DEPARTAMENTO DE BIOLOGIA VEGETAL

TOWARDS CARDIAC CELL THERAPY

Pedro Daniel dos Santos Simões

Tese de Doutoramento em Biologia
na especialidade de Biologia Molecular

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FACULDADE DE CIÊNCIAS UNIVERSIDADE DE LISBOA

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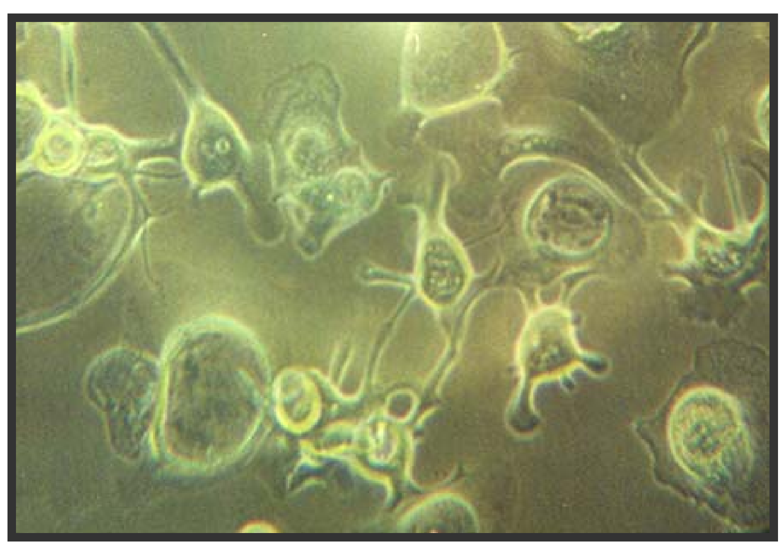
Orientada pela

Professora Doutora Teresa Ramos
e
Professora Doutora Graça Fialho

2007



Primary culture of UCWJ cells

To my son Miguel.

To all who have dedicated their lives to Science …

Resumo

Palavras-chave: Miogénese, apoptose, cardiomiocito, cadeia pesada beta da miosina, progenitores cardíacos

O presente trabalho pretende contribuir para o desenvolvimento de um modelo humano de cardiomiogénese, *in vitro*, o qual, constitui um ponto-chave na implementação clínica e rotineira dos métodos de terapia celular.

Nesta dissertação, a primeira parte do trabalho experimental relata a construção de uma molécula capaz de conferir resistência à geneticina em células humanas que apresentem expressão da cadeia pesada β da miosina (βMyhc). A selecção de cardiomiocitos de entre células estaminais indiferenciadas, utilizando uma estratégia semelhante de biologia molecular, já foi descrita no ratinho. No entanto, tanto quanto sabemos, este tipo de estudos nunca foi aplicado em células humanas.

A segunda parte do trabalho experimental é baseada em estudos de diferenciação com linhas humanas de carcinoma embrionário, com o objectivo de obter claros fenótipos cardíacos, como aqueles já observados no ratinho num modelo similar de carcinoma embrionário.

Este tipo de células é conhecido como constituindo bons modelos celulares onde se podem estudar os mecanismos de diferenciação durante a embriogénese. Neste contexto, foram utilizadas e estudadas a nível morfológico e ultra-estrutural, as linhas celulares humanas de carcinoma embrionário NTERA2cl.D1 (NT2/D1) e PA1. Particularmente, a linha celular NT2/D1, é um conhecido sistema de diferenciação ectodérmica, *in vitro*. Utilizando o Ácido Retinóico e a Proteína Morfogenética do Osso ou a Hexametilenobisacetamida, pode obter-se padrões de diferenciação dorsal (essencialmente neurónios) e padrões de diferenciação ventral, respectivamente. De qualquer modo, muito pouco se sabe acerca destas células humanas poderem também originar derivados da mesoderme como as suas homólogas do ratinho. Assim, neste trabalho, durante o crescimento das células NT2/D1 num meio indutor da angiomiogénese, foram analisadas as características morfológicas e a activação transcricional de genes relevantes na diferenciação cardíaca e endotelial. Os nossos resultados mostram que apesar da tendência natural das células NT2/D1 diferenciarem em linhagens da neuroectoderme, elas também podem activar genes chave de linhagens mesodérmicas.

Abstract

Key Words: Cardiomyocyte, beta myosin heavy chain, cardiac progenitors, pluripotent embryonal carcinomas, angiomyogenesis

The present work aims to contribute to the understanding of a human model of cardiac myogenesis, *in vitro*, as, according to our judgment, this is the vital point in order that future human cardiac cell therapies become frequently used as a clinical practice.

In this dissertation, the first part of the experimental data reports the construction of a molecule capable of confering resistance to geneticin in a human cell line expressing the human beta myosin heavy chain (βMyhc) gene. Selection of cardiomyocytes among undifferentiated stem cells, using a molecular biology approach, has been reported in mice. However, to our knowledge, this strategy has never been applied to human cells.

The second part of the experimental work was based on *in vitro* differentiation studies with pluripotent human embryonal carcinoma (EC) cell lines, with the main goal of obtaining a cardiac phenotype, as previously observed in a mouse pluripotent EC model. Also, it has been reported that these undifferentiated cells are good potential models to study, *in vitro*, the mechanisms that control differentiation during embryogenesis. In this context, the NTERA2cl.D1 (NT2/D1) and the PA1 human embryonal carcinoma cell lines were used and studied at an ultrastructural and morphological level. Particularly, the NT2/D1 cell line is a well known system of ectodermal differentiation. Retinoic acid (RA) induces a dorsal pattern of differentiation (essentially neurons) and bone morphogenetic protein (BMP) or hexamethylenebisacetamide (HMBA) induces a more ventral (epidermal) pattern of differentiation. However, little is known whether these human cells can give rise to mesoderm derivatives as their counterpart in the mouse. In this work, the morphological characteristics and transcriptional activation of genes pertinent in cardiac muscle and endothelium differentiation were analyzed, during the growth of NT2/D1 cells in an inductive angiomyogenic medium. Our results show that, despite the NT2/D1 cells natural tendency to neuroectodermal lineages, they can also activate genes of mesodermal lineages.

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List of abbreviations

ActRII	- Activin type II serine/threonine kinase receptor
ActRI	- Activin type I serine/threonine kinase receptor or Alk2
ActRIB	- Activin type IB serine/threonine kinase receptor or Alk4
Alk2	- Activin receptor-like kinase 2
Alk4	- Activin receptor-like kinase 4
AKT	- The same as Protein Kinase B (PKB)
AMHC	- Atrial myosin heavy chain protein
ANF	- Atrial natriuretic factor
AP	- Anteroposterior
AP1	- Activating protein 1
APH3`	- Aminoglycoside 3' phosphotransferase
APH(4)Ia	- Aminoglycoside 4 phosphotransferase Ia
ASC	- Adult stem cell
ATF	- Activating transcription factor
AV	- Animal-vegetal
AVC	- Atrioventricular canal
AVE	- Anterior visceral endoderm
5-Aza	- 5-Azacytidine
BDNF	- Brain-derived neurotrophic factor
bFGF/FGF2	- Basic fibroblast growth factor
bHLH	- Basic helix-loop-helix (transcription factor)
BM	- Bone marrow
BMC	- Bone marrow cell
2βME	- 2-beta mercaptoethanol
BMP	- Bone morphogenetic protein
BMSC	- Bone marrow stem cell
BNF	- Brain natriuretic factor
BSA	- Bovine serum albumin
βTGF	- Beta transforming growth factor
CaMKII	- Calmodulin kinase II

Car	- Caronte gene
CAT	- Chloramphenicol acetyl transferase
cDNA	- Complementary deoxyribonucleic acid
Cer	- Cerberus gene
CM	- Cardiomyocyte
CMV	- Cytomegalovirus
COUP-TF	- Chicken Ovalbumin Upstream Promoter Transcription Factor
CPC	- Circulating progenitor cells
CPCS	- Cardiac pacemaker and conduction system
CREB	- Cyclic AMP-responsive element-binding protein
CSX	- Cardiac-specific homeobox protein
cTnC	- Cardiac troponin C gene
Cx	- Connexin gene
DBD	- DNA-binding domain
DiI	- Lipophilic fluorescent dye: 1,1-dioctadecyl-3,3,3’3’- tetramethylindocarbocyanine perchlorate
Dkk	- Dickkopf gene
Dll 1	- Delta-like 1 gene
DMSO	- Dimethyl sulfoxide
DNA	- Deoxyribonucleic acid
dpc	- Days post coitum
DSL	- Delta/Serrate/Lag2
DV	- Dorsoventral
E	- Embryonic day
EB	- Embryoid body
EC	- Embryonal carcinoma
ECG	- Electrocardiogram
ECGS	- Endothelial cell growth supplement
ECM	- Extracellular matrix
EG	- Embryonic germ (cells)
EGF	- Epidermal growth factor
EGF-CFC	- Epidermal growth factor - Cripto/FRL1/Cryptic
EmAb	- Embryonic-abembryonic

EPC	- Endothelial progenitor cell
ES	- Embryonic stem
ESC	- Embryonic stem cell
ET1	- Endothelin 1 protein
EtBr	- Ethidium bromide
Ets	- E26 transformation specific
FAST	- Forkhead activin signal transducer protein
FBS	- Foetal bovine serum
FGF	- Fibroblast growth factor
FGFR	- Fibroblast growth factor receptor
FITC	- Fluorescein isothiocyanate
FRL1	- Fibroblast growth factor receptor ligand 1
Fz	- Frizzled gene
GATA	- WGATAA DNA binding transcription factors
GDF	- Growth differentiation factor
GDF1	- Growth differentiation factor 1
HAND	- Heart, autonomic nervous system and neural crest derivatives protein
hASC	- Human adult stem cell
hEC	- Human embryonal carcinoma
hEG	- Human embryonic germ (cell)
hEPC	- Human endothelial progenitor cell
hESC	- Human embryonic stem cell
Hh	- Hedgehog gene
hHSC	- Human hematopoietic stem cell
HFN3β	- Hepatocyte nuclear factor-3β
HGF	- Hepatocyte growth factor
hMSC	- Human mesenchymal stem cell
HSPG	- Heparin sulphate proteoglycan
HSC	- Hematopoietic stem cell
HSV	- Herpes simplex virus
HUVEC	- Human umbilical vein endothelial cell
Hyg^R	- Hygromycin resistance gene
IFT	- Inflow tract

IGF2	- Insulin-like growth factor 2
ITS	- Insulin/transferrin/selenium
IRX	- Iroquois homeobox protein
IVS	- Interventricular septum
JNK	- c-Jun N-terminal kinase
kb	- kilobase = 1000 DNA or RNA base pairs
LBD	- Ligand-binding domain
LEF	- Lymphoid enhancer factor
LIF	- Leukemia inhibitory factor
LPM	- Lateral plate mesoderm
LR	- Left-right
LRP	- Lipoprotein related protein
MADS	- MCM1/Agamous/Deficiens/Serum response factor
MAPK	- Mitogen-activated protein kinase
MCK	- Muscle creatine kinase
mEC	- Mouse embryonal carcinoma
MEF	- Myocyte-enhancer factor
mEG	- Mouse embryonic germ (cell)
mESC	- Mouse embryonic stem cell
MF20	- Antibody against myosin heavy chain
MHC	- Major histocompatibility complex
MIS	- “Müllerian inhibiting substance” protein
MLC2A	- Atrial myosin light chain - 2 protein
MLC2V	- Ventricular myosin light chain - 2 protein
mRNA	- Messenger ribonucleic acid
MSC	- Mesenchymal stem cell
MYH7	- Beta myosin heavy chain; cardiac ventricular myosin; slow fibbers myosin protein
MyHC	- Myosin heavy chain; cardiac ventricular myosin; slow fibbers myosin protein
NEAA	- Non essential aminoacids
Neo^R	- Neomycin resistance gene
NFAT	- Nuclear factor of activated T cells
Oep	- Zebrafish one-eyed pinhead gene
OFT	- Outflow tract

pBK	- pBluescript plasmid from Stratagene
pβMyhc	- Beta myosin heavy chain promoter (human)
PCR	- Polimerase chain reaction
PCP	- Polar cell polarity
PI3K	- Phosphoinositide 3-kinase
PKA	- cAMP-dependent protein kinase or Protein kinase A
PKC	- Calmodulin-dependent protein kinase or Protein kinase C
PLCγ	- Phospholipase C γ isophorm
pSKII(+)	- pBluescript II SacI KpnI (+) plasmid from Stratagene
RA	- Retinoic acid
Raldh2	- Retinaldehyde dehydrogenase type 2
RAR	- *all-trans* retinoic acid receptor
RARE	- RA-response element
RAS	- A low-molecular weight G-protein
RBP-J	- Immunoglobulin J kappa segment recombination signal sequence binding protein
RNA	- Ribonucleic acid
RTK	- Receptor tyrosine kinase
RT-PCR	- Reverse transcriptase – Polimerase chain reaction
RXR	- *9-cis* retinoic acid receptor
SAP	- SAF-A/B/Acinus/PIAS, DNA binding domain
SERCA	- Sarcoendoplasmic reticulum calcium ATPase
SAGE	- Serial analysis of gene expression
sFRP	- secreted Fz-related protein
SHH	- Sonic hedgehog protein
SMAD	- Sma/mothers against decapentaplegic protein
Smyhc3	- Slow myosin heavy chain 3 gene
SRF	- Serum response factor
SURE	- Stop unwanted recombination events
T	- T box transcription factor Brachyuri
TBX	- T-box transcription factor
TCF	- T cell factor
TDGF1	- Teratocarcinoma-derived growth factor 1
TK	- Thymidine kinase

TR	- Thyroid hormone receptor
TRITC	- Tetramethylrhodamine isothiocyanate
UC	- Umbilical cord
UCB	- Umbilical cord blood
UCWJ	- Umbilical cord Whorten`s jelly
UTR	- Untranslated region
VDR	- Vitamin D receptor
VDRE	- Vitamin D response element
VEGF	- Vascular endothelial growth factor
VMHC1	- Ventricle myosin heavy chain 1 protein
WNT	- Wingless/Int protein

Glossary and notes

General glossary

alleles — Different variants of a gene found in the normal population; as individuals carry two copies of each gene, one on each pair of chromosomes, they may have identical (homozygous) or different (heterozygous) alleles.

amniota — Vertebrates which have extraembryonic membranes (amnion, chorion, yolk sac or placenta): reptiles, birds and mammals.

anamniota — Vertebrates which do not have extraembryonic membranes (amnion, chorion, yolk sac or placenta): fishes and amphibian.

antisense RNA — Used in a technique for inactivating a gene using an RNA sequence that complements and hybridizes with the normal RNA made by the gene of interest.

complementary DNA — DNA synthesized by copying mRNA, used for gene cloning and production of large quantities of protein.

DiGeorge's syndrome — A developmental defect caused by malfunction of the cardiac neural crest, resulting in abnormalities in the thymus, parathyroids, cardiac outflow tract, aortic arches and face.

dominant negative protein — Encodes a protein whose structure has been modified, usually by removing some part of it, so that it inhibits the wildtype protein in a dominant fashion. Used experimentally to eliminate the action of proteins such as secreted molecules or receptors.

downregulation — Reduction, not cessation, in the effect being studied, normally, gene expression.

downstream	1. Location of a motif or domain in a gene nearer the 3' end of the sequence than a particular site. Gene sequences are read from the terminal phosphate ($-PO_4^{2-}$), linked to the 5' carbon, also called the 5' end, to the terminal hydroxil (-OH) linked to the 3' carbon, or the 3' end; 2. One may say that the gene "a" that is regulated by the product of the gene "b" it is downstream of that gene; 3. Later reactions in a biochemical cascade.
ectopic expression	Gene expression in cells where that gene is not normally expressed.
expression	Production of mRNA by an activated gene.
growth factor	Polypeptide involved in intercellular signalling, regulating the proliferation and maintenance of cells.
***in situ* hybridization**	Autoradiographic or histochemical method for detecting mRNA in tissue sections.
***lacZ* reporter**	Bacterial *lacZ* gene encoding β-galactosidase, which is detected by a blue histochemical reaction product.
monoclonal antibody	Antibody selected to recognize a specific sequence (epitope) on the target protein.
oligonucleotide	Synthetic DNA or RNA sequence.
polymerase chain reaction	Multifold amplification of selected DNA using small part of sequence as primer.
phalloidin	A toxin from the mushroom *Amanita phalloids* that binds to polymeric and oligomeric actin with high affinity.
phenotype	Physical manifestations of a *wildtype*, mutant or deleted gene.
promoter	Transcription-controlling sequence in a gene.
reporter gene	A sequence that encodes a product that can be easily identified and quantified. Fusion of the reporter gene to a particular gene promoter can be used to detect and measure gene expression.

repressor protein	Product of a regulatory gene that prevents expression of a target gene(s), usually by binding to the promoter sequence of that gene or by interaction with activators or transcription factors.
rescue	Re-establishing a function by adding an exogenous gene or molecule that is either the same or similar to a missing gene or molecule.
reverse transcriptase reaction	Synthesis of cDNA by reverse transcription from mRNA.
signal molecule	Molecule carrying information between cells. Usually a growth factor that activates an intracellular signalling pathway, also known as a ligand.
signalling pathway	Intracellular cascade of biochemical reactions that stimulates an appropriate cellular response as gene transcription; usually activated by a signal molecule binding to its receptor at the cell surface.
transcript	mRNA produced by transcription of a particular gene.
transcription factor	Protein that regulates gene transcription often by binding to a specific DNA sequence. Normally classified by the type of binding domain they contain: homeobox, T box, helix-loop-helix, zinc finger, etc.
upregulation	Opposite of downregulation.
upstream	Opposite direction to downstream.
wildtype	Individual with no known mutation in the gene under study.

Embryological glossary

animal pole — Portion of the early embryo marked by the second oocyte polar body, where the main embryonic structures will develop.

animal–vegetal axis — Line connecting the animal and vegetal poles of the zygote. Whether this corresponds to any particular axis in the adult is still a matter of debate.

anterior visceral endoderm — In mouse: visceral endodermal tissue lining the outer surface of the epiblast at the egg-cylinder stage and is displaced anteriorly at the early primitive-streak stage; probably involved in inducing anterior structures but itself forms extraembryonic structures;
In other mammals and birds, termed the primitive endoderm and the hypoblast, respectively.

blastoderm — The two layers, epiblast and hypoblast at the animal pole of the blastula.

blastula — First stage of embryogenesis when the egg divides several times to form a hollow ball of cells and a two-layered embryo.

cardioblasts — Cells committed to become cardiomyocytes.

cell fate — The full spectrum of tissues that embryonic cells may differentiate into, under normal, unperturbed conditions;
Restriction of a multipotential embryonic cell to producing cells of a single tissue type.

conceptus — The entire product of conception, including the embryo and extraembryonic membranes.

determinants — mRNAs or protein molecules that initiate gene transcription in the early embryo; also known as maternal determinants and dorsal or ventral determinants.

ectoderm — The outermost germ layer; gives rise to epidermis and neural tube, neural crest, and eye and ear placodes.

endoderm — The innermost germ layer; contributes to gut, liver, respiratory system, urogenital system and endocrine glands.

epiblast — During the blastula stage, the blastoderm layer that corresponds spatially to the ectoderm in the gastrulating embryo and gives rise to different germ layers during gastrulation.

extraembryonic mesoderm — Portions of the mesoderm that do not contribute to embryonic structures; in amniotes gives rise to vasculature and supporting membranes of yolk sac or placenta.

fate mapping — Identification of the precursor cells in the early embryo that will give rise to particular tissues or organs.

floor plate — A wedge-shaped group of cells in the ventral neural tube that secretes molecules responsible for the DV organization of particular structures as the spinal cord.

gastrulation — Highly coordinated process involving stereotypic cell movements; results in the establishment of the germ layers; embryo at this stage is termed a gastrula.

germ layers — Three layers of the embryo, ectoderm, endoderm and mesoderm established during gastrulation that give rise to the tissues and organ systems of the late embryo.

Hensen's node — In amniotes: thickening at anterior tip of the primitive streak; organizer inducing axial structures; in the mouse embryo, often referred to as “the node”; homologous to Spemann’s organizer in Amphibia; named after Viktor Hensen, who first described it in 1876.

hypoblast — In chick and rabbit: inner layer of blastoderm; in zebrafish, the equivalent is the yolk syncytial layer, and in mouse, the visceral endoderm; gives rise to extraembryonic structures.

induction — Influence of one group of embryonic cells on the fate of neighbouring cells, usually through the production of diffusible signalling molecules or through direct cell–cell contact.

lateral-plate mesoderm — Sheet of mesoderm further away from the axis, lateral to the paraxial and intermediate mesoderm; contributes to body tissues and extraembryonic structures.

mesendoderm — Embryonic tissue containing a combination of mesoderm and endoderm cells or of cells that contribute with descendants to both germ layers. Also termed endomesoderm.

mesoderm Middle germ layer, gives rise to skeletal muscles, notochord, skeleton, connective tissue, heart and contributes to the gut and urogenital systems formation.

notochord Early mesodermal structure with important role in patterning early embryo; precursor of part of the vertebral column.

neural tube Precursor of the central nervous system. It is formed by the rolling up of the dorsal neuroectoderm, during a stage termed neurulation.

neurula Third stage of embryogenesis when the embryo elongates and the neural tube forms.

neural plate Specialized area of ectoderm that gives rise to the nervous system and neural crest.

notochord A rod of cells forming in the midline dorsal mesoderm. Contributes to the induction of the nervous system; persists in some lower chordates but in all vertebrates is replaced by the vertebral column.

oocyte Unfertilized female gamete (haploid).

organizer A group of cells in the embryo that secretes signal molecules affecting the differentiation of other tissues. Identified by its ability to induce ectopic structures when grafted into a host embryo; see Spemann's organizer, Hensen's node.

paraxial mesoderm Mesoderm on either side of neural tube. Forms presomitic mesoderm, which contributes to the head, and the somites.

precardiogenic mesoderm Also known as cardiogenic plate or cardiac crescent.

primitive streak A groove at the posterior end of the embryo that marks the beginning of gastrulation and the formation of the AP axis. At the posterior end, epiblast cells undergo epithelial to mesenchymal transition to give rise to the mesoderm and definitive endoderm.

situs solitus Normal pattern of LR asymmetry often referred to as "situs". When completed, and total or partial reversal of the internal organs occurs, it is called "situs inversus totalis" or "heterotaxia", respectively.

somites Blocks of paraxial mesoderm that form sequentially from anterior to posterior direction after gastrulation. They are precursors of the axial skeleton, all skeletal muscles and the dermis.

trophoblast A multipotent germ layer that can differentiate into extraembryonic tissues.

vegetal pole Opposite side of early embryo to the animal pole, in some species containing yolky material; gives rise mainly to extraembryonic structures.

zygote Single diploid cell formed by fusion of oocyte and sperm.

Embryological staging references

The following references define the stages of embryological development of the organisms mentioned in this dissertation.

***Drosophila melanogaster (fruit fly)*:**
Taxonomy:
Kingdom: Animalia;
Phylum: Arthropoda;
Class: Insecta;
Order: Diptera;
Family: Drosophilidae

Campos-Ortega, JA and Hartenstein, V:
The Embryonic Development of Drosophila melanogaster.
Springer-Verlag, 1985, Berlin, Heidelberg, New York.

***Danio rerio (zebrafish)*:**
Taxonomy:
Kingdom: Animalia;
Phylum: Chordata;
Subphylum: Vertebrata
Class: Actinopterigi;
Order: Cypriniformes;
Family: Cyprinidae

Kimmel, CB; Ballard, WW; Kimmel, SR; Ullmann, B and Schilling, TF:
Stages of embryonic development of the zebrafish.
Dev Dyn, 1995, 203, 253-310.

Driever, W:
Introduction to the zebrafish. In: Cell Lineage and Fate Determination.
Ed. S. A. Moody, San Diego: Academic Press, 1999, 371-382.

***Xenopus laevis (frog)*:**
Taxonomy:
Kingdom: Animalia;
Phylum: Chordata;
Subphylum: Vertebrata
Class: Amphibia;
Order: Anura;
Family: Pipidae

Nieuwkoop, PD and Faber, J:
Normal Table of Xenopus laevis.
North-Holland Publications, 1967, Amsterdam.

***Gallus gallus (chick)*:**
Taxonomy:
Kingdom: Animalia;
Phylum: Chordata;
Subphylum: Vertebrata
Class: Aves;
Order: Galliformes;
Family: Phasianidae

H. Eyal-Giladi, H and S. Kochav, S:
From cleavage to primitive streak formation: a complementary normal table and a new look at the first stages of the development of the chick. I. General morphology.
Dev Biol, 1976, 49, 321-337.

Hamburger, V and Hamilton, HL:
A series of normal stages in the development of the chick embryo.
J Morph, 1951, 88, 49-67.

***Mus musculus (mouse)*:**

Taxonomy:
Kingdom: Animalia;
Phylum: Chordata;
Subphylum: Vertebrata
Class: Mammalia;
Order: Rodentia;
Family: Muridae

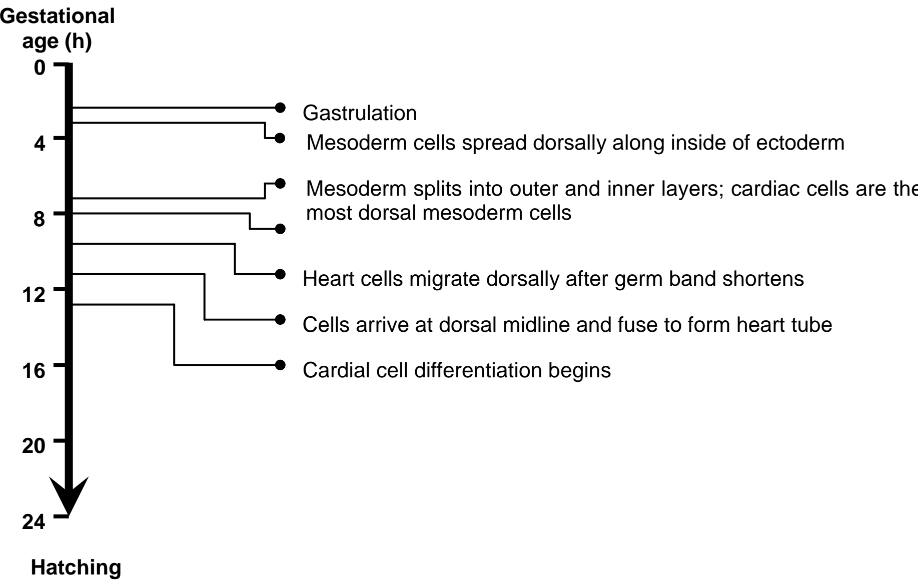
Downs, KM and Davies, T:
Staging of gastrulating mouse embryos by morphological landmarks in the dissecting microscope.
Development, 1993, 118, 1255-1266.

***Homo sapiens (human)*:**
Taxonomy:
Kingdom: Animalia;
Phylum: Chordata;
Subphylum: Vertebrata
Class: Mammalia;
Order: Primates;
Family: Hominidae

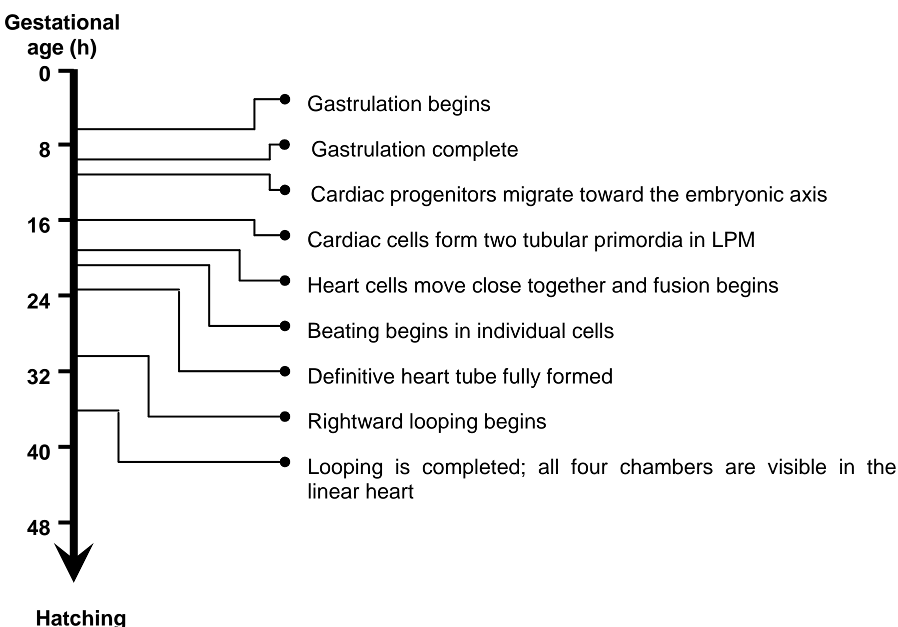
O'Rahilly, R:
Early human development and the chief source of information on staged human embryos.
Eur J Obstet Gynecol Reprod Biol, 1979, 9, 273-280.

Timelines of heart development

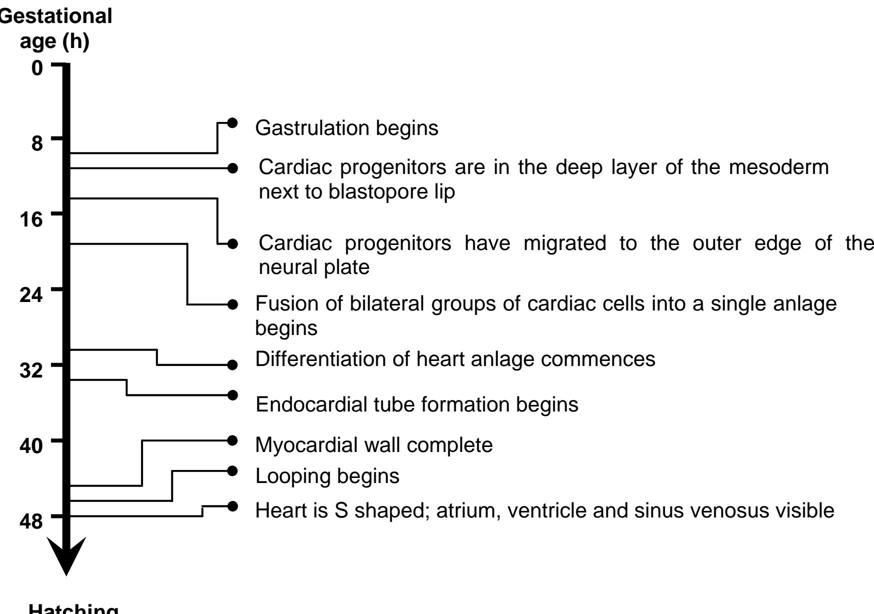
The following diagrams define the main timeline of cardiac development of the organisms mentioned in this dissertation.



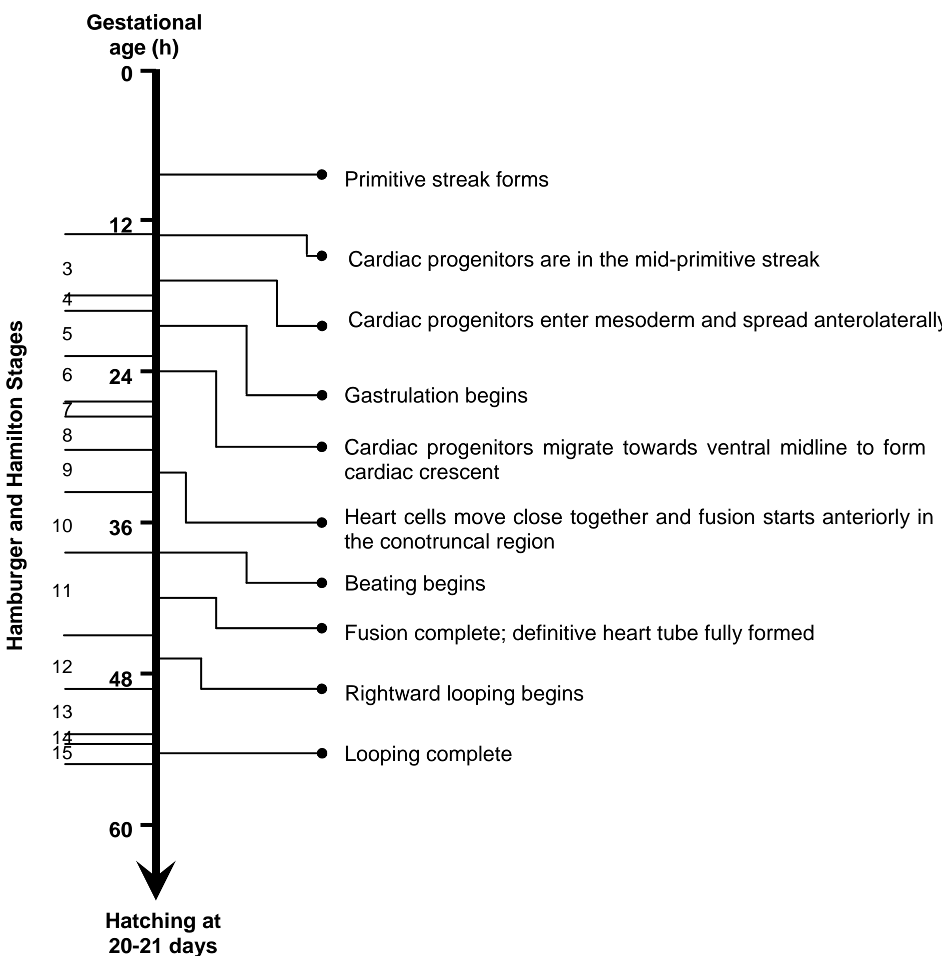
Adapted from "Genetic control of heart development" Ed. J. S. Altman. HFSP, page 16, Strasbourg, 1997.



Adapted from “Genetic control of heart development” Ed. J. S. Altman. HFSP, page 15, Strasbourg, 1997.



Adapted from "Genetic control of heart development" Ed. J. S. Altman. HFSP, page 14, Strasbourg, 1997.



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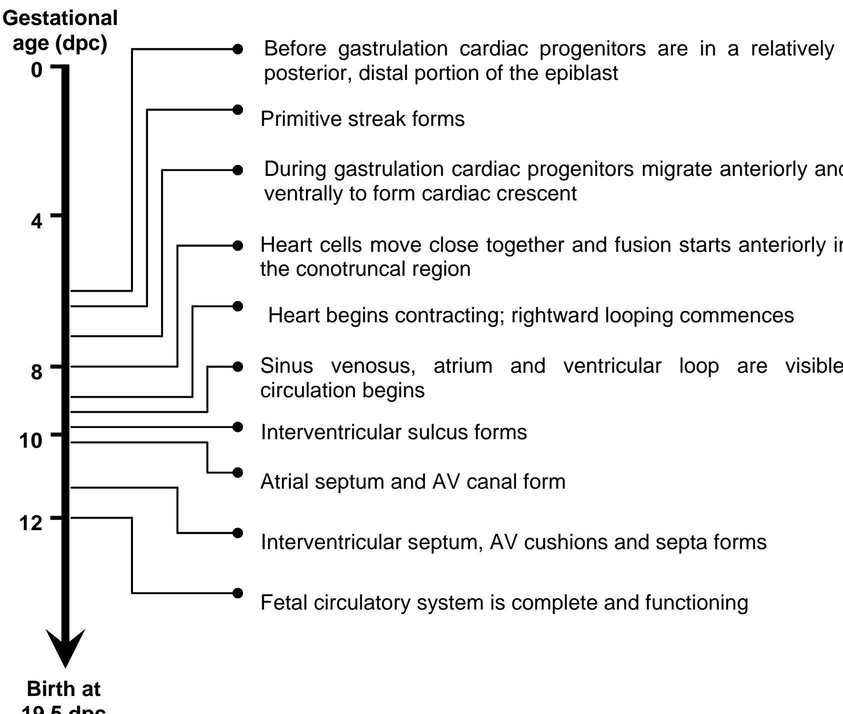
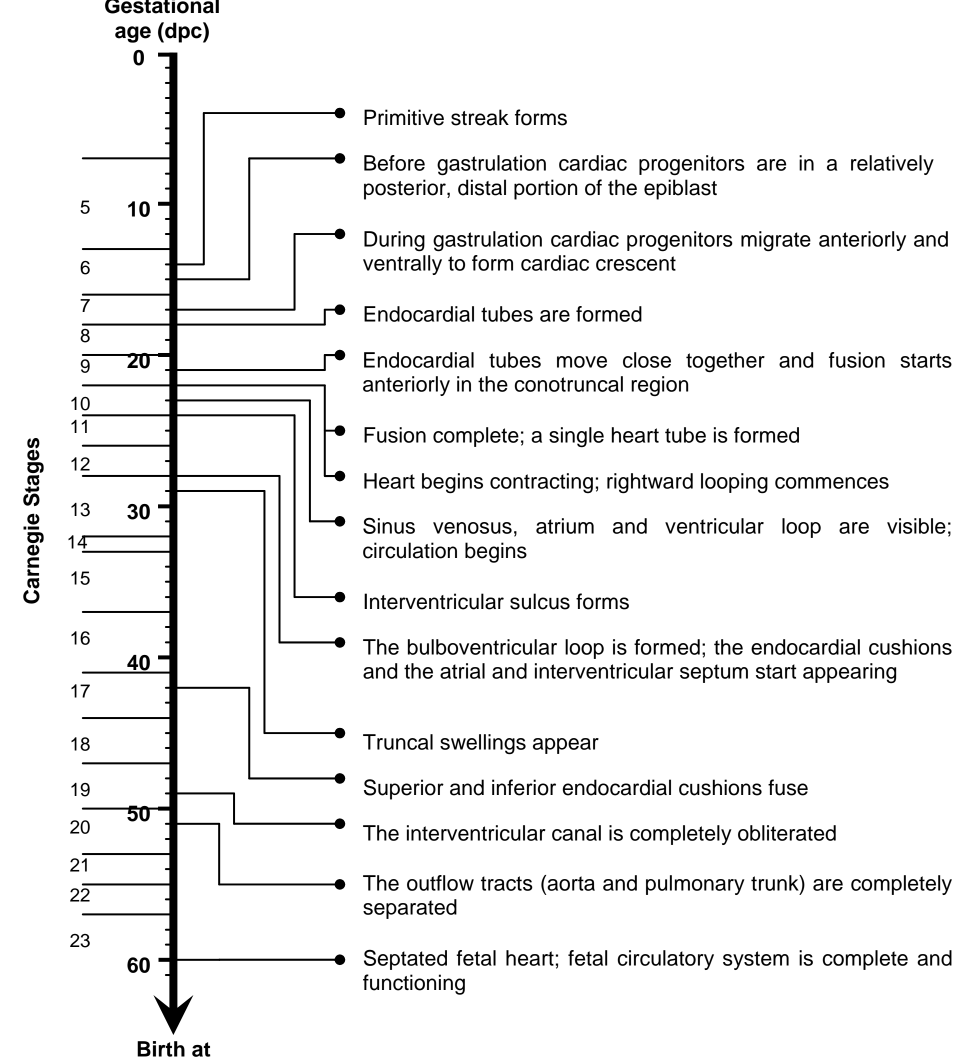
Mouse



Adapted from "Genetic control of heart development" Ed. J. S. Altman. HFSP, page 12, Strasbourg, 1997.

Human

Age (dpc)	Pair of somites
20	1-4
21	4-7
22	7-10
23	10-13
24	13-17
25	17-20
26	20-23
27	23-26
28	26-29
29	29-34
30	34-35



Compiled from:
1. Netter, FH: The Heart - The Ciba Collection of Medical Illustrations vol 5, 5Ed. *The Case-Hoyt Corp*, New York, 1981, 114-126;
2. Sadler, TW: Langman´s Medical Embryology, 8Ed. *Lippincott Williams and Wilkins*, USA, 2000, 214-15;
3. Christa Wellman and John McNulty: Development of the human heart, 1996, *in* http://www.meddean.luc.edu/lumen/MedED/GrossAnatomy/thorax0/heartdev/main_fra.html;
4. Mark Hill: UNSW Embryology: Carnegie Stages, 2007, *in* http://embryology.med.unsw.edu.au/wwwhuman/Stages/Stages.htm;
5. Hunter A, Kaufman MH, McKay A, Baldock R, Simmen MW, Bard JB: An ontology of human developmental anatomy. *J Anat*, 2003, 203(4): 347-55.

Gene nomenclature

The following nomenclature is used for genes described in the text.

Gene names are shown with a first uppercase letter followed by lowercase letters throughout the text; whereas, the “PROTEIN” coded by the gene uses the same name/abbreviation but only in uppercase letters. Ex: Fgf8 gene and FGF8 protein.

Preface

In 2001 an interesting paper was published in *Nature* magazine with the title “Bone marrow cells regenerate infarcted myocardium”. The news of that research caused me such a curiosity that I started to study the state of the art of that area of knowledge.

Among many interesting things, it could be stressed the “New dogma” of heart remodelling, the importance of Insulin growth factors (IGFs), and the recent findings in cardiac progenitors research.

My PhD project had the title: “Mechanisms of transmural scar formation in the surgical treatment of atrial fibrillation with radiofrequency catheter ablation and microwave. Role of heat induced apoptosis.” Although the apoptosis idea is now a well established fact in heart pathology, it did not seem to raise much interest among the portuguese cardiac surgeons, who were more interested in the characterization of physical variables, like the diffusible temperature gradient of the heat induced by radiofrequency, than by genetic ones.

Accordingly, and in a natural way, my work was slowly becoming more engaged with the general field of cardiac progenitors’ research, than with the surgical technique-related apoptotic research. In view of the above, the title “Towards cardiac cell therapy” has been proposed.

Curiously, the two apparently distant research fields were interconnected with a common need, the development of a human cardiomyocyte cell line from undifferentiated adult pluripotent cells. This would allow both the analysis and experiences, at the cellular level, of the conditions and mechanisms underlying cardiomyocyte apoptosis induced by heat and the understanding and the characterization of cell surface antigens involved on cardiac differentiation, *in vitro*.

Developing a cell line from an undifferentiated progenitor cell, can be accomplished in different ways, depending on the information available regarding the developmental steps (growth factors and surface antigens) that lead to the differentiation only into a particular cell type. Because such information has just started to become available, and because differentiating embryonic stem cells never give rise to pure cardiomyocyte cultures, the solution is to transform undifferentiated cells with a bioengineered construct containing one

of the gene promoters known to be specifically activated in the cardiac cell lineage, as the promoter of the human cardiac myosin heavy chain gene linked to an antibiotic resistance gene. This strategy has already been done in mice since 1996 and allowed the selection, in an antibiotic conditioned culture, of mouse cardiac lineage-committed cells, among other type of cells, with a purity of more than 99 %. Accordingly, a similar human construct was created and used in an undifferentiated human myoblastic cell line.

Of course that one of the logical potentialities of such a study would be to extend this cellular approach to clinics, in areas of pathologic heart conditioning, in order to regenerate affected myocardium resorting to a cell-based therapy.

In view of the difficulties raised by the usage of human embryonic stem cells for cardiac differentiation studies, pluripotent human embryonal carcinoma cell lines PA1 and NTERA.clD1 were used.

Ultrastructural and microscopic analyses were performed in defined time points along PA1 and NTERA.clD1 differentiation in pertinent inductive media.

We also analyzed the histochemistry, the cytoskeletal actin organization and the gene expression of genes relevant to cardiac muscle and endothelium differentiation, during the growth of NT2/D1 cells in a reported inductive angiomyogenic medium.

Unfortunately, cardiac differentiation from this cell line was never accomplished, and for that reason, the experiments based on the assumption that the created construct may transform and select human pluripotent stem cells that are cardiac committed, are still on hold.

Introduction

Introduction

General insight

The human cardiac commitment, differentiation and morphogenesis are not completely understood.

In this scenario, the ambitious and already underway “clinical cardiac cell therapy” becomes a procedure without a solid scientific fundament.

A major difficulty in understanding cardiogenesis is related to the use of different vertebrate models to give a descriptive approach of the phenomenon, which contributes, in recent papers, to a confusing variety of terms, including chick, mouse and zebra fish models that do not overlap completely [1]. Following this line of thought, reliable studies based on human embryonic material are limited to few famous collections such as the Carnegie Collection and local collections from some medical universities.

Nevertheless, in the past decade research has progressed from the physiological and functional to the molecular approach, leeding to a deeper understanding of the cardiac functions at genetic and proteomic levels in animal models [2].

In the next pages, the basic aspects of heart development will be shortly described and results of molecular data incorporated whenever relevant.

In order to achieve a more complete integration of the text with the experimental work, a significant effort was made in enlightening the main molecular aspects of embryonic stem cells commitment into cardiac cell lineages. This, we believe, will help to integrate the current state of the art and the development of the goals presented in this thesis. This will also introduce the complex world of cardiac differentiation, a knowledge that is an essential tool when dealing with challenges such as “human cardiac cellular therapy”.

Curiously, the number of possible molecular signalling mechanisms (types or classes) responsible for the formation of an adult heart, are the same, as those responsible for the formation of the whole adult organism, from just one single cell. Furthermore, they are relatively few, when compared to the complex network of embryological processes and to the high number of possible signalling events. In addition, we strongly believe that the sharp molecular cell signaling regulation during development is where researchers should look for, if they want to understand the commitment or the differentiation mechanism of a particular cell lineage, as for instance, the understanding of the appearance of the cardiac cell lineage.

Molecular developmental mechanisms may roughly be classified in four distinct classes: i) cell/cell and cell/ECM related pathways, including the integrins, cadherins, DSL proteins, collagens, fibronectins, laminins, fibrins, elastins and other molecular families; ii) secreted protein factors initiating pathways including Bone morphogenetic proteins (BMPs), Fibroblast growth factors (FGFs), Vascular endothelial growth factors (VEGFs), Wingless/Int (WNT) proteins, Hedgehog (Hh) proteins, Epidermal growth factors (EGFs), *beta* Transforming growth factors (βTGFs) and others; iii) soluble molecules initiating steroid or steroid-like pathways with steroid hormones and Retinoic acid (RA) as the most important members and iv) ion signalling pathways with those Ca^{2+}-mediated as the most important ones.

All of these signalling possibilities have, associated downstream of the respective pathway, specific transcription factor activities, including: i) basic DNA domain, ii) zinc-coordinating DNA binding domain, iii) helix-turn-helix and iv) β-scaffold with minor groove contact transcription factor activities. They will activate or suppress the transcription of specific target genes, which are, ultimately responsible for cell cycle regulation and cell behaviour itself, resulting in proliferative or quiescent cells and migratory or apoptotic cells, respectively.

Since it would not be reasonable to describe all the constitutive members of these four-group's pathways in the present dissertation, only those known to be pertinent to cardiogenesis will be mentioned or illustrated in subsequent sections.

Cardiac embryology

In vertebrates, cardiomyogenesis is a complex and highly regulated process, being each event spatially and temporally ordered. The embryonic stem cells involved in migration and differentiation events are of different types and of several origins and perform precise interactions with each other. It is easy to understand that any irregularity during this development phase will result in congenital heart defects that will be diagnosed during pregnancy, at birth or later in life, according to their respective severity. Importantly, malformations of the heart and great vessels account for the most frequent congenital defects in humans, with an incidence of approximately 1 % [3].

The heart, the first definitive organ to be formed in the embryo and the respective cardiovascular system, starts appearing during gastrulation, in the middle of the third week, when simple diffusion is no longer sufficient to fulfil nutritional requirements.

The actual process of gastrulation might be relatively unimportant considering the cellular commitment to a cardiac fate, owing to the fact that early mesodermal cells can adopt several different phenotypes and contribute to different tissues when grafted into new environments. However, the patterning processes and dynamic cell movements of gastrulation allocate heart mesodermal precursors to specific cell niches in a progressive and spatially complex way, creating the site specific milieu for cardiac cell differentiation and morphogenesis. Accordingly, it is our belief, that at this particular moment, the creation of the "appropriate environment" is more important than the "cardiac cell commitment" itself. In other words the "soil" is more important than the "seed".

Before describing the development of the cardiovascular system, it is useful to mention the appearance of the intraembryonic coelom. It is formed by the confluence of small and initially isolated spaces that appear in the lateral plate mesoderm, resulting in a single horseshoe-shaped cavity, just cranially to the prechordal plate. This event separates the mesoderm into two layers, the parietal or somatopleuric mesoderm in contact with the ectoderm, and the visceral or splanchnopleuric mesoderm in contact with the endoderm.

Cardiac progenitors cells lie on both sides of the midline in the epiblast, immediately lateral to the primitive streak [4, 5]. Then, they migrate throughout the primitive streak, clustering themselves bilaterally in the splanchnopleuric layers of the lateral plate mesoderm [6], in a region known as cardiogenic plates, precardiac mesoderm or primary heart fields.

Recently, it was shown that this group of mesodermal cells express a basic helix-loop-helix transcription factor, the Mesp gene, considered to be the earliest sign of cardiovascular development known to date [7, 8]. This region is located cranial and lateral to the neural plate and will later express other cardiac-related genes like Nkx2.5 (Csx1 in humans) [9, 10], Bmp2 [10, 11], Gata4 [12, 13] and Hand [14]. It is suggested that the helix-turn-helix transcription factor Ets, a RTK activity and a FGF pathway might be the keys for the Mesp1 dependent regulation of Gata4, Nkx2.5 and Hand genes, but that has not yet been proven in mammals [15] (See also *Molecular families in cardiogenesis - The FGF family).*

Although Nkx2.5 is one of the first genes to be detected in the precardiac mesoderm, it is not expressed in the caudal regions of the heart forming zone. By contrast, Bmp2, Gata4 and Hand are detected in the entire primary heart field. Nevertheless, all of them are not cardiac restricted since they are also expressed in regions outside the heart forming zone.

Despite the fact that these genes are many times referred as being cardiac-specific, the specific and restricted early cardiac progenitor marker is still waiting to be discovered.

A recent study in the chick, using two different techniques, fluorescent carbocyanin labelling and gene expression staining, showed that there is no accurate correspondence between the two labellings in the primary heart fields area, highlighting that the final delineation of the exact precardiac regions is not yet completed [10].

Importantly, the first populations of cells that constitute the primary heart fields will not contribute to all the cells of the adult heart or, in other words, it will be the recruitment of other cardiac progenitors, later in development, that will become part of the final outflow and inflow tract-derived structures.

Formation of the endocardial tubes

At the cardiogenic plate in xenopus and chick, BMP2 [16-18], repressors of Wnt signaling [19, 20] and possibly, later FGF signals [21], coming from the endoderm induce cardiac progenitors to form cardiac myoblasts, a complex network that may occur also in mice [22].

The endoderm that is in direct contact with cardiac mesoderm has been considered by some authors as the heart-inducing tissue, because members of the BMP family are expressed in endoderm, as well as in adjacent ectoderm and extraembryonic tissues [23-25]. Accordingly, many studies in different organisms have shown a key role for the BMP signalling, in specifying and maintaining the myocardial lineage [26-29], as well as the BMP-receptor regulated transcription factors of the Smad family which directly activate genes that encode cardiac transcription factors [30-33] (See also *Molecular families in cardiogenesis - The TGFβ superfamily).*

Thinking of BMP2 as a possible candidate to be a cardiac inducer molecule *in vitro* is a particular important point. Indeed, this possibility was the basis for one of the main technical goals of the present work. It will be discussed at lenght in the Discussion section.

During gastrulation, hemangioblasts also appear, in the precardiac splanchnopleuric mesoderm region, ventral to the cranial, horseshoe-shaped portion of the intraembryonic coelom, proliferating and clustering in hemangiogenic islands, known as angiocysts. From this cranial part of the coelom will develop the pericardial cavity. Along in time, those cell clusters will fuse and will form the left and right endocardial tubes, each an endothelial lined tube surrounded by cardiomyoblasts.

Progenitor cells that will be committed to the endocardial and myocardial cell lineages, migrate from within the precardiac mesoderm to become localized just above the endoderm

cell layer and in the developing myocardium, respectively. Curiously, in the chick, these migrating cells may express both myocardial and endocardial markers [34]. These results indicate that cells of the cardiac crescent might be the common precursors for myocardial, endocardial and possibly pericardial cells of the heart. In fact, a study in mice has revealed that all three of the above cell types have activated, in the cardiac crescent, the cardiac transcription factor Nkx2.5 [35]. However, the expression of Nkx2.5 may be more a common characteristic of different populations of cells that coexist in a common region than a marker of a common precursor, which is the same as saying that myocardial and endocardial precursors that reside within the heart-forming region express Nkx2.5. In fact, the endocardial cell layer is constituted by a mosaic of cells with and without Nkx2.5 expression [35]. A possible explanation for this is that endocardial precursors that migrate to the cardiac crescent from Nkx2.5 negative regions will not express Nkx2.5.

Recent experiments in chick, with retrovirus-mediated lineage tagging, have suggested that if there is a common precursor of both myocardial and endocardial cells, then it might be commited to a endocardial and myocardial fate at or before initial primitive streak formation [36]. Conversely, data supporting the existence of a “later” common progenitor comes from studies with the cardiac mesoderm cell line QCE6 [37]. This cell line was derived from precardiac mesoderm of the Japanese quail and exhibits a phenotype consistent with its being a cardiac stem cell. If treated with a combination of RA, bFGF, TGFß2, and TGFß3, the QCE6 cells will differentiate into two distinct phenotypes, a myocardial and an endocardial endothelial within the same culture.

Consistent with these results, two potential models were formulated [36]. One assuming that mesodermal cells reaching at the heart field are equipotent but their fate into either myocyte or endocardial lineage is possibly defined by local, inductive signals from underlying endoderm. Another model presumes that the heart field consists of two subpopulations already restricted to myocardial or endocardial lineage before migration to the heart field. If these two lineages were already separated when their progenitors migrate to the heart field, the role of endoderm-derived factors would be to continue their terminal differentiation, rather than to act as inductive signals.

This still open question regarding the existence of a common endocardial/myocardial progenitor cell had a strong influence on the design of the differentiation studies presented in this thesis. Accordingly, a pluripotent human embryonal carcinoma cell line was used with a previously described angiomyogenic medium [38] in order to obtain cardiac muscle and/or endothelial cell phenotypes.

In the late presomite embryo, other hemangiogenic islands have also developed in the intraembryonic and extraembryonic mesoderm outside the precardiac region. Soon, they will initiate the vasculogenesis of the left and right dorsal aortas that later will connect the cranial pole of the endocardial tubes via the aortic arches, forming the arterial pole of the heart.

Extraembryonically, vasculogenesis has started in the yolk sac mesoderm, forming, sequentially in time, a plexus of vessels in its wall, the vitteline veins, and later the umbilical veins. Together they will connect the most caudal ends of the endocardial heart tubes, establishing the venous pole of the heart. So, each endocardial tube is continuous cranially with a dorsal aorta, the outflow tract, and caudally with a vitteloumbilical vein, the inflow tract (Fig. 1).

Formation of the heart tube

The cranial and lateral folding of the embryo forces the endocardial tubes into the thoracic cavity. As a result of this change, these tubes come to lie closer and parallel to each other, entering more and more into the pericardial cavity.

By this time, CARP, the first cardiac-specific transcriptional regulator to be known, located downstream of the cardiac homeobox gene Nkx 2.5, starts to be expressed. The Carp gene encodes a nuclear coregulator that contains four ankyrin repeats within its carboxyl terminal end and regulates, negatively, the expression of cardiac genes, including Mlc2v and Anf [39]. The subsequent uniform expression of Carp in the heart is due to the presence of distinct cis 5’ flanking sequences of the Carp gene, which are capable of confering specificity regarding the atrial, left ventricular, right ventricular, and conotruncal segments of the heart. Carp may be transcriptional regulated in a cooperative way by Nkx2.5 and GATA4. In fact, the proximal upstream regulatory region of the gene contains an essential GATA4 binding site, which suggests that Nkx2.5 controls CARP expression, at least in part, through GATA4 [40].

At this stage, cardiac progenitors are migrating ventrally, on a graded distribution of fibronectin along the craniocaudal axis, deposited in the endocardial-endodermal interface [41].

The plexiform region of the bilateral cardiac primordia, still separating the right and left endocardial tubes from each other, gradually disappears, resulting in fusion in a craniocaudal direction. At this stage, the single straight heart tube comes to lie, completely, within the pericardial cavity, attached dorsally by a single fold of tissue, the dorsal mesoderm. During this process, correct differentiation of embryonic endoderm is crucial for proper migration

and morphogenesis. Affecting the endoderm-acting genes, as for instance, Gata4, partially disrupt this process, leading to a condition known as cardia bifida [42, 43].

Meanwhile, the mesodermal tissue surrounding the heart tube shows a particular organization in three different layers (Fig. 4). The inner layer, immediately around endothelium, is initially a thick acellular matrix that because of its histological staining (stains lightly) has been called cardiac jelly. The next layer is densely nucleated at first, only a few cells thick. The third and outer layer consists of cuboid epithelial cells which also line the remainder of the pericardial cavity. The second and third layers together are generally referred to as the myocardium, separated from the endocardial tube by the cardiac jelly[44]. The jelly accumulates in the AV junction and in the OFT and progressively disappears from the ventricular and atrial chambers [45].

The human embryo has now 7 somites, is about 2.2 mm long and is approximately 23 days old. It is at about this time, or somewhat earlier that the heart begins to beat.

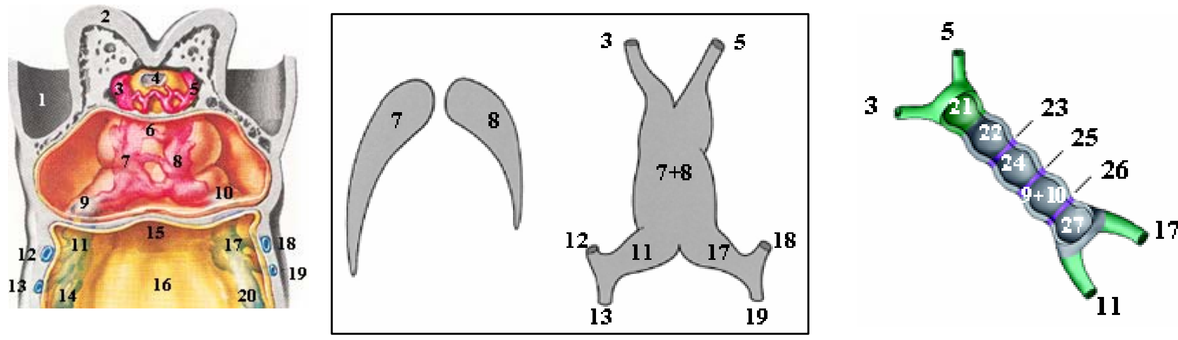


Figure 1 - At left, ventral dissection at the cardiogenic region of a human embryo with approximately 22 days of development (left). Adapted from Netter, FH: The Heart - The Ciba Collection of Medical Illustrations vol 5, 5Ed. *The Case-Hoyt Corp*, New York, page 116, 1981. Schematic representation of the process of heart tube formation originated by the fusion of the endocardial tubes (middle). Adapted from Gittenberger-de Groot, AC *et al,* Pediatric Research, 57(2), page 170, 2005. Schematic representation of segments and rings present in the embryonic linear heart tube (right). Adapted from Moorman, AF and Christoffels, VM, Physiological Reviews, 83, page 1225, 2003.

Legend: 1-Amnion; 2-Forebrain; 3-Right aortic arch; 4-Buccopharyngeal membrane; 5-Left aortic arch; 6-Communication between right and left endocardial tubes; 7-Right endocardial tube; 8-Left endocardial tube; 9-Primitive right atrium; 10-Primitive left atrium; 11-Right sinus horns; 12 and 13-Right vitelline veins; 14-Right umbilical veins 15-Foregut; 18-Yolk sac; 17-Leftt sinus horns; 18 and 19-Left vitelline veins; 20-Left umbilical veins; 21-Aortic sac; 22-Primitive right ventricle; 23-Primary ring; 24-Primitive left ventricle; 25-Atrioventricular ring; 26-Sinoatrial ring; 27-Sinus venosus.

The newly formed heart tube expresses genes that already show a cranial (ventricular) and caudal (atrial) specification, but only during the next stage of cardiac looping, phenotypic differences in myocardium may be observed [1].

It can be divided, from an cranial to caudal direction, into several regions, the left and right dorsal aortas, the aortic sac, the bulbus cordis (truncus arteriosus, conus cordis, the ventriculoarterial ring and the primitive right ventricle), the interventricular primary ring, the primitive left ventricle, the atrioventricular ring, the primitive atria, the sinoatrial ring and the left and right sinus venosus (Fig. 1, right).

The steps that occur after the initial segmentation of the embryonic heart represented in Fig. 1 (right) are interpreted according to two different models, namely the “segmental model” and the “balooning model”. The former defends the concept that the adult atria and ventricles derives, rigorously, from expansion and differentiation of the respective heart tube segment, each of them functioning as somites, morphogenetic units separated by boundary interfaces. The latter, considers that the heart’ s definitive chambers arise from local expansion and differentiation of certain heart tube regions, rather than from an exact tight segmental development [46]. In my opinion, the differences between these two models are more a matter of interpretation of the “segmental model” in a non-classic way. Thus, if each segment of the linear heart tube is not considered to have restricted frontiers and that a single segment may become part of more than one cardiac cavity as well as that the development of a certain chamber may occur by dorsal or ventral expansion of that segment , “the ballooning model”, becomes a flexible interpretation of the “segmental model”. Both models are consistent with studies using whole embryo techniques, which indicate that all of the components and myocardium of the developing heart appear from primary cardiogenic fields [47]. However, later experiments have suggested that the atrioventricular canal, atria and conotruncus are added secondarily to the straight heart tube during looping, concomitantly with addition of new myocardium [48, 49]. The atria are added progressively from the caudal primary heart fields bilaterally, while the myocardium of the conotruncus is elongated from a “secondary heart field” or “anterior heart field” [50-53], located beneath the floor of the foregut, in the prepharyngeal mesoderm.

Cardiac looping

Because the two ends of the heart tube are “fixed”, it is forced to bend in order to adapt itself to the available pericardial space. Meanwhile, perforations appear in the dorsal mesoderm, leading to its disappearance as the openings increase in size, allowing the heart tube to lie free within the pericardial cavity. The cranial portion of the tube bends ventrally and to the right, and the atrial portion shifts dorsally and to the left, regulated by genes that are essential for left-right programming [54]. This is named cardiac looping and is complete by the day 28 (Fig. 2 and 3).

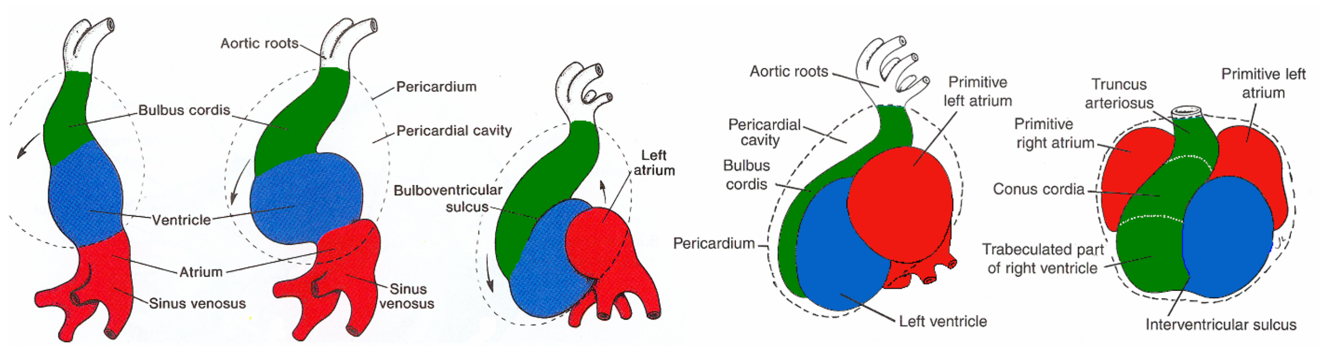


Figure 2 - Schematic representation of the cardiac looping event. Adapted from Sadler, TW: Langman´s Medical Embryology, 8Ed. *Lippincott Williams and Wilkins*, USA, pages 214-15, 2000.

While the cardiac loop is forming, changes in the heart tube are concerned mainly with the development and expansion of the future heart chambers (Fig. 3). The atrial component, initially a paired structure outside the pericardial cavity, forms a common atrium and is incorporated into the pericardial cavity. The atrioventricular junction remains narrow and forms the atrioventricular canal, which connects the common atrium and the early embryonic left ventricle. The bulbus cordis is narrow except for its proximal third that will form the trabeculated part of the right ventricle. The conus cordis will form the outflow tracts of both ventricles. The distal part of the bulbus, the truncus arteriosus, will form the roots and the proximal portion of the aorta and pulmonary artery while the bulboventricular sulcus remains narrow and is called the primary interventricular foramen. At the venous end of the heart, the expanding common atrium forces the convergence of the paired sinus venosus. At the end of loop formation, the heart tube begins to form the primitive trabeculae in two sharply defined areas along the ventral border of the heart tube in the primitive right and left ventricles, which will invade the cardiac jelly and later the myocardium (Fig. 3 and 4).

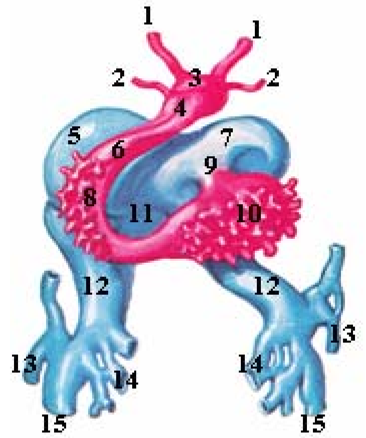
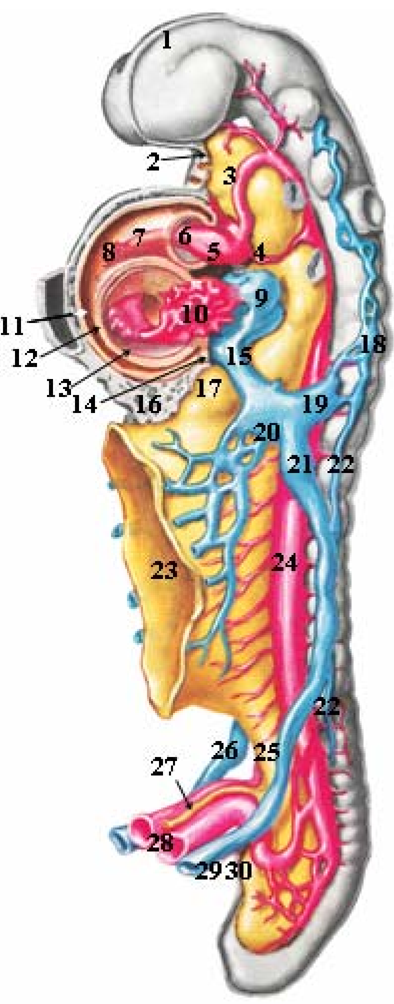


Figure 3 - Schematic drawing of the endocardial tube with myocardium removed. Adapted from Netter, FH: The Heart - The Ciba Collection of Medical Illustrations vol 5, 5Ed. *The Case-Hoyt Corp*, New York, page 118, 1981;
Legend: 1-Aortic arches I; 2-Aortic arches II; 3- Aortic sac; 4-Truncus arteriosus; 5-Right atrium; 6-Conus cordis; 7-Left atrium; 8-primitive right ventricle; 9-Atrioventricular canal; 10-Primitive left ventricle; 11-Sinus venosus; 12-Right and left sinus horns; 13-Right and left posterior cardinal veins; 14-Right and left vitelline veins; 15-Right and left umbilical veins.

Figure 4 - Sagittal dissection of a human embryo with approximately 25 days of development. Adapted from Netter, FH: The Heart - The Ciba Collection of Medical Illustrations vol 5, 5Ed. *The Case-Hoyt Corp*, New York, page 118, 1981;
Legend:
1-Forebrain;
2-Buccopharyngeal membrane;
3-Aortic arch I;
4-Aortic arch II;
5-Aortic sac;
6-Truncus arteriosus;
7-Conus cordis;
8-Primitive right ventricle;
9-Primitive left atrium;
10-Primitive left ventricle;
11-Pericardial cavity;
12-Myocardium;
13-Cardiac jelly;
14-Proepicardial tissue;
15-Sinus venosus;
16-Septum transversum;
17-Hepatic diverticulum;
18-Left anterior cardinal vein;
19-Left common cardinal vein;
20-Left vitelline vein;
21-Left umbilical vein;
22-Left posterior cardinal vein;
23-Yolk sac;
24-Dorsal aorta;
25-Hindgut;
26-Right umbilical vein;
27-Allantois;
28-Umbilical arteries;
29-Left umbilical vein;
30-Cloacal membrane.



The distal portion of the bulbus cordis, initially on the right side of the pericardial cavity, is now located in a more central position, as a result of two dilatations of the atrium, bulging on each side of the bulbus (Fig. 3). At this time, although the heart still consists basically of a single tube, its external appearance already suggests its future four-chambered condition, and now it completely occupies the pericardial cavity.

The human embryo is now about 3.2 mm long, with 20 somites and approximately 25 days old.

Epicardium and coronary vasculogenesis

Around the 24th day of development one can distinguish three cell types in the heart, cardiomyocytes, mesenchymal cells and endocardial cells. Approximately, by this time, a new layer of epithelial cells starts to populate the myocardial surface of the heart which soon will be called the epicardium. This new layer of cells derives from specific proepicardial villi in the proepicardial serosa, as well as from the deeper portions of the adjacent pericardial tissue. These proliferative cells (as shown by simple bromodeoxyuridine experiments) are localized just behind the limit between the liver and the sinus venosus, in a region called "Proepicardial tissue" [55, 56] (Fig. 4). This phase begins when the cardiac tube is already formed and looped. The epicardium-forming cells give rise to the cellular elements of the subepicardial and intermyocardial connective tissues, of the coronary vasculature and blood. These cellular elements include fibroblasts, mesenchymal cells, smooth muscle cells, pericytes, angioblasts, endocardial cells and even a small number of cardiomyocytes [45, 55, 56].

Proepicardial-derived angioblasts differentiate into endothelial cells and assemble into a primitive capillary network that is the basis of the coronary vasculogenesis, which is not yet connected to the systemic circulation. This primitive network subsequently expands in an epi-to-endocardial direction and towards the base of the heart from pre-existing capillaries, the coronary angiogenesis, probably connecting to the developing aorta. Only after perfusion, this plexus become remodelled into larger vessels and respective branches which now become muscularized with proepicardial-derived smooth muscle cells, starting to assume the specific properties of coronary arteries or veins [45].

It is also hypothesied that multipotent proepicardial and/or neural crest-derived cells have an essential role in the induction and differentiation of cardiomyogenic progenitors into cells of the cardiac conduction system [57]. The mechanism by which the proepicardial cells starts invading the myocardium is via the release of groups of cells from the proepicardial

protrusions into the pericardial cavity and subsequent attachment to the myocardial surface [56]. Aditionally, it is important to refer the distal portion of the outflow tract, the truncus arteriosus, a region in which the epicardium does not derive from the proepicardial serosa. Instead, the epicardium is probably derived from the pericardial mesothelium at the junction between the outflow tract and the dorsal wall of the pericardial cavity [45].

Sinus Venosus

Approximatelly around the 25th day of development, the sinus venosus receives three pairs of veins, namelly the vitelline, the umbilical and the common cardinal veins. The first enters the floor of the sinus, medially, the second enters the sinus horns coming from below, and the third also enters the horns but coming from above. At first, communication between the sinus and the atrium is wide (Fig. 3). Later, however, the entrance of the sinus (the sinoatrial opening) shifts to the right, due to the shunting of blood to the right, until the sinus venosus communicates with only the right atrium. The development of anastomotic channels between the right and left systemic veins and the preferential blood flow to the right side, makes the right sinus horn and its proximal cardinal and vitelline veins gain in importance, whereas their left counterparts become greatly reduced in size. At the same time, the right sinus horn attains a more vertical position and becomes incorporated into the right atrium to form the smooth-walled part of it [58]. With obliteration of the left umbilical and vitelline veins and later the left common cardinal vein, all that remains of the left sinus horn is the oblique vein of the left atrium and the coronary sinus. The sinoatrial opening is now flanked in each side by the right and left venous valves. Superiorly, the valves join each other to form a single fold, the septum spurium. As the right sinus horn incorporates into the wall of the atrium, the left valve and septum spurium fuse with the developing atrial septum. The superior portion of the right venous valve disappears entirely and the inferior portion develops into the valve of the inferior vena cava and the valve of the coronary sinus [58, 59].

Cardiac septation and valve formation

During the next period (Fig. 5), that takes approximately 10 days, between the 27th and 37th day of development, no major changes in the external appearance of the heart occurs, however, its relative position keeps changing because of the changing curvature of the embryo and the growth of neighbouring organs.

The development of the various cardiac septa takes place almost simultaneously and are formed, basically, by two embryological processes. One is an active one, in which one or more growing masses of tissue, usually called “endocardial cushions”, approach the opposite structure until they fuse, dividing the lumen into two separate chambers. The other is a passive one, in which the growth of two expanding chambers, on either side of a strip of tissue, that behaves like a “banding”, leads to the approach of the walls of the chambers to each other, eventually merging, thereby forming a septum. Such a septum always leaves a narrow communicating canal between the two expanded chambers, which is usually closed, secondarily, by neighbouring proliferating tissues. TGFβ2 seems to have an essential in role in these septation processes as knockout of this gene leads to defficient septation [60].

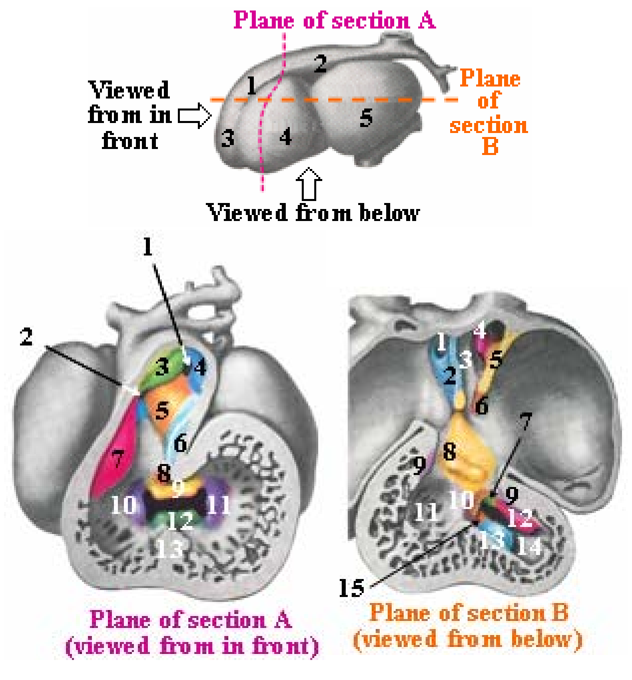


Figure 5 - Frontal (Plane of section A) and transversal (plane of section B) dissections of a human heart with approximately 31 days of development. Adapted from Netter, FH: The Heart - The Ciba Collection of Medical Illustrations vol 5, 5Ed. *The Case-Hoyt Corp*, New York, pages 120, 121 and 123 1981;

In the **image at the top**, it is represented the planes of section and the viewing angles. The numbers refers to: 1-Conus cordis; 2-Truncus arteriosus; 3-Primitive right ventricle; 4-Primitive left ventricle; 5-Primitive left atrium.

In the **plane of section A** (lower left) can be seen:
1-Pulmonary channel; 2-Aortic channel; 3-Right superior truncus cushion; 4-Pulmonary intercalated valve swelling; 5-Left inferior truncus cushion; 6-Left ventral conus cushion; 7-Right dorsal conus cushion; 8-Bulboventricular flange; 9-Superior endocardial cushion; 10- Right endocardial cushion; 11- Left endocardial cushion; 12- Inferior endocardial cushion; 13-Interventricular muscular septum.

In the **plane of section B** (lower right) can be seen: 1-Ostium secundum; 2-Septum primum; 3-Septum secundum; 4-Left venous valve; 5-Right venous valve; 6-Septum spurium; 7-Right superior truncus cushion; 8-Superior endocardial cushion; 9-Left and right endocardial cushions; 10-Bulboventricular flange; 11-Primitive left ventricle; 12-Right dorsal conus cushion; 13-Left ventral conus cushion; 14-Primitive right ventricle; 15-Left inferior truncus cushion.

The whole process of heart partition consists in the formation of several septa in the:

Atria

A sickle-shaped crest descending from the roof of the atrium, the septum primum, begins to divide the atrium into two parts. However, an opening remains, the ostium primum that allows communication between the two chambers. When, later in development, the ostium primum is obliterated by fusion of the septum primum with endocardial cushions, the ostium secundum is formed by cell death, creating an opening in the septum primum. Finally, and lining close to the latter septum, the septum secundum forms also with an opening in it, the oval foramen. Because this last opening is diagonally aligned with the ostium secundum, a communication between the two chambers still occurs. At birth, when the pressure in the left atrium increases, the two septa are pressed against each other, and consequently, the diagonal communication between the two atria disappears [61, 62]. Deficiencies in primary and secondary atrial septation have been linked to a mutation in the Csx1 gene [63].

Atrioventricular canal

Although there are four endocardial cushions surrounding the common atrioventricular canal, the opposing superior and inferior cushions are the ones that present the highest proliferation. They will end up by fusing with each other and resulting in a complete division of the canal into right and left atrioventricular passages. Surrounding cushion tissue then becomes fibrous and forms the mitral valve (bicuspid) on the left and the tricuspid valve on the right [61, 62].

Ventricles

The interventricular septum is formed “passively” (muscular portion) by the development of the medial walls of the expanding ventricles and “actively” (membranous portion) by the left and right conus cushions plus the inferior atrioventricular cushion. Before the complete fusion of the muscular and membranous portions, an opening remains the interventricular foramen [62].

Conotruncal region and myocardialization

The truncus region is divided by the aorticopulmonary septum into the aortic and pulmonary channels, formed by the growth of the right superior truncus cushion and left inferior truncus cushion. When the truncus swellings appears, similar cushions develop along the right dorsal and left ventral walls of the conus cordis that will divide the outflow tracts of the aortic and pulmonary channels [64]. Those conus cordis cushions, with tissue from the inferior endocardial cushion will fuse and close the interventricular foramen. In

this process there is an important contribution of cells derived from an extra cardiac tissue, a specific region of the neural crest, located between the inner ear primordium and the third somite, called cardiac neural crest [65, 66]. Although the majority of these cells go into apoptosis, it has been postulated that neural crest cells are essential in releasing factors that will induce myocardialization of the outflow tract cushions [1]. The formation of myocardial cells within the cushions constitutes a wave of myocardium formation that is considered to be different from the primary myocardium that has been directly formed from the primary mesodermal heart fields. It is suggested that the recruitment of mesenchymal cells into the myocardial lineage and the recruitment of pre-existing cardiomyocytes (myocardialization), or a combination of both, can be considered to constitute a second wave of myocardium formation [67].

In addition to the cardiac neural crest, the "secondary heart field" [50] or "anterior heart field" [53], located beneath the floor of the foregut in the prepharyngeal mesoderm, seems to also have an important cellular contribution with the recruitment of mesenchymal cells into the myocardial lineage in the outflow tract cushions. The expressions "secondary heart field" and "anterior heart field" although defining overlapping regions of embryonic mesoderm and that eventually may be understood as equivalent, are not exactly synonymous. The most restricted is the "secondary heart field" which refers to the prepharyngeal mesoderm caudal to the outflow tract, contributing only to the region of transition from the heart to the base of great arterial vessels, the distal myocardium of the outflow tract and to the smooth muscle in and at the base of the aorta and pulmonary trunk. On the other hand, the "anterior heart field" involves not only the prepharyngeal mesoderm but also the lateral and more cranial splanchnic mesoderm extending itself into the middle of the cranial pharyngeal arches, contributing to the myocardium of the right ventricle, conus and truncus, or, in other words, to the entire ascending segment of the looped heart. Curiously, lineage tracing and expression data [68] have shown that Isl1, a recent postnatal cardiac progenitor's cell marker [69] has an expression pattern which overlaps the anterior heart field described above. Probably, this secondary myocardialization depends, among other things, upon the TBX1 transcription factor activity and also on neural crest migrating cells. This hypothesis is due to the research performed in the DiGeorge's syndrome, a human chromosome 22q11 rearrangement associated syndrome, characterized by malformations of facial features, absence or hypoplasia of the thymus and parathyroid glands, and a specific set of heart defects. All deficiencies in the DiGeorge's syndrome have as cause the deficient migration of specific

populations of neural crest cells into their normal locations in their target developing organs and their role in its respective organogenesis, including the outflow tract of the heart and its septation event. After the observation that TBX1 mutations can give rise to DiGeorge's syndrome phenotype [70-72], TBX1 has became the main primary candidate gene of this pathology. However, TBXI is expressed in pharyngeal arches mesoderm and not in the neural crest cells. So, if the deficient neural crest cells migration is on the basis of DiGeorge's syndrome symptoms, one can say that it depends on a proper TBX1 pathway in neighbouring cells, upstream regulated by the SHH and a downstream regulator of the FGF8 and FGF10. On this line of thought, it is expected that the TBX1 pathway, in the secondary heart field derived cells, would be of great importance for a proper migration of neural crest cells and subsequent induction of septation and myocardialization of the outflow tract cushions [1]. Like the cells of the primary heart fields, cells in the secondary heart field also present the expression of Nkx2.5 and Gata4. Then, when they begin to move and differentiate into the cardiomyogenic cell lineage, they start expressing HNK1, a marker of migrating cells, and later MF20, a marker of cardiomyocyte differentiation. BMP2 and FGFs, expressed in secondary heart field derived cells, are thought to be essential players in myocardial induction, in a manner that mimics induction of myocardium from the primary heart fields [73]. Curiously, induction of myocardium appears to be unnecessary at the inflow pole of the heart and neither FGF8 nor BMP2 are present, as inflow myocardium is added from the primary heart fields at the inflow pole [74].

The cardiac pacemaker and conduction system (CPCS)

The first theories about the cellular embryogenesis of the CPCS formation, assumed that those cells were originally derived from the cardiac neural crest cells, or in other words, from the embryonic neural plate, at the level of the inner ear primordium [75, 76]. However, subsequent studies have given evidence of the existence of a cardiomyogenic cell that can give rise to both cardiac muscle and CPCS cells [77, 78]. It is noteworthy that the neural crest cells, that had migrated to the areas correspondent to the CPCS have only a possible inductive role in the process of differentiation of the cardiomyogenic cell, as the neural crest cells, themeselves are soon apoptotic [79, 80].

Before the pacemaker region shifts to the right sinus primordium, this function is accomplished by cells of the left atrium primordium, that are the first ones to assure polarity

and slow unidirectional waves of contraction in the primitive heart, similar to a intestinal peristaltic contraction [81, 82] and typical of slow voltage-gated calcium ion channels [83, 84]. This peristaltic wave of contraction has a characteristic sinusoidal ECG, the first electric record that can be observed in cardiogenesis [85].

Because the embryonic heart has no valves, a relatively long lag of contraction is observed in the atrioventricular canal and outflow tract, substituting valve function in these areas and being essential in preventing the backflow of blood [86]. Interestingly, the pattern of contraction is closely associated with local differences in calcium-triggered calcium release and with protein levels of sarcoendoplasmic reticulum calcium ATPase (SERCA). Accordingly, immunohistochemistry of human embryo's sections have shown high levels of this protein in the atrium, intermediate levels in the ventricles and low levels in the atrioventricular canal, inner curvature and outflow tract [87].

It is worth mentioning that a very recent study in the embryonic zebra fish heart tube has suggested that the "peristaltic model" of contraction is not suitable to explain the biomechanical properties of such an early stages of cardiac development [88]. Instead, they proposed a "hydroelastic pump model" based on elastic wave propagation and reflection where a single suction activity substitutes the complete synchrony throughout the heart tube mentioned above. However, the discussion of these two models is beyond the scope of the present thesis.

As the right sinus horn is incorporated into the right atrium, pacemaker tissues lies near the opening of the superior vena cava and the sinoatrial node is formed. This occurs around the 5th week of development. The atrioventricular node and the atrioventricular bundle (bundle of His) are derived from cells in the left wall of the sinus venosus and cells from the atrioventricular canal. The first one is connected to the sinoatrial node by the anterior, middle and posterior internodal tracts, all derived from cells in the dorsolateral wall of the right atrium and interatrial septum. A subdivision of the anterior internodal tract will extend along the interatrial region to ramify over the left atrium, constituting the Bachmann´s bundle. The bundle of His passes along the membranous interventricular septum to the apex of the muscular intraventricular septum. At this point, it divides into right and left bundle branches that extend subendocardially along both septal surfaces. Left and right bundle branches further subdivide and form the subendocardial network of Purkinje fibers, which extend into the ventricular walls of the ventricles. Studies performed in chick suggest that Purkinje cells are clonally related to surrounding working myocardium, more precisely, they derive from the trabecular ventricular component and develop in close association with and are induced by

developing coronary arteries [77, 89-91]. Purkinje cell formation can be induced *in vitro* and *in vivo* by the vascular endothelium secreted molecule, endothelin-1 [92].

Although the molecular regulation of these CPCS formation events are far from being understood, other results have revealed that the growth factor Neuroregulin is an important CPCS induction factor [93, 94].

The electrocardiogram (ECG)

The formation of an adult type ECG, is related with the appearance of high conduction velocity cells typical of fast voltage-gated sodium ion channels [83, 95], specific junctional proteins and with the appearance of the atrioventricular sulcus, while no distinct conductive cell system can yet be recognized. The coordinated propagation of the impulse implies a pacemaker region at the inflow tract region of the heart, a rapid conduction in the atrial region, a delay in the slow conductive atrioventricular region, a rapid conduction in the ventricular region and a slow conduction in the outflow tract. Indeed, slow conduction velocities were observed in the sinoatrial region, atrioventricular region and outflow tract of chicken hearts [86].

Although, cells of the conducting system are considered to withdraw from the cell cycle relatively early in development, they attain maturity late in development. Therefore, until sometime after birth, the fastest velocities of conduction do not follow the path observed in the adult. This evidence suggests that the adult type ECG record is independent of the formation of a mature conduction system.

The connexins

Gap junctional proteins consist of 2 connexons, which are hexamers of transmembrane protein subunits called connexins, necessary for electric and metabolic coupling between cells [96-100]. Studies regarding the expression of the main connexin proteins CX40, CX43 and CX45 showed that the first, CX40, was present first in the primitive atria and left ventricle and later in the primitive right ventricle but during further development becomes stronger in atrial myocytes and conductive system and almost undetected in ventricular myocardium. The CX43 was present first in the primitive right ventricle and left ventricle and later in the primitive atria. During further development it becomes also expressed in the atrioventricular node and ventricular bundle branches. The CX45 was a more primary heart tube associated form. It is widely expressed in the linear heart tube and throughout development, the expression extends to all the components of the working

myocardium and conductive system, with a relatively strong expression in the components of the conductive system. Accordingly, expression studies in the atrioventricular bundle and proximal part of the bundle branches of fetal and neonatal mammalian hearts have revealed that the gap junctional proteins CX40 and CX43 are not expressed. In fact, only after birth, can these proteins be detected, in the atrioventricular bundle and proximal part of the bundle branches [101].

By the end of the septation process, around the 40th day of development, the major morphological changes in the forming heart have reached the end. However, during the next weeks and months, all the structures of heart, namelly the CPCS, the muscular and non muscular structures, and the cardiac arteries and veins network, will continuously grow and perfect themselves till adulthood. At birth, the myocardium has attained only a fraction of the thickness it will attain during adulthood, which will be, at least, fourfold larger, essentially due to cardiomyocyte hypertrophy. The capillary density will, as well, increase several times by angiogenic and neovasculogenic mechanisms [102].

Molecular families in cardiogenesis

The NK2 homeobox family

NK2 homeobox genes are conserved across vertebrate species. They are relevant in the present context because they are expressed in early cardiac progenitor cells [103, 104]. These genes encode DNA binding proteins similar to the homeodomain proteins encoded by homeobox containing genes that regulate early embryonic patterning. They all present a 61-aminoacid homeodomain motif. NK2-homeodomain factors recognize the NK-responsive element (NKE) “NAAGTG” on a wide variety of vertebrate target genes, enhancing their transcription [105]. As refered before, the NK2 homeobox gene NKx2.5 (Csx1 in humans) is one of the earliest known markers of development on vertebrate heart and is located upstream several other cardiac genes, including cardiac α-actin and ANF [105, 106]. Most likely, analysis of the role of Nkx2.5 in heart formation of early vertebrate development has been complicated by redundancy of the NK family members [107]. Loss of Nkx2.5 activity in mouse is associated with the inability to perform cardiac looping morphogenesis, even with the primitive heart tube still developing and some cardiac specific genes, including myosin, still being expressed. It is likely that many murine homologs of Nkx2.5 have overlapping and compensatory functions. Therefore, deletion of any one of these genes may not be sufficient

to completely block cardiomyocyte differentiation, although morphogenetic regulation appears defective [33, 107].

The complexity of Nkx2.5 transcription is regulated by different enhancer sequences, suggesting that chamber-specific upstream factors mediate Nkx2.5 expression through its activating and repressing regions, within the DNA surrounding the Nkx2.5 gene [108]. Spatiotemporal regulated enhancers demarcate distinct subpopulations of cardiomyocytes within cardiac compartments, but none can account for the complete expression pattern of Nkx2.5 during development or after birth. (Fig. 7).

The GATA zinc-finger transcription factor family

These transcriptional regulators are a family of DNA binding proteins expressed in a tissue restriceted manner. They all contain one or two highly conserved zinc finger motifs Cys-X_2-Cys-X_{17}-Cys-X_2-Cys that mediate the binding to specific nucleotide sequences (A/T)GATA(A/G) in the correspondent target genes. GATA proteins have been divided into two subgroups, GATA-1, -2 and -3 and GATA-4, -5 and -6, according to their particular expression patterns. As expected, they share a high degree of homology (90 %) in their DNA binding domains.

The representatives of the first subgroup are more associated with regulation of gene expression in haematopoiesis and for that, they will not be further discussed. The second subgroup is expressed in various mesoderm- and endoderm-derived tissues such as heart, liver, lung, gonad, and gut where they play critical roles in regulating tissue-specific gene expression [109] and will receive a little more attention in the present chapter.

Specifically related to cardiogenesis, it appears that representatives of the second subgroup, particularly GATA-4, play an important role in regulating cardiac genes as α-Myhc, TrpC and Anf among others and to cooperate with NKX2.5 in the Nkx2.5 dependent cardiomyocyte differentiation [110-117]. This cooperation is, most likely relevant because, although GATA-4, -5 and -6 bind to GATA motif-containing DNA sequence elements in target genes, their individual affinities for the various promoters depend on flanking nucleotide sequences and on interactions with cofactors and other transcription factors, revealing a more fine specificity in the apparent redundancy among the second subgroup members. The exact mechanism underlying the role of GATA-4 during early cardiogenesis remains to be established, however, it seems that GATA-4 is required for proper visceral endoderm differentiation and for proliferation and migration, rather than for differentiation, of cardiac

cells, a morphogenetic role that may be shared with the other second subgroup members [118]. (Fig. 7).

The MEF2 transcription factor family

The Mef2 genes are members of the yeast mating type-specific transcription factor MCM1, the plant homeotic genes Agamous and Deficiens and the human Serum response factor (MADS) gene family, a family that also includes several homeotic genes and other transcription factors [119]. They all share a conserved DNA-binding domain called MADS-box domain. MEF2 proteins, besides this domain, have an adjacent and characteristic MEF2-domain, which mediates homodimerization, DNA and the formation of heterodimers with myogenic bHLH factors, such as MyoD, myogenin, and ubiquitous bHLH proteins. The latter, known as E proteins, bind a consensus DNA sequence referred to as an E-box (CANNTG) in the control regions of muscle-specific genes [120, 121].

Proteins coded by the Mef2 gene family, bind a DNA target sequence (C/T)TA(A/T)AAATA(A/G) in the regulatory regions of many, if not all, muscle specific genes [122]. In fact, these transcription factors were found to play a pivotal role in the process of differentiation of mesodermal precursor cells to myoblasts of the skeletal, cardiac and smooth muscle lineages [120, 123]. However their role may be more in regulation of muscle differentiation than in specification processes [123]. They also show specific expression patterns in the developing brain [124].

In vertebrates there are four Mef2 genes, Mef2a, Mef2b, Mef2c and Mef2d that have, as other DNA binding proteins, a high homology in the DNA binding domains.

Mef2b and Mef2c are the first members of the family to be expressed in the mouse, with transcripts appearing in the precardiac mesoderm at E7.5 (Fig. 6) prior to the formation of a primitive heart tube. Soon thereafter, contractile protein genes such as myosin and cardiac actin, and the other Mef2 genes Mef2a and -d are expressed throughout the developing heart [124]. Interestingly, the onset of expression of Mef2 genes coincides with that of Nkx2.5 and GATA4 genes, however the exact role of their interactions is still under investigation [103, 104, 125]. After birth, MEf2a, Mef2b and Mef2d

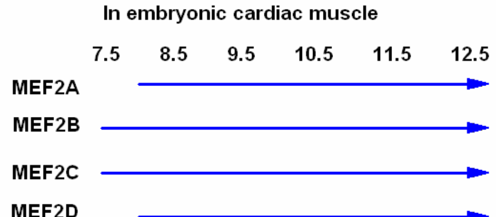


Figure 6 - Temporal patterns of Mef2 genes relative to each other. Expression patterns of each gene during mouse cardiac embryogenesis are indicated by lines. Adapted from Molkentin JD *et al*, Molecular and Cellular Biology, vol.16, 7, page 3823, 1996.

transcripts are expressed ubiquitously, whereas Mef2c transcripts are restricted to skeletal muscle, brain and spleen [124, 126-130]. Despite the widespread reported expression of Mef2 mRNAs in adult tissues and cell lines, a relevant observation is that Mef2 DNA binding activity is highly enriched in muscle cell and neurons.

The regulation of Mef2 expression at the level of 5´noncoding mRNA regions has been very difficult to analyse, much because they present multiple alternatively spliced exons and large introns. Accordingly, no cis-acting regulatory sequences have yet been described for any vertebrate Mef2 gene. However, in *Drosophila melanogaster*, there is only a single Mef2 gene, D-mef2, which encodes a protein with the same DNA binding properties as the vertebrate MEF2 factors. In this easier animal model, it has been possible to define, at least, eight regulatory elements that control the Mef2 transcription. They are independent enhancers within 12 kb upstream of the gene that directly regulate transcription, in an unique temporospatial pattern during development [131]. (Fig. 7).

Despite the fact that Mef2b and Mef2c are the first Mef2 genes to be expressed in early embryo, Mef2d was suggested to represent undifferentiated cardiac cells or cardioblasts [126] and it was the factor chosen among the Mef2 family to be studied in the differentiation studies, presented in this thesis. Curiously, a clinical study in which human female hearts were transplanted into male recipients suggested that Y positive cardiac progenitor cells found in the grafted heart were also MEF2D positive [132].

Another member of the MADS-box family is, as mentioned above, SRF. Binding sites for SRF have the sequence $CC(A/T)_6GG$ and are called CarG boxes. Interestingly, these are present in the regulatory regions of many cardiac genes [133]. SRF is not muscle-specific, however, it can activate muscle-specific genes by recruiting myogenic accessory factors.

Recently, a highly potent transcription factor, myocardin, expressed in cardiac and smooth muscle cells has been isolated [134]. Myocardin belongs to the SAF-A/B, Acinus, PIAS (SAP) family of chromatin-remodeling proteins and associates with SRF in activating cardiac muscle gene promoters. SRF and myocardin appear to be coexpressed with Nkx2.5 and GATA4 in the cardiac crescent and is possible that interactions between them may be one of the main events in regulating cardiogenesis [134, 135]. (Fig. 7).

The TGF beta superfamily

The Transforming Growth Factor β (TGFβ) superfamily of extracellular growth factors regulate a multiplicity of cellular events such as proliferation, differentiation and apoptosis [136-138]. Homo- or hetero-dimers of the TGF-ß family ligands bind to and activate

transmembrane serine/threonine kinase receptors (I and II), which then, stimulate downstream regulatory SMAD proteins that will translocate from the cytoplasm to the nucleus, where they can function as transcriptional regulators [139, 140]. This superfamily of structurally related proteins comprises more than 35 members, including TGFßs, Activins, BMPs, GDFs, Inhibins, MIS, Nodal, and Leftys. Roughly, they can be divided in three subfamilies: TGFßs, Activins and BMPs. Almost all the processes in development, if not all, have the participation of members of these TGFß subfamilies. Since an exaustive description of each one of these subfamilies is beyond the scope of the present thesis, only the main molecules, known to be involved, more specifically, in cardiogenesis will be described.

Interesting studies in avians have revealed two signals regulating cardiogenesis: an early, hypoblast-derived acting on epiblast and mediated by activin and/or TGFβ related molecule [141, 142] and a late, endoderm-derived and possibly ectoderm-derived, acting on mesoderm and involving BMPs [23]. Accordingly, it is suggested that activin or TGFβ can efficiently induce cardiac myogenesis in pregastrula epiblasts, but not in noncardiogenic mesoderm, while BMP2 or BMP4 (in combination with FGF4) can induce cardiac myocytes formation in caudal mesoderm but not in epiblast cells. Activins/TGFβs and BMP2/BMP4, in this way, mimic the tissues in which they are expressed, the hypoblast and cranial lateral plate mesoderm, respectively. However, the all picture is far more complex, because BMP2 or BMP4 were known to inhibit cardiac myogenesis before the mid-gastrula stage in the chick, indicating multiple roles for BMPs in mesoderm induction. Experiments with noggin and follistatin, a specific BMP antagonist and a BMP/Activin inhibitor, respectively, have provided additional clues suggesting that in cardiac induction, BMP signalling follows the activin/TGFβ signal. According to these studies, the hypoblast-derived activin/TGFβ signal was required before and during early stages of gastrulation, regulated spatially and temporally by an interplay between BMPs and their antagonists [26]. Later endoderm-derived signals and possibly ectoderm-derived, mediated in part by BMPs will activate or enhance expression of cardiogenic genes from the GATA and Nkx families among others, conducting to cardiac myocyte differentiation [23, 26]. (Fig. 7).

The angiomyogenic medium described in the chapter "Materials and Methods", as the cell culture medium that we used in our experiments, had BMP2 as the protein that has been described as major cardiomyogenic inductor. See also the "Results - 2.2 Human embryonal carcinomas" and "Discussion" for more details.

The Nodal co-receptor Cripto

Nodal, a TGFβ family ligand, mentioned above, is a gene essential for primitive streak formation in the mouse embryo. Embryos that lack a functional Nodal gene are deficient in rudimentary mesoderm [143, 144]. Nodal activity is required for specification of the endoderm and correct CrCa patterning during gastrulation, as well as contributing to LR asymmetry later in embryogenesis [145] (see next section "*LR patterning*").

Four members define the EGF-CFC gene family: mammalian Cripto and Cryptic, frog FRL1 and zebrafish Oep genes [146]. All these genes encode extracellular proteins that share an N-terminal signals sequence, a variant EGF-like motif, a novel cysteine-rich domain named the CFC motif, and a C-terminal hydrophobic region 7.

Interestingly, the first EGF-CFC gene that was discovered, the human Cripto, also called TDGF1, was unexpectedly isolated as a fusion transcript, in a cDNA library screen performed in the undifferentiated NTERA2.D1 teratocarcinoma cell line [147] **(the cell line used, not innocently, in the cellular differentiation studies presented in this thesis)**. A substantial amount of work in tumour cell growth has indicated that Cripto behaves as a conventional growth factor-like molecule in cell culture systems [148]. Accordingly, 80 % of primary human breast carcinomas, but not normal tissue, overexpresses Cripto [149]. These and other studies have suggested that Cripto is involved in the autocrine and paracrine stimulation of tumour cell growth 43. However, the EGF-like domain is divergent from the canonical EGF motif and Cripto does not bind directly to any of the erbB receptors [150, 151]. Therefore, an EGF-like behaviour cannot account for its growth factor-like activity. Experimental studies suggest that Cripto may activate the PI3K/AKT pathway [152] and/or the RAS/MAPK pathway, through binding to an yet unidentified Cripto-specific receptor complex [151, 153] or through a cross-talk between the TGFβ and EGF receptor pathways. However, the exact mechanisms remain to be elucidated [154, 155].

Cripto was also defined as necessary for cell movements during gastrulation, as well as for CrCa and LR patterning in the mouse embryo [156]. The mutant phenotypic similarity between Cripto and Nodal, including the absence of embryonic mesoderm and definitive endoderm, suggested that they might function in the same pathway. However, unlike the zebrafish Nodal and Cripto gene mutants [157, 158], the murine mutants are not exact phenocopies of each other [159, 160], suggesting that in mammals, Nodal may be able to signal, in some instances, independently of Cripto or that other EGF-CFC members exist that may substitute Cripto in the pre-gastrulation mouse embryo. In fact, chimeric studies in mice

have indicated a role for extraembryonic Nodal, in inducing head formation [161] whereas Cripto mutants readily form cranial neural tissue [156].

Nevertheless, experimental evidence suggests that Cripto acts as a co-receptor for Nodal, recruiting this molecule to an ActRII/Alk4 receptor complex, both serine/threonine kinase receptors that activate the Smad2/3 transcription factors which in turn mediate transcriptional responses [162-165]. Cripto interacts with Nodal through its EGF domain and with Alk4 through its CFC domain [162, 163, 166] and is possible that, in a similar way, Cripto may modulate also other TGFβ family members, including Activin B [167] and GDF1 [168]. Additionally, other recent gene profiling studies have characterized Cripto as a target gene of the canonical β-catenin/LEF/TCF-dependent Wnt pathway during mouse CrCa patterning and mesoderm induction and in colon carcinoma tissues and cell lines [169]. Thus, in the embryo and cancer, EGF-CFC proteins are involved in the complex interplay between fundamental signalling pathways, as a membrane associated or free diffusible proteins.

Particularly, in the embryo, Cripto is firstly uniformly expressed and subsequently, in mouse pre-implantation stages E5.5-E6.25, in the epiblast, as a crescent proximal-distal gradient. In early gastrulation stages, E6.5-E7.0, Cripto expression acquires a crescent CrCa gradient in epiblast and in newly formed mesoderm, becoming restricted to the outflow tract of the heart at the cardiac looping stages, E8.5-E9.5 [156, 170].

Cripto null mouse embryos does not develop beyond the E9.0 stage and show very severe defects, including lack of primitive streak, embryonic mesoderm and definitive endoderm. Therefore, insights for the role of Cripto in cardiomyogenesis had to come from experiments with ESCs. ESCs normally develop as embryoid bodies, showing observable cardiomyocyte contracting regions approximately, after 7 days in the appropriate differentiation medium. Curiously, ESCs Cripto$^{(-/-)}$ did not produce contracting cardiomyocytes even during extended culture periods. In these cells, no expression of cardiac differentiation-related proteins as αMyHC, βMyHC, MLC2a, MLC2v and ANF were detected whereas the expression of the transcription factors GATA4, NKX2.5 and MEF2 appears not to be affected. Cripto re-expression could rescue the ability of the ESCs Cripto$^{(-/-)}$ to differentiate into cardiomyocytes, still the mechanism of the activity of Cripto in cardiac progenitors remains unknown [171, 172].

Relevantly or not, a SAGE study with the highly cardiomyogenic mouse teratocarcinoma cell line P19EC has shown that Cripto expression, while present in undifferentiated cells,

could no longer be detected 3 days after cardiomyogenic induction, suggesting that Cripto may be essential in the early steps of cardiomyogenesis [173]. (Fig. 7).

Taken together, these results have suggested us that Cripto has a very important role in cardiac differentiation processes, at least, when starting from undifferentiated pluripotent cells *in vitro*, an event that is central for "Cardiac cell therapy". Accordingly, we have chosen the NTERA2.D1 (NT2/D1), a human teratocarcinoma cell line that presents a high level of Cripto expression, as the possible cellular model to the angiomyogenic differentiation studies.

The WNT family

The Wnt family of secreted glycoproteins comprises about 19 identified members, which control a variety of development processes including cell fate specification, proliferation, establishment of polarity and migration. Accordingly, deregulation of Wnt signalling can cause severe development defects during embryogenesis and a variety of diseases in the adult [174, 175]. Wnt family members bind to specific cell receptor complexes, composed by a seven-loop transmembrane receptor called Frizzled (Fz) and a low density lipoprotein related protein (LRP) co-receptor. Ligand-receptor interaction triggers intracellular signalling cascades, that lead to nuclear activation/repression of certain genes. Ten members of the Fz family of transmembrane receptors are known [176].

There are at least three signalling Wnt pathways involved in the signal transduction process: i) the canonical or β-catenin/LEF/TCF-dependent, and two non-canonical: ii) the PCP or JNK/AP1-dependent and iii) the Ca^{2+} or PKC/CaMKII/NFAT-dependent signalling pathways. The detailed description of each one of these signalling pathways was reviewed in [177], [178] and [179], respectively.

Wnt molecules have been classified as canonical, the Wnt1 group, which includes Wnt1, Wnt3a and Wnt8, that appear to signal exclusively via the β-catenin/ LEF/TCF pathway and non-canonical, the Wnt5a group, which comprises Wnt4, Wnt5a and Wnt11 that possesses more complex non-canonical signal initiating properties [180, 181]. Interestingly, signals provided by the Wnt5a group have been shown to suppress β-catenin mediated signalling, due to the regulatory inhibition by the non-canonical Wnt pathway. However, changes in Fz isoforms expression may transform the Wnt5a group members from inhibitors into activators of the canonical pathway [182, 183]. There are several types of canonical Wnt inhibitors, including two distinct families of soluble factors, the secreted Fz-related proteins (sFRP) and

the Dickkopf (Dkk) proteins. The former family members exert there activity by binding to Wnts by its Wnt-binding domain and inhibiting the binding of Wnt to their transmembrane receptors by a competitive mechanism [184, 185]. The latter, share with Wnts the ability to bind the cell membrane co-receptors LRP 5/6, exerting their inhibitory action but having also a regulatory role as a consequence [186-189]. Additionally, Dkk proteins may also regulate Wnt activity by binding to the Kremen transmembrane proteins which are then recruited into a complex with LRP6, leading to rapid endocytosis and removal of this Wnt co-receptor from the plasma membrane [190, 191].

In mouse, during early gastrulation, Wnt3, Wnt3a, Wnt5a and Wnt8 are expressed by cells within and immediately lateral to the primitive streak [192-196] (Table 1). Soon thereafter, several Wnts can be found in mesodermal regions overlapping with precardiac mesoderm. Studies in the chick, quail and mouse have confirmed that Wnt11 is either expressed within or in close proximity to the cardiogenic plates [193, 197, 198] and that in mouse, Wnt2 and Wnt2b are expressed in the precardiac mesoderm [199, 200]. On the other hand, at the same stages, several Wnt inhibitors are also expressed. Among those, sFRP1, sFRP2, sFRP3 and crescent (also a sFRP), appear: i) in the cranial portion of the early chick gastrula, in the three germ layers, ii) in chick, in the ectoderm, overlapping with the heart-forming fields, iii) in the three germ layers of mouse and chick, also overlapping with the heart-forming fields and iv) in the cranial portion of the early chick gastrula, but only in endoderm. Additionally, Dkk1 is highly expressed in Hensen´s node, with lower levels in the primitive streak and cranial endoderm [19, 197, 201-205].

Table 1 - Expression of Wnts and Wnt inhibitors at early gastrulation in the mouse embryo, when cardiogenesis is initiated. Adapted from Eisenberg, LM and Eisenberg, CA, Developmental Biology, 293, page 308, 2006.

Embryonic location	
Node	Wnt5a, Wnt11
Primitive streak	Wnt3, Wnt3a, Wnt5a, Wnt8
Precardiac mesoderm	Wnt2, Wnt2b, Wnt11, sFRP3
Ectoderm or endoderm adjacent to precardiac mesoderm	sFRP3

When the linear heart tube begins its formation, low levels of expression of Wnt8a, Wnt11 and sFRP1 can be detected, but, interestingly, only in the myocardium [193, 206]. As cardiac morphogenesis develops, upregulation of both Wnt8a and sFRP1 is observed in the myocardium of the common ventricular and atrial chambers, while Wnt5a and sFRP3 appear only in the ventricle [202, 206]. At the same time, Wnt11 is detected in the outflow tract

myocardium and ventricle conduction system [68, 207-209]. Additional data related to Wnt signaling in the developing heart can be seen in Table 2. Of note, is the expression of Wnt2b, Wnt5a, Wnt6, sFRP3 and Dkk1 in a pharyngeal arches region [196, 202, 204, 210-213], referred to as the anterior or secondary heart field [50-53], possibly suggesting that morphogenesis of the primary and secondary heart-forming fields share a common requirement for Wnt signalling mediation.

Since Dkk1 and crescent were suggested as Wnt inhibitors in the frog and chick [19, 20], the idea that canonical Wnt inhibition is one of the main requirements for heart development

Table 2 - Expression of Wnts and Wnt inhibitors in the developing heart at mid-gestational stages of the chick and mouse embryo. Adapted from Eisenberg, LM and Eisenberg, CA, Developmental Biology, 293, page 309, 2006.

Molecule	
Wnt2b	Secondary heart field; atrioventricular region of the tubular heart
Wnt5a	Outflow tract primordium; outflow tract of the tubular hear; common ventricle
Wnt6	Outflow tract primordium; outflow tract and atrioventricular regions of the tubular heart
Wnt7a	Primitive conduction tissue; outflow tract of the tubular heart
Wnt8a	Newly formed primary heart tube; common ventricular and atrial chambers
Wnt9a	Outflow tract and atrioventricular regions of the tubular heart
Wnt11	Newly formed primary heart tube; outflow tract myocardium; primitive conduction tissue
sFRP1	Newly formed primary heart tube; common ventricular and atrial chambers
sFRP2	Trabeculae of the common ventricle
sFRP3	Outflow tract primordium; outflow tract of the tubular heart; common ventricle
Dkk1	Outflow tract primordium; outflow tract of the tubular heart; common ventricle

has kept a great deal of attention among developmental biologists [214-216] and is still widely accepted. Besides, other developmental studies in frog, chick, mouse as well as in human cardiomyogenic cells, have reported that Wnt11 was able to promote myocardial differentiation not only by inhibiting canonical Wnt signalling but also by triggering a non-canonical JNK/AP1 Wnt pathway [217-220]. In conjuction with the previous idea, the effect of Wnt in the heart formation model may be summarized by saying that the inhibition of canonical Wnt signal transduction and activation of non-canonical pathways are absolute requirements for the specification of the myocardial cell fate. Nevertheless, similarly to what

happens with BMP signalling described above, the hypothesis that whiles some cardiac processes may require Wnt signal inhibition, others may require its activation, should not be excluded. (Fig. 7).

The FGF family

BMPs, Wnt/β-catenin/LEF/TCF antagonism and Wnt/JNK/AP1 activation are known to induce cardiogenic mesoderm, as indicated above. However, this induction does not occur with these pathways alone and the FGF family of signalling molecules, also present in endoderm, appears to be implicated in modulating cardiogenesis too [221].

The FGF family of ligands includes 22 members in humans and mice [222, 223]. FGFs signal by activating four distinct cell surface receptors, encoded in four different genes Fgfr1-4, that, through alternative splicing events, can produce many single-pass transmembrane receptor isoforms, belonging to the RTK family of cellular receptors [224, 225]. Ligand binding to the extracellular domain of the receptor, with the participation of HSPGs, stabilizes the formation of a receptor dimmer and initiates a signal transduction flow. Firstly, phosphorilation of multiple tyrosine kinase residues on the receptors occurs. Secondly, recruitment and assemble of particular signalling complexes takes place. Finally, a cascade of phosphorylation events occurs, that, ultimately, results in modification of gene expression [225]. The best understood FGF pathways are the RAS/MAPK pathway, the PI3K/AKT pathway and the PLCγ pathways [225]. During signal transduction, in many pathways, specific transcription factor families have been identified as the major protein targets that mediate the ligand induced cell responses. As examples the SMAD proteins in the TGFβ pathway and the LEF/TCF proteins in the Wnt signalling can be pointed out. In contrast, no comparable FGF-specific transcription factor mediators have been identified so far. In fact, primary target proteins of the FGF signalling cascades are the ubiquitously expressed transcription factors such as the Ets, AP1 and ATF/CREB proteins, also common to many other signalling pathways [226, 227]. In addition, different FGFs may stimulate the same receptor [228] implying that the FGF pathway has a great deal of redundancy, according to several knockout experiments in the mouse that have shown a complementary role of FGFs in several developmental processes [229-232]. Therefore, in accordance to this, a pertinent question may rise.

Why was this molecular family selected, as one of extreme importance in cardiogenesis?

One fundamental aspect to understand the importance of FGF signalling in cardiogenesis lies upon the nature of the elements controlling transcriptional activation, where enhancers and promoters associate with particular combinations of ubiquitously, cell type-specific and temporarily activated transcription factors to activate transcription. It is here, where the importance of FGF is notorious, because in association with other molecules, the RTK activity induced by FGF, is absolutely essential, as a competence and adjuvant factor, to drive, otherwise non-cardiomyogenic mesodermal cells, into a mitogenic and migratory cardiac pathway (remember the Mesp gene in early gastrulation embryos). However, we cannot rule out the hypothesis that the specific role of FGF in cardiomyogenesis is related to the indirect effects of these signals on heart development, as a result of their involvement in early mesoderm induction and cell migration, during gastrulation [233].

Accordingly, a more direct role of FGF in the specification of heart precursors, has been revealed in experiments in *Drosophila* in which FGF signalling is inhibited only at a time after mesoderm migration is completed [234, 235].

Additionally to *Drosophila*, the clearest data on the *in vivo* function of FGF signalling, directly in cardiac induction, has been obtained in the zebrafish system. Zebrafish FGF8 is expressed in the cardiogeneic fields of the lateral plate mesoderm and in specific regions of the neural tube. Fgf8 mutant embryos display severe heart abnormalities with a particular loss of ventricular structures with strong reductions in Nkx2.5 and Gata4 expression [236].

It has been shown by experiments in the chick system that FGF2 and FGF4 can induce cardiogenesis in non-precardiac mesoderm, although the induction of Nkx2.5, Gata4 and cardiac differentiation needs the presence of BMP2 or BMP4 [26, 237, 238].

Ectopic application of FGF8 in chick, can also prevent the loss of cardiac gene expression in embryos, in which inducing endoderm has been removed and the expression of cardiac markers is expanded when FGF8-soaked beads are placed lateral to the heart fields [21].

Finally, and importantly, cells of the secondary heart-forming field, express Fgf8, Fgf10, Nkx2.5 and GATA4 and may be recruited to a myocardial fate by signals coming from the existing outflow tract [35, 74, 239, 240]. This effect can be blocked by Noggin, an BMP antagonist. Because of the presence of FGFs and dependence on BMPs, it has been suggested that the primary and secondary heart fields share common mechanisms of myogenesis. Interestingly, BMP2 is able to upregulate FGF8 ectopically. It was suggested that FGF8 signaling acts downstream of BMP2 [21]. Taken together these results suggest a possible indispensable role of FGF signalling as a direct or indirect co-specification pathway in

cardiomyogenesis, maintaining and amplifying the expression of cardiac-specific genes, downstream of BMP signaling. (Fig. 7).

A transcriptional network in early cardiogenesis in vertebrates

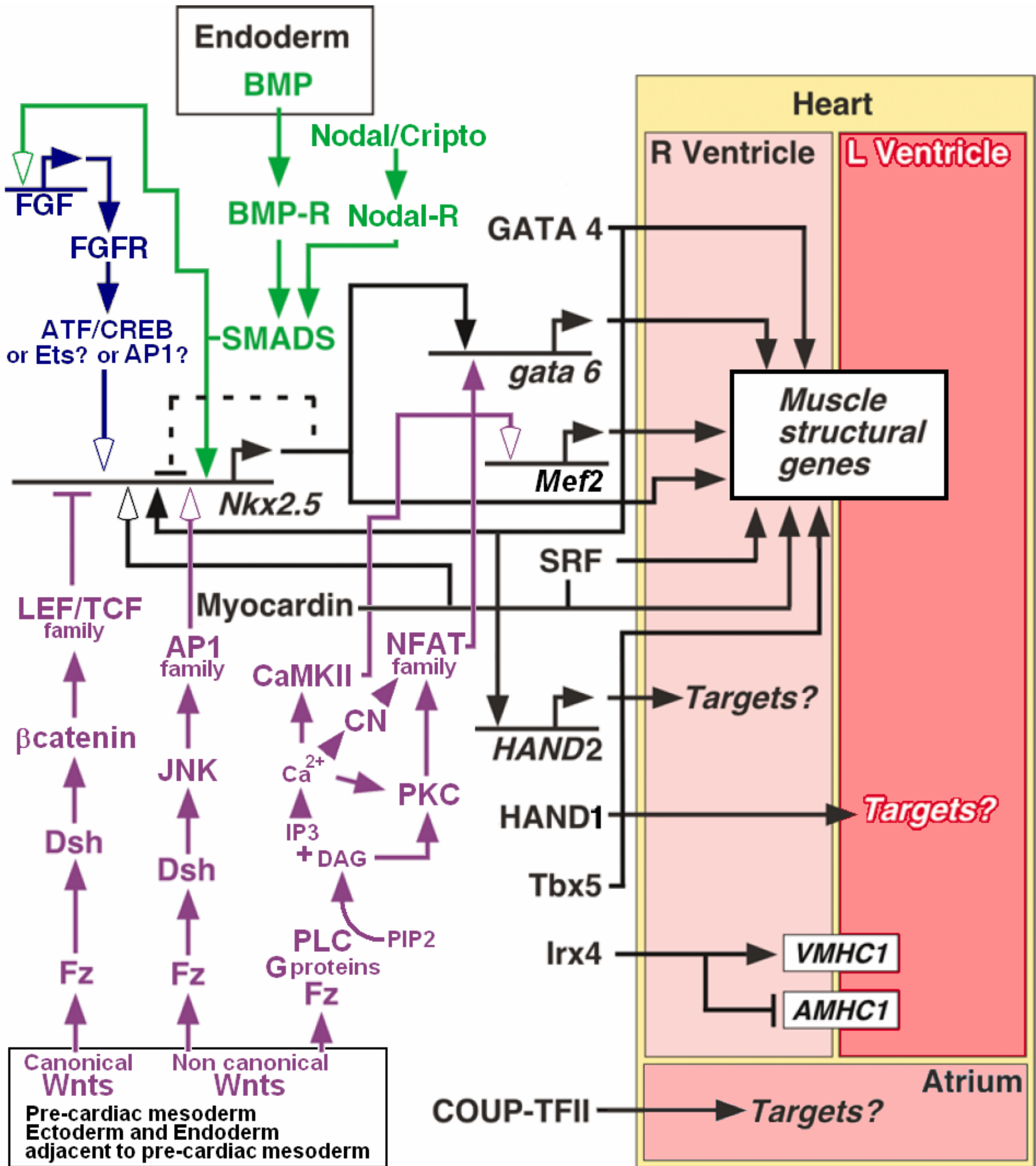


Figure 7 - A transcriptional network in vertebrates, resuming all the main molecular families in early cardiogenesis is mentioned above. The main transcriptional interactions involved in the early steps of vertebrate development are shown. Direct interactions of transcriptional activators with target are represented by solid arrowheads, and positive regulatory effects for which there is no evidence of a direct interaction are indicated with open arrowheads. Repressive direct influences, which have or not experimental evidence, are shown as solid or broken lines, respectively. Target genes are shown as lines with rightward pointing arrows signifying initiation of transcription. Activation of proteins along a signal transduction pathway is also indicated by a close arrow. Members of the TGFβ superfamily are shown in green, Wnt signalling is shown in purple, and FGF pathway in blue (in which the exact final effectors are not known, indicated by ?).
See text for details, but also for references for the respective pathways. Adapted from Cripps, RM and Olson, EN, Developmental Biology, **246**, page 21, 2002.

Axis and gene patternings

Initial mammal zygote cell divisions, without increasing in size, lead to the formation of a morula which soon will be transformed in a blastula. This has an embryonic ectoderm that will produce a middle layer of mesoderm and an inner layer of endoderm through the gastrulation. During this process the radially symmetrical zygote is transformed into an extended three-layered organism with a craniocaudal (CrCa), dorsoventral (DV), and left-right (LR) axis, orienting subsequent developmental stages (Fig. 8).

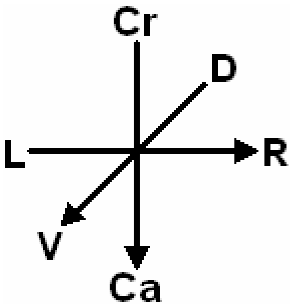


Figure 8 - Body axis

Using morphological and functional criteria, early embryonic development in placental mammals may be divided in five stages. These are: fertilization, cleavage, morula, blastula and gastrulation [241]. All placental mammals have a radially symmetrical ovum, which is polarized along its animal-vegetal (AV) axis, already evident at fertilisation. The animal pole of this axis is usually marked by the second oocyte polar body [242] and the opposite region will be the vegetal pole. The second polar body remains attached to the embryo, acting as an “axial marker” making possible to determine the spatial relationship to the other embryogenic axis, in the next stages of development [243].

After fertilization, maternal determinants, mRNAs or protein molecules that initiate gene transcription in the early embryo, also known as maternal dorsal or ventral determinants, are mobilized from the vegetal pole and migrate to the future dorsocaudal position in the morula, a dense aggregate of cells without a central hollow space. At the late-morula stage, the area in which the maternal determinants have accumulated is activated to form an induction centre.

Soon, asymmetric cell proliferation will originate an accumulation of cells in the embryonic pole, the inner cell mass. When the inner cell mass cells develop into the primitive ectoderm, above, and primitive endoderm, below, they become the blastoderm and the remainant cavity, the blastocyst cavity. It is the blastula stage. Now, the induction center, formed in the previous stage of development, induces the formation of a second induction centre, the mammalian node (Hensen´s node in birds). Also at this moment, the primitive ectoderm initiates the primitive streak. Remarkably, the node and the primitive streak will be very important in defining the definitive caudal end of the developing embryo and are correlated with the appearance of the definitive CrCa and DV axis.

The side of blastocyst on which the inner cell mass is localised, the embryonic pole, and the diametrically opposite side, the abembryonic pole, gives the embryo a distinct asymmetry along a new relevant axis, the embryonic-abembryonic (EmAb) axis. By this time, the inner cell mass, on a plan parallel to the AV axis, no longer forms a perfect round shape, but acquires an oblong form. The long radius defines an axis of bilateral symmetry of the early blastocyst which is normally aligned with the AV axis of the zygote and the orthogonal to its EmAb axis [244].

How the EmAb axis is established is controversial. Some authors have reported that this axis can be traced back to the first cleavage of the embryo [245-247]. According to these, the first cleavage plane coincides with the AV axis of the oocyte and will ultimately coincide with an axis perpendicular to the EmAb axis of the blastocyst. However, other authors have reported that there is no correlation between the first cleavage plane and the EmAb axis [248-250]. It is not clear whether this discrepancy was due to technical differences or differences in the mouse strains used for each study.

In summary, the AV and the EmAb axis will have strong correlation with the definitive CrCa and DV body axis, respectively, which constitute the first definitive axis to be established during the formation of the mammalian body plan, prior to and during the period of gastrulation [243]. The LR axis is specified at last, and is oriented orthogonally to the pre-existing CrCa and DV axes.

To understand the formation of the four-chambered heart is necessary to know which genes or molecules provide the young embryo with the positional and temporal information for the local specific development of subsequent structures of the primary linear heart tube. In line with this, it is pertinent to distinguish along the CrCa, the DV, and the LR axis, the patterns of regulatory genes and molecules that will activate or repress specific downstream targets responsible for differentiation and morphogenesis. To study these regionalized gene programmes, underlying cardiac formation, two strategies have been used, the transgenic animal models, to uncover the site-specific regulatory DNA sequences, and the ectopic inactivation or activation of genes for cardiac transcription factors. Additionally, the construction of quail/chick transplantation chimeras has been of great help in integrating the genomic patterning with fate maps. It should be noted, however, that the extrapolation of results from animal models to the human development might lead to erroneous conclusions and should be taken cautiously. A good example of this is the cup-shaped form of the mouse embryo, a very common animal model, whereas the human embryo is a flat disc.

In the next pages, studies that have provided such kind of insight are reviewed.

CrCa patterning

DiI labelling experiments have revealed, that the localization of cardiac progenitor cells in the primitive streak has poor correlation with the position of their descendents in the tubular heart of the single somite chick embryos. However, in subsequent stages a definitive pattern is formed, since the CrCa position of cardiac progenitors in the cardiac crescent correlates well with positions of cardiomyocytes in the heart tube [10]. For a given stage, these kinds of experiments are very useful in studying migrating cardiac progenitor populations, but include little information about the developmental pathway to which local regions of the heart tube are committed. For that, additional transplant experiments are needed. Accordingly, when early precardiac mesoderm from the cranial bilateral heart forming region is transplanted to more caudal positions, it develops the phenotype of its new location, suggesting that individual commitment may be imposed by regional signals [251]. Once definitive cranial and caudal commitment is achieved in endocardial tubes, just before their fusion, cranial and caudal cells will maintain their characteristics and no longer acquire the phenotype of the new locations in transplant studies [252]. In mammals, there is a very big lack of information about the commitment of cardiac cells. The exception is a study in mice that has shown that, after looping, the specific downregulation of atrial genes, in ventricle cells is apparently impossible to revert, since the latter cells, grafted into the atria no longer acquire the atrial phenotype [253].

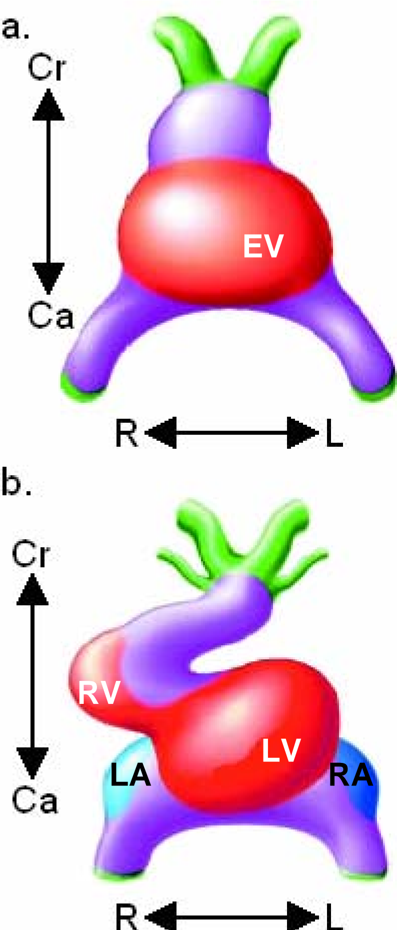


Figure 9 - Schematic representation of the CrCa axis and the LR axis in **a.** the mouse linear heart tube at E8.5 (the prototypical linear heart tube) and **b.** the looping chamber-forming heart at E9.5 (the prototypical looped heart). The primary heart tube is indicated in violet, the atrial chambers in blue or light blue, the ventricular chambers in red and the venous and arterial connections in green. EV - embryonic ventricle, RV - right ventricle, LV - left ventricle, LA - left atrium, RA - right atrium. Adapted from Moorman, AF and Christoffels, VM, Physiological Reviews, 83, page 1248, 2003.

One of the molecules that has been widely used to study cardiac patterning, chamber specification and morphogenesis is the morphogen, Retinoic Acid (RA) [254]. The signaling pathway of retinoids involves specific nuclear receptors that regulate gene expression.

RAR is the receptor for *all-trans* retinoic acid while RXR is the receptor for *9-cis* retinoic acid. RXR and RAR belong to a large superfamily of related nuclear hormone receptors, which share a common architecture. There is a conserved DNA-binding domain (DBD), and a ligand-binding domain (LBD). The sequences of RAR and RXR are related to each other, to the TR, VDR, testosterone receptor, and 150 other hormone/steroid/orphan receptors, all of which form the nuclear receptor family. There are important cross interactions between these nuclear receptors. RXR forms homodimers RXR-RXR or heterodimers as for example RXR-RAR, RXR-VDR or RXR-TR. These receptor dimers are the ligand inducible *trans*-regulators that modulate the transcription of target genes, by interacting with *cis*-acting specific sequences, RAREs, of cellular genes and up/down regulate its transcription. The role of RXR as a promiscuous dimerization partner may be related to the multiple effects of retinoids in mammals [255].

Variation in RA concentrations, during and shortly after gastrulation, have dramatic effects on cardiogenesis, indicating that the development phase that appears to be more responsive to RA, coincides with the time in which mesoderm acquires its cardiogenic fate. This occurs before the CrCa patterning can be observed. The outflow tract, primitive right and left ventricle are considered cranial structures, whereas the atrioventricular canal the atria and the inflow tract should be regarded as caudal (Fig. 9). Curiously, in chicken and mouse, excess of RA causes “caudalization” of the heart tube [256, 257], with the appearance of caudal phenotype in cells that normally would have cranial characteristics. Conversely, deficiency of RA, in quail, causes underdevelopment of the sinus venosus and atria. However, the expression of cardiomyocyte differentiation genes, including atrial and ventricular myosin heavy chains and Nkx2.5 is not altered, reinforcing the idea that a retinoid mediated cardiogenic pathway is unlinked to cardiomyocyte differentiation but involved in the morphogenesis of the caudal heart tube [258, 259].

In chicken embryos, the expression of Raldh2, a key enzyme for the synthesis of RA [260], is also confined, exclusively, to caudal mesoderm, in particular, from the four somite stage onward [261]. In mouse, the Raldh2 expression is initiated shortly after gastrulation and is, as well, characteristic of caudal cardiac progenitors [262]. Interestingly, the sites of RA synthesis colocalizes with its target sites, as observed using a RARE lacZ transgene [263]. This means that when the tubular heart has formed, RA synthesis and response are restricted

to the caudal region of the heart, the sinus venosus. Therefore, it is expected that mutants for Raldh2 show severe caudal cardiac malformations, like impaired atria and sinus venosus. Indeed, that was observed in a recent study in mice [264].

These observations support a model that proposes that the influence of endogenous RA on heart development, depends upon localized synthesis of the ligand, and that the diffusion of this molecule is limited [263]. In this line of thought, it is easy to imagine that the RA gradient, starting with a stronger intensity at the caudal region of the heart, will originate, in early cardiogenesis, a selective contact of caudal cells with RA, constituting one of the main causes of CrCa patterning of the linear heart tube. Accordingly, RA seems to delay the differentiation of cranial cardiomyocytes, as the differentiation of the ventricular myocytes of Raldh2-deficient mouse embryos was more advanced than that of wild-type embryos at E8.5 [264].

Interestingly, the RARE containing gene, the quail Smyhc3, is initially expressed in both cranial and caudal regions. However, once the CrCa patterning becomes established, Smyhc3 expression becomes restricted to caudal region, contributing to the establishment of the caudal phenotype, inflow tract and atria. Because Smyhc3 is downregulated when the RA synthesis is blocked, it was suggested that RA was the important molecule in this "caudalization" process [257, 265].

Recent studies have brought some light into the mechanism underlying the appearance of the atrial-specific expression of Smyhc3. Firstly, it was suggested that the VDR-RXRα receptor binds a VDRE in the Smyhc3 gene promoter and was responsible for the regulation of its atria specific expression. However, this transcriptional interaction also occurs in the ventricles. Thus, this explanation could not be sufficient. In addition, it was discovered, in the chicken, that an Iroquois family homeobox gene, called Irx4, downstream of Nkx2.5 and Hand2, activates the expression of the VMHC1 gene and suppresses the expression of the AMHC1 gene (the chicken homologue of Smyhc3), in ventricles. In addition, Irx4 expression was restricted to the ventricular chambers at all stages of development. Notably, this molecule was found to be the missing link, explaining the atrial-specific expression of Smyhc3. Irx4 interacted with the RXRα component of the VDR-RXRα heterodimer and formed an inhibitory protein complex that binds at the VDRE, inhibiting AMHC1 or Smyhc3 expression in a ventricular specific fashion [266-268].

Other molecules that appear to be involved in the RA signalling pathway are the GATA transcription factors. In RA-treated Xenopus embryos, Gata-4, -5 and -6 are specifically upregulated [117]. Moreover, in quail or chicken embryos, supplementation with RA or its

deficiency induces upregulation or downregulation of Gata-4 expression, respectively [258, 269]. All the three Gata genes are expressed in, and caudal to, the heart-forming region of chick embryos. Accordingly, and with emphasis in Gata-4 expression in mice, they are expressed with higher levels in the caudal heart tube, relatively to the cranial heart tube [13]. Again, in mouse embryos, Gata-4 is expressed in the inflow tract of the fusing heart tube [270]. Taken together, these observations suggest that Gata family members, in particular Gata-4 are possibly morphogenetic players in the establishment of the CrCa patterning of the forming heart. However, they do not appear to interfere with the specification or differentiation of cardiac progenitors. In fact, although heart fusion and folding is severely affected in RA-deficient or Gata-4 deficient embryos, the expression of the cardiomyocyte differentiation marker Nkx2.5 or the chamber-specific marker Anf are not affected in these cells [13, 43, 270]. Nevertheless, Gata factors restrict the Nkx2.5 expression domain to more cranial regions of the embryo, having, in this way, a regulatory role in Nkx2.5 dependent myocardial differentiation [117]. Actually, Nkx2.5 and Gata factors may physically associate and cooperatively activate transcription of target genes. They act within a mutually reinforced transcriptional network to control cardiac gene expression, in cell culture experiments [271-273].

Tbx5 is also a transcription factor that, like Gata4, is upregulated by RA. Therefore, is a good candidate for the regulation of the CrCa patterning linked to RA signalling. As Gata4, it is also expressed in an increasing CrCa gradient in the heart forming region and in the tubular heart [54, 269, 274]. However, Tbx5, contrarily to Gata-4, may also be involved in cardiac specification and differentiation, since normal cardiogenesis was, nearly, completely blocked by an inducible dominant negative isoform of Tbx5, in Xenopus [275]. Additionally, Tbx5 mutant murine embryos also present a downregulation of the expression of Gata-4, Nkx2.5 and of the chamber-specific genes Cx40 and Anf [276]. βMhc-Tbx5 transgenic mice, ectopically expressing Tbx5 in the tubular heart, show a loss of cranial gene expression and a delay in ventricular development [269]. Tbx5 deficiency results in failure of cardiac looping and abnormalities of both atria and ventricle in zebrafish [277]. In mice the latter deficiency leads to severe hypoplasia of caudal heart structures, supporting the role of Tbx5 as a “caudalization” molecule [276]. Accordingly, Tbx5 haplo-insufficiency, causes less severe phenotypes, like defects in the septa and conduction system in humans and mice [276, 278, 279]. As mentioned above, these findings are compatible with a role of Tbx5 in CrCa patterning as a caudal inducer. However, its role is a little more complex than that,

since the murine Tbx5 mutants, surprisingly, also downregulate the expression of cranial heart tube-specific genes Mlc2v, Irx4 and Hey2 [276].

One other class of molecules that appear to be involved in CrCa patterning are the Chicken Ovalbumin Upstream Promoter Transcription Factors (COUP-TFs), which are members of the steroid/thyroid hormone receptor superfamily. They are often called orphan receptors, since their ligands have not been identified. Coup-TF homologs have been cloned in many species, from Drosophila to human. Coup-TFI and Coup-TFII were cloned from the mouse and human, and their genomic organization characterized [280]. Mice with a deletion of the Coup-TFII gene have, as a result, embryonic lethality with defects in angiogenesis and heart development [281]. Curiously, Coup-TFII gene expression is normally restricted to the caudal structures of the heart and, accordingly, these mice have shown underdeveloped atria and sinus venosus. Coup-TFII was suggested to indirectly mediate endothelial-mesenchymal interactions, essential for proper angiogenesis and heart development. This indirect effect was due to the downregulation of Angiopoietin-1, a proangiogenic soluble factor thought to mediate the endothelial-mesenchymal interactions, during heart development and vascular remodeling.

DV patterning

DV differences in transcription regulation can still be detected in the early tubular heart, preceding the stage of chamber formation. They function in conjunction with the CrCa patterning in the control of cardiac looping and chamber expansion. After cardiac looping, the ventral side of the ventricular region and the dorsal side of the atrial region will constitute the outer curvatures of the heart (Fig. 10). The inner curvature corresponds to the original dorsal side of the heart tube, at the ventricular level, and the ventral/caudal [282] side at the atrial level. Actually, there are only a very restricted number of genes known to have a clear differential expression between the ventral and dorsal sides of the heart. Hand1 is one of those that are specifically expressed at the ventral side and caudal half of the linear heart tube, in the E8.5 mouse embryos. In the E9.5 mouse hearts the gene becomes expressed in the outer curvature of the left ventricle, the left side of the atrioventricular canal and weakly in the outer curvature of the right ventricle/outflow tract (original ventral sides) and is absent in the atria and inner curvature [54, 282, 283]. Although Hand1 null mice died very early in development, just before heart looping and expansion, the expression of the chamber specific gene Anf and the cranial heart tube marker Mlc2v could be detected. This indicates that the chamber-specific program and the CrCa patterning were not severely affected [284, 285].

Additionally, when chimeric mice were generated from Hand1-null ES cells and ROSA26 embryos, mutant cells were underrepresented in the left caudal region of the linear heart tube at E8.0. By E9.5, after cardiac looping, mutant cells were almost absent in the outer curvature of the heart tube ventricular myocardium, but did contribute to parts of the left ventricle and to other cardiac chambers [286].

On the other hand, *in vitro*, Hand1-null ES cells were able to differentiate into beating cardiomyocytes that expressed cardiac myosin and several cardiac-specific transcripts including Nkx2.5, α-cardiac actin, and the myofilament genes Mlc2a and Mlc2v [286].

Although the early lethality of Hand1-null mutant mouse embryos has precluded a precise understanding of its function, these results imply that Hand1 is not essential for differentiation of ventricular cardiomyocytes but plays an important role in the morphogenesis of the outer curvature (original ventral side) of the heart tube.

The gene enconding atrial natriuretic factor, Anf, was not expressed in the early linear heart tube. It is first found around E8.25 mouse embryos' stage, on the ventral side of the heart tube [54, 287]. After cardiac looping, Anf was seen strongly in the outer curvature of the left ventricle, more weakly in the outer curvature of the right ventricle and in the atrial primordial regions at the laterodorsal sides of the caudal heart pole. When the Anf-positive regions expand, between E9 and E11, and the atrial regions become positioned cranially to the ventricular region of the tube, the expression of Anf decreases in the right ventricle and become restricted to the auricles of the atria and left ventricular trabeculations [54].

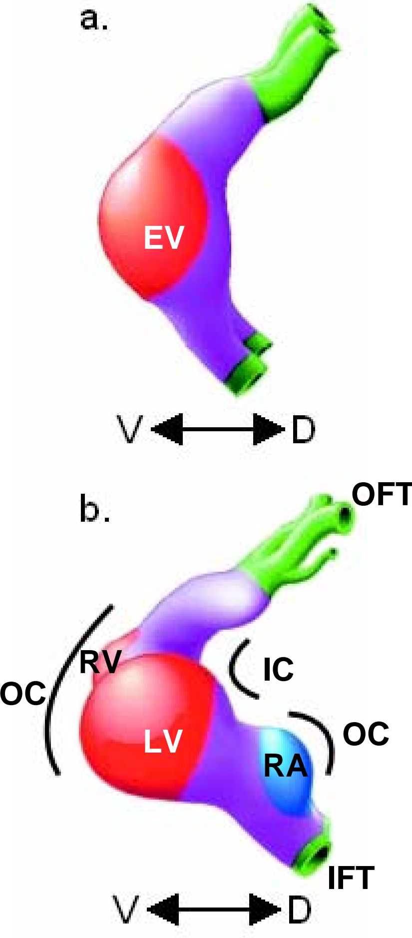


Figure 10- Schematic representation of the DV axis in **a.** the mouse linear heart tube at E8.5 (the prototypical linear heart tube) and **b.** the looping chamber-forming heart at E9.5 (the prototypical looped heart). The primary heart tube is indicated in violet, the atrial chambers in blue and light blue, the ventricular chambers in red and the venous and arterial connections in green. EV - embryonic ventricle, RV - right ventricle, LV - left ventricle, RA - right atrium, OFT - outflow tract, IFT - inflow tract, OC - outer curvature, IC - inner curvature.
Adapted from Moorman, AF and Christoffels, VM, Physiological Reviews, 83, page 1248, 2003.

It is because Anf is expressed solely in the outer curvature of the atrial and ventricular compartments of the early heart tube and not in the inflow tract, atrioventricular canal, outflow tract and inner curvature, that Anf is qualified as an important marker for analysing the formation of chamber myocardium. Similar to Anf, other genes are expressed selectively at the ventral side of the heart tube. Chisel and Cited1 are first expressed in E8.25 and Irx3, in E9 embryos, in the myocardium, whereas Irx5 is expressed in the endocardium of E9 embryos. This confirms the presence of DV patterning in the tubular heart. Because Anf, Chisel, Cited, Irx3 and Irx5 become restricted to the forming chambers, it is easy to understand why DV patterning and chamber formation must be related.

LR patterning

During embryogenesis, an important evidence of increasing complexity is the appearance of the third axis of asymmetry, the LR axis. It is generally accepted that it is in the gastrulation phase that embryo’s symmetry is first broken, precisely in the region of the node (Hensen´s node in birds). The current model that explains these interesting processes, and appears to be common to all vertebrates, is called the “nodal flow model”. On the ventral surface of the mouse node, a specialized cluster of monocilia can be found, which is considered the mammalian equivalent of the early embryonic organizer region, identified through classical transplantation studies in Xenopus. Analysis of that cluster of monocilia has shown that they project themselves into the extraembryonic space surrounding the egg cylinder and exhibit a vertical motion that generates an apparent leftward flow of extraembryonic fluid around the node’s region [288-290]. This flow that is proposed to be responsible for the allocation of one or more extracellular factors, thus triggering the activation of distinct signalling pathways on the left and right sides of the embryo (Fig. 11).

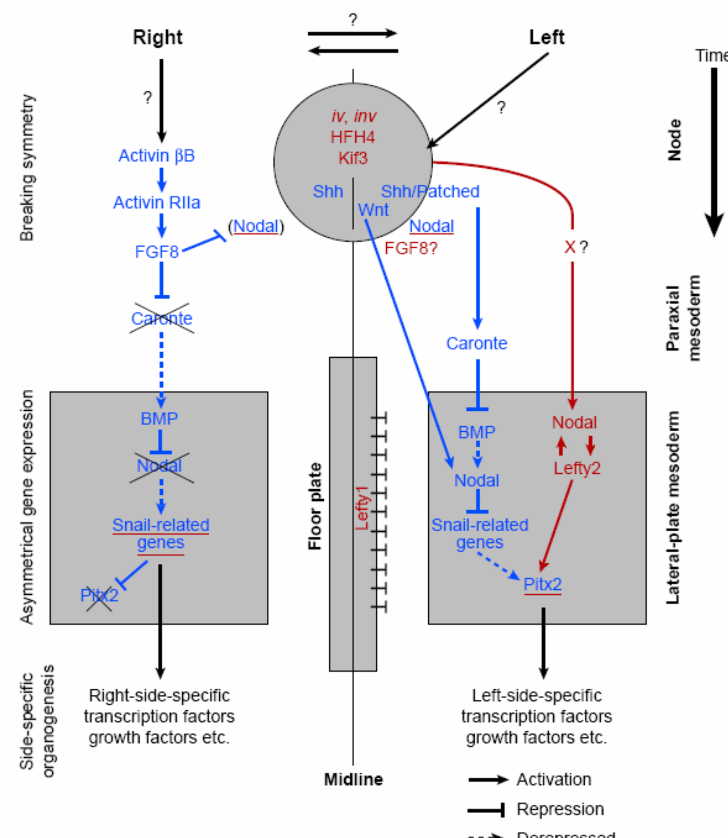


Figure 11 - Schematic representation of the molecular interactions that appear to be involved in the creation of the LR axis in the mouse (red) and in the chick (blue). The establishing of LR asymmetry during development can be divided into three phases, the first is the breaking of LR symmetry, which is thought to take place in the node (Hensen's node in birds) or its progenitors. In the chick node, the asymmetrical expression of Sonic hedgehog (Shh) is established by repression of the gene on the right through an Activin-related signalling pathway that involves the Activin type IIa receptor. Through its interaction with its receptor Patched, Shh induces the expression of nodal at the left side of the node and Caronte (Car), a secreted growth factor, member of the cerberus/dan family, in the paraxial mesoderm. Wnt8c is expressed only on the left. In the mouse node, the inversus viscerum (iv), inversion of embryonic turning (inv) genes, the molecules Kif3 and hepatocyte nuclear factor 4 (HFH4) may be involved in initiating asymmetry. So, nodal is expressed bilaterally at first and then becomes restricted to the left side.

The second phase begins with the induction of genes that are left-side specific in cells of the node, which initiates the transcription of genes coding for a cascade of signalling molecules. In the chick, as the Shh receptor, Patched, is found only close to the node, it seems unlikely that Shh is the molecule carrying the signal from the node to the lateral-plate mesoderm. This role is attributed to Car, that mediates between Shh and the induction of nodal in the lateral-plate mesoderm by suppressing BMP2 and BMP4 signaling. Unilateral expression of Car, stimulated by the left-sided production of Shh in Hensen's node, is reinforced by FGF8, which represses Car expression on the right. A separate signalling pathway involving a protein of the Wnt family, is also involved in maintaining the unilateral expression of nodal. In the mouse, Nodal, Lefty1 and Lefty2 genes are expressed transiently in the lateral-plate mesoderm only on the left. This strongly implicates the Nodal, Lefty1 and Lefty2 proteins in the molecular pathway that sets up the LR axis, however, molecules carrying the signal from the node to the lateral-plate mesoderm are not known. Nevertheless, interactions between Lefty1, Nodal and Lefty2 are essential for the generation of adequate mesoderm.

Finally, the third phase is the conversion of this unilateral gene expression into situs-specific morphogenesis. On the left, Nodal directly or indirectly induces the left-side-specific expression of Pitx2, a bicoid-related homeobox gene encoding a transcription factor that regulates several genes involved in left-side specific organogenesis. On the right, BMP represses Nodal and so the Snail-related gene is derepressed, allowing right-side-specific organogenesis to occur. Adapted from "Axis Formation in Vertebrate Embryos: a Comparative Approach" Eds S.-L. Ang, R. R. Behringer, H. Sasaki, J. S. Altman and C. Coath. HFSP, page 145, Strasbourg, 2001.

Additionally to the scheme presented in the Fig. 11, another relevant developmental pathway, the Notch signaling pathway also controls Nodal transcription. Activation of Notch receptors by the ligand Dll-1, leads to the cleavage and nuclear translocation of the Notch intracellular domain, where it acts as a transcription factor when bound to the DNA-binding protein RBP-J [291]. Loss of function mutations, in components of the Notch pathway, leads to loss of LR asymmetry. Moreover, RBP-J-binding sites found within the Nodal enhancer are required for node-specific expression of Nodal [292, 293]. These data demonstrate that Nodal is a direct target gene of the Notch signaling pathway; however, the relationship between Notch activity and symmetry-breaking events in the node is not clear.

The cascade of signalling molecules that regulate the organization of LR identity of the embryo converges into the expression of snail-related genes in the right side and the homeodomain factor Pitx2 in the left side of visceral organs, including the heart. This appears to be sufficient to establish LR patterning in the heart. In the mouse, the first morphological sign of a break in LR symmetry is the rightward looping of the heart tube at about E8.5, soon followed by the asymmetrical development of the various visceral organs [294, 295]. However, this is preceded, during gastrulation, by a cascade of intercellular molecular interactions and formation of the lateral plate mesoderm in a way that when the left and right endocardial tubes fuse to form the tubular heart, they will have, as their forming region, a left and right identity. Although their contributions to the fused heart are almost the same, the left heart forming region contributes more to the cranial portion of the tube and the right one more to the caudal part [10], a clear evidence of the CrCa patterning influence. Interestingly, in an abnormal situation where one or more of the individual organs systems develops with reversed LR polarity, a condition that results from the embryological failure to properly coordinate the asymmetric development of multiple organ systems, before looping, the heart is normally formed in relation to its CrCa and DV patternings, indicating their independence from the LR specification [283]. Curiously, RA, referred above as related to the CrCa patterning, when in excess or deficiency, can cause also a condition of randomized cardiac looping [296, 297]. This involvement of RA in the LR signalling was associated with its role on the regulation of the level and location of several genes in the LR cascade, namely Lefty1, Nodal and Pitx2 [296, 298, 299]. In fact, the presence of a RARE in the Nodal gene regulatory region, was recently shown [300].

If one follows the Pitx2 expression, from the moment of the appearance of the bilateral cardiogenic plate, it is possible to identify the LR origin of several regions, in the looping heart. Pitx2 transcripts are present in the left portion of the cardiac crescent and in the left side

of the heart tube. Through looping, Pitx2 is present in the left atrium, in the ventral portion of the ventricles and in the left-ventral part of the outflow tract [301]. At first sight, it looks that ventricles do not respond to the LR axis, however, it is useful to remember that the ventricles are aligned along the CrCa and DV axis before looping, and are composed of left and right-forming regions. After looping, the LR arrangement of the heart tube is converted into a DV arrangement, where the left half is found at the ventral side and the right half at the dorsal side of the heart.

Nevertheless, it is important to note that LR patterning is not, by itself, the most important patterning in the heart chamber formation process. Instead, it should be regarded as an accessory signalling pathway that cooperates with the main CrCa and DV patternings for the correct morphogenesis of the four-chambered heart, late in development.

Resuming cardiac myogenesis in a two-step process, one can first consider the formation of a primary linear heart tube, and secondly, the progression of chambers differentiation and morphogenesis in which the patternings along the CrCa and DV axes gives important positional information for the site-specific processes. Curiously, the resultant atrial and ventricular working myocardium are both activated and connected to each other by primary linear heart tube-like myocardium that, ironically, has been named “specialized” several times.

To further understand cardiac development, it is necessary to continue the characterization of its building blocks and then integrate the various molecular pathways imposed on the primary heart field and on the forming heart tube, a task that is far from being complete.

In the next page a list of the most studied and expressed genes in cardiogenesis is given on Table 3.

Table 3 - Gene expression in the linear heart tube, before looping, and in the embryonic heart, after looping. The patterns of expression are schematically represented in the main regions of the looping heart. The levels of expression of a certain gene should be comparable only within one development stage, and because many of these studies are accomplished in slightly different time points in the developing heart and use different animal models, comparison between genes of different studies should be avoidable. For more information about the listed genes, the respective references are mentioned. It is important to note also, that these patterns of gene expression are specific of the developmental moment and thus, may not be maintained in the fetal or adult stages.
x - non detected; • - weak expression; •• - middle expression; ••• - strong expression; Cr→Ca - crescent gradient of gene expression from the cranial region to the caudal region of the linear heart tube; Ca→Cr - crescent gradient of gene expression from the caudal region to the cranial region of the linear heart tube; **?** - no studies were found.

Before Looping | Cranial ← Embryonic Heart → Caudal

Gene	LHT	OFT	RV	LV	AVC	LA	RA	IFT	Refs.
AChE	x	x	x	x	x	•	•	x	[302]
Anf	x	x	•	•••	x	•••	•••	x	[54]
BCK	•••	••	••	••	••	••	••	••	[303]
Bmp2	•••	•••	x	x	•••	x	x	x	[11, 18, 304-306]
Bmp4	•••	•••	x	x	x	x	x	•••	[18, 304, 305, 307]
Bmp6	x	•••	x	x	•••	x	x	x	[306]
Bmp7	•••	•••	•••	•••	•••	•••	•••	•••	[306]
BmprIa (Alk3)	•••	•••	•••	•••	•••	•••	•••	•••	[308, 309]
BmprIb	x	x	x	x	x	x	x	x	[309]
BmprII	•••	•••	•••	•••	•••	•••	•••	•••	[310]
cardiac MyBP	•••	•••	•••	•••	•••	•••	•••	•••	[311]
cardiac TnC	•••	•••	•••	•••	•••	•••	•••	•••	[312]
cardiac TnI	•••	•••	•••	•••	•••	•••	•••	•••	[312]
cardiac TnT	•••	•••	•••	•••	•••	•••	•••	•••	[312]
Carp	••	••	•••	•••	••	•••	•••	••	[39]
Chisel	•••	x	•••	•••	x	•••	•••	x	[54]
Cripto1(Tdgf1)	•••	•••	x	x	x	x	x	x	[170]
cTnI	Cr→Ca	•	••	••	••	•••	•••	•••	[313, 314]
Cx40	x	x	•••	•••	x	•••	•••	x	[99]
Cx43	x	x	•••	•••	x	•••	•••	x	[99]
Desmin	•••	•••	•••	•••	•••	•••	•••	•••	[315]
Desmin-lacZ	••	•••	•••	•	x	x	x	x	[316]
fast TnI	•••	•••	•••	•••	•••	•••	•••	•••	[317]
Fgf10-nlacZ	x	•••	•••	x	x	x	x	x	[318]
Fgf2	•••	x	?	?	?	?	?	x	[319]
Fgf4	x	x	?	?	?	?	?	x	[319]
Fgf8	•••	•••	x	x	x	x	x	x	[68, 320-322]
Fgfr1	•••	•••	•••	•••	•••	•••	•••	•••	[323-325]
Fgfr2	x	x	x	x	•••	x	x	x	[323, 324]
Fgfr3	x	x	x	x	•••	x	x	x	[324]
fgfr4	?	x	x	x	x	x	x	x	[326]
Fibronectin	••	••	••	••	••	••	••	••	[327]
Gata4	••	••	•••	•••	••	•••	•••	•••	[125, 270]
Gata5	••	••	•	•	?	••	••	?	[328]
Gata6	•••	•••	•••	•••	•••	•••	•••	•••	[329]
Gata6-lacZ	•••	••	••	•••	•••	x	x	x	[330]
H19	•••	•••	•••	•••	•••	•••	•••	•••	[331]
Hand1	•••	•••	•••	•	•	•	•	•	[14, 282]
Hand2	•••	•••	•••	•	•	•	•	•	[14, 332]

Hey1	•••	•••	x	x	x	•••	•••	•••	[333, 334]
Hey2	•••	x	•••	•••	x	x	x	x	[333, 334]
Hop	•••	?	•••	•••	?	•••	•••	?	[335]
Irx4	Cr→Ca	•••	•••	•••	•	x	x	x	[54]
Irx5	••	x	••	••	x	••	••	x	[54]
Isl1	x	•••	•••	•	•••	•••	•••	•••	[68]
Jagged1	?	•••	•••	••	••	••	••	••	[336]
MCK	x	•	•••	•••	•	•	•	•	[337]
Mef2a	••	••	••	••	••	••	••	••	[337, 338]
Mef2b	••	••	••	••	••	••	••	••	[337, 338]
Mef2c	••	••	••	••	••	••	••	••	[337, 338]
Mef2d	••	••	••	••	••	••	••	••	[337, 338]
Mlc1a	•••	x	x	x	•	•••	•••	•••	[339]
Mlc1v	•••	•••	•••	•••	•••	x	x	x	[339]
Mlc2a	Ca→Cr	x	x	x	•	•••	•••	•••	[340]
Mlc2v	Cr→Ca	•••	•••	•••	•	x	x	x	[54]
Mlc3f-nlacZ	x	x	•	••	•••	••	•••	••	[341]
Msg1	•••	x	••	•••	x	•	•	x	[342]
Myocardin	•••	•••	•••	•••	?	•••	•••	?	[134]
NaKATPaseα1	•••	•••	•••	•••	•••	•••	•••	•••	[343]
NaKATPaseα2	••	••	••	••	••	••	••	••	[343]
NaKATPaseα3	x	•	•	•	•	•	•	•	[343]
Ncadherin	•••	?	•	•	?	?	?	?	[344, 345]
NCX1	•••	•••	•••	•••	•••	•••	•••	•••	[346]
Nkx2.5	•••	•••	•••	•••	•••	•••	•••	•••	[103]
Noggin	•••	x	x	x	x	x	x	x	[347]
Notch1	•••	•••	•••	•••	•	•	•	•	[336, 348]
Pitx2	•••	•••	•••	•••	•••	•••	x	•••	[349]
PLB	x	•••	•••	•••	•	••	••	•	[350]
RyR2	•	•••	•••	•••	•••	•••	•••	•••	[351]
Serca2a	Cr→Ca	•	••	••	•	•••	•••	•••	[54, 87]
slow TnI	•••	•••	•••	•••	•••	•••	•••	•••	[314]
SM Calponin	•••	•••	•••	•••	•••	•••	•••	•••	[352]
Srf	?	•••	?	?	?	?	•••	?	[353]
Tbx2	•••	•••	x	x	•••	x	x	•••	[354, 355]
Tbx2	x	•••	x	x	•••	x	x	•••	[356, 357]
Tbx20	•••	•••	•••	•••	•••	•••	•••	•••	[358]
Tbx3	x	x	x	x	•••	x	x	•••	[357]
Tbx5	Cr→Ca	x	x	••	•••	•••	•••	•••	[357]
Tef1	?	•••	•••	•••	•••	•••	•••	•••	[359]
Tropomodulin	•••	•••	•••	•••	•••	•••	•••	•••	[360]
Tropomyosin	•••	•••	•••	•••	•••	•••	•••	•••	[361]
Vmhc1	••	•	••	••	•	x	x	x	[10, 362]
α4 integrin	x	•••	x	x	x	x	x	x	[363]
α5 integrin	x	••	•••	•••	••	•••	•••	••	[364]
α6 integrin	•	••	••	••	••	•••	•••	••	[365]
α7 integrin	••	••	••	••	••	••	••	••	[365]
αcardiac actin	Cr→Ca	•	••	•••	•••	•••	•••	•••	[366]
αMhc	Ca→Cr	x	x	x	•	•••	•••	•••	[339]
αSMA	•••	x	x	x	x	x	x	x	[367]
β1 integrin	••	••	••	••	••	••	••	••	[365]
β4 integrin	x	•••	x	x	•••	x	x	•••	[368]
βMhc	•••	•••	•••	•••	•	•	•	•	[54]

The cardiomyocyte

It was clear from the previous sections that endocardium, myocardium and epicardium have, essentially, a mesodermal origin, where the minor contribution of neural crest cells is largely confined to the formation of the aorto-pulmonary septum at the arterial pole of the heart [369] whereas the endoderm only acquires a regulatory role [73]. Moreover, cardiac mesenchymal cells can be endocardium-derived, mainly to the mesenchymal cardiac cushions that will form part of the cardiac septa, which later can be transformed in cardiac muscle, or they can be also contribute to the mesenchymal cardiac cushions. Additionally, however, epicardium-derived cardiac mesenchymal cells, contribute to all of the cellular elements of the subepicardial connective and coronary tissue, namely cardiac fibroblasts, endothelial and smooth muscle cells of the coronary arteries, as well as blood progenitors and even a small number of cardiomyocytes and endocardial cells [45].

Therefore, if all the cardiomyocytes came from mesoderm, it is clear that there are 2 main ways by which thay can arise. Thus, they can either differentiate, directly from mesoderm or come from caudal cardiac tissues. For this reason, it is always a difficult task trying to classify the different types of cardiomyocytes according to their origin. This is, probably, why the two most used criteria used to date to classify the different types of cardiac muscle cells have been based in their electrical and phenotypical characteristics.

Different cardiomyocytes are capable of producing different intrinsic cycles of electrical activity and perform different types of contraction. Accordingly, all cardiac muscle cells have a leading or latent pacemaker activity, are connected by gap junctions, which allow the depolarizing impulse to travel over the myocardium, possess sarcomeres and a more or less developed sarcoplasmic reticulum. Considering these characteristics is possible to classify the cardiac muscle cells in four groups [46], as: nodal cardiomyocytes; primary cardiomyocytes; atrioventricular bundle, bundle branches and peripheral conductive cardiomyocytes and working cardiomyocytes.

Nodal cardiomyocytes

Nodal cardiomyocytes are the cells with the fastest intrinsic rate and responsible for the leading pacemaker activity that stimulates the whole heart to contract and determines its rate. If these cells were to stop, other cardiomyocytes would take over, although at a slower pace. They have a specific arrangement, composition and density of gap junctions and ion channels, characteristic of its slow conduction velocities.

Because automaticity requires poor intracellular coupling, it allows the loading of the cells to a threshold value of electric charge, resulting in depolarization of the adjacent myocardium. Logically, their contractile apparatus is not a well developed structure. All these features are present in the embryonic heart tube. These are the cells often called "specialized".

Primary cardiomyocytes

Vertebrate embryonic heart tube cardiomyocytes have, as the nodal cardiomyocytes, a relatively high automaticity and slow conduction velocities that are absolute prerequisites for peristalsis of the primitive heart. Additionally, they share with all the conductive cardiomyocytes (nodal and others) the poor contractility and contractile apparatus development.

Atrioventricular bundle, bundle branches and peripheral conductive cardiomyocytes

With the development of the synchronous contracting regions of the four-chambered heart, higher conduction velocities of the electric impulse are required, a task that is accomplished by these well-coupled cells. They possess also a relatively high automaticity, however always at a slower rate than the leading pacemaker does.

Additionally, and because contractility is not their main function, they all share a poorly developed contractile and sarcoplasmic reticular apparatus mostly resembling the embryonic cardiomyocyte.

Working cardiomyocytes

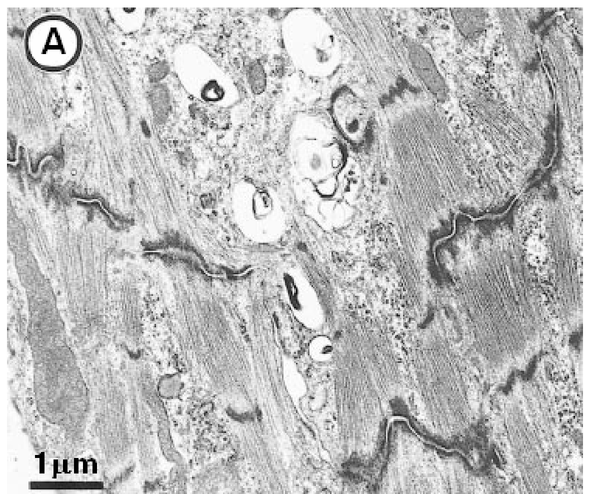
Atrial and ventricular working cardiomyocytes are usually distinguished from their conduction system counterpart by their well developed sarcomeric and sarcoplasmic reticular structures. However, they share with conductive cardiomyocytes the well coupled intracellular organization and the fast conduction velocities of the depolarizing impulse. They are the main contributors for the synchronous high contractility of the four-chambered heart.

In the present context, this last group is the most relevant regarding its fundamental importance in cardiac cellular therapies, because, generally, the main need of a patient with chronic cardiac dysfunction is to assure a reasonable left ventricular ejection fraction, that will only be obtained by regenerating or healing the working myocardium. The current experimental work was conducted with the objective of understanding the

first basic steps in obtaining and selecting the best number of semi- or terminally-differentiated, human working cardiomyocytes that could be used in transplant studies.

Once these steps are understood, and independently of the primary source of the working cardiomyocytes (from embryonic or adult cells), it is very important to rigorously determine the extent to which these cardiac cells home, in an injured myocardium. If their homing capability were exceedingly low, then, the ability to exploit these processes for clinical benefit would likely be quite limited.

One of the crucial points, related to the homing event, is the correct intercellular assembly of the transplanted and the resident cardiomyocytes. The first insights on the capability of adult cardiac cells to form new intercellular contacts came from a study with adult rat ventricular heart cells, *in vitro*, where it was observed that after isolation, these cells undergo a process of dedifferentiation-redifferentiation of the cardiac phenotype [370]. As expected, after isolation and subsequent attachment, adult rat ventricular cells, did not divide or move on the substratum in culture. However, the original elongated shape of ventricular cardiomyocytes turned into a flat polygonal shape and the contractile/cytoskeletal apparatus, along with the intercalated disks, start to disassemble. Accordingly, after 48h in culture, the three distinct types of intercellular junctions present in the intercalated disks, gap junctions, fascia adherens and desmosomes could no longer be detected, with immunofluorescence techniques. This was followed by subsequent regeneration of the myofibrillar and cytoskeletal apparatus and the restoration of mechanical and electrical coupling between redifferentiating cardiomyocytes (Fig. 12). Curiously, the formation of desmosomes and fascia adherens appears to be a prerequisite for CX43 assembly into functional gap junctions. Therefore, it was suggested that all these events might be important in cardiac cell therapy, as the synchronization of mechanical and electrical activity between donor and native regions might be difficult to attain. In spite of the fact that it is not well understood whether similar processes occur in human pathological conditions, cardiac cell therapy clinical trials have been taking place, since the beginning of this century.



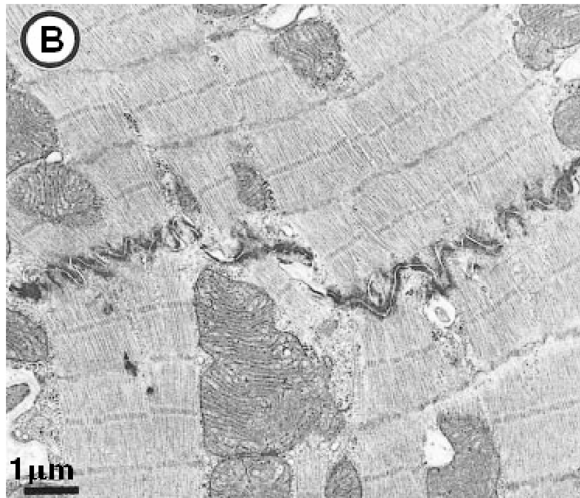


Figure 12 - Ultrastructural images of adult rat cardiomyocytes. **A**, After 2 weeks in culture. **B**, *in situ*.
In Kostin S, Hein S, Bauer EP, Schaper, *Circ Res,* 1999, 85: page 163.
Differences in the morphology of the intercalated disks and intracellular organization can be observed between ventricular cardiomyocytes in culture and *in vivo*. This is a particular relevant point because during the differentiation studies performed with NT2D1 cells in an angiomyogenic medium, we have also performed ultra structural analysis, expecting to find muscle characteristic phenotypes similar to the one observed in the image A.

Regarding the usefulness of a possible *ex vivo* amplification of cardiac cells for transplantation, another relevant issue that is not well understood is the precise molecular events, underlying cell cycle induction in cardiomyocytes.

As generally accepted, cardiac myocytes, unlike their differentiating skeletal counterparts [371, 372], can proliferate throughout embryonic and fetal life [373]. However, shortly after birth, growing occurs, almost exclusively, by hypertrophy [374], with consistent reductions of crucial key cell cycle regulators as cyclins and cdks [375-377]. Contrarily, recent studies suggested that under pathological conditions, adult cardiomyocytes are able not only to re-enter the cell cycle, but also to undergo nuclear mitotic division [378-382]. Rigorous confirmation, with nonambiguous molecular genetic markers, should be a requisite for any new studies assessing the intrinsic proliferative capacity of semi- or terminally-differentiated human working cardiomyocytes, *in vivo*, and *in vitro*. Indeed, if a significative proliferative potential were verified, that knowledge could be applied to cardiomyocyte cultures, *in vitro*, that could then be used, to further analyse the mechanism underlying the formation of the different types of intercellular junctions and the electrical coupling between differentiated cardiac cells.

Meanwhile, in order to obtain the tool required for studying cardiomyocyte differentiation, we are limited to the use of pluripotent undifferentiated cells or cardiac progenitors.

Cardiomyogenic cultures

Starting in 1920s, traditional embryological experiments have used amphibians, chicks and mice to map the mesoderm regions with cardiogenic potential. However, when these tissues were removed from the embryo, before entering the cardiac developmental pathway, and placed in a culture vessel, they did not acquire the expected cardiac phenotype, unless specific tissues, that provide the correct inductive signalling, were present. In mice, such specific inductive tissues seems to be confined to the anterior visceral endoderm, primitive streak and perhaps definitive endoderm [383]. Accordingly, the knowledge of embryologically relevant proteins involved in that specific inductive signalling raises the hope that they may have a similar role in eliciting a procardiogenic response in cultured embryonic or adult stem cells *in vitro*. However, it should be kept in mind that the role of those proteins, during development, depends, mainly, upon the spatiotemporal status of the target cell. Because of that, the studies performed *in vitro* have a high inherent risk of insuccess. In fact, actually, there is not a generally accepted cardiac differentiation protocol and all the methods used to date, should more conveniently, be called "increasing efficiency methods" of, the already present, spontaneous diferentiation potential of the pluripotential cell lines, than truly "inductive" *per se*.

In mouse, pluripotential embryonic cell lines have the capacity to differentiate, *in vitro*, into cells of all three germ layers. They may be isolated from three different sites, namely the inner embryonic stem cell (ESC) mass or epiblast of blastocyst-stage embryos [384, 385], the undifferentiated components of embryonal carcinomas (EC) that arise spontaneously or are experimentally induced by transfer of cells from epiblast to extrauterine sites [386] and the primordial embryonic germ (EG) cells that are first detected in the epiblast and migrate within the growing genital ridges to terminally populate the developing gonads [386]. It should be mentioned that the greatest developmental capacity is restricted to mESCs and some mEG cell lines isolated from early primordial mEG cells. Unlike mEC cell lines, mESCs and mEG cell lines need a feeder layer and the addition of LIF in order to maintain the undifferentiated state, a normal karyotype and the self-renewal ability [387].

Alike the mouse system, ESCs [388], ECs [389] and EGs [390] cell lines have also been derived from humans, but only the hESCs seem to maintain the pluripotent properties established for mESC lines [387]. Nevertheless, some differences between mESCs and hESCs have been observed. As mESCs, hESCs need an embryonic fibroblast feeder layer and serum

supplemented with bFGF. Growth in feeder-free conditions is possible but requires the use of a matrix and conditioned medium [391]. Unlike the mouse system, hESCs do not require LIF [387]. Human ESCs have a population doubling time considerably longer than mESCs, 25-30 hours and, 8-15 hours respectively [392]. Morphologically, hESCs form relatively flat and compact colonies that can be enzymatically dissociated. However, unlike mESCs they present some chromosomal abnormalities. Some authors believe that the latter are triggered by the enzymatic treatment and, therefore, argue that processes of mechanical dissociation should be used for hESCs, to assure a normal karyotype *in vitro*. [393].

Because much of what is currently known about cardiomyocyte differentiation, *in vitro*, has been learned from studies on mESCs, more attention will be given to these systems in the next lines, not forgetting, however, the mouse EC cell line P19 as a particular relevant model of cardiomyocyte differentiation model. Reference will also be made to the human EC line NT2/D1, as the experimental cell model used in the differentiation studies of the present dissertation.

In vitro spontaneous cardiac differentiation of mESCs usually requires an initial aggregation to form structures termed embryoid bodies (EBs). After a few days of culture, under appropriate conditions of cell density, culture medium and serum supplement, cardiomyocytes form between an outer epithelial layer of the EB with characteristics of visceral endoderm and basal mesenchymal cells becoming readily identifiable by spontaneous contraction [394]. Mouse ESCs differentiation recapitulates the programmed expression of cardiac genes observed in the mouse embryo, *in vivo*, both in the kinetics and the sequence in which genes are upregulated. Gata4 and Nkx2.5 transcripts appear before mRNAs encoding ANF, Mlc2v, αMhc and βMhc [394]. Regarding the electrical properties, the rate of contraction has been shown to decrease with differentiation and maturation in culture. From the above, cardiogenesis in vitro was divided in three developmental stages: i) an early stage including pacemaker-like or primary myocardium-like cells, ii) an intermediate stage and iii) a terminal stage including atrial-, ventricular-, nodal-, His-, and Purkkinje-like cells [395] (Fig. 13).

9-11dEBs	12-15dEBs	16-25dEBs **(terminal differentiation stage)**		
early pacemaker	intermediate stage	atrial-like	ventricle-like	sinusnode-like
Ca^{2+} current, transient outward-K^+ current: I_{to}	Na^+ current, Ca^{2+} current, I_f current, K^+ currents: I_K, I_{to}	Na^+ current, Ca^{2+} current, K^+ currents: I_{to}, I_K, I_{K1}, $I_{K,Ach}$, $I_{K,ATP}$	Na^+ current, Ca^{2+} current, K^+ currents: I_{to}, I_K, I_{K1}, $I_{K,ATP}$	Ca^{2+} current, I_f current, K^+ currents: I_{to}, I_K, $I_{K,Ach}$
+60, 0 mV, -80, 1 sec				
spontaneous		triggered		spontaneous

Figure 13 - Typical action potentials recorded in mESC-derived cardiomyocytes at different developmental stages in relation to ionic currents detected in the cells. Adapted from Maltsev, VA; Wobus, AM; Rohwedel, J; Bader, M and Hescheler, J; Circulation Research, 75, page 236, 1994.
Mouse ESC-derived cardiomyocytes express, at any given time during development and within the same EB, different types of action potentials: early pacemaker-, Purkinje-, atrial-, ventricle- and node-like. However, cardiomyocytes of an early differentiation stage (9-11d EBs), mostly reveal pacemaker-like action potentials, whereas cardiomyocytes of the terminal differentiation stage (16-25d EBs) present three major types of action potentials: atrial-, ventricle- and node-like. Cells of atrial and ventricular phenotypes may be characterized by a stable resting potential of about -75 mV and by action potentials of high amplitude and upstroke velocity. Atrial action potentials differ from ventricular action potentials by a less pronounced plateau and by an acetylcholine-induced hyperpolarization. The third type of action potential measured in mESC-derived differentiated cardiomyocytes shows characteristics of sinusnodal pacemaker cells including the typical shape and hormonal regulation. Importantly, the various shapes of action potentials in mESC-derived cardiomyocytes of different developmental stages are well correlated with the expression of specialized types of ion channels and associated ionic currents. While in cardiomyocytes of an early differentiation stage, the primitive pacemaker currents are generated by voltage-dependent L-type Ca^{2+} channels (I_{Ca}) and transient K^+ channels (I_{to}), currents in terminal differentiation cardiomyocytes are generated by various additional types, including voltage-dependent Na^+ channels (I_{Na}), delayed outward rectifying K^+ channels (I_K), inward rectifying K^+ channels (I_{K1}), muscarinic acetylcholine-activated K^+ channels ($I_{K,Ach}$), hyperpolarization-activated pacemaker channels (I_f) and ATP-dependent K^+ channels ($I_{K,ATP}$). Ventricular- and atrial-like cardiomyocytes express I_{Na} and I_{K1} underlying the high upstroke velocity and the stable resting potentials, respectively.

In humans, the first paper reporting an *in vitro* differentiation of hESCs appeared in 2001 [396]. Since then, other similar studies have been published [397-399]. In general, hESCs are dispersed into small clumps of 3-20 cells, using collagenase IV and grow for 7-10 days in suspension to form EB-like structures like mESCs but without the characteristic outer layer of endoderm cells. Then, these EBs are plated onto gelatine-coated culture dishes and after a few days, beating areas may be observed, reaching a maximum number after 20-30 days. The best rate of differentiation was reported to be about 70 % of the EBs showing beating areas [397]. A considerable number of growth factors, chemical compounds and other strategies are currently reported as inducers or adjuvants of cardiomyocyte differentiation of mESCs. The latter include reactive oxygen species [400], TGFβ plus BMP2 [401], TGFβ2

[402], RBP-Jκ inhibition [403], Cripto1 signal transduction [171], ascorbic acid [404], LIF [387, 405], IGF [406], FGFR1 signal transduction [407], cAMP [408], retinoic acid [409, 410], nitric oxide [411], dimethyl sulfoxide (DMSO) [410] and noggin [347]. Although Wnt11 [217] and Wnt3a [412] promote cardiogenesis in mouse P19 EC cells the same effects of Wnt signalling in mouse or human ESCs have not been described. Curiously, 5-azacytidine or 5-aza-2'-deoxycytidine, that induce cardiac differentiation in P19 cells [413] and hESCs [397] appear to have a different effect in mESCs, acting against differentiation [414].

Several of the aforementioned "inducers" have now been tested in the human system. Of those tested, with the exception of 5-aza-2'-deoxycytidine [397], no significant improvement in the differentiation of hESCs to cardiomyocytes has been observed with DMSO, RA or BMP2 [22, 396, 415, 416]. Actually, it is unclear why these factors do not contribute to cardiomyogenesis in hESCs. Optimal concentration, of the various factors, timing and combination with other factors should be considered as possible reasons for that failure.

Although it is clear that hESCs can be differentiated, *in vitro*, towards cardiomyocytes that appropriately respond to different stimuli, suggesting a correct and functional expression of many genes required for excitation-coupling, these cells fail to attain the exact characteristics of adult cardiomyocytes [417].

In addition to the embryonic differentiation system, many studies report the differentiation or transdifferentiation of cardiomyocytes from adult stem, multipotent or progenitor cells, *in vitro*, mainly using culture conditions copied from the embryonic system. These experiments, *in vitro*, include cardiomyocytes differentiation in mice from $CD29^+$ cells from brown adipose tissue [418], bone marrow cells (BMCs) [419], heart-derived $Sca1^+$ cells [420], BM stromal cells [421] and embryonic endothelial cells [422]. In rats, similar results were obtained with mesenchymal stem cells (MSCs) [423], liver stem cells [424] and BM mononuclear cells [425, 426]. In humans cardiomyocytes were obtained from circulating progenitor cells (CPCs) [220], BM lin^- mononuclear cells [427], MSCs [428], endothelial progenitor cells (EPCs) [429], HUVECs [422] and fetal $CD133^+$ cells [38]. However, if these processes can occur significantly, *in vivo*, is still a matter of debate. Experimentally, there is evidence that new cardiomyocytes can be generated in the adult, after a fusion or transdifferentiation event of a multipotent stem cell, as the bone marrow stem cell, or after differentiation from a possible resident cardiac stem cell, in an injured cardiac environment. However, to date, none of this possibilities has been unequivocally supported by experimental data showing regulatory mechanisms underlying the phenomenon or the extension that they occur in adult

organisms [430]. Furthermore, even with these events taking place in an injured heart, they are notably insufficient to revert a pathologic condition, as the estimated number of cells required to regenerate, for instance, a myocardial infarcted area is approximately 10^8-10^9 cells [417].

One of the most important aims of the present work was to develop an, *in vitro*, model of human cardiac differentiation that would lead to a better understanding of cardiac regeneration and, eventually, could be used in cardiac cell therapy.

At the end of this section, is opportune, to mention the facts that contributed to the choice of our "cardiomyogenic cell model". The first conditions that were considered were the availability of the starting material, the reproducibility of the experiments and the maintenance and proliferative capacity of the selected cells. Setting these requirements precluded the use of primary cultures from human sources, as the ones mentioned before. This led us to consider commercially available cell lines. Among them, we have given preference to pluripotent human cell lines. Human ESC lines, although the most logical option, because they may easily acquire a spontaneous or induced cardiac phenotype had several drawbacks. These were the high sensibility to cryopreservation [431], the need for demanding and experienced cell culture techniques [432], legal and ethical issues [433], and, above all, their cost. This has left us with two options. The embryonal carcinoma (EC) or embryonic germ (EG), cell lines, both known for their capability of cardiac differentiation in the mouse system [413, 434]. Although, similar results have not been reported in the humans, we were encouraged by the fact that the mouse EC cell line P19 has proven to be an excellent model of cardiogenesis [413]. To our knowledge, no mouse EG cell line with the cardiac efficiency of the P19 cells has been reported. Results obtained with the EG1 cell line were an illustrative example of that difference [434]. In addition, EG cells, are relatively more difficult to maintain undifferentiated in culture [435]. Curiously, coincidently or not, human EG cells have not attracted much attention as an alternative source of pluripotent stem cells among the research community [390]. Based on this, we chose human embryonal carcinomas PA1 and NT2/D1.

Preliminary studies were performed with this two cell lines, yet the last studies with the reported angiomyogenic medium were performed only in the NT2/D1 cell system, mainly because much more information was available for this cell line. Thus, CD133, a molecule considered to be a stem/progenitor cell marker [436-439] is expressed in the NT2 cell line [436]. The latter is a clonal derivative of NT2/D1 cell line, which also expresses CD133 (our

results). Additionally, fetal liver CD133^{+} cells have successfully differentiated to endothelial and muscle cells (skeletal and cardiac) in a defined angiomyogenic medium [38].

On the other hand, NT2/D1 cells express high levels of Cripto1 [147, 440] and H19 [441, 442] genes, only expressed when in an undifferentiated or differentiated state, respectively. Curiously, the mouse embryonal carcinoma P19 cell line mentioned above, with a high cardiomyogenic potential, expresses Cripto1 only in an undifferentiated state and the H19 gene only after the induction of differentiation to cardiomyocytes [394].

It is generally accepted that Nestin and CD133 are relevant human neural stem/progenitor cells markers [443-445], that mouse and human neural stem cells already have shown the capability to differentiate among non-neurogenic lineages including the myogenic lineage itself [446, 447] and that, in mice those cells can even give rise to cardiomyocytes [448]. Curiously, NT2/D1 cells express CD133 and Nestin and may be regarded as neural progenitor cells [436, 449, 450].

Taken together these results have made us believe that the NT2/D1 cell culture system was an excellent candidate for cardiac differentiation in an angiomyogenic medium.

Cell sources for cardiac therapy

As mentioned before, there is experimental evidence suggesting that natural cardiomyocyte regeneration may occur. However, the controversial evidence of cardiac stem/progenitor cells residing in the myocardium or coming from any other niche such as the bone marrow, and participating in the healing process, as key players, is clearly not sufficient to overcome cardiomyocyte necrosis or apoptosis in an acutely or chronically damaged heart. On the other hand, it was precisely the low ability of myocardium to regenerate and the inhability of the adult cardiomyocyte to proliferate that originated the intense research on this area, in search of a cell that could replace damaged myocardium.

Although there is some considerable knowledge in mouse cardiomyogenesis, *in vitro*, very little is known regarding the molecules or pathways that determine the cardiomyogenic potential, or regulate its commitment, in the human system. This is, most likely due to the difficulties in transferring basic and applied research performed in animal models to humans.

Embryonic stem cells (ESCs)

In an ESC culture from human or mice, it is relatively easy to obtain spontaneous or induced cardiomyocyte differentiation, but with the actual knowledge the process is finalized with insufficient yields or purity to be used in a clinical approach, largely because they differentiate, also, into other undesirable cell lineages. Adressing this problem, Field's group published a study in which, upscaling the production of cardiomyocytes from mouse ESCs was attempted [451], using the same molecular biology strategy they have used before [452] and that we have tried to develop in humans.

An alternative would be to use undifferentiated embryonic stem cells directly into the desirable damaged areas of the heart, but the enormous risk of teratoma formation has put this option aside.

Although, ESCs are, still, the most efficient system that can be used to obtain cardiac differentiation, *in vitro*, their use for therapeutic purposes has two main problems. One, least important, is that in the past few years, the ethical and legal questions regarding the manipulation of hESCs has rendered the use of these cells, almost impossible. The other, more complicated to overcome, without using nuclear transfer, is of immunological nature. Although ESCs express low levels of major histocompatibility complex class I (MHC-I) antigens, once mature cells, they will express normal levels of MHC-I and therefore will be

rejected, if used in other than the autologous context [453]. Both factors have contributed to turn the attention of many scientists from embryonic to adult sources of stem cells.

Adult stem/progenitor cells

There is no worldwide consensus regarding the "stemness" of adult stem cells (ASCs). Furthermore, to our knowledge, experimentally, this concept is supported by a single publication [454]. In spite of this paucity, several scientific papers claim that ASCs can be identified in the brain, bone marrow, peripheral blood, blood vessels, skeletal muscle, epithelial of the skin and digestive system, cornea, dental pulp, retina, liver and pancreas. Furthermore, many of them, alegely, give rise to cardiomyocytes, *in vitro*. However, identifying these adult pluripotential cells as "stem cells" it is in apparent contradiction with the former statement. Therefore we believe that, at this point, is worth clarifying this confusion. The term "stem cell" was originally assigned to embryonic cells that are present early in development, and retain the capacity to develop into any cell type of the body. In contrast, a "progenitor cell" is a cell that has already undergone an irreversible step in differentiation, but is still "pluripotential" within certain limits. A well documented example of the latter is the commonly called "hematopoietic stem cells" that can give rise to all lineages of that system. Some authors believe that they might be able to differentiate into cells belonging to other systems. What is controversial, however, is wether they would be found in all tissues if transferred into, let us say, a morula. Respecting the various opinions, throughout this dissertation we will use the nomenclature used by the respective authors that are cited, although it might not correspond to, what be considered, the correct definition.

To date, among adult "stem/progenitor" cells, the bone marrow-derived stem cells are the most commonly studied. In fact, they are the most logical candidates to be used as source for cell therapies, because of their plasticity, availability and the excellent results obtained in bone marrow transplantation. They naturally appear in the bone marrow but also to a lesser extent in the peripheral blood and peripherical body tissues. Generally, they are divided in three classes: the haematopoietic stem cells (HSCs), the mesenchymal stem cells (MSCs) and the endothelial progenitor cells (EPCs).

In injured heart models, the cardiac differentiation potential of transplanted HSCs remains polemic [455-458]. Less controversy has surrounded the ability of MSCs to differentiate into cardiomyocytes *in vivo* [459-462]. However, more studies are needed, for full clarification of all questions that remain unanswered and that were raised in previous sections, of this thesis.

Regarding EPC transplantation, several studies have also been reported. They suggest that underlying the improvement observed is the expected angiogenesis and, as well, a possible inductive role of EPCs on endogenous myocardial regeneration [463-466].

Resident cardiac stem/progenitor cells

If we could think of an "ideal cell" for cardiac cell therapies, one of the most plausible hypothesis would certainly be a "cardiac progenitor cell". This because they would have a good level of plasticity and proliferation, capacity to adapt themselves, correctly, to the injured heart and would not undergo differentiation into unwanted cell lineages, unlike fully differentiated cardiomyocytes or stem cells, respectively. Actually, there is an enormous interest regarding the cell-surface markers that would identify and characterize a particular cell as a cardiac progenitor cell. Accordingly, it is still to be discovered the cell-surface marker that merits consensual approvement, among the scientific community [467]. However, several populations of cardiac resident of cells, have been suggested as candidates of cardiac progenitors. The latter include, in the mouse system, Lin^{-} + $cKit^{+}$ [468], $Abcg2^{+}$ side population (SP) [469] and Lin^{-} + $cKit^{-}$ + $Sca1^{+}$ cells [470] and in both mouse and human system the $CD34^{+}$ + $cKit^{+}$ (+ $Sca1^{+}$ in mouse) [471] and $cKit^{-}$ + $CD31^{-}$ + $Sca1^{-}$ + $Isl1^{+}$ cells [68]. Unfortunately, these $Isl1^{+}$ cells could not be detected in the human sample examined beyond 8 days of age [68, 69]. Skeletal muscle resident $cKit^{-}$ + $cMet^{-}$ cell population in mice was also proposed as a cardiac progenitor cell pool [472].

Muscle cell populations

Fetal, neonatal and adult cardiomyocytes were successfully engrafted in injured hearts in animal models, whereas availability and immunological reasons limit their use in human patients [473-477]. In small animal models, implantation of smooth muscle cells [478, 479] or skeletal myoblasts [480-483] into the injured heart have resulted in improved contractile cardiac performance, however full electro-mechanical integration is deficient. The latter may contribute to arrhythmogenic events if we think in using them in larger mammals, as humans.

As it will be shown in the next section (Clinical cardiac regeneration - Table 5), bone marrow-derived stem/progenitor cells and skeletal myoblasts have been the sole tried so far, in cardiac cell therapies in humans.

Clinical cardiac regeneration

When the structure and electrophysiologic behaviour of the human heart (Fig. 14) reaches adulthood, it has evolved into two separate but anatomically fused pumping units that work sequencially and provides blood to the systemic and pulmonary vascular circuits of the body. Curiously, it still holds some characteristics of its embryonic development as a muscular tube. For instance, the nodal region, an area of slow conductivity and high automaticity closely resemble the early peristaltic embryonic tubular heart.

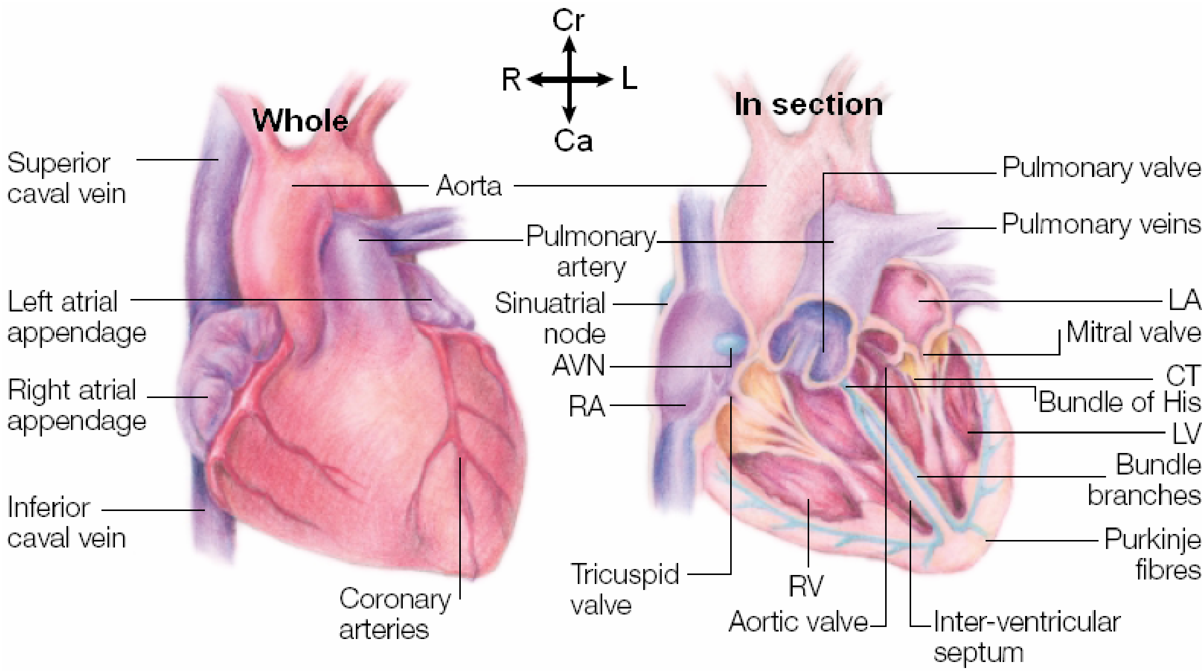


Figure 14 - Structural scheme of the adult heart. In the mature heart, the right atrium (RA) receives venous blood from the body and passes it through the tricuspid valve to the right ventricle (RV), which pumps it through the pulmonary artery to the lungs. Oxygenated blood from the lungs is returned to the left atrium (LA) of the heart through the pulmonary veins, and is passed to the left ventricle (LV) through the mitral valve. From the LV, blood is pumped through the aorta to the arterial vascular circuit of the body and to the coronary circulation branches from the proximal portion of the aorta. The electrical impulse is initiated at the sinoatrial node at the junction between the RA and the superior caval vein and propagated throughout the atria to the atrioventricular node (AVN), which is the stream for the impulse to pass, after a delay, to the ventricles. Rapid conduction occurs along the bundle of His and its bundle branches to the ventricular apex and then, throughout the ventricles by the Purkinje fibres. Cr - cranial, Ca - caudal, L - left, R - right, CT - Chordae tendineae. Adapted from Harvey RP, Nature Reviews Genetics, 3(7), page 545, 2002.

In addition, during adulthood, as mentioned before, cardiomyocytes have withdrawn irreversibly from the cell cycle and from birth, the mechanism of heart size modulation is mainly confined to hypertrophy instead of the hyperplasia seen before [484].

Historically, the adult heart has been viewed as a terminally differentiated organ, without the capacity for self-renewal or regeneration. Recently, cardiac myocytes division was observed in patients with end-stage idiopathic dilated cardiomyopathy [485], as well as in adjacent regions of infarcted myocardium [380]. Furthermore, in sex-mismatch cardiac allografts, in humans, there was a small contribution of host cell-derived cardiomyocytes in the resultant cardiac chimerism [132, 486-489]. Once more, the exact source of the regenerating cells remains unclear.

Curiously, this restricted capacity of regeneration does not seem to be an inerent characteristic of cardiac tissue, because the hearts of newts [490], zebra fish [491] and the MRL mouse strain [492] are able to regenerate following injury. It is interesting to note that when the mechanisms that promote this blockade of proliferation in human adult cardiomyocytes are known, we will probably be able to understand why cardiac tumours are amazingly rare.

Nevertheless, one new polemic idea is undoubtly proven. There is in fact, although limited, cellular restorative potential in the adult human heart.

Available treatments of heart failure including heart transplantation, ventricular assisted devices and the use of artificial hearts, are not readily available to most patients and even to those that can benefit from them the efficiency and quality of life are far from satisfactory. Therefore, and while the knowledge that allows the complete control of the regeneration process does not exist, there is a tremendous need for the development of novel strategies for treatment of heart failure, including the implantation of additional cells into the injured myocardium.

Adequately or not, clinical trials in humans with cardiac cell therapy have started.

In the majority, if not all, of the clinical studies, muscle regeneration was not obvious, in spite of the fact that, in most of them, other improvements were reported (see below). These trials have, however, contributed to emphasize that fundamental issues have to be adressed experimentally, to answer, at least, the following questions: What is the ideal cell type for the cell-based repair? How is a pertinent level of homing efficiency reached? Is transient homing sufficient? What is the fate of the injected cells? How long do they survive after grafting? What is the differentiation potential of donor cells and how do they integrate electrophysiologically with the host's myocardium? What is the best cellular delivery method? (Table 4) What is the optimal timing for repair? What is the ideal number of cells to transplant? Finally, what is the relative contribution, for the improved outcome, of angiogenesis and cardiogenesis, when bone marrow-derived cells are used?

When all these questions are answered we may then talk of real abrogation of adverse remodelling and scar formation as well as restoration of the ejection fraction, in patients treated for cardiac pathological conditions.

Table 4 - Comparison of cell delivery methods.
Adapted from Balsam LB and Robbins RC, Clinical Science, 109, page 489, 2005.

Method	Advantages	Disadvantages
Intramyocardial	Precise delivery to the area of injury.	Invasive: through an open surgery or transendocardial catheter-based delivey; Risk of arrhythmias.
Intracoronary	Precise delivery to the area of injury.	Invasive: through an open surgery or percutaneously; Risk of coronary flow impairment.
Intravenous	Non-invasive.	Low homing efficiency: requires innate homing mechanisms to reach injured myocardium.

The main clinical trials published in scientific papers to date and those still ongoing are presented on Table 5.

Table 5 - Cardiac Cell Therapy Clinical Trials**.** MNC indicates bone marrow - derived mononuclear cells; CPC, circulating blood-derived progenitor cells; NC, bone marrow - derived nucleated cells; MSC, bone marrow - derived mesenchymal stem cells; $CD133^+$, bone marrow - derived $CD133^+$ cells; IC, intracoronary; AMI, acute myocardial infarction; LVEF, left ventricular ejection fraction; LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; PI, principal investigator; $CD34^+/CXCR4^+$, bone marrow - derived $CD34^+/CXCR4^+$ cells; PTCA, percutaneous transluminal coronary angioplasty; BM, bone marrow; LVDV, left ventricular diastolic volume; LVSV, left ventricular systolic volume; LVDD, left ventricular diastolic diameter; BMDC, bone marrow - derived cells; TransEndo, transendocardial; TransEp, Transepicardial; CABG, coronary artery bypass grafting; EMM, electromechanical mapping; NYHA, New York Heart Association; CAD, Coronary artery disease; ADRC, Adipose-derived stem and regenerative cells; BMPC, bone marrow-derived progenitor cells; DCM, Dilated cardiomyppathy. Values are means ± SD. The abbreviations were employed trying to maintain the same nomenclature used by the authors in their studies.
The first double-blind, randomized, placebo-controlled trials to assess intracoronary infusion of progenitor cells after AMI are represented in green.

In Patients With Acute Myocardial Infarction								
Year	**[n]**	**Cell Type**	**Dose**	**Delivery**	**Time After AMI**	**No Change Outcome**	**Outcome**	**Ref.**
2002	10 treated, 10 controls	MNC	$2.8{\pm}2.2x10^7$	IC	5-9 days	Global LVEF LVEDV	Regional wall motion ↑; Infarct size ↓; Perfusion ↑	[493]
TOPCARE-AMI, 2004	29 MNC, 30 CPC, 11 controls	MNC CPC	$2.1{\pm}0.8x10^4$ $1.6{\pm}1.2x10^4$	IC	5±2 days	LVEDV	Regional wall motion ↑; Global LVEF ↑; Infarct size ↓; Coronary flow ↑	[494]
2004	20 treated, 13 controls	MNC	$7.8{\pm}4.1x10^4$	IC	14±6 days	LVEDV	Regional wall motion ↑; Global LVEF ↑	[495]
2004	5 treated **no control**	MNC	$3.9{\pm}2.3x10^4$	IC	6 days	Reg. wall motion Global LVEF	no improvements	[496]
BOOST, 2004	30 treated, 30 controls	NC	$2.5{\pm}0.9x10^4$	IC	6±1 day	LVEDV Infarct size	Regional wall motion ↑; Global LVEF ↑	[497]
2004	34 treated, 35 controls	MSC	$4.8{\pm}6.0x10^4$	IC	18 days	-	Regional wall motion ↑; Global LVEF ↑; Infarct size ↓ LVEDV↓	[498]

2004	12 treated, 10 controls	$CD133^+$	$6.6{\pm}1.4x10^4$	IC	14±6 days	-	Regional wall motion ↑; Global LVEF ↑; Perfusion ↑	[499]
ASTAMI, 2005	50 treated 50 control	MNC	from 50 ml BM	IC	5-8 days	LV function	no improvements	[500]
2005	19 treated 16 control	$CD133^+$	$12.6{\pm}2.2x10^6$	IC	recent		Regional wall motion ↑; Global LVEF ↑; Perfusion ↑	[501]
2006	**33 treated, 34 controls**	**NC**	**$304{\pm}128x10^6$**	**IC**	**<29h**	**Global LV function**	**Infarct size ↓; Regional systolic function ↑**	**[502]**
REPAIR-AMI, 2006	**101 treated, 103 controls**	**MNC**	**$236{\pm}174x10^8$**	**IC**	**3-6 days**	**-**	**Global LVEF ↑; LVESV↓**	**[503]**
Ongoing Studies (PI)	**expected [n]**	**Cell Type**	**Dose**	**Delivery**	**Time after AMI**	**LVEF**		
Lemarchand, P	50 treated 50 controls	MNC	-	IC	7-10 days after PCI	<45 %		
Kassem, M	10	BMDC	-	IC	-	>45 %		
Traverse, J	60	MNC	-	IC	48-96h	30 - 50 %		
Tendera, M	200	BMDC $CD34^+$/ $CXCR4^+$	-	IC	PTCA successful	<40 %		
Piepoli, M	50	MNC	-	IC	<72h	-		
Dohman, HF	300	MNC	-	IC	-	<40 %		

In Patients With Myocardial Ischemia

Year	[n]	Cell Type	Dose	Delivery	LVEF	Time after AMI	Outcome	Ref.
2001	5 treated **no control**	MNC	$0.3 \text{-} 2.2x10^9$	TransEp (during CABG)		-	Perfusion ↑	[504]
2003	8 treated **no control**	MNC	from 40 mL BM	TransEndo (by EMM)	58±11 %	-	Angina ↓; Perfusion ↑; Regional wall motion ↑;	[505]
2003	10 treated **no control**	NC	$7.8{\pm}\ 6.6x10^7$	TransEndo (by EMM)	47±10 %	-	Angina ↓; Perfusion↑	[506]
2004	14 treated, 7 controls	MNC	$3.0{\pm}\ 0.4x10^7$	TransEndo (by EMM)	30±6 %	-	Angina ↓; NYHA class ↓; Perfusion ↑; Global LVEF ↑ Regional wall motion ↑;	[507]
2004	12 treated **no control**	$CD133^+$	$1.0\text{-}2.8x10^6$	TransEp (during CABG)	36±11 %	3–12 weeks	Global LVEF ↑; LVEDV ↓; Perfusion ↑	[508]
2005	5 treated 10 control	CPC MNC $CD34^+$	$2.0x10^7$ $6.0x10^5$	TransEp (during CABG)	<45 %	12 months	Global LVEF ↑; LVDV ↓; LVSV ↓; LVDD ↓;	[509]
IACT, 2005	18 treated 18 control	MNC	$6.0\text{-}13.2x10^7$	IC	<69 %	5-102 months	Global LVEF ↑; Regional wall motion ↑; Infarct area ↓	[510]
2006	35 MNC, 34 CPC, 23 controls	MNC CPC	$2.0{\pm}1.1x10^8$ $2.2{\pm}1.1x10^7$	IC	40±11 %	3–144 months	Global LVEF ↑; (only in MNC group)	[511]
Ongoing Studies (PI)	**expected [n]**	**Cell Type**	**Dose**	**Delivery**	**LVEF**	**Time after AMI**		
Losordo, D (sponser, not PI)	18 treated 6 controls	$CD34^+$	-	TransEndo	-	-		
Asahara, T	10	$CD34^+$	-	TransEndo	<50 % and (CAD)	>6 months		
Diederichsen, AC	35	BMDC	-	IC	<40 %	-		
Kastrup, J	40	MSC	-	TransEp	-	-		
de Oliveira, SA	300	BMDC	-	TransEp (during CABG)				
Zeiher, AM	100	MNC	-	IC	<50 %	>3 months		
Liu Sheng	50	BMDC	-	TransEp (during CABG)	40-50 %	>3 months		
Milstein, AM Fernandez-Aviles, F Perin, E	36	ADRC	-	TransEp	-	-		

In Patients With Chronic Heart Failure								
Year	**[n]**	**Cell Type**	**Dose**	**Delivery**	**LVEF**	**Time after AMI**	**Outcome**	**Ref.**
2003	10 treated **no control**	Myoblast	$8.7\pm1.9x10^8$	TransEp (during CABG)	24±4 %	3–228 months	Regional wall motion ↑; Global LVEF ↑	[512]
2003	11 treated **no control**	Myoblast	$1.9\pm1.2x10^8$	TransEp (during CABG)	36±8 %	3–168 months	Regional wall motion ↑; Global LVEF ↑; Viability in infarct area ↑	[513]
2003	5 treated **no control**	Myoblast	$2.0\pm1.1x10^8$	TransEndo (by EMM)	36±11 %	24–132 months	Regional wall motion ↑; Global LVEF ↑	[514]
2004	10 treated **no control**	Myoblast	$0.04\text{-}5.0x10^7$	TransEp (during CABG)	25±40 %	4–108 months	Regional wall motion ↑; Global LVEF ↑	[515]
2004	20 treated **no control**	Myoblast	$3.0\pm0.2x10^8$	TransEp (during CABG)	28±3 %	-	Regional wall motion ↑; Global LVEF ↑; Viability in infarct area ↑	[516]
2005	24 treated (CABG) 6 treated (VAD) **no control**	Myoblast	$1.0\text{-}30x10^7$	TransEp (during CABG or VAD)	<40 %	-	Global LVEF ↑; Viability in infarct area ↑	[517]
2005	10 treated 10 controls	MNC $CD34^+$ (70 %)	$2.2x10^7$	TransEp (during CABG)	<35 %	not reported not all had AMI	Global LVEF ↑; NYHA class ↓	[518]
Ongoing Studies (PI)	**expected [n]**	**Cell Type**	**Dose**	**Delivery**	**LVEF**	**Time after AMI**		
Patel, AN	10	BMPC	-	TransEp (during VAD)	<35 %	-		
Patel, AN	75	BMPC	-	TransEp (during CABG)	<40 %	-		

In Patients With Dilated Cardiomyopathy					
Ongoing Studies (PI)	**expected [n]**	**Cell Type**	**Dose**	**Delivery**	**LVEF**
Zeiher, AM TOPCARE-DCM	30	BMDC	-	IC	<40 % (DCM)
Bozza, AZ	300	BMDC	-	-	≤35 %

In Patients With Chagas Cardiomyopathy					
Ongoing Studies (PI)	**expected [n]**	**Cell Type**	**Dose**	**Delivery**	**LVEF**
Santos, RR Feitosa, G	300	BMDC	-	-	≤35 % (DCM)

As could be seen in Table 5 many of the clinical studies exploring the safety and feasibility of stem cells therapy have been conducted in non-controled patient groups whereas the vast majority of the controlled clinical studies have used non randomized control groups. They have used different cell types and preparations and have been performed in a too small number of patients with different pathological stages.

Neverthelless, in the era of evidence-based medicine, these preliminary clinical studies suggest that stem cell therapy might work.

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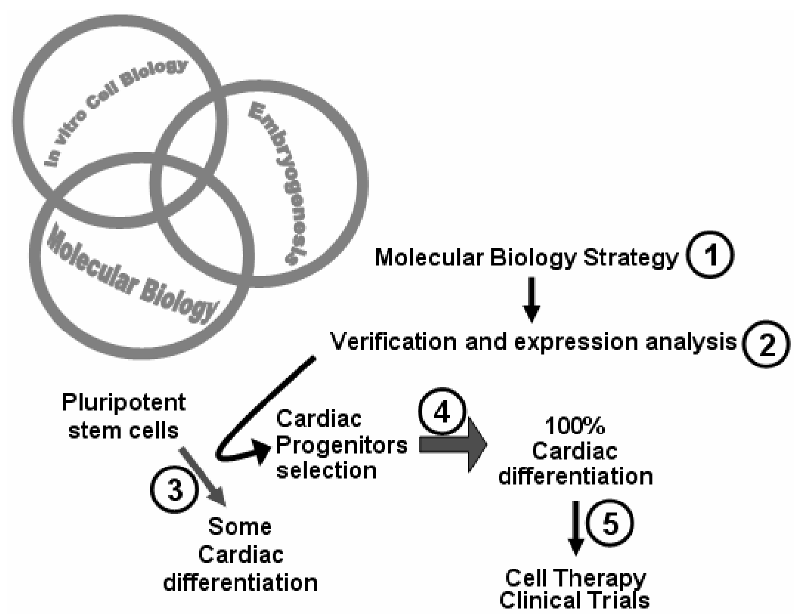
Aims and technical goals

Aims



The process of adult cardiac differentiation, from undifferentiated progenitors, is not well understood. Studying *in vitro* adult cardiomyocytes is limited by the inability of such cells to divide and attach in standard culture conditions. These are, among others, the reasons why a cardiac differentiating cell model is so desirable.

To approach this issue, one alternative would be to select, *in vitro*, the desired cardiac progenitor cells, among undifferentiated pluripotent or multipotent stem cells, from embryonic or adult human sources, with a verified molecular biology strategy. Such methodology would make possible the characterization of cell surface markers of that progenitor cells, the optimization of cell culture conditions and, eventually, the achievement of a 100 % pure, cardiomyogenic-comitted cell cultures, that could be used in clinical pathological conditions.

The present work tried to bring forth the first steps ①, ② and ③ of such experimental model, in a way that, when overtaking all those demanding steps, science will have the possibility of studying, *in vitro*, cardiac cell progenitor’s biology, cardiac commitment and adult cardiomyocyte adherent cell cultures ④. Then, the time for the common practice of clinical cardiac cell therapies will be reached ⑤.

It is reasonable to admit that a goal like this roughly lays on the intersection of three main areas of basic research, namely, embryology, molecular biology and *in vitro* cell biology. Molecular and development biologists are constantly disclosing the molecular signalling required for recruiting undifferentiated embryonic or possible adult cells to cardiomyogenic cell lineages. When a precise understanding of their regulation becomes available, the present difficulties regarding the selection and expansion of human cardiac progenitors or cardiomyogenic induction in embryonic or adult stem cells for cell-based therapies will be overcomed.

In view of the above, the present dissertation constitutes a contribution to that complex and ambitious purpose.

Technical goals

1. To construct a Neomycin/geneticin selection vector under the control of the human cardiac beta Myosin Heavy Chain (βMyhc) gene promoter.

    1.1. To validate the vector in undifferentiated human myogenic cells expressing the βMyhc gene.

    1.2. To validate the vector in human embryonic stem cells or fetal cardiomyocytes.

2. Using the embryonal carcinoma or teratocarcinoma NT2/D1 cell line, cultured in angiomyogenic medium and supplemented with bone morphogenetic protein-2 (BMP2), to generate a functional human model, of cardiomyogenesis, *in vitro*.

Materials and Methods

Materials and Methods

Nucleic acids extraction

DNA extraction from human tissues and cells in culture

We have chosen to use a rapid, non-hazardous (without organic solvents) and inexpensive method to extract DNA from biological samples [1]. This method involves salting out of the cellular proteins by dehydration and precipitation with a saturated NaCl solution.

Briefly, chopped tissue samples (up to 50 mg) or cellular pellets (up to 10^6 cells) were resuspended in 1.5 ml Eppendorf tubes with 400 µl of lysis buffer (10 mM Tris-Cl pH 8.0, 400 mM NaCl, 1 % SDS and 2 mM Na_2EDTA, pH 8.2). The lysates were digested for 2 h at 56°C with 10 µl of a 20 mg/ml Proteinase K solution (C_f = 0.5 mg/ml). After digestion and tube's spinning down was complete, 385 µl of each tube were transferred to clean Eppendorfs. 125 µl of saturated NaCl (~ 6 M) was added to each tube and shaken vigorously for 15 seconds, followed by centrifugation at 13000 rpm for 15 minutes in a Biofuge 13 microcentrifuge from Heraeus Instruments. The precipitated protein pellet was left at the bottom of the tube and the supernatant containing the DNA was transferred again to clean Eppendorf tubes. 2 volumes of room temperature absolute ethanol were added and the tubes inverted several times until the DNA precipitated. The visible precipitated DNA strands were removed with a sterile pipette tip and transferred to a clean tubes containing 70 % ethanol. DNA was pellet air-dried, dissolved in 50 - 200 µl sterile DEPC - treated bidestiled water and quantified in a GeneQuant II photometer from Pharmacia Biothec.

It is documented that the DNA obtained from this or similar technique yield quantities comparable to those obtained from phenol-chloroform extractions and that the 260/280 ratios were consistently 1.8 - 2.0, demonstrating good deproteinization [1, 2].

RNA extraction from human tissues and cells in culture

For the extraction of RNA from biological samples, the TRIZOL reagent (Invitrogen) extraction method was used.

TRIZOL Reagent is a ready-to-use reagent for the isolation of total RNA from cells and tissues. The reagent, a mono-phasic solution of phenol and guanidine isothiocyanate, is an improvement to the traditional single-step RNA isolation method [3].

In the presence of TRIZOL Reagent, RNA is protected from RNase degradation, however, downstream sample handling steps required that non disposable glassware or plasticware were

RNase-free. Accordingly, during the subsequent steps, we used sterile, disposable plastic ware and automatic pipettes tips reserved for RNA work to prevent cross-contamination with RNases from shared equipment.

Tissue samples were homogenized, in average with 1 ml of TRIZOL per 100 mg of tissue, using a Tissue Tearor, Model 985370 from Biospec products Inc. Or alternatively, cells in culture flasks or well-plates were lysed directly, with 0.1 ml of TRIZOL per cm^2 of culture dish, after the culture medium was removed. The homogenized samples remained for 5 minutes at RT to permit the complete dissociation of nucleoprotein complexes.

Addition of 0.2 ml of chloroform per ml of TRIZOL, followed by shaken and centrifugation at 12000 g for 15 min at 4º C, separated the sample solution into an aqueous phase and a lower phenol organic phase. The RNA remains exclusively in the aqueous phase. Each aqueous phase was then transferred to fresh tubes, and each organic phase was saved if isolation of DNA or protein was desired. To precipitate the RNA from the aqueous phase, 0.5 ml of isopropyl alcohol per 1 ml of TRIZOL was used. After incubation at RT for 10 minutes and centrifugation at 12000 g for 10 minutes at 4º C, the RNA precipitated as a gel-like pellet on the side and bottom of the tube. The supernatant was discarded and the RNA pellet washed once with 1ml of 75 % ethanol and centrifuged at 7500 g for 5 minutes at 4º C.

At the end of the procedure, the RNA pellet was air-dried, dissolved in DEPC-treated water by incubation for 10 minutes at 55°C and stored at -20º C until used.

Plasmid DNA extraction with the lysozyme/boiling miniprep protocol

After transformation and proliferation (see *Cloning*), bacteria carrying recombinant plasmids formed white colonies in a semi-solid chromogenic selective medium, because they are no longer capable of α-complementation. Positive transformants were directly analysed by PCR. A picked white colony could be directly ressuspended on a PCR tube, in the PCR final mix as an usual DNA template. The only difference between this approach and the normal PCR protocol (see *Polymerase Chain Reaction*) was the initial extra denaturating step, for 10 min at 94º C in the Biometra UNO Thermoblock thermo cycler.

When the expected PCR product was obtained, the respective white colony was picked again and grown overnight in 3 ml of LB broth plus ampicillin (50 µg/ml). One and half ml of the culture was pelleted in a microcentrifuge at 12000 g for 1 minute at 4º C, the supernatant removed by gentle aspiration and the pellet ressuspended in 350 µl of STET buffer [0.1 M NaCl, 10 mM Tris.Cl pH 8.0, 1 mM EDTA (pH 8.0), 5 % Triton® X-100]. After

vortexing, 25 µl of a freshly prepared solution of lysosyme 10 mg/ml in Tris.Cl pH 8.0 was added. The mixture was vortexed again, incubated for 1 minute at RT and placed in a boiling water bath for 40 seconds. The bacterial lysate was centrifuged at 12000 g for 10 minutes at RT and the pellet of bacterial debris removed and discarded with a sterile toothpick. To the supernatant
1 µl of RNase 10 mg/ml was added for 5 minutes followed by 5 µl of Proteinase K 20 mg/ml for 30 minutes at 56º C. After the enzymatic digestions, 1 volume of phenol: chloroform: isoamylic alchool (25:24:1) was mixed with the sample. The emulsion was centrifuged at 12000 g for 1 minute at RT and the upper aqueous phase was transferred to a clean microcentrifuge tube. Thereafter, 1 volume of chloroform was added and the emulsion centrifuged at 12000 g for 1 minute at RT. Again, the upper aqueous phase was transferred to a clean tube, 1 vol of isopropanol was added and incubated for 30 min at -20º C. The DNA precipitate was recovered by centrifugation at 12000 g for 15 minutes, washed with 70 % ethanol, air dried and ressuspended in 30 µl of DEPC-treated distilled water.

Alternatively, for higher yields, the selected white colony was inoculated and grown overnight in 25 ml of LB broth plus ampicillin (50 µg/ml) and the plasmid DNA recovered using the QIAfilter Plasmid Midi protocol from Qiagen.

DNA extraction from agarose gels

Rotinely, the expected PCR products were verified with sequenciation (see *Sequencing*) after a QIAquick PCR purification Kit protocol from QIAgen as described by the manufacturer. However, when a PCR product was contaminated with unspecific amplicons, a 0.8 % Nusieve Seaplaque® (Cambrex) low melting agarose gel electrophoresis without EtBr was performed, in order to separate the expected PCR products from the unwanted ones. Subsequently, the wanted gel band was cut with a sterile bistoury and a QIAquick Gel extraction Kit protocol was followed as described by the manufacturer to extract and purify the DNA from the agarose gel.

Molecular Biology

Polymerase Chain Reaction

All PCR reactions were performed using the Platinum Taq DNA Polymerase High Fidelity from Invitrogen according to manufacture intructions.

Platinum® *Taq* DNA Polymerase High Fidelity is an enzyme mixture composed of recombinant *Taq* DNA polymerase, *Pyrococcus species* GB-D polymerase, and Platinum® *Taq* Antibody [4, 5]. *Pyrococcus species* GB-D polymerase possesses a proofreading ability by virtue of its 3' to 5' exonuclease activity [6]. Mixture of the proofreading enzyme with *Taq* DNA polymerase increases fidelity approximately six times over that of *Taq* DNA polymerase alone and allows amplification of simple and complex DNA templates over a large range of target sizes. The anti-*Taq* DNA polymerase antibody complexes with and inhibits polymerase activity at room temperature. Activity is restored after the initial denaturation step in PCR cycling at 95º C, providing an automatic "hot start" for increased specificity, sensitivity, and yield [7, 8]. Briefly, the following components (Table 1) were added individually, on ice, to autoclaved DNase/RNase free PCR tubes:

Table 1 - Final mix used in PCR

Components	Final concentration
ddH_2O (treated with DEPC)	-
10X High Fidelity PCR Buffer	1x
10 mM dNTPs mix	200 µM
Taq + GB-D	1.0-2.5 U / tube
50 mM $MgSO_4$ or $MgCl_2$	2 mM
Upper primer	0.2 µM
Lower primer	0.2 µM
DNA template	~ 100 ng
Final volume	25 or 50 µl

After they have been mixed and spanned down, they were placed in a Biometra UNO Thermoblock thermal cycler and run according to the respective optimized program from Step 1. In general the program is:

Step 1 - 95º C, 2 min.
35-40 cycles:
Step 2 - 94º C, 1 min.
Step 3 - 50 - 68º C, 1 min.
Step 4 - 68º C, 2 - 10 min.
Step 5 - 68º C, 5 - 10 min.
Step 6 - 4º C, pause

After cycling, the samples could be stored at -20º C until used or analysed by agarose gel electrophoresis and visualized by ethidium bromide staining [9].

Digestion with restriction endonucleases

Alternatively, for directional cloning procedures, the PCR reaction tube containing the insert (measured in picomole ends), was purified using the Qiaquick PCR purification kit from Quiagen and digested with 0.5 U/µl of two particular endonucleases (Table 2), for 18 hours at 37º C according to Table 3 (Tube 2), where they are generally named endonuclease 1 and 2. As note, each endonuclease restriction site is present in the upper primer or in the lower primer sequence.

Table 2 - Restriction endonucleases used in directional cloning

Name	Restriction site	Incubation	Supplier Incubation buffer	Inactivation	Target
10U/µl SalI (Roche)	g/tcgac	18 h 37º C	1X Enz buffer H	15 min 65º C	pSKII; pβMyhc; Hyg^R
10U/µl BamHI (Roche)	g/gatcc	18 h 37º C	1X Enz buffer B	15 min 65º C	pSKII; pβMyhc; Neo^R
10U/µl XbaI (Roche)	t/ctaga	18 h 37º C	1X Enz buffer H	15 min 65º C	pSKII; Neo^R; Hyg^R
10U/µl NotI (Roche)	gc/ggccgc	18 h 37º C	1X Enz buffer H	15 min 65º C	pSKII; Hyg^R

Table 3 - Restriction enzyme digestion of the insert and/or plasmid

Name	**Tube 1**	**Tube 2**	f_d	C_f
vector 0.1 pmol ends/µl	10 µl	-	0.100	0.01 pmol ends/µl
insert 0.2 pmol ends/µl	-	10 µl	0.100	0.02 pmol ends/µl
Enz Buffer 10X	10 µl	10 µl	0.100	1X
ddH_2O	70 µl	70 µl	-	-
Endonuclease 1 10U/µl	5 µl	5 µl	0.050	0.5U/µl
Endonuclease 2 10U/µl	5 µl	5 µl	0.050	0.5U/µl
Final Volume	100 µl	100 µl	-	-

To determine the picomole ends the following equation was used:

$$\text{pmol ends/µg DNA of } x \text{ bp} = 2 / x \text{ bp} \cdot 660 \cdot 10^{-6}$$

(Note: 1 pmol of 1000 bp DNA = 0.66 µg of 1000 bp DNA)

Ligation

Thereafter, the enzymes have been inactivated by heat for 15 minutes at 65º C and the samples were maintained on ice. Again, they were purified with the Quiagen kit to a final volume of 10 µl, quantified and ligated to the plasmid, also previously purified with the same kit and digested with the same enzymes, for 2 hours at RT according to Table 4. For the ligation reaction, the ideal ratio of insert-to-vector DNA varies, however, a reasonable general ratio was 2:1 insert-to-vector ratio, measured in picomole ends.

Table 4 - Ligation reaction of insert + plasmid

			Controls	
Tube	**1**	**2**	**3**	**4**
Prepared vector 0.1 pmol ends/µl	1 µl	1 µl	1 µl	0 µl
Prepared insert 0.2 pmol ends/µl	2 µl	3 µl	0 µl	1 µl
10 mM ATP pH 7.0	1 µl	1 µl	1 µl	1 µl
10X Ligase Buffer	1 µl	1 µl	1 µl	1 µl
ddH_2O	4.5 µl	3.5 µl	7.0 µl	6.5 µl
T4 DNA Ligase 4 U/µl	0.5 µl	0.5 µl	0 µl	0.5 µl
Final volume	10 µl	10 µl	10 µl	10 µl

10X Ligase Buffer
500 mM Tris.Cl (pH 7.5)
70 mM $MgCl_2$
10 mM DTT

Two microliters of each tube were used in the transformation of the appropriate competent bacteria and plated on the respective selective media (see *Cloning*).

The β - Myosin heavy chain gene promoter (pβMyhc or pMyh7)

The myosin 5’ flanking region of the human pβMyhc, exons 1 through 3, including the initiation codon and carrying the SalI, BamHI endonuclease restriction sites (5.3 kb), was generated by PCR from human DNA and inserted, using direct cloning, in the multiple cloning site (MCS) of the pBluescript II SK(+) (pSKII) phagemid vector from Stratagene (Fig. 2). The primers used may be seen in the Table 5 and the respective amplicon in the Fig. 1.

Table 5 - Primers used in the amplification of the human pβMyhc.

Gene	Expected product	GeneBank	Primer	Primer sequence	nt	Amplicon
pβMyhc	**gDNA (bp)**	NT_019583	Upper	5' ggg ggg g/TC gAC TgA Agg CAA gAT CAA CCT gCC CTA ACT AC 3' (SalI site underlined)	(84899-84939)	**OK**
	5284		Lower	5' AAA AAA Ag/g ATC CCA Tgg CTg TgC CTg gAg TgA gCA 3' (BamHI site underlined)	(90147-90182)	

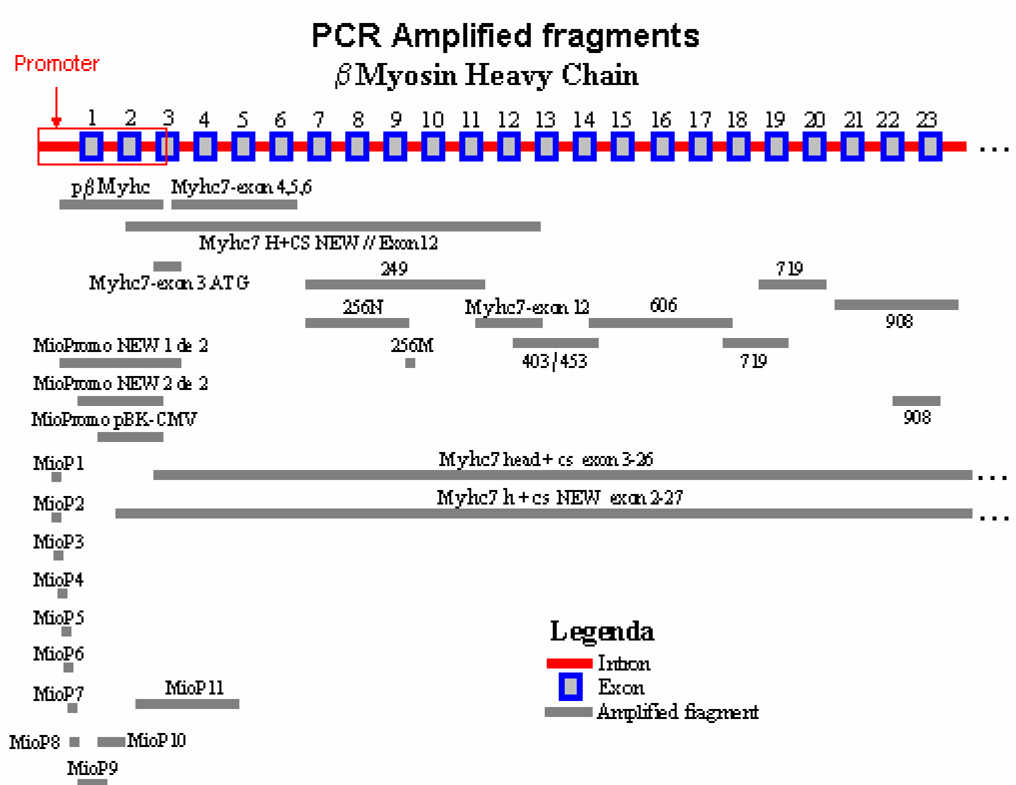
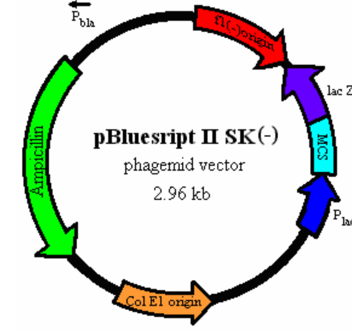


Figure 1 - Scheme of the βMyhc PCR amplified fragments. During the time course of the present work, it was possible to construct several primer pairs that have made possible the analyses of many regions of the βMyHC gene sequence, however, only the "pβMyhc" and the "Myh7 H+CS NEW // Exon12" amplicons were used in the construct creation procedure and in the cellular expression studies, respectively, and accordingly, only their associated primer pairs are mentioned in this section.

Figure 2 - The pBluescript II SK(+) phagemid vector



The Neomycin resistance gene (Neo^R) (Stratagene)

The aminoglycoside 3' phosphotransferase G418 resistance gene (Neo^R) carrying the BamHI, XbaI endonuclease restriction sites (1.3 kb) from the pBK-CMV phagemid vector from Stratagene (Fig. 3) was also generated by PCR and inserted at 3' of the pβMyhc fragment in the MCS of the pSKII phagemid vector. The primers used are shown in the Table 6.

Table 6 - Primers used in the amplification of Neo^R.

Gene	Expected product	Sequence Source	Primer	Primer sequence	nt	Amplicon
Neo^R	**cDNA (bp)** 1250	pBK-CMV Stratagene	Upper	5' ggg ggg g/gA TCC gCC ATT gAA CAA gAT ggA TTg CAC gCA 3' (BamHI site underlined)	(507-545)	**OK**
			Lower	5' AAA AAA AAT /CTA gAC CgC CCC gAC gTT ggC TgC gAg C 3' (XbaI site underlined)	(1720-1756)	

The Hygromycin resistance gene (Hyg^R) (Stratagene)

The aminoglycoside 4 phosphotransferase Ia Hygromycin resistance gene (Hyg^R) carrying the XbaI, NotI endonuclease restriction sites (2.0 kb) from the pExchange Module EC Hyg from Stratagene (Fig. 4), was also generated by PCR and inserted at 3' of the pβMyhc-Neo^R fragment in the MCS of the pSKII phagemid vector. The primers used are shown in the Table 7.

Table 7 - Primers used in the amplification of Hyg^R.

Gene	Expected product	Sequence Source	Primer	Primer sequence	nt	Amplicon
Hyg^R	**cDNA (bp)** 1979	EC-Hyg Stratagene	Upper	5' CCC CCC T/CT AgA TCC CTg CTT CAT CCC CgT ggC 3' (XbaI site underlined)	(59-90)	**OK**
			Lower	5' AAA AAA AAg C/gg CCg CCA gTT TgC TCA ggC TCT CC 3' (NotI site underlined)	(2002-2036)	

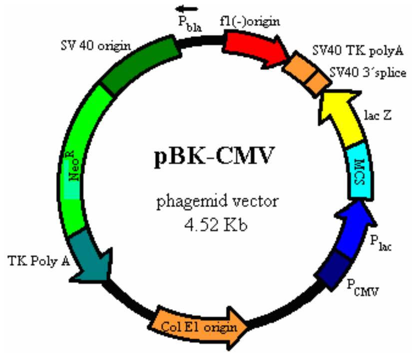


Figure 3 - The pBK-CMV phagemid vector.

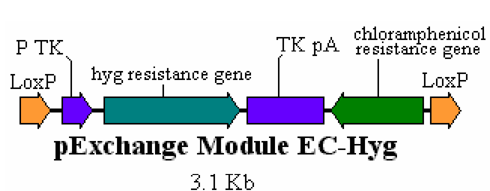


Figure 4 - The pExchange Module EC Hyg.

The pSKII Multiple Cloning Site (MCS) primers

The MCS is shown, schematically, in the Fig. 2. Its sequence is located inside the α-subunit sequence of the β-galactosidase (lacZ) gene. Thus, of the 357 nucleotides that code for the 119 amino acids that constitute the lacZ protein present in the pSKII phagemid, 108 constitute the MCS.

Depending upon the presence or absence of inserts in the MCS, appropriate strains of bacteria transformed with the pSKII phagemid and grown in selective medium, will produce white or blue colonies, respectively. Flanking the pSKII MCS are the T3 and T7 RNA polymerase promoter sequences, where the T3 and T7 primers (Table 8) anneal.

Amplification of the MCS from a white colony, with the latter primers, made it possible to analyze, whether the size of the inserted DNA fragment was the expected one.

Table 8 - Primers used in the amplification of the pSKII MCS.

pSKII	Expected product	Sequence Source	Primer	Primer sequence	nt
MCS (empty)	**DNA (bp)** 164	pSKII Stratagene	T3	5’ gTA ATA CgA CTC ACT ATA ggg C 3’	(625-646)
			T7	5’ TAA CCC TCA CTA AAg ggA AC 3’	(769-788)

Cloning

For cloning, the ultracompetent *E. coli* strain that contains the “Stop Unwanted Recombination Events” (SURE) genotype (Stratagene), was used. All cloning procedures were done according to accepted molecular biology techniques [9]. First, the pSKII vector containing the pβMyhc was cloned. Subsequently, the Neo^R gene was ligated 3’ of the pβMyhc and the Hyg^R gene 3’ of the Neo^R gene.

Bacterial growth and selection was performed using LB-tetracycline (20 g/L LB broth, 12.5 mg/L tetracycline) and LB-tetracycline/ampicillin (LB-tetracycline medium with 50 mg/L ampicillin; all from Sigma), respectively.

To prepare competent cells for transformation, 10 ml of SURE cells were culture overnight.

Subsequently, 200 µl of this culture were diluted in 30 ml of 31 g/L 2xYT medium (Sigma) pH 7.0 and incubated for 3 hours at 37° C with vigorous shaking (225 rpm).

Meanwhile, all the material and solutions of the subsequent steps had been placed at 4° C. They are the 50 ml centrifuge tubes, pipettes and competent solutions RF1 (100 mM RbCl,

50 mM $MnCl_2$, 30 mM KCH_2COOH, 10 mM $CaCl_2$, 15 % (v/v) glycerol, pH 5.8; all from Sigma) and RF2 (10 mM MOPS, 10 mM RbCl, 75 mM $CaCl_2$, 15 % (v/v) glycerol, pH 6.8; all from Sigma).

When the OD_{540nm} value was between 0.45 - 0.55, the cells were transferred to a new sterile, disposable, ice-colded 50 ml conic tube and incubated on ice for 15 minutes. Afterwards, they were centrifuged at 3000 g for 15 minutes at 4º C and the supernatant decanted from the cell pellet. The cells were ressuspended with 10 ml of cold RF1 and incubated on ice for another 15 minutes. Again, they were centrifuged as before, ressuspended with 2.4 ml of cold RF2 and incubated on ice for more 15 minutes. At this time, they could be processed immediately for transformation or to be stored in 200 µl aliquots at -80º C.

Precisely, 2 µl of each of the 4 ligation products (see *Ligation* and Table 4*)* were placed in microcentrifuge tubes and left on ice. To each tube, 200 µl of competent cells were added, gently mixed and incubated for more 45 minutes on ice. Then, the tubes were transferred to a rack previously placed in a circulating water bath at 42º C and left there for exactly 35 seconds. After that, the tubes were rapidly transferred to an ice bath and incubated for 5 minutes. To each tube, 800 µl of LB medium preheated to 37º C was added. Before plating, the cultures were incubated for 60 minutes at 37º C with vigorous shaking (225 rpm) to allow the bacteria to recover and to express the antibiotic resistance marker encoded by the plasmid.

Importantly, during this recovering time, 100 µl of 10 mM IPTG in sterile H_2O and 100 µl of 2 % (w/v) X-gal in dimethylformamide (Sigma) were spread in the surface of previously prepared LB tetracyclin-ampicillin agar medium (20 g/L LB broth, 20 g/L of agar, 12.5 mg/L tetracycline, 50 mg/L ampicillin; all from Sigma) 90 mm plates.

Exactly 200 µl of transformed competent cell culture were gently spread over the surface of each agar plate using a sterile bent glass rod.

The plates were left at RT until the liquid medium was absorbed and incubated for overnight at 37º C in an inverted position. In the next day the plates were sealed with parafilm and stored at 4º C for the future analysis.

Colonies containing the pSKII without insert appeared blue after the overnight incubation at 37º C and the colonies with the pSKII containing insert appeared white.

Further enhancement of the blue colour was obtained by placing the plates at 4º C for 2 hours following the overnight growth at 37º C.

Reverse Transcription and Polymerase Chain Reaction (RT and PCR) analysis

Total RNA was extracted from NT2/D1 cells, according to Trizol total RNA extraction protocol (Invitrogen). cDNA first strand synthesis was performed on 1 µg total RNA, by using the ThermoScript RT-PCR System (Invitrogen) with an oligo dT primer.

As a second step, cDNA samples were subjected to PCR amplification, in a separate tube, using primers specific for the gene of interest (see below), and Platinum Taq DNA Polymerase High Fidelity (see *Polymerase Chain Reaction*) using 40 cycles at 56º C.

For each gene, the DNA primers were derived from different exons, allowing the distinction between cDNA and contaminating genomic DNA amplifications.

Primers were synthesized for the following human genes: Actb, Vlc1, Vlc2, Myh7, Csx1, Gata4, Mef2d, BmprIa, Cripto (or Tdgf1), H19, Nestin, Flt1, CD133, Kdr, BmprIb, and BmprII. The primers used may be seen on Table 9 and the amplicons in Fig. 5.

Table 9 - Primers used in the NT2/D1 expression studies.

Gene	Expected product	Gene Bank	Primer	Primer sequence	nt	Amplicon
β - Actin	**cDNA (bp)** 513 **gDNA (bp)** 1080	M10277.1	Upper Lower	5' ATA TCg CCg CgC TCg TCg TC 3' 5' Tgg CAT ggg ggA ggg CAT AC 3'	(1103-22) (2169-88)	**OK**
Vlc1	**cDNA (bp)** 591 **gDNA (bp)** 5150	NT_022517.17	Upper Lower	5' ATg gCC CCC AAA AAg CCA gAg 3' 5' ggT TTA gCT ggA CAT gAT gTg 3'	(52-72) (5181-201)	**OK**
Vlc2	**cDNA (bp)** 505 **gDNA (bp)** 9397	L01652.1	Upper Lower	5' ATg gCA CCT AAg AAA gCA AAg 3' 5' CCC CTC CTA gTC CTT CTC TTC 3'	(1272/4)-(2591/2609) (10648-68)	**OK**
Myh7	**cDNA (bp)** 1216 **gDNA (bp)** 4915	M57965.1	Upper Lower	5´ CCT Tgg CCC CTT TCC TCA TCT gT 3' 5' CAT gAg gTA ggC AgA CTT gT 3'	(5206-28) (94558-77)	**OK**
Csx1 [10]	**cDNA (bp)** 418 **gDNA (bp)** 418	NC_000005.7	Upper Lower	5´ TAT AAC gCC TAC CCC gCC TAT 3' 5' TAA TCg CCg CCA CAA ACT CTC C 3'	(662-42) (266-45)	**OK**
Gata4	**cDNA (bp)** 823 **gDNA (bp)** 2032	NC_000008.8	Upper Lower	5' ggC CTg TCA TCT CAC TAC gg 3' 5' TgA TgA ggC TgT gCT gTg gT 3'	(52799-818) (54811-30)	**OK**
Mef2d	**cDNA (bp)** 185/206 **gDNA (bp)** 8046	NC_000001.7	Upper Lower	5' CAg gAA Agg ggT TAA TgC ATC AC 3' 5' ggA gAg CTC TgC ACT ggT CAA CTg 3'	(13311-289) (5289-66)	**OK**
BmprIa	**cDNA (bp)** 369 **gDNA (bp)** 2136	NC000010.8	Upper Lower	5' gTg ggC ACC AAA CgC TAC AT 3' 5' CAT CTT ggC AAg CgT CTT CTT A 3'	(164919-38) (00-00)	**OK**

Table 9 - Primers used in the NT2/D1 expression studies (cont.).

Tdgf1	**cDNA (bp)** 1199 **gDNA (bp)** 2376	M96955.1	Upper Lower	5’ TgT gCC TgC CCT CCC TCC TT 3’ 5’ ggA AAC TTg CCC TTC CAT TTA gCC 3’	(4305-24) (6657-80)	**OK**
H19 [12]	**cDNA (bp)** 575 **gDNA (bp)** 655	M32053.1	Upper Lower	5’ TAC AAC CAC TgC ACT ACC T 3’ 5’ Tgg AAT gCT TgA Agg CTg CT 3’	(2754-72) (3389-408)	**OK**
Nestin	**cDNA (bp)** 389 **gDNA (bp)** 3466	NC_000001.7	Upper Lower	5’ CAg CgT Tgg AAC AgA ggT Tgg 3’ 5’ Tgg CAC Agg TgT CTC AAg ggT Ag 3’	(7784-64) (4341-19)	**OK**
Flt1	**cDNA (bp)** 1010 **gDNA (bp)** 4319	NC_000013.8	Upper Lower	5’ ACC CAA ggC CTC gCT CAA gA 3’ 5’ ACA AAT CAA AAC ATg CCA CgA ATg 3’	(188395-414) (191690-192713)	**OK**
CD133	**cDNA (bp)** 762 **gDNA (bp)** 10748	NC_000004.8	Upper Lower	5’ TCA TAA AgA TCA TgT ATA Tgg TAT TC 3’ 5’ TTg TCA gAT ggA gTT ACg CA 3’	(11206-181) (478-59)	**OK**
Flk1/Kdr	**cDNA (bp)** 630 **gDNA (bp)** 4655	NC_000004.9	Upper Lower	5’ gTg ACC AAC ATg gAg TCg Tg 3’ 5’ CCA gAg ATT CCA TgC CAC TT 3’	(10215-34) (14850-69)	**OK**
BmprIb	**cDNA (bp)** 453 **gDNA (bp)** 2229	NC_000004.9	Upper Lower	5’ gTg CCC AgT gAC CCC TCT TA 3’ 5’ ACA AgT TAC CCA AgC ggT TTC T 3’	(276852-71) (279059-80)	**OK**
BmprII	**cDNA (bp)** 297/1577 **gDNA (bp)** 7104	NC_000002.9	Upper Lower	5’ gAT gCA gAg gCT Cgg CTT AC 3’ 5’ TTg gAA TgA ACT gCC CTg TTA C 3’	(175886-905) (182968-89)	**OK**

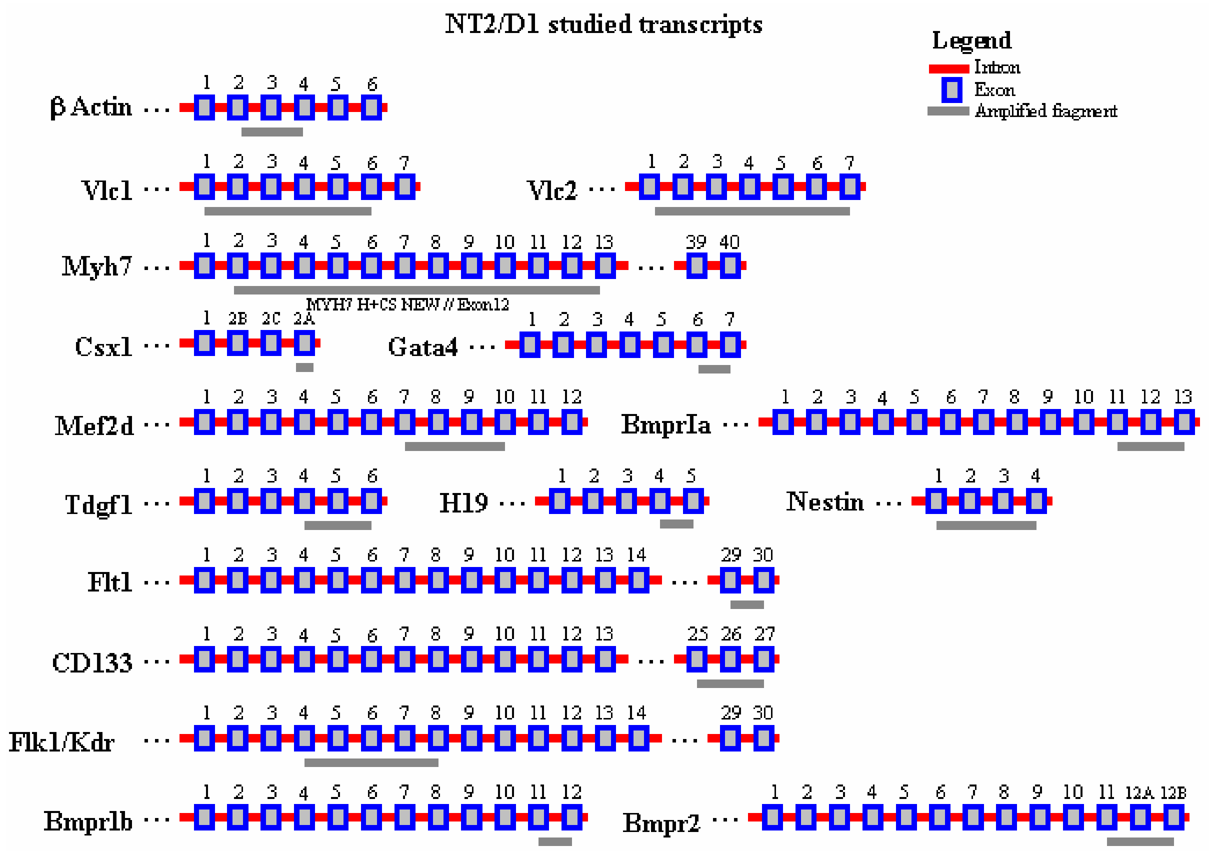


Figure 5 - Scheme of the NT2/D1 transcripts amplified by PCR.

Expression studies in human rhabdomyosarcoma cells

The alveolar rhabdomyosarcoma cell line RH30 from “Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmbh” (DSMZ) was used for the expression studies.

Cells were seeded at 5.5 x 10^4 cells/cm^2 in 3 of 24 well cluster plates, with glass coverslips coated with 2 % Gelatin (Sigma), in RPMI 1640 medium without Glutamine, with 10 mM HEPES, 2 mM Glutamine, 10 % FBS, 1 % (v/v) Penicillin 10000 IU/ml + Streptomycin 10 mg/ml (all from Invitrogen) and 20 µM 2-β-Mercaptoetanol (Sigma) and left for growth to 80 % confluence. Control wells, without cells or without DNA and test samples were performed in separate plates.

The pβMyhc-Neo^R-Hyg^R final construct was gently mixed with Fugene 6 (Roche) in microcentrifuge Eppendorf tubes at Fugene 6: DNA ratios of 3:2, 3:1 and 6:1 according to the manufacturer instructions (Table 10 and 11). One unit of DNA corresponded to 0.4 µg and 1 unit of Fugene 6 to 0.2 µl. After incubation at RT for 15 minutes, 10 µl of the mixture was added to each well and the plates returned immediately to the incubator. Control wells received

either 1 unit of DNA, 3 units of Fugene 6 or none. Fugene 6 manufacturer reports that the exposure of most common laboratory cell types, COS-1, CHO-K1, HEK-293 and HeLa to the Fugene 6: DNA complex until the time of the reporter gene assay (normally, 48 hours later), has produced no adverse effects. Accordingly, we observed no negative effects regarding RH30 cells. Therefore, after the transfection, the medium containing the Fugene/DNA complex was left unchanged until the cells needed to be fed.

Table 10 - Recommended Fugene 6 starting volumes and DNA masses.
The recommended values for the 24 well cluster plates are shown in bold.

Type of dish or plate	**Surface area per well or plate (cm2)**	**Total media volume per well or plate (ml)**	**Starting volume of Fugene 6 Reagent (µl/well or plate)**	**Starting mass of DNA (µg/well or plate)**
60 mm	21	4	6.0	2.0
35 mm	8	2	3.0	1.0
6-well	9.4	2	3.0	1.0
12-well	3.8	1	1.5	0.5
24-well	**1.9**	**0.5**	**0.6**	**0.2**
96-well	0.3	0.1	0.15	0.05

Table 11 - Fugene 6 transfection reaction master mix.
The final volume of the mixture added to each well was 10 µl.

	Cell control	**Reagent control**	**DNA control**			
Reagents \ Tubes	**01**	**02**	**03**	**1**	**2**	**3**
Fugene 6: DNA Ratio	-	-	-	3:2	3:1	6:1
Serum-free media (µl)	100	94	99	90	92	86
Fugene 6 Reagent (µl)	-	6	-	6	6	12
DNA at 1µg/µl (µl)	-	-	1	4	2	2
Total (µl)	100	100	100	100	100	100

Three days after transfection, Hygromycin B (Invitrogen) was added at a final lethal concentration of 600 µg/ml. Five days later, the medium was replaced with fresh medium containing Geneticin (Sigma) at the final concentration of 500 µg/ml, and left for additional 5 days. Thereafter, the medium was removed in all samples, and those that would be processed for immunofluorescence were fixed in 2 % Formaldehyde for 25 minutes at RT. Wells in which the cells would be used for RNA extraction, were treated with the Trizol Reagent and processed according to the manufacturer instructions protocol (see *RNA extraction from human tissues and cells in culture*).

Primary cultures

Fragments of human left ventricle myocardium or Wharton's jelly were cultured in 20 cm^2 flasks in RPMI 1640 medium without Glutamine, with 10 mM HEPES, 20 µM 2-β-Mercaptoetanol, 2 mM Glutamine 10 % FBS and 1 % (v/v) Penicillin 10000 IU/ml + Streptomycin 10 mg/ml.

In some cases, adherent cells from umbilical cord´s Wharton's jelly cultures were treated with 3 µM 5-Azacytidine for 24h, washed and ressuspended in the same initial medium.

Alternatively, umbilical cord Wharton's jelly cultures were performed in a Defined Keratinocyte basal medium (Invitrogen) supplemented with defined growth additives including insulin, EGF and FGF. All the cell cultures of the present work used as cell incubator the IG150 from Jouan in a 5.0 % CO_2 atmosphere at 37º C.

In some experiments tissue fragments were digested with 85-400 U/ml of collagenase type XI (Sigma) in HBSS (Invitrogen) and washed before culturing.

Embryonal carcinoma and rhabdomyosarcoma cell cultures

Two human embryonal carcinoma, PA1 and NT2/D1 and one human rhabdomyosarcoma, RH30 cell lines from Interlab Cell Lab Collection (ICLC), Stratagene and "Deutsche Sammlung von Mikroorganismen und Zellkulturen Gmbh" (DSMZ), respectively, were used. Cells were cultured and expanded in 80 cm^2 flasks in RPMI 1640 medium without Glutamine, with 10 mM HEPES, 20 µM 2-β-Mercaptoetanol, 2 mM Glutamine 10 % FBS and 1 % (v/v) Penicillin 10000 IU/ml + Streptomycin 10 mg/ml and left for growth to 80 % confluence, harvested by scraping and reseeded 1:4.

Preliminary embryonal carcinoma differentiation studies

Several conditions were tested in order to identify the best combination of factors that could potentiate the hypothetic cardiogenic differentiation of PA1 and NT2/D1 cells.

Because these were preliminary studies, we only look to the morphologic and ultrastructural characteristics of the cultured cells during the time course of the experiments trying to find signals of cardiac muscle differentiation as for instance the presence of sarcomeres or expected automatic contractility associated with embryoid body-like structures.

Briefly, the cells were scraped from an 80 % confluent culture, divide between two 50 ml Falcon tubes, centrifuged at 200 g for 10 minutes, washed one time with HBSS, resuspended in control or inductive medium (IM) and distributed on 24 well plates. The next table (Table 12) intends to resume the main components of the cell culture medium.

Table 12 -
Preliminary embryonal carcinoma differentiation studies.
Legend: ✓ - used in the cell culture medium;
× - not used in the cell culture medium;
×✓ - used or not in different wells.

		45 days cell cultures in 24 well plates		
		PA1 cells	**NT2/D1 cells**	
Inductive Medium (IM) components	**Control medium**	2500 cells/cm^2	2500 cells/cm^2	1000 cells/cm^2
RPMI 1640 (Invitrogen)	✓	✓	✓	✓
10 mM HEPES (Invitrogen)	✓	✓	✓	✓
20 µM 2βME (Sigma)	✓	✓	✓	✓
2 mM Glutamine (Invitrogen)	✓	✓	✓	✓
1X ITS (Invitrogen)	×	✓	✓	✓
1X NEAA (Invitrogen)	×	✓	✓	✓
1 mM Na Pyruvate (Sigma)	×	×	×	✓
1 % FBS (Invitrogen)	×	✓	✓	✓
10 % FBS (Invitrogen)	✓	×	×	×
1000U/ml Heparin Na salt (Sigma)	×	✓	✓	✓
1 % (v/v) 10000IU/ml Penicillin + 10 mg/ml Streptomycin (Invitrogen)	✓	✓	✓	✓
50 ng/ml BDNF (Invitrogen)	×	✓	✓	✓
5 ng/ml FGF2 (Sigma)	×	✓	✓	✓
10 ng/ml $VEGF_{165}$ (Sigma)	×	×	×	✓
10 ng/ml IGF2 (Sigma)	×	✓	✓	✓
150 µg/ml ECGS (Sigma)	×	✓	✓	×
3.75 µg/cm^2 Fibronectin (Sigma)	×	×	×	×✓
3 µM 5-Azacytidine treatment (Sigma)	×	×	×	×✓
100 ng/ml BMP2 (Sigma)	×	×	×	×✓

The NT2/DI cell cultures in inductive angiomyogenic medium (IAM)

In angiomyogenic induction, NT2/D1 cells were suspended in inductive angiomyogenic medium (IAM) with Endothelial Cell Growth Supplement (ECGS) as described in [14]. Briefly, cells were cultured in M199 Medium (Invitrogen) supplemented with 20 % FBS, 10 mM HEPES, 1U Heparin (Sigma), 1 % (v/v) Penicillin 10000 IU/ml + Streptomycin 10 mg/ml, 10 ng/ml VEGF (Sigma), 5 ng/ml bFGF (Sigma), 50 ng/ml BDNF (Invitrogen) and 150 µg/ml ECGS (Sigma) and seeded at 1000 cells/cm^2 on glass or thermanox coverslips coated with 2 % gelatin, in 24-well dishes. The seeded cells were left for the first 24h in 10 µM 5-Azacytidine and then washed and resuspended in the same initial medium. Additionally, BMP2, at the concentration of 100 ng/ml was added to IAM (IAM + BMP2). Medium without VEGF, bFGF, BDNF and ECGS was used as control. Cells were harvested on day 1, 5, 10, 20 and 30 after initiation of culture and used for histological, ultrastructural and RT-PCR analysis.

Histochemistry and Immunofluorescence

Glass coverslips, containing the NT2/D1 cells, were fixed for 30 min with the addition of buffered formaldehyde pH 7.0 into the cell culture wells at a 2 % final concentration, washed in D-PBS pH 7.2-7.4 (Invitrogen) and stored at 4º C until processed.

Hematoxylin-eosin staining

Coverslips were washed in water, immersed in Harris´s hematoxylin (Merck) for 10 minutes, washed in water, differentiated in 70 % EtOH with 1 % HCl for 10 seconds, immersed in eosin Y (Merck) for 10 seconds and washed again. Stained cells in coverslips were dehydrated in graded series of ethanol, followed by xylol (Merck), and mounted on glass microscope slides, cell side down, using Entellan (Merck).

Phalloidin staining

Dilution of phalloidin-TRITC stock solution (Sigma) and washing of the glass coverslips were performed in D-PBS pH 7.2-7.4 containing 0.05 % saponin (Sigma). Coverslips were fixed for 30 min with the addition of buffered formaldehyde pH 7.0 into the cell culture wells at a 2 % final concentration, incubated with 1 µg/ml phalloidin-TRITC solution for 90 min at RT and washed 3 times. The coverslips were then mounted on microscope slides, cell side down, using Glicerol:D-PBS (1:1) and visualized in a Olympus BX50 microscope.

Immunofluorescence

Coverslips were washed for 30 min, incubated with the primary antibody for 60 min at RT, washed 3 times, incubated with the secondary antibody for 60 min at RT and washed another 3 times. The coverslips were then mounted on microscope slides, cell side down, using Glicerol:D-PBS (1:1) and visualized in a Olympus BX50 microscope. All dilutions of the antibodies and washing of the coverslips containing the transfected cells were performed in D-PBS pH 7.2-7.4 (Invitrogen) containing 1 % HSA and 0.05 % Saponin.

The primary antibodies used in this study were: Anti-Neomycin Phosphotransferase II (rabbit polyclonal IgGs from Upstate), anti-Hygromycin B (MicroPharm) and the MAB1628 (Chemicon), that reacts with the βMyhc human protein. Secondary antibodies were: Goat anti-rabbit TRITC (Sigma), Donkey anti-sheep FITC (Sigma) and Rabitt anti-mouse FITC (Dako).

Ultrastructural analysis

Thermanox coverslips (Nunc) from day 20, were fixed in 0.1 M sodium cacodylate buffer (EMS) pH 7.3 with 3 % gluteraldeyde (EMS) for 1h, washed in 0.1 M sodium cacodylate, followed by 1 % osmium tetroxide (EMS) in the same buffer for 1 h and washed again, first in cacodylate buffer and then in 0.1 M sodium acetate buffer (Merck) pH 5.0. Fixed cells were incubated with 1 % uranyl acetate (EMS) in 0.1 M sodium acetate buffer pH 5.0 for 1h, washed in the same buffer, dehydrated in graded series of ethanol, followed by propylene oxide (EMS), embedded in epon (EMS) and left for 3 days at 70º C. Thin sections cut on a LKB III microtome with a diamond knife for transmission electron microscopy (TEM) were stained on 300 mesh square copper grids with 2 % uranyl acetate and lead citrate (Fluka) and observed in a Philips CN10 transmission electron microscope. All the steps were made at room temperature, except when otherwise documented.

Sequencing

The PCR products were purified using Qiaquick PCR purification kit (Quiagen) as described by the manufacturer and visualized by ethidium bromide after electrophoresis in a 1 % agarose gel. A molecular weight standard a 1kb DNA ladder (Invitrogen,) was used. All sequences were determined in an automated DNA capillary sequencer CEQ 2000-XL (Beckman Coulter, USA, in ICAT-Lisbon Faculty of Sciences Sequencing Services) by a dye-labeled dideoxy termination method (DTCS, Dye Terminator Cycle sequencer start kit, Beckman Coulter). Two

sequencing reactions were performed using the two primers used for PCR amplification. The sequences obtained, were compared with existing data from the National Centre for Biotechnology Information GenBank entries for the pβMyhc and for all the amplified genes or from Stratagene Database for Neo^R and Hyg^R inserts.

References

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Results

Results

The next pages resume all the experimental work performed in the persecution of the main aim of the thesis; the effort of establishment of a human cardiac differentiation cell model in order to obtain new scientific insights suitable to be applied in strategies of clinical cardiac cell therapies.

With the intent to facilitate the understanding and description of the experimental data this chapter was organized in two major fields:

A first one, of traditional molecular biology techniques that reports the construction of an aminoglycoside phosphotransferase resistance gene under the control of the human β myosin heavy chain promoter and its validation in a human rhabdomyosarcoma cell line (Fig. 1.1). Studies of the best cell culture conditions to the expression of the βMyHC are also reported. This section is named: **Construction / validation of the pβMyhc-Neo^R-Hyg^R molecule.**

A second one, of human cell culture based methods, initially analyzing human primary cultures of ventricular myocardium and umbilical cord Wharthon's jelly at the level of cell expansion and possible muscle differentiation, respectively. Subsequently an attempt to reach the best culture conditions in order to assure a mesodermal phenotype (preferentially cardiac muscle) from differentiating human pluripotent cells *in vitro* is reported. This section has the name: **Human cell culture studies.**

1. Construction / validation of the pβMyhc-Neo^R-Hyg^R molecule

Although clinical improvement has been reported in patients with acute myocardial infarction submitted to "stem cell" therapy, the benefits of this therapy still needs to be validated in further large-size double blinded controlled clinical trials [1]. Furthermore, the specific postnatal cardiac left ventricular progenitors have not yet been identified [2], the homing and differentiation mechanism remain unclear and the route of delivery and optimal dose still require optimization [3].

With a few exceptions, in which CD34$^+$ or CD133$^+$ cells were used [4, 5] in patients with myocardial ischemia, in the majority of clinical trials performed to date, specific subpopulations of bone marrow cells were not used. Instead, the mononuclear or the mesenchymal stem cell fraction was used [6-11]. In patients with chronic heart failure cell therapy was performed with adult skeletal muscular progenitor [12-17].

Considering that the heart is composed of many different cell types as atrial, ventricular, sinus nodal-like and Purkinje-like cells, the best cell type to be used in stem cell therapy, would be the one that could differentiate into the desired cardiac cell type (right or left atrial and right or left ventricular) without causing arrhythmogenic events. In spite of the tremendous advances in the stem cells research and the identification of postnatal human cardiac progenitors in right ventricle, atria and outflow tract, the progenitors of left ventricle are still unknown [2].

While a non selective cell therapy approach may be justified in some conditions, in others, as for instance chronic heart failure, is crucial to transplant cells that become left ventricular cardiac muscle with high efficacy, in order to improve systolic and diastolic mechanical function [1].

Therefore, the selection process between potential progenitor cells is essential, in order to obtain good levels of safety, homing and electromechanical integration. It is therefore, imperative to define methods that allow the isolation of cells, that will become left ventricular cardiomyocytes, at a stage of none terminal differentiation. Such tool would support identification of cell markers of the required cells and thus, became an important instrument in stem cell therapeutics.

In these results, undifferentiated human muscle cells were genetically modified to access the feasibility of that selection.

It is our assumption that human pluripotent stem cells that start to express the human β- myosin heavy chain (βMyhc) gene are likely to be left ventricle progenitors, as less differentiated cells have a higher probability of developing into skeletal muscle.

The method we used is similar to the one that has already been performed in mice [18]. Briefly, a fragment carrying the 5' flanking region of the human βMyhc promoter (pβMyhc), the G418 resistance gene (Neo^R) and the Hygromycin resistance gene were linked together in the pBluesript II SK(+) vector. The final relevant fragment that could be used for mammalian transformation was obtained by PCR from this construct. Because the Neo^R does not carry a promoter, its expression depends upon the activation of the βMyhc gene and will, thus, be responsible for the selection of βMyhc expressing cells from an undifferentiated cell pool. The Hygromycin resistance gene, containing the HSV-TK promoter, will be expressed in all transformed mammalian cells despite their lineage commitment, and used as transformation reporter gene. This strategy is summarized in Fig. 1.1.

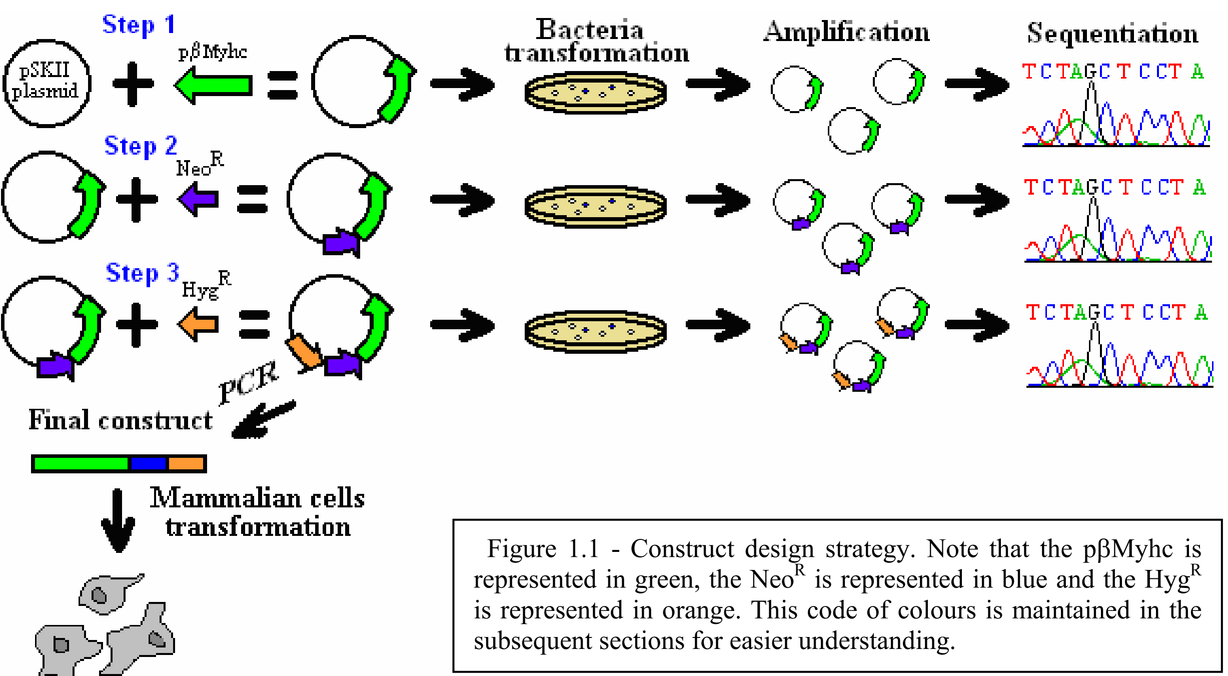

Figure 1.1 - Construct design strategy. Note that the pβMyhc is represented in green, the Neo^R is represented in blue and the Hyg^R is represented in orange. This code of colours is maintained in the subsequent sections for easier understanding.

The pβMyhc SalI/BamHI, the Neo^R BamHI/XbaI and the Hyg^R XbaI/NotI fragments

All inserts were generated by PCR (Fig. 1.2) and processed sequentially. As mentioned before, first they were purified with the PCR purification kit and double digested with the restriction endonucleases. The vector was also double digested with the respective restriction endonucleases. Inserts and vector were, then, purified with the same Quiagen kit and submitted to the ligation reaction with T4 DNA ligase (Roche) at room temperature for 2 hours and at different vector/insert ratios in final volume of 10 µl. After that, 2 µl of each ligation product was used in the bacteria transformation protocol.

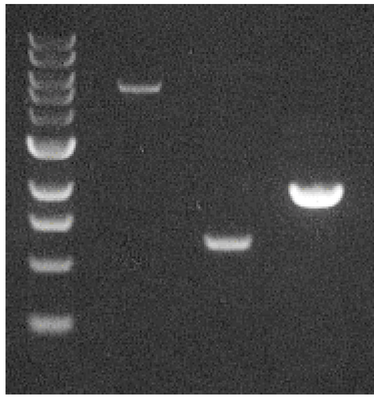

Figure 1.2 - 0.8 % Agarose gel electrophoresis of the three inserts that were generated by PCR before the sequential insertion in the plasmid pSKII(+).
From left to right:
Lane 1: 1Kb DNA Ladder (10; 8; 6; 5; 4; 3; 2; 1.5; 1; 0.5 Kb);
Lane 2: pβMyhc SalI/BamHI (5284 bp);
Lane 3: Neo^R BamHI/XbaI (1250 bp);
Lane 4: Hyg^R XbaI/NotI (1979 bp).

Construction steps

The first phase of this strategy was to ligate the βMyhc promoter to the pBluesript II SK(+) vector (step 1). Secondly was the ligation of the Neo^R and finally the Hyg^R (steps 2 and 3, respectively; Figs. 1.1, 1.3, 1.4, 1.5 and Table 1.1). We used a special ultracompetent *E. coli* strain that contains the "Stop Unwanted Recombination Events" (SURE) genotype, in the cloning procedures. Initial problems in the pβMyhc cloning procedure using XL1B *E. coli* supercompetent cells, such as, the ability of the none-expressed regions to create secondary structures, their interaction with the host bacterial genome by homologous or heterologous recombination, their hydrolysis by host endonucleases and possible interactions with the transcriptional machinery of the host, were overcomed with the use of the SURE phenotype.

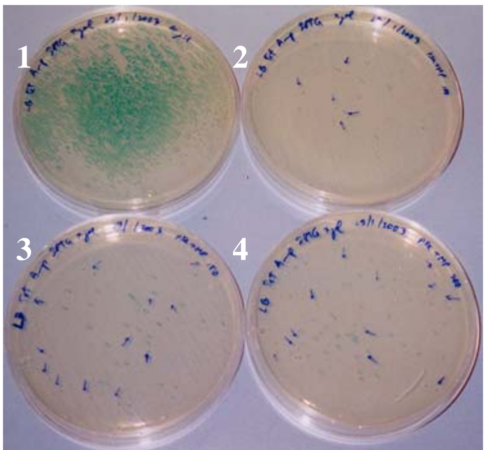

Figure 1.3 - An illustrative image of SURE plates representing one of the many transformation events performed on step 1.
After the transformation protocol (See Material and Methods, *Cloning*), the plates were incubated overnight at 37º C in an inverted position. In the next morning, colonies containing the pSKII without insert appeared blue and the colonies with the pSKII containing the insert appeared white. On plate 1 (control), only blue colonies may be observed.
The small blue arrows represent the white colonies after overnight growth using 100, 150 and 200 µl of transformed competent cell culture, plates 2, 3 and 4, respectively. Blue colonies may also be seen on these plates.

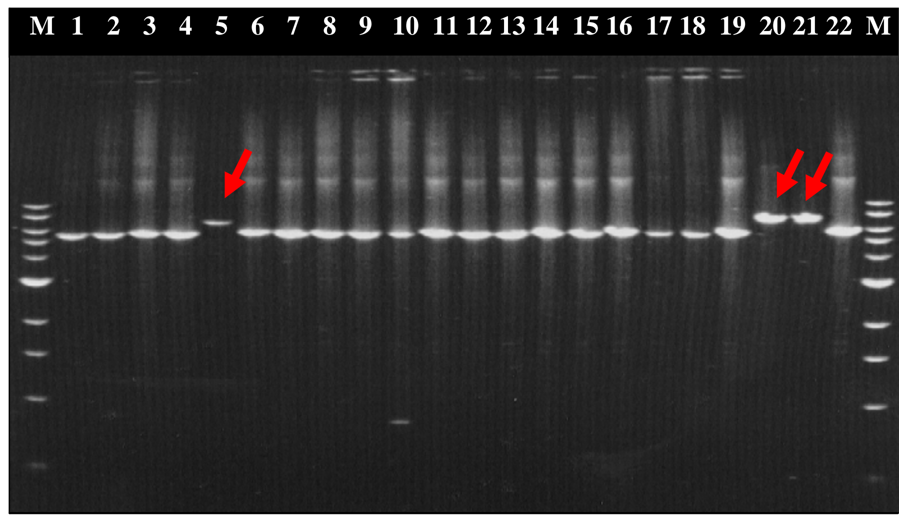

Figure 1.4 - Representative 0.8 % Agarose gel electrophoresis image, during step 2, of a direct PCR analyses with T3/T7 primers on positive transformants (see Material and Methods, *The pSKII Multiple Cloning Site primers*). Part of a selected white colony was directly ressuspended on a PCR tube, in the PCR final mix as an usual DNA template. The only difference between this approach and the normal PCR protocol (see Material and Methods, *Polymerase Chain Reaction*) was the initial extra denaturating step at 94º C for 10 minutes. White colonies with the expected insert give rise to heavier bands after PCR and may easily be identified with this procedure (red arrows). Once identified, these were amplified and their plasmids purified and verified by sequentiation.
Legend:
M: 1Kb DNA Ladder (10; 8; 6; 5; 4; 3; 2; 1.5; 1; 0.5 Kb);
Lanes 1-22: T3/T7 PCR amplification of pSKII(+)-pβMyhc-Neo^R (6602 bp) in different white colonies; Lanes 5, 20 and 21 have the expected insert (red arrows).

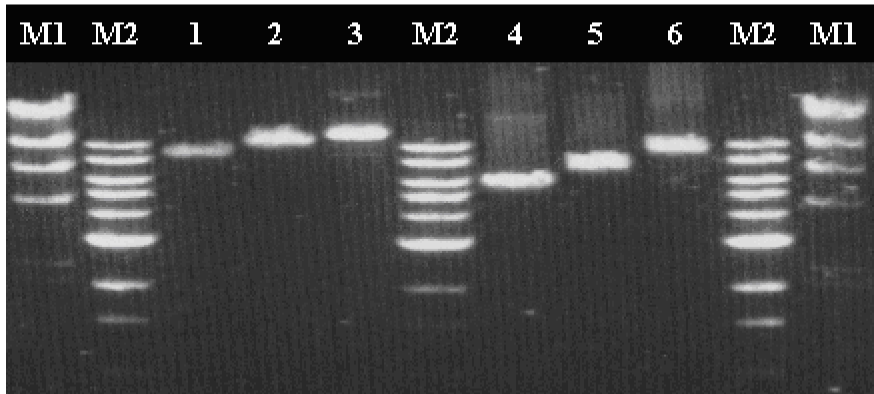

Figure 1.5 - Construct creation steps.
Construct design Step 1: Lanes 1 and 4; Step 2: Lanes 2 and 5; Step 3: Lanes 3 and 6 in a 0.8 % Agarose gel stained with EtBr. In the diagrams bellow green arrows represent the βMyhc promoter, blue arrows the Neo^R, orange arrows the Hyg^R and black lines the pSKII backbone:
M1: λ DNA HindIII digest Ladder (23.1; 9.4; 6.6; 4.4; 2.3; 2.0; 0.6; 0.5; 0.1 Kb);
M2: 1Kb DNA Ladder (10; 8; 6; 5; 4; 3; 2; 1.5; 1; 0.5 Kb);
1. BamHI digested pSKII(+)- pβMyhc (8181 bp); ( )
2. BamHI digested pSKII(+)-pβMyhc-Neo^R (9399 bp); ( )
3. NotI digested pSKII-pβMyhc-Neo^R-Hyg^R (11350 bp); ( )
M2. 1Kb DNA Ladder;
4. T3/T7 PCR amplification of pSKII(+)-pβMyhc (5384 bp); ( )
5. T3/T7 PCR amplification of pSKII(+)-pβMyhcC-Neo^R (6602 bp); ( )
6. T3/T7 PCR amplification of pSKII(+)-pβMyhc-Neo^R-Hyg^R (8553 bp); ( )
M2. 1Kb DNA Ladder;
M1. λ DNA HindIII digest Ladder.

Table 1.1 - Construct design base pairs.

Name	**Sizes (bp)**
vector pSKII(+)	2961
pβMyhc SalI/BamHI	5284
Neo^R BamHI/XbaI	1250
Hyg^R XbaI/NotI	1979
Step 1	
digested pβMyhc SalI/BamHI	5265
digested pSKII(+) SalI/BamHI	2916
ligated pSKII(+) SalI/BamHI + pβMyhc SalI/BamHI	2916 + 5265 = 8181
pSKII(+)-pβMyhc PCR with primers T3/T7	5384
Step 2	
digested pSKII(+)-pβMyHhc BamHI/XbaI	8169
digested Neo^R BamHI/XbaI	1230
ligated pSKII(+)-pβMyhc BamHI/XbaI + Neo^R BamHI/XbaI	8169 + 1230 = 9399
pSKII(+)-pβMyhc-Neo^R PCR with primers T3/T7	6602
Step 3	
digested pSKII(+)-pβMyhc-Neo^R XbaI/NotI	9392
digested Hyg^R XbaI/NotI	1958
ligated pSKII(+)-pβMyhc-Neo^R XbaI/NotI + Hyg^R XbaI/NotI	9392 + 1958 = 11350
pSKII(+)-pβMyhc-Neo^R-Hyg^R PCR with primers T3/T7	8553

The pβMyhc-Neo^R-Hyg^R insert details in the plasmid pSKII

Fig. 1.6 shows the relevant fragment that was used for mammalian transformation (8.4 kb) in the plasmid MCS and the relevant restriction sites used in this strategy. Note the putative MYOGENIN recognition sites in the pβMyhc.

The sequence of the construct was verified for accuracy, with physical data, only in the critical regions (Fig. 1.7, 1.8 and Table 1.2). Because, during the construction steps, all the sample sizes have resulted as expected, the remainder sequences (middle construct regions and plasmid sequence) from the final molecule were obtained from sequences already registed in literature, namely, the sequence of chromosome 14 from GeneBank NT_019583 GI:14751918 that includes the pβMyhc and the sequences for Neo^R, Hyg^R and pSKII(+) from the Stratagene

web site. The final total sequence may be seen in Fig. 1.9.

The PCR band of the construct amplification with the primers T3/T7 (Fig. 1.7 Lane 1) was used as target in eight sequentiation reactions, with the primers for the critical regions (Table 1.2), after processed for the removal of PCR reagents with the PCR purification kit from Qiagen. Four of those eight chromatograms can be seen in Fig. 1.8. This has made possible the verification of the accuracy of the boundaries that resulted from the ligation of the different fragments in the present method.

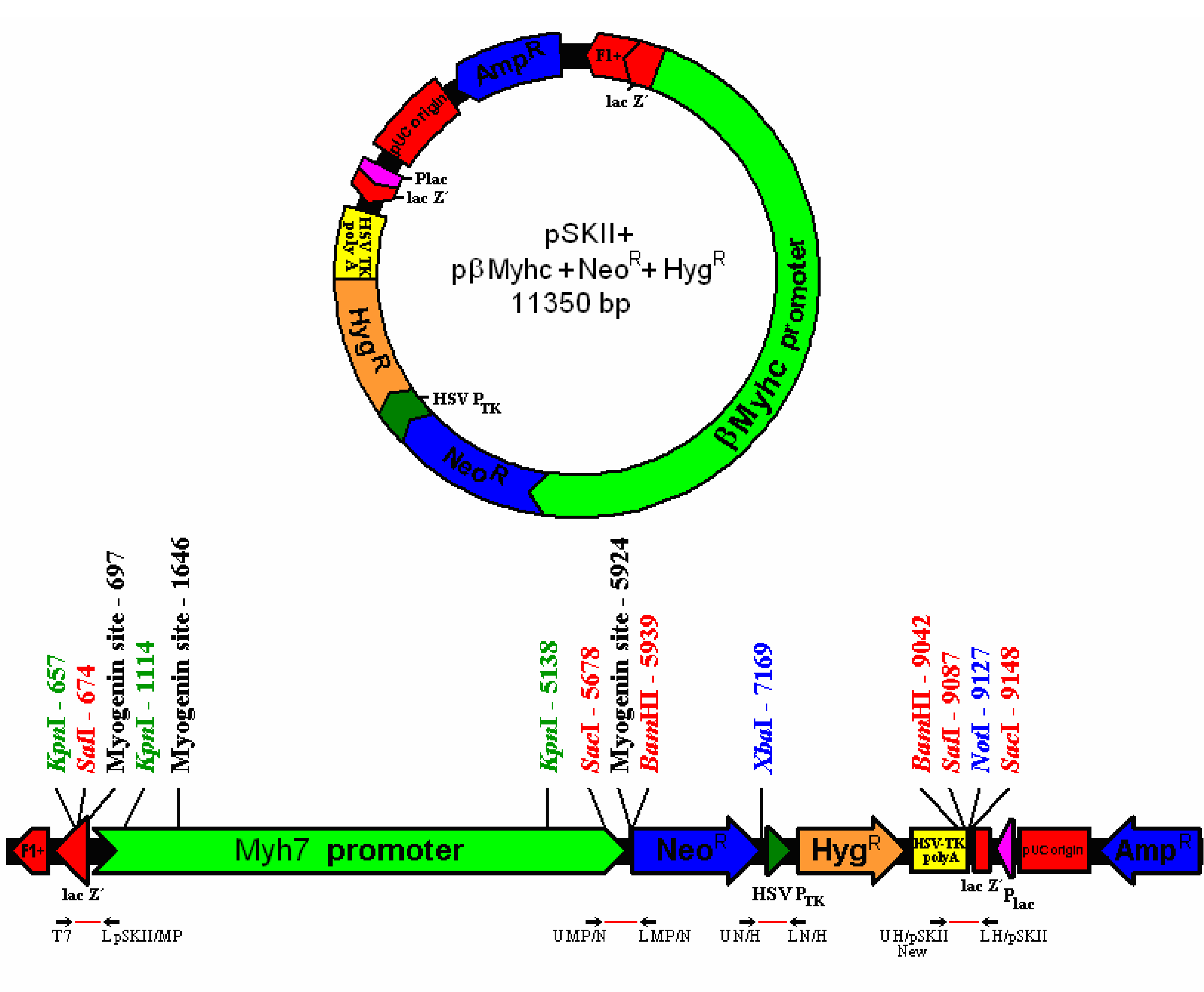

Figure 1.6 - pβMyhc-Neo^R-Hyg^R insert details. The plasmid is represented in the circular form (above) and in the linear form (below) with the localization of essential regions, endonucleases restriction sites, myogenin sites and the PCR amplified fragments and primers relative to the critical regions referred in table II and represented here by short red lines. The critical regions were named, from left to right, pSKII(+)-pβMyhc, pβMyhc-Neo^R, Neo^R-Hyg^R and Hyg^R-pSKII(+) junctions. See also Fig. 1.7.

Table 1.2 - Sequences of the primers used in amplification and sequenciation of the critical regions.

	5´primers		
Critical Region	**Name**	**Sequence 5´-3´**	**nt**
pSKII(+)-pβMyhc	**T7**	gTAATACgACTCACTATAgggC	625-646
pβMyhc-Neo^R	**U MP/N**	ggggTCCAgggATAgATgAg	5615-5634
Neo^R-Hyg^R	**U N/H**	ggggATCTCATgCTggAgTT	6880-6899
Hyg^R-pSKII(+)	**U H/pSKII New**	CgTgggggTTATTATTTTgg	8795-8814
	3´primers		
Critical Region	**Name**	**Sequence 5´-3´**	**nt**
pSKII(+)-pβMyhc	**L pSKII/MP**	gCggTAgTTCAgAggAAAAgT	1232-1212
pβMyhc-Neo^R	**L MP/N**	AACACggCggCATCAgAgCA	6054-6035
Neo^R-Hyg^R	**L N/H**	gCggggTTTgTgTCATCATA	7275-7256
Hyg^R-pSKII(+)	**L H/pSKII**	TggAATTgTgAgCggATAAC	9246-9227

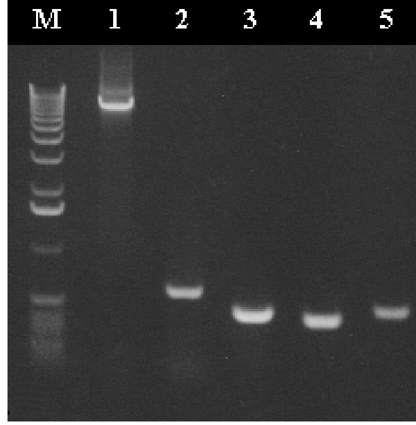

Figure 1.7 - DNA 0.8 % agarose gel of the construct in PCR amplified critical points, i.e.: pSKII(+)/pβMyhc; pβMyhc/Neo^R; Neo^R/Hyg^R and Hyg^R/pSKII(+) regions.
Legend:
M. Gibco 1Kb DNA Ladder (12.22; 8.14; 7.13; 6.12; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.52; 0.40; 0.34; 0.30; 0.22; 0.20 Kb);
1. T3/T7 PCR amplification of the pSKII(+)-pβMyhc-Neo^R-Hyg^R (8553 bp);
2. pSKII(+)-pβMyhc junction (608 bp);
3. pβMyhc-Neo^R junction (440 bp);
4. Neo^R-Hyg^R junction (396 bp);
5. Hyg^R-pSKII(+) junction (452 bp).

Figure 1.8 - Physical data of the critical regions. The PCR band of the construct amplification with the primers T3/T7 (Fig. 5 Lane 1) was used as target for sequenciation, after the removal of PCR reagents, with the primers for the critical regions. The chromatogram files are shown for **a.** L pSKII/MP, **b.** U MP/N, **c.** U N/H and **d.** U H/pSKII.
Two putative MYOGENIN binding sites and the four endonuclease restriction sites used in this construction are represented in yellow and the exact point of hydrolysis with a short red line.

```
   1 ctaaattgta agcgttaata ttttgttaaa attcgcgtta aatttttgtt aaatcagctc attttttaac caataggccg aaatcggcaa
  91 aatcccttat aaatcaaaag aatagaccga gatagggttg agtgttgttc cagtttggaa caagagtcca ctattaaaga acgtggactc
 181 caacgtcaaa gggcgaaaaa ccgtctatca gggcgatggc ccactacgtg aaccatcacc ctaatcaagt tttttggggt cgaggtgccg
 271 taaagcacta aatcggaacc ctaaagggag cccccgattt agagcttgac ggggaaagcc ggcgaacgtg gcgagaaagg aagggaagaa
 361 agcgaaagga gcgggcgcta gggcgctggc aagtgtagcg gtcacgctgc gcgtaaccac cacacccgcc gcgcttaatg cgccgctaca
 451 gggcgcgtcc cattcgccat tcaggctgcg caactgttgg gaagggcgat cggtgcgggc ctcttcgcta ttacgccagc tggcgaaagg
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 631 cgactcacta tagggcgaat tgggtaccgg gccccccctc gaggtcgact gaaggcaaga tcaacctgcc ctaactaccc caaagtgaac
 721 taggccagcc ccaagcctgt ctccggccct ccatctctct ctttgttcac atccatcaga aagcagagac ctagagtagg gaatgagagg
 811 gggtgcacaa gtacaaaaga ttttgaagcc agcaggaaga caaaagaatg acaagaagga gcagctgttt gtaaagggaa atctgagaca
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5041 gactgagtag gagaaagtgc attgaagtgc ttttagactg gaaagtgccg aacaaatgtg accattagtt gtctctcaga tggctcaaag
5131 aaaggtacca ctagatgggt acaggcaggg gtgggaccat ctcgcaggag tgaagaggat tagggttcag gttgagttga caccaggaag
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5311 ttttggaaga gggtggatgc tgaagaacag agccagggag aagcaggaag gtgggactgg tcaggttggg cacagcctgc cctgacacag
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5851 cccactgaag gggagagccc agggagggaa gggaagaatg ggcagatggg agggcagcca gcttctgctc actccaggca cagccatggg
5941 atccgccatt gaacaagatg gattgcacgc aggttctccg gccgcttggg tggagaggct attcggctat gactgggcac aacagacaat
6031 cggctgctct gatgccgccg tgttccggct gtcagcgcag gggcgcccgg ttctttttgt caagaccgac ctgtccggtg ccctgaatga
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```

Figure 1.9 - Continue

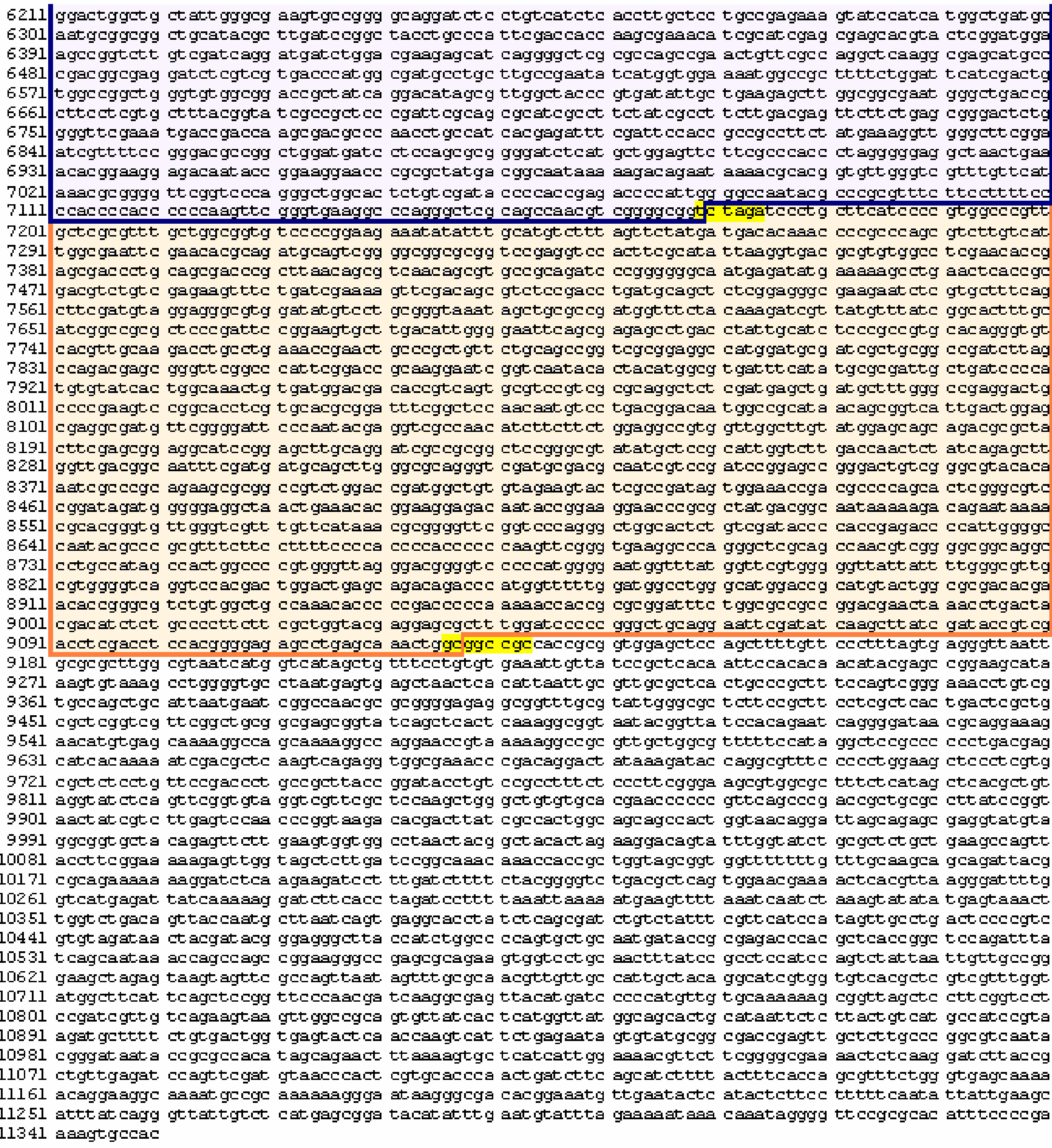


Figure 1.9 - Final total sequence of the construct + vector molecule. The scheme of this sequence may be seen in Figure 4. The four endonuclease restriction sites used in this construction are represented in yellow and the Neo^R start codon in pink. The other colors presented in the figure use the same code as Figure 1.1, the pβMyhc, Neo^R and Hyg^R sequences in green, blue and orange boxes, respectively.

βMyhc (or Myh7) expression in RH30 cells

Our construct carries an aminoglycoside phosphotransferase resistance gene under the control of the human β myosin heavy chain promoter. Therefore, it was relevant to obtain a human cell line which expressed that gene.

With the lack of a human commercially available cell line expressing high levels of βMyhc, the alternatives to test the construct functionality were few. One could have used human primary cell lines from human skeletal muscle; however the variability of such approach was not reasonable for this kind of tests. Another hypothesis was to transfect an immortal human cell line with the βMyhc gene, but even that has never been done before. Consequently, the choice had to fall into a commercially available human cell line, with some βMyhc expression and if possible with high MYOGENIN expression, because our construct carries three putative MYOGENIN binding sites (accordingly to SIGNALSCAN analysis [19]). We have used the rhabdomyosarcoma cell line, RH30, that is known to fail to complete the skeletal muscle differentiation program, express high levels of MYOGENIN, muscle promoting factor MYOD and MYOD repressor MYOSTATIN, the PAX3/FKHR fusion protein [20-26] and the βMyHC (see below).

Several different culture conditions were tested in, in order to obtain the maxim expression of the βMyhc gene. Approximately 5.6 x 10^3 RH30 cells were plated in 30 different conditions for 10 days as shown in Table 1.3. We tested several factors that are known to influence the level of differentiation of cells in culture, such as, different serum concentrations, different cell substrates and in particular the presence or absence of the differentiation agent for RH30 cells, the IFNα2a [27]. Thereafter, RNA was extracted from the cells in each culture and studied with the RT+PCR method. Each culture was also analysed with phase contrast microscopy in order to obtain additional morphologic information (Table 1.3).

On the 10^{th} day, we used the TRIZOL protocol to extract the total RNA and analyse the βMyhc gene expression by RT+PCR with primers for the same gene (see Materials and Methods, *Reverse Transcription and Polymerase Chain Reaction Analysis).* At first sight, it seemed that βMyhc gene expression could indeed be alterated by different cell culture conditions even presenting a possible alternative splicing event. This because βMyhc RT+PCR gel electrophoresis has revealed not only one but several bands (Fig. 1.10). However, the supposed βMyhc band was in fact an unspecific product from the polymerase chain reaction with 1258 bp which may be confused in a 0.8 % agarose gel electrophoresis,

with the true βMyhc gene single product (1216 bp) (Fig. 1.11). This was, in fact, realized after the sequentiation results (Figs. 1.12-1.15). Furthermore, the microscope images did not reveal any clear sign of muscle maturation, as no fusion events among the muscle undifferentiated cells were observed (Table 1.3).

Table 1.3 - RH30 cells phase contrast microscope images after 10 days in different culture conditions.

RPMI 1640, 10 mM HEPES, 20 µM 2βME, 2 mM Glutamine,
1 % (v/v) Penicillin 10000 IU/ml / Streptomycin 10 mg/ml

	①	②	③	④	⑤
	10 % FBS + IFNα2a 10U/ml	**1 % FBS + IFNα2a 10U/ml**	**10 % FBS**	**1 % FBS**	**no serum**
Glass coverslip					
Thermanox coverslips					
2 % Gelatin					
10 µg/cm^2 Collagen I					
3.75 µg/cm^2 Fibronectin					
Polystyrene					

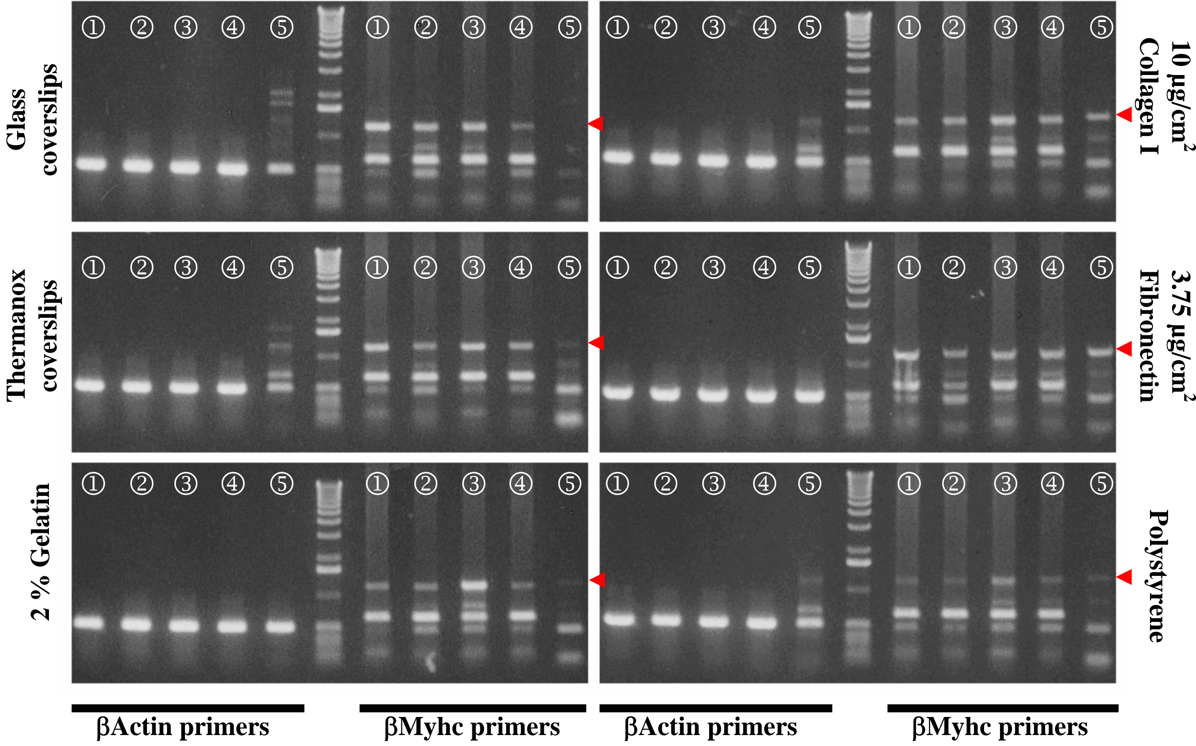


Figure 1.10 - EtBr visualization of several 0.8 % agarose gel electrophoresis of respective RT+PCRs from RH30 extracted RNAs in different culture conditions, amplified by PCR with the βMyhc primers and control βActin primers. In the vertical text boxes are indicated the different substrates used. The horizontal numbers (① to ⑤) are the same as in Table 1.3 and they refer to the different culture conditions studied.
In each run the Gibco 1Kb DNA Ladder (12.22; 8.14; 7.13; 6.12; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.52; 0.40; 0.34; 0.30; 0.22; 0.20 Kb) divides the control βActin bands from the products amplified with the βMyhc primers.

Note that, in each gel, the band that may be confused with the expected βMyhc band (1216 bp) is the heavier band of all (1258 bp) in the lanes at right of the molecular marker, indicated in the Fig. 1.10 with small red arrow heads (see also Fig. 1.11). What at the first sight, we thought to be the βMyhc gel band, has revealed to be, after sequentiation and BLAST, an unspecific product related with a Tubulin Tyrosine-like Ligase 9 (TTLL9) gene (Figs. 1.12 -1.15). The βActin band was correct.

Before sequentiation we were convinced that the βMyhc RT+PCR band from RH30 cells was the expected one (Fig. 1.11, red arrow). In the gel of Fig. 1.11 the βMyhc RT+PCR band from human left ventricle was run in parallel with the βMyhc RT+PCR band from RH30 cells. Looking at the figure becomes clear why, at first sight, we were erroneously induced to think that we were working with the same transcript.

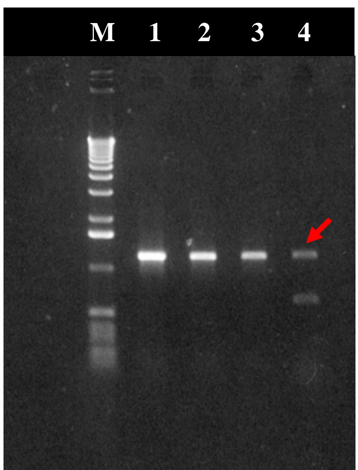


Figure 1.11 - 0.8 % agarose gel electrophoresis of the βMyhc RT+PCR products from human left ventricle and RH30 cells
Legend:
M- Gibco 1Kb DNA Ladder (12.22; 8.14; 7.13; 6.12; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.52; 0.40; 0.34; 0.30; 0.22; 0.20 Kb);
1 - βMyhc from human left ventricle, 1216 bp;
2 - 1:2 dilution of lane 2;
3 - 1:4 dilution of lane 2;
4 - βMyhc from RH30 cells, 1258 bp
(the same as figure 1.10, 10 μg/cm^2 Collagen I, lane ②)

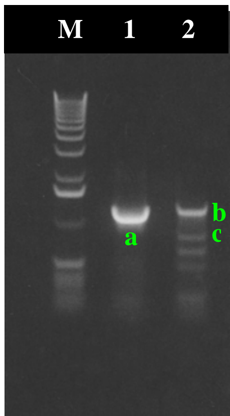


Figure 1.12 - 0.8 % agarose gel electrophoresis of the βMyhc RT+PCR products from human left ventricle and RH30 cells that were prepared for sequentiation.
Legend:
M - Gibco 1Kb DNA Ladder (12.22; 8.14; 7.13; 6.12; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.52; 0.40; 0.34; 0.30; 0.22; 0.20 Kb);
1 - βMyhc from human left ventricle, (one band);
2 - βMyhc from RH30 cells, (4 bands). This sample refers to culture conditions in 10 % FBS and 2 % Gelatin (Fig. 1.10, 2 % Gelatin, lane ③).

a, **b** and **c** refers to the samples seen in Fig. 1.13.

We have cut for sequentiation gel bands of the βMyhc RT+PCR products (Fig. 1.12), one band from lane 1, 1216 bp **(a)** and the first two bands of the product from lane 2, 1258 bp **(b)** and 896 bp **(c)**, respectively. In Fig. 1.13 are visualized the purified samples (5 μl applied in the gel). Each product, with a final volume of 50 μl, was purified from the respective agarose gel band. The initial sample volume was 150 μl.

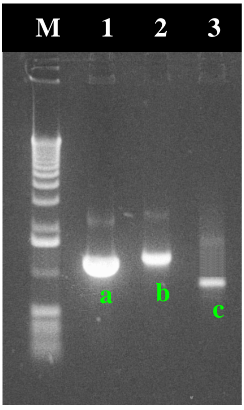


Figure 1.13 - 0.8 % agarose gel electrophoresis of the RT+PCR products seen in Figure 1.12 (**a**, **b** and **c**), isolated in agarose slices and purified with the Qiaquick Gel purification kit.

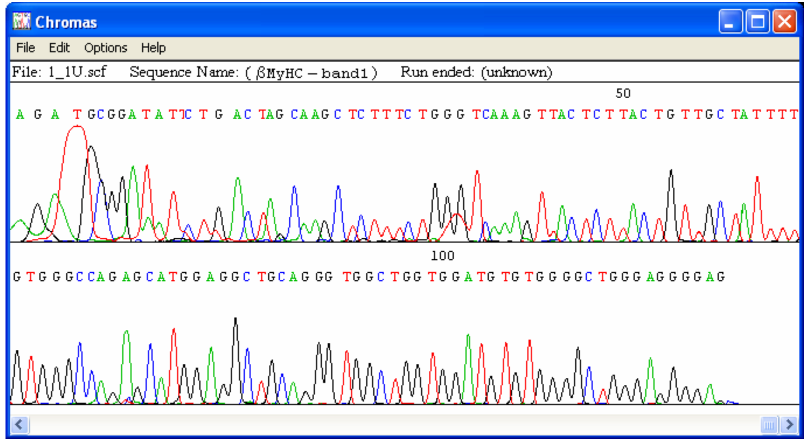


Figure 1.14 - Forward sequence (123 nt) of the βMyhc product from RH30 cells, 1258 bp, Figure 1.13, lane 2.

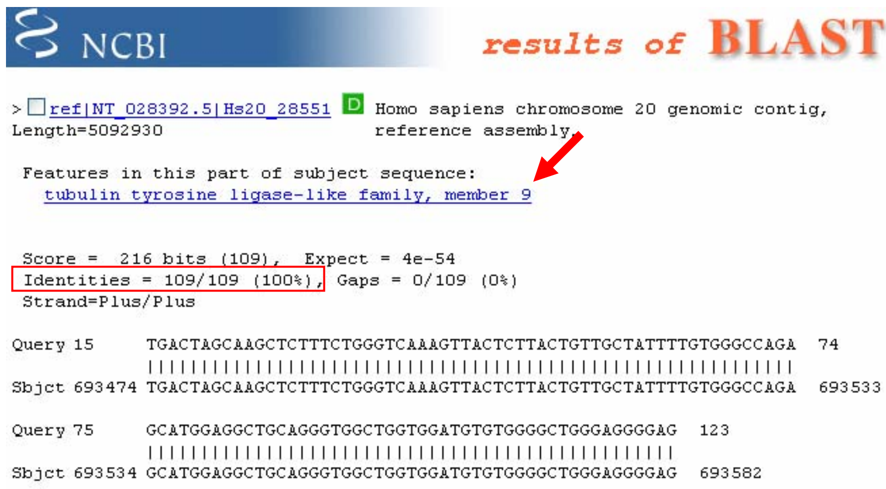


Figure 1.15 -BLAST of the (forward) sequence from figure 1.14.

The βMyhc RT+PCR product (1258 bp) from RH30 cells gave as the most probable match the TTLL9 gene (Fig. 1.15, red arrow) with an 100 % identity among 109 nt (red box). The lower sequence also gave as the most probable match the TTLL9 gene (results not shown).

As these results were inconclusive regarding the establishment of the best conditions for βMyhc expression, we chose to proceed using the reported expansion medium and gelatine-coated well plates, a common cell substrate used in many models of myogenesis, *in vitro* [28-32]. To our knowledge, the expression of the βMyhc in this cell line was never documented in the literature, however, we could observe some βMyHC positive cells in RH30 cell cultures by immunohistochemistry (Fig. 1.16a - d) but not by RT-PCR (see above).

A possible explanation of these results is that the βMyhc transcript in this cell line may undergo different post-transcriptional splicing processing in it 5' untranslated region (UTR) when comparing with adult cardiomyocytes. Accordingly, and remembering that the upper primer used in the βMyhc RT+PCR anneals in the βMyhc untranslated exon 2, a different 5' UTR may be the reason for deficient upper primer annealing and favouring of unspecific amplifications.

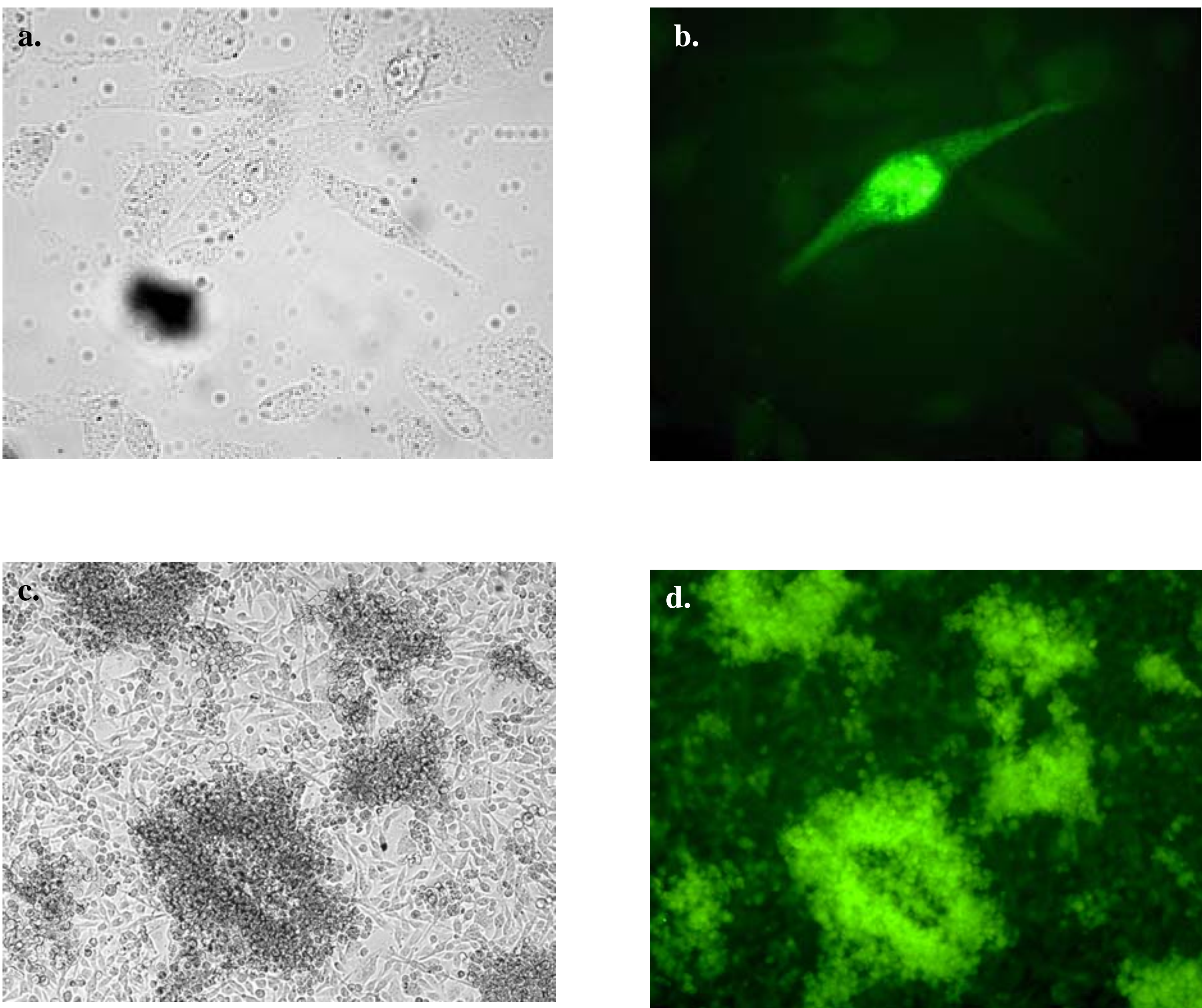
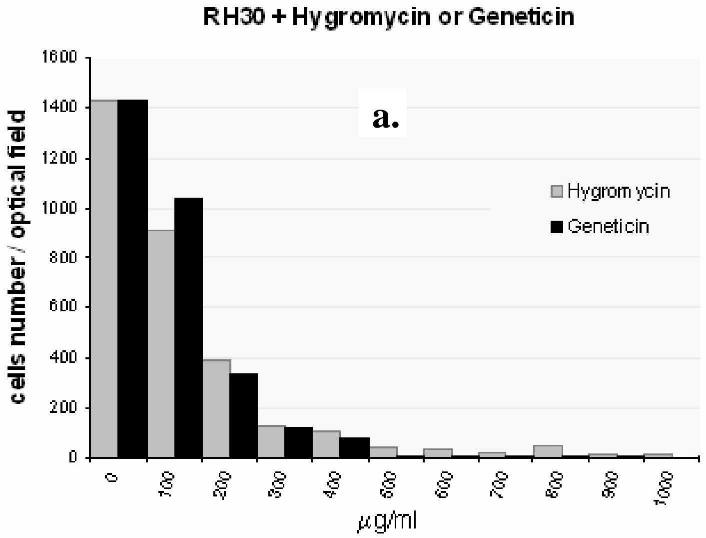


Figure 1.16 **(a. - b.) -** RH30 rhabdomyosarcoma cells, cultured in RPMI 1640 medium with FBS and antibiotics, expressing the βMyhc gene. We have used the anti human βMyhc MAB1628 and a FITC conjugated secondary antibody in the immunofluorescence method;
a. and **b.** - visible light, 400X and FITC fluorescence, 400X of the same optical field, respectively;
c. and **d.** - visible light, 100X and FITC fluorescence, 100X of the same optical field, respectively.

Note that the frequency that adherent cells express the slow twitch myosin is very low, at least with immunofluorescence as the detection method.

Expression studies with the construct pβMyhc-Neo^R-Hyg^R

Before any transfection experiments could be done, we have experimentally determined the lethal concentration of the antibiotics geneticin (neomycin analogue) and hygromycin that were used as selective agents in the reporter gene screening. We have individually determined that a final hygromycin concentration of 600 μg/ml followed by a concentration of 500 μg/ml geneticin would be more than sufficient to assure a strong selective pressure among RH30 tranfected cells in culture (Fig. 1.17). Accordingly, when RH30 cells are incubated with 600 μg/ml hygromycin for five days followed by a concentration of 500 μg/ml geneticin for another five days, no viable cells could be visualized (results not shown).



RH30 cells
(No antibiotic)

antibiotic μg/ml	RH30 + Hygromycin	RH30 + Geneticin
100		
200		
300		
400		
500		
600		
700		
800		
900		
1000		

b.

Figure 1.17 - Five days RH30 cell assay determining the working concentration of hygromycin and geneticin as 600 μg/ml and 500 μg/ml respectively.
a - Graphical view of RH30 cells per optical field versus the respective antibiotic concentration. Each determination is a mean from triplicate cell culture experiments.
b - Representative RH30 cell culture images for each antibiotic concentration.

The RH30 cells were transfected with different DNA/Fugene 6 ratios with 10 μl of the transfection mixture according to Table 1.3, where "DNA" refers to the pβMyhc-Neo^R-Hyg^R final construct. For determination of the pβMyhc-Neo^R-Hyg^R concentration a gel electrophoresis and a comparative analysis between our sample and a standard 1 Kb Mass profile DNA Ladder was done (Fig. 1.18).

Table 1.3 - Mixtures used in the transfection method.
Only 10 μl of each transfection mixture was added to different wells of the cell cluster plate.

Tubes	**01**	**02**	**03**	**1**	**2**	**3**	**Controls:**
Reagent:DNA Ratio	-	-	-	3:2	3:1	6:1	**01** Cell control: no reagent; no DNA
Add serum free media (μl)	100	94	99	90	92	86	**02** Reagent control: 6 μl reagent; no DNA
Add FUGENE6 Reagent (μl)	-	6	-	6	6	12	**03** DNA control: no reagent; 1 μg DNA
Add DNA at 1μg/μl (μl)	-	-	1	4	2	2	
Total (μl)	100	100	100	100	100	100	

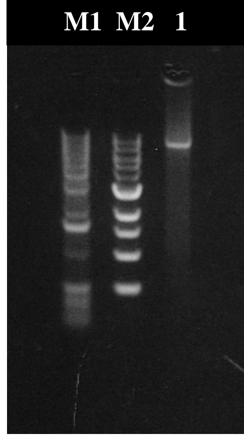


Figure 1.18 - Gel-based quantification of the pβMyhc-Neo^R-Hyg^R final construct. Legend:
M1 - Gibco 1Kb DNA Ladder: 12.22; 8.14; 7.13; 6.12; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.52; 0.40; 0.34; 0.30; 0.22; 0.20 Kb;
M2 - NEB 1Kb DNA Ladder:
10 Kb (42 ng); 8 Kb (42 ng); 6 Kb (50 ng); 5 Kb (42 ng); 4 Kb (33 ng); 3 Kb (125 ng); 2 Kb (48 ng); 1.5 Kb (36 ng); 1 Kb (42 ng); 0.5 Kb (42 ng);
1 - 1/20 pβMyhc-Neo^R-Hyg^R final construct (1 μl).

The DNA ladder in lane 2 has an established mass profile for sample quantification. Comparatively, we estimate that our sample (lane 1) has a mass of approximately 50 ng which makes its initial concentration to be approximately 1 μg/μl.

After three days, hygromycin B was added and left for five days. After washing, geneticin was added, for five additional days. Thereafter, RNA was extracted from each sample, first strand cDNA synthesized and the expression of βActin, Neo^R and Hyg^R analyzed by PCR (Fig. 1.19, Table 1.4 and Fig. 1.20a and b).

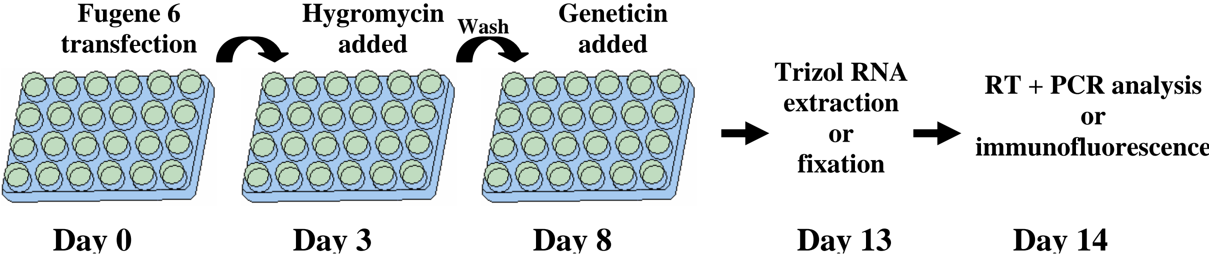


Figure 1.19 - Timeline of one complete transfection experiment.

Table 1.4 - PCR primers used in the PCR performed on day 14 of the transfection experiment. See also Fig. 1.20a and b.

Gene	**source**	**5´primers (5´-3´)**	**nt**	**3´primers (5´-3´)**	**nt**	**PCR product**
βActin	M10277.1	ATATCgCCgCgCTCgTCgTC	1103-1122	TggCATgggggAgggCATAC	2169-2188	513 bp (cDNA) 1080 bp (gDNA)
Neo^R	Stratagene	CTCTgATgCCgCCgTgTTCC	6037-6056	CCCCAgAgTCCCgCTCAgAA	6753-6734	717 bp
Hyg^R	Stratagene	CgATgTAggAgggCgTggAT	7564-7583	gCgACCTCgTATTgggAATC	8136-8117	573 bp

In Fig. 1.20 is showed a representative figure of four similar transfection experiments. The day 13 pos-transfection, RNA was extracted and cDNA first strand synthesized and amplified using selected PCR primers for βActin, Neo^R and Hyg^R. Additional controls were done to assure that the amplified PCR bands were not due to free insert present in the cell wells. We add our insert to cell free wells of the 24 wells cell cluster plate and those were processed in the same conditions as all the others. After the transfection day the tube 03 was stored at -20º C until the end of the experiment. As expected, the insert was not degraded within the course of the experiment (Fig. 1.20a, "Tube 03" lanes) however, due to the cell culture medium changes, no free insert was present in the end of the experiment, co-precipitating with RNA in the Trizol protocol and giving rise to false positive results. (Fig. 1.20a, "Tube 03 without cells" lanes).

Relevant expression of the desired gene products could only be seen in the expected tubes (Fig. 1.20b, tubes 1, 2 and 3). Note that the insert could also have entered the cells without the Fugene 6 Reagent as one can see in Fig. 1.20b, lane 03 regarding the Neo^R expression.

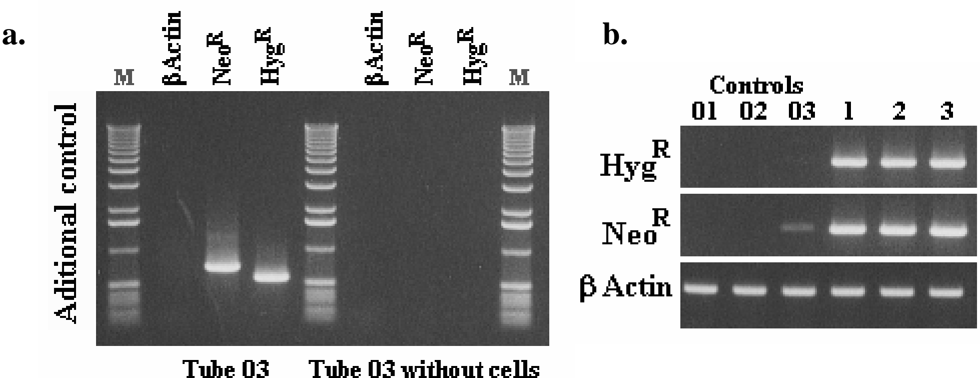
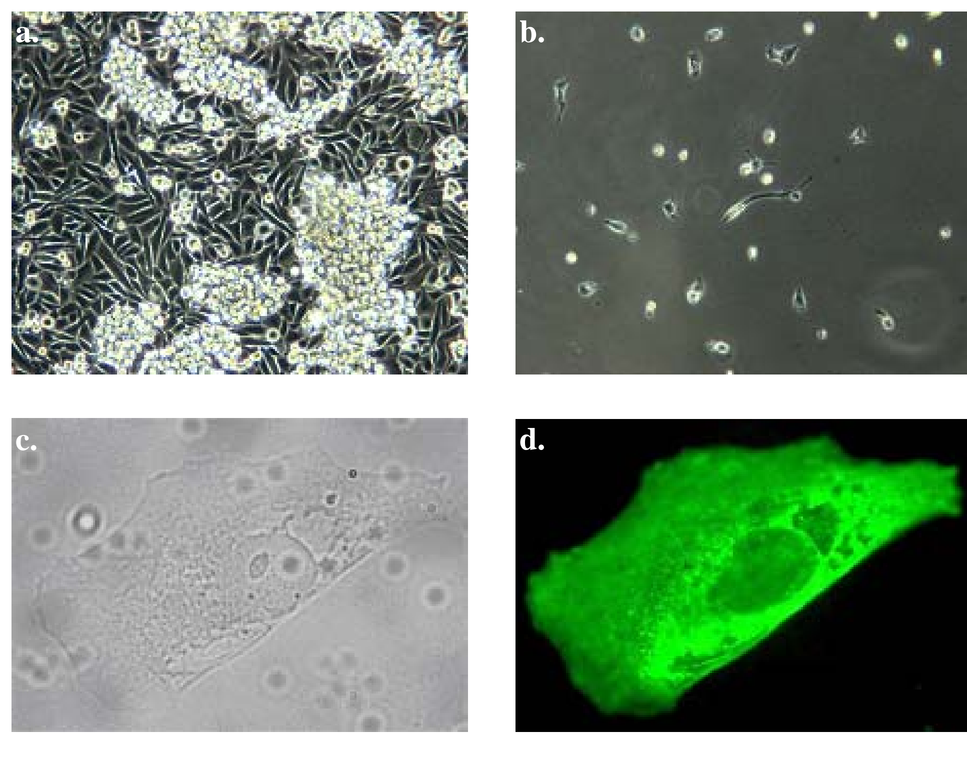


Figure 1.20 - The day 13 pos-transfection, RNA was extracted from each well using Trizol protocol, and cDNA first strand synthesized using the ThermoScript RT-PCR system with the oligo dT primer. Then each cDNA was amplified using selected PCR primers for βActin, Neo^R and Hyg^R.

a. Additional controls (see text for details);

b. 0.8 % agarose gel of the PCR for βActin, Neo^R and Hyg^R (see Material and Methods, Expression studies in human rhabdomyosarcoma cells).

In addition to RT+PCR, immunofluorescence analysis was also performed, in order to confirm the data presented above (Fig. 1.21a - f). In fact, it was possible to detect hygromycin and geneticin resistant cells, however with a very low frequency. We estimate that just one cell in 10^5 geneticin resistant cells were identifiable by immunofluorescence, althoug the RT+PCR results had indicated a higher frequency of positive cells. Obviously, another cell model was needed to confirm all these experiments, as for instance fetal human cardiomyocytes. This work is currently being done in the “Hubrecht Laboratorium” in The Neatherlands.



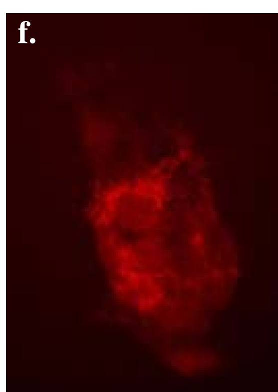


Figure 1.21 **-** Microscope analysis of the complete transfection experiment.

a. Phase contrast image of RH30 cells in day 3 after transfection and before the addition of the hygromycin B antibiotic. Amplification 100X **b.** Phase contrast image of RH30 cells in day 8 after transfection and before the addition of the geneticin antibiotic. Note that all the cells that were hygromycin B sensitive have died. Amplification 100X.

c. and d. - An hygromycin B positive cell, in day 14 after transfection, showing a high intracellular concentration of antibiotic (a good indicator of cellular resistance to it). The secondary antibody used was FITC conjugated. The visible light image, 400X amplified and the fluorescence image, 400X amplified refers to **c.** and **d.** respectively.

e. and f. - A rhabdomyosarcoma cell, at the end of the experiment, showing expression of the Neo^{R} gene. The secondary antibody was TRITC conjugated. One can see in **e.** the visible light image and in **d.** the fluorescence image, both 400X amplified.

Expression of the Neo^{R} gene, which confers geneticin resistance, was detected in RH30 cells in culture (Fig. 1.21f). These results suggested us that the construct was functional.

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2. Human cell culture studies

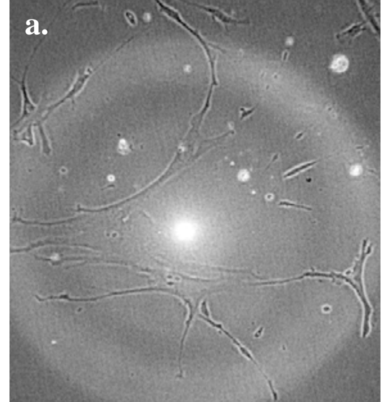
The general goal of the present project was to develop a cardiac differentiating cell model.

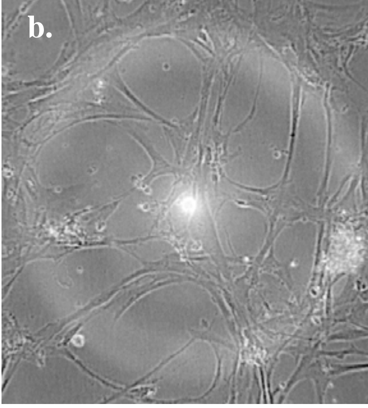
To achieve this goal the most plausible strategy was to differentiate, *in vitro*, cardiac muscle from undifferentiated progenitor/stem cells.

2.1 Primary cultures

From human myocardium

Recent findings indicate that human adult cardiomyocytes may have some potential of proliferation and regeneration in the human pathological adult heart [1, 2]. This evidence leaded us to attempt to establish a primary culture of human cardiomyocytes, in standard culture conditions. We have faced very important and expected limitations, mainly the inability of such cells to divide, to attach on culture vessels and high levels of cell death. Furthermore, when some proliferating cells could be observed *in vitro*, their typical morphology was similar to that of neuronal and myofibrobalst, Fig. 2.1.1, and also myoepithelial cells in culture, Fig. 2.1.2 and 2.1.3.





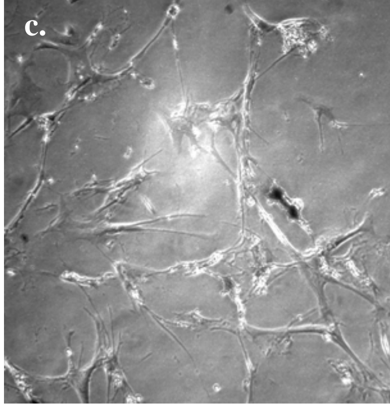


Figure 2.1.1 - Human myocardium primary cultures.
a. and **b.** Phase contrast images, 200X amplified, of cells harvested from a 44 years old female transplanted heart. The cells were cultured for 22 (**a.**) and 28 days (**b.**) in a RPMI medium supplemented with 10 % FBS and incubated at 37º C in a humidified atmosphere containing 5 % CO_2. They present a neuronal-like morphology.
c. Phase contrast images, 40X amplified, of cells harvested from a post-mortem 4 years old male myocardium and culture in a RPMI medium supplemented with 10 % FBS for 3 months at 37º C in a humidified atmosphere containing 5 % CO_2. They show a myofibroblast-like morphology.
See also Fig. 2.1.2.

Additionally, with the help of histochemical and immunohistochemical techniques we have further characterized these cells (Fig. 2.1.2). In fact, all the results indicated that we were dealing with non muscle populations from human myocardium, namely the absence of muscle

phenotypes and the positive immunostaining with a fibroblast and/or myoepithelial specific marker, the β prolyl 4-hydroxylase subunit.

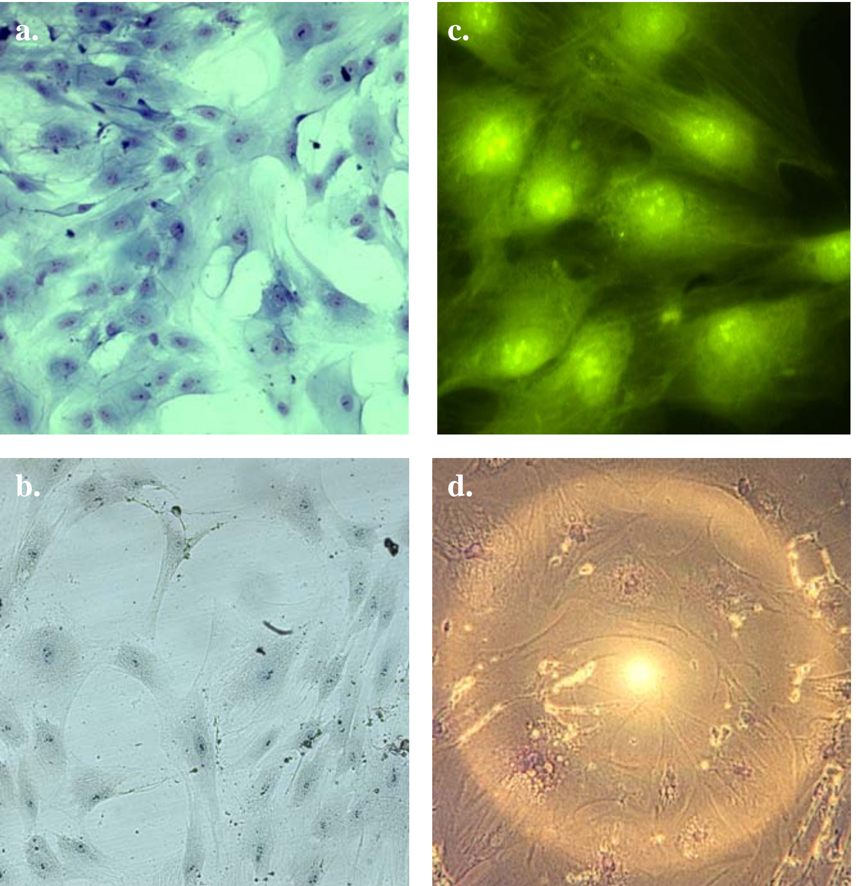


Figure 2.1.2 - Human myocardium primary cultures.
a. Hematoxilin/eosin and **b.** Mason's Trichrome staining images, 100X amplified, of cells harvested post-mortem from a 30 year old male myocardium **(a.)** and during an open chest heart surgery from a 69 year old male **(b.)**. The cells were cultured for 1 month and 1 month and an half, respectively, in a RPMI medium supplemented with 10 % FBS and incubated at 37º C in a humidified atmosphere containing 5 % CO_2. They both present a myoepithelial-like morphology.
c. Immunoflurescence image, 100X amplified, of β prolyl 4-hydroxylase positive cells from a post-mortem 4 year old male myocardium, grown in a RPMI medium supplemented with 10 % FBS for 3 months at 37º C in a humidified atmosphere containing 5 % CO_2. The monoclonal mouse 5B5 anti-human fibroblast IgG1 (Dako) was used as primary antibody. Note the myoepithelial-like morphology.
d. Phase contrast image of an hematoxilin/eosin staining, 100X amplified, of the same cells as **c.**

During all the cell culture experiments in this and in subsequent sections, one of the strategies used for detecting muscular cells in culture was the phalloidin-TRITC staining. Phalloidin is a fungal toxin isolated from the poisonous mushroom *Amanita phalloides* that binds with a high affinity to polymeric actin. When conjugated with a fluorescence molecule, as TRITC, it can be used to label actin filaments in histological preparations for fluorescence microscopy. With this method, muscle cells may be easily identified because sarcomeres have a high content of polymeric actin which gives a typical fluorescence pattern (Fig. 2.1.3).

Accordingly, several phalloidin-TRITC incubations were done in primary cultures from human myocardium. However, no sarcomeres could be detected among these cultures, (Fig. 2.1.3).

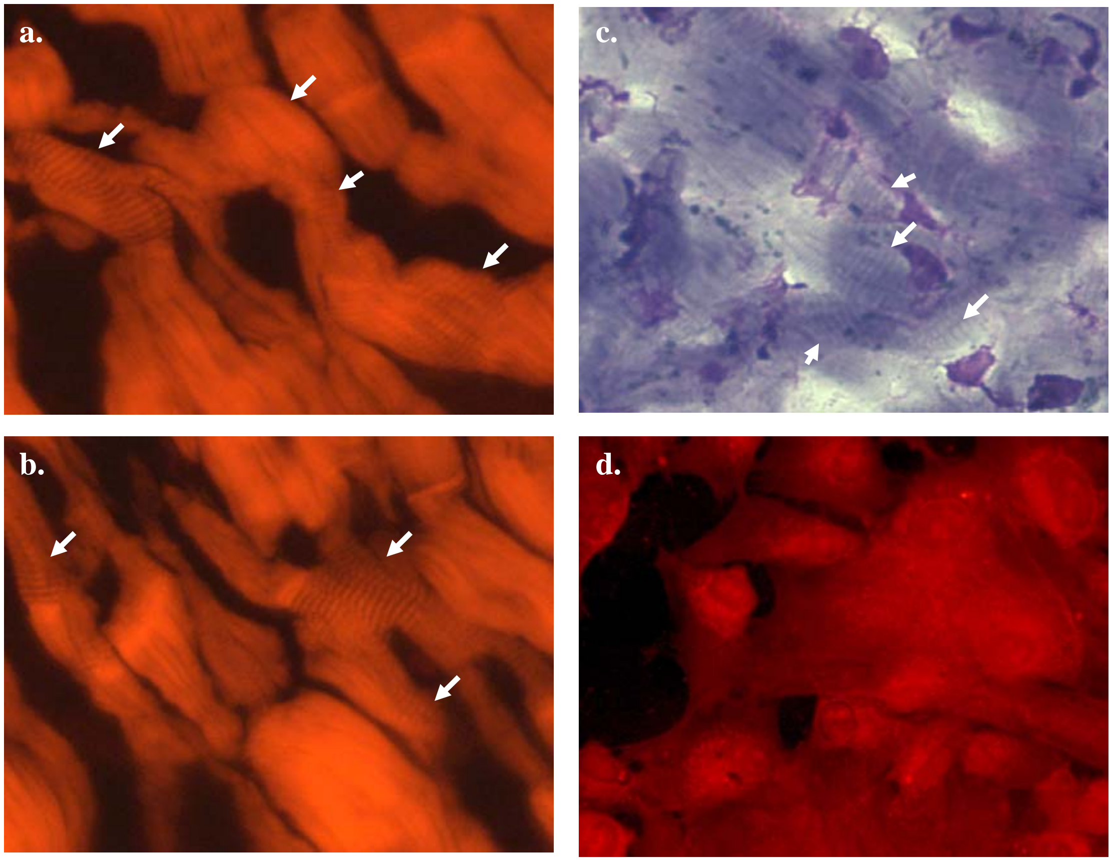


Figure 2.1.3 - Human myocardium cryosections and primary culture.
a., b. and c. Phalloidin **(a. and b.)** and hematoxilin/eosin **(c.)** staining images, 400X amplified, of post-mortem cryosections from a 52 years old female myocardium. Evidence for the presence of cardiac muscle cells was verified when several sarcomeres were observed (white arrows). **d.** Phalloidin staining images, 100X amplified, of cells harvested post-mortem from a 30 years old male myocardium. The cells were cultured for 1 month in a RPMI medium supplemented with 10 % FBS and incubated at 37º C in a humidified atmosphere containing 5 % CO_2. They present a myoepithelial-like morphology but sarcomeres could not be detected.

As mentioned before, another fact that was normally observed in human myocardium primary cultures was the high incidence of cell death. It is reasonable to admit that a myocardium sample harvested some hours post-mortem or even during surgery, has undergone a drastic change in its physiological milieu, mainly due to prolonged hypoxia and hypothermia. These states are known to cause necrosis in cardiac cells [3]. Necrosis is a process characterized by irreversible cellular edema associated with thickening of mitochondria, lysosomal rupture, relatively late DNA degradation by nonspecific lysosomal nucleases and rupture of the surface membrane with complete desintegration of the cell [4, 5].

In our opinion, necrosis would be the main cause of cardiomyocyte cell death before the inclusion of myocardium samples in a more physiological state as RPMI medium supplemented with 10 % FBS and incubated at 37º C in a humidified atmosphere containing 5 % CO_2. We speculate that, in these conditions, it would be apoptosis the most probably cause of cell death, because apoptosis is an active and energy-consuming process that occurs under physiological conditions. It is characterized by initial morphological cell nucleus changes, where the chromatin becomes condensed and subjected to the action of activated nucleases while mitochondria and the surface membranes remain intact. Fragmentation of the cell follows associated with the formation of apoptotic bodies with still intact surface membranes [6].

Among the many markers of apoptosis, DNA fragmentation is considered an hallmark in many, if not all, apoptotic pathways [7, 8]. During this process endonucleases are activated, cleaving histone unprotected internucleosomal DNA strands generating fragments of 200 bp multiples, that will run electrophoretically as a characteristic DNA ladder [5]. On the contrary, in necrosis, DNA is not usually cut in an orderly manner, which is explained by the activation of nonspecific lysosomal nucleases and evidenced by a smear in agarose gel electrophoresis [5]. Thus, a demonstrable presence of DNA laddering in a given cell population is indicative of ongoing apoptosis, whereas the presence of DNA smear is indicative of necrosis.

These differences in the structure of DNA damage are frequently used to discriminate between apoptosis and necrosis. However, it should be noted that mechanistic boundaries between apoptosis and necrosis are becoming more and more vague. A recent report demonstrated the occurrence of the apoptotic ladder-type DNA fragmentation in the early necrosis of thymocytes [9].

To verify if apoptotic or necrotic conditions were already present in the harvested samples or if they were induced after some days in our culture medium, we extracted DNA from the same human myocardium before and after the cell culture procedures and compared their agarose gel electrophoresis migration profiles, Fig. 2.1.4.

As compared to fresh cells, in cells cultured in standard culture conditions for 6 days, the increase in smear and the appearance of an apoptotic ladder was notorious. This is both indicative of chaotic DNA hydrolysis (necrosis) and internucleosomic hydrolysis (apoptosis), respectively. The red arrows in Fig. 2.1.4 represent the apoptotic DNA bands.

Therefore, we concluded that both processes are increased in primary cultures of human myocardium.

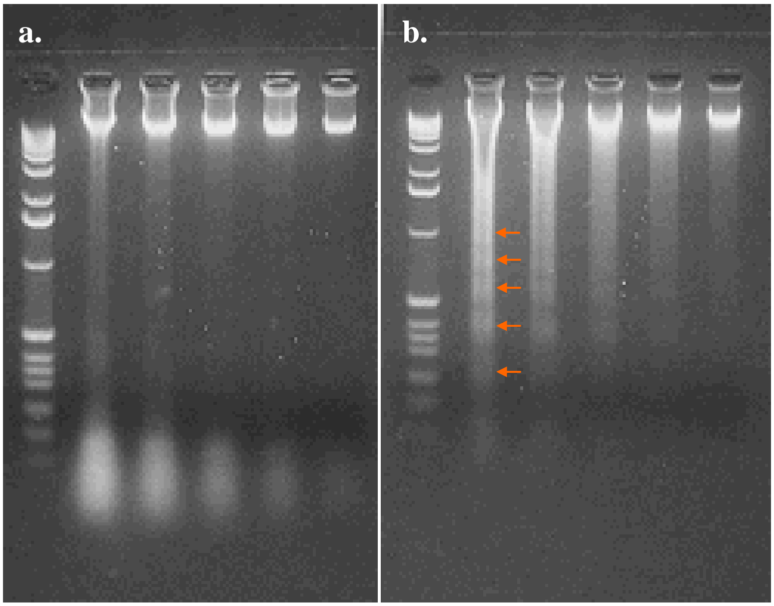


Figure 2.1.4 - Evidence of apoptosis and necrosis in human myocardium primary cultures.
Small myocardium fragments from a 51 years old female, harvested 7h post-mortem were processed for DNA extraction. The purified DNA ran in a 1.8 % agarose gel electrophoresis with EtBr and visualised on an UV transiluminator (**a.**). Similarly, some fragments were culture in a RPMI medium supplemented with 10 % FBS for 6 days at 37º C in a humidified atmosphere containing 5 % CO_2 and then processed for DNA extraction and DNA agarose gel electrophoresis (**b.**) as mentioned before.
Lane 1, 1Kb DNA Ladder from Gibco (12.22; 8.14; 7.13; 6.12; 5.09; 4.07; 3.05; 2.04; 1.64; 1.02; 0.52; 0.40; 0.34; 0.30; 0.22; 0.20 Kb); Lane 2, purified DNA (800 µg/ml); Lane 3, purified DNA (400 µg/ml); Lane 4, purified DNA (200 µg/ml); Lane 5, purified DNA (100 µg/ml); Lane 6, purified DNA (50 µg/ml); The red arrows represent the apoptotic DNA bands.

Discrimination between apoptotic and necrotic cell death was important for evaluation of the effect of cell culture conditions on harvested cardiomyocytes. Taken together, our results show that, human adult cardiomyocytes do not proliferate in culture and that our standard cell culture conditions do not create the best environment for human cardiomyocyte survival. Therefore, the use of more undifferentiated cells was the logical next step.

From human umbilical cord Wharton`s jelly

The Wharton`s jelly (Fig. 2.1.5) is an hyaluronic acid rich gelatinous intercellular substance which is the primitive mucoid connective tissue of the umbilical cord. It may be regarded as a non invasive attainable source of primitive, potentially multipotent, stem cells.

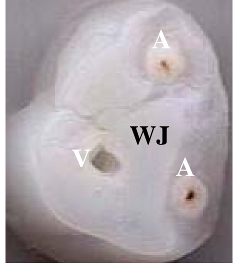


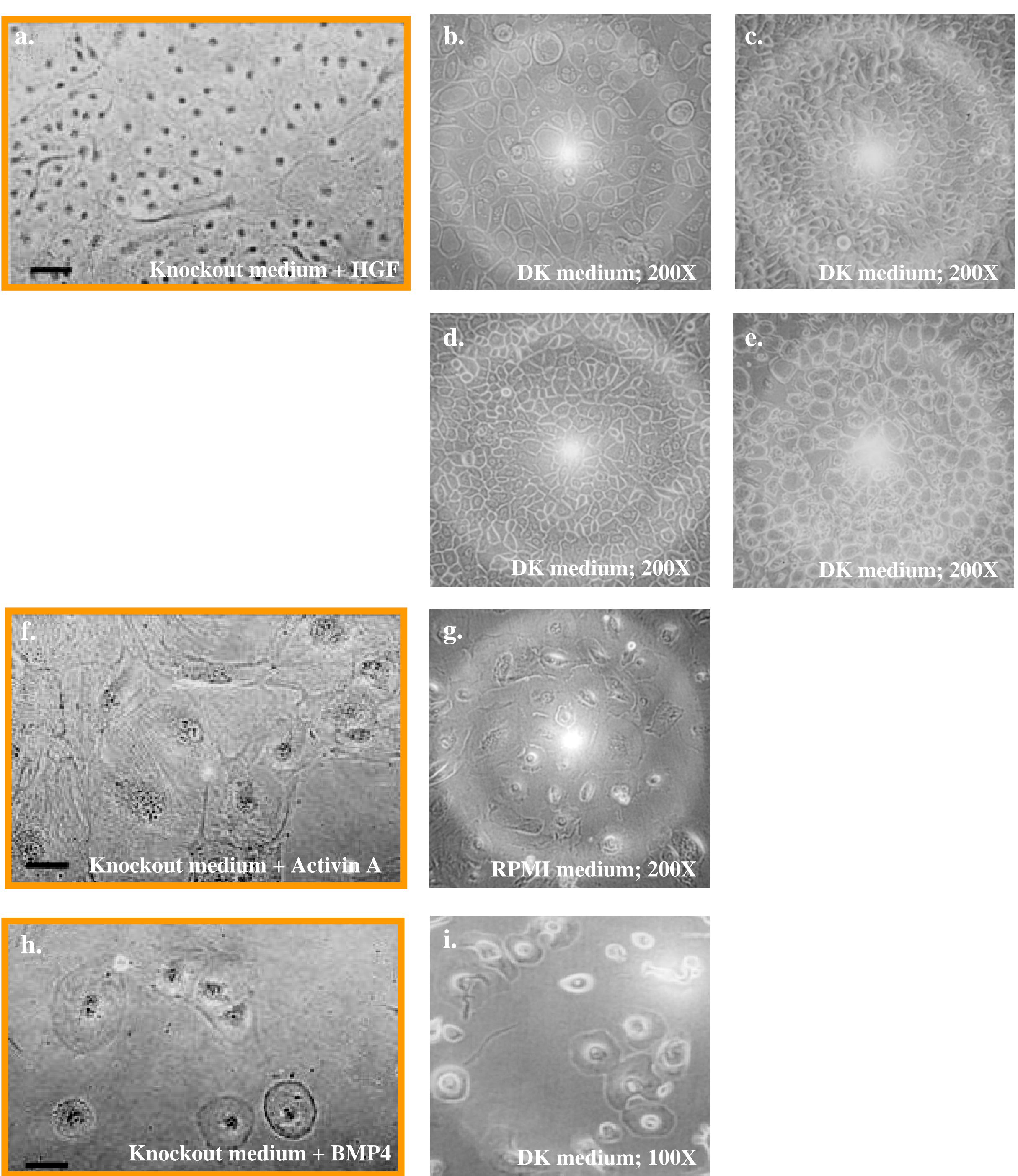
Figure 2.1.5 - Transversal section of a human umbilical cord. In the image, it can be observed the blood vessels of the umbilical cord, namely the two arteries **(A)** and the single vein **(V)** that transport the deoxygenated blood from the embryo and the oxygenated blood from the mother, respectively. These vessels are surrounded by the gelatinous Wharton's jelly **(WJ)**.

The presence of stem cells in the umbilical cord Wharton`s jelly, is now a well established fact [10-14]. We have tried to induce differentiation of these stem cells into cardiac muscle, first in standard culture media (Fig. 2.1.6) and subsequently with 5-azacytidine (Fig. 2.1.7). The latter is a commonly used cardiomyogenic inducer of undifferentiated cells, including bone marrow-derived MSCs of mouse [15, 16], rat [17] and human [18], human umbilical cord blood-derived MSCs [19] and hESCs [20]. Although we found very interesting cell morphologies in all Wharton`s jelly cell cultures, such as epithelioid-like cells, fibroblastoid-like cells, neuronal-like cells and even unknown phenotypes, auto-contractile or sarcomeric muscular cells have not been detected along the time course of the experiments (see Fig.s 2.1.6 and 2.1.7).

In order to facilitate the integration of the several observed cell morphologies with the current research, several cell images from hESCs differentiated cell cultures by Schuldiner *et al*. [21] are shown for comparison (orange frames, Fig. 2.1.6). That latter study describes the individual effect of several different growth factors on directing, in culture, the differentiation of human pluripotent stem cells, derived from the inner cell mass of *in vitro* fertilized human blastocysts.

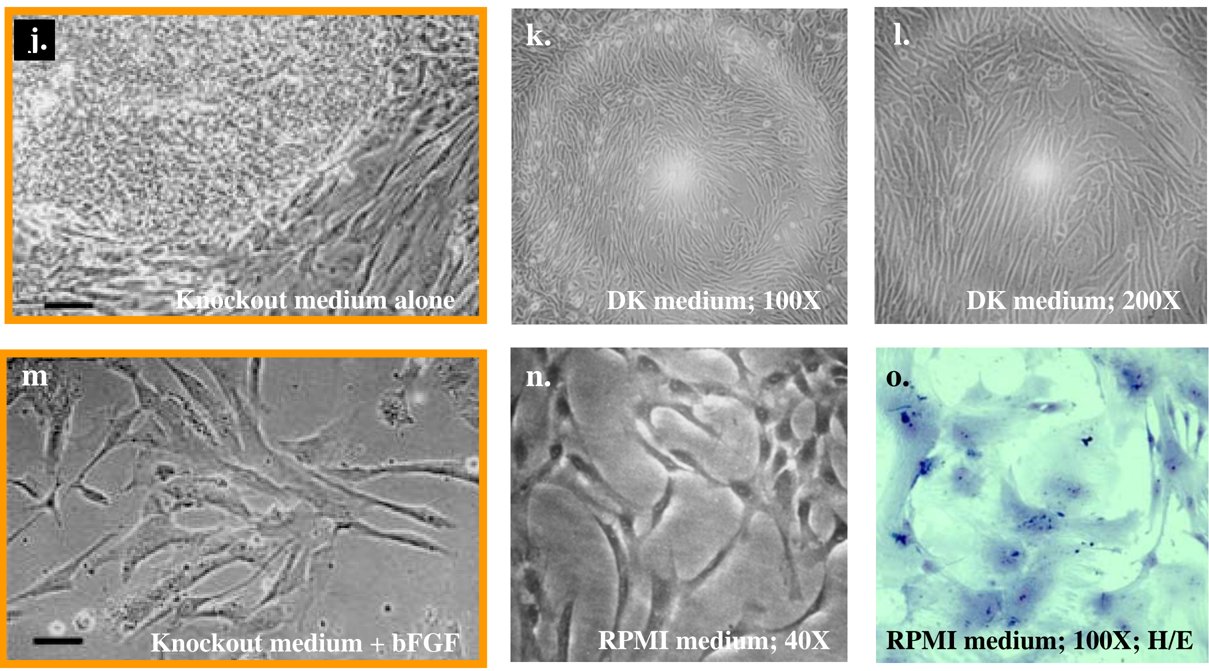
Because the differentiation of hESCs into different phenotypes is spontaneous and uncontrolled, *in vitro*, this study has constituted an important first step toward understanding the controlled differentiation of hESCs in culture. However, regarding Wharton's jelly-derived stem cells no similar studies exist.

Epithelioid-like cells

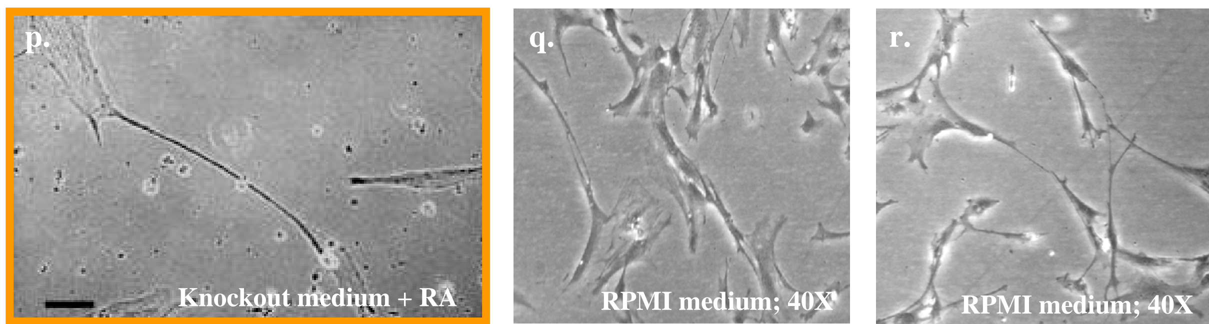


(Figure 2.1.6)

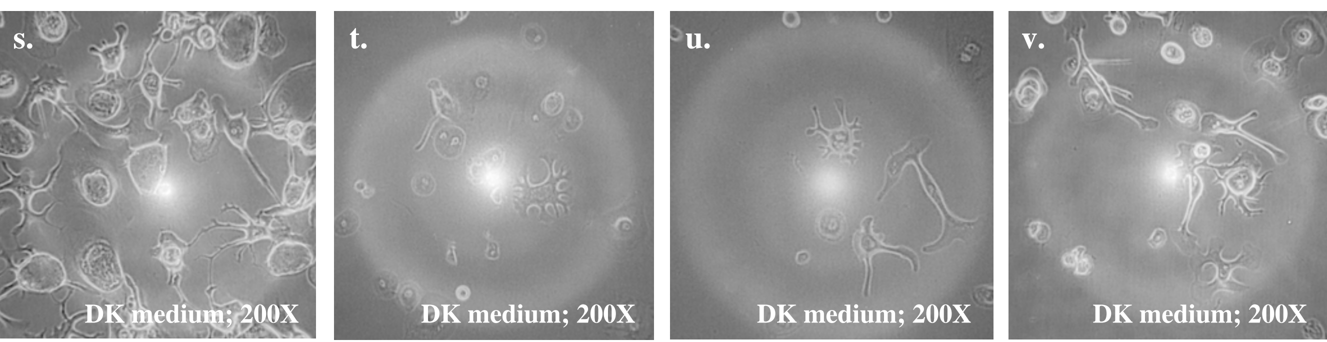
Fibroblastoid-like cells



Neuronal-like cells



Unkown morphology



(Figure 2.1.6 - cont.)

Figure 2.1.6 - hESCs and human UC Wharton's jelly morphologic analogies.

Published hESCs images **(a, f, h, j, m, p)** adapted from Schuldiner *et al*, PNAS, 97, page 11308, 2000, are represented with an orange frame, scale bar = 100 µm. The authors have grown hESCs H9 on mouse embryo fibroblasts in 80 % KnockOut DMEM, 20 % KnockOut SR, 1 mM glutamine, 0.1 mM 2βME, 1 % NEAA, 4 ng/ml bFGF, 10^3 units/ml LIF and 0.1 % gelatin to cover the tissue culture plates. To induce formation of EBs, hESCs were transferred by using trypsin/EDTA to plastic Petri dishes to allow their aggregation and prevent adherence to the plate. Human EBs were grown in the same culture medium without LIF and bFGF for 5 days, then dissociated with trypsin and plated on tissue culture plates coated with 50 µg/ml Fibronectin. The cells were grown in the absence **(j)** or presence of each of the following human recombinant growth factors: 20 ng/ml HGF **(a)**, 20 ng/ml activin-A **(f)**, 10 ng/ml BMP4 **(h)**, 10 ng/ml bFGF **(m)**, and 1 µM RA **(p)**. Under these conditions, the differentiated embryonic cells were grown for another 10 days.

Our results: pieces of human UC Wharton's jelly were digested with 200 U/ml of collagenase type XI in HBSS, washed and cultured in 20 cm^2 flasks in RPMI 1640 medium with 10 mM Hepes, 20 µM 2βME, 2 mM Glutamine, 10 % FBS and 1 % (v/v) Penicillin 10000 IU/ml + Streptomycin 10 mg/ml, in media, for 30 days **(g, n, o, q, r)** or in a Defined Keratinocyte basal medium (DK) supplemented with defined growth additives including insulin, EGF and FGF, in media, for 45 days **(b-e; i, k, l, s-v)**.

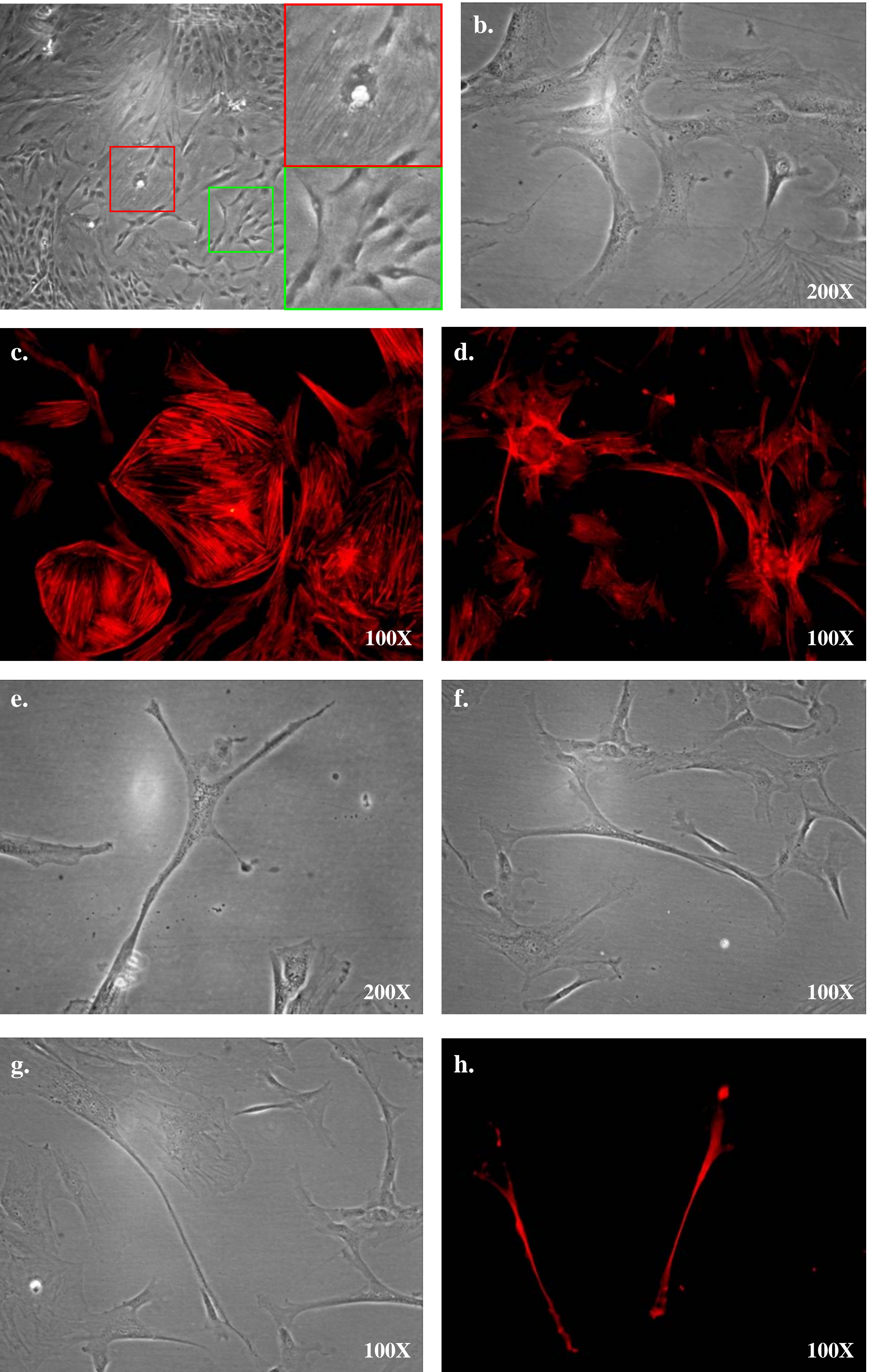
Hematoxilin/eosin (H/E) staining of one of the cultures **(o)**.

For the human UC Wharton's jelly-derived primary cultures the microscope amplification is shown at bottom right of each image.

Cell morphologies of embryonic-derived or umbilical cord-derived cells, were divided in four classes: epithelioid-like **(a-i)**, fibroblastoid-like **(j-o)**, neuronal-like **(p-r)** and unknown phenotypes **(s-v)**.

As mentioned above, some adherent cells from umbilical cord Wharton's jelly cultures, were treated with 5-azacytidine (5-Aza) for 24h, washed and ressuspended in the same initial medium. 5-Aza is a cytidine analogue with a nitrogen atom replacing the carbon at the 5^{th} position of the pyrimidine ring and may be transported into cells by the facilitated nucleoside transport system for uridine and cytidine [22]. This molecule was initially developed as a cytotoxic agent [23], but it was subsequently discovered that it also had a non cytotoxic role, when in relatively low concentrations. Under this conditions, 5-Aza is a powerful inhibitor of DNA methylation and a gene expression and differentiation inducer, in cultured cells [24, 25]. It can be converted into deoxynucleotide triphosphates and incorporated, instead of cytosine, into replicating DNA. Therefore, its mechanism of action depends upon the phase S of the cell cycle [24]. Once incorporated into DNA it can bind DNA methyltransferases in an irreversible (covalent) manner, thus, sequestering the enzyme and preventing maintenance of local and downstream methylation states, influencing the heritable epigenetic cellular regulation [22, 26]. As said above, this blockage of cytosine methylation occurs at noncytotoxic concentrations which do not inhibit DNA synthesis, whereas at high concentrations it provokes the covalent binding to DNA of an excessively high number of DNA methyltransferases, an event that it is thought to be responsible for the citotoxic effect of this agent [27]. Therefore, 5-Aza was used at a relatively low final concentration of 3 µM in cell cultures (Fig. 2.1.7).

Wharton`s jelly-derived cells without 5-Aza treatment (Control)



(Figure 2.1.7)

Wharton`s jelly-derived cells with 5-Aza treatment

i. 40X
j. 40X
k. 40X
l. 100X
m. 100X
n. 100X

Figure 2.1.7 - Wharton's jelly-derived primary cultures treated with 5-Azacytidine (5-Aza), phase contrast **(a, b, e, f, g, i, j, k)** and fluorescence images **(c, d, h, l, m, n)**.
Pieces of human umbilical cord Wharton's jelly were digested overnight with 400 U/ml of collagenase type XI in HBSS, filtered in a 70 µm nylon mesh, washed and cultured in 20 cm^2 flasks in RPMI 1640, with 10 mM Hepes, 20 µM 2βME, 2 mM Glutamine, 10 % FBS, and 1 % (v/v) Penicillin 10000 IU/ml + Streptomycin 10 mg/ml in the absence **(a-h)** or presence of 3 µM 5-Aza **(i-n)** for 24 h. Thereafter, cells were washed and ressuspended in the same initial medium. Both, 5-Aza treated and untreated cells were incubated at 37º C in a humidified atmosphere containing 5 % CO_2 for two weeks.
Phalloidin-TRITC fluorescence staining in 5-Aza untreated **(c, d, h)** and treated cells **(l, m, n)**.
In **a)**, magnified images at right, correspond to the colored boxes on the culture area. Note two different cell morphologies in the same culture flask.
5-Aza untreated cells presented several different morphologies in culture: epithelioid-like cells **(a -** red boxes and **c)**, fibroblastoid-like cells **(a -** green boxes**, b and d)** and neuronal-like cells **(e-h)**; 5-Aza treated cells shown predominantly a fibroblastoid phenotype **(i-n)**. Image amplifications at bottom right of each figure.

It is worth notice that these experiments were performed long before the reported of the angiomyogenic medium which will be described in the next section.

We believe that it would be interesting to repeat some of these human myocardium and umbilical cord Wharton's jelly primary cultures, using as growth medium, an angiomyogenic medium as the one used with the teratocarcinoma cells.

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2.2 Human embryonal carcinomas

The experiments with human teratocarcinoma cell lines PA1 and NT2/D1 were started with the believe that with these kind of cells and the use of the correct inductive cell culture medium we would obtain differentiation of muscle and/or endothelial phenotypes *in vitro*, although we were more interested in the muscle lineages. The results from these experiments can be divided in three subsections:

i) Preliminary cultures of PA1 and NT2/D1 in inductive medium (IM);

ii) Cultures of NT2/D1 in inductive medium (IM) with fibronectin, 5-Aza and BMP2;

iii) Cultures of NT2/D1 in inductive angiomyogenic medium (IAM) with BMP2.

In all experiments, and in spite of the similarity in formulation of the culture media, we decided to call "angiomyogenic medium" only when its composition was exactly the same as the cell culture medium used by Shmelkov *et al*. in their angiomyogenic differentiation studies [1]. In the other cases we refer to the cell culture medium as "inductive medium (IM)".

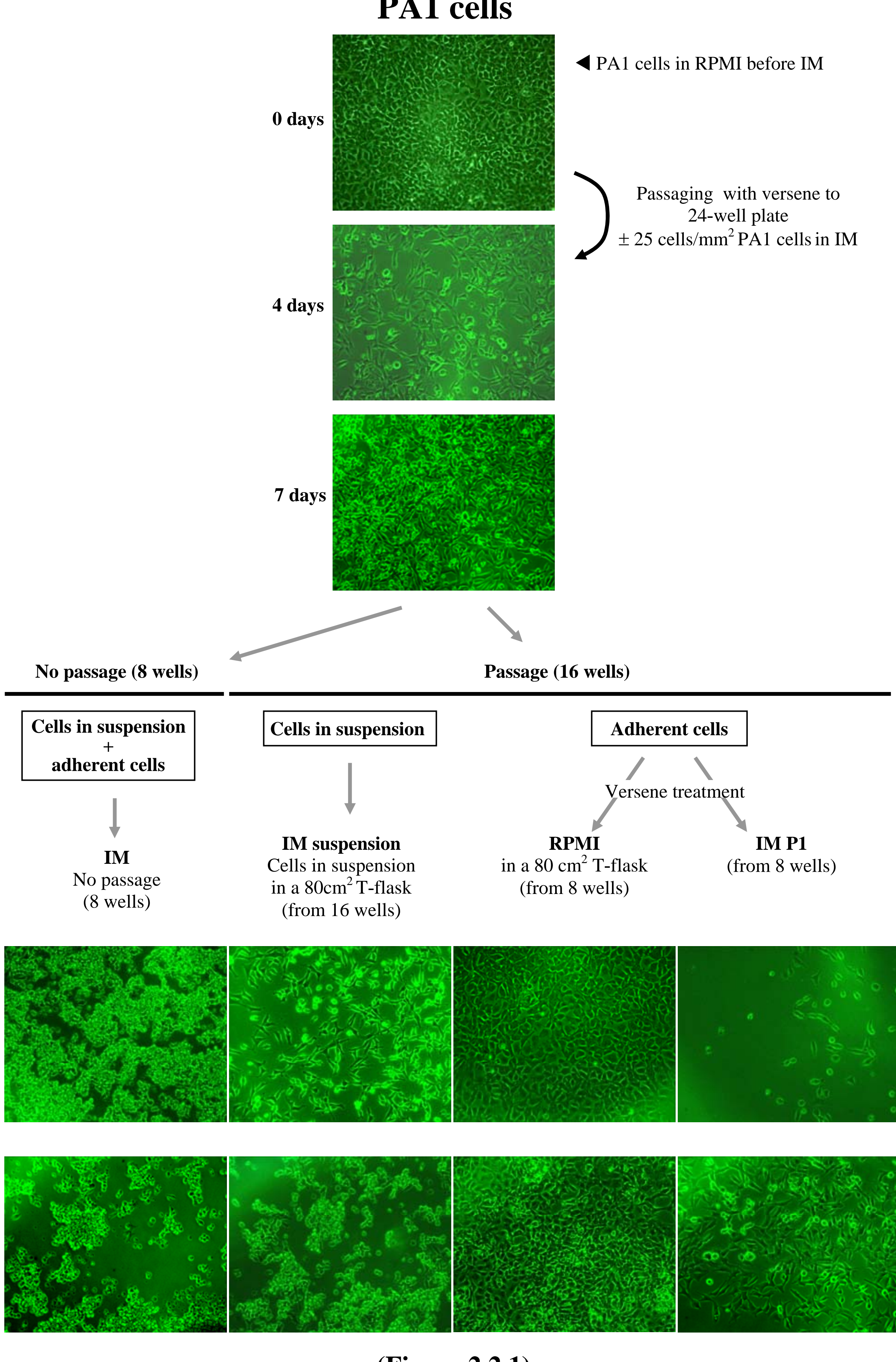
i) Preliminary cultures of PA1 and NT2/D1 in IM

PA1 and NT2/D1 cells have a very characteristic epithelioid-like morphology when growing in standard media. However, when cultured in inductive medium a clear change in phenotype was observed with the cells acquiring a fibroblastoid-like or espheric morphology (Fig. 2.2.1). Interestingly, if they were plated again in the expansion medium, a return to the previous phenotype was seen, suggesting that the inductive media had indeed the ability to induce other paths of differentiation.

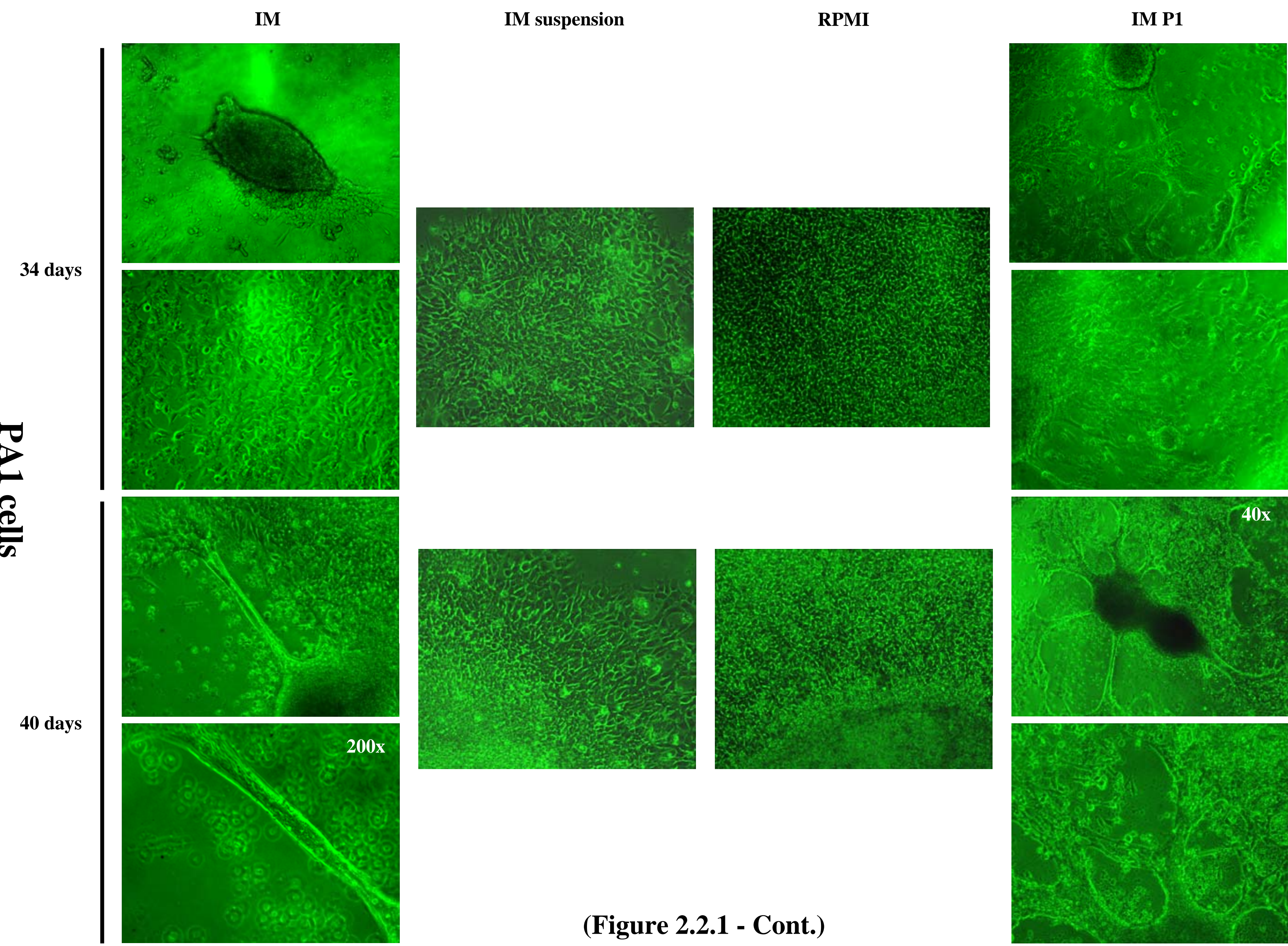
To evaluate which cell subpopulation had the most promising plasticity, non adherent cells were separated from the adherent and ressuspended in the same inductive medium, in both cell lines. It was clear from the microscope images of PA1 and NT2/D1 cells in culture, that it was in the adherent subpopulation that the most pluripotential cells resided. One very interesting fact was that PA1 cells could be passaged without loosing any ability to proliferate and differentiate *in vitro* whereas NT2/D1 cells were clearly affected by this procedure (even not using trypsin), presenting a enormous level of cell death when placed back in the same initial inductive medium.

Both cell lines could be maintained in inductive medium for at least 77 days showing, along the culture period, promisings morphologies as embryoid body-like and angiogenic network-like shapes (see Fig. 2.2.1). Furthermore, some of this cell aggregates were very similar to some hESCs derived structures *in vitro.* However, and contrarely to the mouse model, no autocontractile regions could be observed in either cell lines, even when primed with 20 μg/ml adhrenalin or subjected to defined voltage pulses (results not shown).

After 77 days in culture, the cells were processed for transmission electron microscopy (TEM). Several cell aggregates were analysed to see if there was any muscle-like phenotype among them (Fig. 2.2.2). Althoug a well defined desmosome was seen in NT2/D1 cells, no organized myofibers could be detected. TEM images for both cell lines showed that they were much more directed to form the typical epithelial-like aggregates. The NT2/D1 presented typical microvilosities, and a clear asymetric organization between their apical and basal regions, well known characteristics of these cells, when differentiating through a pathway that leads to the extraembryonic endoderm phenotype.



(Figure 2.2.1)



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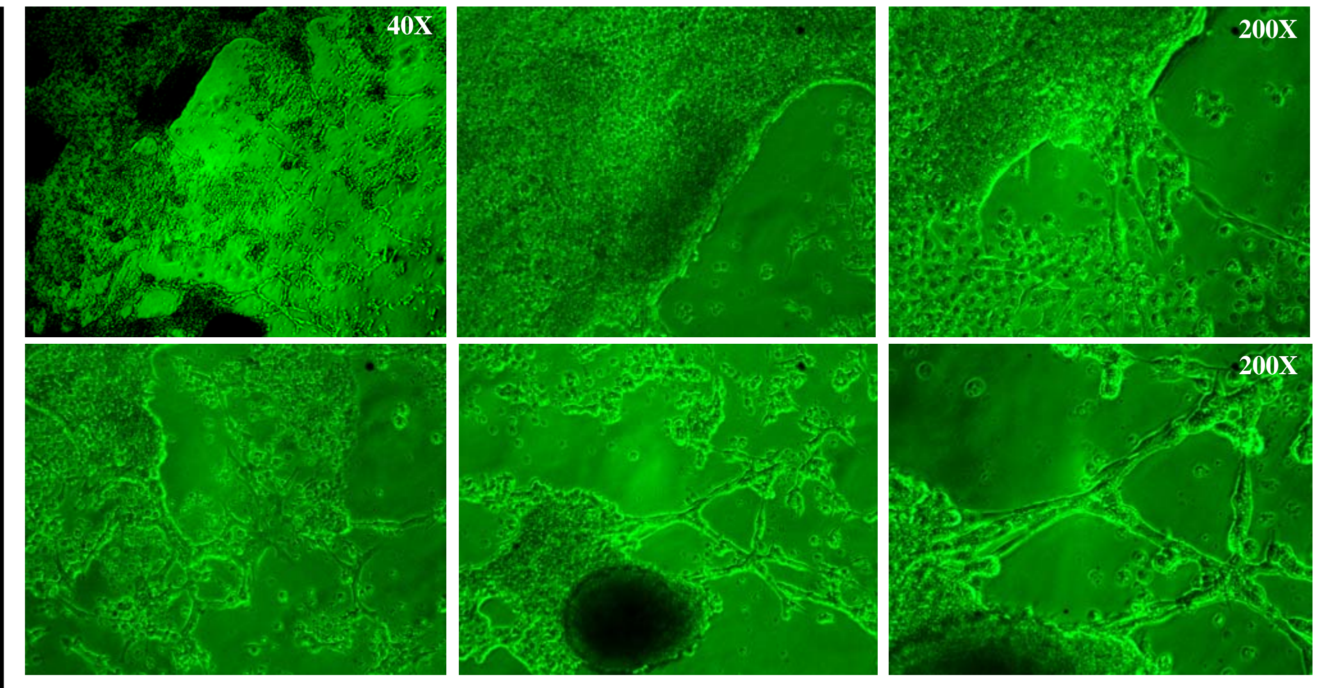
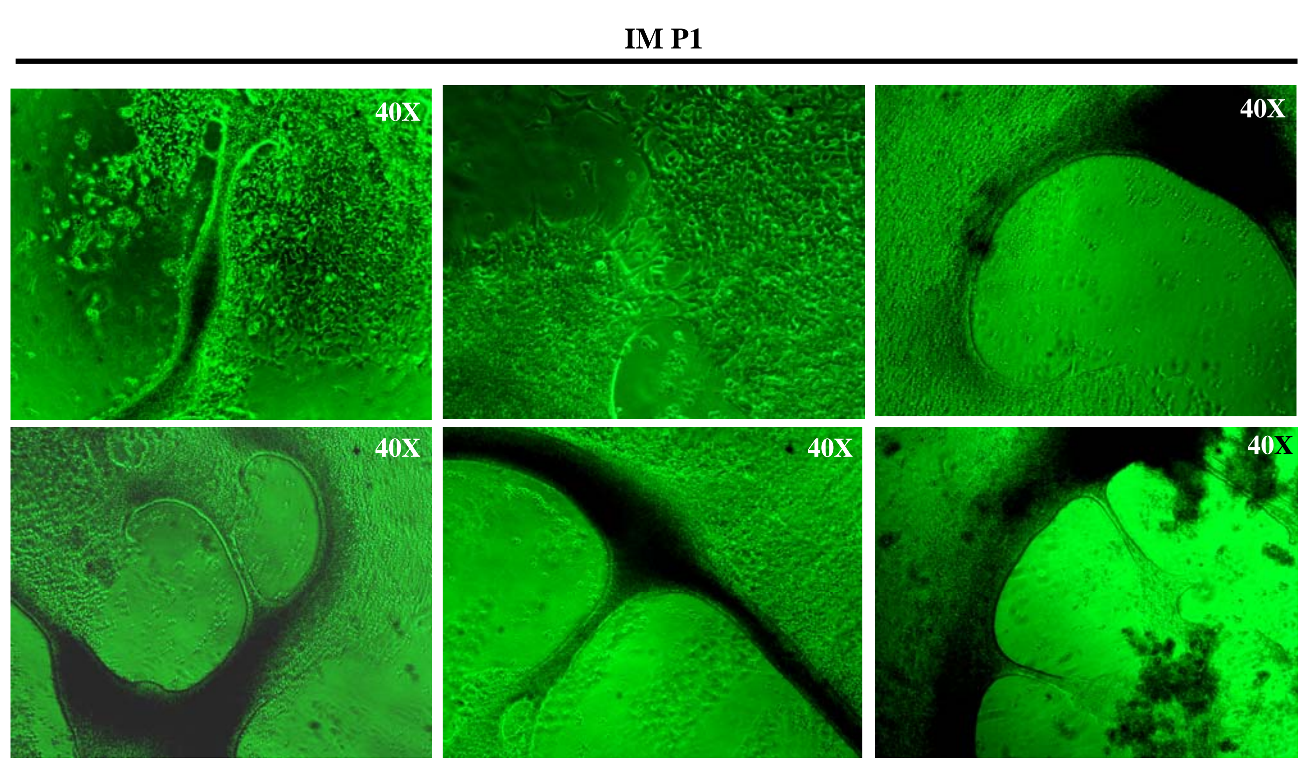
PA1 cells

IM



77 days

IM P1



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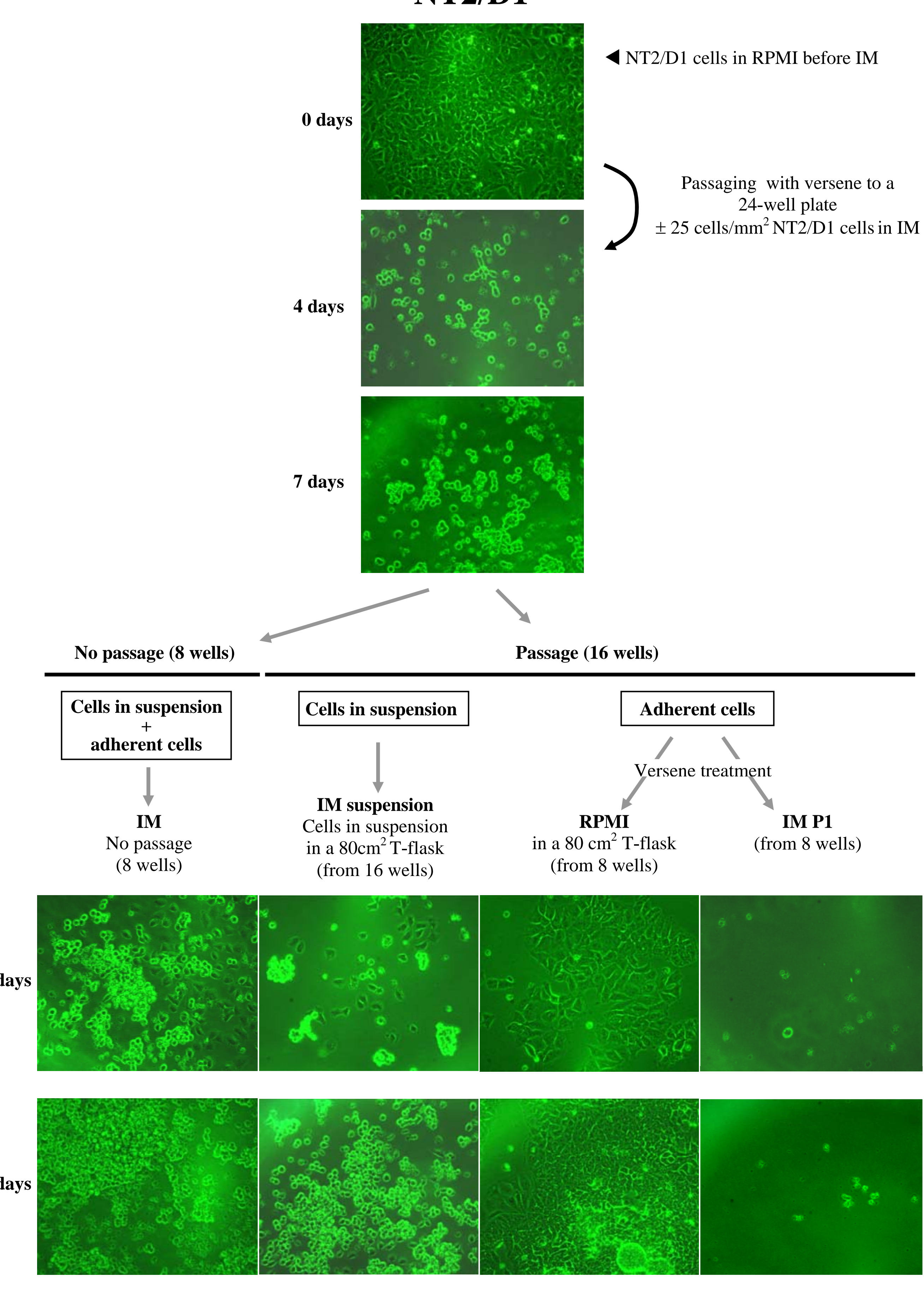

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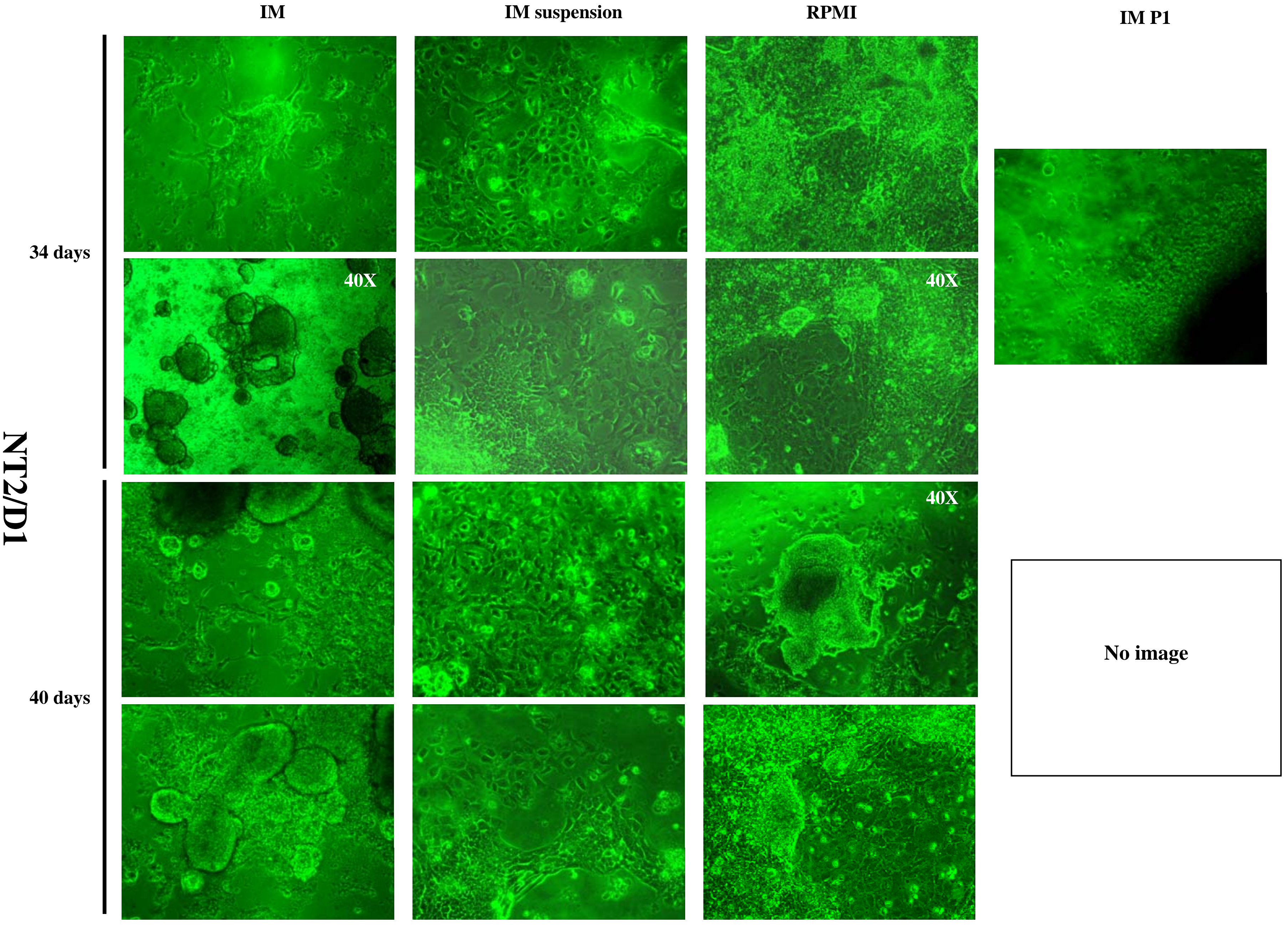
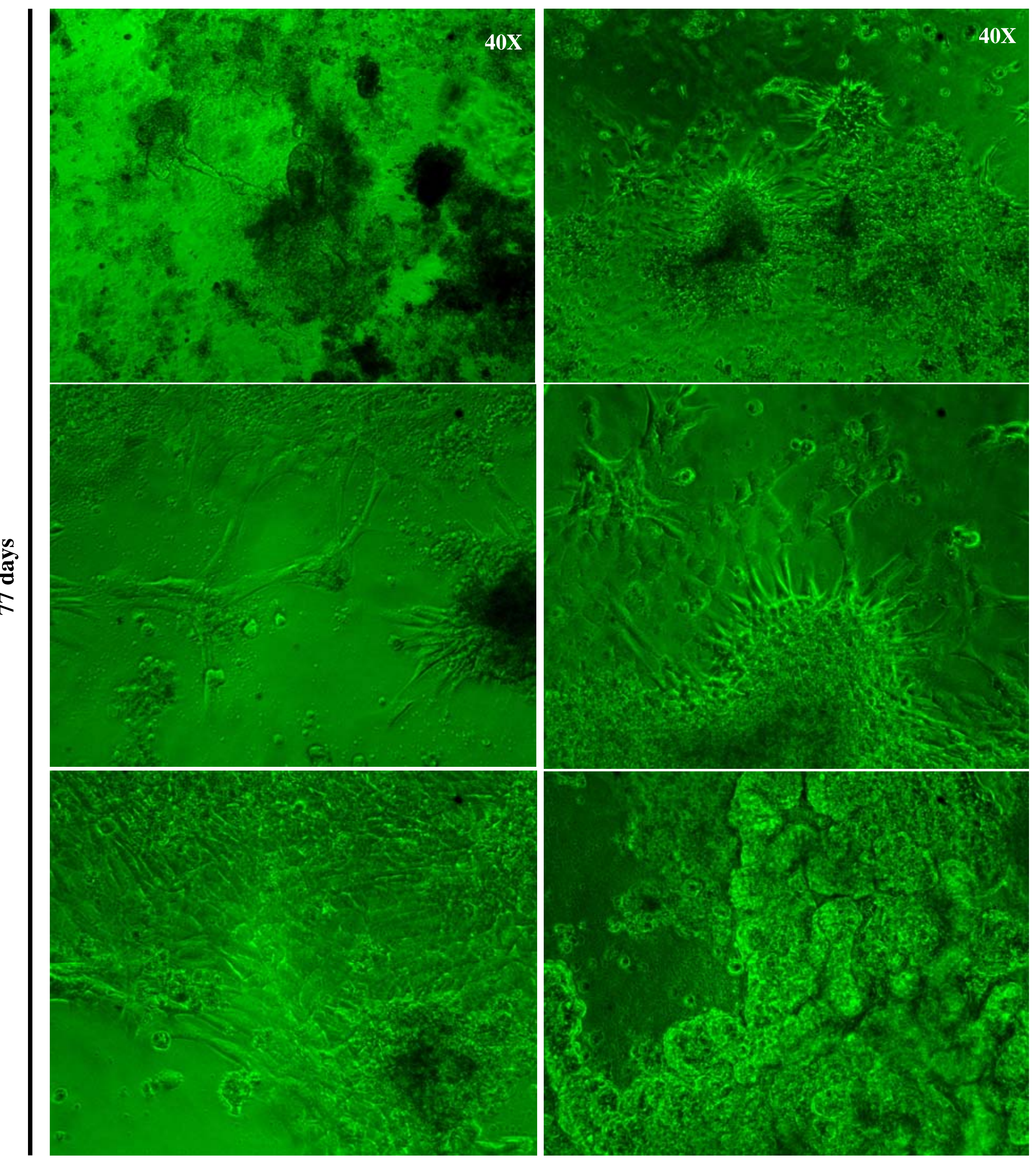


(Figure 2.2.1 - Cont.)

NT2/D1

IM



(Figure 2.2.1 - Cont.)

Figure 2.2.1 - Human teratocarcinoma cell lines PA1 and NT2/D1 in inductive medium (IM).
PA1 and NT2/D1 cell lines grown to confluence in a standard expansion medium designated as "RPMI", composed of RPMI 1640 Medium, 10 mM Hepes, 20 µM 2βME, 2 mM Glutamine, 10 % FBS, 1 % (v/v) Penicillin 10000 IU/ml + Streptomycin 10000 mg/ml. The cells were then detached with versene and transferred to a 24-well plate in the inductive medium (IM) at a concentration of 25 cells/mm^2. The IM was composed of RPMI 1640 Medium, 10 mM Hepes, 20 µM 2βME, 2 mM Glutamine, 1X NEAA, 1X ITS, 1 % FBS, 150 µg/ml ECGS, 50 ng/ml BDNF, 10 ng/ml $VEGF_{165}$, 5 ng/ml FGF2, 10 ng/ml IGF2, 1000U/ml Heparin Sodium salt, 1 % (v/v) Penicillin 10000 IU/ml + Streptomycin 10 mg/ml.
After 7 days, the culture medium carrying the non adherent cells was collected from 16 wells and distributed in a new 24-well plate, "IM suspension". Adherent cells that remained in the 16 wells from which the culture medium was collected, were detached with versene and replated into new wells. Half of them were ressuspended again in RPMI medium, "RPMI" and the other half in IM, "IM P1" (P1 refers to "passage 1").
Cells in eight wells of the initial 24-well plate were never passaged "IM".
This distribution and nomenclature was maintained through the experiment. 100X magnification is shown, except when otherwise indicated.

PA1 and NT2/D1 cells - TEM images

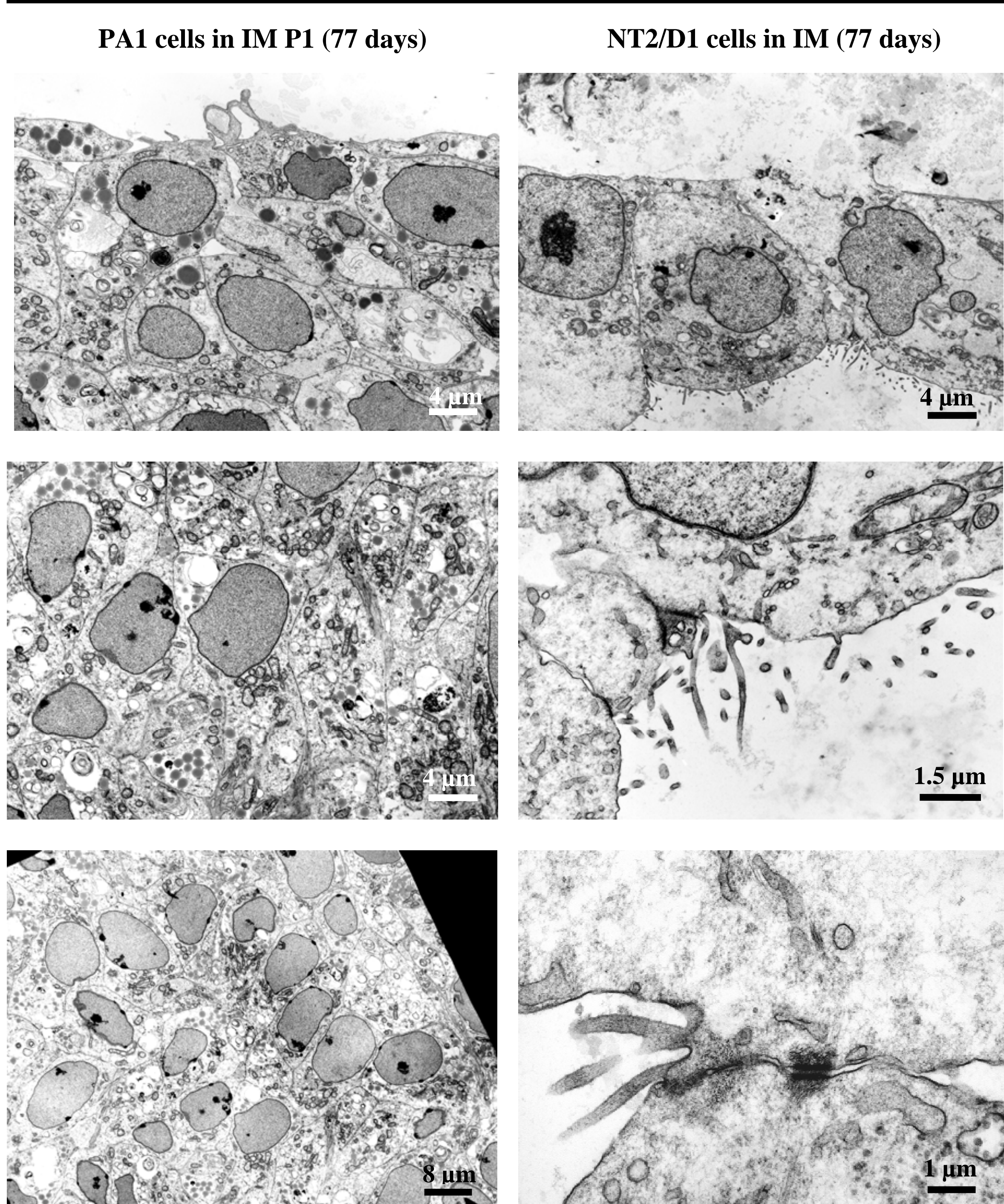


Figure 2.2.2 - TEM images of the teratocarcinoma cell lines PA1 (left column) and NT2/D1 (right column) after growth for 77 days in inductive medium (see also Fig. 2.2.1).

After these preliminary studies we chose to proceed only with the NT2/D1 cell line. Among the reasons that were mandatory for making a selection was the need to reduce to a minimum the volumes of the very expensive inductive medium. This cost that was further increased by the inclusion, in the study, of fibronectin as a cell culture substrate and of 5-Aza and BMP2 as differentiation inducers. Therefore, the price of each experiment was an important factor that would have made the study of two cell lines unreasonable. Also, the logical evolution of this study with analysis of gene expression, histochemistry and immunohistochemistry would have made the use of two cell lines not practicable.

Our preference regarding this cell line was obvious influenced by the accumulated experimental evidence that made us believe that NT2/D1 was the teratocarcinoma cell line that had the best probability of differentiating into cardiac muscle. Namely, its similarities with the mouse P19 teratocarcinoma cell line and its characteristic expression of CD133, Nestin and Cripto, all “signals” compatible with an hypothetical cardiomyogenic potential (See *Introduction - Cardiomyogenic cultures*).

The experiments were organized in a way that no cell passages were needed, as we had seen that NT2/D1 lost its ability to differentiate after cell passages when in inductive medium.

ii) Cultures of NT2/D1 in IM with fibronectin, 5-Azacytidine and BMP2

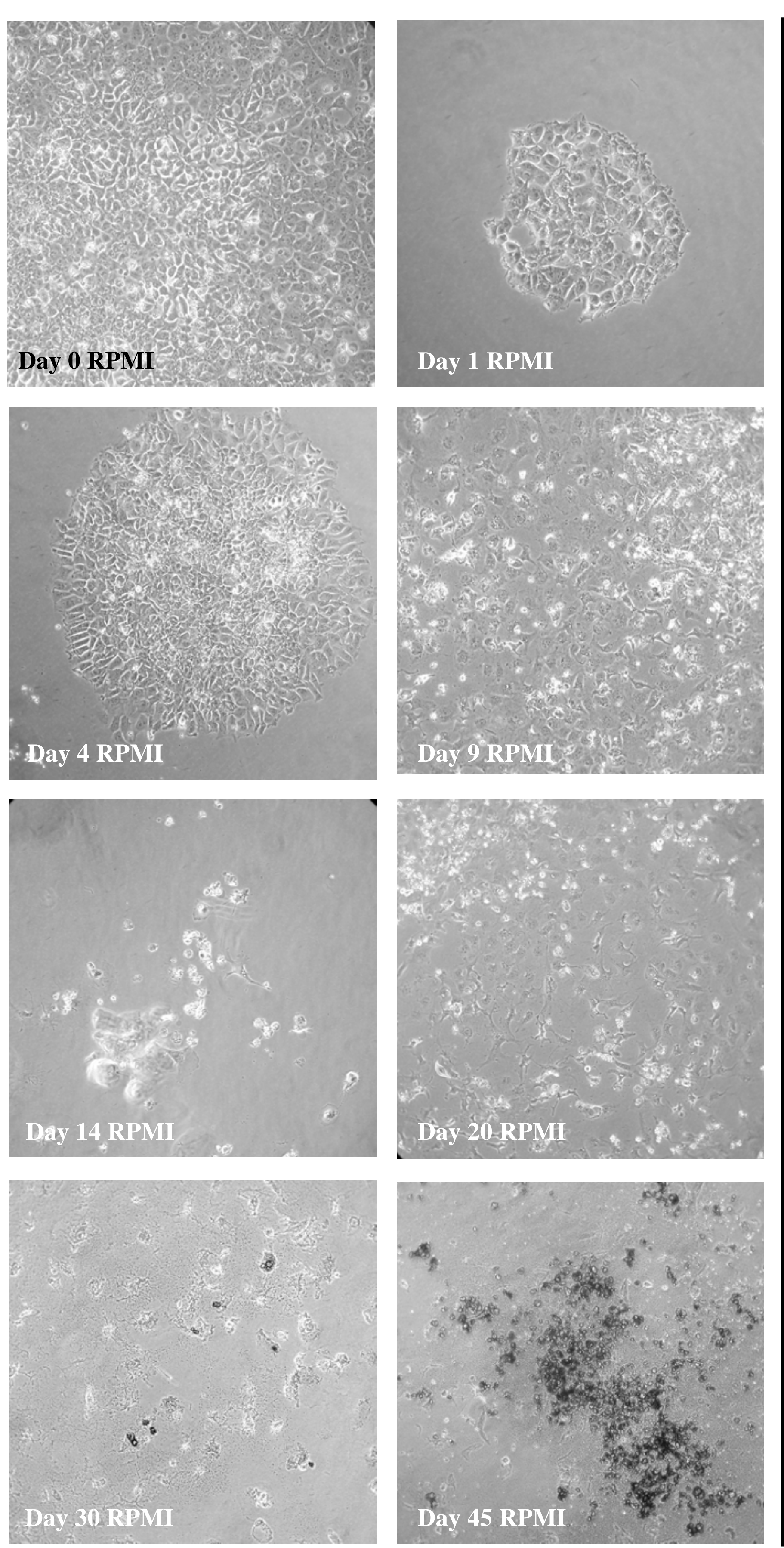
Cell culture substrates and differentiation inducers are among the most important variables to consider when designing a cellular differentiation experiment, *in vitro*. Accordingly, in subsequent experiments with NT2/D1, we tested an inductive medium, two inducer molecules, with reported relevance in cardiogenesis and one extracellular matrix (ECM) culture substrate (Fig. 2.2.3).

Fibronectin, is a glycoprotein and one of the components of the ECM to which integrins bind at specific adhesion sequences. Integrins are transmembrane receptor glycoproteins involved in regulation of gene expression, by their association with the cytoskeleton and through signal transduction pathways. The cross-talk between fibronectin and resident cells, through integrins, initiate the characteristic phenotypic changes associated with cardiomyocyte differentiation [2] as well as other cells. In fact, fibronectin is present in the ECM of the developing atria and ventricles [3]. Furthermore, if the molecule is absent, adherens junctions between myocardial precursors do not form properly [4]. It has also been suggested that this molecule plays a relevant role in the hypothetical cardiac stem cell niche, in the mouse adult heart [5].

5-Aza, as mentioned before, is a commonly used cardiomyogenic inducer of undifferentiated cells. The cells upon which 5-Aza has been shown to act include bone marrow-derived MSCs of mouse [6, 7], rat [8] and humans [9], human umbilical cord blood-derived MSCs [10] and hESCs [11].

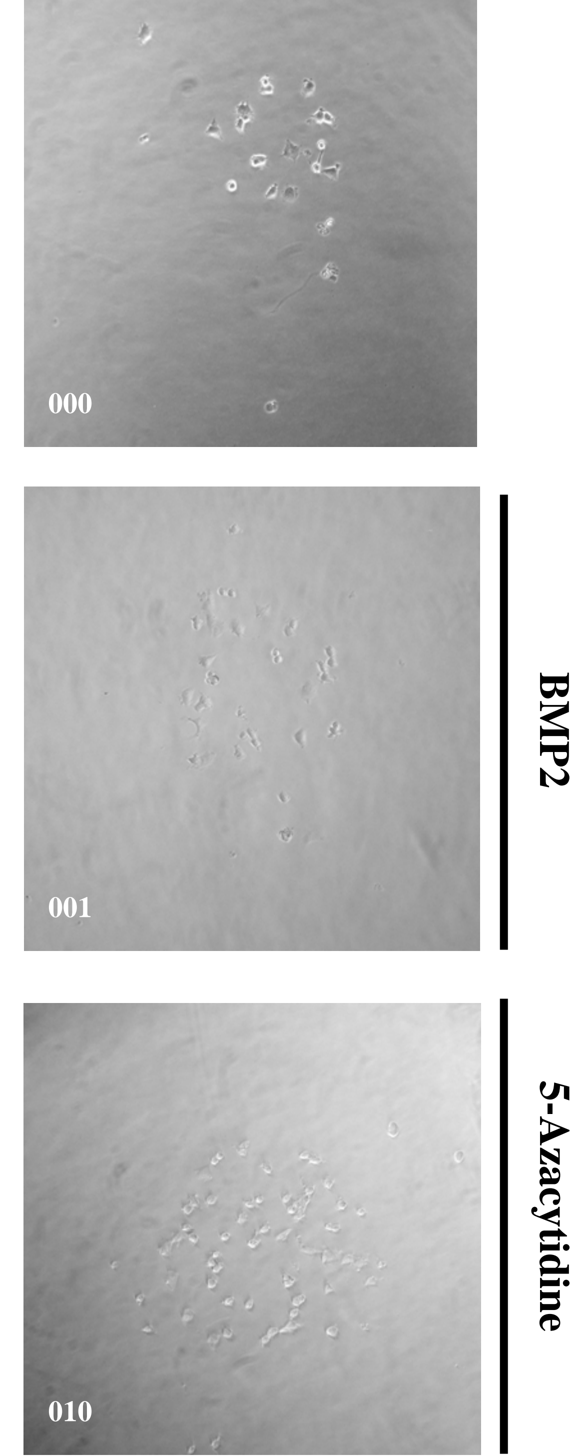
BMP2, a transforming growth factor, is an unquestionable cardiomyogenic inducer in the mouse teratocarcinoma cell line P19 [12]. In our opinion this cell line has many similarities with the NT2/D1, as for instance the ability to differentiate into extraembryonic endoderm. The latter cells that present, among others, many microvillosities as those observed in NT2/D1 (Fig. 2.2.2).

Based on the above assumptions a 45-day cell culture was performed, expecting to obtain clear differences between the various culture conditions and that at the end of the experiment we would have sufficient healthy cells that allowed gene expression analysis. Unfortunately, the results obtained were not conclusive regarding the development of new phenotypes (Fig. 2.2.3). Despite the many embryoid body-like structures that could be observed, no autocontractile regions were detected. Additionally, the RNA extracted from the cells at defined time points during the time course of the experiment did not give rise to the expected RT+PCR bands in agarose gel electrophoresis.



(Figure 2.2.3)

NT2/D1 IM Day 1



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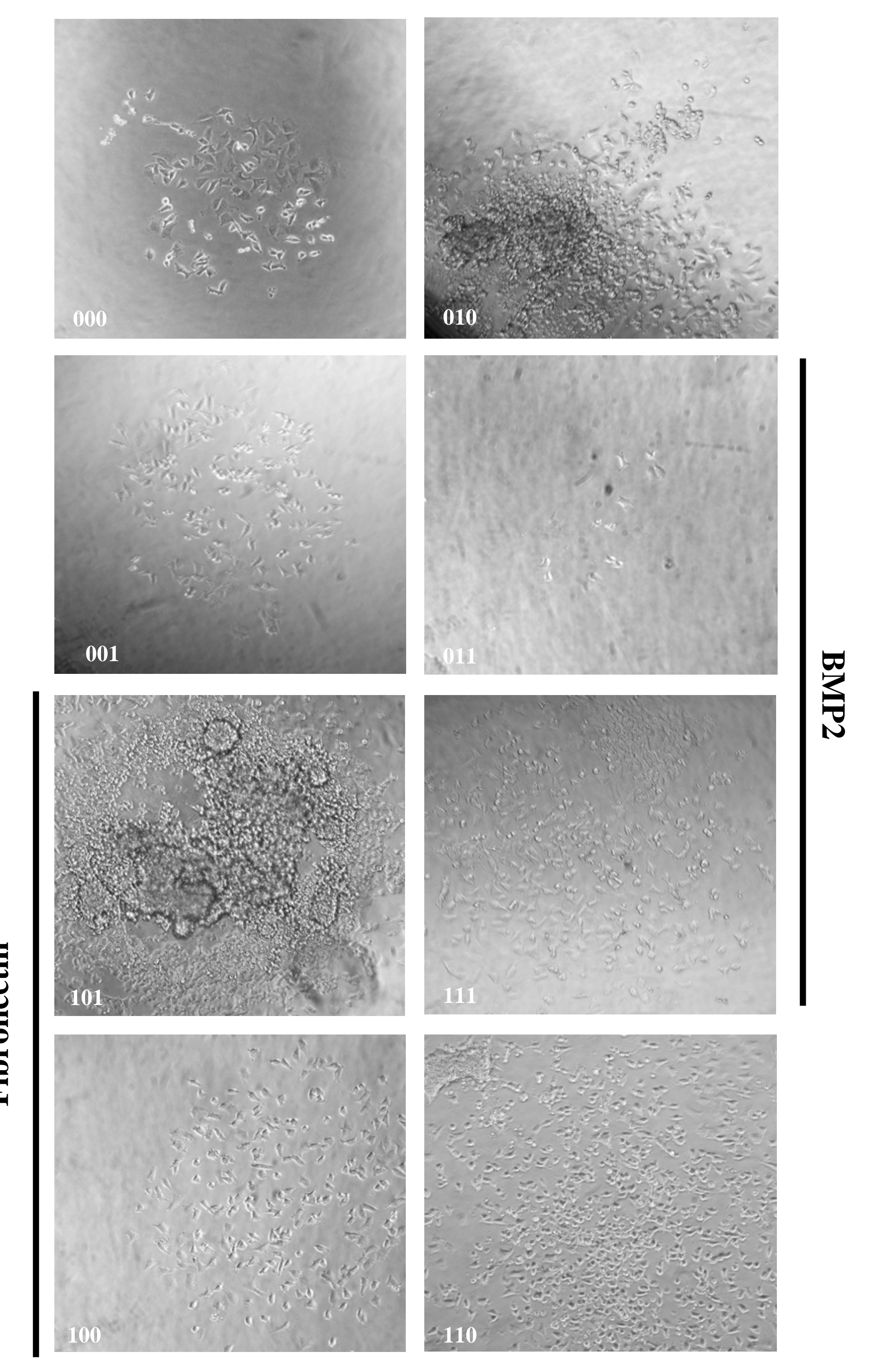

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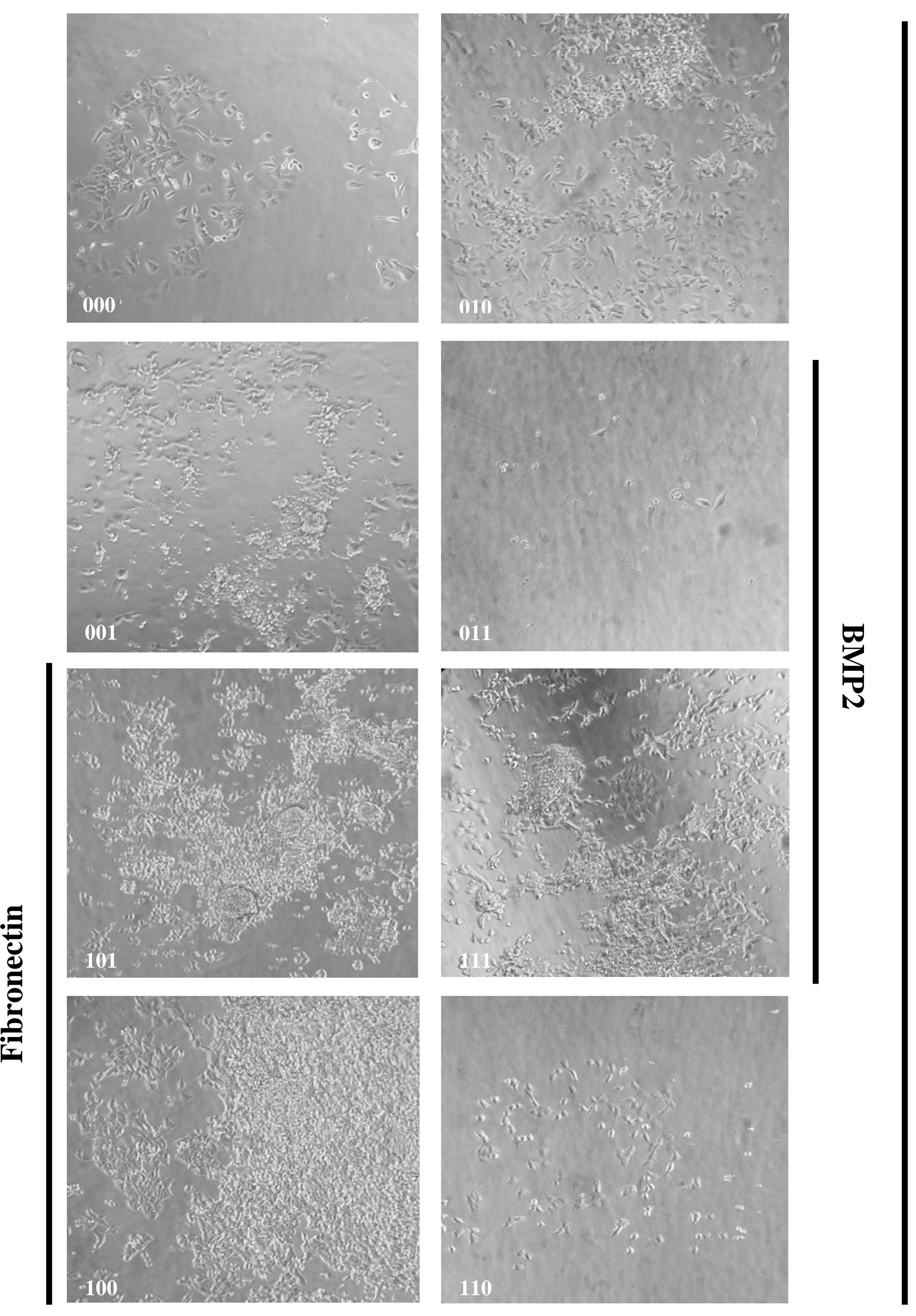

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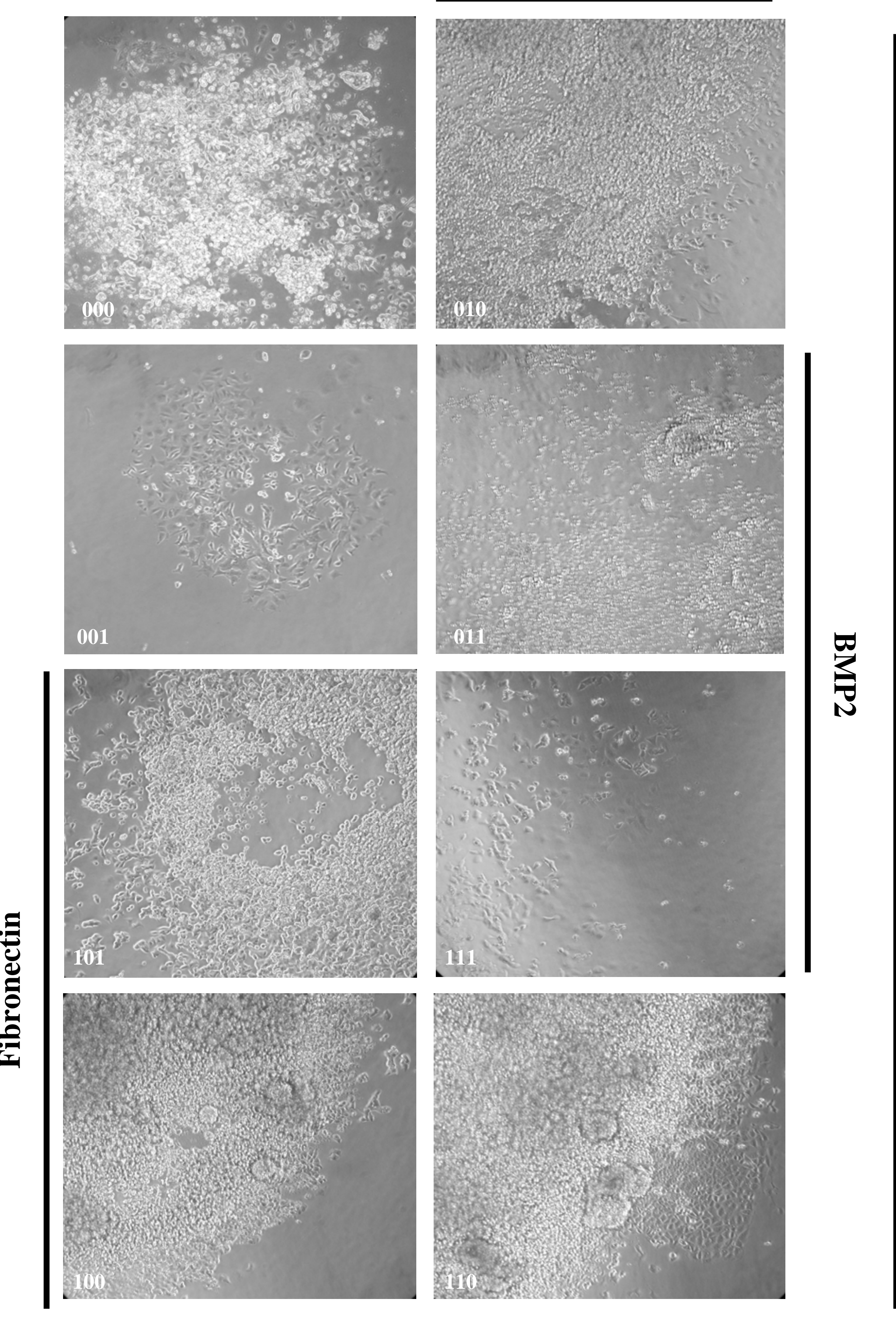

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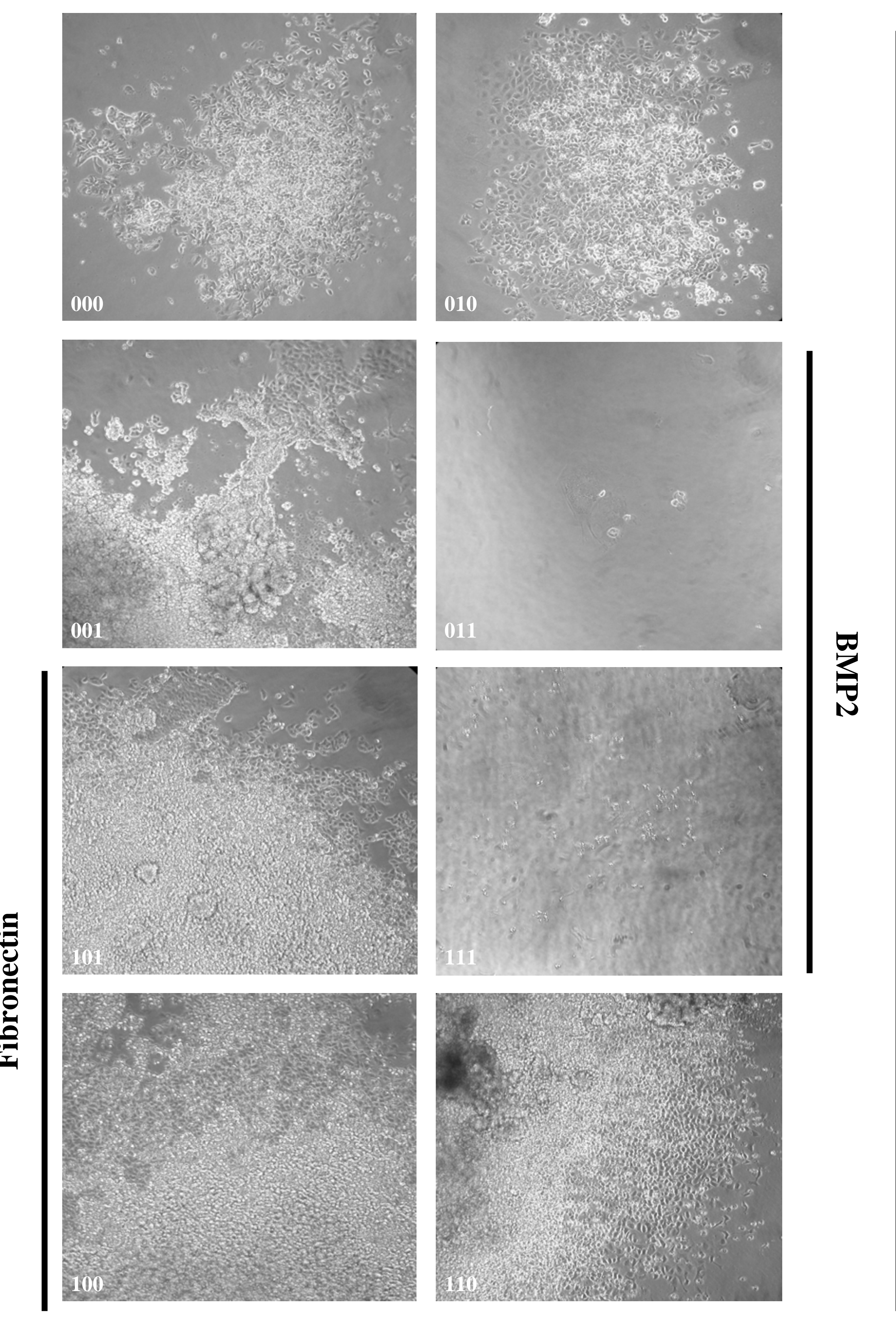

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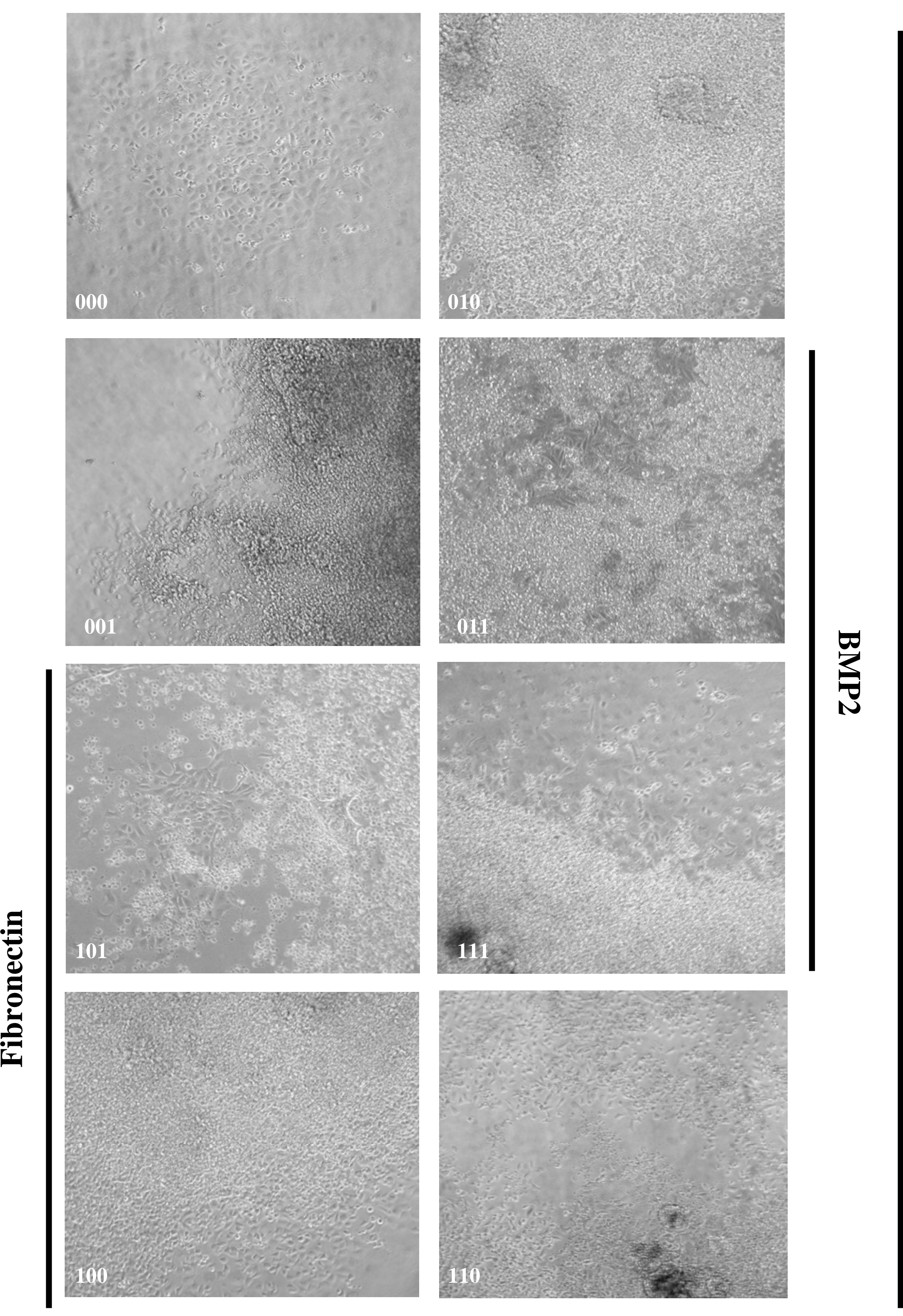

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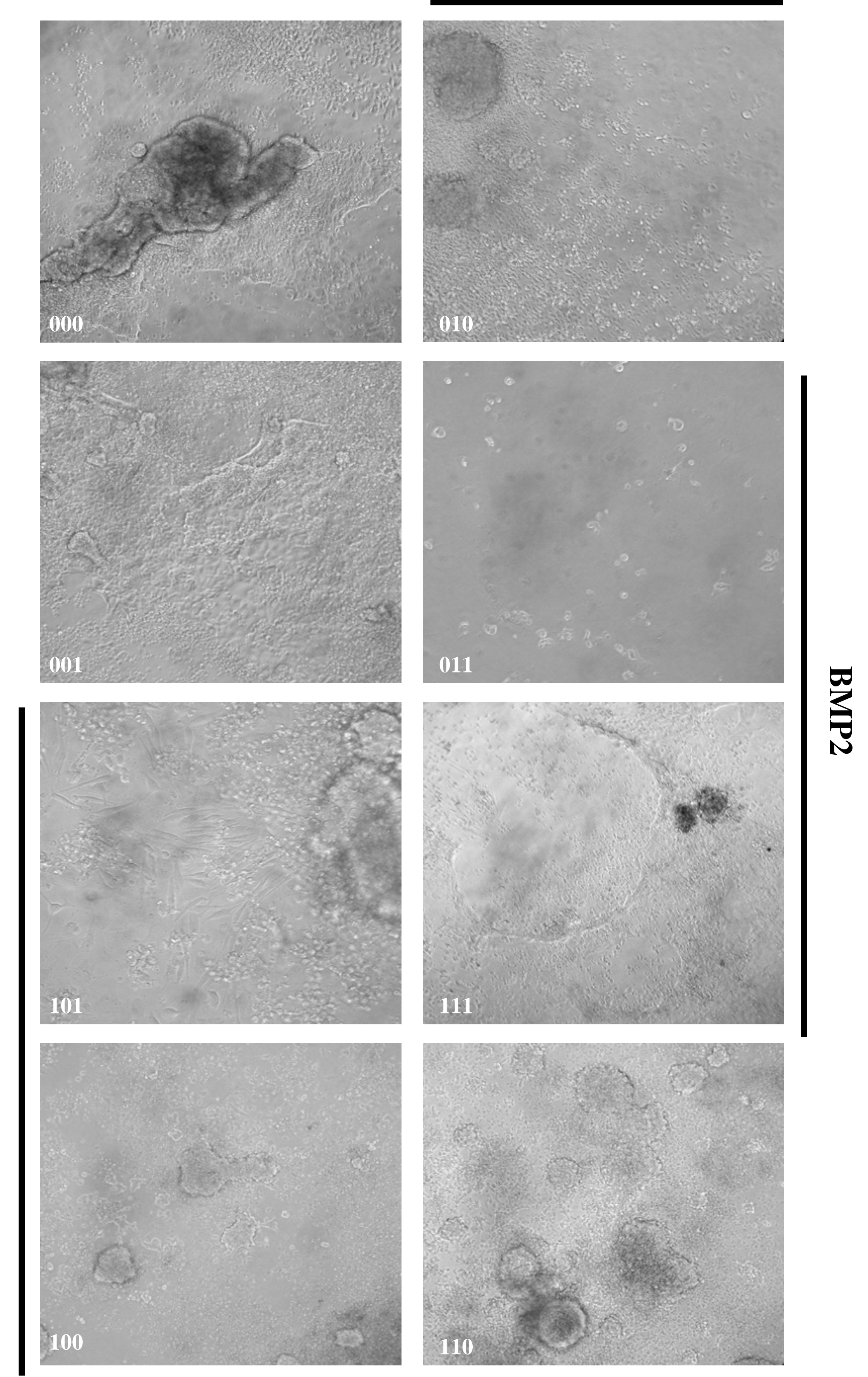


(Figure 2.2.3 Cont.)

Figure 2.2.3 - Human teratocarcinoma NT2/D1cells in inductive medium with 5-Aza and BMP2.
NT2/D1 cells grown to confluence in a standard expansion medium (Day 0, RPMI). "RPMI" was composed of RPMI 1640 Medium, 10 mM Hepes, 20 µM 2βME, 2 mM Glutamine, 10 % FBS, 1 % (v/v) Penicillin 10000 IU/ml + Streptomycin 10000 mg/ml. The cells were then detached with versene and transferred to one 24-well plate in RPMI and to six 24-well plates in the inductive medium (IM) with or without 5-Aza, fibronectin and BMP2 at a concentration of 10 cells/mm^2, according to the table below. The IM is constituted by RPMI 1640 Medium, 10 mM Hepes, 20 µM 2βME, 2 mM Glutamine, 1 mM NaPyruvate, 1X NEAA, 1X ITS, 1 % FBS, 50 ng/ml BDNF, 10 ng/ml $VEGF_{165}$, 5 ng/ml FGF2, 10 ng/ml IGF2, 1000U/ml Heparin Sodium salt, 1 % (v/v) Penicillin 10000 IU/ ml + Streptomycin 10 mg/ml.

Absence or presence of Fibronectin, 5-Aza, and BMP2 (absence = 0; presence = 1)			
Numbers at the bottom left corner of cell culture images	IM and:		
	Fibronectin C_f = 3.75 µg/cm^2	5-Azacytidine C_f = 3 µM	BMP2 C_f = 100 ng/ml
000	absent	absent	absent
001	absent	absent	present
010	absent	present	absent
011	absent	present	present
100	present	absent	absent
101	present	absent	present
110	present	present	absent
111	present	present	present

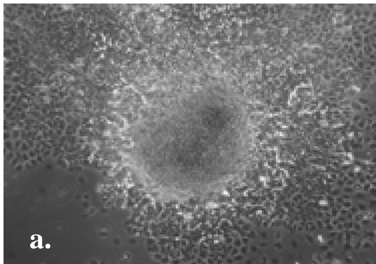
The medium was changed periodically during the next 45 days and microscope phase contrast images were recorded at several defined time points during the time course of the cultures.
Cell cultures in the six 24 well-plates were stopped at 4, 9, 14, 20, 30 and 45 days, respectively, with the removal of cell culture medium from each well and the addition of 500 µl of Trizol reagent for the subsequent RNA extraction protocol. After the last time point the cells were processed for RNA extraction.
All of the images have an amplification of 100X.

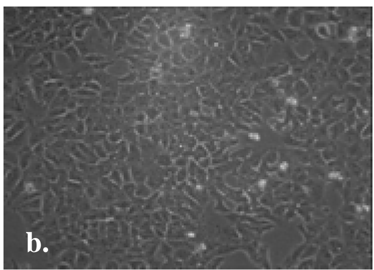
iii) Cultures of NT2/D1 in IAM with BMP2

The following series of experiments performed with NT2/D1 cells was conducted taking into account not only the accumulated experience but also the recently published results showing that a cell culture medium, very similar to the one we had been previously using, has driven human fetal liver $CD133^{+}$ cells to differentiate into endothelial and skeletal and cardiac muscle [1]. Accordingly, we cultured NT2/D1 cells, under the same culture conditions used in the referred study, with the believe that small differences could be decisive on the induction of differentiation of the NT2/D1 cells into mesoderm fates. Additionally, tested again the effects of BMP2 in some cell samples.

In summation, we analyzed, once more, the morphological characteristics and transcriptional activation of genes pertinent in cardiac muscle and endothelium differentiation, during the growth of NT2/D1 cells in inductive angiomyogenic medium with or without BMP2. Analysis of ultrastructural organization, actin cytoskeleton and histochemistry were also performed in this set of experiments.

As before, the typical NT2/D1 morphological characteristics, as the increase in nuclear/cytoplasm volume ratio, the flattening of the cells, the prominent nucleoli (Fig. 2.2.4), the polymerized actin organization (phalloidin staining) and ultrastructural analysis were used as the morphological criteria to monitor cell differentiation.





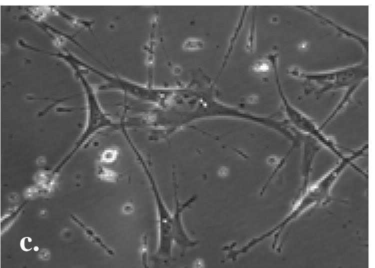


Figure 2.2.4 – Phase contrast images of three typical morphologies of NT2/D1 cells in expansion medium, (RPMI, FBS and antibiotics) analyzed in a CK2 Olympus microscope: a. Embryoid body-like structures, magnification 40X; b. epithelioid-like phenotype, magnification 100X; c. neuronal-like phenotype, magnification 200X.

As can be seen in Fig. 2.2.5, after 20 days in culture, in control as well as in IAM medium, NT2/D1 cells can form many embryonic body-like structures (white arrows), surrounded by an epithelioid squamous layer of adherent flattened cells, with the interesting fact of presenting many microvillosities and endocytic vacuoles (see ultrastructural images, Fig. 2.2.5), resembling a visceral endoderm phenotype [13]. When in an overconfluency state, many structures presented some similarities with an endothelial angiogenic network. In contrast, neuronal-like cells could not be seen, indicating a possible inhibition of that phenotype. Muscular structures were never detected during the time course of the experiment. The microvillosities that could be detected in TEM (Fig. 2.2.5, TEM, control) were consistent with the known tendency that NT2/D1 cells have to differentiate through an epithelial path. Although desmosomes that were visible between adjacent cells (Fig. 2.2.5, black arrow) were consistent with a muscular phenotype, morphologies specific of this tissue, such as sarcomeres, were never found.

To analyse wether NT2/D1 were predisposed to differentiate into the cardiac muscle or endothelial branch, specific genes for these tissues and other classes were studied (Fig. 2.2.6 and 2.2.8):

Endothelial progenitor related genes

Transcripts of CD133 and the VEGF receptor 2, Kdr, could be detected at all time points of the experiment. This finding could reflect the angiogenic potential of these cells [14, 15]. In fact, there are some images in the differentiation cultures that remind angiogenic like networks, but further studies are needed to prove this concept.

Other progenitor related genes

Knowing that NT2/D1 expresses the CD133 and that these cells easily undergo neural differentiation, we would expect to detect expression of Nestin [16, 17]. That was indeed the case. Nestin expression was detected from the first day of the experiment. On the other hand, the expression of Flt1 indicated that these cells could be considered not only endothelial but also haematopoietic progenitor cells as described by others [18, 19].

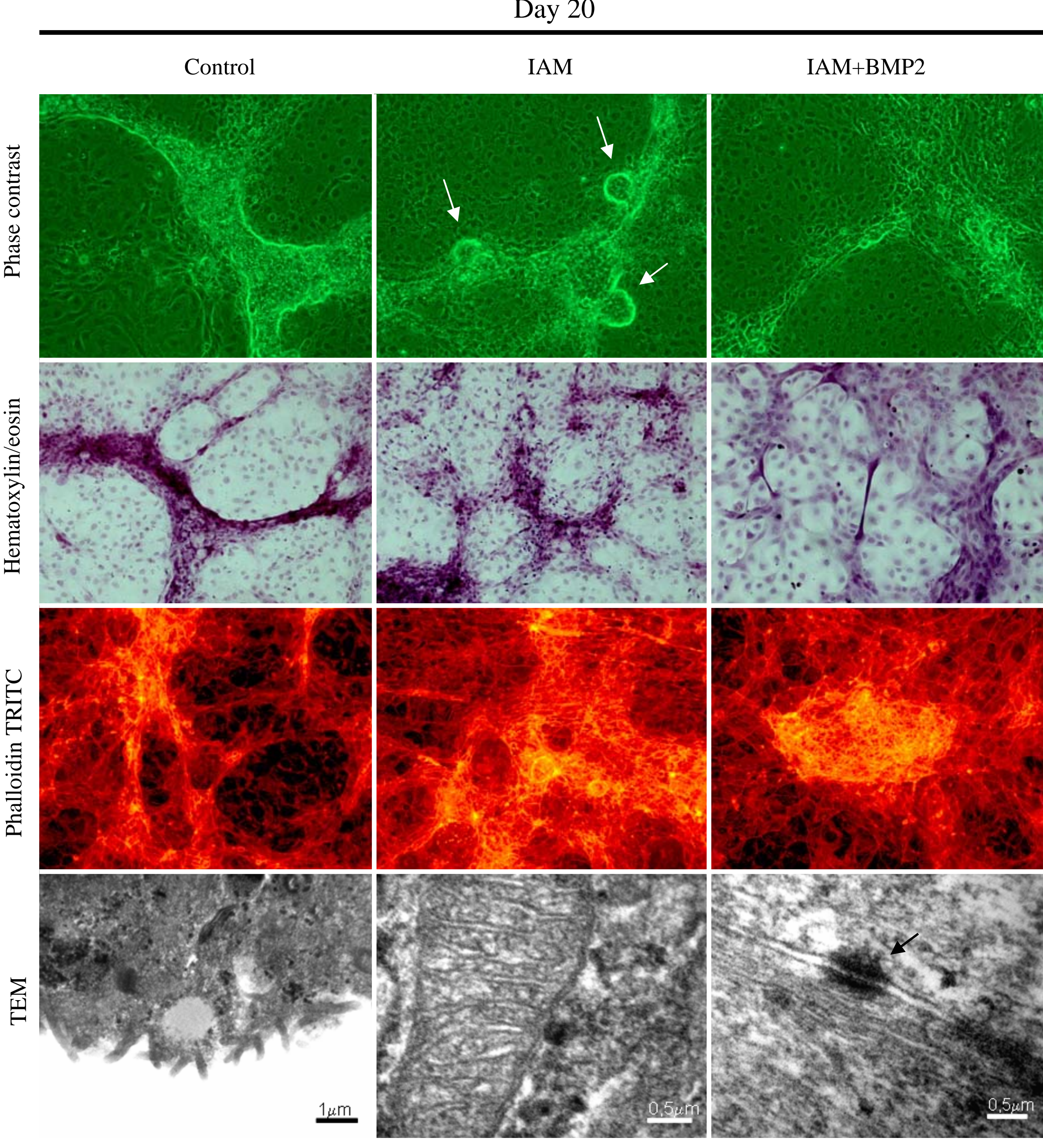


Figure 2.2.5 – Cell culture images of NT2/D1 cells after 20 days growing in control, IAM and IAM + BMP2 medium. All images are 100X magnified. Ultrastructural images have a scale bar in the bottom right corner. (To see images of days 1, 5, 10 and 30, see Fig. 2.2.8). NT2/D1 cells can form many embryonic body-like structures (white arrows), surrounded by an epithelioid squamous layer of adherent flattened cells. Desmosomes can be seen between adjacent cells (TEM black arrow).

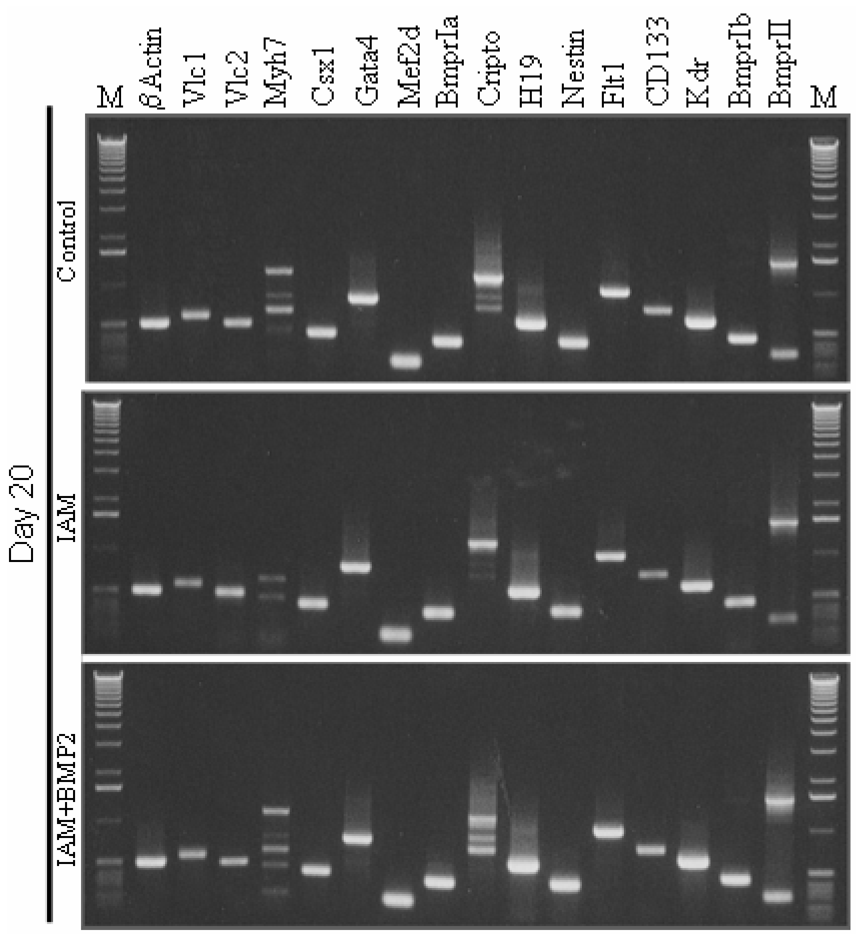


Figure 2.2.6 – PCR amplified gene products of NT2/D1 cells cDNA.
Images of 0.8 % agarose gels with EtBr.
In the first and last lanes - 1 Kb DNA ladder (Invitrogen).
To see gel images of the days 1, 5, 10 and 30, see Fig. 2.2.8.

Bone morphogenetic protein receptors (BMPRs)

Because one of our main assumptions was that BMP2 was an inducer of mesodermal differentiation in NT2/D1, we analyzed whether the main BMP receptor genes, namely BmprIa, BmprIb and BmprII [20, 21] were transcriptionally active. Type II receptors can bind ligands in the absence of type I receptors, but they need their respective type I receptors to transduce the signal [22, 23]. BmprIa, BmprIb and BmprII transcripts could be detected at all time points of the experiment. However, the heavy isoform of BmprIb was only detected after 30 days in the inductive angiomyogenic medium. The mechanism underlying this phenomenon remains to bee clarified (Fig. 2.2.8).

Muscle related genes

At all time points analyzed, NT2/D1 cells expressed genes specific for undifferentiated and differentiated cardiac cells like Gata4, CsxX1 and Mef2d and Vlc1 and Vlc2, respectively. The slow muscle myosin heavy chain gene Myh7, found in cardiac but also in some skeletal muscles, was the sole gene, of those analyzed that was not detected in this study.

The Myh7 primers were constructed and verified for accuracy using cDNA samples of human masseter and human left ventricle, which are muscle tissues known to express this gene in a relatively high level. With the latter samples we could obtain single PCR bands that were verified by sequencing (Fig. 2.2.7). The first time we used the Mhy7 primers, in NT2/D1 (Fig. 2.2.7), we obtained an interesting pattern of expression that reminded the images obtained with molecules that can undergo alternative splicing. However, sequencing of the different bands obtained by RT+PCR, with the Myh7 primers, showed that the products were unrelated to Myh7 (Fig. 2.2.7). The five unspecific bands from NT2/D1 are marked 1 to 5 and the respective identified sequences are shown at left of the gel. Thus, although the primers for the Myh7 were specific for this gene, the bands found were nonspecific products of the polymerase reaction. Curiously, other non muscular tissues (Fig. 2.2.7) also gave rise to nonspecific products with the specific Myh7 primers. (Remember the case of Myh7 expression in RH30 cells in *Results section 1*)

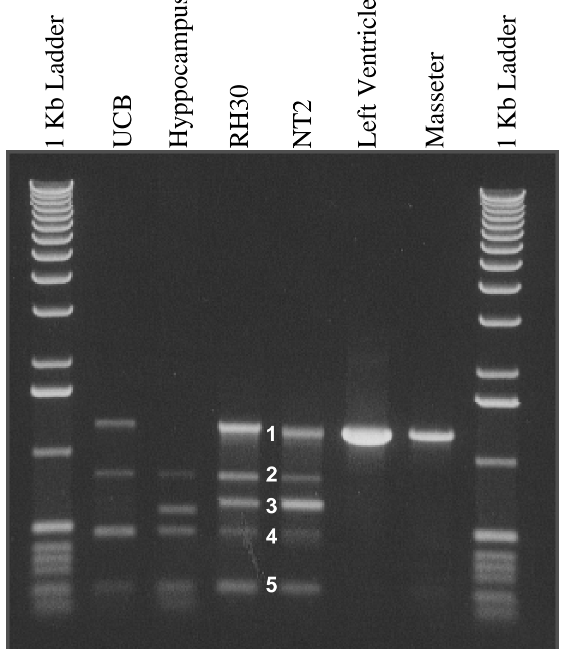


Figure 2.2.7 - An agarose gel electrophoresis of RT+PCR results with the Myh7 primers.
Tissues, cell lines and 1 Kb DNA ladder (Invitrogen) are legended above.
Samples of human masseter and human left ventricle, were confirmed by sequencing. The five non specific bands in the NT2/D1 lane are marked from 1 to 5 with the respective sequentiation results shown below.

Band	**Gene**	**Chr**
1	Tubulin Tyrosin Ligase Like 9 (TTLL9) intron	20
2	Repeat elements (MIR, AluSx, L2)	8
3	Fatty Acid Desaturase 1(FADS1)	11
4	Unknown region	2
5	Not sequenced	-

NT2/D1 related genes

H19 is a gene that is expressed in a vast number of organs during fetal development. In adults, however, expression is confined to lung, thymus, skeletal and cardiac muscle [24]. Accordingly, in EC cells it is expressed after induction of differentiation [24-28]. Our experiments showed that NT2/D1 expressed H19 throughout the time course of the experiment (see Fig. 2.2.8 for further details).

As mentioned before, one of the genes highly expressed in P19 cells, a murine EC, with cardiac differentiation potential is Cripto, that disappears when differentiation is completed [25]. As expected, we detected in the NT2/D1 cell line relatively good levels of Cripto expression that changed to a more unspecific pattern in the last days of the experiment. This suggested that NT2/D1 had differentiated [29]. The expected band for Cripto was 1199 bp. The significance of the several unexpected bands that appeared in NT2/D1 after some days in culture remains unclear (see Fig. 2.2.8 for further details). This similarity between P19 and NT2/D1 cells supported our initial suggestion, that in the appropriate conditions, NT2/D1 cells could give rise to mesodermal derivatives, such as skeletal or cardiac muscle.

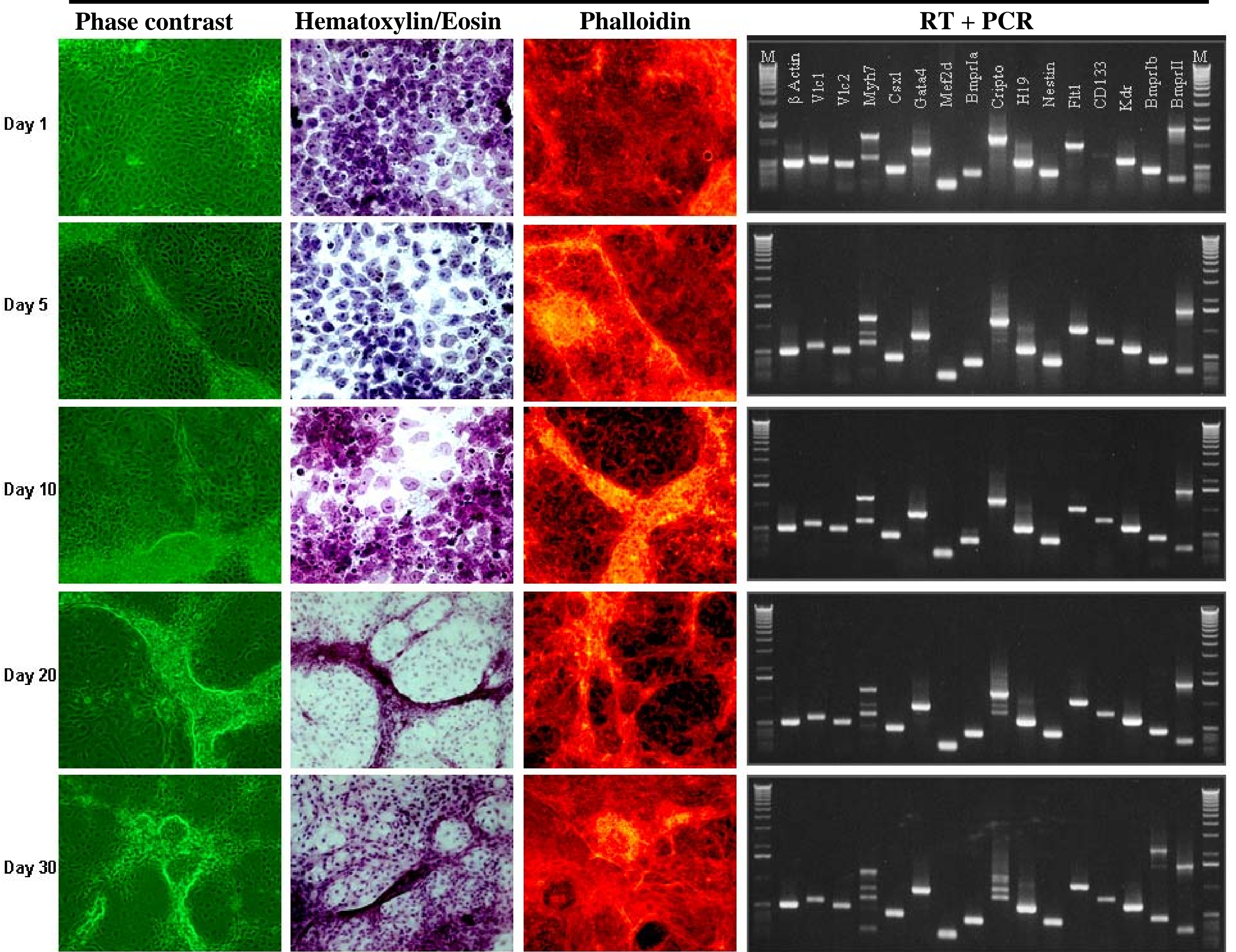
At the end of this experiments we could not compare transcripts of different genes because quantitative PCR was not performed, nevertheless, other observations could be made:

1. Contrarily to what we have expected, in general, all genes were expressed in the different time points analyzed, with the exception of the Myh7 that was never detected;

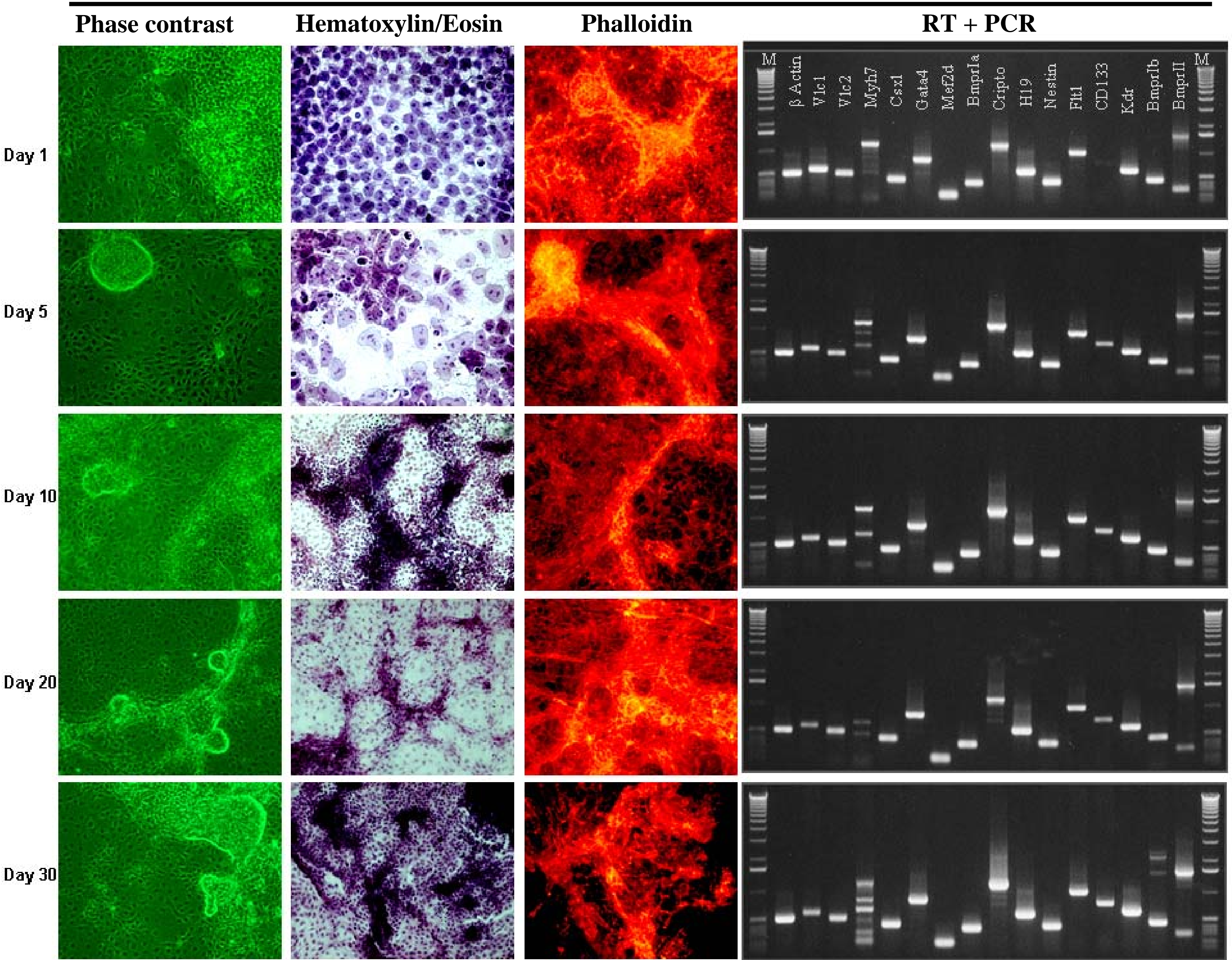
2. The gel band of Cripto has the tendency to change to a specific pattern during NT2/D1 differentiation;

3. A DNA band of BmprIb appears in the late stages of NT2/D1 differentiation. We cannot exclude DNA contamination in the PCR reaction, but if so, and because all the other reactions used the same cDNA, why was this gene the only one presenting a DNA amplified band? We do not have a explanation for this;

4. The heavy band of BmprII appears only in late stages of NT2/D1 differentiation.



(Figure 2.2.8)



(Figure 2.2.8 Cont.)

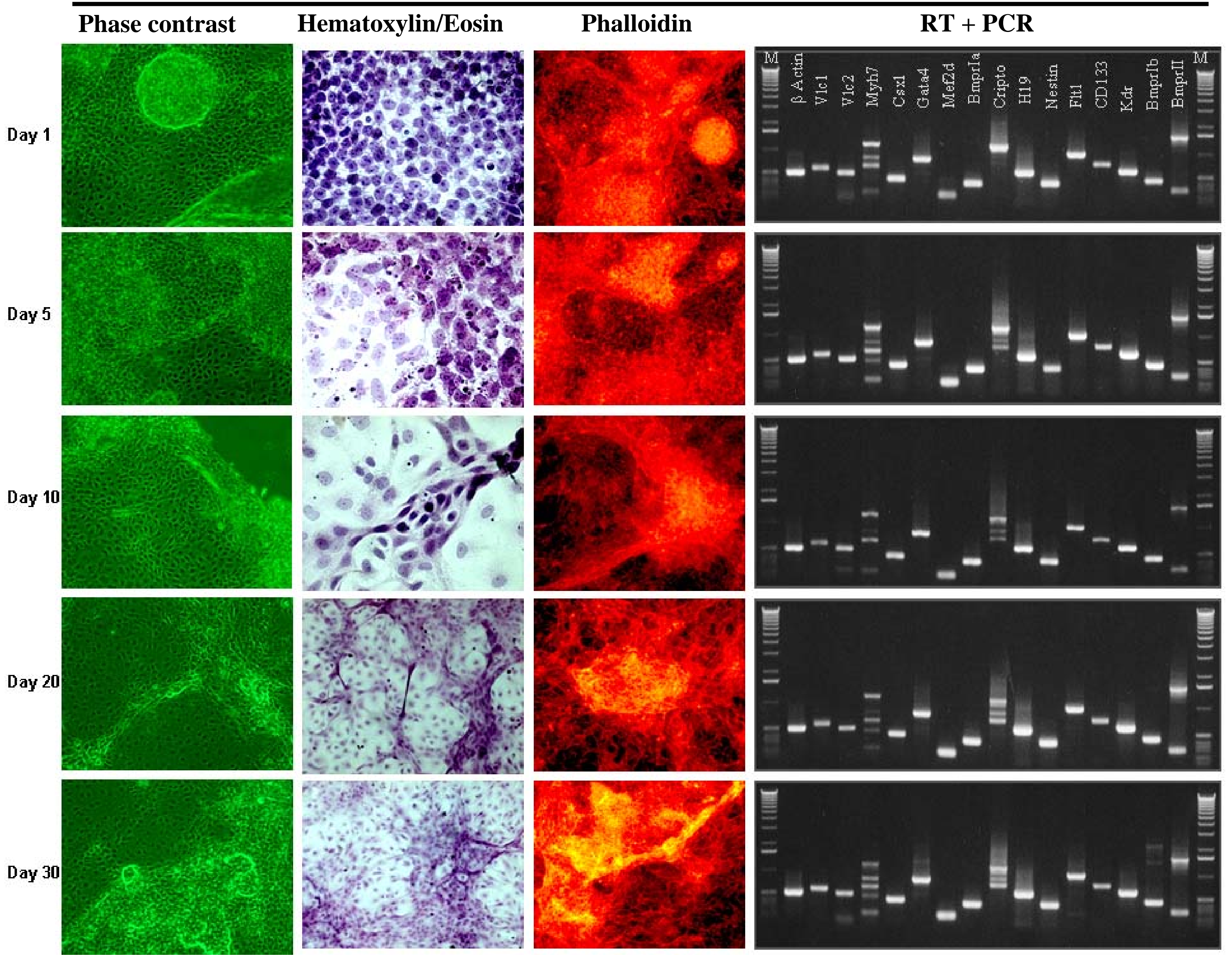


Figure 2.2.8 – Cell culture images of NT2/D1 and their amplified gene products from cDNA after 1, 5, 10, 20 and 30 days growing in Control, IAM and IAM + BMP2 medium.
All images are 100X magnified.
In gels, the first and last lanes show the 1 Kb DNA ladder (Invitrogen).

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Discussion

Discussion

Postinfarction coronary heart disease was one of the first non-hematopoietic human pathological conditions in which stem cell-based therapy was used [1]. However, and in spite of the tremendous advances in the stem cell research, with the detection of postnatal human cardiac progenitors in right ventricle, atria and outflow tract, progenitors of the left ventricle remain elusive [2]. With a few exceptions, in which $CD34^+$ or $CD133^+$ cells were used [3, 4] in patients with myocardial ischemia, in the majority of the clinical trials performed to date, specific subpopulations of bone marrow cells were not employed. Instead, the mononuclear or the mesenchymal stem cell fraction was used [1, 5-9] or, in patients with chronic heart failure, adult skeletal muscular progenitors [10-15].

Differentiation protocols that can give rise to a good number of cardiac progenitors from the embryonic or adult stem cell pool, assuring a good homing outcome, are not yet available [16]. In fact, using only undifferentiated stem cells in cardiac cellular therapies gave low rates of muscle regeneration, possibly due to the small number of specific cardiac progenitor cells that were transplanted [17].

In spite of the big boom of research in cardiac cellular therapeutics, it is urgent to design methods that can be used in selection processes, thus assuring that a good number of cardiac progenitor cells are transplanted into the affected regions of the heart.

Comparing the applied research performed in mice and the clinical trials in humans one can conclude that very little of what was learned from the former can be directly applied to the latter. The differences between small and big mammals [18] is so relevant, regarding differentiation of cardiomyocytes from stem cells, that much work is still required.

The present work was intended to contribute to a better understanding of those differences and, thus provide new ways of obtaining the appropriate cells to be transplanted.

One possible way of obtaining a high number of cardiac immature cells to transplant, would be to use the biotechnological tools that are presently available to select, among the differentiating embryonic human cultures, the cells that will became cardiomyocytes, as was done in mice [19]. This would allow the selection of an almost 100 % pure cell population.

As it was mentioned above and shown in the previous chapter (*Clinical cardiac regeneration* - Table 5), up to now, bone marrow-derived stem/progenitor cells and skeletal myoblasts were the only cells tested for myocardial regeneration in humans. The selection process, among the potential cardiac progenitor cells, is therefore essential in order to obtain

good levels of safety, homing and electromechanical integration.

With the research that has been presented herein, we have attempted to develop a method that allows the isolation of cells, that will become left ventricular cardiomyocytes, at a stage of none terminal differentiation. Such tool would support identification of cell markers of the required cells and thus, when validated, become an important instrument to use in stem cell therapy.

For that purpose, a molecular biology strategy was used. First, a fragment carrying the 5' flanking region of the human βMyhc promoter (pβMyhc), the G418 resistance gene (Neo^R) and the Hygromycin resistance gene (Hyg^R) were linked together in the pBluesript II SK(+) vector. Then the final relevant fragment, used for mammalian cell transfection, was obtained by PCR from this construct. Because the Neo^R does not carry a promoter, its expression depends on the activation of the βMyhc gene and will, thus, be responsible for the selection of βMyhc expressing cells from an undifferentiated cell pool, in a geneticin containing cell culture medium. On the other hand, the Hyg^R, contains the HSV-TK promoter and will be expressed in all transformed mammalian cells despite their lineage of commitment. Consequently it will serve as the transformation reporter gene and will allow for selection in an hygromycin containing cell culture medium.

Among the many possible cardiac muscle-specific promoters that could have been chosen to be responsible for the selection of a cardiac progenitor cell, we have favored the βMyhc promoter. The heart is composed of many different cell types as atrial, ventricular, sinus nodal-like and Purkinje-like cells. Therefore, when considering the "cardiac progenitor cell", we thought that special care should be taken, because what we wanted was to select cells that could differentiate into the desired cardiac cell type, namely ventricular cardiomyocytes, without unwanted differentiation events. Cells that are not ventricular cardiomyocytes have a high probability to cause arrhythmogenic events when transplanted into a ventricular region, in eventual clinical studies. The ventricular muscle cell may be considered as a working cardiomyocyte, which in the present context, is the most relevant cell because the main need of a patient with chronic cardiac dysfunction is to assure a reasonable left ventricular ejection fraction. This can only be obtained with regeneration of the working myocardium. Thus, the ventricular cardiomyocyte assumes a fundamental importance, in cardiac cellular therapies. Therefore, one of the main aims of the current work was to understand of the first basic steps required to obtain, and select, the best number of semi- or terminally-differentiated human working cardiomyocytes. Because the βMyhc gene is the predominant myosin isoform

expressed in the human ventricular myocardium throughout embryonic, fetal and adult life [20-22] and one of the first contractile genes that are expressed during embryonic stem cell differentiation into cardiac muscle in mice [23, 24], a ventricle muscle progenitor cell has a high, if not total, probability of expressing this gene. Hence, the choice of the βMyhc gene promoter for this study. There was, however a disadvantage in using this gene for it. The βMyhc is also the major isoform found in normal slow twitch skeletal muscle throughout development [25, 26]. So, apparently, skeletal muscle progenitor cells could also be selected. To our advantage was the fact that such problem had not been reported in studies that have used a similar strategy, in mice [19]. Furthermore, studies with mESCs suggested that it did not seem reasonable that a cell culture medium that induced mESCs to differentiate into cardiomyocytes would also induce differentiation of skeletal muscle [27, 28]. Additionally, *in vivo*, embryonic cardiac βMyhc is the first predominant myosin heavy chain gene to be expressed, followed by its later expression in the somites and slow fibers of skeletal muscle [21, 29]. Taken together, these results showed that the existence of different βMyhc transcriptional regulation programmes between cardiac and skeletal muscle progenitors is an highly probable hypothesis. This would explain the different βMyhc transcription in these two kinds of muscle progenitor cells when in the same culture conditions. In fact, the members of the helix-loop-helix myogenic lineage determining factors, MYOD-related proteins, which act as transcriptional regulators for almost all skeletal muscle-specific genes analysed to date, are notably absent from cardiac tissue at any stage of development [30, 31]. Thus, one might expect that divergent transcriptional programs regulate the expression of sarcomeric genes, as the βMyhc, in cardiac and skeletal muscle.

Nevertheless, each experiment should be considered in a separate analysis and whether embryonic, embryonal carcinoma, fetal or adult undifferentiated cells are being used, this question should not be underestimated until more data becomes available.

Detailed studies of the mouse [23, 29, 32-34], rat [22] and human βMyhc gene promoter [20, 22, 35], described several important regions involved in the transcriptional regulation of this gene. They may be divided in three main regulatory regions, the basal promoter and the proximal and the distal enhancer elements. It was possible to identify in the βMyhc basal promoter and proximal enhancer several protein-biding sites, using the DNaseI protection assay [22]. They are, numbered relatively to the human βMyhc transcription starting site: a TATA box (-30/-35), six DNaseI protected regions called βe6 (-74/-90), βe5 (-102/-134), βe4 (-148/-167), βe3 (-186/-208), βe2 (-279/-296) and βe1 (-311/-341), a C-rich region

(-233/-241) between βe3 and βe2 (which was not footprinted by DNaseI) and a classical E-box (-476/-481).

The basal promoter is constituted by the TATA box and by DNA regions βe6 to βe4 and was suggested to account for very low levels of muscle-specific gene expression. The proximal enhancer element includes βe3, C-rich regulatory sequence, βe2 and βe1 and appears to be responsible for high levels of gene expression in muscle cells. In addition, lying 5' of this proximal enhancer is the classical E-box.

The activity of the intact proximal enhancer exceeds the sum of the activities of its components, namely, βe2, C-rich regulatory sequence and βe3. This showed that the cooperative interaction between these subelements was required for maximal enhancer activity in muscle cells. Unexpectedly, the βe1 region did not contribute to the activity of the proximal enhancer in those cells.

βe2 was suggested to be a strong positive element that was required for high levels of expression of the human βMyhc gene in fetal rat cardiac muscle cell. It was shown to bind to βF1, a protein present in nuclear extracts of rabbit hearts and related to the M-CAT factor which controls muscle-specific expression of the cTnT gene [36]. It is worth notice that, it contains a binding site for the Transcriptional Enhancer Factor-1 (TEF1), shown to be important for gene expression, induced by adrenergic stimuli during the hypertrophic response [37, 38]. In addition to βe2, also the βe3 subelement, is sufficient to maintain significant levels of muscle-specific expression in myocytes. One may say that βe2 and βe3 subelements, by themselves, confer skeletal and cardiac muscle-specific activity. Together, they account for maximal activity of the βMyhc proximal enhancer in skeletal myocytes. However, in cardiac but not skeletal muscle cells, the C-rich motif element was also required for full activity. Because the proximal enhancer activity is reduced to similar levels when the C-rich motif, βe2 or both together were mutated or deleted, it was suggested that the C-rich motif may interact cooperatively with βe2 in cardiomyocytes. Additionally, deletion of βe2, C-rich regulatory sequence or βe3, did not permit expression of βMyhc in HeLa cells above the background level. Hence, all these three subregions of the proximal enhancer were considered to be positive muscle-specific regulatory elements. Interestingly, the level in which they may positively activate gene expression varies among species. Full activation of the human βMyhc proximal enhancer element seems to require adjacent sequences containing a potential stem-loop structure. The sequence of this distal human element is conserved in the 5'-flanking sequences of the rabbit and rat βMyhc genes but is not found in the αMyhc genes

of these species. A 5.6 kb upstream region that directs position independent and copy number dependent high levels of gene expression was suggested to reveal a Locus Control Region (LCR) of the βMyhc gene and to constitute the referred distal enhancer element. An LCR is a regulatory element distinct from classical enhancers in its ability to confer position-independent regulation of gene expression of linked genes [39]. Constructs containing 5.6 kb of upstream sequence to the transcriptional start site and linked to a reporter gene encoding a chloramphenicol acetyltranferase are expressed, throughout early development, similarly to the endogenous gene in skeletal myogenesis but not in the heart, when using an *in vivo* transgenic approach. This indicates that complete cardiac-specific regulation of the βMyhc gene expression, during development, involves elements outside the 5.6 kb fragment. This concept is further supported by the findings performed with the upstream region of the βMyhc gene (-5518/-2490) linked to the luciferase reporter gene and transfected to mESCs before they were induced to differentiate to cardiomyocytes. It was found that this region directed high levels of transcriptional activity during cardiogenesis, *in vitro*, but only when the construct was stably integrated into DNA, and not when expressed extrachromosomally in transient assays. This indicated that chromosomal integration was necessary for appropriate function of the βMyhc distal enhancer element in the regulation of βMyhc gene expression [23].

In resume, the basal promoter and the proximal and distal enhancer elements can drive individually, muscle-specific gene expression in both skeletal and cardiac environments with the basal promoter being the weakest inducer and the distal promoter the strongest one. However, it is important to note that, while these cis-acting regions may explain all the regulation of the βMyhc gene expression during skeletal myogenesis with regard to cardiac differentiation, there are still other cis-acting elements waiting to be found.

In our experiments we have designed a 3.3 kb upstream sequence to the transcriptional start site of the human βMyhc gene, carrying the basal promoter and the complete proximal enhancer but not the distal enhancer element, intended to control the expression of the Neo^R gene. Linked 3’ from these elements is the Hyg^R controlled by the HSV-TK promoter.

In this way, we have the possibility to study Neo^R expression in a skeletal and/or cardiac environment without the need of stably construct integration into DNA. If we remember that the goal of our construct was to select transiently the maximal number of βMyhc expressing cells among undifferentiated and cardiac committed cells, in a cardiogenic environment, the need of no integration is, in our opinion, an advantage in the overall efficiency of the method.

On the other hand, the possibility of testing the functionality of our construct in a human skeletal undifferentiated myogenic cell line was an important advantage because no human cardiac cell lines are available. Additionally, the construct carries important positive cis-acting elements in skeletal myogenesis. Among these is the classical E-box, known to bind important trans-acting myogenic HLH factors as MYOGENIN, which may facilitate its expression in skeletal muscle cells.

We have used the rhabdomyosarcoma cell line RH30 in the transfection experiments.

These cells are known to fail to complete the skeletal muscle differentiation program, express high levels of MYOGENIN, the muscle promoting factor MYOD and the MYOD repressor MYOSTATIN and the PAX3/FKHR fusion protein [40-46]. Because, to our knowledge, the expression of the βMyhc in this cell line had never been documented in the literature, we tested by immunofluorescence wether βMyHC positive cells were present in the RH30 cell cultures. Indeed, some positive cells could be found. However, no amplification of the gene's RNA could be found by RT-PCR. We believe this was due to the fact that the 5' UTR might be somewhat different between skeletal and cardiac βMyhc cDNA, and consequently the unexpected results, may result from the inability of the 5' primer for the 5' UTR of the cardiac βMyhc cDNA, to efficiently anneal with the skeletal βMyhc cDNA. There is some controversy regarding the expression of myosin heavy chain isoforms in rhabdomyosarcomas. Some authors detected the slow isoforms expression in their samples [47-50] while others do not [51, 52]. This is possibly due to the different gene expression signatures presented by the different rhabdomyosarcoma subtypes [53].

Despite the knowledge of high levels of βMyhc gene expression in human left ventricle and in some skeletal muscles, and contrarily to what happens in mice, there were no human cell lines known to express this gene. Therefore, the finding that RH30 expressed βMyhc was very important because it made our preliminary validation experiments possible. Obviously this does not exclude the need for further studies, with other cell lines that express higher levels of βMyhc, allowing the revalidation of the method.

The results from the transfection experiments showed strong RT+PCR gel bands for the Neo^R and Hyg^R in the transfected cells, but not in control cells, whereas βActin bands were found in all cell samples (transfected and not transfected). Taken together these results suggest that our construct was functional.

In order to confirm the molecular biology data immunofluorescence analysis was also performed. Although with a very low frequency, hygromycin and geneticin resistant cells,

coud be detected. We estimate that just one cell in 10^5 cells were identifiable, by immunofluorescence, as a geneticin resistant cell, although the RT+PCR results suggested a higher frequency of positive cells.

It is our belief that a molecule such as pβMyhc-Neo^R-Hyg^R will be of great help in overcoming the problems mentioned in the beginning of this chapter, not only in a direct approach, but also, in conjunction with a proteomic research. Furthermore, this method might help to disclose the protein markers of cardiac progenitor cells. Such progress would make the selection of cells for clinical applications possible, giving rise to higher yields of regeneration.

Our work showed that it is possible to select cells that express the cardiac myosin heavy chain gene, among undifferentiated cells. This may open a door for finding the true left ventricle progenitor cell in future studies where cardiac-committed embryonic stem cells are used.

Because we did not have the possibility to work with hESCs, we have tried to create a human cellular model of cardiac differentiation based in other cell types, as primary cultures of undifferentiated pluripotent human Wharton's jelly cells and teratocarcinoma cells. Another possibility that we have experimented was to develop primary cultures of human myocardium. However, we soon verified that such a goal was almost impossible to achieve *in vitro*, mainly due to the high level of cell death in the cultures.

Because no cardiac cell phenotype was obtained with human myocardium or with umbilical cord Wharton's jelly primary cultures, and because these kinds of cultures depend upon the availability of human samples, which sometimes may be time consuming, we decided to continue with the human undifferentiated embryonal carcinoma cell lines PA1 and NT2/D1 in Inductive Medium (IM).

Teratocarcinomas are highly malignant tumour, cells that have a big potential to differentiate into other cell types. They are considered to recapitulate many events occurring in early embryogenesis, but with a lesser degree of organization and regulation, being also called embryonal carcinomas (EC) [54-58]. The "undifferentiated elements" of these tumors are composed of EC cells or malignant pluripotent stem cells that, in culture, appear as embryoid body-like structures. After differentiation, they can also be histologically positive for many somatic tissues such as bone, muscle, nerve and others, these constituting the "differentiated elements" of these tumors or the teratoma components [59].

After preliminary studies with these two cell lines we chose to proceed only with the NT2/D1 cell line. Because: firstly, with the logical saving of time and money we could have the possibility to perform other, in our opinion, indispensable studies as gene expression

analysis and histochemical techniques that otherwise would have been impracticable. Secondly, our preference regarding this cell line was obvious influenced by the accumulated experimental evidence that made us believe that NT2/D1 was the teratocarcinoma cell line that had the most probability of differentiating to cardiac muscle. Namely, the similarities of NT2/D1 cells with the mouse P19 teratocarcinoma cell line and its characteristic expression of CD133, Nestin and Cripto, all compatible "signals" with a hypothetical cardiomyogenic potential (Remember *Introduction - Cardiomyogenic cultures*). This kind of information was not available for the PA1 cells. The experiments were organized in a way that no cell passages were needed, since had been seen that NT2/D1 lost its ability to differentiate after cell passage when cultured in inductive medium. The NT2/D1 cell line is a EC cell line that does not require any feeder layer to preserve its undifferentiated potential and it may form embryoid body-like structures *in vitro*, a feature also observed in other EC tumours [60-62]. The presence of some differentiating agents as RA, in the culture medium, seems to activate the neuroectodermal differentiating program [63], where the NT2/D1 cells give rise to neural cells but also nonneural cells. However, even without this agent, NT2/D1 can originate some neuronal-like phenotypes [64].

When NT2/D1 cells give rise to neurons, less than 10 % of the cells adopt this phenotype whereas more than 90 % of the cells, considered the nonneural populations, remain to be further analyzed and identified. Thus, the hypothesis of mesodermal derivatives from NT2/D1 cannot be excluded [63, 65, 66]. Nonneural cells were suggested to be primarily epithelial in nature, by immunocytochemical staining, but the exactly identity of such cells has never been documented [64]. It is suggested that this cell line although expressing mesodermal genes, does not adopt a mesodermal differentiation program [66]. Most authors agree that this cell line does not give rise to mesodermal derivatives; however, opposite evidence has been recently documented in a BMP2 induced differentiation [67].

Much of what is currently known about cardiomyocyte differentiation *in vitro* has been learned from studies on mESCs and subsequently applied to hESC studies. Using the same line of thought, we speculate that the mouse EC cell line P19 as a particular relevant model of cardiomyocyte differentiation model could have in the EC line NT2/D1 its human counterpart.

It is generally accepted that CD133 is a stem/progenitor cell marker [68-71]. Interestingly, fetal liver CD133^{+} cells have successfully differentiated into endothelial and muscle cells (skeletal and cardiac) in a defined angiomyogenic medium [72]. When coexpressed with Nestin, CD133 is a relevant human neural stem/progenitor cell marker [73-75]. Mouse and

human neural stem cells have the capability to differentiate among non-neurogenic lineages including the myogenic lineage itself [76, 77] and in mice those cells can give rise to cardiomyocytes [78]. Curiously, NT2/D1 cells express CD133 and Nestin and may be regarded as neural progenitor cells as well [65, 68, 79].

One of the genes highly expressed in P19, that disappears after the differentiation is finished, is *Cripto* [24]. This gene codes for a small cysteine-rich protein that contains an epidermal growth factor (EGF)-like motif and a CRIPTO/FRL1/Cryptic (CFC) motif. CRIPTO is anchored in the cell membrane by a glycosyl-phosphatidylinositol linkage where it functions as a coreceptor for Nodal, a member of the TGF-β family [80]. It can be detected in the trophoblast and in the inner cell mass of mouse blastocysts, becoming, thereafter restricted to developing myocardium. Cripto knockout is lethal in mouse and its inactivation *in vivo* results in the loss of detection of some cardiac transcripts, such as, αMyhc, βMyhc, Mlc2a, Mlc2v and Anp [81]. In ESCs, its loss does not affect cell commitment to the mesenchymal, endodermal or ectodermal lineage, but results also in the absence of transcription of cardiac specific genes. For all this, it has been recently suggested that Cripto is a master gene regulator in cardiomyogenesis. The mouse embryonal carcinoma P19 cell line, which, as mentioned above, has a high cardiomyogenic potential, expresses Cripto only in an undifferentiated state and the H19 gene only after the induction of differentiation to cardiomyocytes [24]. Similarly, NT2/D1 cells, express high levels of Cripto when in an undifferentiated state[82, 83] and the H19 gene [84, 85] when differentiated.

Taken together these results have made us to believe in the NT2/D1 cell culture system as an excellent candidate to give a cardiac differentiated phenotype in an angiomyogenic medium. Furthermore, finding a human embryonic cell line that could differentiate, with high frequency, into myocardium and to identify the factor(s) that would lead the cells through that path, would be a powerful tool to study cardiomyogenesis, *in vitro*.

Here, we have studied the cell morphology and the transcription profile of the NT2/D1 cells cultured for several days in fibronectin or gelatin coated plates, in two different cell culture media with and without BMP2 or other conditioning factors. The gene expression analyzed was particularly focused on EC, endothelial, neural progenitors and early and late cardiac differentiation genes. Importantly, an angiomyogenic medium was tested as differentiation culture medium.

Recent experiments in chick, with retrovirus-mediated lineage tagging have suggested that, if there is a common precursor for both myocardial and endocardial cells, then it might be committed to a endocardial and myocardial fate, at or before initial primitive streak formation

[86]. Conversely, data supporting the existence of a "later" common progenitor comes from studies with the cardiac mesoderm cell line QCE6 [87]. This cell line was derived from precardiac mesoderm of the Japanese quail and exhibits a phenotype consistent with a cardiac stem cell. If treated with a combination of RA, bFGF, TGFβ2, and TGFβ3, the QCE6 cells will differentiate into two distinct phenotypes, a myocardial and an endocardial endothelial within the same culture. In accordance with these results, two potential models were formulated [86]. One assumed that mesodermal cells reaching at the heart field were equipotent but their fate into either myocyte or endocardial lineage was possibly defined by local inductive signals from underlying endoderm. Another model presumed that the heart field consisted of two subpopulations already restricted to myocardial or endocardial lineage before migration to the heart field. If these two lineages were already separated when their progenitors migrate to the heart field, the role of endoderm-derived factors would be to continue their terminal differentiation, rather than to act as inductive signals. These models regarding the existence, or not, of a common endocardial/myocardial progenitor cell had some influence on the design of the differentiation studies presented in this thesis. Accordingly, NT2/D1 was used with an already verified angiomyogenic medium [72] in order to obtain cardiac muscle and/or endothelial cell phenotypes.

Despite the differences between human and mouse EC, at the level of cell surface markers, they share some common characteristics, such as, the morphological appearance with little cytoplasm and prominent nucleoli, the growth pattern with preferential clustering and the potential to originate neuroectodermal derivatives in the presence of RA. Together with the other similarities mentioned above one is tempted to believe that the NT2/D1 is the human counterpart of the mouse teratocarcinoma cell line, P19. However, in the presence of RA P19 but not NT2/D1 can still give rise to some mesodermal derivatives, such as smooth muscle, skeletal muscle and cardiac muscle [88, 89]. The latter two cell types, can also be induced by dimethylsulphoxide (DMSO) [90]. and the cardiac differentiation inhibited by noggin [91], a BMP2 antagonist, indicating that probably, this growth factor is essential in this inductive process. BMP2, belongs to the transforming growth factor superfamily and is involved in many important processes in early human and mouse development [92-98] and have a variety of effects in human and mouse teratocarcinomas. It can induce epithelial differentiation in NT2/D1 [99] and endodermal differentiation in GCT27X-1 EC and human ESCs [93, 100]. In P19 cells it can also give rise to cardiomyocytes [91].

Contrarily to human ESCs, treatment of mouse ESCs with BMP2 and/or FGF2 efficiently enhanced cardiomyogenesis [101].

Because of the above-mentioned effects, BMP2 has been considered to be an inhibitor of the neural differentiation program and an inducer of embryonic ectoderm [64], of epidermal extra-embryonic endoderm in human pluripotent stem cells [93], and of mesoderm in mouse pluripotent stem cells [102]. Only recently, has the latter inductive role been documented in humans [67].

We also believe that BMP2 can indirectly induce NT2/D1 cells into mesodermal derivatives like cardiac muscle, because: 1) observations from amphibians, chicks and more recently from mice, have proposed that the factors produced by embryonic endoderm are good candidates to be cardiogenic inducers [103]; 2) BMPs, as BMP2, have a role in visceral endoderm differentiation in the mouse [104] and in the human ESCs [105]; 3) the mouse P19CL6noggin EC cell line that overexpresses the BMP2 antagonist noggin and has lost it potential to differentiate into cardiomyocytes, can regain that property when in the presence of excess BMP2 protein [91]; 4) the mouse visceral endoderm-like cell line END2, derived from P19 EC, when cocultured with the parental cells [106] or mouse ESCs [27] induces spontaneous aggregation and differentiation into cardiac muscle, in the absence of BMP2. The same effect could be obtained with END2 conditioned medium [27]. Curiously, in humans one can also obtain cardiomyocytes by coculturing human ESCs with mouse END2 [16, 27]. The latter findings indicate that the visceral endoderm-like cells and not the BMP2, are the primary key for myocardial differentiation. Apparently, END2 cells produce one or more factors that promote cardiac differentiation.

Hence, one can say that the main potential of P19 cells to spontaneously differentiate into cardiac derivatives may come from the factors produced by the cells that have undergone visceral endoderm-like differentiation, thus inducing other cells to the mesodermal lineage. Therefore, we theorize that when the BMP2 is present it may contribute indirectly to cardiomyogenesis by increasing the number of visceral endoderm like cells in culture.

Consequently, if BMP2 could inhibit the neural fate and induce the mesodermal and the visceral endoderm-like phenotype in NT2/D1 cells, it would be possibly to induce some cells to the cardiac lineage.

Consistent with this assumption were the good levels of CRIPTO expression, in the NT2/D1 cell line. Furthermore, the kinetics of expression of CRIPTO transcripts showed a reduction in intensity of the bands in later time periods while H19 transcripts became more intense indicating that NT2/D1 were differentiating, at least regarding this genes, in a similar manner as their mouse counterpart P19. In addition, transcripts for the cardiomyogenic (Vlc1, Vlc2, Mef2d, Gata4 and Csx1) and endothelial (kdr and CD133) mesodermal lineages, bone

morphogenetic protein receptors (BmprIa, BmprIb and BmprII) and NT2/D1 specific, were detected, in a qualitative and constitutive manner during the time course of the experiment.

However, morphological signals of muscle differentiation as myotube formation and sarcomeric intracellular actin organization were not detected in our experiments, as we did not see any muscle beating areas at any the time point of the experiment. This might indicate that either the NT2/D1 are not committed to mesodermal lineages or that the culture conditions we did no meet the necessary requirements for the cells to differentiate into that lineages. A third alternative would be that the apparent blockage of the differentiation pathways through a mesodermal commitment, *in vitro*, would be due to yet undefined pos-transcriptional or pos-translational mechanisms. Finally, it is possible that the aneuploid state of NT2/D1 cells inhibits the key differentiating genes, and what once was an advantage, regarding the tumor clonal selection of differentiation-resistant cells, is now a relevant obstacle in differentiation studies like this one.

Because of the pattern of gene expression and because, in some cultures, we observed some angiogenic like structures, we believe that our initial premise might be correct, but further studies will be needed to confirm this assumption.

Identifying the factors capable of regulating differentiation of pluripotent human EC cell lines, will give us the clues how to manipulate human stem cells, an important raw material for *in vitro* generation of tissues for transplantation therapy.

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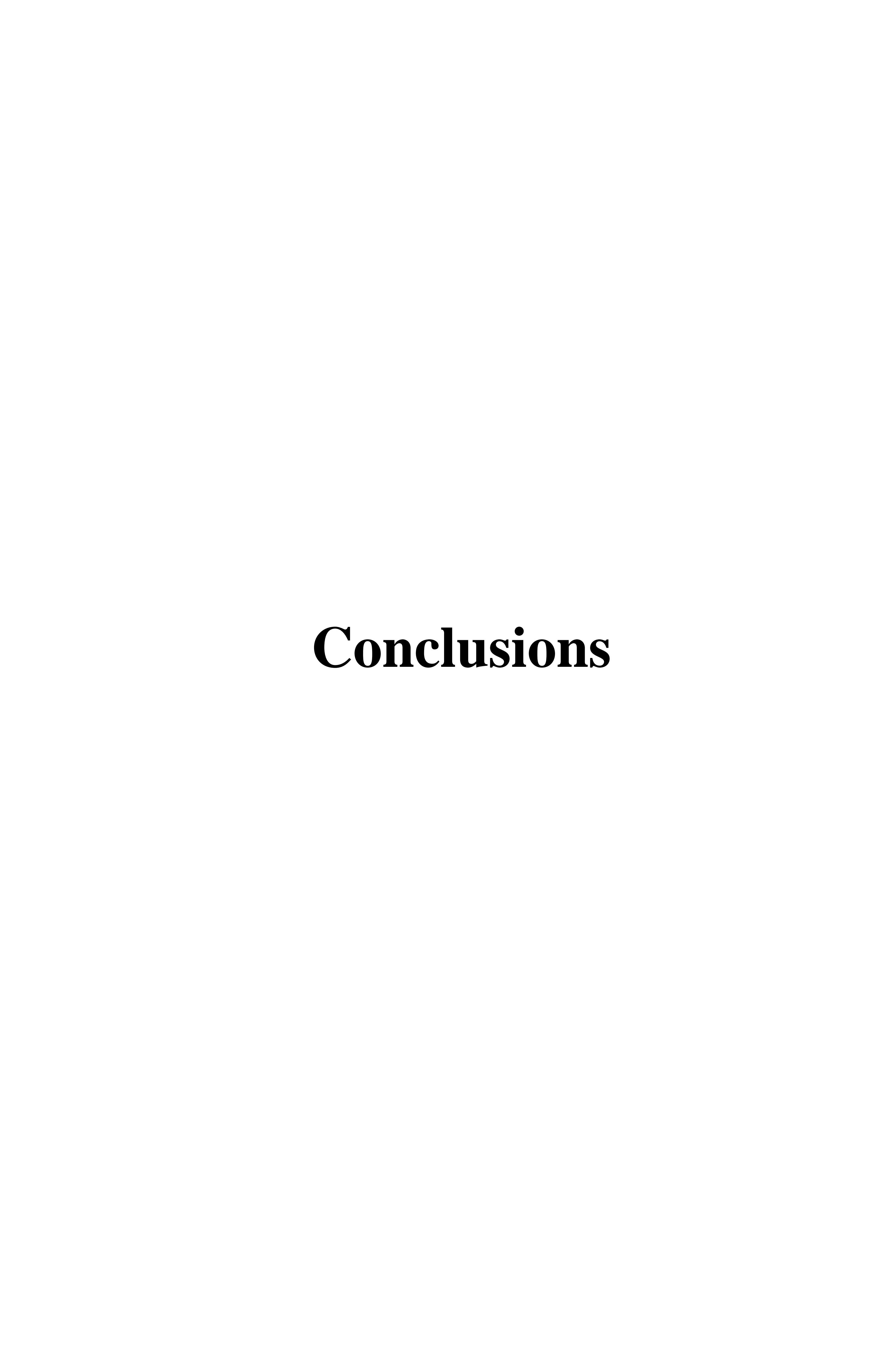

Conclusions

Conclusions

- The RH30 cells express the MYH7 gene. To our knowledge, this is the first time that such expression is reported. It is possible to identify MYH7 expressing cells among the adherent RH30 cell population, although with a low frequency of differentiation.
- The pβMyhc-Neo^R-Hyg^R construct is functional in RH30 cells;

However, more studies are necessary in other cell lines to recheck the functionality of this method.

- NT2/D1 cells differentiate spontaneously or in the presence of inductive media towards an epithelioid morphology, as is referred in the literature and with vilosities and tight junctions, with similarities to a glandular cell. In a less extent neural and fibroblastoid morphologies can also be detected;
- Despite the presence of desmosomes in some TEM images, a muscular morphology was never observed;
- There was no clear effect of 5-Azacytidine and of BMP2 in NT2/D1 typical phenotype;
- In the presence of IAM+BMP2, NT2/D1 cells create structures with some similarity with an angiogenic network;
- In general all the genes analysed are expressed in NT2/D1 with the exception of the MYH7 gene that was never detected;
- The Cripto has tendency to decrease and the H19 has tendency to increase during growth in IAM, indicating that NT2/D1 cells differentiate, at least, in some extent similarly to the P19 cells;
- NT2 cells express genes from muscular (Vlc1, Vlc2, Mef2d, Gata4, Csx1 and BmprIa) and endothelial lineages (CD133 and Kdr), but a morphological positive analysis of such derivatives still have to be detected;
- The inhibition of mesodermal phenotypes may consist in post transcriptional or post translational mechanisms.

Future Studies

Future Studies

1. Looking to the importance of having a high level of reproducible MYH7 expressing cells, a commercially available, such human cell line, is required.

To immortalize a human primary culture of slow skeletal muscle or human myocardium it would be an interesting future work.

2. Human umbilical cord Wharton's jelly is in our opinion an extraordinarily pertinent autologous tissue (as the umbilical cord blood) that presents several undifferentiated pluripotential cells not yet fully explored. Primary cultures of these cells in the IAM would be an interesting next step.

3. Given the similarity between the NT2 cells and the P19 cells, a conclusive explanation regarding the open of the mesodermal differentiation in the P19 cells and the closure in the NT2 cells has not been found yet. So, other culture conditions with for instance END2 conditioned medium would be relevant.

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Publications that resulted from this work

Simões PD, Ramos, T*, Construction of an aminoglycoside phosphotransferase resistance gene under the control of the human beta myosin heavy chain promoter*, Biomolecular Engineering, 2007, *Submitted*;

Simões PD, Ramos, T*, Human pluripotent embryonal carcinoma NTERA2 cl.D1 cells maintain their typical morphology in an angiomyogenic medium,* The Journal of Negative Results in Biomedicine, 2007, 6:5 (18 April 2007).

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