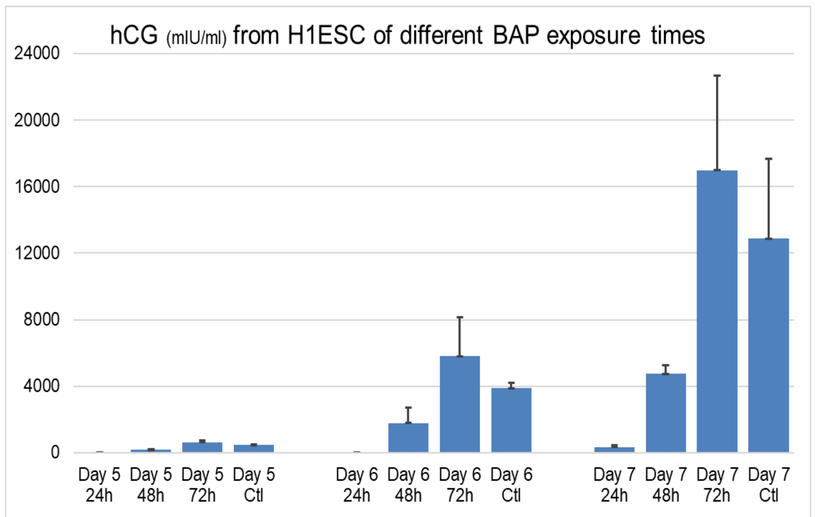
Day 7- 72 Hour BAP



Day 7- Control BAP

The 72 hour sample shows very similar morphology to the control sample. However, 24 and 48 hour samples showed almost undetectable or clearly reduced syncytialization. Because multinucleated syncytiotrophoblasts secret major amount of pregnancy hormones, such morphological differences are consistent with hCG production amounts (in the graph below).

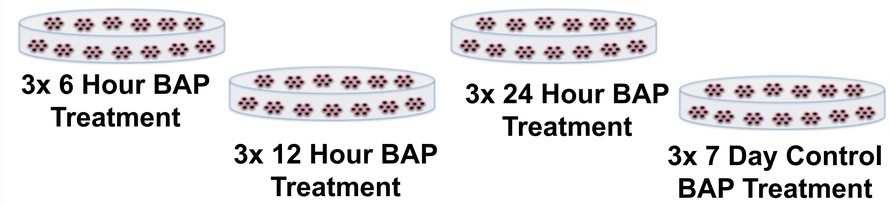
Human Chorionic Gonadotropin ELISA



The graph above shows the ELISA run on human chorionic gonadotropin in the collected medium of the experiment. Overall, the 72 hour sample shows even more hCG production than the control on days 5, 6, and 7.

Secondary Experiment and Results

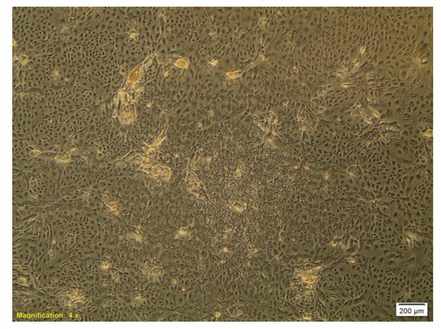
As seen above, the 48h BAP treatment was necessary to lead equivalent differentiation without supplementation of inhibitors, A83 and PD. Next we tested an idea that if the two inhibitors are maintained, the BMP4 treatment time can be shortened further. The BAP medium was replaced with control medium (i.e. minus the BMP4 factor, but still containing the two inhibitors) at different times during a seven-day culture period. The hESC were cultured under BAP conditions for either 6 h, 12 h, 24 h, or for the full seven days (control 1). After each set period, 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.



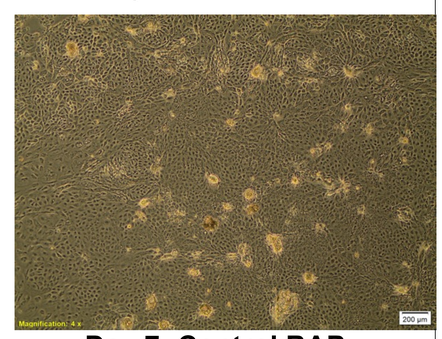
After set amount of time, the BMP4 was removed from the medium. Collected medium on days 5, 6, and 7 to run human chorionic gonadotropin ELISAs. Took images of the cells on days 5, 6, and 7. Collected DNA for normalization on day 7.

H1 ESC Passage 52

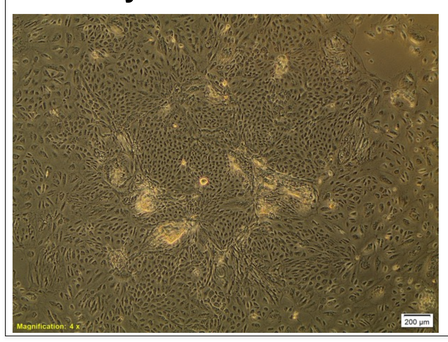
Day 7-6 Hour BAP



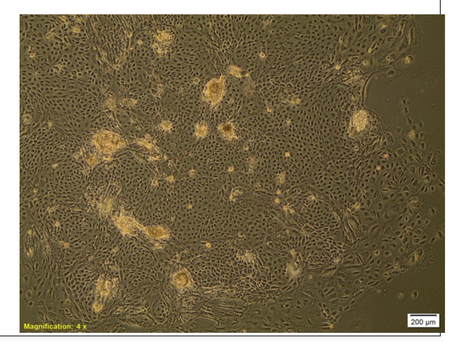
Day 7- 12 Hour BAP



Day 7- 24 Hour BAP



Day 7- Control BAP



Conclusions and Further Testing

Although our ability to conduct the hormone assays has been interrupted by COVID19, the emerging appearance of the cells exposed to BMP4 for as short a time as 6 hours was indistinguishable from the control held under BAP conditions for 7 days. Colony expansion, an epithelioid-like morphology to the cells, and colony ruffling (indicative of areas beginning syncytialization) were indistinguishable between treatments, as shown by the images above. These outcomes suggest, but do not prove, that a BMP4 exposure of only 6 hours is sufficient to prime the hESC for differentiation to trophoblast.
Further strengthening of this hypothesis is expected to come from the hormone measurements conducted on the media samples and accurate assessment of colony areas. In the future, we plan to do an immunohistochemical analysis for trophoblast markers (KRT7, HLA-G), to find colony growth rates, and to do RNA extraction at different days to assess marker gene expression by qPCR.

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. By keeping two inhibitors in cultures, cells with BAP for 24 h, 12 h and even 6 h showed similar morphological changes and syncytialization to the. It seems that 6 hours of BMP4 exposure is sufficient to drive the differentiation, but further testing will confirm this finding.

Funding Resources

Pluripotent human stem cells as models for normal and abnormal trophoblast at implantation
R01HD094937 (PD/PI: Roberts) 12/1/2018 – 11/30/2023 NIH/NICHD