Characterisation of mesoderm and endoderm in differentiating EPL cells

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Eindverhandeling voorgedragen tot het bekomen van de graad
Master in de biomedische wetenschappen afstudeerrichting
klinische en moleculaire wetenschappen

Universiteit Maastricht

universiteit hasselt

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List of abbreviations

BMP4	bone morphogenetic protein 4
dpc	days post coitum
EB	embryoid bodies (an attached number indicates days in culture)
EBM	embryoid bodies in MEDII (an attached number indicates days in culture)
EPL	early primitive ectoderm-like (an attached number indicates days in culture)
EPLEB	early primitive ectoderm-like embryoid bodies (an attached number indicates days in culture)
ES	embryonic stem
FCS	foetal calf serum
ICM	inner cell mass
LIF	leukaemia inhibiting factor
PI	propidium iodide
STREP-PE	phycoerithrin conjugated streptavidin
STREP-FITC	Fluorescein conjugated Streptavidin

Foreword

A great many people have helped me both in being able to come to Adelaide and in completing my internship; they all deserve my heartfelt thanks. First I would like to thank my Australian promoter Dr. Joy Rathjen for giving me the opportunity to do my internship in the Rathjen lab, presenting me with an interesting project for research and providing the expert knowledge and guidance to attain and interpret my results. I would also like to thank my Belgian promoter Prof. Dr. Piet Stinissen for stimulating me to do my intership abroad and for all the necessary feedback from back home. Further thanks should go to James Hughes for tirelessly assisting me both on the theoretical and practical front. I probably would not have lasted a day without him. Robert Moyer deserves my gratitude for helping me to get into contact with Joy Rathjen; without his mediating role I would not have gotten this great opportunity. Also thanks to the entire Rathjen Lab for all the help and the great working environment. My parents have supported me greatly before and during my internship. In general, I am grateful to the University of Hasselt and to Adelaide University for this rewarding learning experience.

Abstract

Embryonic stem cells have been the focus of a lot of attention because of their potential to differentiate into the tissues of the adult body and their progenitors. To study embryonic stem cells, the cells that are derived from them and the mechanisms involved in this differentiation, in vitro models can be set up. Mesoderm and definitive endoderm cells are two kinds of cells that are of interest because of the wide range of cells that are derived from them. Mesoderm leads to muscle, bone and haematopoietic cells and tissues, while endoderm is the progenitor of the tissues found in the gastrointestinal tract. There are no markers known that are specific for either of these cell types however. With markers, endoderm and mesoderm growth patterns could be visualised and studied in embryo's and pure colonies of these cell types could be produced, studied and characterised in more detail, possibly leading to information that in the future could be used in therapeutic therapies. In this study a few possible markers are introduced and cxcr4 is proposed as a candidate marker for definitive endoderm.

1 Introduction

The potential of embryonic stem (ES) cells to differentiate into a whole range of different cell types has prompted a lot of interest because of its potential in therapeutic applications. Extensive research has led to the characterisation of different cell types and many cellular processes that take part in the differentiation pathways. Much of this research was done on mouse derived stem cells because mouse and human embryogenesis, both at cellular and genetic level is fairly well known [1]. This information has led to the development of procedures that can influence cell properties and guide pluripotent cell differentiation [2].

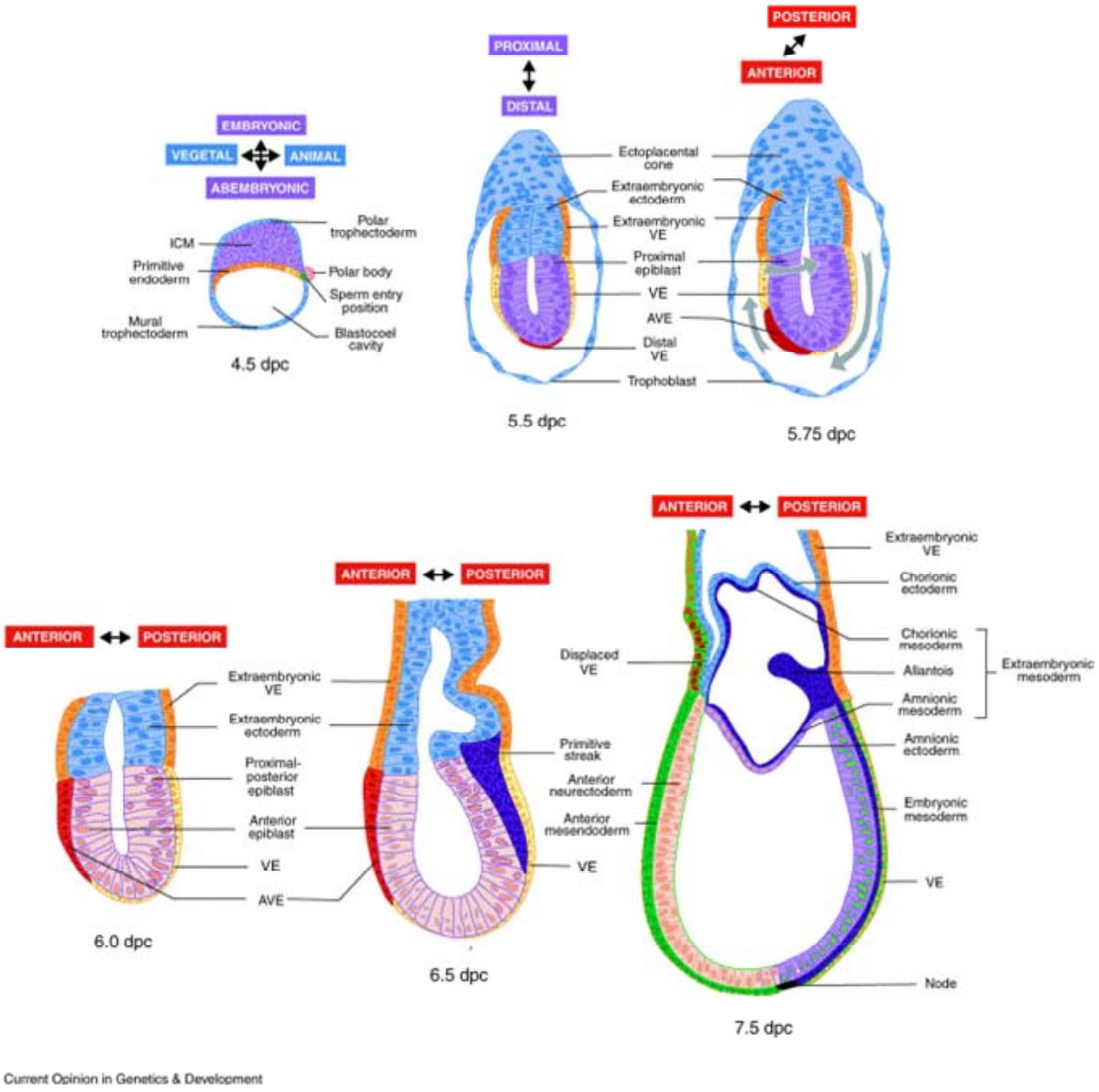

Figure 1: Embryogenesis. The proliferation and differentiation of the embryonal cells are depicted going from blastocyst through gastrulation. Time after conception is shown in days post coitum (dpc). [3]

The fertilized egg or zygote has the capacity to give rise to an entire organism. Because these cells can differentiate into all intra- and extraembryonic cell types, they are called totipotent cells. This ability is maintained until the fertilised egg hat split into 8 cells, called the morula. The cells of the morula differentiate and give rise to the blastocyst. The blastocyst is composed of an outer layer of trophectoderm and an inner cell mass (ICM), containing undifferentiated cells with a covering layer of primitive endoderm [4]. (Fig.1, 4.5dpc) The trophectoderm differentiates into the different cell types of the placenta and the primitive endoderm develops into the visceral and parietal endoderm of the yolk sac. The ICM becomes the primitive ectoderm. (Fig.1, 5.5-6.0) From the primitive ectoderm all embryonic and some extraembryonic tissues are formed. Mesendoderm, ectoderm, which are embryonic cell types, and extraembryonic mesoderm are formed from the primitive ectoderm during gastrulation [5]. (Fig.1, 6.5-7.5) The mesendoderm will differentiate into mesoderm and definitive endoderm. The mesoderm, definitive endoderm and ectoderm will all give rise to distinct cell lineages in the maturing embryo, which together comprise all the tissues in adults [6]. Mesoderm develops into hematopoietic cells and muscle and bone tissue, definitive endoderm gives rise to the tissues of the gastrointestinal tract and ectoderm forms the cells of the nervous system [5].

ES cells can be derived from the ICM and through addition of leukaemia inhibiting factor (LIF) the pluripotancy of the ES cells can be maintained. Methods of controlled, regulated and reproducible differentiation of ES cells in vitro are needed to better study their properties. One such model is the culturing of embryoid bodies (EB). These EB's are cellular aggregates wherein differentiation takes place. The outer cells of the EB differentiate into extraembryonic endoderm and the inner cells form cells equivalent to primitive ectoderm. From those cells mesendoderm and ectoderm are formed. A range of other cell types are derived from these germ lines through further differentiation. A possible problem with this model is that visceral endoderm, formed within the EB's, can influence the pattern of differentiation of other cell types. This could modify responses to different experimental conditions. To gain a clear picture of cellular responses to, for instance, growth factors it is necessary to have as little outside influence as possible [7].

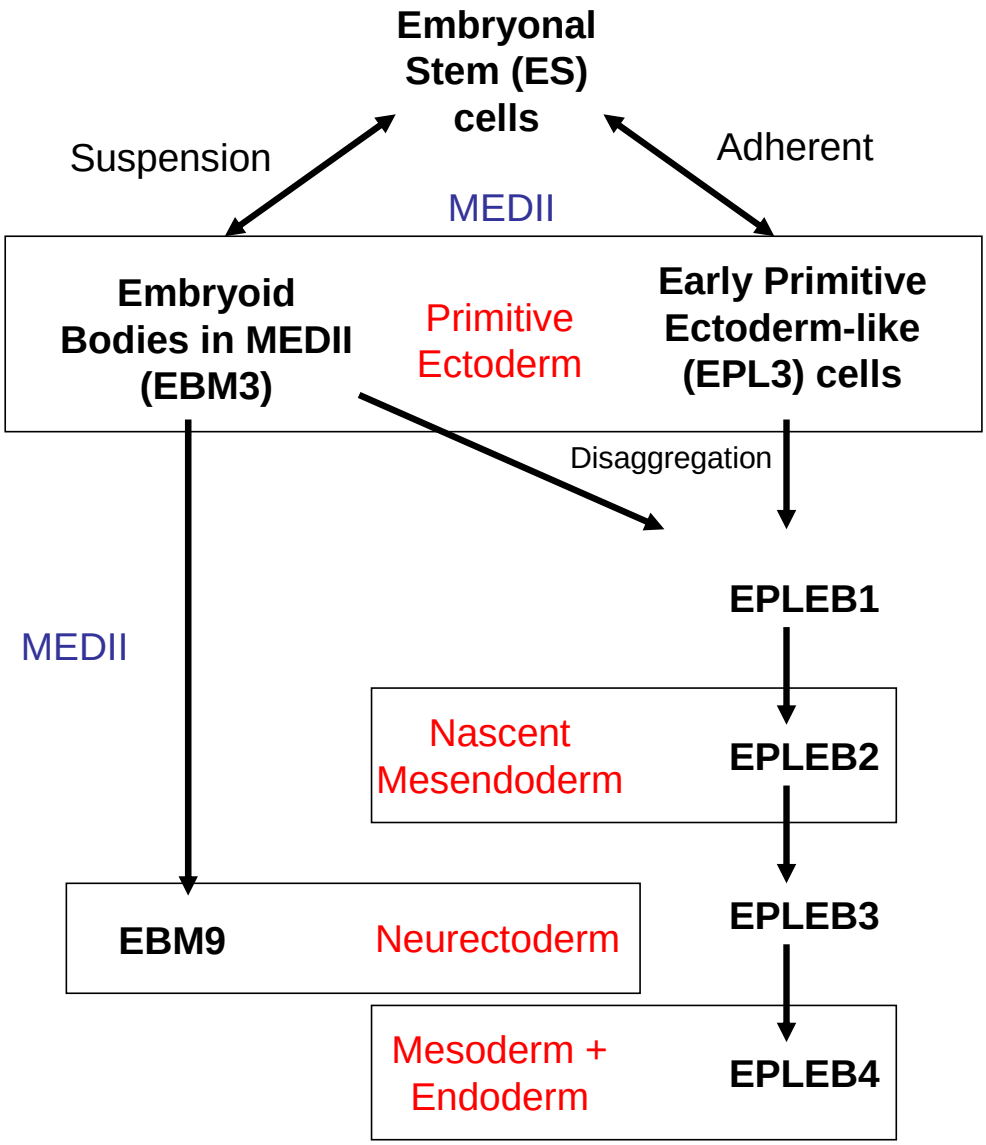

Figure 2:Cell culture and differentiation. ES cells can be cultured in different ways to guide differentiation. Additives to the media are shown in blue and the cell types at certain time points are shown in red. Numbers refer to the days in culture.

MEDII is a medium that has been conditioned by exposure to the hepatocellularcarcinoma cell line HepG2. When ES cells are cultured in the presence of this medium they reversibly [8] differentiate to early primitive ectoderm-like (EPL) cells. These cells are equivalent to the primitive ectoderm formed from the ICM. When differentiated, EPL cells do not form visceral endoderm and in this way prevent the signalling interference from these cells [9]. EPL cells can be grown both in adherent culture or as cellular aggregates, in which case they are called embryoid bodies in MEDII (EBM) [10]. The cells derived from these cultures are identical however. When EBM's are continued to be cultured with MEDII, they differentiate into a neural precursor population [11]. When MEDII is removed from the medium and the EPL cells are cultured in aggregates, called early primitive ectoderm-like embryoid bodies

(EPLEB), they differentiate into mesendoderm and subsequently into mesoderm and definitive endoderm. Because EPL and EBM cultures result in the same cells, both can be used to form EPLEB's.[10] (Fig.2)

Up to this point no marker has been found that can distinguish between definitive endoderm and mesoderm. Because of the different cell types that are derived from mesoderm or definitive endoderm, such a marker would be of interest. With it the cell type it is specific for could be studied in more detail without interference from other cell lines. A more accurate understanding of the underlying mechanisms of cellular functions could lead in advances in the field of therapeutic treatments of certain diseases. The aim of this project is to search for potential markers, test them and identify mesoderm or definitive endoderm specific molecules.

A microarray was performed in which expression of 22 thousand genes between EBM3, EPLEB2 and EPLEB4 was compared. From this data and from the literature potential markers were obtained. Hand1, Cdkn1c, Mest, Nrp1, PTPRD and Dlk1 are the microarray genes which are examined in this study and from the literature Cxcr4 was chosen.

2 Materials and methods

2.1 cell culture

D3 ES cells [12] were cultured in ESC media (90% DMEM with high glucose and no Hepes (Gibco-BRL, Cat.# 11995-065), 10% Foetal Calf Serum (FCS, Gibco-BRL, Cat.# 10099-141) supplemented with 1mM L-glutamine (Gibco-BRL, Cat.# 25030-081), approximately 10 units/ml leukaemia inhibitor factor (LIF), 0.1mM β-mercaptoethanol, 10 units/ml Penicillin/Streptomycin (Gibco-BRL, Cat.# 15140-122)). Cells were passaged after 2 days incubation at 37°C in 10% CO_2 when seeded at $1x10^6$ cells in 10cm dishes which have been specially treated to promote cell adhesion (tissue culture grade plasticware, BD Falcon) or after 3 days when seeded at $0.5x10^6$ cells. Media was changed daily. Single cell suspensions were made using trypsin (0.5% Trypsin/EDTA.4Na, Gibco-BRL, Cat.# 15400-054). The media was aspirated after which the cells were washed with PBS. 2ml trypsin was added and pipetted over the surface of the dish. After pipetting up and down for no more than one minute the trypsin was stopped using 2ml EB/differentiation medium (90%DMEM, 10%FCS supplemented with 1mM L-glutamine, 0.1mM β-mercaptoethanol, 10 units/ml Penicillin/Streptomycin). ES cells were centrifuged (1200xg for 2min) and the supernatant was aspirated. The cells were then resuspended in 1ml of EB/differentiation medium and counted using a haemocytometer.

Adherent EPL cells were formed by seeding $0.25x10^6$ ES cells in gelatinised 10cm dishes in 50%MEDII (50% MEDII [7], 45% DMEM, 5% FCS supplemented with 1mM L-glutamine, 0.1mM β-mercaptoethanol, 10 units/ml Penicillin/Streptomycin). Media was changed on day 2 and on day 3 the EPL cells were collected using trypsin in the same way as described above and then filtered using a 70μm nylon cell strainer (BD Falcon). The EPL cells were peletted by centrifugation (1200xg for 2min), resuspended in 1ml EB/differentiation medium and counted using a haemocytometer.

To form EBM's, ES cells were seeded at $1x10^6$cells in 10cm bacterial dishes (Techno-plas) in 50%MEDII. On day 2 media was changed and the bodies were split 1 in 2. On day 3 the bodies were collected or made into a single cell suspension. In the case of single cell suspensions, the medium containing the bodies was centrifuged (500xg for 1min), the medium was taken off and the bodies were washed with PBS. After the centrifugation (5OOxg for 1min) and aspiration of the PBS, 2ml of trypsin was added and the bodies were broken up by pipetting the trypsin and bodies for no more than 1min. The reaction was

stopped by adding the same amount of EB/differentiation medium. The cells were filtered with a 70μm nylon cell strainer (BD Falcon), pelleted by centrifugation, resuspended in EB/differentiation medium and counted with a haemocytometer. In case of a longer EBM series (up to day 8), the bodies were split 1 in 4 on day 2 and 1 in 2 on day 4 and media was changed on day 2, 4, 6 and 7.

EPLEB's are embryoid bodies formed from EPL3 or EBM3 cells. They were formed by seeding $1x10^6$ EPL3 orEBM3 cells in EB/differentiation medium. In bacterial dishes the bodies were split 1 in 2 bacterial plates on day 2 and the media was changed on day 2 and 4.

When cells were cultured in the presence of BMP4, the media was changed every day and human recombinant BMP (R&D Systems, Cat.# 314-BP-010) was added to a plate of 10ml to a final concentration of 10ng/ml.

2.2 RNA extraction and cDNA synthesis

RNA was extracted from EBM3 (EPL) cells, from ES3 cells, and from EPLEB's and EB's on days 1-5 of differentiation. The bodies were collected by centrifugation (1200xg for 2min), the supernatants were removed and the pellets were stored at -20°C until required. The ES cells were first made into a single cell suspension, collected by centrifugation (1200xg for 2min) and frozen. The RNA extraction was done with TRIzol Reagent (Invitrogen). Approximately $5x10^6$ cells were lysed in 1ml TRIzol by pipetting. The samples were incubated for 5min at room temperature. 0.2ml chloroform per 1ml TRIzol was added, after which the mixture was shaken for 15 sec and incubated for 2-3 min at room temperature. The samples were centrifuged for 15 min at 12000xg and 4°C. After centrifugation the mixture has split in two phases. The upper aqueous phase, which contains the RNA, was transferred to a clean 1-5ml tube and the RNA was precipitated by adding 0.5ml of isopropanol per 1ml of TRIzol used. After an incubation of 10 min at room temperature, the RNA precipitate was peletted by centrifugation (12000xg at 4°C). The supernatant was removed and the pellet washed with 1ml 75% ethanol per 1ml TRIzol used. The sample was mixed by vortexing and centrifuged at 7500xg for 5 min at 4°C. The RNA pellet was airdried, dissolved in RNase-free water and incubated for 10 min at 55°C. The RNA concentration was ascertained with a BioPhotometer (Eppendorf). The RNA was stored at -20°C.

For cDNA synthesis the Omniscript reverse transcription protocol and reagents (Qiagen, Cat.# 205113) were used, in addition to Oligo-dT primers (Invitrogen, Cat.# Y01212) and Superasin (Ambion, Cat.# 2694). A mix of Buffer RT (1x), dNTP Mix (0.5 mM of each dNTP),

Oligo-dT primer (1 µM), Superasin (10 units per 20µl reaction), Omniscript Reverse Transcriptase (4 units per 20µl reaction) and template RNA (up to 2µg per 20µl reaction) was incubated at 37°C for 60 min and stored at -20°C.

2.3 qRT-PCR PCR

Reactions of 25µl containing 12,5µl Platinum SYBRGreen qPCR Supermix (Invitrogen), 10ng of cDNA and 0.2µM of forward and reverse primers were incubated at 50°C for 2 min, denaturated at 95°C for 2 min and cycled at 60°C for 30 sec and 95°C for 15 sec for 40 cycles. Every reaction was done in triplicate and control reactions for Actin and noRT were included. A PTC-2000 Peltier Thermal Cycler coupled with a Chroma 4 Continuous Fluorescence Detector (MJ Research) was used for thermo cycling and detection. The primers flank one or more exon-exon boundaries. The primers used are described in Table 1.

Tabel 1: Primers used in qRT PCR

Gene	Primer sequences		Product length
Actin	Forward	5'-CTGCCTGACGGCCAGG-3'	89bp
	Reverse	5'-GATTCCATACCCAAGGAAGG-3'	
Hand1	Forward	5'-GAAAGCAAGCGGAAAAGG-3'	140bp
	Reverse	5'-GCCTGGTCTCACTGGTTTAGC-3'	
Cdkn1c	Forward	5'-TGATGAGCTGGGAACTGAGC-3'	187bp
	Reverse	5'-GCGCTGCTCTTGATTCTCG-3'	
Mest	Forward	5,-CCCTGTGATCCGCAATCC-3'	132bp
	Reverse	5'-GGTACGCAGCCAGCAAGG-3'	
Nrp1	Forward	5'-CTGTGCAAAACCAACAGACC-3'	112bp
	Reverse	5'-CCTGGAGATGTTCTTGTCACC-3'	
PTPRD	Forward	5'-TCGAACAGAGCGAAGAATCC-3'	136bp
	Reverse	5'-GAGAATCTTGGTGGGACACG-3'	
Dlk1	Forward	5'-AGGACTGCCAGCACAAGG-3'	138bp
	Reverse	5'-CACAGAAGTTGCCTGAGAAGC-3'	
Brachyury	Forward	5'-TGCTGCCTGTGAGTCATAAC-3'	143bp
	Reverse	5'-GCCTCGAAAGAACTGAGCTC-3'	
Mixl1	Forward	5'-CTTCCGACAGACCATGTACCC-3'	145bp
	Reverse	5'-GATAAGGGCTGAAATGACTTCCC-3'	

2.4 Cloning PCR fragments for in situ hybridisation

PCR fragments for in situ probes have to be longer than DNA fragments used for qRT-PCR, so a different set of primers was used. The primers used are described in Table 2.

Tabel 2: Primers used in PCR

Gene	Primer sequences		Product lenght
Hand 1	Forward	5'-CCTCGTTGCCTACAGAAACC-3'	844bp
	Reverse	5'-GCCTGGTCTCACTGGTTTAGC-3'	
Cdkn1c	Forward	5'-TGATGAGCTGGGAACTGAGC-3'	968bp
	Reverse	5'-GCTTACAGTGTCCCCCACT-3'	
Mest	Forward	5'-TCAGTACTCCATATTTGAGC-3'	528bp
	Reverse	5'-AACTCTGGATACGGATTTAT-3'	
Nrp1	Forward	5'-GGAGCTACTGGGCTGTGAAG-3'	802bp
	Reverse	5'-CCTGGAGATGTTCTTGTCACC-3'	
Dlk1	Forward	5'-CAACTTCTGTGAGATCGTAG-3'	503bp
	Reverse	5'-ACTTGTTGAGAAAGACGAT-3'	
Ttr	Forward	5'-AGTCCTGGATGCTGTCCGAG-3'	441bp
	Reverse	5'-TTCCTGAGCTGCTAACACGG-3'	

PCR was performed on EPLEB4 or EPLEB5 cDNA to obtain DNA fragments for insertion into vectors. The PCR mix consisted of 10µl Platinum PCR Supermix (Invitrogen, Cat.# 11306-016), 1µl of each primer (5µM concentration) and 1µl cDNA. This mixture was incubated at 94°C for 1 min and cycled 33 times at 94°C for 30 sec, 60°C for 30 sec and 72°C for 45 sec in a PTC-200 Peltier Thermal Cycler (MJ Research).

This fragment was introduced into a pGEM-T Easy vector (Promega, Cat.# A1360) by mixing with a ligation reaction mix that consisted of 5µl 2x Rapid Ligation Buffer, 1µl pGEM-T Easy Vector (50ng), 2µl PCR product, 1µl T4 DNA Ligase and 1µl MQ water. The mixture was incubated for 1 hour at room temperature.

2µl of the product was added to 50µl DH5α competent cells and gently mixed. The cells were incubated on ice for 20 min. Next the cells were heat-shocked for 45-50 sec at 42°C and returned to ice for 2 min. 950µl Luria Broth/ampicillin was added to the cells and the cells were incubated for 1.5 hours at 37°C with shaking. 100µl of the culture was then plated out on a LB/ampicillin/IPTG/x-Gal plate and incubated overnight at 37°C. White colonies were then introduced into a liquid LB/ampicillin medium and incubated overnight with shaking.

Plasmids were extracted from the cells using a QIAprep Spin Miniprep Kit (Qiagen, Cat.# 27106). Plasmids were then tested by PCR to examine if the correct DNA fragment was inserted and the amount of plasmid was analysed with a BioPhotometer (Eppendorf). Using a combination of the gene primers and the M13-M4 and M13-RV primers (see Table 3) and PCR the orientation of the fragment in the vector was determined.

Tabel 3: Plasmid primers

	Primer Sequence
M13-M4 (forward)	5'-GTTTTCCAGTCACGAC-3'
M13-RV (reverse)	5'-CAGGAAACAGCTATGAC-3'

A PCR was then performed using the M13-M4 and M13-RV primers and the amount of PCR product was analysed with gel electrophoresis using the Spp1 ladder.

2.5 Probe manufacture

Probes for in situs were produced using the DIG RNA Labeling Mix (Roche, Cat.# 1277073). 100-200ng PCR product was mixed with 2µl DIG RNA labelling mix, 10x, 2µl Transcription Buffer, 10x, 2µl RNA polymerase 20 U/µl (Sp6 or T7 depending on the orientation of the fragment), 2µl DTT, 1µl Superasin and MQ up till 20µl. The mixture was incubated for two hours at 37°C. 2µl DNase I was added and incubated for 15 min at 37°C. The reaction was stopped by adding 2µl 0.2 M EDTA (pH 8.0).

2.6 In situ hybridisation

The cells were washed 3x5 min in PBT (PBS + 0.1%TritonX100). The cells were then permeabilised with three washes of 20 min in RIPA buffer (150mM NaCl, 1% NP-40, 0.5% NaDOL, 0.1% SDS, 1mM EDTA, 50mM Tris pH8.0) Next the cells were fixed for 20 min in 4% formaldehyde in PBT + 0.2% glutaraldehyde and washed 3x5 min in PBT. The cells were washed 5 min in 50% hybridisation buffer (50% formamide, 5x SSC, 0.1% Tween 20 (Sigma)) in PBT and 5 min in hybridisation buffer. 100µg/ml herring sperm DNA that was denaturated at 80°C for 10 min and then snap cooled on ice was added together with 100µg/ml tRNA to hybridisation buffer. This was added to the cells and the cells incubated for 1 hour at 65°C in a box humidified with soaked towels. The cells were split into anti-sense and sense wells and hybridisation buffer/hsDNA/tRNA with anti-sense or sense probes (1/500 dilution) that was denaturated for 10 min at 80°C and snap cooled was added to the cells. The cells were returned to the box, wrapped and incubated overnight at 65°C.

On day 2 the cells were washed 5min in Post Hybridisation Wash Buffer (PHWB: 2x SSC, 50% formamide, 0.1% Tween 20 (Sigma)) followed by 3x30 min washes in PHWB at 65°C. The cells were washed 3x5 min in 1x TBST (1.37M NaCl, 27mM KCl, 0.25M Tris-HCl pH7.5, 1% Tween 20 (Sigma) in H_2O) and 1 hour in 10% FCS in TBST. AP-conjugated anti-Digoxigenin (150U/200µl, Roche, Cat.# 1093274) was added to 1% FCS in TBST (1/2000 dilution), added to the cells and incubated over night at 4°C.

On day 3 the cells were washed 3x5 min in TBST and 2 hours using 3 washes of TBST. Next the cells were washed 3x10 min in AP buffer (100mM NaCl, 50mM $MgCl_2$, 0.1% Tween 20 (Sigma), 100mM Tris pH9.5). A development solution, consisting of 450µg/ml NBT (Roche, Cat.# 1383213) and 175µg/ml PCIP (Roche, Cat.# 1383221) in AP buffer, was added to the cells. The cells were then wrapped with foil and incubated until the staining became visible. The reaction was stopped by washing the cells 3x5 min in 1mM EDTA in PBT.

2.7 Sequencing

Sequencing of DNA fragments was done by mixing approximately 500ng DNA with 4µl Big Dye Terminaror Mix v3.1 (Applied Biosystems, Cat.# 4336913), 1µl of primer and MQ up to 20µl. The mixture was incubated at 96°C for 1 min and cycled for 25 times at 96°C for 10sec, 50°C for 5 sec and 60°C for 6 min in a PTC-200 Peltier Thermal Cycler (MJ Research). The PCR reaction was transferred to an eppendorf tube and mixed with 80µl 75% isopropanol and incubated for 20 min at room temperature. The tube was centrifuged for 20 min at 13200rpm and 4°C. The supernatant was removed and the pellet resuspended by vortexing in 250µl 75% isopropanol. After a centrifugation of 5min at 13200 the supernatant was removed and dried for 5 min at 55°C. The sample was brought to the Institute for Veterinary Medicine (IMVS) for sequencing.

2.8 Flow cytomety

2.8.1 Single cell suspensions

Single cell suspensions were obtained by trypsin as described above in 2.1. Alternitavely, cells were disassociated with Collagenase Type IV(1mg/ml EB/differentiation medium, Gibco-BRL, Cat.# 17104-019), DispaseII (1mg/ml EB/differentiation medium, Roche, Cat.# 165859) and enzyme free cell dissociation buffer (Gibco-BRL, Cat.# 13150-016). The protocols were as follows.

The bodies were pelleted by centrifugation (500xg for 1min) and the supernatant aspirated. The bodies were washed with PBS and after centrifugation (500xg for 1min) and aspiration of

the PBS, 2ml Collagenase IV, Dispase II or dissociation buffer was added. The bodies were incubated for 30min at 37°C. In the case of Collagenase IV and Dispase II, the bodies were spun down (500xg for 1min). The bodies were then washed with PBS and pelleted by centrifugation (500xg for 1min). 1ml of enzyme free dissociation buffer was added and the bodies were broken up by pipetting for no more than 1min. In the case of enzyme free dissociation buffer the wash with PBS was not necessary and the bodies were broken up using pipetting and the dissociation buffer already present. Once the bodies were broken up the cells were put through a 70µm nylon cell strainer and pelleted by centrifugation (1200xg for 2min). The supernatant was aspirated and the cells were resuspended in 1ml 1% BSA/PBS. The cells were then counted using a haemocytometer.

2.8.2 Cell staining

During the staining and washing steps, the cells were kept on ice. The cells were divided in tubes (about $2x10^5$ cells per tube) and pelleted by centrifugation (1400xg for 5min).

If the cells were to be fixed, they were incubated in a 1% Paraformaldehyde/PBS solution for 15 min. After this the cells were pelleted by centrifugation (1400xg for 5min) and resuspended.

If the cells were stained for Cxcr4, cells were blocked in 10%BSA/PBS for 15min. Primary antibody (4.5µg/ml) was added to $2x10^5$ cells and incubated on ice for 30min. The primary anti-bodies were either Biotinilated anti-mouse Cxcr4 (anti-Cxcr4, BD Biosciences Pharmingen, Cat.# 551968) or Biotinilated rat $IgG_{2b,\kappa}$ (IgG, BD Biosciences Pharmingen, Cat.# 553987) as an isotype control. The cells were pelleted by centrifugation (1400xg for 5min) and washed 2x5min in PBS. The cells were resuspended in streptavidin conjugated secondary fluorophore (9µg/ml) and incubated for 30min at 4°C in the dark. Phycoerithrin conjugated Streptavidin (STREP-PE, Rockland, Cat.# S000-02) and Fluorescein conjugated Streptavidin (STREP-FITC, Rockland, Cat.# S000-08) were both used as secondary fluorophore. After 20min of incubation 5µl of a 5µg/ml solution of propidium iodide was added, to appropriate tubes to indicate dead cells. After the 30min incubation the cells were pelleted by centrifugation (1400xg for 5min), washed 3x5min in PBS, resuspended in 300µl PBS and analysed using a BD FACSCanto.

3 Results

EPL cells cultured under the right conditions form aggregated cell bodies that are composed of mesoderm and definitive endoderm. These two cell lineages have the capacity to develop a great range of different cell types. To study these two cell types in vitro it would be useful to develop a technique to distinguish between them. The aim of the following experiments is finding a marker for either mesoderm or endoderm.

3.1 Microarray candidates

The basic data used for identifying potential markers was obtained through microarray. In this microarray the expression of 22 thousand genes was compared between EBM3, EPLEB2 and EPLEB4 cells. The microarray was done by Joy Rathjen.

3.1.1 Identification of potential markers

The cell types of interest are postulated to exist around day 3 in EPLEB's. A potential marker would therefore have an expression pattern that is up regulated in EPLEB4 compared to EBM3 or EPLEB2 in the microarray. When analysing the microarray data, genes that showed an up regulation in EPLEB4 relative to EBM3 and EPLEB2 were selected. Out of this list of potential candidates, six genes were chosen for further analysis because of two defining characteristics, either a very high fold up regulation or genes whose protein product was membrane bound. The gene product being membrane bound would make staining with anti-bodies and flow cytometry on live cells possible. The transcription factor Heart and neural crest derivatives expressed transcript 1 (Hand 1), Cyclin dependent kinase inhibitor 1c (Cdkn1c) and Mesoderm specific transcript (Mest) were chosen as transcripts highly up regulated and Neuropilin1 (Nrp1), Protein tyrosine phosphatase receptor D (PTPRD) and Delta-like homolog (Dlk1) were chosen out of the membrane bound group. (Fig.3)

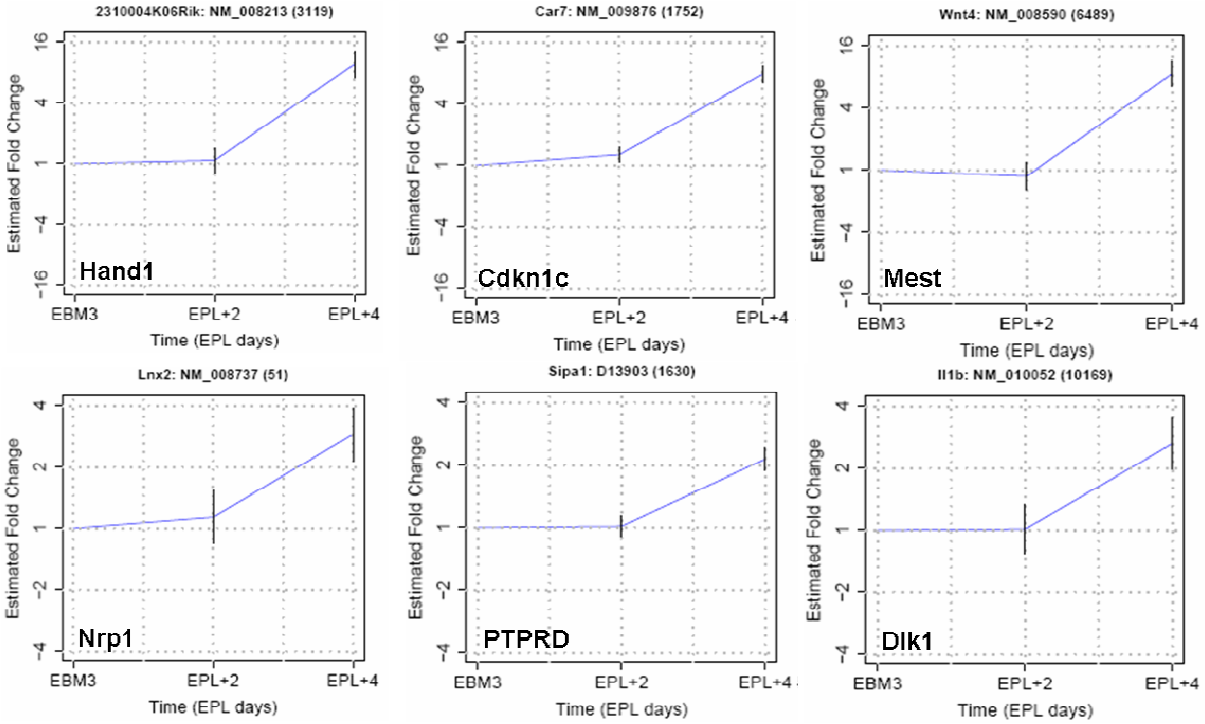

Figure 3: Microarray results for candidate genes. Fold change in expression between EBM3, EPLEB2 (EPL+2) and EPLEB4 (EPL+4) of the candidate genes.

3.1.2 Set up system for verification

To test possible markers, an in vitro model had to be set up. EPLEB's contain both mesoderm and endoderm, and would therefore be ideal for examining candidate markers. EPLEB's and EB's were collected on days 1-5 and the RNA was extracted. EPLEB's form mesendoderm earlier than EB's with a transcient peak of expression of early mesendoderm markers Brachyury and Mixl1 seen on day 2-3 for EPLEB's compared to day 4-5 in EB's. [9, 13,14]

To check whether the EPLEB and EB series obtained through cell culture conformed to this model, quantitative real time PCR was performed for Brachyury and Mixl1. In the EB series Brachyury came up on day 4 and went down again on day 5. In the EPLEB series the expression of Brachyury reached a peak on day 2 and went down again after that. The expression of Mixl1 in the EB series increased on day 4 and continued to rise on day 5. In contrast the Mixl1 expression in the EPLEB series was up regulated on day 2 after which it went down again. (Fig.4)

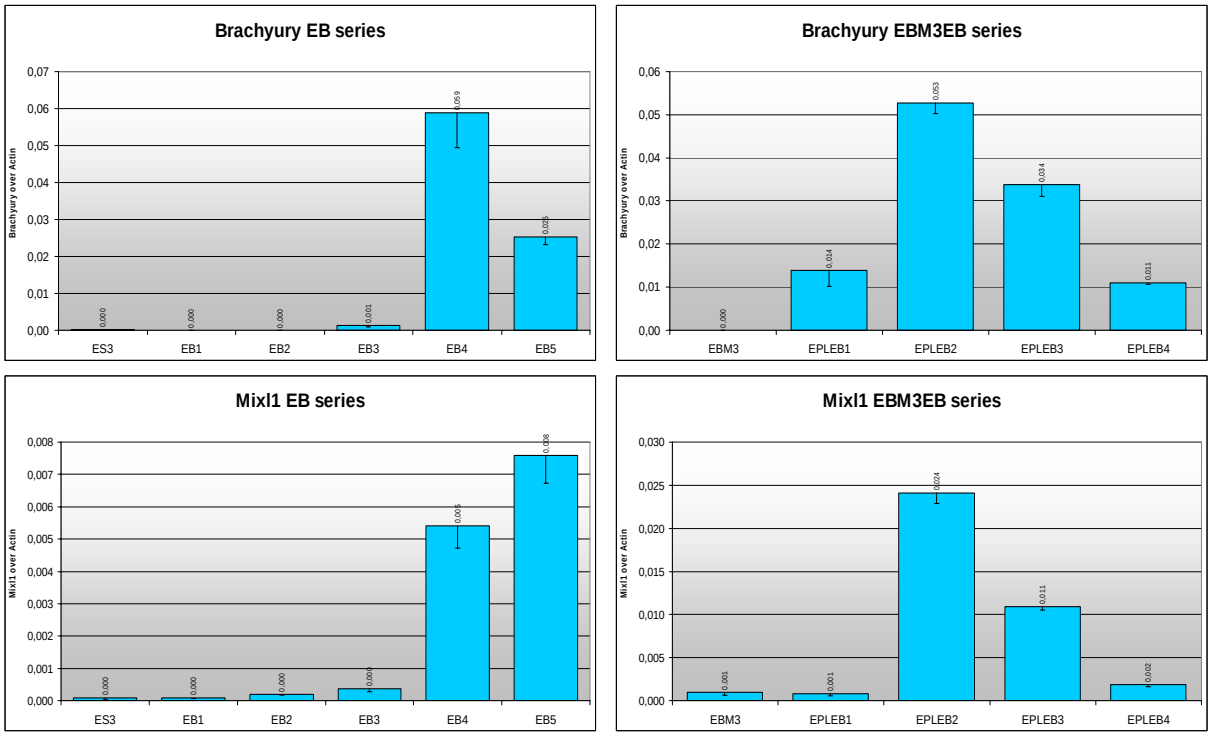

Figure 4: Brachyury and Mixl1 expression over an EB and EPLEB series. Expression of the mesendoderm markers Brachyury and Mixl1 over an EB and EPLEB series were examined by qRT-PCR. The measurements were compared with actin expression which is constant.

3.1.3 Verification of microarray data for the candidate markers

To confirm the gene transcription data obtained through microarray and to study it in more detail, qRT-PCR was performed for Hand1, Nrp1, Mest, PTPRD, Dlk1 and Cdkn1c using cDNA obtained from an EPLEB series. For Mest and Dlk1 the series was composed of cDNA from EBM3 and EPLEB1 till EPLEB5. For the other genes the series stopped at EPLEB4. In the microarray the RNA expression of EBM3, EPLEB2 and EPLEB4 was compared.

All genes that were examined here were up regulated on day 4 in the microarray data. By qRT-PCR Hand1, Cdkn1c, Mest and Nrp1 were up regulated on day 3 and day 4. Dlk1 appeared to be modestly up regulated by day 3 go down again on day 4 and up regulate on day 5. PTPRD was up regulated on day 2. (Fig.5) So, except for PTPRD the qRT-PCR data showed a good correlation with the microarray data.

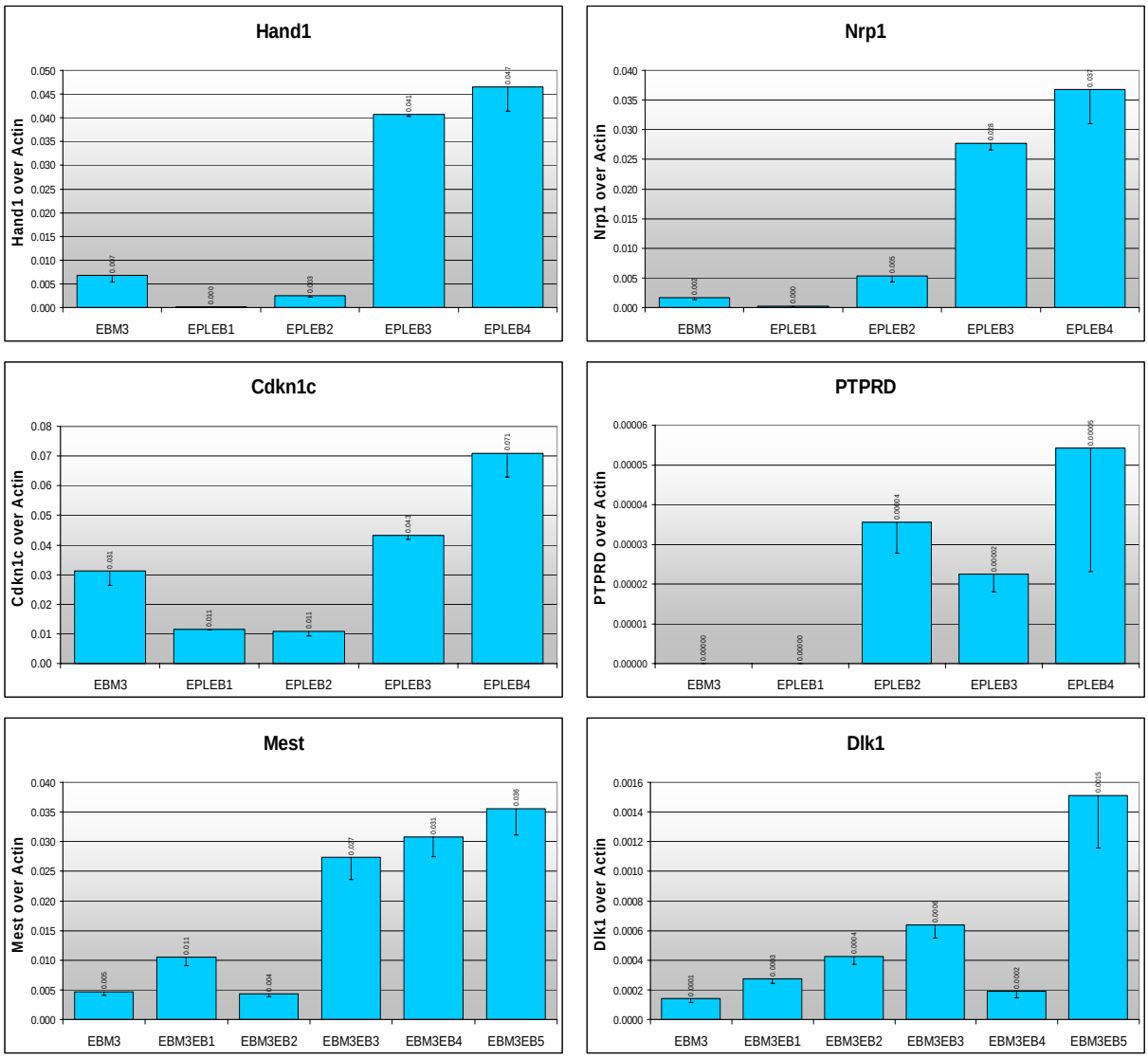

Figure 5: Expression of candidate genes over an EPLEB series. The expression relative to Actin of the candidate genes over an EPLEB series was examined by qRT-PCR. Mest and Dlk1 include one more day than the other genes.

3.1.4 Cloning PCR fragments for in situ analysis

To study the candidate genes further, probes were designed for in situ hybridisation. First, appropriate DNA fragments had to be cloned. A new gene, Ttr, was included in this experiment as control. Ttr is a marker for visceral endoderm which should not be present in EPLEB's. Nanog, a transcription factor with a role in maintaining pluripotency and shown to be present in ES cells and the ICM and re-expressed in late posterior primitive ectoderm [15], was used as a test for the technique.

Due to technical difficulties no DNA fragment could be produced or obtained in sufficient quantities to synthesise in situ probes. The primers for Cdkn1c didn't produce a PCR product

when run on cDNA from EPLEB4's and EPLEB5's. The Dlk1 primers produced multiple bands. Hand1, Nrp1, Mest and Ttr were inserted into pGEM-T Easy vectors and transvected into competent cells. The cells were cultured and the plasmids were extracted. Cells containing plasmids with Hand1 and Nrp 1 fragments only yielded low amounts of plasmid. When PCR was performed for the plasmids, fragments of the right size were obtained for Nrp1, Mest and Ttr, but not for Hand1. These PCR fragments could not be amplified in sufficient quantity for probemaking. From the genes above only Nrp1 and Ttr could be identified by sequencing. The other sequencing reactions were unsuccesful.
The plasmids containing Nanog fragments were already prepared and the PCR resulted in a fragment of about 1200bp.

3.1.5 Probe making and nanog in situ

Because of insufficient template no probes were made for the candidate genes. Nanog was used as a test for both probemaking and in situ hybridisation. When the probes were loaded on an agarose gel and after electrophoresis multiple bands were seen.
The in situ hybridisation was done on a series of EPLEB's. The expression of Nanog, a transcription factor important in maintaining cell pluripotency, in these bodies was examined. The resulting staining showed little staining in EBM3, after which expression was up regulated in EPLEB1 and EPLEB2. In EPLEB3 the expression became patchy, with a few clear regions inside bodies with higher expression than surrounding cells. Heterogeneous staining was still evident in EPLEB4's, and EPLEB5's. Staining appeared greater in EPLEB5 in the smaller bodies. The big bodies on day 5 seemed to stain les compared with day 4. (Fig.6) The bodies incubated with the sense probe did not show any visible staining.

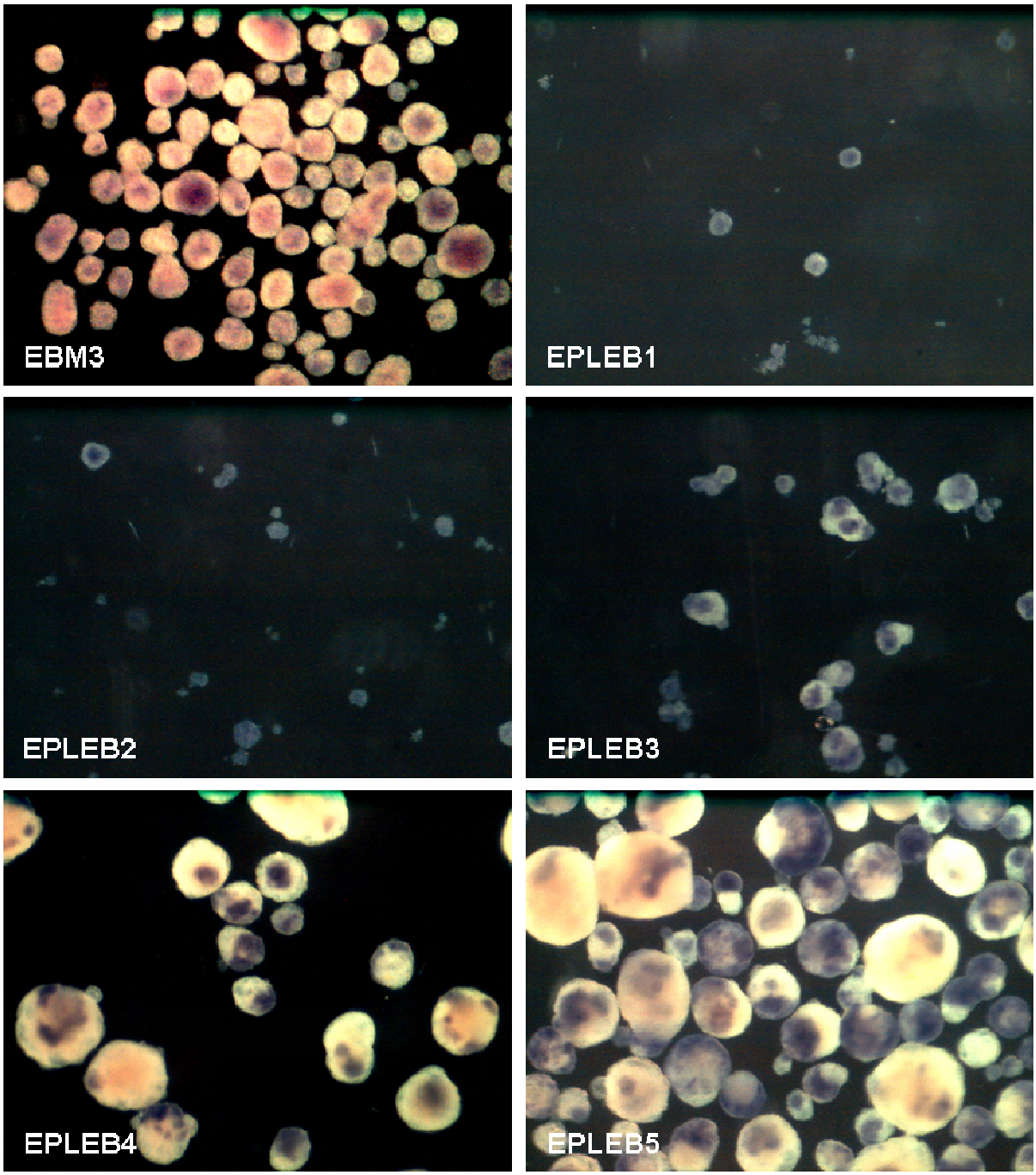

Figure 6: In situ hybridisation of Nanog. An in situ hybridisation was done on an EPLEB series. The bodies were stained with Nanog anti-sense probes. The sense control probes showed no staining (data not shown).

3.2 Cxcr4

From the literature a candidate marker for definitive endoderm, called Cxcr4, was identified [16]. This gene, a chemokine receptor, has been used as a marker to distinguish definitive from visceral endoderm. Here, we wanted to establish Cxcr4 as a marker that could distinguish definitive endoderm from mesoderm. Anti-bodies for Cxcr4 were available so

flow cytometry was used for this experiment. First a suitable protocol had to be designed after which Cxcr4 expression was examined over a series of EPLEB's and EBM's.

3.2.1 Anti-Cxcr4 compared with isotype control

Fixed EPLEB4 cells were incubated with anti-Cxcr4 to test if the antibody bound Cxcr4 with this system. As control, both unstained cells and isotype control were included in the experiment. The primary anti-bodies in this experiment were added at 9µg/ml and the secondary fluorophore at 18µg/ml. 87% of cells stained with the anti-Cxcr4 showed higher fluorescence than unstained cells. However isotype control showed 68% cells with fluorescence above unstained cells. The high level of staining in the isotype control makes it difficult to measure the percentage of positive cells, although the presence of 64% cells obove isotype fluorescence indicates the antibody is working. (Fig.7)

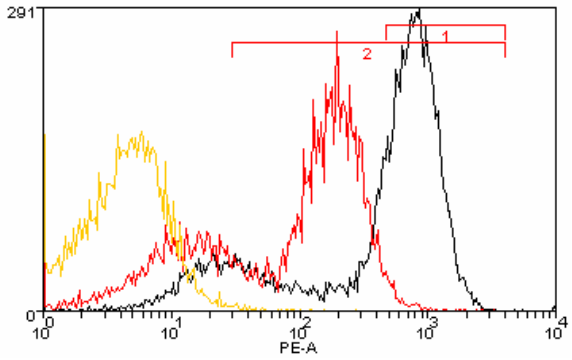

Figure 7: Cxcr4 staining of EPLEB's. A single cell suspension of EPLEB4's was stained with anti-Cxcr4 (black curve) and compared with cells stained with IgG (red curve) and unstained cells (yellow curve). The secondary fluorophore used was STREP-PE. The histogram shows the amount of staining of the cells. Gate one contains the fraction of cells that show staining above IgG background. Gate 2 contains all positive cells.

3.2.2 Live or fixed cells

To determine whether cells would be easiest to analyse when they were fixed or unfixed, an experiment with EBM3 cells was set up using PI staining to detect dead cells. When the scatter plots for live and fixed cells are compared a difference in cell size distribution is noticeable. In the unfixed cells two distinct populations are seen (Fig.8 A and B), while fixed cells resolve into single population (Fig.8 C and D). The group of cells with high forward scatter is composed mainly of cells with a low PI staining, indicating that this group contains mostly live cells. The group of cells with lower forward scatter contains mostly PI positive cells. There are no PI negative cells when the cells are fixed before staining. Both scatter plots

have a group of dots with very low forward and side scatter that have very little PI staining, indicative of cellular debris.

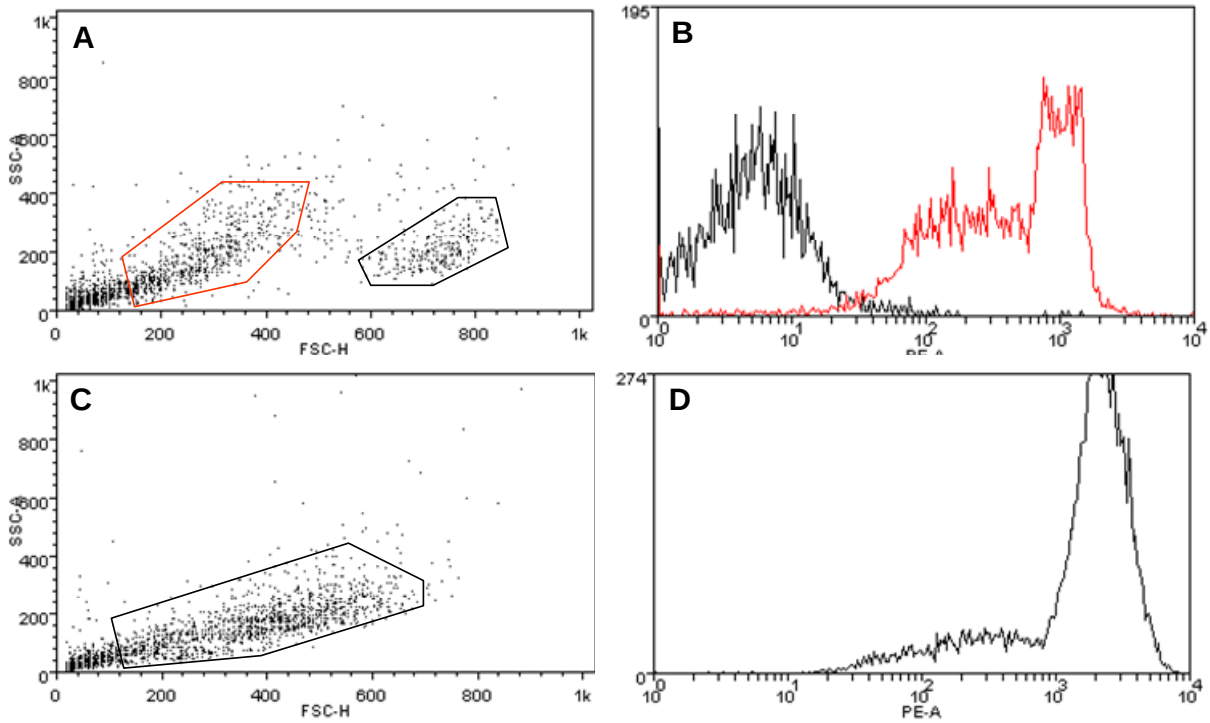

Figure 8: Unfixed versus fixed cells. A single cell suspension of EPLEB5's was either PE stained directly without fixing (A and B) or stained after fixing with 1% Paraformaldehyde (C and D). The dot plot represents the forward and side scatter of the cells. The unfixed cells could be split in two groups and accordingly gated, while only one gate was used in the fixed group. The histogram shows the degree of staining of the cells.

This experiment was repeated with EPLEB2 cells. Unfixed cells resolve into two distinct regions, while the fixed cells again form a single large group. Gate 1 shows no PI staining, gate 2 and contains PI positive cells. Gate 3 shows only PI positive cells. The results are not shown because they are very similar to those of the last experiment.

3.2.3 Cell dissociation and Cxcr4 on unfixed cells

To examine whether different cell dissociation techniques might influence the result, different products for making single cell suspensions were tested. EPLEB5 bodies were dissociated with collagenase IV, Dispase II, enzyme free cell dissociation buffer and trypsin. When the cells were counted immediately after dissociation the viability was best in Dispase II. (Table)

If the region earlier identified as live cells is gated anti-Cxcr4 and isotype control binding can be compared. Dead cells can not be controlled for because no PI staining was used. When the different dissociation techniques are ranked according to IgG background staining, Dispase II

and Trypsin are the lowest, with Collagenase IV and enzyme free cell dissociation buffer following.

The Cxcr4 staining leads to an emergence of a positive and negative population in all the techniques used except when using Trypsin. Trypsin only shows a slight raise in positive cells. (Fig.9 and Tabel 4)

Tabel 4: Summary of cell dissociation results.

	Viability (%)	IgG background	Poistive staining (%)
Collagenase IV	40	intermediate	42
Dispase II	80	low	40.6
EFDB	37	high	36
Trypsin	50	low	16.8

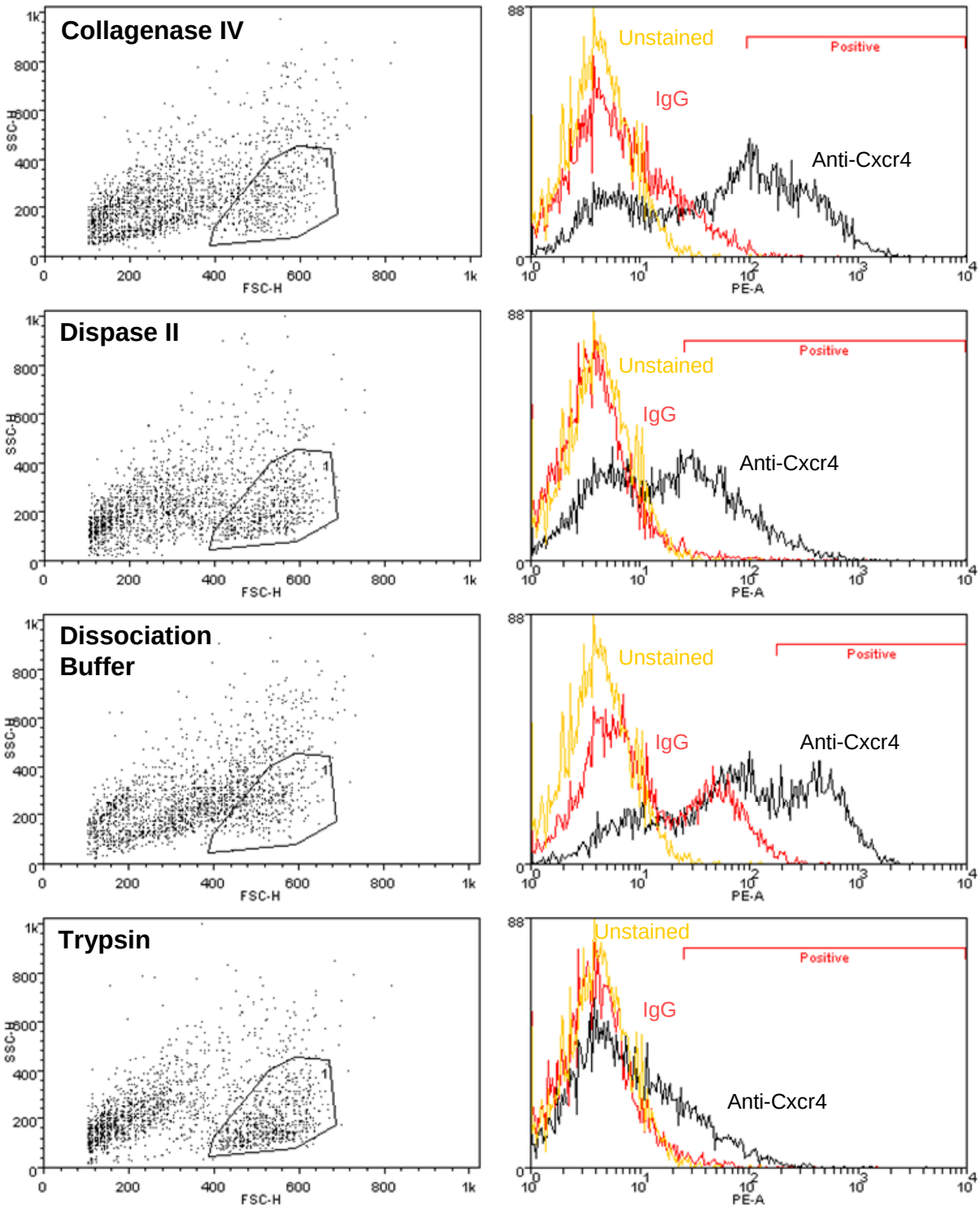

Figure 9: Cell dissociation methods. Different cell dissociation methods were used on EPLEB5's and stained with anti-cxcr4 (black curve) or IgG (red curve). The secondary fluorophore used was STREP-PE. No PI staining was used so a gate for live cells was chosen according to information obtained in previous experiments (Gate 1 in the dot plots). Only cells within this gate were included in the experiment. The staining intensity of both groups were compared with each other and unstained control cells (yellow curve).

3.2.4 Secondary fluorophore

A secondary fluorophore had to be tested that could be used in conjunction with PI so viability could be visualised. STREP-FITC was chosen. At the same time different concentrations of the secondary were used to test its maximise the staining of positive cells.

Dead cells seem to have a large positive population when stained with anti-Cxcr4. This population is absent in the isotype control. Isotype control in the lower concentration (10µg/ml) is approximately equal to the PI only control group. When a higher concentration of secondary fluorophore (40µg/ml) was used, both the isotype control and Cxcr4 stained groups showed a higher positive cell count when the same gate as with the lower concentration was used. When the gate was changed to exclude positive staining in isotype control, the percentage of positive staining in the Cxcr4 stained group became much lower than when a low concentration of secondary fluorophore was used. The percentage of positive live cells in the group with a lower concentration of secondary staining is 23.9%. (Fig.10)

STREP-FITC works, but a low concentration needs to be used to avoid either too much background staining or lack of staining.

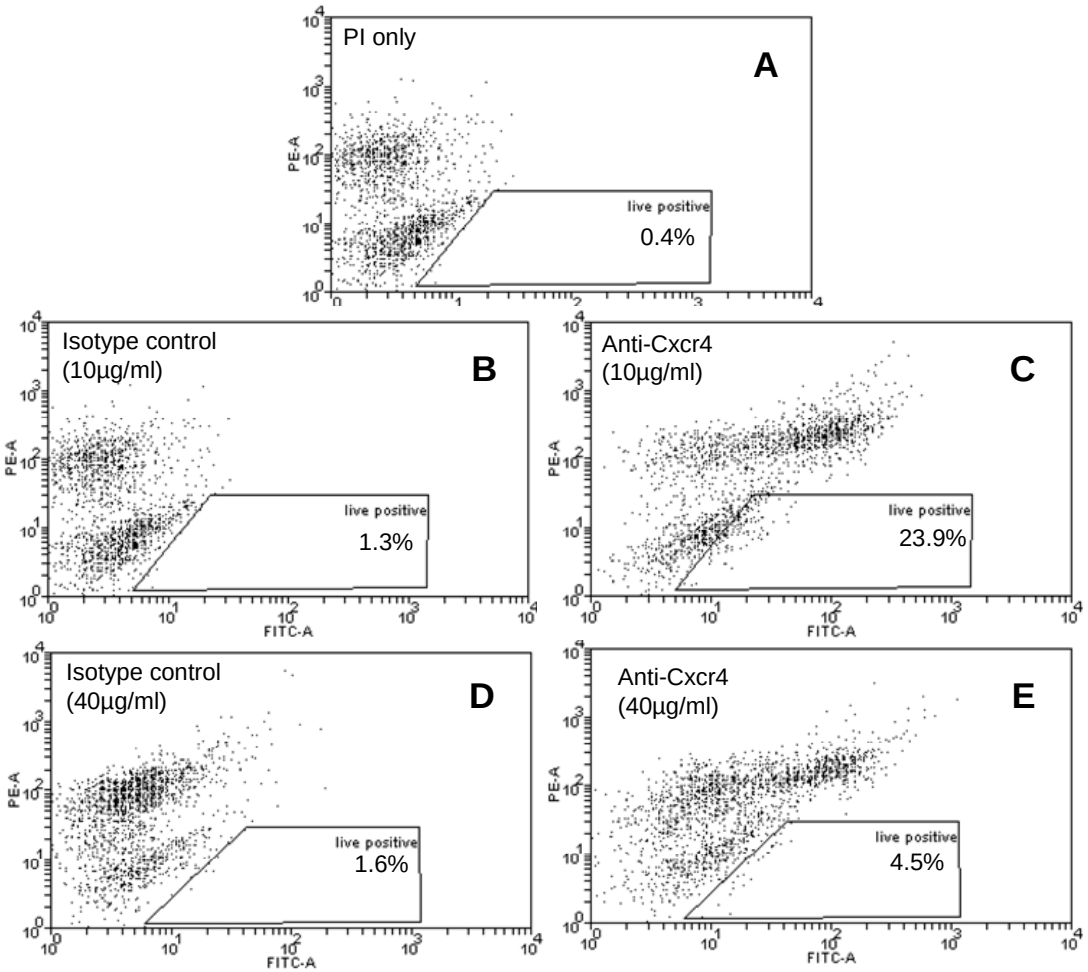

Figure 10: STREP-FITC Secondary fluorophore. Two different concentrations of secondary fluorophore were tested. A PI only staining was used as control (A). For both concentrations the isotype control and Cxcr4 stained live positive fractions were compared. B and C show the results for 10µg/ml and D and E for 40 µg/ml secondary fluorophore.

3.2.5 The expression of Cxcr4 over an EPLEB series

Differentiation of mesendoderm into mesoderm and endoderm in EPLEB is postulated to happen around day 3 in EPLEB's. If Cxcr4 were a marker for endoderm and not mesoderm two different populations should arise around this time. One population expressing Cxcr4 and no expression in the other. An EPLEB series was set up to test this. As a control an EBM series was set up at the same time. EBM's do not differentiate into mesoderm or endoderm, so no expression should be detected. EPLEB1 wasn't included in this experiment, because no staining was expected on this day.

The gate for PI positive or negative was chosen using a gate around all cells (EPLEB4) excluding cell debris and then choosing the point in the middle between the two peaks. The border between FITC positive and negative seems to run diagonally in stead of vertically in live cells. Because of this a gate with a diagonal border was chosen.

EBM3 and EPL3 cells show a moderately increased Cxcr4 live positive count (3.9% and 7.1% of live cells respectively). In the EBM series the expression of Cxcr4 seems to go down again after the first measurement on day 2, before a moderate rise to 8.3% on EBM8. (Fig.11 and Tabel 5)

In the EPLEB series the expression goes down right after EPL3. On day 3 the expression rises again and reaches a peak on day 4 with more than 40% of the cells expressing Cxcr4. The expression on day 5 is lower than on day 4. The amount of dead cells in both the series is high (more than 50%) even though the cell debris has been gated out. (Fig.12 and Tabel 5)

Tabel 5: Comparison of Cxcr4 expressing cells between an EBM series and an EPLEB series.

Celltype	Percentage of live positive cells	Celtype	Percentage of live positive cells
EBM3	3.9%	EPL3	7.1%
EBM5	1.8%	EPLEB2	1.1%
EBM6	2.3%	EPLEB3	27.7%
EBM7	2.5%	EPLEB4	44.1%
EBM8	8.3%	EPLEB5	23.9%

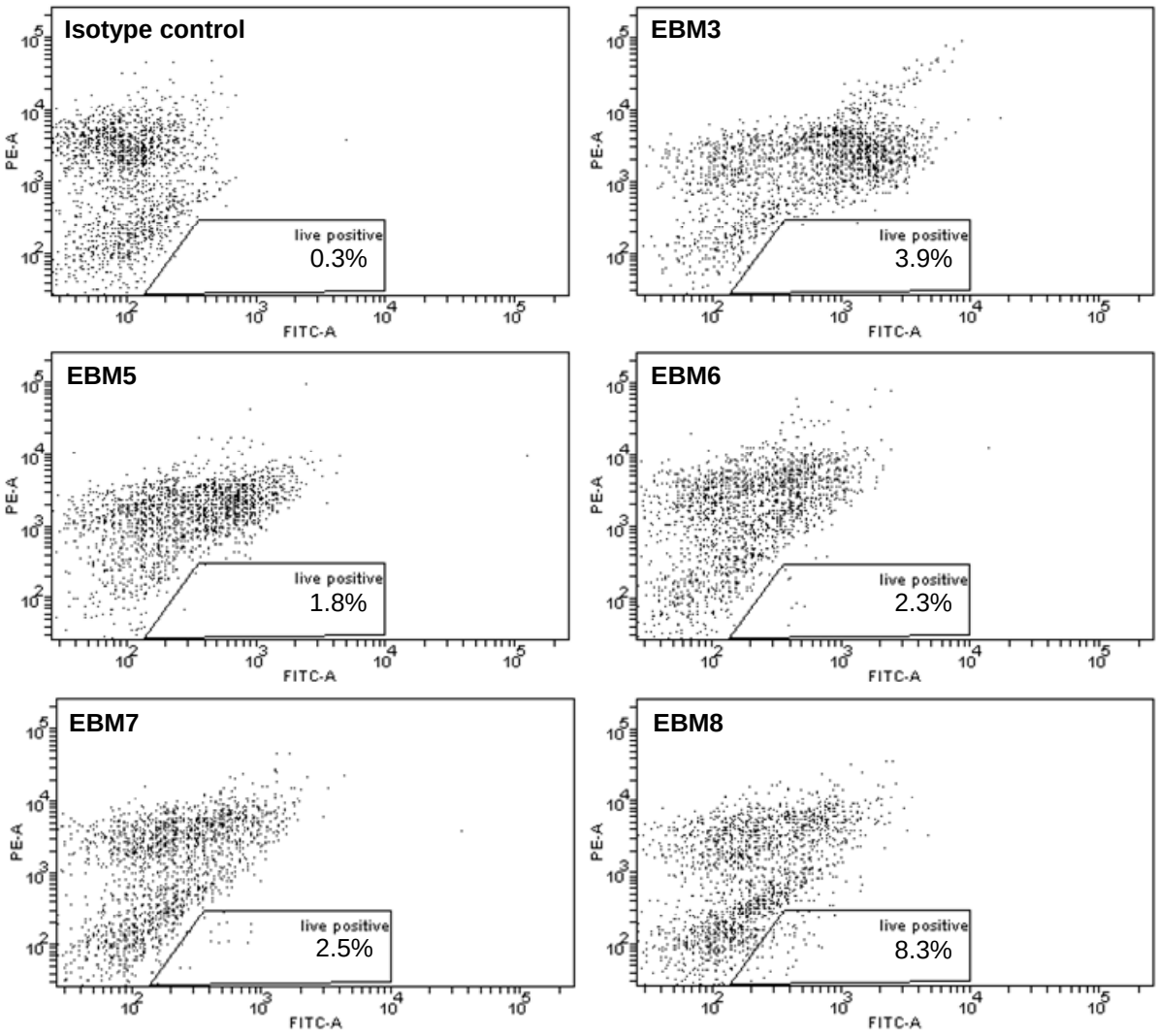

Figure 11: Cxcr4 expression in an EBM series. An EBM series was stained with anti-Cxcr4 to observe the dynamics of the protein within this series. The cells were stained with PI to exclude dead cells. STREP-FITC was used as secondary fluorophore. IgG was used as isotype control. The percentage shown in the dot plots is the percentage of live cells that stain positive. The isotype control shown is for EBM7 and illustrates the diagonal boundary between positive and negative. All the results have been modified according to their isotype control.

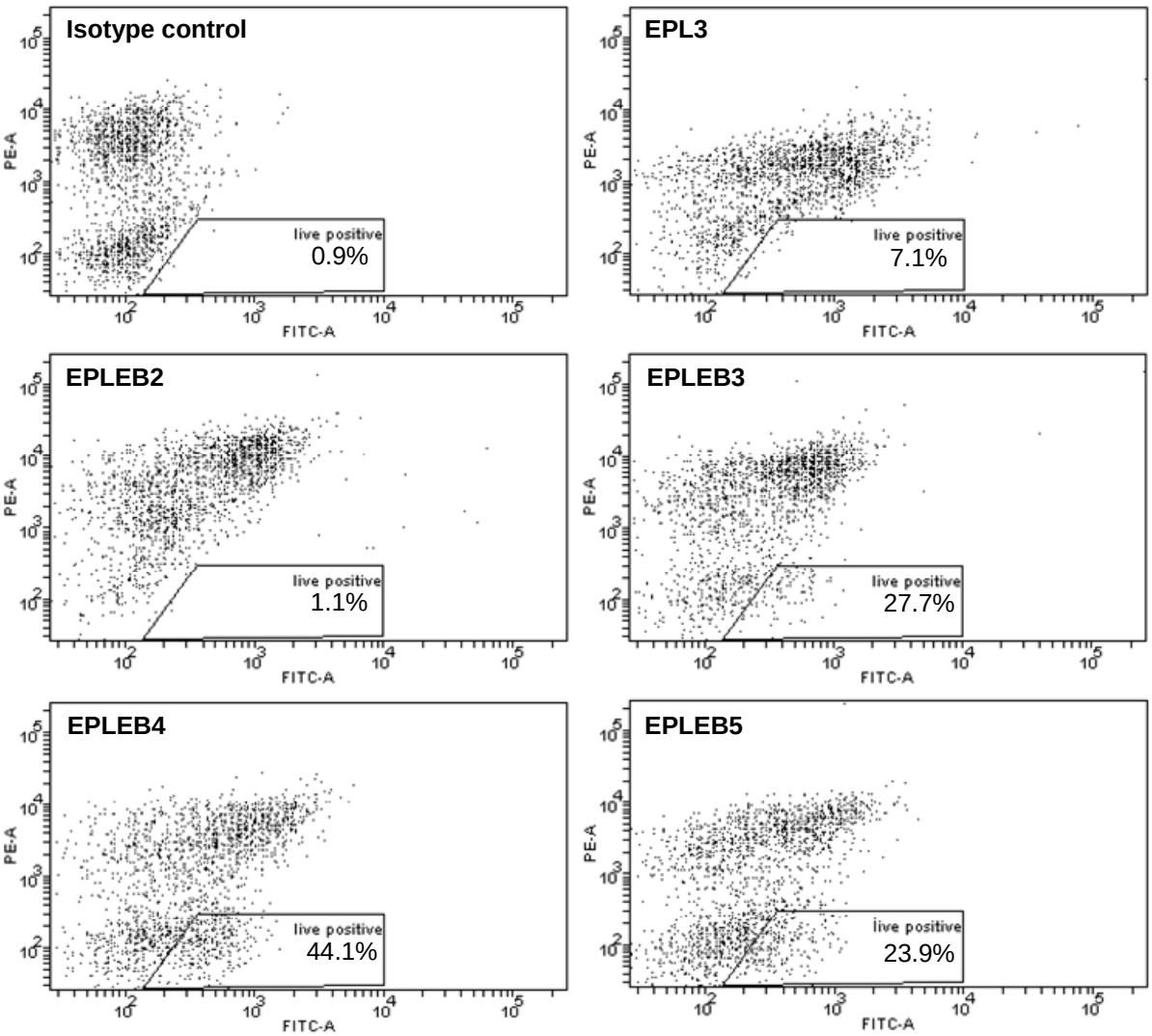

Figure 12: Cxcr4 expression in an EPLEB series. An EPLEB series was stained with anti-Cxcr4 to observe the dynamics of the gene within this series. The cells were stained with PI to control for dead cells. STREP-FITC was used as secondary fluorophore. IgG was used as isotype control. The percentage shown in the dot plots is the percentage of live cells that stain positive. The isotype control shown is for EPLEB4 and illustrates the diagonal boundary between positive and negative. All the results have been modified according to their isotype control.

3.2.6 The effect of BMP4 on Cxcr4 expression

To further examine the expression pattern of Cxcr4, EPLEB's were cultured with BMP4 added to the culture medium. BMP4 has the effect of inducing mesoderm formation from mesendoderm cells. [5] If Cxcr4 is specific for definitive endoderm, a rise in the mesoderm fraction within EPLEB's would result in a fall in Cxcr4 expression. In this experiment the effect of BMP4 on EPLEB5's was examined.

There seems to be a drop of 28% in Cxcr4 expression compared to the EPLEB5's that weren't treated with BMP4. (Fig.13)

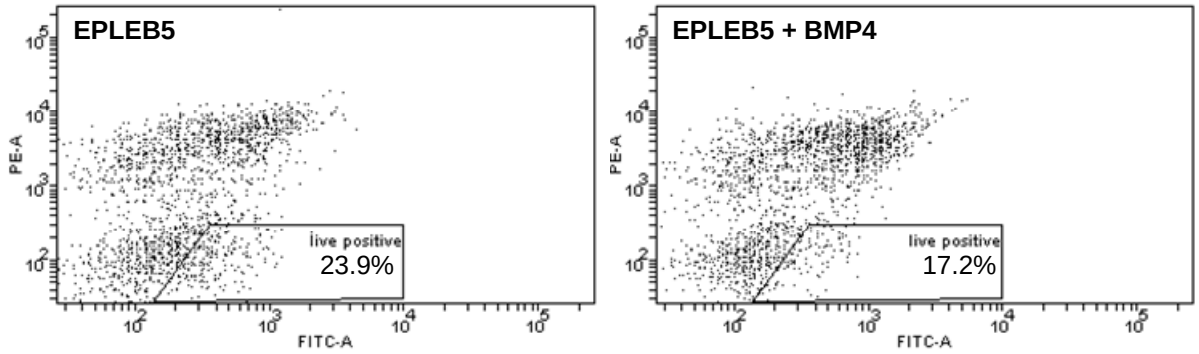

Figure 13: The effect of BMP4 on Cxcr4 expression in EPLEB5's. EPLEB's were cultured in the presence or absence of BMP4 and made into a single cell suspension on day 5. The cells were stained with anti-Cxcr4 and STREP-FITC was used as secondary fluorophore. PI was used as a measure of viable cells. IgG was used as isotype control. The percentage shown in the dot plots is the percentage of live cells that stain positive.

4 Discussion

4.1 qRT-PCR

To verify the that the cDNA used in the experiments was representative of a EPLEB series, the expression of brachyury and Mixl1 in the cell series was compared with that of an EB series by qRT-PCR. The expression of both genes was up regulated earlier in the EPLEB series than in the EB series. This result for brachyury matches previous findings [9]. Mixl1 like Brachyury is a marker for cells destined to become mesoderm and endoderm [14] so a similar expression pattern would be expected and was seen in the qRT-PCR data. The up regulation of these genes indicate a change in cell type. In this case it is a switch from primitive ectoderm to mesendoderm, a precursor of mesoderm and endoderm. These results suggest that the cells cultured and examined here are indeed EPLEB's.

Microarray data was used to identify a few possible candidates for mesoderm or endoderm markers. The data from the microarray was compared with qRT-PCR data from a EPLEB series. Hand1, Cdkn1c, Mest and Nrp1 were all up regulated from day 3 onward. This confirms the microarray data. When these results are compared with the brachyury and Mixl1 results shown earlier, the up-regulation of the marker candidates is a day after mesoderm and endoderm precursor markers are up regulated. This suggests that the candidates are up regulated immediately following mesendoderm when we propose mesoderm and endoderm might exist. Dlk1 follows the same pattern, but has a dip in expression on day 4 after which it is up regulated again on day 5. Because of this up regulation on day 5 it can still be considered a viable candidate. Whether the dip in expression on day 4 is an error or not could be established by repeating the experiment. PTPRD expression comes up on day 2 coinciding with the up regulation of mesendoderm markers. PTPRD expression also seems to be low which might explain earlier observations of inconsistent expression. Because of these two things PTPRD wasn't examined further as a candidate.

The qRT-PCR data is only a first step in identifying markers for mesoderm or definitive endoderm. Though the expression pattern shows an up regulation on the right time it doesn't say anything about the cell types in which this up regulation takes place. To investigate this further the next step would be in situ hybridisation of EPLEB cells. Definitive endoderm forms an outer layer of cells on EPLEB's [Vasilieva S, unpublished], so staining of the bodies on the outside would indicate a specificity of the gene up regulation for endoderm, the opposite is true when the outer layer seems to not stain at all. As a control an EBM series

could be used because there should be no definitive or endoderm present in EBM's. It would provide some information about lineage specificity of the up-regulation. If the results of the previous experiment would be good, staining of an entire embryo could supply final confirmation in a setting where special information allows reliable prediction of endoderm and mesoderm arrangement.

4.2 Cloning PCR fragments for in situ analysis

PCR fragments from the candidate marker genes were cloned for the production of probes for in situ hybridisation of EPLEB's. Due to various technical difficulties most of the DNA fragments didn't reach the stage where they could be used for probe production and those that did (Nrp1, Mest and Ttr) couldn't be obtained in sufficient quantity to start the probe making process.

Depending on the particular technical problem, a different solution to overcome it could be proposed. Problems with primers could be solved by using different sets of primers or, in the case of multiple product lengths, a temperature curve could be used to optimalise the primers. Other problems could be helped by starting the entire process over. This might be an option if the genes that did make the final stage but couldn't be amplified continue to yield low quantities, though using different concentrations of the template in the PCR reaction might still provide a solution.

When sufficient amounts of DNA fragments are obtained they can be used as templates for probes and used for in situ. For the goal of this project, the in situ's should be done on EPLEB's because they contain both mesoderm and endoderm and so would provide a first impression about whether the genes are expressed in one or both the cell types. In this study the former would be required. As stated in the previous segment, the next step would be staining of the entire embryo.

4.3 Probemaking and nanog in situ

The probes produced for nanog in situ's show more than one band when run on a gel. This is not necessarily a problem. Because the template DNA fragment, when run on a agarose gel, only shows one band, the multiple bands of the probes might indicate differential folding of the probe, explaining the multiple bands.

The pattern of expression seen in the nanog in situ partly corresponds with previous findings [15]. Nanog is found in ES cells and in the ICM after which it is re-expressed in the posterior primitive ectoderm, which is the place where mesendoderm is postulated to emerge. The

expression peak of nanog in the EPLEB series in the in situ corresponds with the emergence of mesendoderm. The up regulation of nanog in EPLEB5 has not yet been described and could mean different things. It could be possible that nanog is re-expressed in a later cell type or, because most of the staining seems to be in smaller bodies, the stained bodies differentiated later than the other bigger bodies present. Additional research is required to get an answer for what is happening.

4.4 Flow cytometry

The aim of the flow cytometry experiment was to determine whether Cxcr4, which is described in the literature as a marker to distinguish between visceral and definitive endoderm, [16] could be used to differentiate between mesoderm and definitive endoderm. To this end a series of experiments was set up to design a protocol for flow cytometry.

The first experiment was a test of the Cxcr4 antibody. Though the anti-Cxcr4 had a noticeable effect compared to the unstained cells, the difference between anti-Cxcr4 and isotype control was not so explicit. This seems to suggest that the anti-bodies work, but that the concentrations at which they were used still needs some optimisation.

The next experiment showed a change in morphology of cells when they were fixed in paraformaldehyde. Where before fixing there were two populations of cells, of which one contained mostly live cells, one larger population of mixed live and dead cells was formed. The fixed cells also take up PI staining, meaning dead cells cannot be controlled for. A possible solution for this might be to stain before fixing, but this has not been tested here. Due to the morphological advantage and better PI staining of the unfixed cells, unfixed cells were used for the following experiments.

Cells dissociated using dispase II had a higher viability (80%) compared to collagenase IV(40%), enzyme free cell dissociation buffer (37%) and trypsin (50%). Dispase had the best percentage of Cxcr4 stained cells compared to the other dissocation techniques. The isotope control of cells dissociated with cell dissociation buffer showed a positive peak. It would be useful to stain for dead cells at the same time as staining for Cxcr4 so the effect of staining on dead cells can be compensated for. Trypsin treated cells had a slight shift in Cxcr4 staining compared to the isotype control, but no separate peaks in expression were visible. A possible explanation for this is that trypsin, while breaking up cell bodies, also damages Cxcr4. This would lead to less antibody binding. These results suggest that, because of the high viability and good Cxcr4 staining, dispase II is the best product of the four when dissociating cells for Cxcr4 staining.

When STREP-FITC was used in stead of STREP-PE it becomes possible to simultaneously stain with PI. This makes it possible to control for dead cells. It appears to be important to do this because the results indicate that dead cells have a high affinity for anti-Cxcr4. It could be explained by dead cells externalising Cxcr4 or an other molecule that binds the antibody, or the cell becoming permeable to the antibody and trapping it, though the last is less probable because the isotype control would most likely also be trapped in that case and the results show no increased fraction positive cells in the dead cells.

Changing the concentration of the secondary fluorophore only has a slight effect on the results. A higher concentration also slightly raises the positive cell count in the isotype control. This indicates that it would be best to use a low concentration of secondary fluorophore with the advantage of reducing background.

With the results shown above it was possible to conduct a first test on a series of EPLEB's and a series of EBM's as a control. Because mesendoderm formation has been shown to begin on day 3 of a EPLEB series it would be expected that mesoderm and endoderm closely follow this event. If Cxcr4 were a marker for definitive endoderm, the formation of two groups with different Cxcr4 expression would be expected to arise around this time. EBM's which differentiate into neurectoderm when MEDII is kept as a part of their culture media, would in that case not show any Cxcr4 positive cells.

The EBM series does seem to have some Cxcr4 staining. Especially the EBM3 cells have a high expression which corresponds with the expression of the EPL3 cells from the EPLEB series. It is unclear whether the higher expression is due to unspecific binding of the anti-bodies to a molecule expressed by these cells or because they actually express Cxcr4. The last would indicate that Cxcr4 is not specific for definitive endoderm. It might also be possible that the EBM's do form the occasional definitive endoderm cell, which would also influence the result. In any case the staining procedure will have to be further refined to get a clearer picture of what is happening here.

In the EPLEB series there is an up regulation of Cxcr4 on day 3 and even more so on day 4. Because there are also cells present that show no anti-Cxcr4 staining this would indicate the emergence of two different populations of cells starting on day 3. Cxcr4 staining goes down on EPLEB5, which might indicate that Cxcr4 expression in this cell type is transient or that different cell types are starting to emerge that do not express Cxcr4.

A separate group of EPLEB's was set up with BMP4 added to the culture media. BMP4 induces mesoderm formation from mesendoderm, so if Cxcr4 were a marker for definitive endoderm a reduction in Cxcr4 positive cells would be expected.

A drop of 28% was seen between the EPLEB5's that were cultured without BMP4 and those that were. The experiment was only conducted once and to be able to ascertain whether or not this drop is repeatable and significant the experiment will have to be repeated. Cxcr4 expression seems to be highest on day 4, while cells examined after culturing with BMP4 were from EPLEB5's. This data was not yet available when this experiment was run, so when the experiment is repeated it might be of interest to do a time course for the cells cultured with BMP4 as well as those cultured without.

A possible mistake might have happened during the cell culture on day 4. During the changing of the media of the cells that had to be collected on day 5, BMP4 might have been added to the wrong plate of cells. This would result in a lowering of Cxcr4 positive cells in the EPLEB series without BMP4 and a rising of the positive cell count in the BMP4 treated group. This is not certain however and as said above the experiment will have to be repeated to confirm this.

The results seem to indicate different levels of Cxcr4 expression which would justify further investigation. The visualisation of the expression of Cxcr4 on EPLEB's and EBM's would provide additional information about the distribution of Cxcr4 expression. If Cxcr4 were specific for definitive endoderm the staining of an entire embryo's could supply interesting information about the patterns of differentiation into definitive endoderm during embryogenesis. Through FACS it would be possible to obtain pure populations of Cxcr4 positive cells, which could then be further studied without the interference of other cell types. For example it could be attempted to reculture the cells obtained through FACS. If successful, pure colonies of endoderm and its descendants would be predicted and this could be analysed with techniques like PCR.

An other experiment that could lead to interesting information is immunofluorescence of EPLEB's and later full embryo's. The anti-bodies are available so they would not have to be designed from scrach. The resulting images could result in a better understanding of the growthpattern of endoderm within EPLEB's and embryo's.

From the results presented above it can not yet be deduced with certainty that any of the proteins are markers for mesoderm or endoderm. Further research is certainly justified however. Especially Cxcr4 shows great potential. If a marker for either cell type were found it would be one step closer to understanding the mechanisms underlying cell pluripotency and differentiation. Understanding of these mechanisms could lead to advances in therapeutic

treatments of a wide range of diseases. Because of this goal research in this field will certainly remain a focus of interest in the future.

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