Aus dem Institut für Herz- und Kreislaufphysiologie

der Heinrich-Heine-Universität Düsseldorf.

Direktor: Prof. Dr. med. Jürgen Schrader

”Differentiation of Mouse Embryonic Stem Cells in a Monolayer Environment towards Cardiac Lineages”

Dissertation

zur Erlangung des Grades eines Doktors der Medizin
Der Medizinischen Fakultät der Heinrich-Heine-Universität
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vorgelegt von
Oliver Wernet

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Preface

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Gez.: Univ.-Prof. Dr. med. Joachim Windolf

Dekan

Referent: Univ.-Prof. Dr. med. Schrader

Korefferrent: PD. Dr. med. Dr. rer. nat. Giers

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1 Introduction:

Despite improvements in drug treatment and advanced surgical therapies cardiac disease, along with heart failure, is one of the leading causes of death today in the western world. In socioeconomic aspects it makes up for a major part of the health care cost in every country of the modernized world. [1] Since the beginning of advanced medical treatment along with the wide establishment of public hygiene and sanitation, which greatly increased the average population age, cardiovascular complications have a major influence on the limitation of further longevity.

Even though a vast amount of funding has been committed to better understand the various mechanisms of genetic influence, environmental factors, pathopysiology of acute events as well as degenerative diseases of the cardiovascular system, a lot of mechanisms need to be better understood or discovered. Partially the success of ongoing research has been hampered by the complication of effective and cost efficient model systems for cardiac disease. Even though mouse models are widely used, the heart as an organ presents a wider challenge as the physiology of the heart encompasses not only cellular-molecular interactions but also electromechanical coupling, reaction and adaptation. The heart of a mouse for instance has a much faster heart rate than a human heart and the chambers are refilled from proportionally much larger prechambers. Therefore it is clear that the mouse as an animal model has limited applications in the field of cardiac medical research. However, the alternatives being larger animal models such as pigs, dogs or monkeys make the research extremely difficult to handle and expensive. Human cardiomyocites are hard to culture, extremely sensitive and will age and decay in vitro cultures easily which make them unsuitable for longer studies in addition to the regular challenges of in vitro tissue and cellular cultures.

During the recent decades stem cell science has developed embryonic stem cells, cells that are harvested from the inner cell mass of blastocysts, which can differentiate into various different tissue types such as bone, cartilage, neurons, β-islet cells as well as cardiac cells. [2] Furthermore with ongoing research it was discovered that certain subsets of cells in the mammalian organisms do not fully differentiate and stay as precursor cells in various tissue regions. These precursor cells also derive from pluri – or multipotent stem cells. [3].

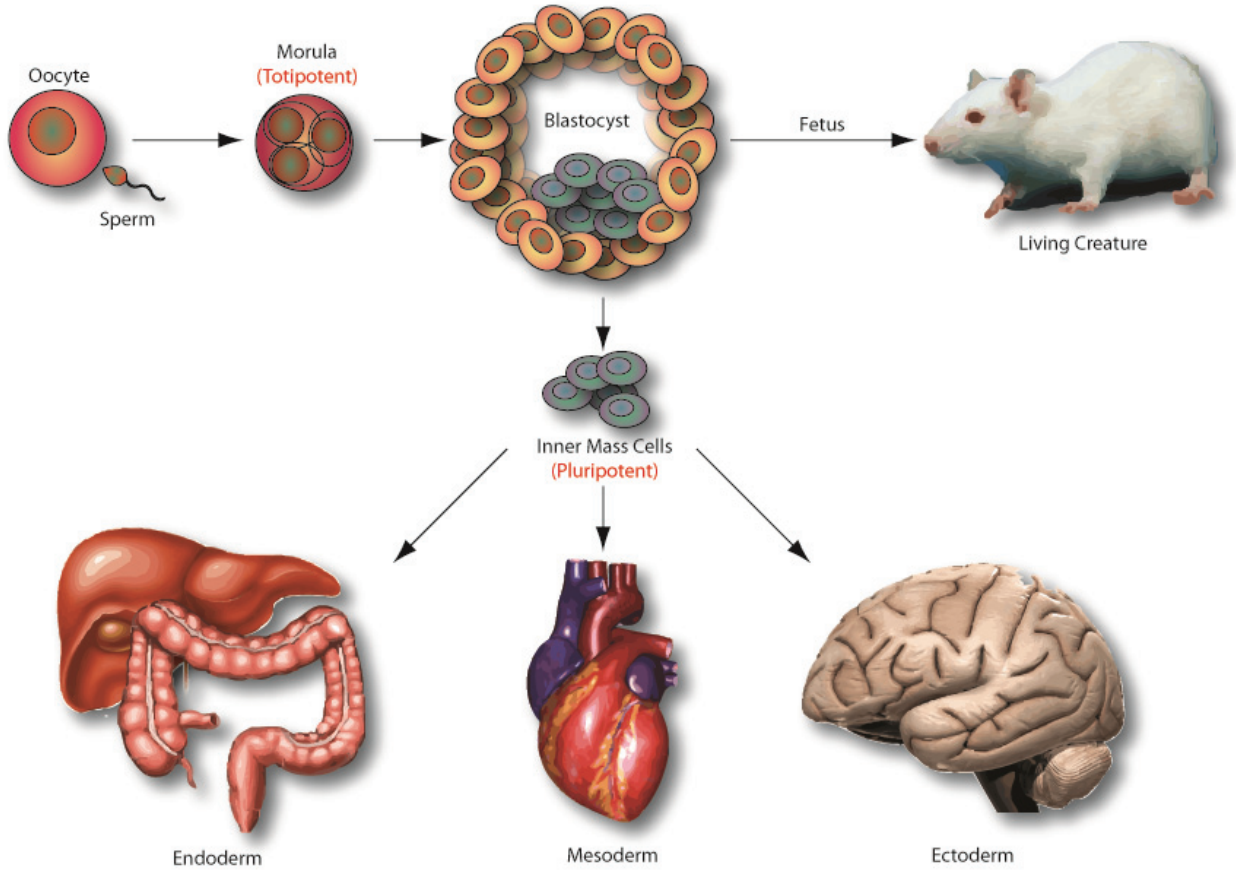


Figure 1: Development of a fertilized oocyte to a blastocyst along with examples of tissue derived from the three germ layers that can be generated from it.

The ability of stem cells to be pluripotent and maintain such pluripotency in tissue culture for longer periods of time as well as the increasing knowledge of the molecular mechanisms and pathways of their differentiation have made stem cells an important tool to generate better in vitro assays for various tissue types. [4]

Recently novel cardiogenic progenitors have been discovered and described by several laboratories around the world. It has been hypothesized that these cardiogenic progenitors contribute to the heart growth and also pertain in the adult heart but are intrinsically unable to efficiently repair significant tissue damage. [5]

Especially in the field of cardiac research this has reveled offers an abundant amount of new possibilities to better understand the mechanisms and pathways which lead to the formation of the mammalian heart. Now it is possible to generate cardiac cells as well as their precursors from mouse embryonic stem cells in vitro on demand and thus better examine cardiac development at the a cellular level.

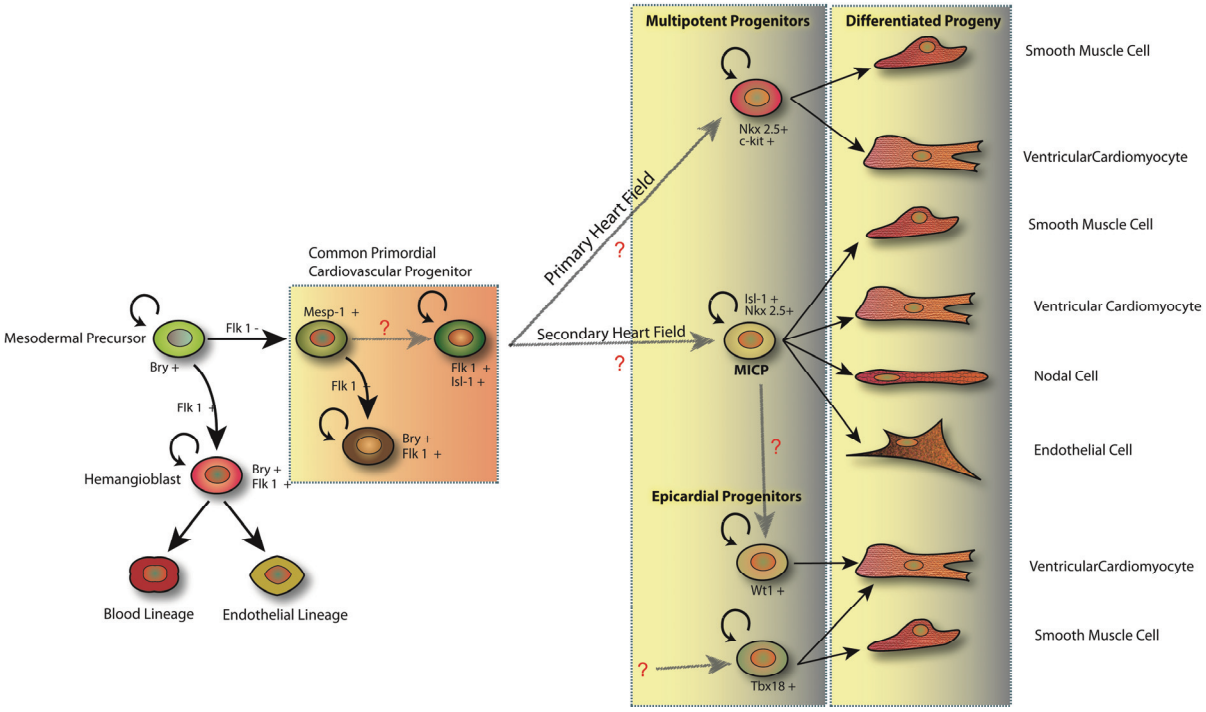


Figure 2: The different progenitor and precursor cells which give rise to various cardiac cells. (Adaptation from [5] and [6])

In parallel to these discoveries the development of screening assays and their automation occurred. With the advances in robotics and computer technologies large amounts of assays could now be exposed to various bioactive factors and tested and evaluated in an almost fully automated environment. [7] This enables researchers to rapidly test different experimental variables in numerous settings and made experimental designs feasible which before where too labor intensive, time consuming or cost inefficient. [8] In addition to that elements with previously known effects on cell models could be further investigated to better understand the molecular mechanisms underlying these changes and to improve their beneficial effects as well as reduce unwanted side effects.

Figure 3 shows a fully automated screening setup including a multi well plate magazine along with fluorescence evaluation. The plates are stacked and then transported on a conveyer belt where a robot arm transfers them to the evaluation machine.

Figure 3: A fully automated high throughput screening setup. The readout includes fluorescence intensity and polarization, luminescence and ELISAs

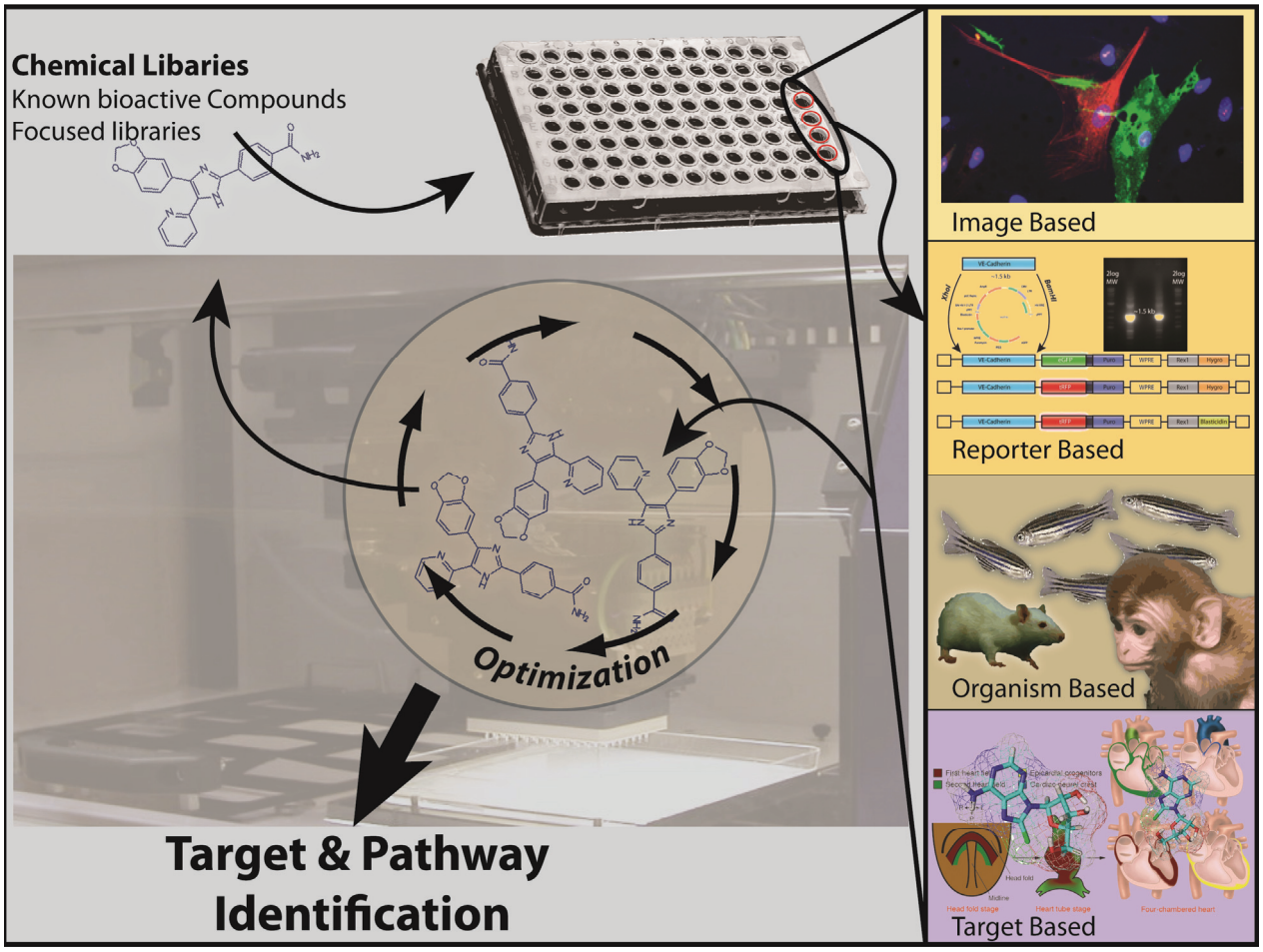


Figure 4: An overview of chemical screening and small bioactive molecule screening. Various libraries can be tested on the screening assays. The target cells are then evaluated and compared to a control group. Substances of impact are then further investigated and the effect is optimized so that the underlying biological mechanism can be better studied.

Figure 4 shows the broad setup of various screening methods. Possible scoring targets are image, reporter, target or even organism based. These results can then be used to further improve and adapt the libraries to use them in further screening approaches. Once candidates of impact have been identified, underlying molecular mechanisms can be further examined in detail utilizing state of the art biochemical methods.

Embryonic stem cells differentiate in vitro in three dimensional formations in media suspension, the “Embryoid Bodies” (EB), and are subject to variance during this differentiation which might be due to changes of the microenvironment inside the bodies. [9] Since EB are three dimensional and of varying size the diffusion of chemicals, gases and factors in the medium might vary greatly and affect the outcome of the differentiation. [10] Therefore even though embryonic stem cells offer excellent models for understanding tissue and organ development as well as understanding cellular interactions the common culturing method through the stage of the EB is not ideally applicable for large scale screening designs as constant experimental parameters such as factor concentrations are vital for robust data reproducibility. [11]

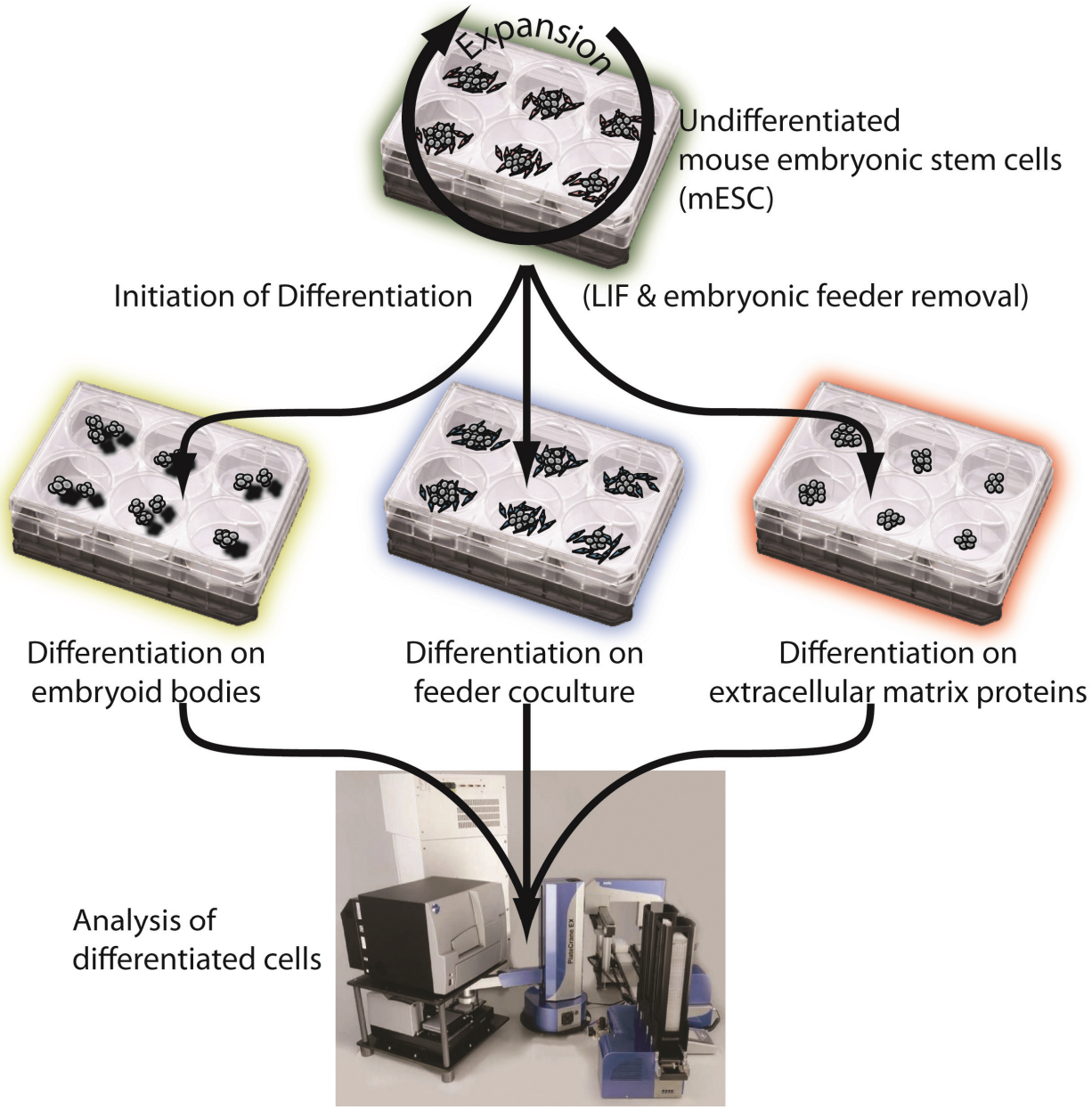


Figure 5: Culturing methods of mouse embryonic stem cells. Undifferentiated mESC are cultured on mouse embryonic fibroblasts with Leukemia inhibitory factor (LIF) to prevent differentiation. After the removal of LIF the cells can be differentiated as embryoid bodies, on feeder cells or on extracellular matrix proteins.

Figure 5 gives an overview of different methods to culture, maintain and differentiate embryonic stem cells utilizing various conditions and feeders.

Recently first advances were made in culturing and differentiating stem cells in vitro while bypassing these Embryoid bodies. These assays, culturing the stem cells on a monolayer seeded on feeder cells or extracellular matrix proteins, negate some of the difficulties for the large scale screening and make it an interesting field of research. By developing a robust, accurate and efficient protocol for cardiomyocite differentiation in vitro on a monolayer environment here the aim of this thesis was to develop and examine appropriate tools for such large scale screening experiments. These screenings should then serve as a promising platform to a greater understanding and insight into several aspects of cellular differentiation and commitment in the cardiac lineage, molecular and cellular mechanisms of cardiogenesis as well as the generation of eventual drug treatments for cardiac diseases such as cardiac insufficiency or hypertrophy.

2 Material and Methods:

Described in chapter two are the exact materials used for the experiments as well as the methods which facilitated the experiments. Equipment and software used for this study are also listed.

2.1 Material:

Vendor	Product	Cat#
USA Scientific	1-20ul filter tip	1120-1810
	1-200ul filter tip	1120-8810
	100-1000ul filter tip	1126-7810
	1-200ul yellow, beveled tip	1111-1206
	1-200ul natural, beveled tip	1111-1810
	101-1000ul blue, graduated tip	1111-2831
	1.5ml tubes	1615-5500
	1.5ml tubes, sterilized	1615-5510
	2ml tubes	1620-2700
	8 cap strips	1400-3800
	0.2ml tube strips without caps	1402-3500
	TempPlate 96-well plates	1402-9596

Vendor	Product	Cat#
Fisher	Kimwipe-Small	06-666A
	Kimwipe-Large	06-666C
	Multi-fold towel	1804
	5ml Round Bottom FACS Tube w/top	14-959-2A
	14ml Round Bottom Tube w/top	14-959-11B
	Tubes-15ml Conical Falcon	14-959-49B
	Tubes-50ml Conical Falcon	14-432-22
	Cryotube-1ml	377224
	Cryotube-1.8ml	368632
	Plate-6 well	3516
	Plate-12well	3513

Fisher	Plate-24 well	3524
	Plate-48 well	3548
	Plate-96 well	3596
	TC Dish-150mm	08-774-9
	TC Dish-100mm	3296
	TC Dish-60mm	3295
	TC Dish-35mm	3294
	Basin-Sterile	13-681-101
	Petri Dish-60mm	08-757-100B
	Petri Dish-100mm	08-757-100D
	Petri Dish-150mm	08-757-148
	Steriflip filters-50ml 0.22um	SCGP-005-25
	Filter-150ml 0.22um PVDF	SCGVU01RE
	Filter-250ml 0.22um PVDF	SCGVU02RE
	Filter-500ml 0.22um PVDF	SCGVU05RE
	Filter-1000ml 0.22um PVDF	SCGVU11RE
	Pipet-1ml individual	13-675-15B
	Pipet-2ml individual	13-675-17
	Pipet-5ml individual	13-675-22
	Pipet-10ml individual	13-675-20
	Pipet-25ml individual	13-675-30
	Pipet-50ml individual	13-675-27
	Pipet-2ml Aspirating	13-675-16
	Pipet-2ml	13-675-10DD
	Pipet-5ml	13-675-21
	Pipet-10ml	13-675-18
	Pipet-25ml	13-675-10EE
	Pipet-50ml	13-675-26
	Underpad	14-206-62
	Parafilm	PM-996
	Aluminim Foil	01-213-100
	Plastic Wrap	22-305-654
	Cell Strainer 40um	08-771-1
	Cell Strainer 70um	08-771-2
	Cell Strainer 100um	08-771-19
	Box-Fiberboard Standard microtube storage	11-678-24A
	Box-Chipboard brown	12-565-183
	Divider Grid for Box-81 slot	13-989-218
	Razor Blades	12-640
	Tape-Aqua 1 inch x 60yd	15-901-25H

Fisher	Tape-Blue 1 inch x 60yd	15-901-25G
	Tape-green 1 inch x 60yd	15-901-25C
	Tape-Orange 1 inch x 60yd	15-901-25F
	Tape-pink 1 inch x 60yd	15-901-25D
	Tape-red 1 inch x 60yd	15-901-25E
	Tape-white 1 inch x 60yd	15-935
	Autoclave Tape white ¾ in x 60 yd	11-889-11
	Storage Bottle-150ml	09-761-140
	Storage Bottle-250ml	09-761-4
	Storage Bottle-500ml	09-761-10
	Storage Bottle-1000ml	09-761-11
	Chamber Slides-2well	177429
	Chamber Slides-4well	177437
	Chamber Slides-8well	177445
	Microscope Slides	12-550-15
	Glove-Latex exam Small	2393
	Glove-Latex exam Medium	2395
	Glove-Latex exam Large	2397
	Glove-purple Nitrile X-Small	19-149-863
	Glove-purple Nitrile Small	19-149-863A
	Glove-purple Nitrile Medium	19-149—863B
	Glove-purple Nitrile Large	19-149-863C
	70% Isopropyl Alcohol	I-170 20Liter
	Tissue Path Paraplast	23021400
	Capacity: Hold 100 Slides; Color: Red	03-448-3
	Towel Scott Multi-Fold	06-666-32A
	2l Medi Vac Suction Canisters	NC9516182

Vendor	Product	Cat#
Invitrogen	Non-AA	11140076
	Hepes	15630-114
	0.25% Trypsin/EDTA	25200-106
	0.05% Trypsin/EDTA	25300112
	Newborn Calf Serum	26010074
	FBS10082147	10082147
	PBS w Ca &mg	14040182
	PBS w/o Ca&Mg	14190250

Invitrogen	DMEM	11971025
	IMDM	12440061
	DMEM/F12	11330065
	Horse Serum	26050088
	Sodium Pyruvate	11360070
	Medium199	11150067
	HBSS	14170120
	P/S	15140122
	L-G	25030164
	Knockout Serum Replacement	10828028

Vendor	Product	Cat#
Bio-Rad	iQ SYBR GRN	1708884
	iScript cDNA Synthes kit	1708891
	Multiplate-96, NATL 25/BX	MLP9601
	Microseal B ADHES SEAL, 100/PK	MSB1001
	Agarose	161-3102

Vendor	Product	Cat#
Qiagen	Gel Extraction Kit (250)	28706
	Miniprep Kit (250)	27106
	Plasmid Maxi Kit (25)	12263
	RNeasy Mini kit (50)	74104
	PCR Purification Kit	28106

Vendor	Product	Cat#
HyClone	ES FBS	SH30071

Vendor	Product	Cat#
Sigma	Red Extract-N-Amp PCR Mix 12ml	R4775
	X-Gal	B4252-1mg
	Mitomycin C	M0503-2mg

2.2 Primary Antibodies:

cat. #	antigen	Isotype	company
A2547	mouse alpha-SMA	monoclonal	Sigma
9792	MEF-2c	poly, rabbit	cell signaling
A 7811	a-actinin (sacrcomeric)	mono, mouse	Sigma
AB5754	Isl1	poly, rabbit	Chemicon
AB21700	Ki67	poly, rabbit	AbCAM
553141	mouse CD16/CD32	mono, Rat	BD Pharmingen
SC-19714	HCN4 (C-16)	poly, goat	Santa Cruz
SC25310	GATA 4	mono, mouse	Santa Cruz
550274	mouse CD31 (PECAM)	mono, Rat	BD Pharmingen
MS-295-p1	Troponin T	mono,	NeoMarkers
REC-RCAB0002PF	mouse Nanog	poly	Cosmo Bio Co.
39.4D5	Isl1	Mono, mouse	Hybrodoma Bank
555308	mouse Flk-1	mono,rat	BD Pharmingen
sc-14033	Nkx2.5 (H-114)	poly, rabbit	Santa Cruz
c6219	connexin-43	poly, rabbit	Sigma
40.2D6	Isl1	Mono, mouse	Hybrodoma Bank
07-716	KDR/Flk-1/VEGFR2	Poly, rabbit	Upstate
MAB3242	Smoothelin	Mono, mouse	Chemicon Internt
ab45932	cardiac Troponin T	poly, rabbit	Abcam
sc-9053 (H-112)	GATA-4	Poly, rabbit	Santa Cruz
sc-1237 (C-20)	GATA-4	Poly, goat	Santa Cruz
sc-8697	Nkx2.5 (N-19)	Poly,goat	Santa Cruz
sc-8121	Troponin T-C (C-19)	Poly, goat	Santa Cruz
ab5694-100	Smooth Muscle Actin	Poly, rabbit	Abcam
555307	Flk-1	Mono, rat	BD Pharmingen
sc-8118	TroponinI (C-19)	Poly, goat	Santa Cruz
ab13970	Chicken poly to GFP	Poly, chicken	Abcam
ab290	GFP	Poly, rabbit	Abcam
BC-1150S-L001	Myosin	Mono, mouse	Alexis Biocytex
sc-28562	Smoothelin	Poly, rabbit	Santa Cruz
AF357	Flk1	Poly, goat	R&D
ab28364	CD31	Poly, rabbit	Abcam
BD 555308	Flk-1	Mono, mouse	BD Pharmingen
553731	CD34	mono, Rat	BD Pharmingen
ab5968	Nestin	poly, rabbit	Abcam
APC-052	HCN4	poly, rabbit	Alomone labs

557659	CD45	Rat, mono	BD Pharmingen
ab6142	Nestin	Mono, mouse	Abcam
ab20670	Isl1	poly, rabbit	Abcam
ab19615	cardiac Troponin I	Mono, mouse	Abcam
550539	Anti CD45	Mono, rat	BD Pharmingen
BT-562	Smooth Muscle-Myosin	Poly,rabbit	Biomedical Technologies

2.3 Secondary Antibodies:

Name	Catalog Number	Vendor	Color
Alexa Fluor® 350 donkey anti-goat IgG (H+L) *2 mg/mL*	A-21081	Invitrogen	Alexa Fluor® 350
Alexa Fluor® 350 donkey anti-mouse IgG (H+L) *2 mg/mL*	A-11045	Invitrogen	Alexa Fluor® 350
Alexa Fluor® 350 donkey anti-rabbit IgG (H+L) *2 mg/mL*	A-11046	Invitrogen	Alexa Fluor® 350
Alexa Fluor® 350 donkey anti-rat IgG (H+L) *2 mg/mL*	A-21093	Invitrogen	Alexa Fluor® 350
Alexa Fluor® 488 donkey anti-goat IgG (H+L) *2 mg/mL*	A-11055	Invitrogen	Alexa Fluor® 488
Alexa Fluor® 488 donkey anti-mouse IgG (H+L) *2 mg/mL*	A-21202	Invitrogen	Alexa Fluor® 488
Alexa Fluor® 488 donkey anti-rabbit IgG (H+L) *2 mg/mL*	A-21206	Invitrogen	Alexa Fluor® 488
Alexa Fluor® 488 donkey anti-rat IgG (H+L) *2 mg/mL*	A-21208	Invitrogen	Alexa Fluor® 488
Alexa Fluor® 488 goat anti-chicken IgG (H+L) *2 mg/mL*	A-11039	Invitrogen	Alexa Fluor® 488
Alexa Fluor® 488 goat anti-mouse IgG (H+L) *2 mg/mL*	A-10667	Invitrogen	Alexa Fluor® 488
Alexa Fluor® 488 goat anti-rabbit IgG (H+L) *2 mg/mL*	A-11008	Invitrogen	Alexa Fluor® 488

Alexa Fluor® 488 goat anti-rat IgG (H+L) *2 mg/mL*	A-11006	Invitrogen	Alexa Fluor® 488
Alexa Fluor® 594 donkey anti-goat IgG (H+L) *2 mg/mL*	A-11058	Invitrogen	Alexa Fluor® 594
Alexa Fluor® 594 donkey anti-mouse IgG (H+L) *2 mg/mL*	A-21203	Invitrogen	Alexa Fluor® 594
Alexa Fluor® 594 donkey anti-rabbit IgG (H+L) *2 mg/mL*	A-21207	Invitrogen	Alexa Fluor® 594
Alexa Fluor® 594 donkey anti-rat IgG (H+L) *2 mg/mL*	A-21209	Invitrogen	Alexa Fluor® 594
Alexa Fluor® 594 goat anti-chicken IgG (H+L) *2 mg/mL*	A-11042	Invitrogen	Alexa Fluor® 594
Alexa Fluor® 594 goat anti-mouse IgG (H+L) *2 mg/mL*	A-11005	Invitrogen	Alexa Fluor® 594
Alexa Fluor® 594 goat anti-rabbit IgG (H+L) *2 mg/mL*	A-11012	Invitrogen	Alexa Fluor® 594
Alexa Fluor® 594 goat anti-rat IgG (H+L) *2 mg/mL*	A-11007	Invitrogen	Alexa Fluor® 594
Alexa Fluor® 660 donkey anti-goat IgG (H+L) *2 mg/mL*	A-21083	Invitrogen	Alexa Fluor® 660
Alexa Fluor® 660 goat anti-mouse IgG (H+L) *2 mg/mL*	A-21054	Invitrogen	Alexa Fluor® 660
Alexa Fluor® 660 goat anti-rabbit IgG (H+L) *2 mg/mL*	A-21073	Invitrogen	Alexa Fluor® 660
Donkey anti-Chicken, Cy3 conjugate	AP194C	Millipore	Cy3

2.4 Chemicals:

Chemical full name	Company	Catalog no.
2,2,2,-Tribromethanol	Sigma	T48402-25g
2-Propanol	Fisher	A464-4
3-Methylbutanol	Sigma	19392
Acetone	Fisher	A18-4
Acid Fuchsin	Sigma	F8129
Agarose, Molecular Biology	BioRad	161-3102
Ammonium Hydroxide	Sigma	338818-1
Ascorbic Acid	Sigma	A4544-100g
b-Mercaptoethanol	Fisher	M6250
Bacto Agar	BD	214010
Bacto Tryptone	BD	211705
Bacto Yeast extract	BD	212750
Brilliant Blue R-250	Fisher	BP101-25g
Bromphenol blue	Sigma	B8026-5g
Calcium chloride anhydrous	Sigma	C4901-500g
Calcium chloride dihydrate	Sigma	C3306-250g
Cesium chloride	Sigma	C3032-100g
Chloroform	Fisher	BP1145-1
Citric Acid	Sigma	251275-100g
Copper sulfate pentahydrate	Sigma	C7631-250g
Corn oil	Sigma	C8267
D-Galactose	Fisher	BP656-500g
D-Glucose	Sigma	G5400-250g
D-Sorbitol	Sigma	S3889-500g
EDTA	Fisher	BP148-500g
EGTA	Sigma	E3889-25g
Eosin Yellow	Fluka	45242
Ethanol	Fisher	S73985
Fast Green FCF	Sigma	F7258-25g
Ficoll 400, Type 400 DL	Sigma	F9338-25g
Formaldehyde	Fisher	F79-4
Formamide	Sigma	185906
Gelatin, from porcine skin	Sigma	G1890
Glycerol, >99%	Sigma	G5150-1L
Glycine	Fluka	BP381-500g
Glycine, electrophoresis grade	Sigma	G8898-1kg

Hepes, Sigma Ultra	Sigma	H7523-50g
Hematoxylin solution	Sigma	HHS16
Hydrochloric Acid	Fisher	A144-500g
Isopropanol	Big Red Bird	I-270
K3Fe(CN)6	Sigma	244023-500g
L(+)-Arabinose	Fluka	10840
LB Agar	Fisher	BP14225-500g
Lithium chloride	Sigma	L9650-100g
Magnesium chloride anhydrous	Sigma	M8266-1kg
Magnesium chloride hexahydrate	Fluka	63072-1kg
Magnesium chloride hexahydrate, Sigma Ultra	Sigma	M2670-500g
Maleic Acid, >99%	Sigma	M153-1kg
Manganese chloride	Acros Organics	205895000
Methylgreen	Vector	H3402
Methanol	Fisher	BP1145-1
Methylorange Mischindikator Loesung	Riedel de Haen	32940
MOPS	Fisher	BP308-500g
N,N-Dimethyl Formamide	Sigma	D4551
NP40 Substitute Igepal CA-630	USB	19628
Permount	Fisher	SP15-500g
Phosphomolybdic acid hydrate	Fluka	79560
Phosphotungstic acid	Sigma	P4006-100g
PIPES	Sigma	P6757-100g
Polyvinylalcohol 4-88	Fluka	81381
Polyvinylpyrrolidone	Sigma	P5288-500g
Potassium Acetate	Fisher	BP364-500g
Potassium chloride	Fisher	P217-500g
Potassium dichromate	Sigma	P2588-1kg
Potassium hexacyanoferrate(II) trihydrate	Sigma	P3289-500g
Potassium Hydroxide	Fisher	P251-500g
Potassium phosphate dibasic	Fisher	BP363-1kg
Potassium phosphate monobasic	Fisher	P285-500g
Potassium phosphate monobasic	Fisher	P285-500g
Saponin	Fluka	84510
Sodium acetate	Sigma	S2889-250g
Sodium azide	Sigma	S2002-25g
Sodium bicarbonate	Sigma	S5761-500g
Sodium chloride	Fisher	S271-10kg
Sodium citrate	Fisher	BP327-1kg
Sodium citrate trihydrate	Sigma	S-1804

Sodium deoxycholate	Sigma	D6750-25g
Sodium dodecylsulfate	Fisher	BP166-500g
Sodium Hydroxide	Fisher	BP359-500g
Sodium Hydroxide, 10N	Fisher	S5255-1kg
Sodium phosphate dibasic	Sigma	S7907
Sodium phosphate monobasic	Sigma	S-8282
Sodium tetraborate decahydrate	Sigma	B9876
Sucrose, 99%+	Sigma	S0389
Tris Base	Fisher	BP152-5kg
Triton X-100	Sigma	T8787
Tween 20	Sigma	P5927-500ml
Xylene cyanol FF	Sigma	X4126-10g
Xylenes	JT Baker	9493-03

2.5 Software:

Program	Company	Version
Adobe Creative Suit 4	Adobe	CS4
Antivir	Avira	2009
Blackberry Desktop Manager	RIM	5.0.1
Cisco AnyConnect	Cisco	2.3.254.0
Cisco Clean Access	Cisco	4.1.0.10
Endnote X1	Thomson Reuters	X1.0.1 [Bld. 2682]
Firefox	Mozilla	39205
Flow Jo	Tree Star, Inc.	7.6
Harvard eCommons	Harvard Medical School	2009
Internet Explorer 8 64-Bit	Microsoft	8.0.6001.18882
IrfanView	Irfan Skiljan	4.25
MS Office 2007 Enterprise with OneNote	Microsoft	2007 SP2
Ms Windows Vista Business	Microsoft	SP2
Nero Smart Essentials	Nero Inc.	9.4.12.3
Open Office	Open Source	3.2 RC 5
Skype	Skype Limited	4.0.0.227
Syncplicity	Syncplicity Inc.	2.0.3632.29994
Thunderbird	Mozilla	2.0.0.23
VLC	VideoLAN	1.0.3

2.6 Laboratory Equipment:

Item	Vendor
AB 104-S / FACT	Mettler Toledo
Centrifuge 5417 R	Eppendorf
Centrifuge 5424	Eppendorf
Centrifuge 5810 R	Eppendorf
Freezer -20 ˚ C	Kenmore
Freezer -80 ˚ C	Revco
Hera Cell 150	Thermo Electronic
Isotemp 125 D	Fisher Scientific
Isotemp 205	Fisher Scientific
Kryos N2 Liquid	Taylor-Wharton
Leica DMI 4000 B	Leica
Leica DM IL	Leica
Master Cycler Gradient S	Eppendorf
Medicool	Sanyo
Micro Pipet 1000 µl	Eppendorf
Micro Pipet 2.5 µl	Eppendorf
Micro Pipet 20 µl	Eppendorf
Micro Pipet 200 µl	Eppendorf
Microcentrifuge	Fisher Scientific
Milli-Q	Millipore
Orbital Water Bath	Daiki
PB 3002-S / FACT	Mettler Toledo
Platform Shaker	New Brunswick Scientific
Precision Water Bath	Thermo Electron
Realplex4	Eppendorf
Refridgerator 4 ˚ C	Kenmore
Seven Easy	Mettler Toledo
Smart Spec Plus	Bio Rad
Steril GARD III Advance	The Baker Company
Sub Cell GT	Bio Rad
Universal UV Gel Hood II	Bio Rad
Vortex Mixer	Fisher Scientific

2.7 Cell Medium Mixtures:

RPMI / B27 (# I) (2%)		
RPMI (Advanced)	500 ml	100 ml
B27	10 ml	2 ml
FBS	11,6 ml	2,4 ml
Pen / Strep	1 ml	0,2 ml
L-Glutamin	5,8 ml	1,2 ml

DMEM (# V)		
DMEM (High Glucose)	500 ml	100 ml
FBS	46,4 ml	12 ml
Pen / Strep	5,8 ml	1,2 ml
L-Glutamin	5,8 ml	1,2 ml
Sodium Pyruvate	5,8 ml	1,2 ml
NEAA	5,8 ml	1,2 ml
β-Mercapto-Ethanol	4,4 µl	1 µl
Ascorbate (0,1M)	1 ml	0,2 ml

DMEM / F12 (# II)		
DMEM-F12	500 ml	100 ml
FBS	11,6 ml	2,4 ml
Pen / Strep	5,8 ml	1,2 ml
L-Glutamin	5,8 ml	1,2 ml
Sodium Pyruvate	5,8 ml	1,2 ml
NEAA	5,8 ml	1,2 ml
Ascorbate (0,1M)	1 ml	0,2 ml

RPMI / B27 (# I) (0,2%)		
RPMI (Advanced)	500 ml	100 ml
B27	10 ml	2 ml
FBS	1,16 ml	0,24 ml
Pen / Strep	1 ml	0,2 ml
L-Glutamin	5,8 ml	1,2 ml

DMEM / B27 (#III)		
DMEM (High Glucose)	500 ml	100 ml
B27	10 ml	2 ml
FBS	1,16 ml	0,2 ml
Pen / Strep	5,8 ml	1,2 ml
L-Glutamin	5,8 ml	1,2 ml
Sodium Pyruvate	5,8 ml	1,2 ml

RPMI / B27 (# I) (8%)		
RPMI (Advanced)	500 ml	100 ml
B27	10 ml	2 ml
FBS	46.4 ml	9.28 ml
Pen / Strep	1 ml	0,2 ml
L-Glutamin	5.8 ml	1,2 ml

DMEM / B27 (#IV)		
DMEM (High Glucose)	500 ml	100 ml
B27	10 ml	2 ml
FBS	11,6 ml	2 ml
Pen / Strep	5,8 ml	1,2 ml
L-Glutamin	5,8 ml	1,2 ml
Sodium Pyruvate	5,8 ml	1,2 ml

mES		
DMEM (High Glucose)	500 ml	1000 ml
FBS	94 ml	188 ml
L-Glutamin	6,25 ml	12,5 ml
Pen / Strep	6,25 ml	12,5 ml
NEAA	6,25 ml	12,5 ml
Nucleoside Mix	6,25 ml	12,5 ml
LIF	1 ml	2 ml
β -Mercapto Ethanol	4,4 µl	8,8 µl

MEFs		
DMEM (High Glucose)	500 ml	1000 ml
FBS	58 ml	116 ml
L-Glutamin	5,8 ml	11,6 ml
Pen / Strep	5,8 ml	11,6 ml
Sodium Pyruvate	5,8 ml	11,6 ml
β -Mercapto Ethanol	4,4 µl	8,8 µl

Cardiomyocytes		
DMEM-F12	500 ml	1000 ml
FBS	11,6 ml	23,2 ml
L-Glutamin	5,8 ml	11,6 ml
Pen / Strep	5,8 ml	11,6 ml
NEAA	5,8 ml	11,6 ml
Sodium Pyruvate	5,8 ml	11,6 ml
BovineInsulin(10mg/ml)	58 µl	116 µl

Differentiation		
DMEM (High Glucose)	500 ml	1000 ml
FBS	94 ml	188 ml
L-Glutamin	6,25 ml	12,5 ml
NEAA	6,25 ml	12,5 ml
Ascorbate (0,1M)	625 µl	1250 µl
β -Mercapto Ethanol	4,4 µl	8,8 µl

mES Adaptation		
IMDM (High Glucose)	500 ml	1000 ml
FBS	94 ml	188 ml
L-Glutamin	6,25 ml	12,5 ml
Pen / Strep	6,25 ml	12,5 ml
NEAA	6,25 ml	12,5 ml
Nucleoside Mix	6,25 ml	12,5 ml
LIF	1 ml	2 ml
β -Mercapto Ethanol	4,4 µl	8,8 µl

Freezing Medium		
Respective Medium	50 %	50 ml
FBS	40 %	40 ml
DMSO	10 %	10 ml

2.8 Assay Cell Seeding Count and Densities:

Plate	Seeding Cell Count	Cells / cm2 (rounded)
10 cm dish	1.200.000	21800
6 well plate	200.000	21000
12 well plate	100.000	26300
96 well plate	12.000	37500
384 well plate	2000	35700

2.9 Differentiation Factors:

Factor	Company	Catalogue Number	Stock Concentration	Volume used
Activin A	R&D Systems	338-AC	100 ng/ml	5 µl / ml
BMP-4	R&D Systems	5020-BP	10 ng/ml	1 µl / ml
Wnt3a	R&D Systems	1324-WN	3.5 ng/ml	0.875 µl / ml

2.10 Differentiation Assays:

The following cell lines where used for the experiments:

- **Isl1$^{Cre/+}$ & Rosa26$^{LacZ/+}$** (Isl1 linked to LacZ;)
- **Isl1$^{Cre/+}$ & Rosa26$^{YFP/+}$** (Isl1 linked to yellow fluorescent protein (YFP);)
- **Mef2c-AHF$^{GFP/+}$** (Mef2c linked to green fluorescent protein (GFP); Mesoderm-Anterior Heart Field (AHF) specific enhancer)
- **Bry$^{GFP/+}$** (Brachyury linked to GFP; Early Mesoderm)

2.10.1 Composition:

The above mentioned mouse embryonic stem cells (mESC) where cultured in medium without Leukemia Inhibitory Factor (LIF) at day 0 and treated with cytokines known to influence differentiation of stem cells. LIF is necessary to prevent mESC from differentiating. The diagram below shows the setup of the cytokine treatment during the differentiation assay.

Figure 6: Shown above are the various protocols for the combinations of the cytokine mix. Protocol (A) shows Activin A starting at day 0 and ending at day2 followed by Bmp4 along with Wnt3a until day 6. Protocol (B) shows Activin A starting at day 0 and ending at day2 followed by Bmp4 only until day 6. Protocol (C) shows Activin A along with Wnt3a at day 0 whereas only Activin A is removed at day 2 and Wnt3a is supplemented with Bmp4 at day 2. Both factors are removed at day6. Protocol (D) shows Activin A along with Wnt3a at day 0 whereas only Activin A is removed at day 2 and Wnt3a is continued until day 6.

2.10.2 Timelines:

During the experiments the length of exposure for the most promising cytokine mix was altered to further optimize the efficiency. The timelines below show the culturing of mESC without LIF from day 0 and the exposure length of the cytokines.

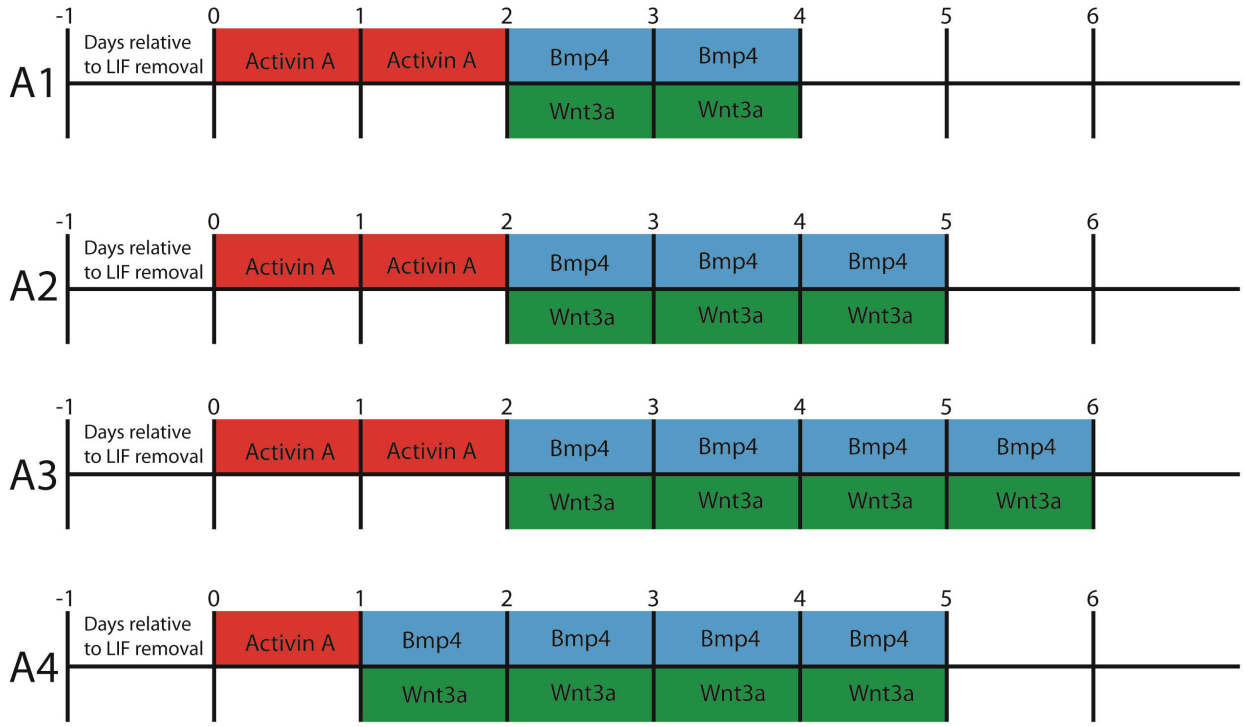


Figure 7: Shown above are the various protocols for the start and stop time points of the cytokine exposure. Protocol (A1) shows a short combination of Activin A from day 0 until day 2 with Bmp4 and Wnt3a starting together at day 2 until day 4. Protocol (A2) shows the same combination of Activin A from day 0 until day 2 with Bmp4 and Wnt3a starting together at day 2 but ending at day day 5. Protocol (A3) is similar to (A2) but with the exposure to Bmp4 and Wnt3a until day 6. Protocol (A4) has only one day of Activin A from day 0 until day 1 and four days of Bmp4 and Wnt3a from day 1 until day 5.

2.11 Surface Coating of Culture Dishes:

Tissue culture dishes and plates were coated with various extracellular matrix proteins and supportive cells such as Gelatin, Matrigel, Fibronectin or mouse cardiac mesenchymal cells (CMC).

Component	Concentration	Incubation	Solvent
Gelatin	0.1 %	1h @ 37˚C	PBS
Fibronectin	100 µl / 10 ml (1:100)	overnight @ 4 ˚ C	PBS
Matrigel	100 µl / 10 ml (1:100)	overnight @ 4 ˚ C	DMEM (cold)
CMC	90% confluent	density dependant	Medium

2.12 Transgenic mESC:

2.12.1 **Isl1$^{Cre/+}$ & Rosa26$^{LacZ/+}$**

This mES cell line was available from the Kenneth Chen laboratory. It is genetically altered to contain a Cre-LoxP system [12] which is triggered by the expression of Isl1. When Isl1 is expressed Cre is also expressed and excises the genetic information between the loxP sites. Here a stop command which hindered lacZ from being expressed. This mechanism is a helpful tool for lineage tracing since it will label all cells which have expressed Isl1 at some point in development. [13]

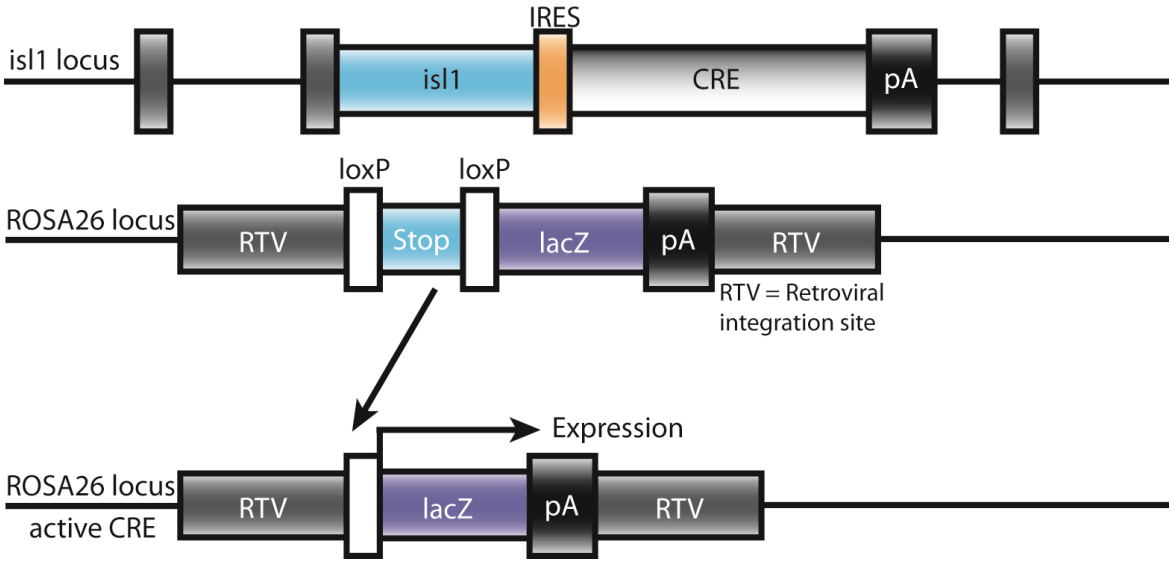


Figure 8: The schematic above shows the Cre-LoxP system in its two loci, (Isl1 and Rosa26). Once Isl1 is expressed the activated Cre system cuts at the loxP sites and a ligase closes the gap. With the excision of the stop command reporter is expressed. (In this case lacZ)

2.12.2 **Isl1$^{Cre/+}$ & Rosa26$^{YFP/+}$**

These transgenic mESC where obtained from Lei Bu from the Kenneth Chen laboratory.

Just like the Isl1$^{Cre/+}$ & Rosa26$^{LacZ/+}$ cell line this line had the Cre-LoxP system tied to Isl1 inserted. The reporter in this cell line however was yellow fluorescent protein (YFP).

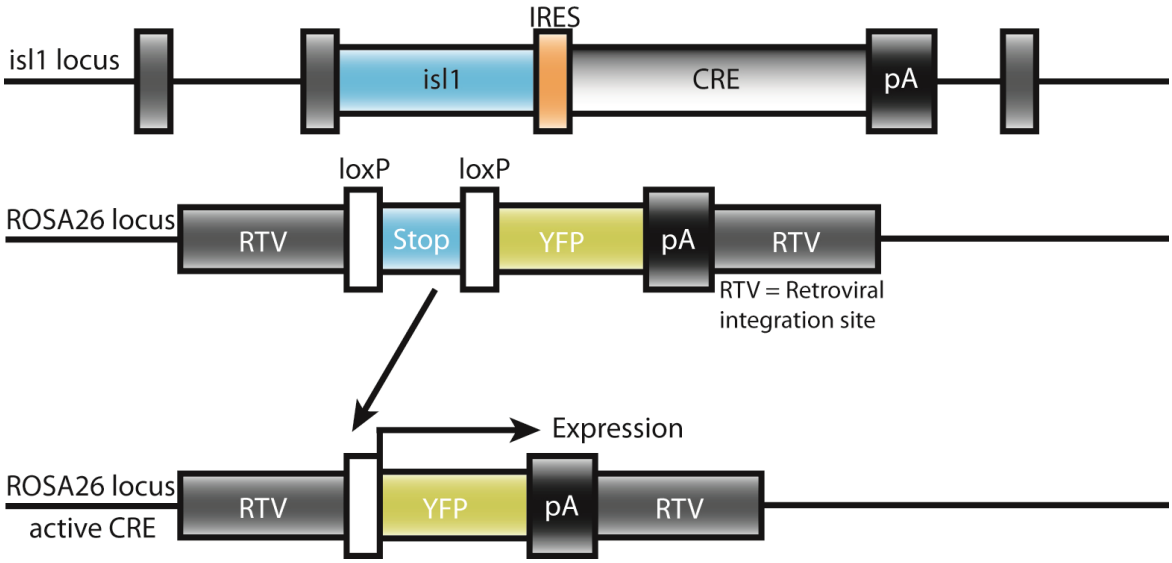


Figure 9: The schematic above shows the Cre-LoxP system in its two loci, (Isl1 and Rosa26). This cell line is similar to the one mentioned before in all aspects besides the reporter system. This cell line contains a YFP in the Rosa26 locus after the loxP sites.

2.12.3 **Mef2c-AHF$^{GFP/+}$**

The Mef2c-AHF$^{GFP/+}$ mESC were obtained from the Ibrahim Domian laboratory.

Figure 10: Above a schematic of the cenetic alteration of the Mef2c-AHF$^{GFP/+}$ cell line as described in the original publication by Domian et al. [14]

An enhancer fragment from the 5' regulatory region of murine Mef2Cgene was inserted into a promoterless GFP expression vector (Invitrogen). The DNAinsert, including the GFP expression sequence, was introduced into the pronucleus from C57Bl/6 mice (Charles River Laboratories, Wilmington, MA). (Adaptation from supplement of Domian et al. . [14]

2.12.4 **Bry$^{GFP/+}$**

As the fourth cell line the BryGFP/+ mESC, kindly donated by Gordon Keller's laboratory, was employed here.

Mouse embryonic stem cells (E14.1, 129/Ola) were electroporated with the *Not*I-linearized targeting vector. Clones that had undergone a homologous recombination event were identified by PCR with one primer (5′-CAGGTAGAACCCACAACTCCGAC-3′) annealing to genomic sequences in the 5′ region of the brachyury gene, upstream of the 'short arm of homology', the other primer (5′- CCGGACACGCTGAACTTGTGGC-3′) to the 5′ region of EGFP. Correctly targeted clones were confirmed by Southern blot analysis. Out of 384 singly selected and 80 doubly selected colonies, four and three correctly targeted clones were identified, respectively. Two positive clones (#164 and #201) were transiently transfected with a modified Cre recombinase expression vector to excise the *neo* gene. Neo-deficient clones were identified due to loss of G418 resistance. The intactness of the targeted locus before and after Cre-mediated excision of Neo was confirmed by Southern blot analysis. The absence of the *neo* cassette in Cre-treated G418- sensitive clones was verified by Southern blotting using the Neo cassette as probe. (Adaptation from supplement of [15])

2.13 Tissue Culture Methods:

2.13.1 Medium Generation:

All medium was prepared under a sterile hood depending on cells of interest according to the recepies described earlier in this section. All medium was then filtered through a Millipore Stericup 0.22 µm filtration system and kept in the bottom container and stored at 4˚C.

2.13.2 Cell Washing:

Unless otherwise noted cells where washed three times with PBS with Ca^{2+} if cells were supposed to stay attached and with PBS without Ca^{2+} if they were removed or passaged.

2.13.3 Passaging Cells:

Cells were washed three times with PBS without Ca^{2+} and then exposed to warm 0.25% or 0.05% Trypsin for three to five minutes depending on cell status. The Trypsin activity was blocked by the corresponding medium or with mES medium case of low serum medium. The cells were flushed up and down with a pipet in order to further disassociate cell clusters and collected in a 50 ml Falcon tube. After spinning down the cells in an Eppendorf Centrifuge 5810 R at 1000 RPM for five minutes the cells where resuspended in PBS without Ca2+ and spun down once more. Cells were then counted in a Neubauer Chamber and seeded according to assay density or plated in dilution for amplification.

2.13.3 Freezing Cells:

Cells were trypsinized, washed and collected in a Falcon tube. After removing the supernatant they were resuspended in 0.5 ml of medium for each cryotube used. Cryotubes where labeled with cryoresistant labels or marked by alcohol proof felt pen and precooled in an alcohol cryosafe to ensure the steady, continous and non rapid freezing of the cells. Cryotubes were then loaded with 0.5 ml of freezing medium consistent of 80% fetal bovine serum (FBS) and 20% dimethyl sulfoxide (DMSO). 0.5 ml of cells suspension with medium was then added to ach crytube, sealed in the cryosafe and quickly stored in a -80˚C freezer over night to ensure the best cell survival. The cryotubes were then moved to liquid nitrogen at the next day for long term storage.

2.13.4 Thawing and Reculturing Cells:

Cells were taken from liquid nitrogen and quickly brought to a 37˚C waterbath and defrosted under mild shaking of the tube until only a small pellet of ice was left in the tube. The cells were then collected in a Falcon tube with 5 ml of medium per cryotube and spun down in an Eppendorf Centrifuge 5810 R at 1000 RPM for five minutes. After resuspesion in PBS with Ca2+ they were then spun down once more in an Eppendorf Centrifuge 5810 R at 1000 RPM for five minutes counted in a Neubauer Chamber and seeded according to necessary assay density or plated in dilution for amplification.

2.13.5 Changing Medium:

Medium was changed every day for mESCs and every second day for mouse embryonic fibroblast (MEF) cultures as well as every day after passaging or reculturing after freezing in liquid nitrogen. If medium contained differentiation factors such medium was only changed every second day to reduce the cost of the assays. Old medium was sucked out by vacuum and the cells were gently washed with phosphate buffered saline (PBS) with Ca^{2+}. New pre warmed medium at 37˚C was then added back to the tissue culture plates or the tissue culture dishes. The normal volume of medium was 25 ml per 15 cm dish, 15 ml per 10 cm dish, 2 ml per 6 well plate per well, 1 ml per 12 well plate per well, 0.5 ml per 96 well plate per well and 200 µl per 384 well plate per well.

2.13.5 MEF Inactivation:

Live MEF cells were inactivated after amplification at passage three by different means. One option was to expose the MEFs to Mitomycin C for three hours (10 µg / ml of MEF medium) and then washing the cells twice with MEF medium. A more practical alternative was gamma ray irradiation with a Gamma Cell Irradiator for an hour on ice with a dosage of 60 Gray. Since the irradiation proved to be more cost efficient and less labor intensive this method was mainly used. After inactivation of the MEF cells the cells were frozen in one cryotube per 15 cm dish and defrosted to be used for three 10 cm dishes depending on quality and density of the MEF cells.

2.14 MEF Derivation:

CD-1 mice where ordered with about 12 pups per mother at day 12.5 gestation. Mice were anesthetized and euthanized by cervical dislocation or CO2 exposure. Mice where then soaked in alcohol 80% for cleaning and sterilization. The abdominal cavity was opened with sterile tools under a tissue culture hood designated for primary mouse work and the uterine horns were collected and stored in a petri dish with cold PBS. After washing of the uterine horns with PBS for three times the embryonic sacs were collected and transferred to a new petri dish. Embryonic sacs were opened and the embryos were collected. Visceral tissue as well as the head, arms, legs and tail were removed from the embryo and the remaining part was transferred to a new petri dish and washed with PBS three times. After having processed all embryos this way they were collected and minced in a new petri dish without PBS. Sterile razor blades as well as vigorously pipeting up and down with a 1000 µl pipet was utilized to mince the tissue, alternatively soft tearing and tweezing with forceps can also be used. The now sludgy mass was cultured on 15 cm dishes in MEF media with about 3 embryos per dish. To harvest more cells the work dish was washed out with PBS and the collected cells added to the tissue culture. After culturing under standard conditions for 24 hours the medium was changed and not attached cells as well as tissue clumps were removed. Once the passage 0 MEFs where fully confluent they were trypsinized and passaged to 15 cm culture dishes in a one to three dilution. The trypsinization was done with 0.25% Tripsin for three to five minutes after washing with PBS for three times. Once the MEFs reached passage three they were collected and inactivated with Mytomicin or gamma ray irradiation and frozen down at -80˚C to be later on stored in liquid nitrogen.

2.15 Cardiac Mesenchymal Cell Derivation:

For feeder cardiac mesenchymal cells (CMC) CD-1 mice with newly born litter were obtained. The litter was euthanized by CO2 bath, hearts were collected and placed in cold HBSS in a 6 well plate and the pericard was removed. About eight hearts were collected per well of the 6 well plate and excessive non myocardial tissue was removed such as blood vessels. The hearts are then cut into quarters so that liquid can better reach the inner parts of the organs. The hearts were then transferred to new wells with cold Hank's Buffered Salt Solution (HBSS) for washing and where washed this way three times. After the washing the hearts were transferred to a small bottle with HBSS and 0.5 mg / ml of Trypsin to predigest over night at 4˚C on an orbital shaker spinning at 80 RPM. At Day 2 the Trypsin is reduced by removing the liquid and the reaction is blocked with warm light medium (see below) for three to four minutes on an orbital shaker at 150 RPM.

Light Medium	Volume
DMEM	375 ml
M-199	125 ml
100x Pen / Strep / Glut	5 ml
HEPES (1M)	5 ml

Dark Medium	Volume
DMEM	375 ml
M-199	125 ml
100x Pen / Strep / Glut	5 ml
HEPES (1M)	5 ml
Horse Serum	60 ml
FBS	30 ml

After that all solution was carefully aspirated and HBSS with collagenase (80 mg / 100 ml) was added and, once again, the container was incubated on an orbital shaker at 37˚C (150 RPM) for two minutes only. This constitutes the pre digest and is discarded as it contains too much debris, dead cells and red blood cells. Once more HBSS with collagenase (80 mg / 100 ml) was added and then incubated on an orbital shaker at 37˚C for ten minutes rotating at 150 RPM. The supernatant was collected and added to a separate Falcon tube containing dark medium (see above) to block the digestion. This digestion step was then repeated four times and the collected supernatant with dark serum was pooled aand collected in Falcon tubes. The tubes where then spun down in

an Eppendorf centrifuge Centrifuge 5417 R at 600 RPM and the supernatant, which holds the CMC, was collected. The pellet holds neonatal cardiomyocytes which can be used for other experiments if needed. The supernatant was once again spun down in an Eppendorf centrifuge centrifuge 5417 R but this time at 1000 RPM to pellet the CMC at the bottom of the Falcon tube. The cells where then replated and cultured in CMC medium (see below) for an hour at standard conditions in a tissue culture plate of choice.

CMC Medium	Volume
DMEM	500 ml
NCS	50 ml
FBS	25 ml
Pen / Strep	5 ml

Afterwards the supernatant was recultured at the same conditions for the same duration on a tissue culture plate of choice for another hour. The adherently growing cells are the CMC and can later be used as feeder cells in further experiments

2.16 mESC Differentiation by Embryoid Body:

The respective mESC were recultured from liquid nitrogen and cultured under standard conditions on inactivated MEF feeder layers for two days with mES medium. After two days the cells were trypsinized and brought into single cell status, resuspended in differentiation medium (see below) containing Activin A.

Differentiation		
DMEM (High Glucose)	500 ml	1000 ml
FBS	94 ml	188 ml
L-Glutamin	6,25 ml	12,5 ml
NEAA	6,25 ml	12,5 ml
Ascorbate (0,1M)	625 µl	1250 µl
β -Mercapto Ethanol	4,4 µl	8,8 µl

Droplets were attached to the lid of an uncoated low attachment tissue culture plate with a multi pipet and a concentration of 100,000 cells / ml. The hanging drops were

incubated under standard conditions of 37˚C and 4% CO2 for two days and then collected to be further cultured in the various differentiation media following the various protocols. At the day of interest, depending on experiment, the embryoid bodies were disassociated with collagenase or fixed with 2 % Paraformaldehyde (PFA) for 20 minutes and stained with antibodies.

2.17 mESC Differentiation by Monolayer Culture:

The respective mESC were recultured from liquid nitrogen and cultured under standard conditions on inactivated MEF feeder layers for two days with mES medium. After those two days the mES cells were trypsinized and recultured to the assay plates after a depletion step. To deplete the MEF cells the cell suspension after trypsinization was cultured on 10 cm tissue culture dishes under standard conditions for one hour and the supernatant was recollected since most of the MEFcells had reattached to the dish in this time. This depletion step was repeated once. The cells were then counted in a Neubauer Chamber and depending on the assay seeded accordingly. Various conditions changed depending on the assay such as the composition of the differentiation factors, initial cell seeding number, culture plates, surface proteins and feeder cells, transgenic cell lines, exposure to the differentiation factors, medium composition, serum concentration in the medium and days after the beginning of differentiation for readout and fixation. In the final assay which was later used in the high throughput screening Isl1Cre/+ & Rosa26YFP/+ mESC where cultured with Medium Mix I and 0.2 % FBS, plated on gelatinized 384 well plates at a cell count of 2000 cells / well and treated with two days of Activin A at a concentration of 5 µl / ml. After two days the medium was changed, again Medium Mix I and 0.2 % FBS, and BMP4 (1 µl / ml) and Wnt3a (0.875 µl / ml) where added for four days whereas the medium was renewed after two days respectively. After day six post differentiation the treatment with differentiation factors was stopped and the cells were cultured in untreated Medium Mix I

and 0.2 % FBS. The cells were evaluated for beating clusters or fixed for immunostaining at different days post differentiation but predominantly at day 10.

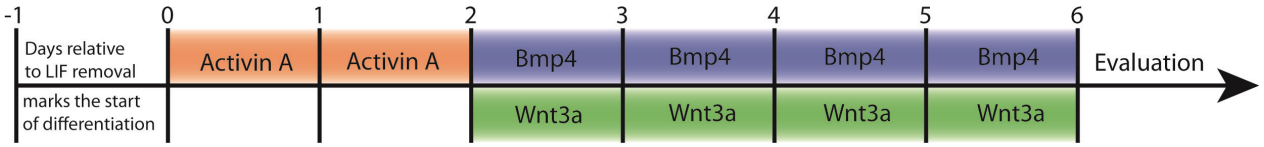


Figure 11: The figure above displays a schematic of an example of a differentiation assay protocol. Here Activin A is used from day 0 until day 2 to be then replaced by Bmp4 with Wnt3a until day 6.

2.18 mESC Evaluation

2.18.1 mESC Morphology and Beating Clusters by Light Microscopy:

Morphology of the mESC as well as the area of beating clusters was checked by light microscopy using a Leica DM IL as well as a Leica DMI 4000 B and evaluated for changes and general condition of the cells. For beating clusters the size was taken relation to the well size as well as the intensety and freaquency of the beats. Light microscopy with said microscopes was also used to check the intensity of LacZ during different timepoints and compared to other read out systems such as YFP or GFP.

3 Results:

The experimental work presented here examines different possibilities and varying set ups for the culturing of mouse embryonic stem cells with the goal of establishing an assay for large scale screening with small molecules, chemicals and RNAi libraries in the field of cardiac development. Due to this the main focus was on a two dimensional system in which mouse embryonic stem cells where cultured mostly without cellular feeder layers and a simple and cost efficient differentiation protocol with various chemicals was employed in order to promote directed differentiation into cardiac lineages. These different developmental assays where then scored and compared among each other as well as to the classical differentiation protocol utilizing “Embryoid Bodies”.

During the experimental work the following points have been examined, tested and optimized:

- Readout systems
- Feeder cells and extracellular matrix proteins
- Cell density
- Culture medium composition and fetal bovine serum concentrations
- Differentiation factors
- Differentiation factor timelines and exposure length

3.1 Readout Systems:

Genetically altered mouse embryonic stem cells, derived from transgenic mice, taken from the laboratory stock or obtained from other laboratories, where employed in the assays to determine which system would give the most reliable, sensitive and robust read out. The mouse embryonic stem cells have genetic constructs of genes important to cardiogenesis linked to color markers. The mayor focus was the Isl1 gene since the Multipotent $Isl1^{+}$ Cardiogenic Progenitor has been shown to be an important progenitor in the development of the heart. Isl1 however is not cardiac specific and exists in other celltypes during development, such as in neuronal and pancreatic cells for instance. [16-18]

The following cell lines were used and scored for the detection time of the marker as well as intensity, as specifically outlined in Materials and Methods:

- **$Isl1^{Cre/+}$ & $Rosa26^{LacZ/+}$** (Isl1 linked to LacZ;)
- **$Isl1^{Cre/+}$ & $Rosa26^{YFP/+}$** (Isl1 linked to yellow fluorescent protein (YFP);)
- **Mef2c-$AHF^{GFP/+}$** (Mef2c linked to green fluorescent protein (GFP); Mesoderm-Anterior Heart Field (AHF) specific enhancer)
- **$Bry^{GFP/+}$** (Brachyury linked to GFP; Early Mesoderm)

Cell Line:	Marker detection: (Days after start of differentiation)		Signal strength
	First day of sighting	Mean day of sighting	
$Isl1^{Cre/+}$ & $Rosa26^{LacZ/+}$	Day 8	Day 10	Staining dependant
$Isl1^{Cre/+}$ & $Rosa26^{YFP/+}$	Day 4	Day 6	Good
Mef2c-$AHF^{GFP/+}$	Day 8	Day 10	Weak
$Bry^{GFP/+}$	Day 3	Day 6	Weak

Table 1: The different cell lines used during the assay optimization.

Since the cell line Isl1$^{Cre/+}$ & Rosa26$^{YFPR/+}$ had high signal strength, an early detection time after the beginning of the differentiation as well as the advantage of the possibility of screening for the marker by fluorescent light microscopy, the majority of further assays used this cell line. Overall the Isl1$^{Cre/+}$ & Rosa26$^{YFPR/+}$ proved to be the most functional tool for monitoring the expression of Isl1 in a lineage tracing system.

3.2 Feeder Cells and Extracellular Matrix Proteins:

Key influences on the development and differentiation of embryonic stem cells are cell to cell contact as well as the contact with extracellular matrix proteins. [19] In regard to the Multipotent Isl1^{+} Cardiogenic Progenitor a previous paper from our laboratory has shown that utilizing neonatal CMC can increase the amount of Isl1+ progenitors but will inhibit their differentiation. [3]

The comparison of various extracellular matrix proteins such as Matrigel, Fibronectin and Gelatin as well as co culture with cardiac mesenchymal cells and mouse embryonic fibroblasts has shown distinct differences in the amount of YFP positive cells after the start of differentiation.

12 well plate assay	Beating clusters			
	Day 7	Day 10	Day 12	Day 13
Matrigel	None	+	++	+
Gelatin	None	+	++	+++
Fibronectin	None	++	++	++
CMC	None	+	+	+

Table 2: Occurrence of beating cell clusters as detected by light microscopy on a 12 well plate. the plus signs relate to the amount of beating clusters whereas (+) equals low occurrence, (++) medium occurrence and (+++) a high occurrence.

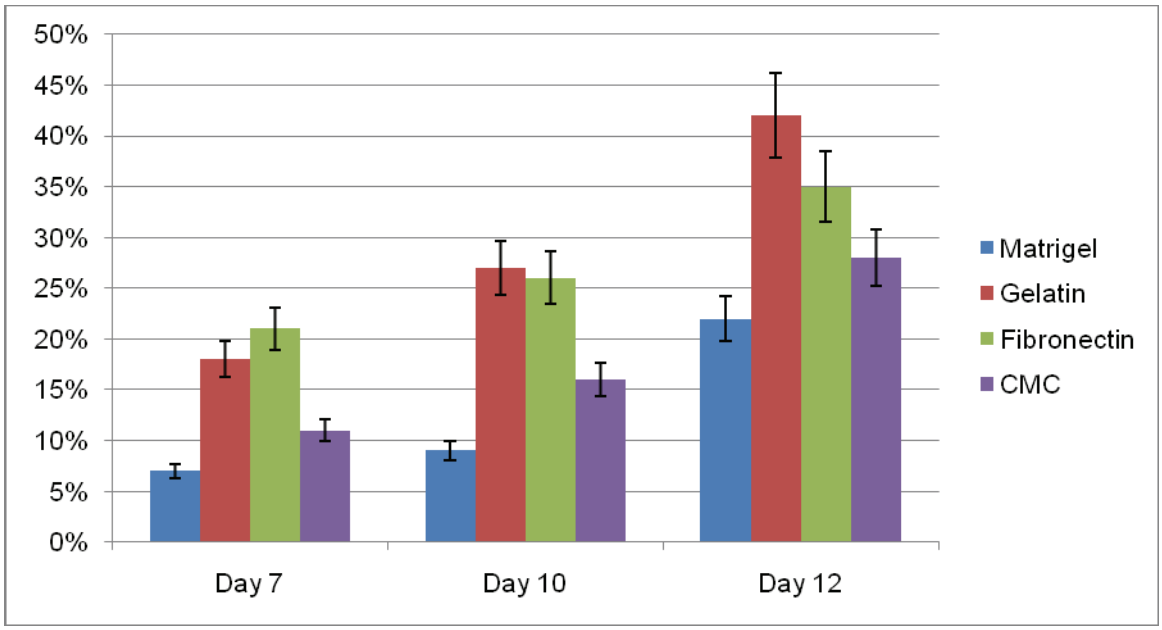


Figure 12: The score represents the amount of YFP positive cells in percentage of the examined wells. (Data of the 12 well plate assay)

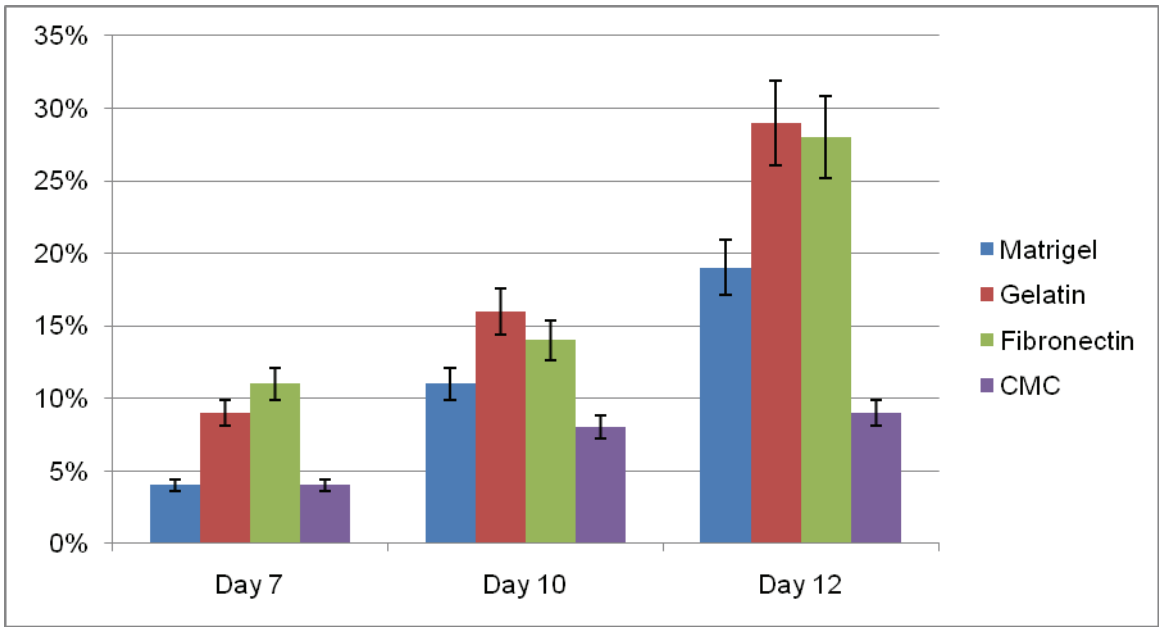


Figure 13: The score represents the amount of YFP positive cells in percentage of the examined wells. (Data of the 96 well plate assay)

As the table and the diagrams show Gelatin and Fibronectin were the most efficient extracellular matrix proteins. Matrigel did not show an advantage towards cardiac commitment compared to Gelatin and Fibronectin. Also as expected cardiac mesenchymal cells [3] did hinder the differentiation of the cells and the occurrence of beating clusters. Since Gelatin is more cost efficient and easier to create and store than Fibronectin further assays focused on Gelatin as an extracellular matrix.

3.3 Cell Density:

Previous work, especially in the field of neuronal differentiation, has revealed that cell density plays an important role in differentiation [20] and can impact on the direction of development. Therefore different cell densities were examined and tested to discover which density would be best suitable for cardiac commitment.

The cell density assay, described in the material and methods section, was scored for the intensity of fluorescence of YFP during different time points as well as the existence of beating cell clusters.

Cell density (12 well plate)	Beating clusters			
	Day 5	Day 6	Day 8	Day 9
50,000 / well	None	None	+	++
100,000 / well	None	+	++	+++
200,000 / well	None	++	++	+

Table 3: Occurrence of beating cell clusters as detected by light microscopy on a 12 well plate. the plus signs relate to the amount of beating clusters whereas (+) equals low occurrence, (++) medium occurrence and (+++) a high occurrence.

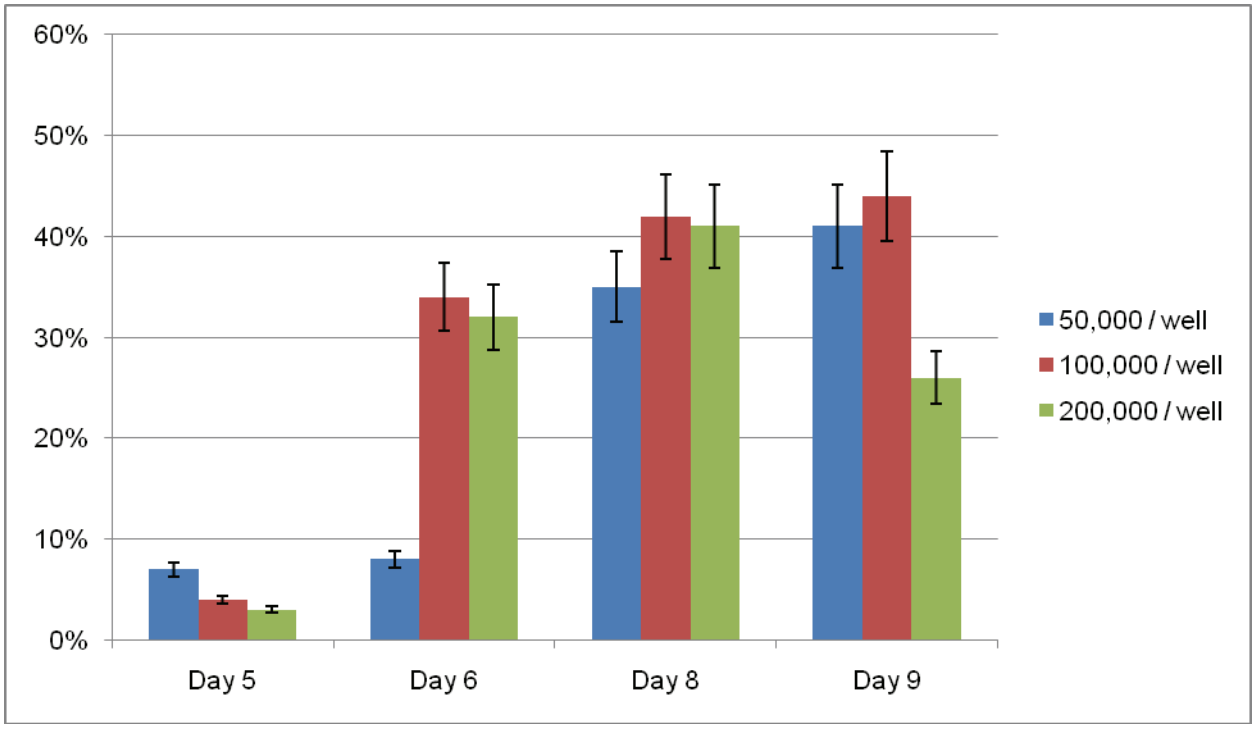


Figure 14: The score represents the amount of YFP positive mES cells in percentage of the examined wells. (Data of the 12 well plate assay)

The data above shows that a cell density of 100,000 cells per 12 well plate well will yield the best results for the monolayer environment since the YFP signal will be stronger earlier and will be slightly more abundant compared to the 50,000 cells per 12 well plate well at differentiation day 9. Since the well of a 12 well plate has a surface area of 3.8 cm^2 a cell density of 100,000 cells per 12 well plate well is a density of about 26,000 cells per cm^2.

Based on this calculation the following cell densities where selected for further assays and tested in different plate setups. The final cell densities per well used are:

10 cm culture dish	6 well plate	12 well plate	96 well plate	384 well plate
1,200,000 cells	200,000 cells	100,000 cells	16,000 cells	2000 cells

Table 4: The Table above shows the optimum cell density for the start of the differentiation assays for the different culture plates and dishes.

3.4 Culture Medium Composition and Fetal Bovine Serum Concentrations:

3.4.1 Culture Medium Composition:

Since the cell growth and differentiation potential is depending on different culture conditions several assays had to be dedicated to the optimization of the most efficient culture conditions for the mouse embryonic stem cells in regard to cardiac commitment and differentiation. An important part of the conditions are set by the choice of culture medium and the composition of the supplements as well as the concentration of fetal bovine serum in the medium. As defined in material and methods five different media mixtures were tested out of which three were examined further since the yielded the best result. After the preliminary assays Medium Mix I, III and V were tested further while Medium Mix II and IV did not have a beneficial influence on the development.

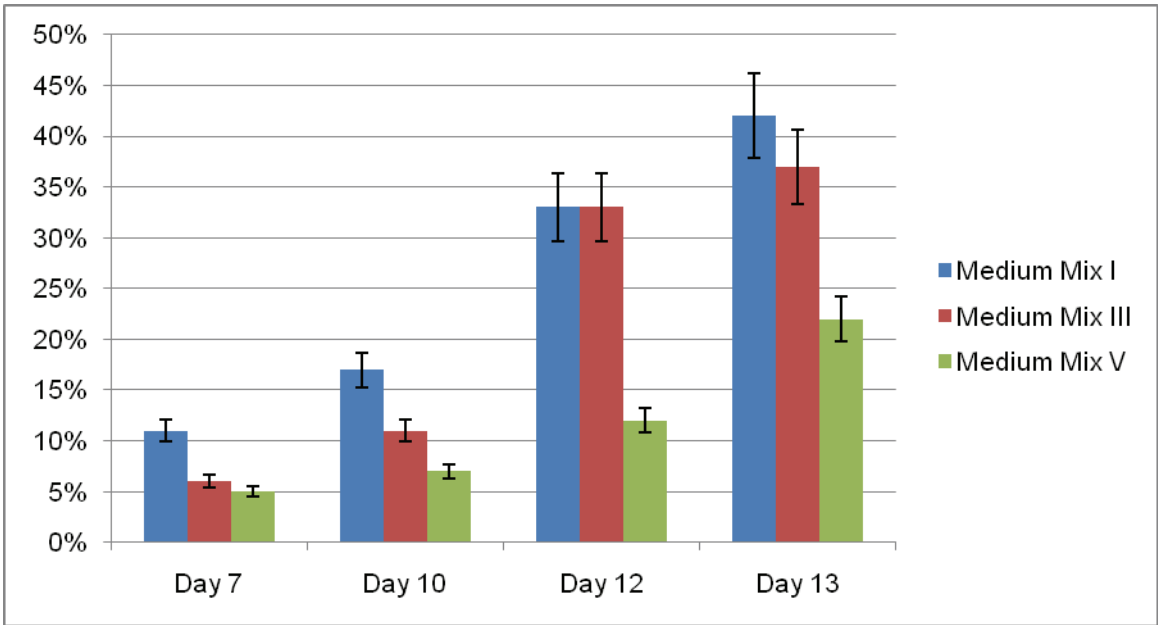


Figure 15: The score represents the amount of YFP positive cells in percentage of total cells per wells. (Data of the 12 well plate assay) Compared here are Medium Mix I, III and V.

12 well plate assay	Beating clusters			
	Day 7	Day 10	Day 12	Day 13
Medium Mix I	+	+	++	++
Medium Mix III	None	None	+	None
Medium Mix V	None	None	None	None

Table 5: Occurrence of beating cell clusters as detected by light microscopy on a 12 well plate. the plus signs relate to the amount of beating clusters whereas (+) equals low occurrence, (++) medium occurrence and (+++) a high occurrence.

96 well plate assay	Beating clusters			
	Day 7	Day 10	Day 12	Day 13
Medium Mix I	+	++	+++	++
Medium Mix III	None	None	+	None
Medium Mix V	None	None	None	None

Table 6: Occurrence of beating cell clusters as detected by light microscopy on a 12 well plate. the plus signs relate to the amount of beating clusters whereas (+) equals low occurrence, (++) medium occurrence and (+++) a high occurrence.

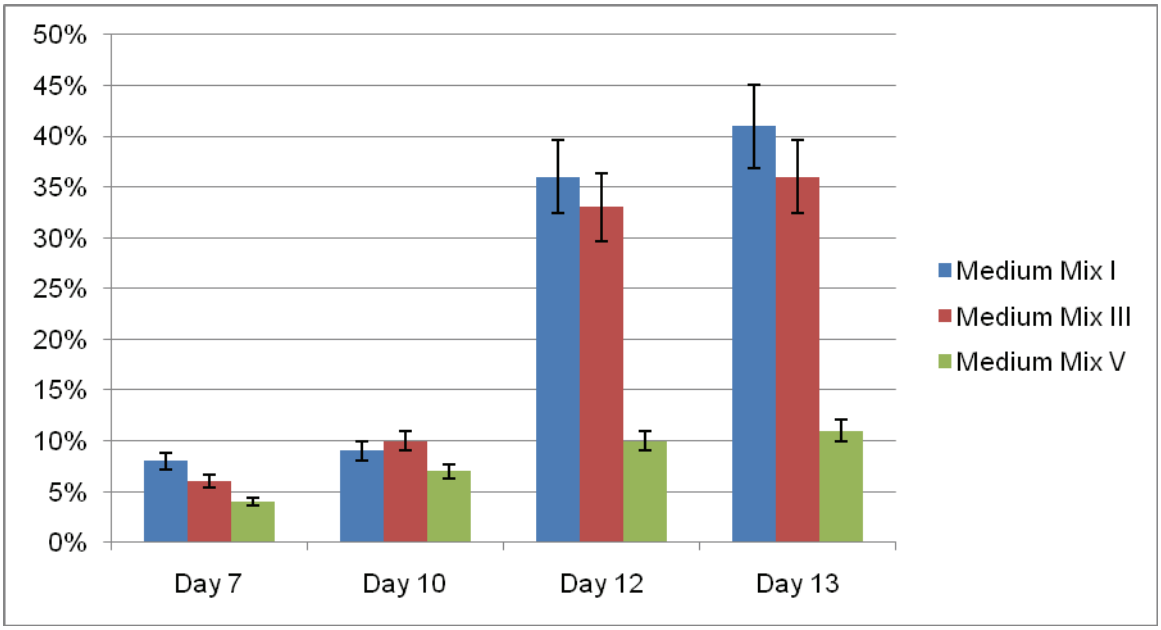


Figure 16: Relative percentage of YFP positive mESCs per well. Examined at different time points for the individual culture media. (Data of the 96 well plate assay)

As the diagrams 4 and 5 show, both Medium Mix I and III show similar levels of YFP expression with Medium Mix I being slightly more efficient around day 12 and day 13. Also the amount of beating clusters, as described in the tables, is far different between Medium Mix I and III where Medium Mix I has more beating clusters. Also the cells started beating about five days earlier than with Media Mix III.

Given the results of the medium composition further experiments focused on testing out different fetal bovine concentrations using Medium Mix 1 as a standard.

3.4.2 Fetal Bovine Serum Concentration:

Media Mix I was used as a base for the testing of different serum concentrations, concentrations of 0.2% FBS, 2% FBS and 8% FBS where tested. Since during the experiments a low serum concentration of 0.2 % showed several days earlier signal of YFP the time points for evaluation where chosen sooner after the beginning of the differentiation. Also at day 10 FACS data was collected and is shown in comparison to the other serum concentrations as well as other differentiation factor assays which are presented FACS data in section 3.8.

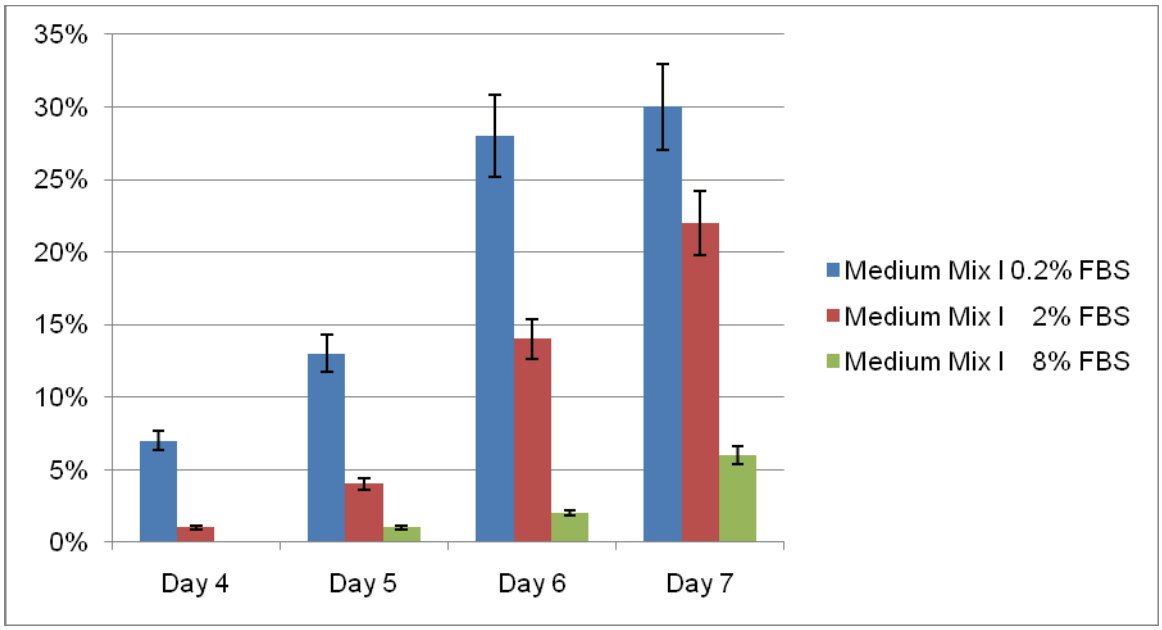


Figure 17: The score represents the amount of YFP positive cells in percentage of the examined wells. (Data of the 12 well plate assay)

12 well plate assay	Beating clusters			
	Day 4	Day 5	Day 6	Day 7
Medium Mix I (0.2% FBS)	None	+	++	++
Medium Mix I (2% FBS)	None	None	None	+
Medium Mix I (8% FBS)	None	None	None	None

Table 7: Occurrence of beating cell clusters as detected by light microscopy on a 12 well plate. the plus signs relate to the amount of beating clusters whereas (+) equals low occurrence, (++) medium occurrence and (+++) a high occurrence.

Since the improvement of speed of differentiation and the increased intensity of YFP staining during culture with Medium Mix I 0.2% FBS the standard was further amended by employing these experimental conditions.

3.5 Differentiation Factors:

Since the Multipotent Isl1^{+} Cardiogenic Progenitors are of mesodermal origin a variety of factors were tested to first commit the embryonic stem cells to the mesodermal lineage and then further increase the percentage of cardiac progenitors in the assays. Several pathways influence the differentiation of stem cells by known and unknown ligands. One of these pathways is the wnt/β-catenin pathway as described in a publication by Eldad Tzahor [21] and further investigated in regard to the Multipotent Isl1^{+} Cardiogenic Progenitor by our laboratory. [3] Since it was know that Activin A, Wnt3a and BMP4 had an influence on the mesodermal differentiation as well as the commitment towards cardiac progenitors various assays where created for different exposure patterns.

3.5.1 Differentiation Factor Composition:

The first batch of assays consisted of different factors added during timepoints shown in the figure below:

Figure 18: The differentiation assays with the various factors. Day -1 is the plating day whereas day 0 is the start of the ligand treatment.

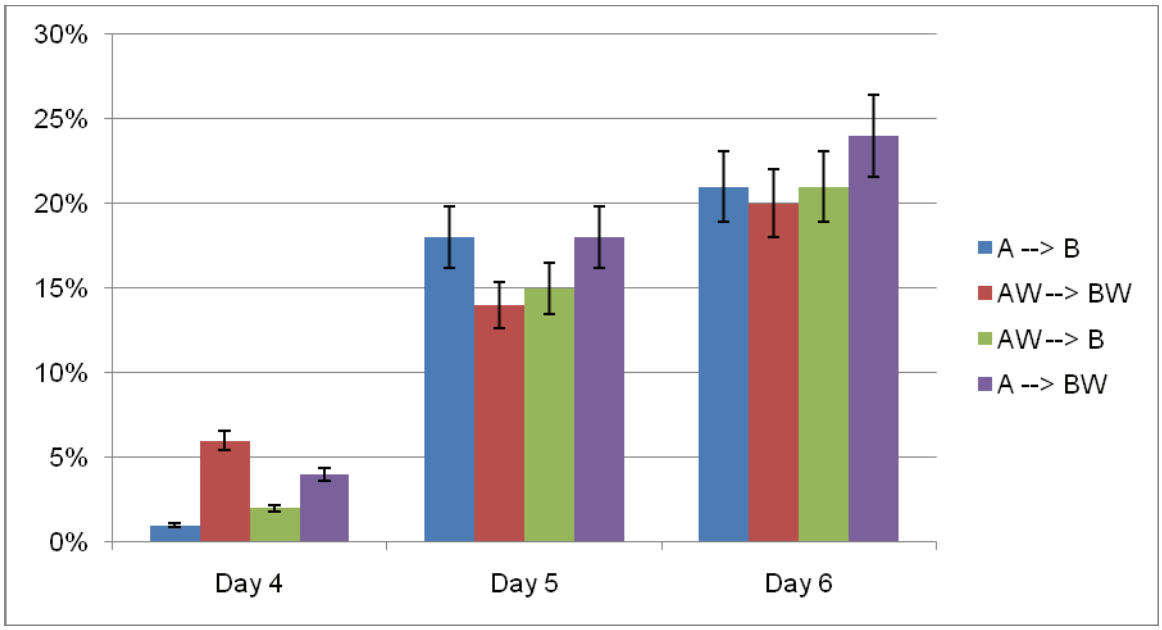


Figure 19: The score represents the percentage of YFP positive cells of the examined wells. (Data of the 12 well plate assay) (A = Activin A; B = Bmp4; W = Wnt3a)

Since the treatment of Activin A for two days and the combined treatment of Wnt3a with BMP4 resulted in the best results among the different assays further experiments were designed to find the best exposure time of these factors. Also to better capture the influence of the factors after the exposure as well as to test for the occurrence of beating cell clusters the time points of evaluation where taken at later days.

3.5.2 Differentiation Factor Exposure Length:

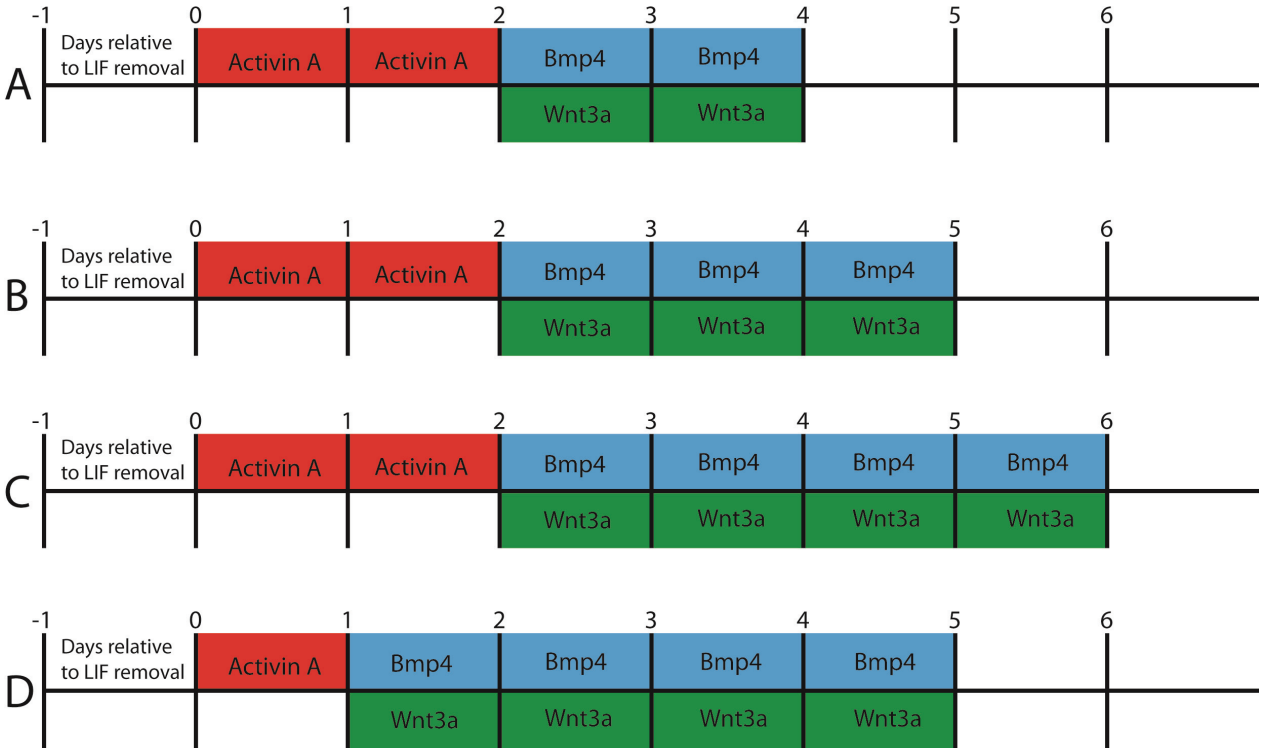


Figure 20: The differentiation assays with various exposure lengths. Day -1 is the plating day whereas day 0 is the start of the ligand treatment.

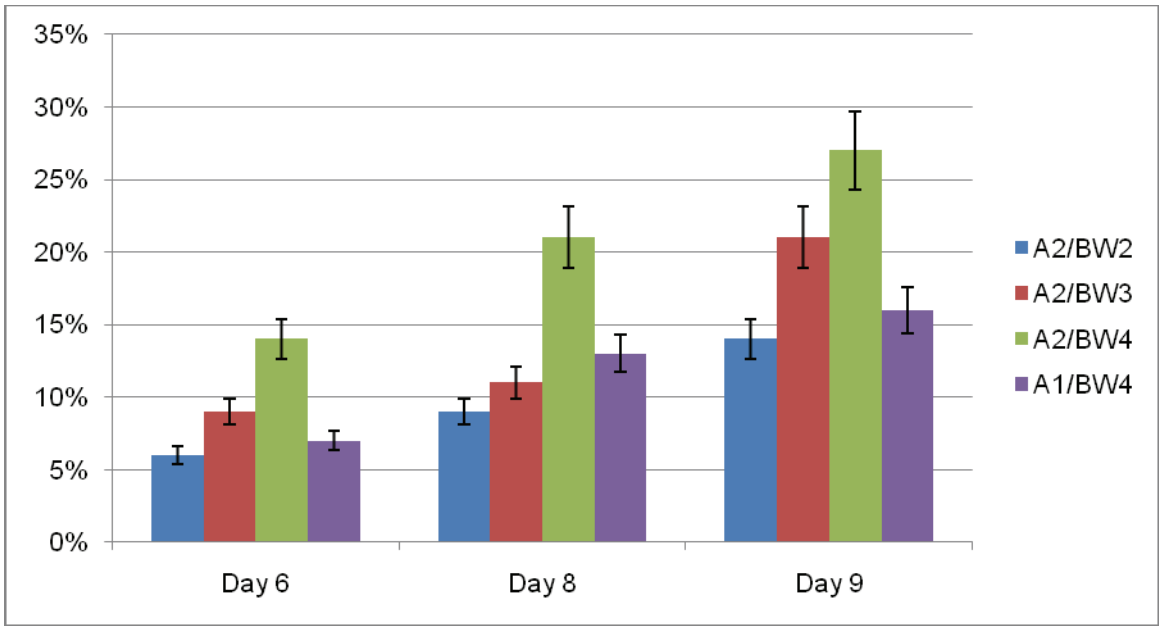


Figure 21: The score represents the amount of YFP positive cells in percentage of the examined wells. (Data of the 12 well plate assay) (A = Activin A; B = Bmp4; W = Wnt3a)

After evaluating the new results the exposure template of two days of Activin A and 4 days of Wnt3a and BMP4 yielded the amount of 27% YFP positive cells. Also in this template the cells started to beat a day earlier and form beating clusters more frequently.

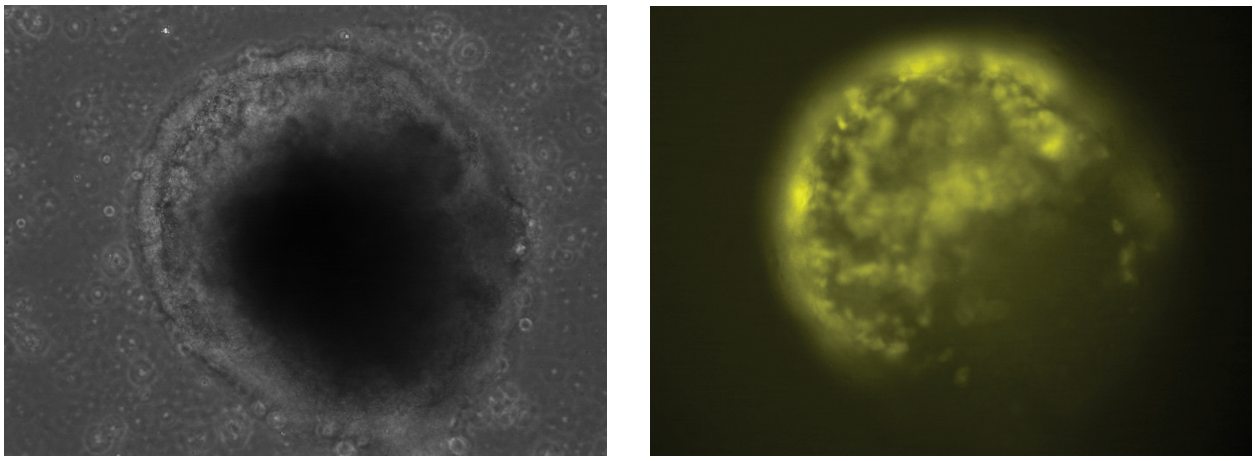
3.6 Markers of Differentiated Cells by analysis via FACS and Immunocytochemistry:

To prove that the mouse embryonic stem cells differentiate in vitro into the different germlayers and various cardiac cells several assays were run and these cells were stained at certain days after the beginning of the differentiation protocol with antibodies representative for known differentiation stages. The antibodies had been conjugated with fluorochromes and evaluated using a fluorescence microscope. The figures below show representative selections of the data collected:

3.6.1 Differentiation of Mouse Embryonic Stem Cell Lines using Embryoid Body Differentiation and the Monolayer Differentiation:

To show that the monolayer differentiation is a generally applicable method and not just tied to a specific cell line various mES cell lines where cultured, differentiated and characterized by immunocytochemistry.

3.6.1.1 Embryoid Body Differentiation at Day 5+2 (**Isl1$^{Cre/+}$ & Rosa26$^{YFPR/+}$** Mouse Embryonic Stem Cells):



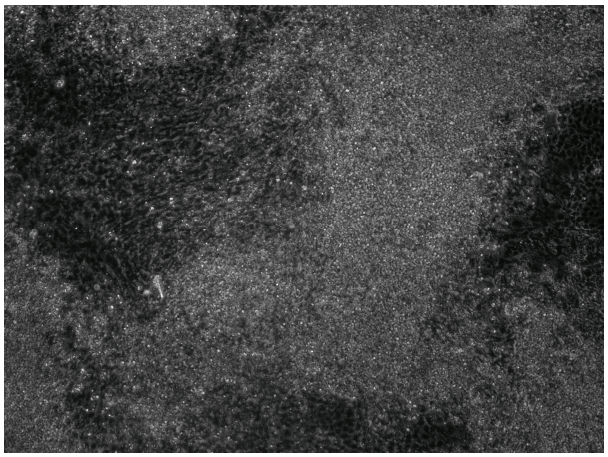
A: Embryoid body at day 5+2; bright field ***B: Transgenic Isl1-YFP***

Figure 22: The panels A and B above show an embryoid body of the Isl1$^{Cre/+}$ & Rosa26$^{YFPR/+}$ mES cell line at day 5+2 (Cultured 5 days in hanging drops and 2 days cultured on gelatin coated plates) (50x magnification).

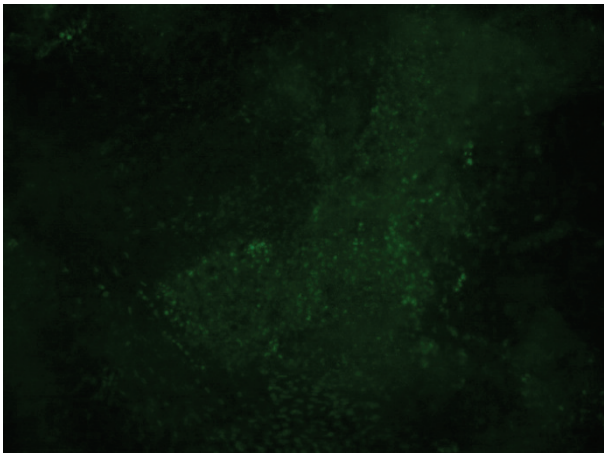
To prove that the Isl1$^{Cre/+}$ & Rosa26$^{YFPR/+}$ mES cell line would differentiated regularly after genetic alteration and to establish a comparable control differentiation the mES were differentiated by embryoid body differentiation and analyzed for YFP signal under a fluorescent microscope.

3.6.1.2 Monolayer Differentiation at Day 10 (Wild Type Mouse Embryonic Stem Cells):

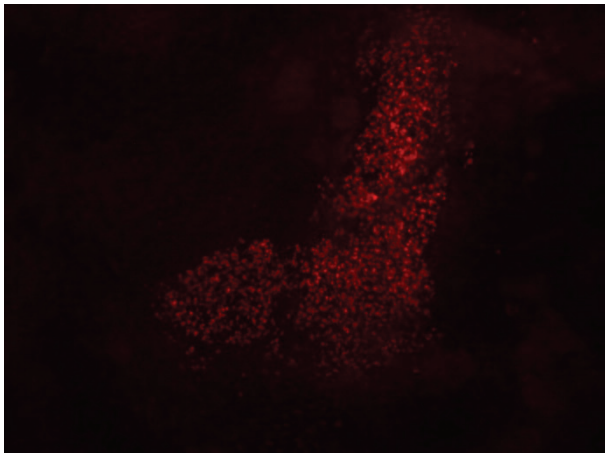
Below are figures of wild type mESCs cultured, differentiated as a monolayer and fixed at day 10 after the differentiation. Bright field as well as staining with antibodies and “4',6-diamidino-2-phenylindole” (DAPI) are shown. The composite shows the overlap of the green and the red channel whereas green is the antibody for Isl1 and red is the antibody for Nkx 2.5. The multipotent islet one cardiogenic progenitor is Isl1 and Nkx 2.5 positive.



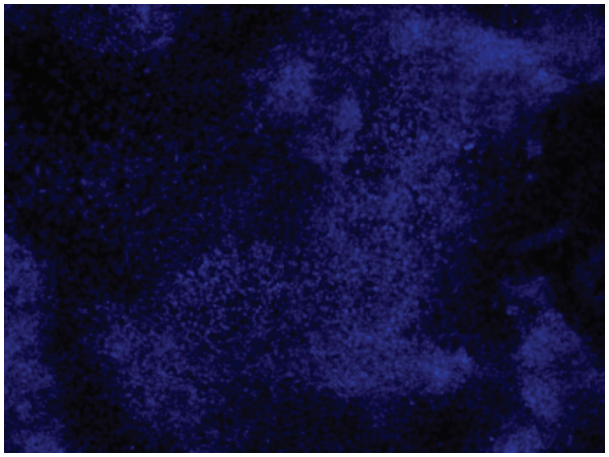
A: Bright field



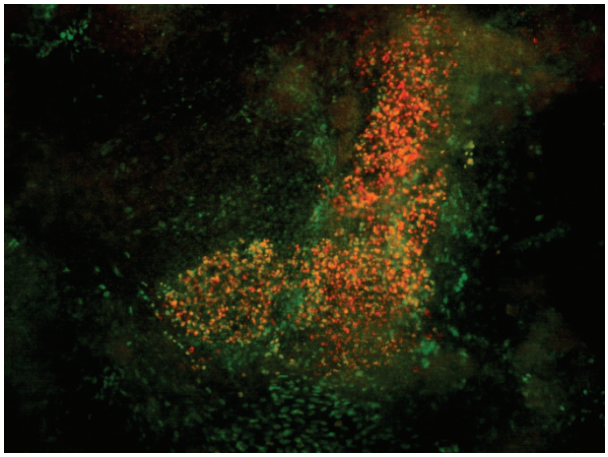
B: Isl1 antibody



C: Nkx 2.5 Antibody



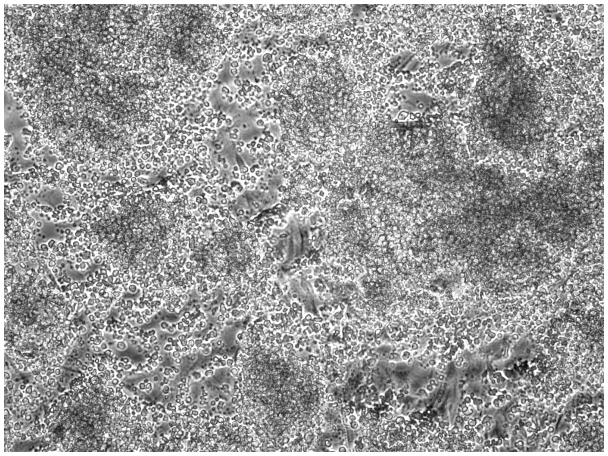
D: DAPI staining



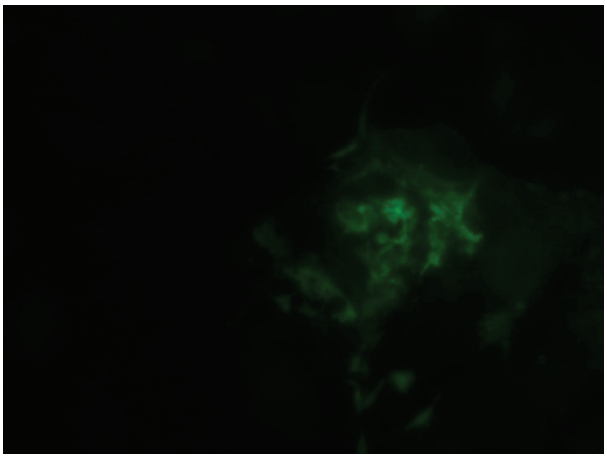
E: Composite of Isl1 and Nkx 2.5

Figure 23: The panels A – E above show a wild type mES cell line in bright field and stained for Isl1 and Nkx 2.5, two markers of the multipotent Isl1 cardiogenic progenitor which can give rise to various cardiac cell types (50x magnification).

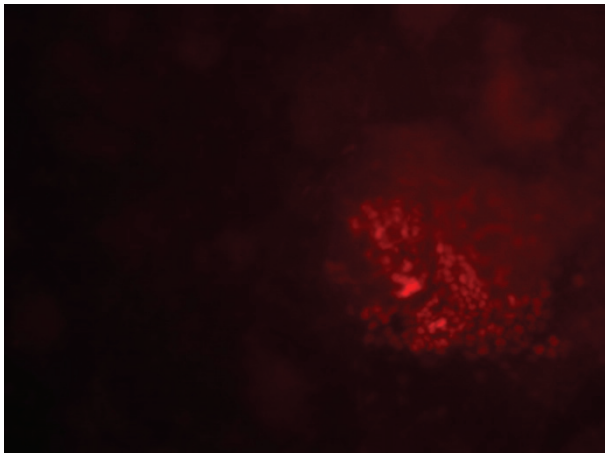
3.6.1.3 Monolayer Differentiation at Day 10 (**Mef2c-AHF$^{GFP/+}$** Mouse Embryonic Stem Cells):



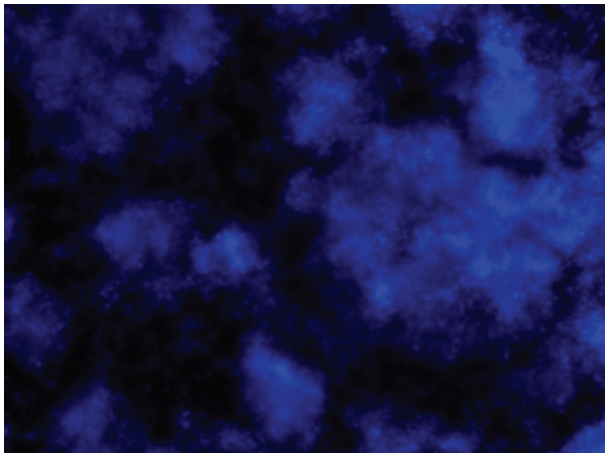
A: Bright field



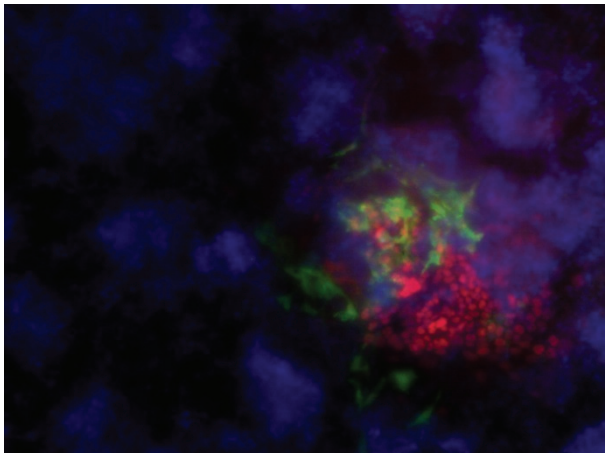
B: GFP Antibody



C: Nkx 2.5 Antibody



D: DAPI staining



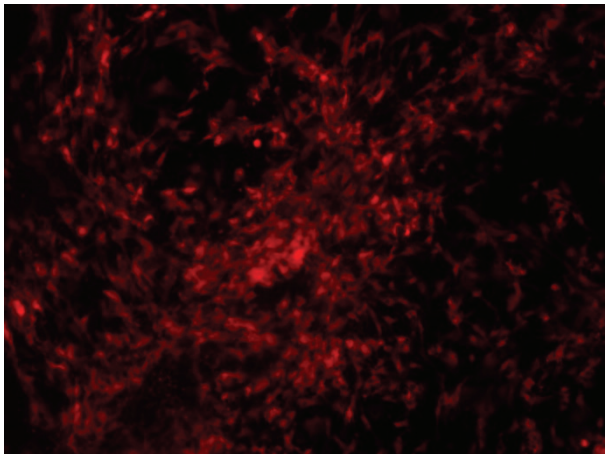
E: Composite of GFP antibody, Nkx 2.5 and DAPI

Figure 24: The panels A – E above show the Mef2c-AHF$^{GFP/+}$ mES cell line in bright field and stained for GFP and Nkx 2.5 (50x magnification).

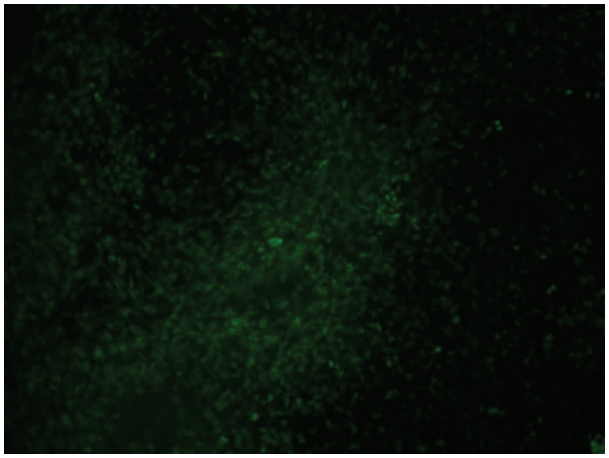
Above are panels of Mef2c-AHF$^{GFP/+}$ mESCs cultured, differentiated as a monolayer and fixed at day 10 after the differentiation. Bright field as well as staining with antibodies and DAPI are shown. The composite shows the overlap of the green and the red channel whereas green is the antibody for GFP and red is the antibody for Nkx 2.5. Again, the multipotent islet one cardiogenic progenitor is Isl1 and Nkx 2.5 positive.

3.6.1.4 Monolayer Differentiation at Day 10 (**Isl1$^{Cre/+}$ & Rosa26$^{YFPR/+}$**):

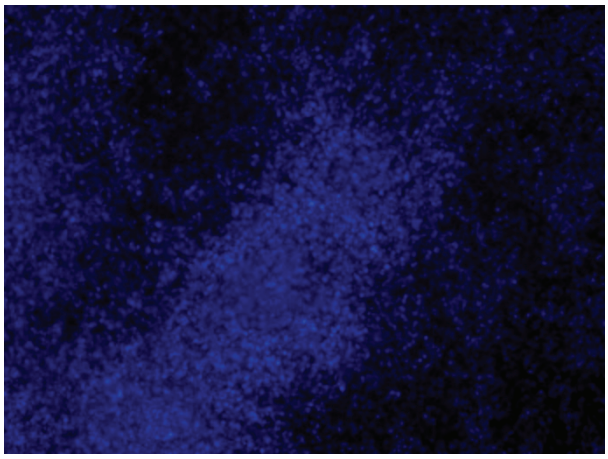
To show that the endogenous YFP expression of the mESC Isl1$^{Cre/+}$ & Rosa26$^{YFPR/+}$ corresponds to Isl1 positive cells assays where fixed and stained at day ten after differentiation. In these panels red is the GFP antibody which also binds to YFP and green correlates to the ISl1 antibody.



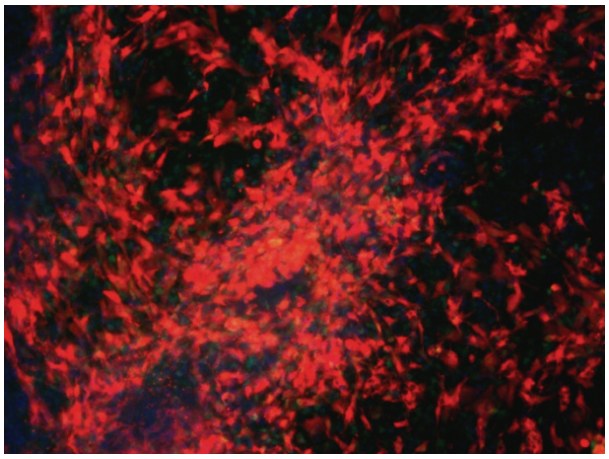
A: GFP Antibody



B: Isl1 Antibody



C: DAPI staining



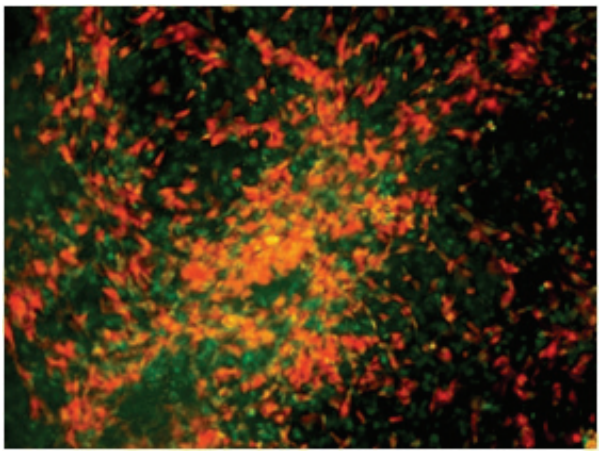
D: Composite of GFP, Isl1 and DAPI

Figure 25: The panels A – D above show the Isl1$^{Cre/+}$ & Rosa26$^{YFPR/+}$ mES cell line in stained for GFP and Isl1 (50x magnification).

Figure 25 (A – D) demonstrates the colocalization of the transgenic Isl1-YFP expression, here stained by GFP antibody, to Isl1 antibody staining.

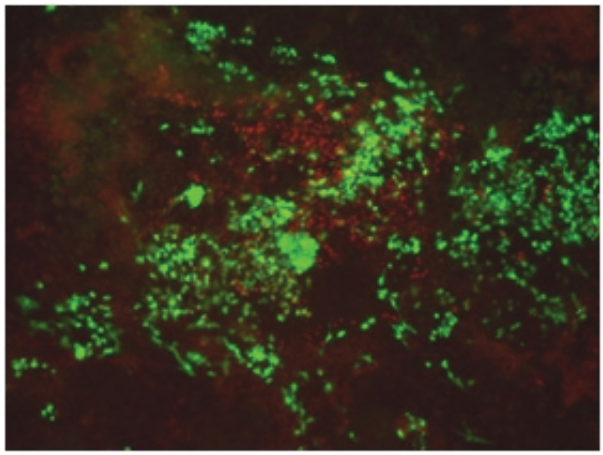
3.6.2 Monolayer Differentiation Potential (**Isl1$^{Cre/+}$ & Rosa26$^{YFPR/+}$**):

To show that the monolayer system with the standard conditions could differentiate into various tissue types more culture assays where performaed and stained with various antibodies at day 10 after the start of differentiation.



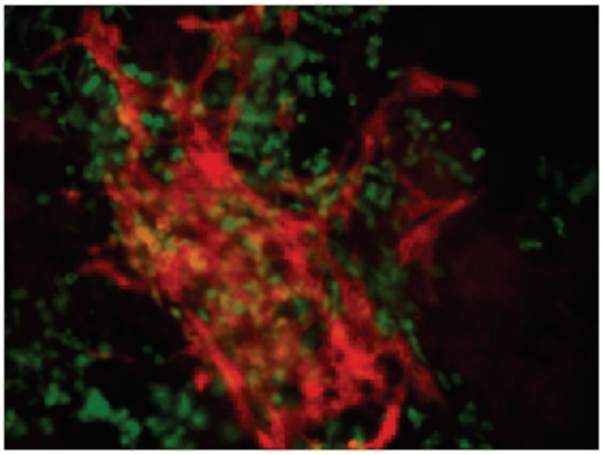
A: Transgenic YFP in red;

Isl1 antibody in green

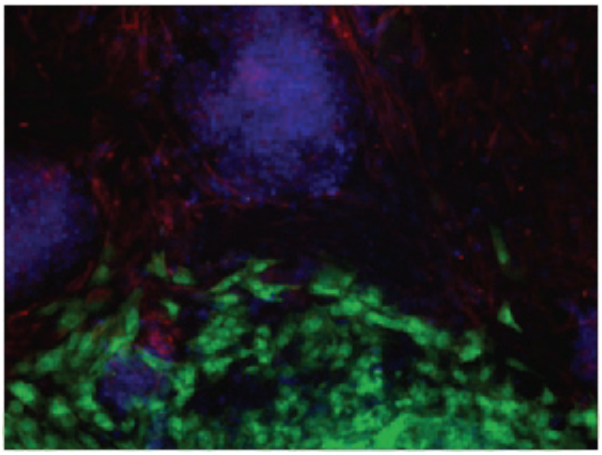


B: GFP antibody in green;

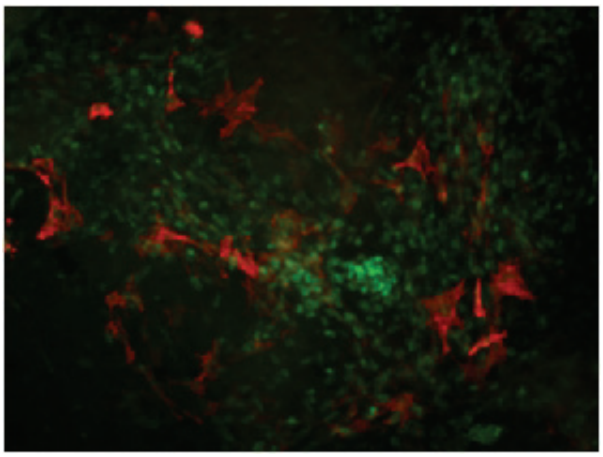
Nkx 2.5 antibody in red



C: GFP antibody in green; α-actinin antibody in red

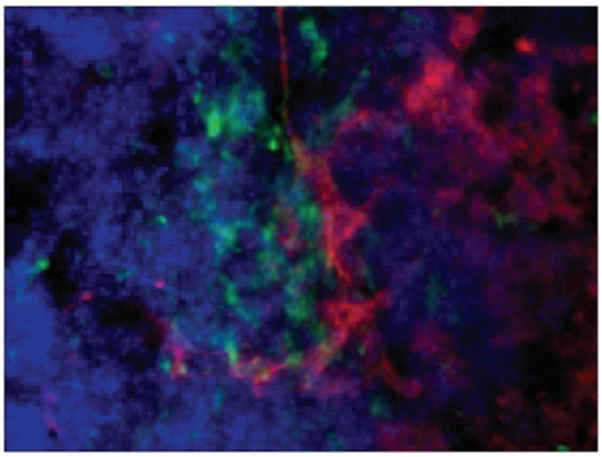


D: GFP antibody in green; nestin antibody in red; DAPI staining



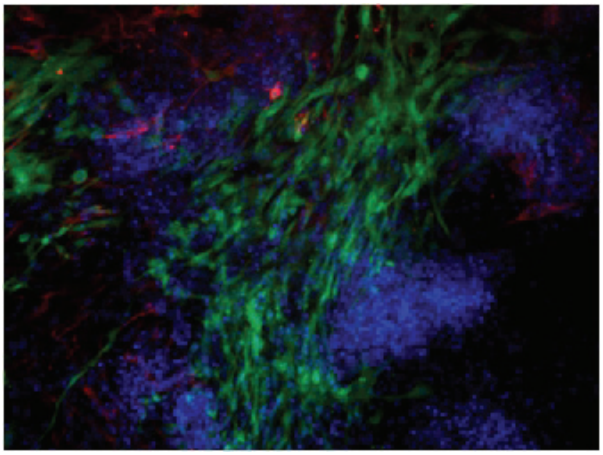
E: GFP antibody in green;

α-smooth muscle actin antibody in red



F: GFP antibody in green;

CD31 antibody in red; DAPI staining



G: GFP antibody in green;

Neurofilament antibody in red; DAPI staining

Figure 26: The panels above show composites of staining for various antibodies and transgenic YFP fluorescence signal such as Isl1, Nkx 2.5, α-smooth muscle actin, CD 31 and DAPI (50x magnification).

All figures shown are composites of dual staining or expression.

Panel (A) shows the transgenic YFP in red along with the Isl1 antibody in green.

Panel (B) shows the GFP antibody in green along with the Nkx 2.5 antibody in red.

Panel (C) shows the GFP antibody in green along with α-actinin antibody in red.

Panel (D) shows the GFP antibody in green along with nestin antibody in red.

Panel (E) shows the GFP antibody in green along with α-smooth muscle actin antibody in red.

Panel (F) shows the GFP antibody in green along with C31 antibody in red.

Panel (G) shows the GFP antibody in green along with Neurofilament antibody in red.

3.6.3 Monolayer Differentiation Potential (Wild Type Mouse Embryonic Stem Cells without Reporter System) Scoring for Isl1-YFP by FACS analysis:

In order to be able to better quantify the results FACS analysis was performed and scored for YFP signal which emits in the wavelength overlapping with FITC.

The timeline below shows the mean percentage of YFP positive viable cells during different time points.

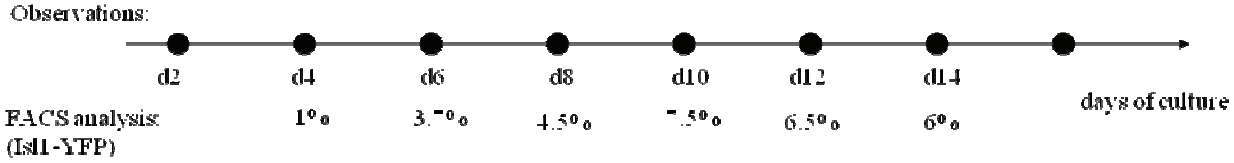


Figure 27: The timeline above shows the mean percentage of Isl1 positive cells stained by antibody conjugated to YFP during different timepoints (GFP and YFP show up in the FITC channel of the FACS analyzer).

The plots below are a sample figure from the FACS analysis at day 12. In the first plot the side scatter area is compared to the forward scatter area and used to gate out dead cells and debris. The second plot represents the gate from the first plot and is a comparison of the APC area channel against the FITC area channel. The wavelength of YFP overlaps with the detection wavelength of FITC and therefore the FITC channel can be used to detect YFP. The APC channel is used as a negative channel and represents the cells autoflourescence.

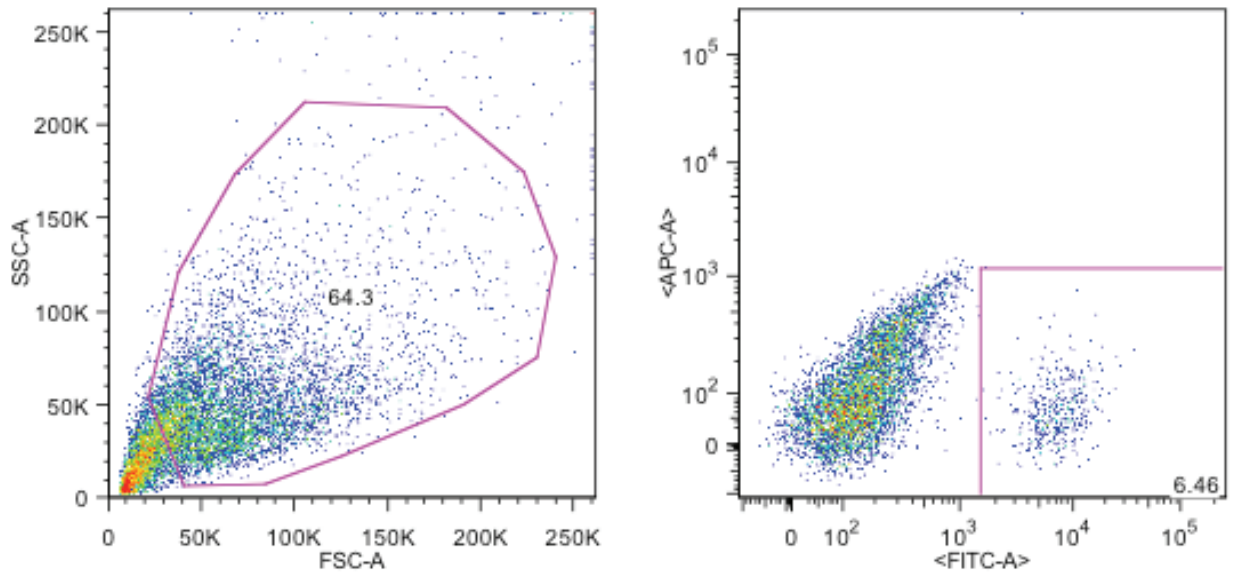


Figure 28: FACS plot at day 12. The left side shows the gating of the viable cells whereas the right side shows the YFP positive cells in the FITC channel. The APC channel is a negative channel which corresponds to auto fluorescence of the cells.

Also in the same batch of assays the area of beating cells was quantified on a separate plate. Below is the diagram which shows a steady increase of beating cells up to differentiation day 13.

Figure 29 shows a steady increase in the percentage of beating clusters peaking at day 13. The first beating clusters where observed at day 7. Between day 13 and day 15 there is a decline in the percentage of beating clusters which might be explained by the difficulty of culturing cardiomyocytes in vitro under these conditions for prolonged durations.

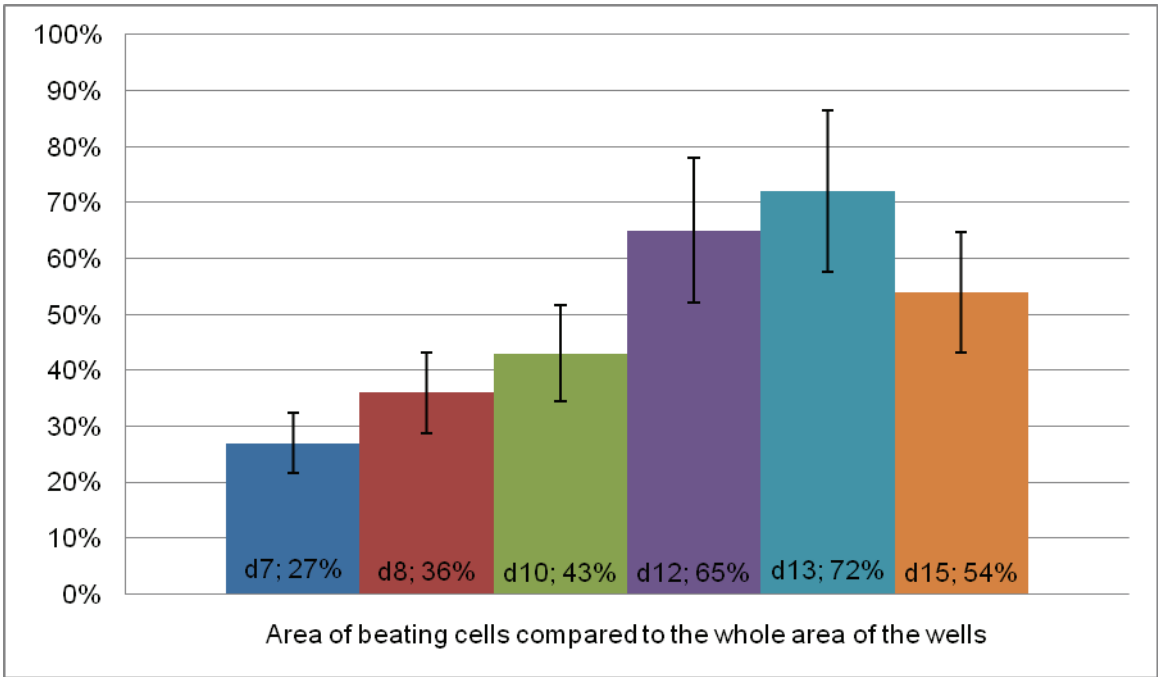


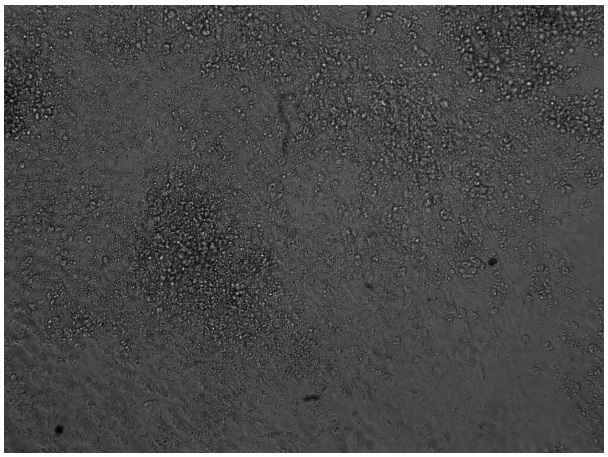
Figure 29: The diagram shows an increase in the area of beating cells up to differentiation day 13. The mESC (Isl1$^{Cre/+}$ & Rosa26$^{YFPR/+}$) where cultured on a 12 well plate.

3.7 High Throughput Screening Assays on 384 Well Plates:

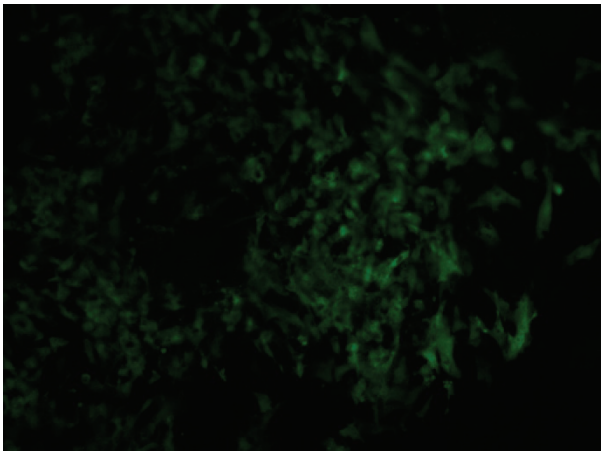
After having established the most beneficial conditions for cardiac differentiation in a monolayer setting, these conditions were applied to mESC (Isl1$^{Cre/+}$ & Rosa26$^{YFPR/+}$) in 384 well plates. Since 384 well plates in combination with automated mechanized readout systems enable the collection of vast and repetitive data points it was important for the system to be robust and accurate in these high throughput experiments.

3.7.1 Immunocytochemistry in the 384 Well Plate Setup at Day 10 after the Beginning of Differentiation:

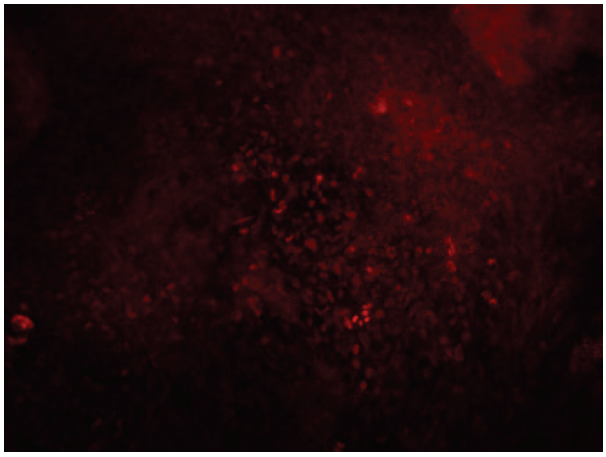
To prove that the differentiation towards cardiac cell types was not negatively affected by the much smaller wells of a 384 well plate further differentiation assays where performed.



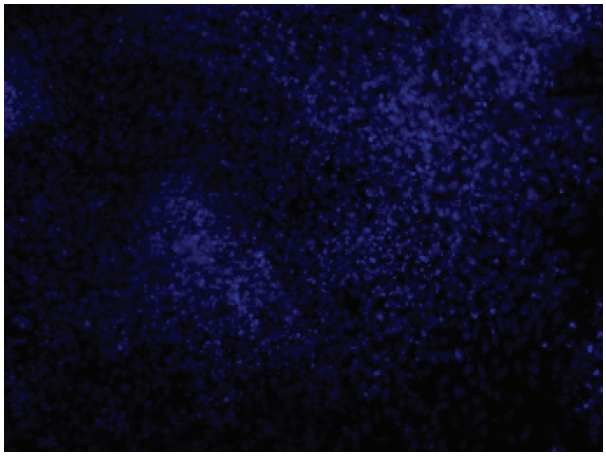
A: Bright field



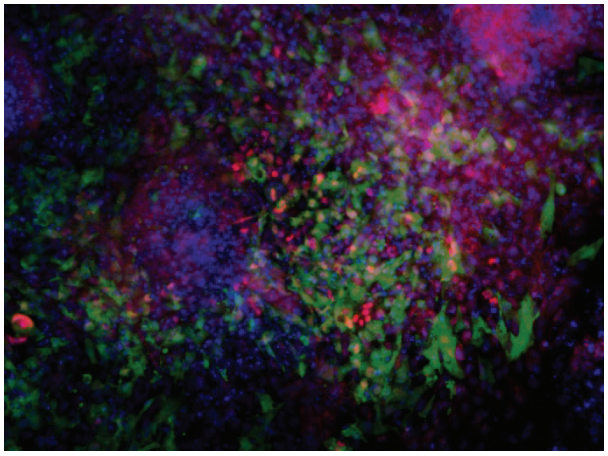
B: GFP antibody in green;



C: Nkx 2.5 antibody in red



D: DAPI staining



E: Composite of the GFP, Nkx 2.5 and DAPI staining.

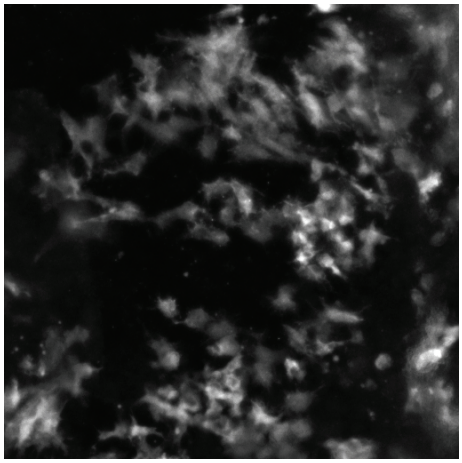
Figure 30: The panels A – E above show antibody staining and transgenic YFP signal. The cells were cultured on a 384 well plate which is the basis for high throughput screening.Panel A shows the bright field, panel B shows staining for GFP in green, panel C shows staining for Nkx2.5 in red, panel D shows the DAPI staining and panel E shows the composite of all three fluorescent channels. (50x magnification).

The panels A – E show the mESC ($Isl1^{Cre/+}$ & $Rosa26^{YFPR/+}$) at day 10 after differentiation. The GFP antibody is colored green whereas the Nkx 2.5 antibody is colored red. The yellow cells are the GFP / Nkx 2.5 double positive cells and are likely to be multipotent islet one cardiogenic progenitors.

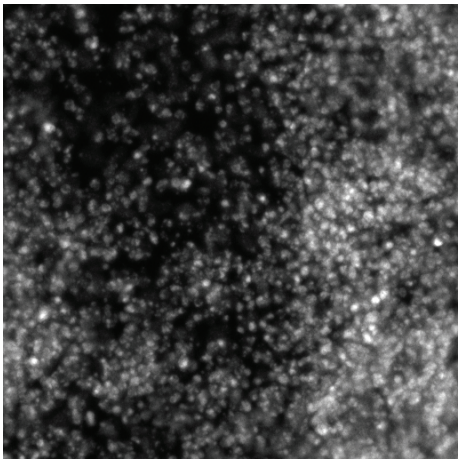
3.7.2 Results Obtained in Collaboration with the Laboratory of Lee Rubin Specialized in Automated High Throughput Screening:

This preliminary data with the automated readout system were performed in collaboration with Lee Rubin. Assays on 384 well plates where analyzed at day 10 past the beginning of differentiation. The use of methodological facilities to automatically detect fluorescent signals over distinct time courses and their ability to add various

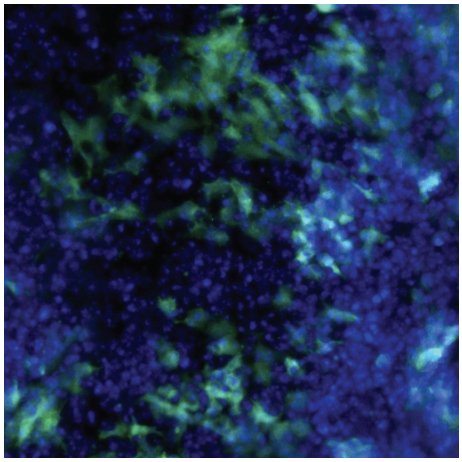
additional components to the culture conditions present a powerful and unique tool to further research in vitro cardiac development and gain insight into the cardiac progenitor cells.



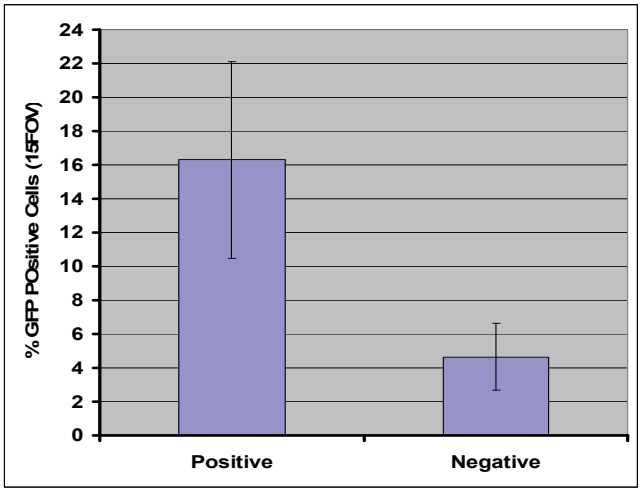
A: GFP antibody



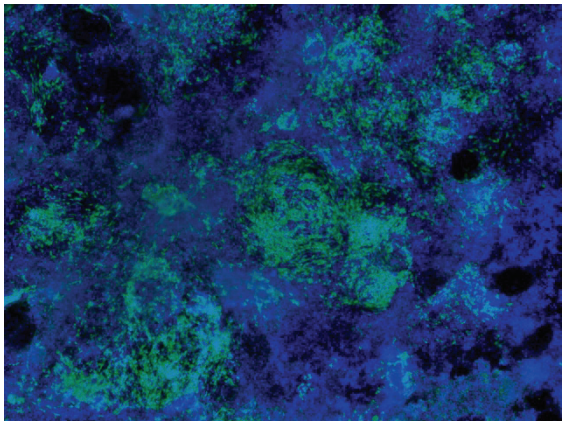
B: DAPI staining



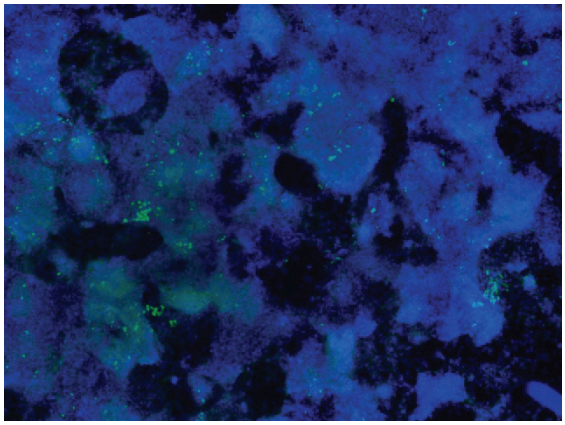
C: Composite of GFP antibody and DAPI staining.



D: Percentage of GFP positive cells in relation to the total amount of cells. (DAPI)



E: Positive control of the Isl1 - Cre & Rosa26 – YFP cell line with the standard treatment of Activin A for two days followed by four days of Wnt3a and BMP4 along with the screening substance in DMSO.



F: Untreated control of the Isl1 - Cre & Rosa26 – YFP cell line with no treatment of Activin A, Wnt3a or BMP4 but with DMSO only.

Figure 31: The panels A – F above show data from the automated high throughput screening set up in Lee Rubin's laboratory. Panel A shows GFP, panel B DAPI for the cell count and panel C is the composite of A and B. D shows the automatically generated count with the percentage of GFP for the positive and negative control. Panel E is a composite of the positive control with the established cytokine driven differentiation and panel F is the negative control with the established cytokine driven differentiation but only DMSO added during screening (100x magnification).

The panels A - F show the immunocytochemistry as well as the percentages of the initial test run of the high throughput screening. Panel A shows the GFP antibody, panel B shows DAPI staining and panel C is the overlay of panel A and B. Panel D is the statistic of the quantification of panel A and F and represents the standard treatment as the positive control and the same established culture conditions without cytokines and DMSO only as negative control. Panel E is the composite of the positive control with the GFP antibody in green and blue DAPI staining. Panel F shows the same but for the negative control with DMSO only treatment.

4 Discussion:

The goal of this thesis was to establish and fine tune a robust and high yield differentiation assay towards cardiac lineages of mouse embryonic stem cells in a monolayer culturing condition. The assay was designed and evaluated experimentally in order to be applicable in a fully automated high throughput screening setup. Therefore an emphasis was placed on simple per formability and cost efficiency. To achieve this, different transgenic mouse embryonic cell lines where induced to the cardiomyocyte lineage by Activin A, BMP4 and Wnt3a in a monolayer culture environment in contrast to the classical embryoid body culture system. These assays have been evaluated for the best possible signal from different read out systems as well as the amount of cardiac progenitors and beating cells at certain time points.

Since mouse embryonic stem cells normally differentiated through an embryoid body step, these are difficult to expose to environmental factors with constant diffusion efficiency. A monolayer culture method was thought to be advantageous for subsequent screening of many different compounds in order to determine their potential on these cardiogenic precursor cells. While the seeding number of cells that would generate the embryoid bodies can be defined and a qualitive analysis can be made after differentiation using immune fluorescent markers, a quantitive analysis, especially an automated one, was technically extremely challenging since the three dimensional structure of an embryoid body hampers precise image evaluation. While it is possible to disassociate the embryoid bodies and FACS sort and analyze them the survival rate of such sorted cells is low and thus reculturing them difficult, time and cost extensive. Therefore this would require greater starting numbers, more time and would increase the cost per screening run.

Here mouse embryonic stem cells derived from blastocysts where cultured under several different conditions and variables such as cell density, feeder layers, extracellular matrix proteins, culturing media compositions and serum concentrations. Also factors that were known to influence the cardiac development in vivo were tested in vitro with regard to timekinetics, concentration and combination. Furthermore these assays have then been translated to 384 well multiplates and evaluated comparatively among each other as well as to the embryoid body culture system. After the robust functionality and superiority of the monolayer culture system evaluated here had been elaborated, this latter experimental setup has been employed in an automated screening design.

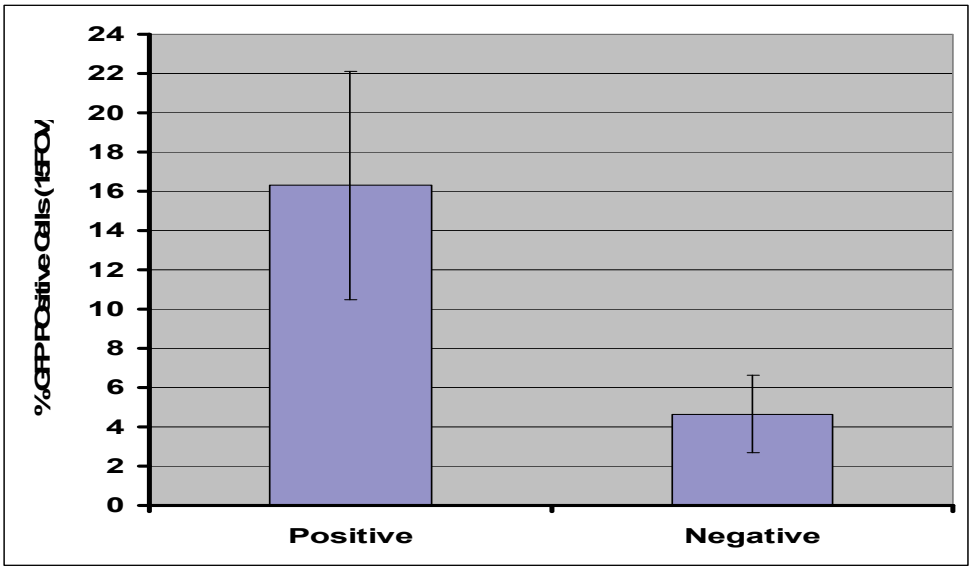


Figure 32: The amount of GFP positive cells compared to the amount of cells. Positive is the assay with 2 days of Activin A followed by 4 days of Wnt3a and BMP4. Negative is also after 6 days with treatment of DMSO only.

While the variables of setting up such an assay have been shown to be extensive and the experimental refining of the quality of these variables was pursued to great lengths, it became apparent that the preliminary screening setup may not be a perfect one. A representation of a sufficient amount of target cells which are to be exposed to the screening factors are important so that the impacts of these, may it be an increase or a

decrease in number or a change in later fully differentiated cell function, can be clearly distinguished from the background noise.

Given the preliminary data of our assay standard of 2 days of Activin A followed by 4 days of Wnt3a and BMP4 compared to a DMSO control we can see an increase in GFP positive cells from 5% to 16%.

Using this setup should enable us to now test several factors, for instance small molecules, known drugs or RNAi fragments, to see if there is an impact on the development and differentiation of the multipotent isl1 cardiac progenitors.

While the mouse embryonic stem cells are easier to harvest, more robust in culture and much easier to maintain and handle in vitro one should not forget that, even though mouse embryonic stem cells are a good in vitro model on the cellular level, human stem cells might react different to the exact same conditions. Therefore one next step would be to amend this system for human embryonic stem cells and fine tune the experimental conditions for optimum progenitor yield. Given the nature and frailty of human embryonic stem cells along with the increased challenge of using genetic tools in the human system this task, while definitely worthwhile, might be much harder.

Additionally, with the rise of induced pluripotent stem cells during the last year and the recent development of patient specific and disease specific induced pluripotent stem cells a lot of new possibilities have become available for further understanding mechanisms of cardiac development under disease conditions. This could lead to results that would be comparable to heart development under normal conditions and could thus be invaluable in the effort to understand the underlying key points of heart development and even heart failure.

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