

**CHARACTERISATION OF CHAMBER-SPECIFIC
FIBROBLASTS FROM ADULT HUMAN HEART AND
INVESTIGATION OF THEIR POTENTIAL CONTRIBUTION
TO THE FUNCTION OF ENGINEERED HEART TISSUE**

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I certify that this dissertation and the research to which it refers, are the product of my work, conducted during the year (2015, 2016) in the Clinical Medical Research at Imperial College London and Heart Science Centre at Harefield Hospital. All work, observation, and findings presented is my work. Any ideas or quotation from the work of other people, published or otherwise, data from ongoing parallel projects in the institute, or from my previous work are fully acknowledged in accordance with the standard referencing practices of the discipline.

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For the pilot data, the human myocardial samples were collected from Imperial College Healthcare NHS Trust with JRCO-Imperial approval number R14011. The human ventricular samples were taken from the native heart of the transplant recipients. This study was supported by the supply of human tissue samples from the Cardiovascular Disease Biomedical Research Unit Biobank at the Royal Brompton and Harefield NHS Foundation Trust (NRES ethics number for biobank samples: 09/H0504/104+5; Biobank approval number: SH001-01-2013). Informed consent was obtained from each patient involved in this study. The Ethics Approval for this project was granted by NRES committee London – Harrow. (Reference no. 15/LO/0098) The IRAS Project ID number of this study is 164456. The human cardiac biopsy samples used in main research were obtained under the title of DISCOVER III from Imperial College Healthcare NHS Trust with JRCO-Imperial approval number 14HH2440. The project was funded by BHF and the Clinical Research Training Fellowship number was FS/15/79/31736 (CRM: 00001126).

DISCLAIMER

All human myocardial samples were taken from the patients undergoing cardiac surgical procedures following an informed and signed consent. A minimum period of 12 hours was offered to the patients to consider their participation in the project. The patient safety and choice were prioritised over participation in the project. A signed copy of the consent form and patient information sheet was offered to all the participants.

In a few instances, although the patient was consented for participation in the study but based on the intra-operative circumstances, the myocardial biopsies were not taken. The patient was informed about it, and it was documented in the clinical notes.

None of the biopsy samples taken during the study incurred additional harm to the patient under any circumstances and had not led to any untoward events. The patient who refused to participate in the study were not discriminated for any pre-operative and post-operative care package.

PUBLICATIONS

PAPERS IN PREPARATION

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ABSTRACT

Research in the past 15 years has shown that the adult heart, including human, contains cardiac fibroblasts exhibiting regenerative capacity. Little is known about the precise characteristics, properties, and differentiation potential of endogenous cardiac fibroblasts (CF) in the four cardiac chambers of the human heart, and how these are affected by the disease process. The fibroblast population in the heart is being redefined from an inert scar-forming cell type into one, which has an active role in terms of paracrine effects and cardiomyocyte coupling. Fibroblast characterisation shows overlap with the variously described mesenchymal stem cell populations, and these cells can be reprogrammed directly to cardiomyocytes. 3D engineered heart tissue (EHT) is made using cardiomyocytes differentiated from human induced pluripotent stem cells (hiPSC-CM). The addition of human foetal cardiac fibroblasts to the EHTs provided structural support, enhanced force output, improved calcium handling and induced expression of more mature sarcomeric proteins.

In this thesis, for the first time the human CFs from all four chambers in patients with preserved biventricular function are investigated. A comparison is made between all four chambers and between left and right side of the heart. Fibroblasts identified by a range of markers, are characterised by growth pattern, rate of proliferative, secreted factors and expression of cardiogenic transcription factors. Moreover, the effect of chamber-specific fibroblasts on the function and gene expression of the iPSC-CM in EHT is studied. Finally, for the first time the secretory profile of EHT constructs is elucidated by the proteomic analysis of the medium bathing EHTs. This project aims to investigate the mechanism of this effect regarding cell coupling and paracrine effectors.

The in-vitro growth of a truly defined human all four-chamber cardiac fibroblast population depicted that atrial fibroblasts have a better rate of proliferation, growth pattern and lesser predilection for myofibroblastic transformation as compared to ventricular fibroblasts. The transcription factors expression of atrial fibroblasts is different from ventricular fibroblasts hence pointing towards their different embryological origin. The co-expression of chamber-specific fibroblasts in EHTs with cardiomyocytes of same origin showed that right ventricular fibroblasts based EHTs had the best kinetics. Finally, the proteomic expression of EHTs is mainly dependent on presence of cardiomyocytes. The right ventricular fibroblasts based EHTs showed least amount of proteins expressed in conjunction with best kinetics eluding to the fact that in-vivo many proteins expressed by cardiomyocytes and non-cardiomyocytes may have an inhibitory effect.

This thesis provides an insight into phenotypic variability, transcription factor expression, impact on kinetics and protein co-expression in EHTs of chamber-specific fibroblasts with cardiomyocytes. Further investigations into the role of chamber-specific fibroblasts in EHTs, in patients with impaired ventricular function can be explored subsequently.

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LIST OF ABBREVIATIONS

ACE	Angiotensin Converting Enzyme
ACTS-2	Actin Alpha-2
ACTN-2	Alpha Actinin-2
ADH	Antidiuretic Hormone
ANOVA	Analysis of Variance
ANG	Angiogenin
Ang-I	Angiotensin-I
Ang-II	Angiotensin-II
ANOVA	Analysis of Variance
ANP	Atrial Natriuretic Peptide
AO/PI	Acridine orange/Propidine iodine
AT ₁ R	Angiotensin 1 Receptor
AVR	Aortic Valve Replacement
B-27	Human Leucocyte Antigen B-27
BAFF	B-cell Activating Factor
BCA	Bicinchoninic Acid
BHF	British Heart Foundation
bHLH	Basic Helix-Loop-Helix
BNDF	Brian-derived Neurotrophic Factor
BNP	Brain Natriuretic Peptide
BSA	Bovine Serum Albumin
C5/C5a	Complement Component C5/C5a
CABG	Coronary Artery Bypass Grafting
cAMP	Cyclic Adenosine Mono Phosphate
CAD	Coronary Artery Disease
CCD	Charge-Couple-Device Image
CCR-2	Chemokine Receptor Type 2
CDi	Cellular Dynamic iCell
CD-31	Cluster of Differentiation-31
CF	Cardiac Fibroblast
CFD	Complement Factor D
CH13L1	Chitinase 3-Like 1
CHD	Coronary Heart Disease

CHIR	6-[[2-[[4-(2,4-Dichlorophenyl)-5-(5-methyl-1H-imidazol-2-yl)-2-pyrimidinyl] amino] ethyl] amino] -3-pyridinecarbonitrile
CICR	Calcium-induced calcium release
CM	Cardiomyocytes
CM-only	Cardiomyocytes-only
CMs	Cardiomyocytes
CMTV	Comparative Map and Trait Viewer system
c-NP	c-Type Natriuretic Peptide
CO ₂	Carbon Dioxide
CO	Cardiac Output
COX-2	Cyclo-Oxygenase-2
CPC	Cardiac Progenitor Cell
CRP	C-Reactive Protein
CST3	Cystatin 3
CVD	Cardiovascular Disease
CuSO ₄	Copper Sulphate
DAPI	4',6-diamidino-2-phenylindole
DCM	Dilated Cardiomyopathy
DDR - 2	Discoidin Domain Receptor – Type 2
Dkk-1	Dickkopf-1
DMEM	Dulbecco Modified Eagles Medium
DMSO	Dimethyl sulfoxide
DPPIV	Dipeptidyl-peptidase IV
DTT	1,4 Dithiothreitol
EC	Endothelial cell
ECGM	Endothelial Cell Growth Medium
ECL	Enhanced chemiluminescence
ECM	Extracellular Matrix
ECM	Extracellular membrane
EDTA	Ethylenediaminetetraacetic acid
EDV	End Diastolic Volume
EF	Ejection Fraction
EGF	Epidermal Growth Factor
EHT	Engineered Heart Tissue
EHT	Engineered Heart Tissue

EHTs	Engineered Heart Tissue
ELISA	Enzyme-linked Immunosorbent Assay
EMT	Endothelial-to-Mesenchymal Transition
ENF-78	Epithelial-derived Neutrophil-activating Peptide 78
ESV	End Systolic Volume
FAP	Fibroblast Activation Protein
FB	Fibroblast
FB-only	Fibroblasts-Only
FBS	Foetal Bovine Serum
FCS	Foetal Calf Serum
FDAR	Frequency Dependent Acceleration of Relaxation
FF	Foetal fibroblasts
F-FBs	Foetal Fibroblasts
FFR	Force-Frequency Response
FGM	Fibroblast Growth Medium
FOC	Force of contraction
FS	Fractional Shortening
FSP – 1	Fibroblasts Specific Protein 1
GATA 4	Transcription Factor GATA-4
GATA 5	Transcription Factor GATA-5
GATA 6	Transcription Factor GATA-6
G-CSF	Granulocyte Colony Stimulating Factor
GDF-15	Growth Differentiation Factor 15
GFR	Glomerular Filtration Rate
GH	Growth Factor
GM-CSF	Granulocyte Monocyte Colony Stimulating Factor
HAND2	Heart and Neural Crest Derivative-expressed Protein 2
HBSS	Hank's Balanced Salt Solution
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HF	Heart Failure
HGF	Hepatic Growth Factor
hESC-CM	Human Embryonic Stem Cell Derived Cardiomyocytes
hiPSC-CM	Human Induced Pluripotent Stem Cell-derived Cardiomyocytes
HRP	Horseradish Peroxidase
HS	Horse Serum

HTN	Hypertension
ICAM-1	Intercellular Adhesion Molecule-1
ICTEM	Imperial Centre for Translational and Experimental Medicine
IRES	Integrated Research Application System
IF	Immunofluorescence
IFN γ	Interferon- γ
IGFBP-2	Insulin-like Growth Factor-Binding Protein-2
IL-1	Interleukin-1
IP-10	Interferon Gamma-induced Protein 10
iPSCs	Induced Pluripotent Stem Cells
IRAS	The Integrated Research Application System
I-TAC	T-cell Alpha Chemoattractant
JRCO	Joint Research Compliance Office
JVP	Jugular Venous Pressure
KLK3	Kallikrein 3
LA	Left Atrium
LA-FBs	Left Atrial based Fibroblasts
LDS	Lithium dodecyl sulphate
LIF	Leukaemia Inhibitory Factor
Luna FL	Luna Florescent Light
LV	Left Ventricle
LVEDP	Left Ventricular End Diastolic Pressure
LVEDV	Left Ventricular End Diastolic Volume
LVESV	Left Ventricular End Systolic Volume
LV-FBs	Left Ventricular based Fibroblasts
MAP	Mean Arteria Pressure
MCP	Monocyte Chemoattractant Protein
M-CSF	Monocyte Colony Stimulating Factor
Mef-2	Myocyte Enhancer Factor-2
MESP-1	Mesodermal Posterior 1 Homolog
MF	Myofibroblast
MHC	Myosin Heavy Chain
MI	Myocardial Infarction
MIF	Monocyte Migration Inhibitory Factor
MIG	Monokine Induced by Gamma Interferon

MIP-1	Macrophage Inflammatory Protein 1-Alpha
miR-1	Micro Ribonucleic Acid-1
miR-133	Micro Ribonucleic Acid-133
miRNA	Micro Ribonucleic Acid
MMP	Matrix Metalloproteinases
MOPS	3-(N-morpholino) Propanesulfonic Acid
MPD	Mean of pixel density
MPO	Myeloperoxidases
MS	Mass Spectrophotometry
MVR	Mitral Valve Replacement
MYO-D	Myocardin Transcription Factor
Na ⁺ /K ⁺	Sodium ion/Potassium ion
NCX	Sodium Calcium exchanger
NRES	National Research Ethics Service
NRVC	Neonatal Rat Ventricular Cardiomyocytes
O ₂	Molecular Oxygen
OCT	Optimal cutting temperature formulation
OPN	Osteopontin
P/S	Penicillin/Streptomycin
P1	Passage no.1
P2	Passage no. 2
PBS	Phosphate Buffer Saline
PBS-T	Phosphate Buffer Saline with Tween
PCR	Polymerase chain reaction
PDGFR α	Platelet Derived Growth Factor Receptor – Alpha
PE	Pro-epicardium
PF4	Platelet Activation Factor 4
PFA	Paraformaldehyde
PRP	Post-rest potentiation
PTX3	Pentraxin 3
RA	Right Atrium
RAS	Renin Angiotensin System
RAAS	Renin-Angiotensin-Aldosterone System
RA-FBs	Right Atrial based Fibroblasts
RAGE	Receptor for Advanced Glycation Endproducts

RANTES	Regulated on activation, normal T-cell expressed and secreted
RAP	Right Atrial Pressure
RBP-4	Retinol Binding Protein-4
RETN	Resistin
REC	Research and Ethics Committee
RIPA-lysis	Radio Immuno Precipitation Assay - Lysis Buffer
RLN2	Relaxin-2
ROCKi	Rho-associated, coiled-coil containing protein kinase inhibitor
POS	Reactive Oxygen Species
RA	Right Atrium
RA	Retinoic Acid
RAGE	Receptor for advanced glycation end products
RANTES	Regulated on activation, normal T-cell expressed and secreted
RBP 4	Retinol Binding Protein 4
ROCK	Rho-Associated Coiled-coil Kinases
RPM	Revolutions per minutes
RPMI	Roswell Park Memorial Institute Medium
RV	Right Ventricle
RV-FBs	Right Ventricular based Fibroblasts
RyR2	Ryanodine receptor 2
SAN	Single Nucleotide Polymorphism
SAN	Sino-atrial Node
SD	Standard Deviation
SDF-1 α	Stromal Cell-derived Factor-1 α
SERCA	Sarco/endoplasmic reticulum Ca ²⁺ -ATPase,
SEM	Standard error of Mean
SHBG	Sex Hormone-binding Globulin
SMC	Smooth Muscle Cells
SNS	Sympathetic Nervous System
SR	Sarcoplasmic Reticulum
SV	Stroke Volume
TARC	Thymus Activation Regulated Chemokine
Tcf-21	Transcription Factor 21
TBX-2	T-Box Transcription Factor-2
TBX-5	T-Box Transcription Factor-5

TBX-20	T-Box Transcription Factor-20
TFF3	Trefoil factor-3
TfR cells	Follicular Regulatory T-cells
TGF- β 1	Transforming Growth Factor-Beta 1
THY – 1	Thymocyte Antigen 1
TIM-3	T-cell Immunoglobulin and Mucin-domain Containing-3
TNF- α	Tumour Necrosing Factor Alpha
TLR-4	Toll-like Receptor 4
TNNI3	Troponin I Cardiac Muscle 3 gene
TPR	Total Peripheral Resistance
TSP-1	Thrombospondin-1
UEA	Ulex Europaeus Agglutinin I
VADs	Ventricular Assist Devices
VCAM-1	Vascular Cell Adhesion Molecule-1
VDBP	Vitamin D BP
VEGF	Vascular Endothelial Growth Factor
VF	Valvular fibroblasts
VIC	Valve Interstitial Cells
VIM gene	Vimentin gene
VLC-1	Ventricular Light Chain -1
vWF	Von Willebrand Factor
VSFP	Voltage Sensitive Fluorescent Proteins
v/v	volume/volume
w/v	weight/volume
WT-1	Wilms' Tumour 1
XL	Extra Large
α 1	Alpha-1 Receptor
α -Actinin	Alpha Cardiac Actinin
α -SMA	Alpha-smooth Muscle Actin
β -1	Beta-1 Receptor
β -2	Beta-2 Receptor
μ PAR	Urokinase-type Plasminogen Activator Receptor

CHAPTER 1: INTRODUCTION

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1.1 OVERVIEW

Heart failure is a syndrome prevalent in 1 in 125 patients presenting with Cardiovascular disease (Heart et al., 2017). HF is a progressive, symptom complex clinical syndrome secondary to any structural or functional cardiac injury that impairs the ability of the ventricle to fill with or eject blood (Hunt, 2005). It is characterised by progressively worsening shortness of breath, angina, fatigue, decrease exercise tolerance and dependent limb oedema and has a poor outcome. The estimated survival rate following diagnosis is 50% and 10% at 5 and 10 years, respectively (Ambrosy et al., 2014). The left ventricular dysfunction is associated with increased mortality as this imposes an increased risk of sudden death (Chugh et al., 2009). The cost incurred by the treatment for HF is 2% of the total budget for NHS and 5% of all the emergency hospital admissions (Polly Mitchell, Damian Marle, Akosua Donkor, Aminat Shote, Theresa McDonagh, Suzanna Hardman, Henry Dargie, John Cleland, 2013).

Almost 70% of the patients presenting with HF have ischaemic heart disease. Impaired blood supply to the myocardial tissue leads to the myocardial infarction. This irreversible damage to the myocardial tissue causes scar formation, and the thick muscular myocardial wall is replaced with thin fibrotic scar tissue. All existing medical and surgical therapies are palliatives only with a primary focus on limiting disease progression and improving the functional efficacy of the remaining myocytes, but they do not restore myocardial structural integrity and functional capacity (Rathi and Deedwania, 2012) (Deedwania and Carbajal, 2012). The only treatment modalities available to-date for end-stage heart failure are mechanical circulatory support or cardiac transplant (Akhmedov and Marín-García, 2013). Myocardial regeneration is being actively pursued in the hope that it can offer a curative treatment for post-infarct myocardial tissue and potential reversal of impaired myocardial dysfunction back to normal.

1.2 HEART FAILURE

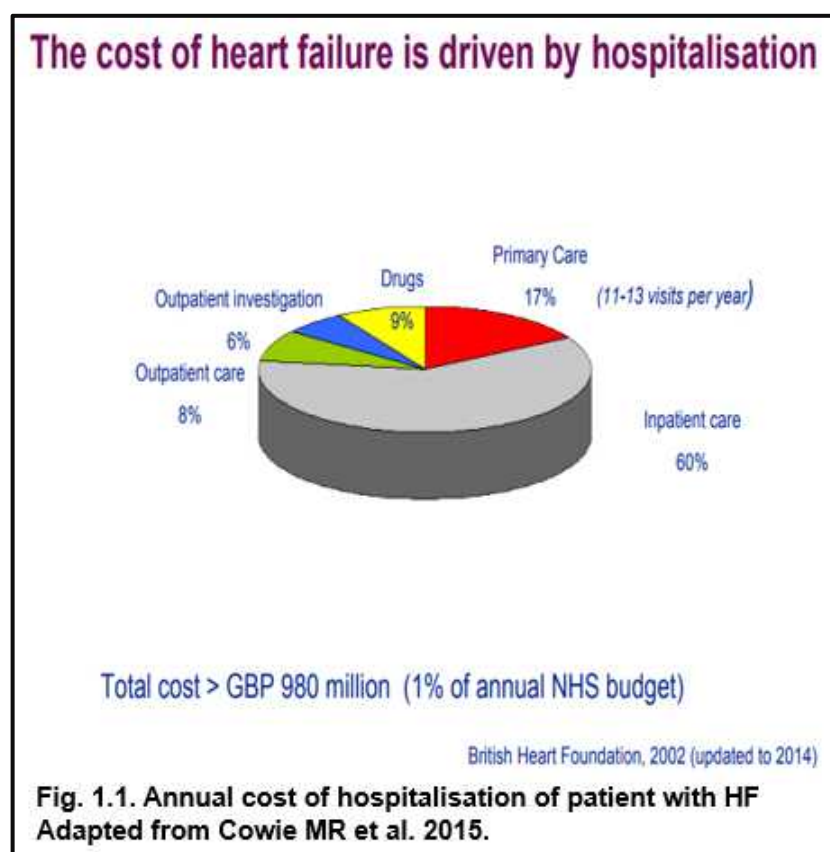
Cardiovascular diseases (CVD) covers a range of disease patterns from uncomplicated coronary artery disease to complex valvular dysfunctions. If the primary pathology is left untreated, heart failure ensues and has a poor prognosis. HF is a clinical syndrome secondary to any structural or functional cardiac injury that impairs the diastolic filling and systolic pumping action of the heart hence leading to defective functioning as a pump. It characterises a profound imbalance of otherwise finely adjusted physiologic mechanisms to maintain cardiac output, blood pressure, and fluid balance. In the absence of anatomical or functional impairment, the normal adult heart is a stable mechanical tissue pump adjusting performance to changes in loading conditions and inotropic state. The defective pumping action of the heart causes poor forward flow leading to poor tissue perfusion/oxygenation and eventually leads to multi-organ failure. The common cascade of events is impaired renal function, fluid retention, and pulmonary congestion, manifesting as progressively worsening fatigue, breathlessness and dependent oedema of the lower limbs. As the disease progresses, it causes a major adverse effect on quality of life.

Coronary artery disease (CAD) is a large part of the pathology ultimately triggering heart failure. Almost two third of the cases of heart failure are considered to be secondary to ischaemic heart disease. Due to an ageing population, better health care facilities and diagnostic tools, improved survival rate after acute coronary syndrome and more effective treatment, there is a steep global rise in both the incidence and survival of the heart failure patients.

1.3 INCIDENCE OF HEART FAILURE

Globally there are 26 million patients diagnosed to have different stages of heart failure. HF is responsible for more than 1 million hospital admission per year in Europe and united states (Ambrosy et al., 2014) and accounts for 1-4% of all hospital admissions (Cowie, 2015). Nearly two-thirds of the population in the group is affected by at least one significant comorbidity which can likely worsen the overall health status of the patient (Van Deursen VM et al. 2014). Altogether the cost incurred by heart failure is almost 1-2% of the total health care expenditures (Cowie MR et al. 2014). The prevalence of HF is anticipated to increase further because of the ageing of the population and the improved survival of those with acute MI, hypertension, and other cardiovascular conditions.

Currently, almost 0.5 million people in the UK are diagnosed to have heart failure, and half of this patient population will die within 4-5 years from the time of initial diagnosis. CHD accounts for almost 27% of all-cause mortality in the UK leading to 1.5 million deaths per year. The



latest UK patient data and cardiovascular health specific surveys reveal an average of 7 million people affected by the disease process with no gender preponderance. Nearly 2.3 million people are affected by CHD leading to 70,000 deaths per year, and most deaths are caused by an ischaemic cardiac event (Melorose, Perroy and Careas, 2015).

The incidence of HF will decrease only with the implementation of preventative and curative strategies, but until then HF will continue to persist as a major contributor to increased morbidity and mortality in cardiac patients.

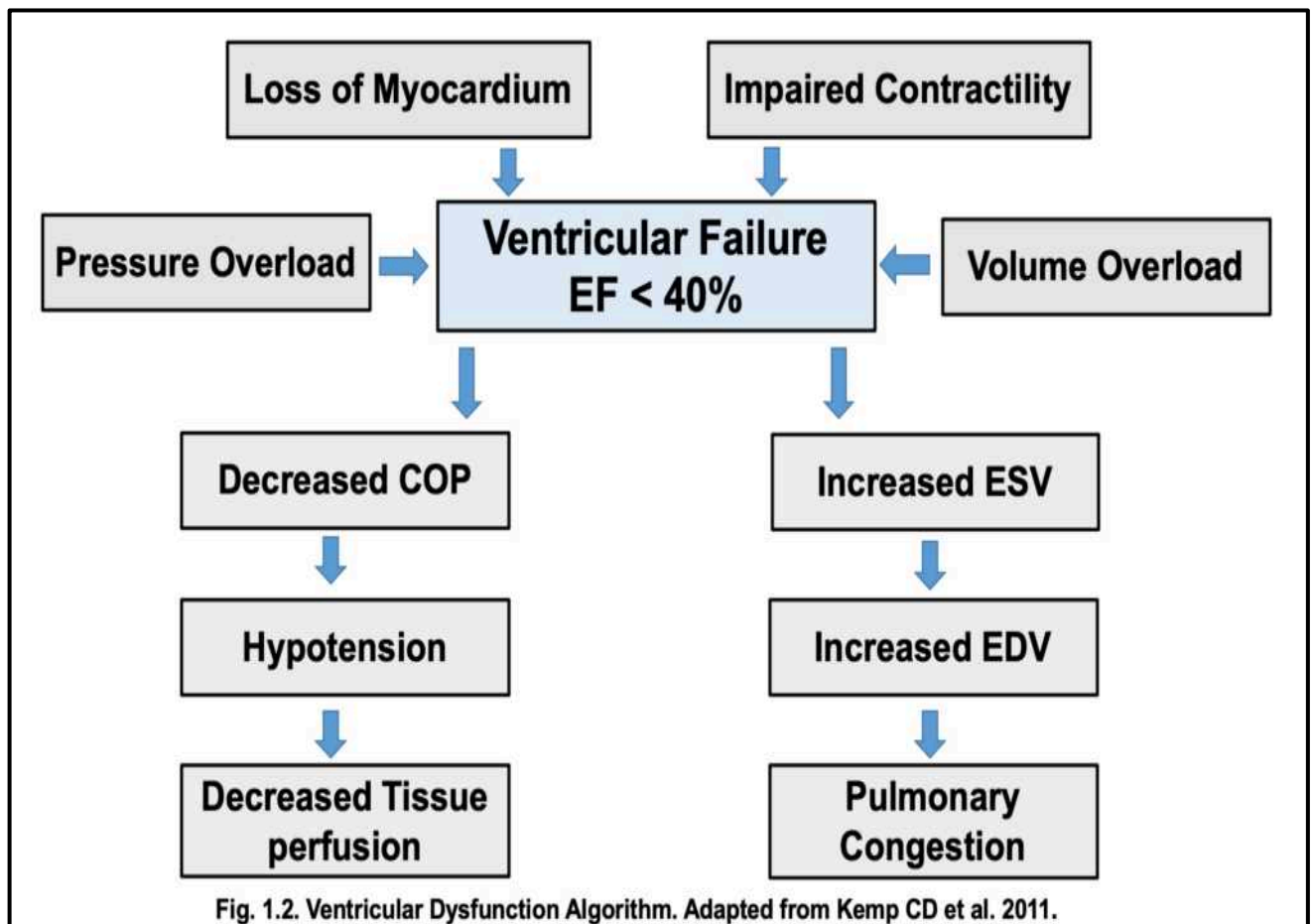
1.4 PATHOPHYSIOLOGY OF HEART FAILURE

Acute myocardial infarctions interrupt the blood supply to cardiomyocytes, leading to their death by necrosis and the necrotic area is replaced by a scar. Myocardial injury alters the loading and biochemical environment of both impaired and uninjured cardiac cells. As a consequence of such an injury, the heart failure can be due to left, right or biventricular failure. Left ventricular dysfunction is further divided into systolic failure (impaired systolic contraction leading to poor ejection fraction) and diastolic dysfunction (impaired relaxation leading to poor diastolic filling). The LV dysfunction is caused by ischaemic injury, uncontrolled hypertension and cardiotoxic drugs and RV dysfunction are predominantly caused by the LV impairment/failure.

Some aetiologies affect one ventricle more than the others with 70% of patients presenting with left ventricular failure and only 30% present with right heart failure. However, the patients presenting with systolic dysfunction can also have a component of diastolic dysfunction as well. Ventricular failure is defined by the reduction in the ejection fraction (EF). An EF less than 40% refers to systolic impairment and an EF more than 40% with features of heart failure represents diastolic dysfunction (Lilly LS et al. 2011).

The impaired LV function causes decreased cardiac output (CO) and hence decreased end-organ perfusion which leads to multiple organ failures. In addition to this, the raised end-diastolic volume is increased which in turn reflects as raised left ventricular end diastolic pressure (LVEDP). The increased LVEDP causes a rise in the left atrial pressure (LAP), and

this leads to pulmonary oedema and increased right-sided pressures causing pulmonary hypertension over a period.



Endocrine, paracrine (on adjacent cells), autocrine (on the same cell), or intracrine (internally within the same cell without extracellular secretion) mechanisms all can contribute to a subsequent compensatory biologic response. Mean arterial pressure (MAP) is defined as the product of CO and total peripheral resistance (TPR) and therefore directly dependent on CO. During the acute phase of HF, the CO decreases which in turn leads to the reduction in the MAP and therefore tissue perfusion is compromised. The initial response of the body is to maintain adequate tissue perfusion and attempt to bring the MAP back to normal.

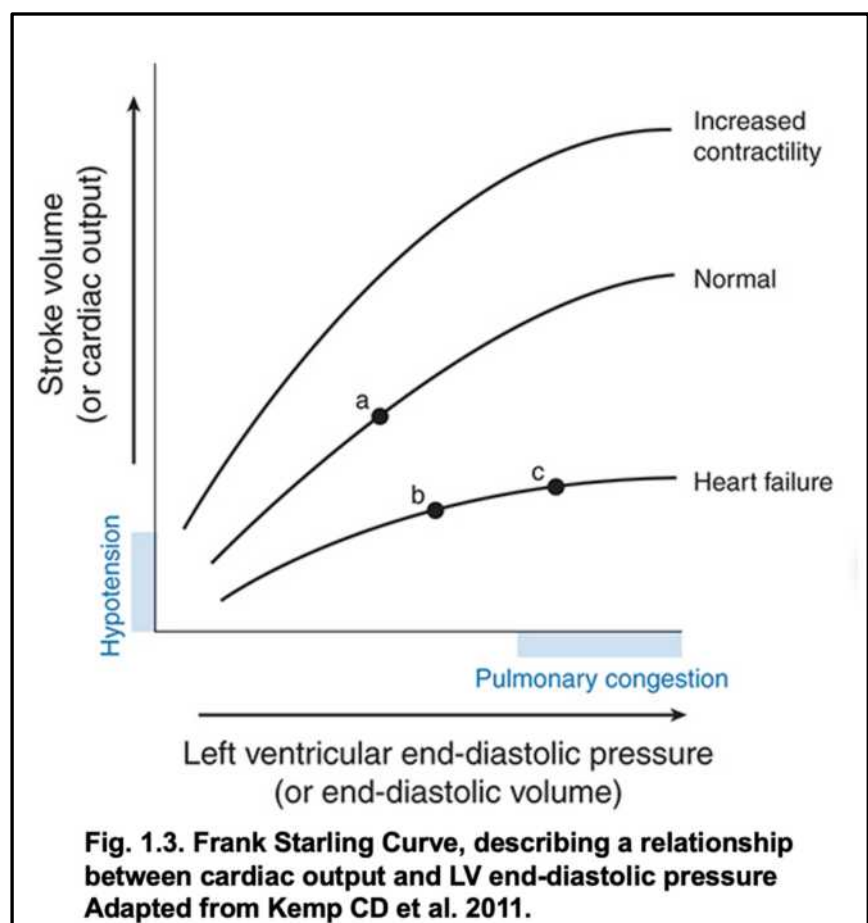
1.5 COMPENSATORY MECHANISM OF HEART FAILURE

The mechanisms, vital in this compensatory phase include the Frank-Starling mechanism, neurohormonal activation, and ventricular remodelling. Although all these mechanisms play a pivotal role in the initial compensatory phase of heart failure in due course, these mechanisms serve to worsen HF in a vicious cycle.

1.5.1 FRANK-STARLING MECHANISM

The Frank-Starling law/mechanism plays an important compensatory role in the acute stages of HF. Within the confines of elastic limit, this law correlates the force of contraction of a myocardial syncytium to the initial length of the myofibril. The smaller the initial length of myofibrils, the lesser will be the force of contraction and vice versa. The initial length of the myofibrils is dependent on preload or LVEDV which in turn increases the LVEDP and hence the CO. This graphical representation of this curve is called a Frank-Starling curve. This graph

depicts CO as a function of LVEDP which is directly related to preload/LVEDP. Point A on the curve represents a healthy patient with a preserved ventricular heart. The curve depicts a directly proportional relationship between LVEDP and CO. The raised LVEDP causes a stretch in the myocardium which responds with an increase



in CO. There is a limit to this increase in CO and beyond that limit the increase in preload or LVEDP does not increase CO rather it decreases as the curve flattens out. Points B and C on the other curve represents a patient with systolic dysfunction. The reason for this curve to be lower than that of the normal heart is because the failing heart has impaired contractile reserves and fractional shortening. As the force of contraction of the myofibril decreases, the SV falls with a resultant increase in LVEDV (preload).

Point B, which is on the ascending portion of the curve, represents the extent to which the failing heart compensates for the increase in preload by increasing the CO. In a preserved ventricular function patient, a similar increase in preload does not show the same response. The progression of HF as indicated by point C shows that beyond this point, for any given increase in LVEDV there is no effect on the CO as the curve flattens. The reason for this response is decompensation of heart muscle as all compensatory mechanisms are exhausted. Clinically, beyond this point, an increase in LVEDP has rather a detrimental effect on the outcomes as it causes pulmonary congestion.

1.5.2 NEUROHORMONAL COMPENSATORY MECHANISMS

The Neurohormonal axis activation plays a vital role in the maintenance of MAP and compensation of early stages of HF (Lee CS et al. 2008). Low MAP and CO direct the activation of this pathway. Once activated this pathway promotes vasoconstriction, sodium and water retention, which improves SV and augments CO. The drop in MAP leads to a stimulation of the sympathetic nervous system (SNS) and release of catecholamines (epinephrine and norepinephrine). This stimulation has a direct chronotropic effect on heart mediated through β_1 , β_2 receptors, and vasoconstriction to increase the preload of the heart via α_1 receptors. In HF patients, activation of both β_1 and β_2 receptors along with the α_1 receptors, eventually lead to myocardial toxicity. The effects of this cardiotoxicity are

manifested by decreased EF, arrhythmias, and tachycardia due to overstimulation by the SNS (Chaggar PS et al. 2009).

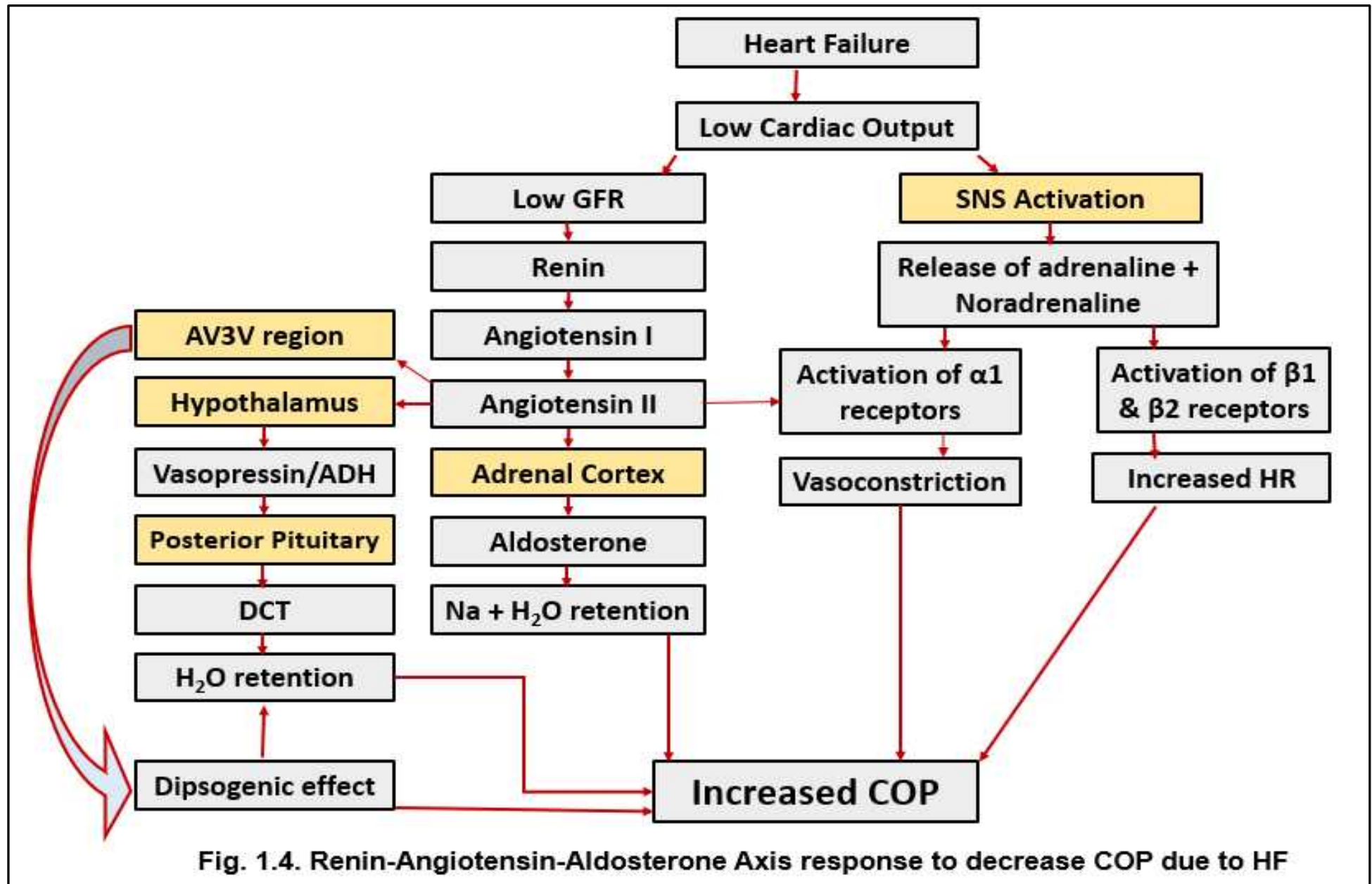
Renin, a serine protease is secreted from the juxtaglomerular apparatus from the kidneys in response to the low MAP and reduced glomerular filtration rate (GFR). In addition to this, the sympathetic activation stimulates the release of renin from the kidneys. Once activated, Renin then acts on its substrate, angiotensinogen I in the liver to convert this into its activated form, angiotensin I. Circulating angiotensin I is converted to angiotensin II by angiotensin-converting enzyme (ACE) in the lungs. Angiotensin II is a potent vasoconstrictor, which directly increases vasomotor tone in the peripheral blood vessels and promotes the release of aldosterone from zona glomerulosa. In the peripheral vasculature, activation of the α_1 and β_1 receptors leads to activation of the renin-angiotensin-aldosterone system (RAAS) which causes vasoconstriction, sodium retention, a positive dipsogenic effect and augments MAP (Lee CS et al. 2008) (Chaggar PS et al. 2009).

Angiotensin II is an octapeptide, which acts on posterior pituitary gland and releases Antidiuretic Hormone (ADH) or vasopressin. It is also controlled via a negative feedback loop. ADH is synthesised in the hypothalamus and secreted by the posterior pituitary gland. In response to a drop in the MAP, central baroreceptors decrease their negative feedback impulses to the hypothalamus, and this leads to an increase in ADH/vasopressin secretion. ADH acts on the distal convoluted tubules in the kidneys to promote the absorption of water. The effects of the activation of the final common pathway of this particular neurohormonal system are the release of norepinephrine, vasoconstriction, sodium reabsorption, water retention, increased thirst, stimulating the release of vasopressin and increasing contractility. All these pathways augment an increase in MAP and perfusion pressure (Rea ME et al. 2008). Although the activation of this neurohormonal pathway is an essential compensatory

mechanism in the early stages of heart failure, the long-term results of RAAS activation cause ventricular remodelling which further hastens myocardial dysfunction. Along with the systemic RAAS, individual local organ based RAAS has been identified more recently. The functioning of the local RAAS is diverse in different organs, and these RAAS can work autonomously as in brain and adrenal gland or conjunction with circulation RAAS as in heart and kidneys (van Kats et al., 1998) (Aldred et al., 1993) (Kobori et al., 2004). More recently, an intracellular RAAS is identified as well. (De Mello, 2003) (Re and Cook, 2006).

The other significant neurohormonal mechanisms augmenting and interacting with RAAS activity are natriuretic peptides. These include atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and c-type natriuretic peptide, (CNP) which serve to counteract the vasoconstriction effects of RAAS cascade. ANP and BNP are secreted by the atria and ventricles respectively in response to the stretch of the chamber wall while CNP is found predominantly in the central nervous system. These hormones act primarily on peripheral blood vessels to cause vasodilation, sodium and water excretion and inhibit secretion of RAAS activation hormones renin, aldosterone, and vasopressin. Elevated BNP, in particular, is considered to be one of the first signs of HF and is being used to follow the progression of disease and response to the treatment modalities (Kim HN et al., 2011).

Vascular endothelium produces different vasoactive substances that act in a paracrine fashion to promote vasoconstriction (endothelin I) or vasodilation (prostacyclin, bradykinin, and nitric oxide) (Boulanger CM et al. 1999) (Bauersachs J et al. 2004). The production of cytokines, i.e., interleukin 1 α (IL-1 α), interleukin 6 (IL-6), interferon α (INF- α) and tumour necrosis factor α (TNF- α) are also increased in HF. The elevated levels of these small molecules are associated with worse clinical outcomes (Chen D et al. 2008).

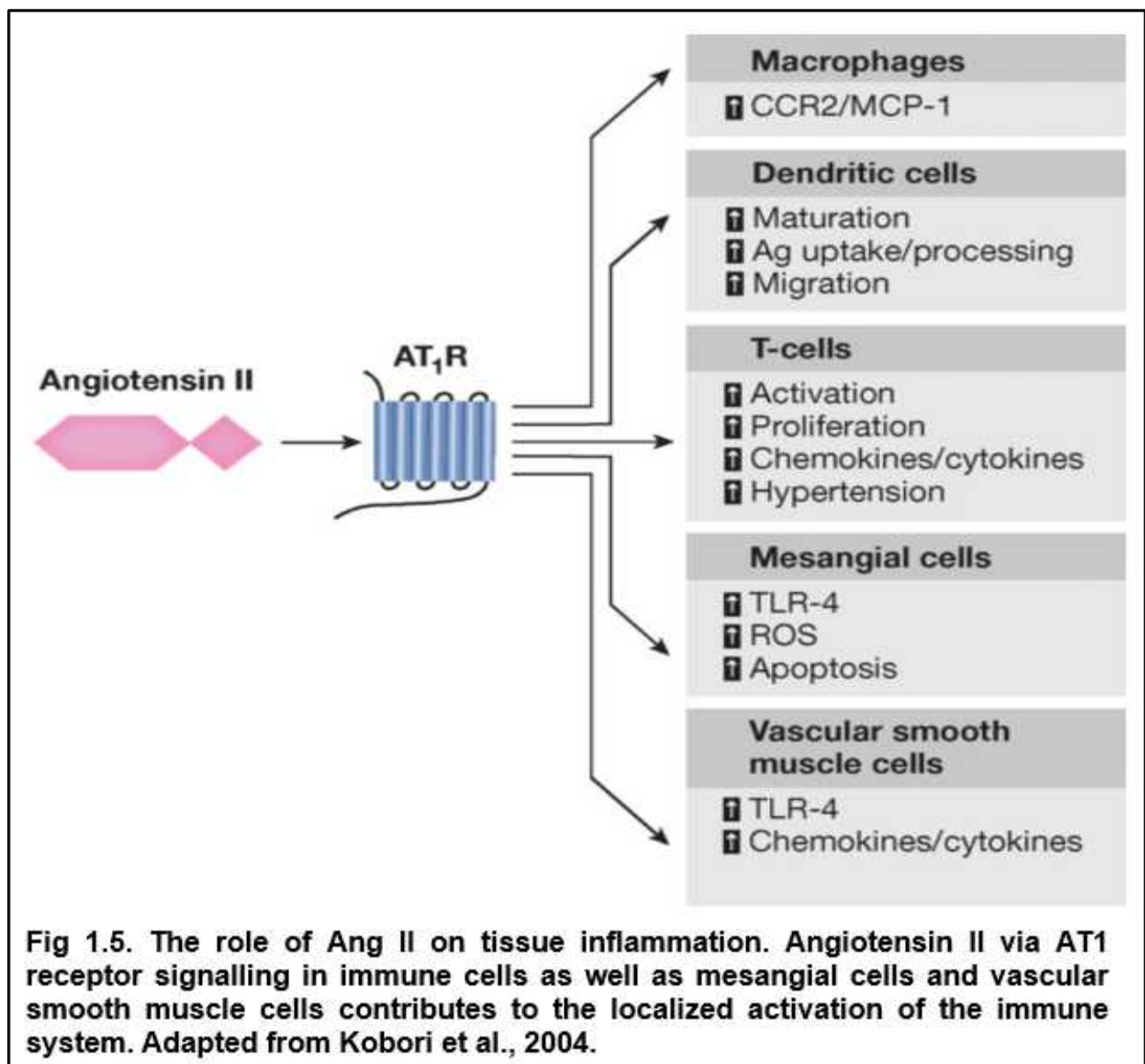


Angiotensin II has been identified to play some vital non-hemodynamic role as well. The cellular and local tissue-based RAS and Angiotensin II lead to inflammation, fibrosis and cellular proliferation. These actions are mediated by activation of multiple intracellular signalling pathways, production of reactive oxygen species (ROS), cell growth, cell migration, and differentiation, regulates gene expression, extracellular matrix remodelling, and apoptosis leading to tissue injury. In tissues, Angiotensin II can induce an inflammatory response by stimulating and mediating the production of specific pro-inflammatory cytokine/chemokines, recruitment of infiltrating inflammatory cells responsible for the accumulation of immunocompetent cells in the tissues. The activation of local RAS and Angiotensin II synthesis increases vascular permeability by promoting the expression and secretion of VEGF (Kitayama et al., 2006; Suzuki et al., 2003; Chua et al., 1998). This further induces the expression of endothelial adhesive molecules including selectins (P-Selectin and L-selectin), intercellular adhesion molecules-1 (ICAM-1), vascular cell adhesion molecules-1 (VCAM-1) and integrins (Alvarez et al., 2004; Pueyo et al., 2000). Ang II also causes endothelial dysfunction through COX-2 activation by generating vasoactive prostaglandins and ROS (Welch, 2008; Wu et al., 2005).

In murine models, an increase in Angiotensin II secondary to hypertension is associated with increased infiltration of the macrophages into the aortic wall. This is mediated by enhanced expression of MCP-1 (Monocyte chemoattractant) and CCR-2 receptor (Chemokine receptor) and blocking of these receptors with ACE inhibitors is associated with markedly decreased macrophages infiltration in the aortic wall (Dai et al., 2007). The inflammatory effect of Angiotensin II is mediated via T cells which contain intracellular RAS. The activation of RAS in T cells leads to reengagement of the cytoskeletal which leads to the release of cytokines and chemokines which favours further recruitment of T cells. The infiltration of tissues with T cells leads to hypertension and blocking of the pathways leads

to blunted hypertensive response (Guzik et al., 2007).

Angiotensin II is also linked to T cell response in transplantation as blockage of their receptor is associated with improved allograft survival as compared to the untreated patients (Dragun et al., 2005). The modulation of the T cell response by Angiotensin II has alluded to the notion of the possible role of the octapeptide in the autoimmune response. Therefore, the RAS antagonist can not only be used as antihypertensive agents and ventricular remodelling agents but possibly can also be used as anti-inflammatory and immunomodulatory agents (Kobori et al., 2004).



1.5.3 VENTRICULAR REMODELLING

The alterations in the histological and biochemical characteristics of the myocardial tissue necessitate an ordered sequence of cell proliferation, apoptosis, hypertrophy or atrophy; a process called ventricular remodelling (Curry CW et al. 2000). As remodelling ensues, there are changes in ventricular volume, composition, mass and its overall geometry changes as it becomes more spherical from its original elliptical shape. These geometric changes initially are compensatory as, despite a reduced EF, a failing heart enlarges to increase ventricular volume which leads to greater stroke volume and higher cardiac output. Like other tissues in the body, these signals potentially reinstate a foetal growth pattern of transcription and translation (Lionetti et al., 2010). In due course, myocardial wall thickness and overall ventricular mass are also increased, which in the interim improves the myocardial contractility. Although the diseased heart undergoes the process of remodelling, scarred myocardium causes progressive functional deterioration of ventricular systolic or diastolic function.

The remodelling process in HF is progressive and compensatory but eventually becomes detrimental. In the absence of injury, the normal adult heart is a stable mechanical tissue pump adjusting performance to changes in loading conditions and inotropic state (Chien and Olson, 2002). As the ventricle continues to dilate and the myocardium hypertrophies, there is a steady increase in the wall tension and fibrosis, which eventually impairs contractility. The continuous process of ventricular remodelling also leads to an increase in apoptosis. Due to the hypertrophy and patchy apoptosis, there is significant contractile desynchrony in the dilated and remodelled ventricle leading to less efficient pumping and further reduction in the EF.

1.6 CONSEQUENCES OF HEART FAILURE

HF usually start when the compensatory mechanisms are exhausted, and the heart is no longer able to maintain a cardiac output to meet the metabolic requirement of the peripheral tissues. The symptoms of left-sided heart failure different from the right side and can be classically detected only when HF effect one side of the heart sparing the other side. However, in the majority of the cases, both ventricles are affected by the disease process simultaneously (Kemp and Conte, 2012) (Dharmarajan and Rich, 2017).

Heart failure eventually leads to poor quality of life, multi-organ failure and increased risk of mortality within five years of development of initial symptoms and diagnosis (Lloyd-Jones DM et al. 2002) (Nieminen MS et al. 2006) (Ambrosy et al., 2014). Almost, two third of the patients diagnosed with HF are above the age of 65 and therefore with an anticipated increase in the ageing population the incidence and prevalence of HF will increase as well (Bui AL et al. 2011) (Jugdutt BI. 2012) (Ponikowski et al., 2014). Once a diagnosis of HF is established, the treatment of the underlying pathology delays the progression of symptomatic deterioration, but it does not offer any cure or reversal of the underlying disease progression nor does it show any significant prognostic benefits.

The aetiology of heart failure has been investigated extensively but is still poorly understood at the cellular and molecular level. It is important to understand the cellular architectural changes and microenvironment alterations surrounding the cells following a pathology leading to HF. Although many murine and canine models have attempted to explore the cellular and molecular basis of diseases leading to HF anatomically and physiologically the murine and canine heart is different from an adult human heart. It is challenging as the acquisition of the human myocardial samples from living normal or failing heart is not feasible in most cases.

1.7. GROWTH POTENTIAL OF ADULT MAMMALIAN HEART

Contemporary data is suggestive that throughout its lifespan, the mammalian heart, including that of humans has some limited regenerative capacity. However, post-MI, this phenomenon is not efficacious enough to substitute lost myocardial mass (Nadal-Ginard and Fuster, 2007), (Beltrami et al., 2003), (Ellison et al., 2013). Cell therapy has a curative potential for human disease that can be achieved by administration of cells that have been selected, multiplied and pharmacologically altered outside the body (Preston et al., 2003), (Mason C, 2001). It is postulated that after an early myocardial injury, cell therapy has the potential to prevent the development of end-stage heart failure.

Current clinical trials have produced modest results by using bone marrow cells or mesenchymal cells of different origins as the therapeutic agent but the desired of myocardial muscle mass regeneration is not achieved (Nadal-Ginard and Fuster, 2007), (Martin-Rendon et al., 2008). To date many robust protocols are established that can successfully generate hESC-CM or hiPSC-CM ($n = 10^9$) by differentiating of human pluripotent stem cells (embryonic (ESC) or induced pluripotent cells (iPSC)) (Chong JHJ et al. 2014).

1.8. COMPOSITION OF ADULT HUMAN HEART

The normal human heart is composed largely of cardiomyocytes, vascular cells, and cardiac fibroblasts. Although the non-myocytes play a significant role in cardiac wellbeing and disease, there are fundamental gaps in our comprehension about them. In spite of the consensus that cardiomyocytes constitute 30 - 40% of the cells in the mammalian heart, the estimates of the frequency of non-cardiomyocytes vary substantially. Insufficient tools and lack of reliable specific markers for fibroblasts have contributed to this uncertainty (Walsh et al., 2010) (Zhou and Pu, 2016) (Raulf et al., 2015). The cellular composition of the mammalian heart has attracted a lot of attention more recently.

Historically, the cardiac fibroblasts are believed to comprise more than 50% of all the cells in the heart and replace the dead cardiomyocytes after an ischaemic myocardial injury (Camelliti et al. 2005) (Snider et al., 2009). In some cases, cardiac fibroblasts have been quantified by excluding cells that lack structural elements corresponding to cardiomyocytes, endothelial cells or vascular smooth muscular cells (Ng AC 1980).

Bergmann and co-authors have analysed the cellular composition of human hearts by both stereological and flow cytometric methods. The cardiomyocyte and endothelial cell nuclei were marked with PCM1 and lectin *Ulex Europaeus* Agglutinin I (UEA), while unmarked cells were classified as mesenchymal. Stereological analysis of histological sections estimates of the frequency of cardiomyocyte, endothelial cells, and mesenchymal populations was 18%, 24%, and 58%, respectively. However, the flow cytometry of isolated cell nuclei estimated frequencies of 33%, 24%, and 43%, correspondingly. Based on the assumption that majority of the mesenchymal cells are fibroblasts, the study concluded that fibroblasts are the predominant cardiac cell type in human hearts (Bregmann et al. 2015).

More recently based on histologic and flow cytometric analysis, it has been reported in the murine model that among the non-cardiomyocytes population the endothelial cells supersede the fibroblast population. More so, the fibroblast population is much less than reported in the previous studies. The study incorporated several refinements that make the most comprehensive quantification of the cellular composition to date. The study lists that 32% of all nuclei in the murine heart belonged to cardiomyocytes, 55% to Endothelial cells, and 13% to fibroblasts. The cause of preceding over-estimation of the fibroblast population was attributed to lack of a clear morphological definition for fibroblasts, non-cardiomyocyte-favouring isolation protocols, lack of true representative markers while quantifying and tracking these cells in vitro. The pericytes and leukocytes make almost 5% and 7-10%

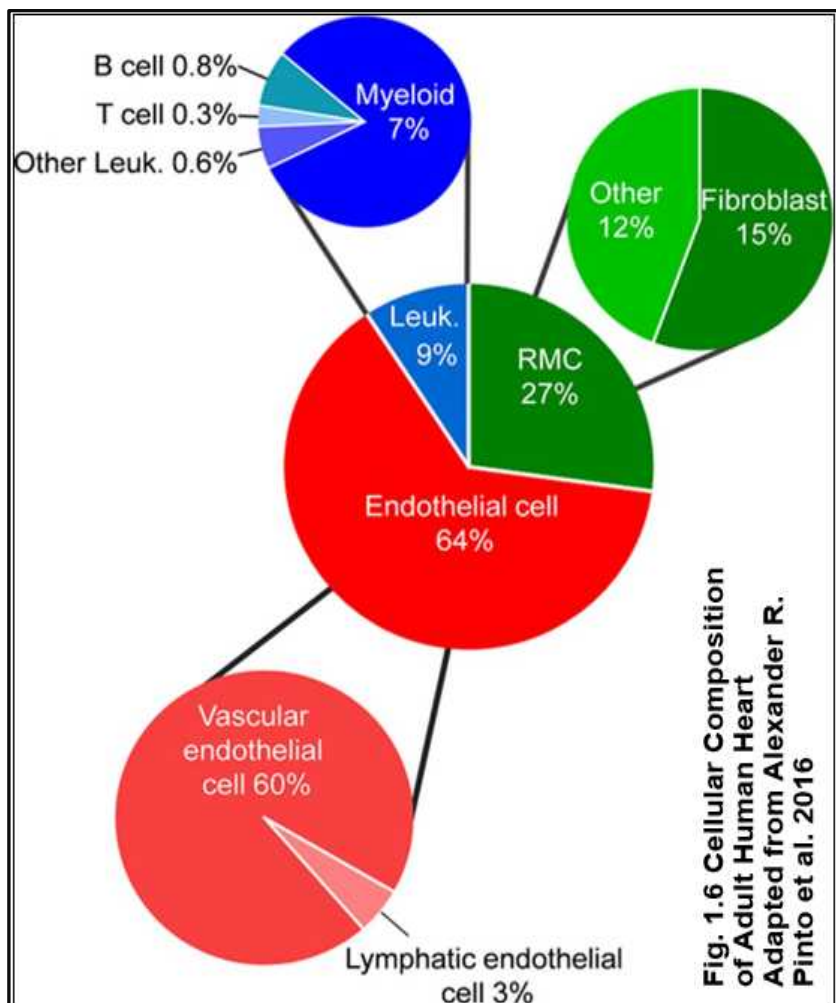
respectively of the cardiac cell population were possibly counted previously towards the fibroblasts population as well (Pinto et al., 2016).

However, having employed the same dissociation protocols and quantification methods to measure the cellular composition of the heart, it has been demonstrated that cardiomyocyte to non-cardiomyocytes cell ratio may vary significantly among different species (Banerjee et al., 2007).

1.9 ORIGIN OF HUMAN CARDIAC FIBROBLASTS

1.9.1 EPICARDIAL ORIGIN

Epicardium which is necessarily a thin layer of epithelial cells, arise from an extra-cardiac source of cells known as the proepicardium (PE) and give rise to vascular endothelial cells, smooth muscle cells, and fibroblasts. Proepicardium is an ephemeral organ, contributing a heterogeneous population of cells leading to the development of various structures of the heart. The proepicardium is a cauliflower-shaped accumulation of villous or bleb-like protrusions of the pericardial mesothelium forming a demarcated region of the embryonic pericardial wall near the venous pole of



the embryonic heart tube and protrudes to the pericardial cavity (Manner et al. 2005) (Viragh *et al.*, 1993). The proepicardium is considered not only as a significant source of the epicardial epithelium delivering cellular components of the vascular wall and interstitial tissue fibroblasts but also partly contributes to a fibrous tissue skeleton of the heart. Epicardial-derived cells also play an inductive role in differentiation of cardiac myocytes into the conductive tissue of the heart (Ratajska, Czarnowska and Ciszek, 2008).

The epicardium does exhibit a cardiogenic potential (Snider et al., 2009). The epicardium is formed over the mid-gestation heart and is maintained through the adult life. It has been acknowledged as a leading source of cardiac fibroblasts in various development models and studies, and the very first observation was made in experiments with chick embryos utilizing retroviral tagging of pro-epicardial progenitors (Cai et al., 2008) (Gittenberger-de Groot et al., 1998) (Smith et al., 2011) (Mikawa and Gourdie, 1996). The expression of the transcription factor TCF-21 in epicardial cells is mandatory for cardiac fibroblast development (Acharya et al., 2012a). In addition to fibroblasts, the epicardium gives rise to another cell including pericytes, smooth muscle cells endothelial cells and myocytes (Zhou et al., 2008) (Cai et al., 2008) (Mikawa and Gourdie, 1996).

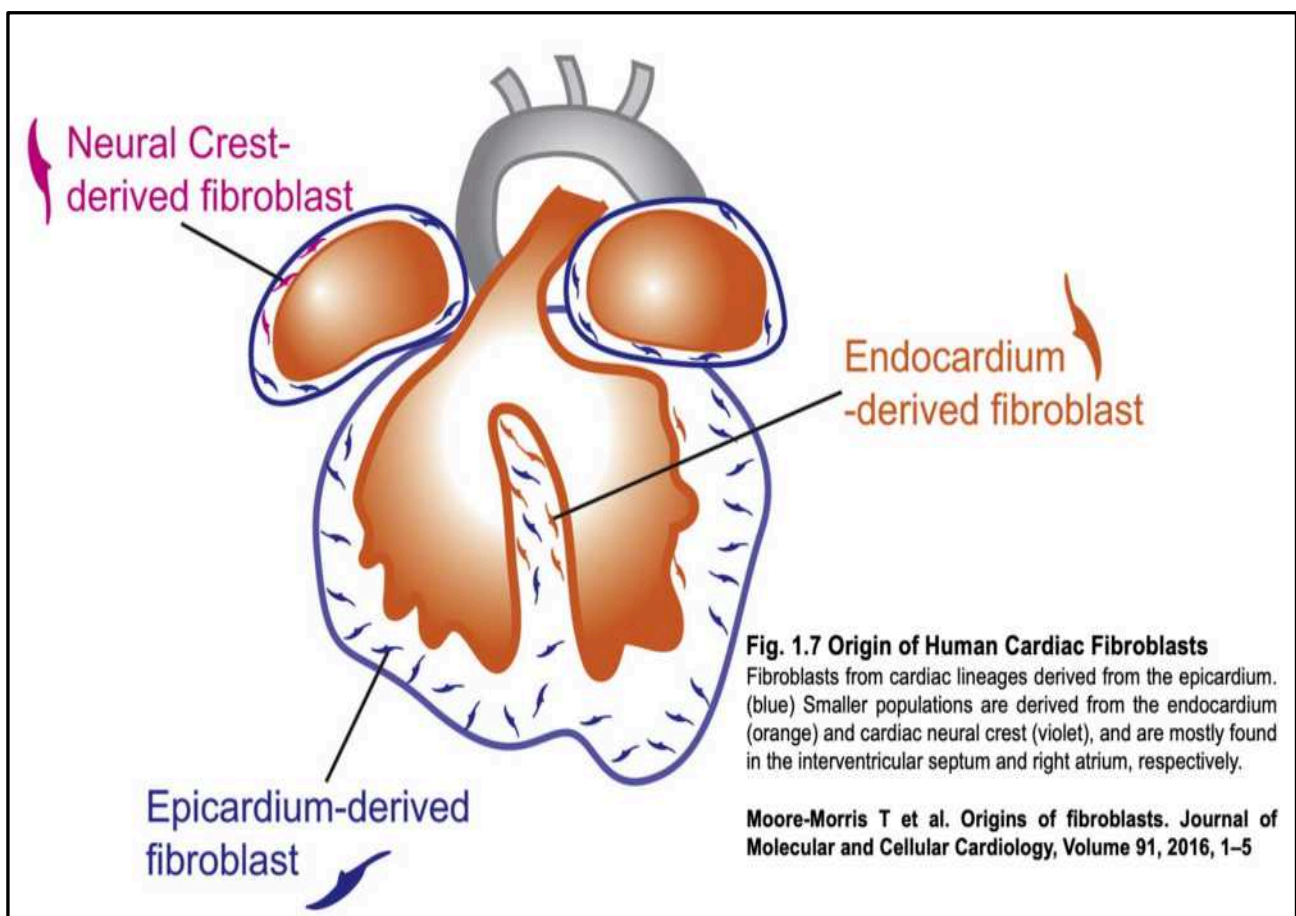
1.9.2 ENDOCARDIAL ORIGIN

More recently it has been advocated in the murine models that endothelial-to-mesenchymal transition (EMT) of endocardium that occurs during the development of the endocardial cushions of heart, contributes towards the fibroblast-akin cells of the mural leaflets of the cardiac valves. Based on their location they are labelled as valve interstitial cells (VICs), or valvular fibroblasts (VFs). The fibroblasts produced during this development phase not only populate the valves but also invade the surrounding myocardium, resulting in the presence of a considerable number of endothelial-derived fibroblasts. This population of fibroblasts of

endocardial origin is mostly located within the interventricular septum, overall presenting a distribution pattern complementing to that of epicardial-derived fibroblasts (Wessels et al., 2012) (Combs and Yutzey, 2009). However, the majority of the fibroblasts population invading and occupying the myocardium is of epicardial origin (Moore-morris et al., 2014).

1.9.3 NEURAL CREST ORIGIN

A small subgroup of fibroblasts within the myocardium, populating the right atrium, emerge from the neural crest. Neural crest cells are a migratory cluster of cells originating from the dorsal aspect of the neural tube (Ali et al., 2014) (Moore-Morris et al., 2016). They represent a very heterogeneous population giving rise to various lineages including neurons, melanocytes and mesenchymal cells (Dupin and Sommer, 2012) (Dupin and Sommer, 2012). The myocardium is of epicardial origin (Moore-morris et al., 2014). (Fig.1.7)



An individual niche of neural crest cells contributes to mesenchyme of the semilunar valves of the outflow region of the heart, as well as melanocytes. These cells migrate from the caudal pharyngeal arches to the base of the heart and the outflow tract during the process of outflow tract septation. They are deemed to play an active role in the septation of the outflow tract. Cardiac neural crest cells are also dispersed to regions correlated with sites of remodelling, such as the aortic sac transforming into the base of the ascending aorta and pulmonary trunk, the primitive distal truncus patterned into the two semilunar valves and in the proximal trunco-conal cushion, which is the site of formation of the muscular septum. Additionally, cardiac neural crest cells reside at the site of closure of the ventricular septum, the wall of the pulmonary infundibulum, and transiently in the wall of the aortic vestibule (Jiang et al., 2000) (Waldo et al., 1998). In the semilunar valves, neural crest-derived mesenchyme is eventually replaced by endocardium derived mesenchyme, leaving melanocytes as the only neural crest contribution to adult valves (Nakamura, Colbert and Robbins, 2006). Nevertheless, the fibroblast niche residing in the right atrial myocardium seem to persist to some extent into adulthood (Ali et al., 2014).

1.9.4 ORIGIN OF FIBROBLASTS IN FAILING HEART

The origin of fibroblasts forming scarred tissues and fibrotic lesions in failing hearts has been controversial. Some reports are suggestive that fibroblasts contributing to scar formation are derived solely from native neighbouring resident fibroblast lineages, while others have proposed that other cell types transdifferentiate into fibroblasts and significantly contribute to fibrosis and matrix deposition (Moore-Morris et al., 2016). The latter point of view has been supported in many studies reporting that endothelial to mesenchymal transition and recruitment of circulating fibroblast progenitors contributed significantly to cardiac fibrosis. These studies advocate the endothelial to mesenchymal transition and blood progenitor cell recruitment as general mechanisms of cardiac fibrosis. Importantly, these processes

represent potential therapeutic targets as they reveal that inhibiting the endothelial to mesenchymal transition would promote the preservation of the vasculature while reducing fibroblast accumulation (Zeisberg et al., 2007) (Aisagbonhi et al., 2011) (Xu et al., 2011) (Widyantoro et al., 2010).

Recently the studies have attributed the heterogeneity in fibroblast lineages in pathological conditions to the activation of separate developmentally derived lineages. It is noted that before and during fibrosis; two main developmentally derived fibroblast lineages, of epicardial and endocardial/endothelial origin, accounted for the vast majority of fibroblasts populating the fibrotic area. Moreover, the use of bone marrow transplants and adult endothelial-lineage tracing, in conjunction with markers such as PDGFR α have provided strong evidence against fibroblastic contributions from blood and endothelium to fibrosis (Moore-morris et al., 2014) (Ali et al., 2014).

Although the origins of cardiac fibroblasts during the development phase and pathological situations has become more explicit, the extent to which the origin of a fibroblast regulates their functional properties remains ambiguous. In adult hearts, fibroblasts of endothelial and epicardial origin, exhibit similar hypertrophic response and transcription factors expression in response to a pathological condition. Regardless of their origin, the location of fibroblasts within the myocardium concerning the vasculature dictates the expression of specific markers in fibrotic conditions. Hence this concludes that the phenotype of fibroblasts is dependent more on their location rather than their developmental origin (Braitsch et al., 2013) (Moore-morris et al., 2014).

1.10 HISTOLOGICAL FEATURE OF CARDIAC FIBROBLASTS

Historically fibroblast is an equivocal but suitable term coined for an ill-defined connective tissue cell derived from the primitive mesoderm. Formerly, fibroblasts were described in the late 19th century based on their location and morphological criteria. Classically, fibroblasts are recognised by their flattened spindle-shaped morphology, ability to adhere to culture plates, and absence of markers of specific for epithelial, smooth muscle, endothelial, perineural, and histiocytic cells (Kalluri and Zeisberg, 2006) (Chang et al., 2002).

1.10.1 CELLULAR HISTOLOGY OF FIBROBLASTS

Human cardiac fibroblasts are a cluster of heterogeneous flat spindle-shaped cells with multiple processes. The cardiac fibroblasts lack a basement membrane, a feature that distinguishes them from the other cardiac cells (Camelliti et al. 2005). When active they tend to display a prominent Golgi apparatus, extensive rough endoplasmic reticulum while the resting fibroblast phenotype lacks intracytoplasmic actin and desmin filaments (a hallmark feature of myofibroblasts). Human cardiac fibroblasts are mono-nucleated with nucleus occupying the centre of the cell. The senescent cells tend to display a zone of prominent dense granular material aggregation surrounding the nucleus. Small wire-like extensions between adjacent cells and colonies called as nanotubes (Koyanagi et al., 2005) (Plotnikov et al., 2008) (Davis and Sowinski, 2008) or filopodia (Goldsmith et al., 2004). These nanotubes/filopodia not only mediate cell continuity but are also permit direct long distance transfer of cell signals, cell components (He et al., 2011) and intracellular organelles such as endosomes and mitochondria (Plotnikov et al., 2008) as well as other constituents, e.g., prions (Gousset et al., 2009). The diameter of these nanotubes ranges from 50-200 μm (Chinnery, Pearlman and McMenamin, 2008) and the cytoskeleton is composed of microtubules and F-actin (Onfelt et al., 2006) (He et al., 2011). The transfer of cytoplasmic organelles and cellular component through the tubules is selective (Davis and Sowinski,

2008) but is bidirectional (Dixon and Davies, 2011). This local intercellular communication is thought to be important to maintain homeostasis is critical in maintaining and synchronising cellular activities. Nanotube mediated intercellular interaction has been recorded during in-vitro growth of human atrial and ventricular cardiac fibroblasts at different stages.

Despite being considered as uniform cells in different organs, the fibroblasts display phenotypic heterogeneity and gene expression in different tissues and within a tissue under different physiological conditions (Chang et al., 2002) (Fries KM et al. 1994). Identification of fibroblasts is often based solely on morphological characteristics but is subjected to variance with location and overall activity of the tissue.

1.10.2 ARCHITECTURAL ARRANGEMENT OF FIBROBLASTS

The fibroblasts in the cardiac tissues are organised into a three-dimensional interconnected network analogous to their arrangement in collagen gels (Grinnell, 2000). In vivo fibroblasts are arranged and enmeshed in a collagenous endomysial weave network that surrounds the assemblies of myocytes. This laminar structure comprising of two to five myocytes in thickness is fundamental to the organisation and function of the heart (Legrice et al. 1997). In vitro, the fibroblasts can migrate within the system and can cause the contraction of the collagen gel surrounding them. Cardiac fibroblasts are established components of the ECM in the heart because of their ability to synthesise and express several types of collagens (Carver et al. 1993), MMPs (Spinale 2002), growth factors (Diaz-Araya et al. 2003). They play an important role in cytokine signalling. Indeed, fibroblasts have been termed sentinel cells because of the many functions they mediate in the heart (Smith et al. 1997).

1.11 HUMAN CARDIAC FIBROBLAST MARKERS

Cardiac fibroblasts are a group of heterogeneous cells of different lineages. To date, there is no one marker identified that encompasses the combination of sensitivity and specificity required to determine the cardiac fibroblasts in vivo and vitro. A few of the commonly used CF markers in different studies include Discoidin domain collagen receptor 2 (DDR-2), THY-1 cell surface antigen (THY-1), Vimentin, Periostin, Fibroblast-specific protein 1 (FSP-1), Fibroblast activation protein (FAP) and Platelet-derived growth factor receptor alpha (PDGFR α). No definitive cardiac fibroblast lineage marker has been identified yet.

DDR-2 is a member of the receptor tyrosine kinases family of proteins and represents a good marker for identifying cardiac fibroblasts in vivo. Receptor tyrosine kinases convert the extracellular stimuli into a cellular response and are helpful in a variety of cell functions including growth, migration, morphology, and differentiation (Schlessinger 1997). Although some studies have proposed DDR2 receptor as specific for cardiac fibroblasts and not expressed by myocytes, endothelial cells or vascular smooth muscle cells (Camelliti, BORG and KOHL, 2005), in other murine studies, the expression of DDR-2 is limited to only a small population of CFs (Krenning, Zeisberg and Kalluri, 2010) (Zeisberg and Kalluri, 2010).

Vimentin is an intermediate filament protein considered to be one of the most sensitive markers. However, it is co-expressed by the entire endothelial lineage making it less useful as a definite CF marker. THY-1 (CD90) is a membrane glycoprotein expressed on the surface of cardiac fibroblasts but is also detectable on some endothelial cells surface as well (Acharya et al., 2012a). Likewise one of the widely used markers of CFs FSP-1 is co-expressed within vascular cells and inflammatory leukocytes in murine models; thus jeopardising its official role as a CF-specific marker (Zeisberg et al., 2007) (Kong et al., 2013).

Periostin, a 90 kDa matricellular protein is strongly up-regulated in post-infarct activated cardiac fibroblasts but in quiescent or resting CFs is relatively downregulated (Kong et al., 2013) (Kudo, 2011) (Snider et al., 2009). Based on intranatal and postnatal developmental studies Periostin is considered to be a reliable marker of activated cardiac fibroblasts (Qian et al., 2012). Other markers such as FSP-1 and cadherin – 11 are only relatively specific.

Besides CFs specific markers, other genes are highly expressed by cardiac fibroblasts including beta1-integrin, fibronectin, connexins, and the fasciclin gene. Tomasek et al. have shown that under appropriate conditions, resting or quiescent fibroblasts can acquire an active, synthetic, contractile phenotype and express several smooth muscle cell markers and genes that are not typically expressed in fibroblasts (Tomasek et al., 2002).

In the absence of a comprehensive cardiac fibroblast marker, it is difficult to fully understand the interaction between fibroblasts and adjacent resident cardiac cells including cardiomyocytes, endothelial cells, and lymphocytic cells. Since there is no ideal way to identify the CFs with a single marker, therefore, depending upon the variable expression of commonly used markers at the different developmental stage or the cellular environment (i.e., recent ischemic injury), a combination of markers can be used to identify the fibroblasts (Lajiness and Conway, 2014).

1.12 PHYSIOLOGICAL ROLE OF CARDIAC FIBROBLASTS

Cardiac fibroblasts are fully differentiated somatic cells that provide support, structure, generate signals and contribute to scar formation upon cardiac damage (Camelliti, BORG and KOHL, 2005) (Snider et al., 2009) (Ieda et al., 2009). Under physiological conditions, fibroblasts are sustentacular cells that help to synthesise and maintain a 3D scaffold for cardiomyocytes which ensures the functional integrity of the myocardium. Every cardiomyocyte is in direct contact with one or more fibroblasts which enable CFs to contribute to the structural, biochemical, mechanical, and electric properties of the functional myocardial syncytium (Miragoli, Gaudesius and Rohr, 2006) (Dobaczewski et al., 2010). CFs play an active role in secretion, maintenance, and remodelling of the extracellular matrix (ECM). The ECM also provides structural support for cardiac myocytes and formation of the elaborate cardiac skeleton.

In addition to this, CFs are involved in paracrine communication with myocytes as well as with endothelial cells. These contacts affect the electrophysiological properties; coordinate pump function of the heart, secretion of growth factors and cytokines, as well as potentiating blood vessel formation. Under ischemic conditions, CFs secrete factors that have shown to have cardioprotective effects in vitro and ex vivo models (Baudino et al. 2006).

Cardiac fibroblasts play a pivotal role in the post-myocardial infarction fibrosis and myocardial remodelling. Recently it has been shown in murine models that in spite of the same mesenchymal origin, fibroblasts within the same tissue display the functional diversity when exposed to distinctive environmental conditions. The two discrete dermal lineages were found to be contributing uniquely towards the tissue repair and regeneration, in response to injury. This functional assortment can be possibly attributed to the differentiation hierarchy or the origin from diverse lineages (Driskell et al. 2013). This alludes to the

possibility of distinctive fibroblast lineage in other organs and species. It was hypothesised that due to structural and functional differences of the human cardiac chambers, the fibroblasts in these chambers might exhibit distinctive phenotypes and functional spectra. The different mechanical environment may induce growth factor secretion triggering various autocrine and paracrine effects that can modulate the physiological status of cells. This may alter the precise phenotypic characteristics; accentuate the inherent phenotypic disparities and growth pattern of the CFs and the expression of the transcription factors exhibited by these cells (MacKenna et al. 2000).

In diseased hearts, fibroblasts play a pivotal role in modulating cardiac function. Subsequent to ischemic cardiac injury leading to necrotic cell death, the cardiac fibroblasts, together with inflammatory cells, infiltrate the ischemic area to initiate healing and scar formation, thereby maintaining the structural integrity of the myocardium (Weber et al., 2013). The ECM and growth factors secreted from fibroblasts under these circumstances is exaggerated and promote cardiomyocyte hypertrophy, leading to myocardial remodelling (Sano et al., 2000) (Weber et al. 1991). Also, during acute tissue injury, mesenchymal and inflammatory cells secrete TGF- β 1 to induce fibroblast to myofibroblast transformation.

1.13 MYOFIBROBLASTS

Myofibroblasts (MF) are phenotypically modulated cells, characterised by the presence of a micro-filamentous contractile apparatus enriched with α -smooth muscle actin (α -SMA). Myofibroblasts secrete growth factors, cytokines, ECM, and proteases and have a critical role in tissue repair, blood vessel formation, fibrosis, and organogenesis. Except for heart valve leaflets, the normal healthy heart does not harbour myofibroblasts. Following injury, the mechanical and chemical microenvironment integrate to promote tissue remodelling by myofibroblasts. The myofibroblasts are formed by fibroblasts acquiring a migratory phenotype by developing the contractile filaments and hence are referred as proto-myofibroblasts. This term was coined to differentiate activated fibroblasts from quiescent fibroblasts that are devoid of any contractile apparatus in most intact tissues (Tomasek et al., 2002).

This initial phenotypic change occurs in response to changes in the composition, organisation, mechanical property of the extracellular matrix (ECM) (Hinz et al. 2003) and locally released cytokines by inflammatory and resident cells (Werner et al. 2003). An important stimulus for this phenotypic transition is the change of the mechanical microenvironment. The fibroblasts in an intact tissue are stress-shielded by the crosslinked ECM; this protective structure is lost in the continuously remodelled ECM of injured tissue (Tomasek et al., 2002). In response to the mechanical challenge, fibroblasts acquire contractile stress fibres that are first composed of cytoplasmic actin, hallmarking the “protomyofibroblast.” Stress fibres are connected to fibrous ECM proteins at sites of integrin-containing cell-matrix junctions (Hinz et al. 2006) and between cells via de novo established N-cadherin-type adherents junctions (Hinz et al. 2004). With increasing stress due to their remodelling activity, proto-myofibroblasts further develop into “differentiated myofibroblasts” by expressing smooth muscle actin (α -SMA). α -SMA is the most widely used MF marker.

These features, closely resemble those of cultured fibroblasts that have been mechanically activated by the rigid plastic substrate, whereas stress fibres do not form on very soft culture substrate hydrogels or in compliant collagen gels. In culture, the proto-myofibroblast is a stable phenotype, representing an intermediate step in most in vivo conditions where it proceeds toward the “differentiated myofibroblast” that is characterised by de novo expression of α -SMA (Hinz et al., 2007).

1.13.1 ORIGIN OF MYOFIBROBLASTS

The classical view on dermal wound healing implies local recruitment of fibroblasts from the dermis of the intact adjacent skin to sites of inflammation (Desmouliere et al., 2005). Perivascular and vascular smooth muscle cells and pericytes have been proposed as another local myofibroblast source in scleroderma (Rajkumar et al. 2005) as well as liver and glomerular fibrosis (Desmouliere et al. 2003). Also, a small proportion of fibroblasts may originate from fibrocytes, a sub-population of bone marrow-derived leukocytes with fibroblast characteristics (Abe et al. 2001). This concept has been revisited and confirmed by transplanting bone marrow from male to irradiated female mice and identifying the Y chromosome by in situ hybridisation (Direkze et al. 2003) and from transgenic green fluorescent mice to irradiated wild-type animals (Hashimoto et al. 2004).

Some studies have advocated that fibrocystic progenitors can potentially contribute towards a surprisingly high fraction of 30–50% of the wound myofibroblasts (Direkze et al. 2003) (Ishii et al. 2005) (Mori et al. 2005). Comparable proportions have been demonstrated for myofibroblasts appearing during fibrosis of the liver (Forbes et al. 2004), kidney (Direkze et al. 2003), and lung (Hashimoto et al. 2004) and during the stroma reaction to epithelial tumours (Ishii et al. 2003). It is still unfolded whether myofibroblasts from different origins exhibit different characteristics and functions during tissue repair as suggested for liver

fibrosis (Guyot et al. 2006) or whether under pathological conditions the myofibroblasts are recruited from different sources to meet the temporarily high demand of contractile cells, which may all follow a similar differentiation program.

1.13.2 HISTOLOGICAL FEATURES OF MYOFIBROBLASTS

Microscopically the fundamental ultrastructures, defining the true population of myofibroblasts are cytoplasmic contractile filaments (actin & desmin), lack of filopodia, extensive cell to matrix attachment sites (cellular digitation) as well as intercellular adherence and gap junctions (Eyden et al. 2005; Schurch et al. 2006). However, these features are only applicable in distinguishing the myofibroblasts from quiescent fibroblasts as other contractile cells such as smooth muscles cells may mimic various feature attributed to myofibroblasts (Hinz et al. 2007).

1.13.3 MYOFIBROBLASTS IN PATHOLOGICAL CONDITIONS

The interstitial and adventitial cardiac fibroblasts transform into myofibroblasts, and improper regulation of these cells is associated with destructive remodelling (Melchior-Becker et al., 2011). The persistent myofibroblastic activity is related to excessive scarring, loss of tissue compliance, and an extensive fibrotic response that is the basis for fibrotic disorders in numerous organs (Hecker et al., 2009) (Davis and Molkentin, 2014). Myofibroblasts have been shown as transient phenotype and can be switched back to fibroblasts. Fibroblast growth factor can block and reverse the myofibroblast back to fibroblasts. Fibroblast growth factor can block and reverse the myofibroblast phenotype.

1.14 CARDIOMYOCYTE AND NON-CARDIOMYOCYTE INTERACTION

Recently studies based on the embryonic cardiac development in mammals and non-mammals have shown that a complex and crucial interplay of the epicardial and endocardial fibroblast paracrine signalling are important for differentiation and postnatal functioning of cardiomyocytes. The epicardium responsible for the generation of fibroblasts is ectodermal in origin, and the endocardium along with myocardium are of mesenchymal origin. In these models, paracrine pathways such as Wnt-1, FGF and retinoic acid (RA) are identified as playing a crucial role in harmonising the proliferative and differentiation processes in the developing myocardium (Tian Y et al. 2012). However, the activation of these pathways in the adult mammals is an area that is still unknown. In non-mammalian species like zebrafish, following a critical myocardial injury, epicardium at a distance is capable of generating signal pathways which can help to restore the damaged myocardium (Duan J et al. 2011). In contrast to the this, the adult mammalian hearts after sustaining a myocardial injury repair the infarcted zone by a scar formation through activation and expansion of cardiac fibroblasts. There is no myocardial regeneration, and hence the functional capacity of the heart is compromised.

The adult human heart is comprised of cardiomyocyte and non-cardiomyocyte populations with non-cardiomyocytes out numbering the cardiomyocytes. In a normal healthy heart, the cardiomyocytes are arranged as myocardial syncytium in which the cardiomyocytes are arranged as flat sheets and are coupled in an end to end manner. The conclusion to end union is marked with intercalated discs and gap junctions. This architectural continuity allows the conduction of electrical impulses generated by the action potential and helps the mechanical activity of cardiac syncytium during contraction and relaxation phase (Rhett & Gourdie et al. 2012).

The cardiac fibroblast is the scaffolding cells which are important to maintain the structural integrity of the heart. Cardiac fibroblasts play essential roles in postnatal cardiac remodelling after injury and stress by helping to stabilise the myocardial structure and form scar tissue. Under normal physiological conditions, cardiac fibroblasts are responsible for the architectural support and maintain the homeostatic environment for the heart. Following myocardial injury and remodelling, there is a loss of gap junction between adjacent cardiomyocytes. There sporadic interspread of non-cardiomyocytes between the damaged cardiomyocytes and gap junctions lead to desynchronized contractility and slow conduction (Gaudesius et al. 2003) (Miragoli et al., 2006) (Rohr et al. 2009).

Conventionally, the cardiomyocytes are labelled as the electrical impulse and action potential generator cells, and non-cardiomyocytes are thought to be a non-excitabile cell population and a barrier to the propagation of electrical impulses. However, the anatomical connection between the non-excitabile and excitable cell population (cardiomyocytes and non-cardiomyocytes are connected to each other via connexin proteins) has prompted the notion of a possible contribution of non-excitabile cell population toward the action potential propagation. This is supported by the clinical observations such as conduction of impulses through the scarred tissues, repaired congenital cardiac defects with fibrotic margins, post-infarct scarred tissue and generation of impulses in the myocardial tissue after thermic and cryoablations. All these clinic findings allude to the possible contribution of non-cardiomyocytes toward the generation of electrical impulses. Recently, it has been shown with the use of voltage sensitive fluorescent proteins (VSFP) to measure the cell-specific electrical activity that non-cardiomyocytes show cardiomyocytes like action potential signals in the tissue surrounding the cryoablation induced infarcted zones. Connexin proteins and nanotubules mediated connections between the cardiomyocytes and non-cardiomyocytes are postulated to be responsible for it. This opens a new interesting avenue of the possible

passive contribution of non-cardiomyocytes toward the generation of the action potential (Quinn et al., 2016).

The extensive autocrine and paracrine effects of fibroblasts are affected by the nature of the disease. The studies on murine models have shown that α -SMA negative CFs increase cardiomyocyte Ca^{+2} transient amplitude, whereas cultured fibroblasts and myofibroblasts from pressure-overloaded hearts decrease Ca^{2+} transient amplitude. In turn, cardiomyocytes affect fibroblast proliferation in a paracrine fashion. It was shown that TGF- β directly causes cardiomyocyte hypertrophy and participates in bi-directional regulatory signalling between fibroblasts and cardiomyocytes (Cartledge et al., 2015).

1.15 TRANSCRIPTION FACTOR EXPRESSION OF CARDIAC FIBROBLASTS

Human atrial and ventricular fibroblasts (from different patient groups) have recently been identified, characterised and compared with the murine cardiac and non-cardiac fibroblasts extensively, for specific markers and gene expression. Murine and human cardiac fibroblasts are negative for the mature cardiomyocyte markers (MHY6, MHY7, and TNNT2) but show surprising enrichment in cardiogenic transcription factors, i.e., Tbx-20 (variant 1 & 2), GATA 4, GATA 6, Hand-2 as well as epicardial transcription factors, i.e., Tcf21 and Wt1. It was also observed that GATA4, GATA6 are present in all chambers of the heart unlike Tcf21, Hand2, and Tbx20, which are preferentially expressed in the ventricles. In contrast, GATA5 and Wt1 exhibited atrial predilection in the human heart. This augments our hypothesis of functional paracrine interaction with the cardiomyocyte. Interestingly the Heat Map study have demonstrated an overlap of expression of a transcription factor in fibroblasts from different chambers, which have been successful in directing reprogramming in the other studies. It suggests that some chamber specific fibroblast populations may be more amenable to reprogramming than others (Furtado et al., 2014).

The therapeutic strategy of generating cells from autologous human tissue to replenish the diseased and dead cells and circumvent the immunogenic issues has shown promising outcomes. The discovery that a minimal number of transcription factors can reprogram somatic cells to induced pluripotent stem cells (iPSCs), which resemble ESCs regarding totipotency, toppled the view that development can only proceed unidirectionally. Further, fibroblasts have also shown the potential to be directly reprogrammed into cardiomyocytes, muscle cells, and neurons by combinations of lineage-enriched transcription factors in murine models (Kalani and Martirosyan, 2012) (Takahashi and Yamanaka, 2006) (Pang et al., 2011) (Caiazzo et al., 2011) (Qian et al., 2012). Reprogramming by cardiac transcription

factors and miRNAs seems to involve the direct conversion of fibroblasts toward a cardiomyocytes-like fate without transition through a stem cell intermediate. Direct cardiac reprogramming of fibroblasts also circumvents potential teratogenicity and immunogenicity of iPSCs. Cardiac fibroblasts account for almost 60% cells in the heart and represent a potential cellular source for restoration of cardiac function following injury through phenotypic reprogramming to myocardial cell fate.

It has been reported in a murine model that fibroblasts can be reprogrammed to contractile cardiac-like myocytes cells by expressing developmental transcription factors, MEF2, HAND2, GATA4, and TBX5 or by treatment with a combination of miRNA's (miRNAs 1, 133, 208 and 499). (Jayawardena et al., 2012). Wada et al. has elaborated this concept and has demonstrated that addition of Mesp1 and Myocd to GMT can up-regulate a broader spectrum of cardiac genes in human cardiac fibroblasts more efficiently compared with GMT alone (Wada et al., 2013). Recently Young-Jae et al. have shown that transcription factors (GATA4, Hand2, Tbx5) and microRNA's (miR-1, miR-133) can successfully direct the human dermal fibroblasts to cardiac-like myocytes (Nam et al., 2013).

1.16 ENGINEERED HEART TISSUE

Since endogenous regenerative mechanisms do not suffice to compensate for cardiomyocytes death after MI, the exogenous regenerative strategies have gained increasing attention and offer the promise of creating functional tissue replacements for use in the failing heart. In vitro approaches are aimed at organising cardiomyocytes into a functional tissue capable of generating contraction and propagating electrical signals as normal myocardium. An engineered cardiac tissue of clinical relevance must exhibit functional and morphological properties similar to that of native myocardium and remain viable after implantation. Eschenhagen and Zimmermann pioneered the concept of generated engineered heart tissue (EHT) by seeding a mix of collagen or fibrin, extracellular matrix proteins (Matrigel) and neonatal rat or human iPSC-derived cardiomyocytes (iPSC-CM) into lattices or circular moulds (Zimmermann et al., 2002). The continuous contraction of these spontaneously active constructs against the flexible silicone posts aligns and matures the iPSC-CM. Zimmerman et al. also showed that EHTs could be fused together to create large contracting constructs and grafting of these constructs into the infarcted wall in murine models induced thickening and improved fractional shortening. It has been established that the improvement of systolic function resulted from cardiomyocytes engraftment and not merely from scar stabilisation or paracrine effects (Zimmermann et al., 2006).

Lately, Kensah et al. have shown a considerable improvement in the construction of EHTs by developing constructs that contract with forces only 5-fold lower than the human myocardium (Kensah et al., 2013). It was postulated that the addition of fibroblasts and the extracellular matrix produced by them would improve for otherwise distant connection between cardiomyocytes. Hence, the addition of these extra cell-cell connections between cardiomyocytes allows for greater force development and subsequent maturation of the

tissue due to the beating of the construct (Parrag, Zandstra and Woodhouse, 2012) (Liau et al. 2012). The concept of construction of EHTs was first introduced by Eschenhagen et al. who cultured the cardiomyocytes derived from embryonic chick ventricle with collagen matrix solution for the generation of spontaneously beating, three-dimensional models of heart tissues (Eschenhagen et al. 1997). The dissociated cardiomyocytes laden hydrogel-like matrices were attached to silicon posts to maintain a defined preload. Cardiomyocytes dispersed in the surrounding hydrogel not only remodel and align along the line of force but also show growth in size to form a cardiac syncytium. These matrices exhibited anatomical physiological and histological features analogous to the myocardial syncytium. Moreover, it was possible to measure the force and rate of contraction exhibited by this model (Eschenhagen et al. 2012, Hirt et al. 2014) (Zimmermann et al., 2002). The individual EHT constructs can be fused to generate larger constructs and these constructs exhibit force of contraction comparable to the human myocardial tissue (Zimmermann et al. 2006). The construction methodology and the kinetics depicted by the EHTs have improved ever since. Lately, Kensah et al. have established a model of the generation of EHTs with force of contraction only 5-fold less than that of the normal human myocardium (Kensah et al., 2013).

In our lab, (BHF Regeneration Medical Centre of Imperial College) the methodology of EHTs had been developed and tested. The preliminary data was collected from EHT constructs with cardiomyocytes only and later on fibroblasts were added to the constructs alongside cardiomyocytes. Murine ventricular fibroblasts were used to study the effect of fibroblasts on iPSC-derived cardiomyocytes initially, and this was followed by human foetal fibroblasts and adult atrial and ventricular fibroblasts. It had been shown that addition of the fibroblasts to the EHT construct leads to enhanced inotropic effect, sarcomeric gene expression, calcium transient acceleration and exhibits further maturation of troponin and myosin (Owen T, Harding S). (Fig 1.8) This data reiterated the hypothesis of previous studies that

fibroblasts improve the connections between the cardiomyocytes and hence improves the contractile force alongside the maturation of the tissue (Parrag, Zandstra and Woodhouse, 2012) (Liau et al. 2012).

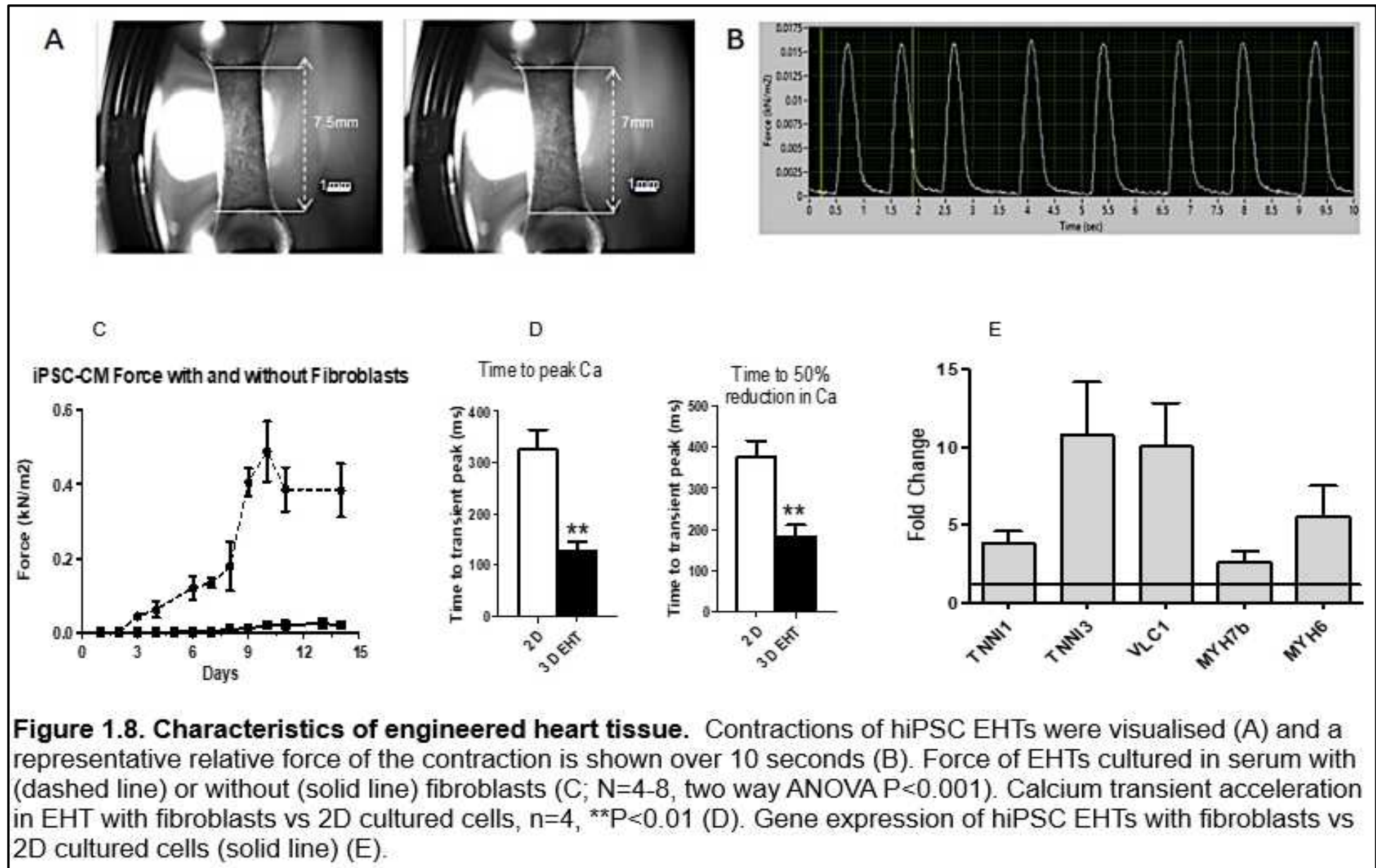
In the adult, the human heart, not only do the chambers have different anatomy, but they are subjected to different pressures and biochemical environment. Moreover, the same disease pattern affecting different chambers leads to variable outcomes clinically, and the pathological features vary as well. The ventricular fibroblasts used for the pilot data were harvested from the native heart (the patients were having end stage cardiac failure either due to dilated or ischaemic myopathy and hence were on the transplant list) of transplants recipients. The myocardial tissue was extremely hypertrophied with extensive fibrosis all through the muscle layers. The hypertrophy and fibrosis had a bearing on the cellular characteristics of the native fibroblast population. It was presumed that the addition of these fibroblasts to the EHTs could affect their morphology and physiological characteristics. This necessitated the study to investigate the role and impact of fibroblasts from the normal functioning heart in EHTs. In this study, for the first time, the human cardiac fibroblasts from different chambers from the same patient are going to be added to the EHTs to compare their effectiveness. However, there are some limitations as tissue samples obtained from the ventricular biopsies were very small and only a few samples could generate enough fibroblast count at the P2 stage so that it can be used to construct EHTs. The results obtained from these chamber specific fibroblasts co-cultured with iPSC-CM were still able to bring forward some valuable information with regards to any chamber specific behaviours of the fibroblasts.

The other limiting factor was the difficulty in obtaining and isolating cardiomyocytes from the adult human heart and using them for these constructs. Hence, human iPSC-CM were

primarily used to generate the human EHTs. Human iPSC-CM had been used in different studies to construct EHTs that exhibit morphological and functional characteristics mirroring human myocardial tissue (Tulloch et al. 2011) (Schaaf et al. 2011) (Kensah et al., 2013) (Thanvandiran et al. 2013) (Nunes et al. 2013). Although iPSC-CM are extensively used in the many studies their morphological feature, functional characteristics, phenotype, gene expression and calcium transients are more comparable to foetal cardiomyocytes than adult cardiomyocytes (Gai et al. 2009) (Itzhaki et al. 2011) (Ma et al. 2011) (Rao et al. 2013).

The BHF Regenerative Medicine Centre based at Imperial College has developed the EHT methodology. Fig 1.8 shows the morphology of the EHT and an example trace (A, B). Recently we showed that introduction of human foetal cardiac fibroblasts into the EHT with human iPSC-CM led to the markedly enhanced force of contraction (Fig 1.8-C). This was not due only to structural effects since calcium transients were markedly accelerated (Fig 1.8-D). Importantly, when iPSC-CM were co-cultured with fibroblasts, there was a strong induction of sarcomeric genes in EHTs, as well as a shift to the more mature cardiac forms of troponin I and myosin (Fig 1.8-E). All isoforms showed an induction over what is seen in fibroblast-depleted 2D culture. Ventricular light chain (VLC-1) was highly induced around 15-fold. Moreover, both the cardiac isoforms of troponin I and myosin heavy chain (TNNI3, 15-fold, and MYH6, 9-fold, respectively) were induced over their immature counterparts (TNNI 5-fold and MYH7 3-fold, respectively. (Adapted from on-going lab work of S, Harding, Owen T)

Similarly, other studies have shown the beneficial effects of stromal cells on the survival, spreading and functional maturation of murine and iPSC-derived cardiomyocytes (Caspi et al., 2007) (Xi et al., 2011). The force exhibited by EHTs is based on the contractile potential of cardiomyocytes. The cardiomyocytes contract due to a well-defined interplay of intrinsic



electrical activity, myofilament activation, and calcium current handling (Mannhardt et al., 2016).

Different models depict the automaticity and chronotropy as a surrogate of the Membrane Clock Model and other as Calcium Clock Model. In “Membrane Clock” model, the inward directed Na^+/K^+ current is activated at negative voltages by membrane hyperpolarisation and is directly regulated by cAMP (Brown, DiFrancesco and Noble, 1979) (O’Regan and Gratzel, 1991). In the “Calcium Clock” model, the diastolic depolarisation is a spontaneous recurrent phenomenon secondary to the release of Ca^{2+} from the sarcoplasmic reticulum, activating the $\text{Na}^+/\text{Ca}^{2+}$ exchanger. This NCX symport mediated inward current depolarises the membrane voltage-gated channels to the threshold of the action potential (Vinogradova et al., 2002) (O. Huang, L.H. Christopher, R. Solaro, 2016). The T-Type and L-Type calcium channels are known to play an important role in the Calcium Clock model. Recently, an updated “Coupled-Clock” model has amalgamated both previous models. In the coupled-clock model, the membrane ionic channels mediated depolarisation and spontaneous release of Ca^{2+} from SR equally participate to generate automaticity. The dynamic and synergistic coupling of both the clocks is crucial for the generation of the normal chronotropic response of the individual myocytes. The sarcolemmal membrane and sarcoplasmic reticulum (SR) are designated calcium and membrane chronometers of the cells. There is compelling evidence suggestive of crosstalk of the clocks works on a beat-to-beat basis and determines the action potential, rate and rhythm (Yaniv, Lakatta and Maltsev, 2015) (Lakatta, Maltsev and Vinogradova, 2011).

In sinoatrial node (SAN) cells, the electrical rhythm is generated by a complex process of ionic fluxes across the sarcolemmal membrane. At the cellular level, different classes of ionic channels of the cell membrane, sarcoplasmic reticulum and mitochondria contribute to

the generation, propagation, and regulation of impulses. The intrinsic chronotropic response of different cardiomyocyte groups is variable and is dependent on the various signalling mechanisms originating at multiple levels. These include subcellular, cellular, neurohormonal, brain-heart signalling and are modulated by chemical, mechanical, environmental and thermal factors. The rate of contraction of the SAN is more than the Purkinje fibres. The precise functional roles and interactions of all these mechanisms are still not fully understood (Mesirca, Torrente and Mangoni, 2015) (Yaniv, Lakatta and Maltsev, 2015).

Similarly, the mechanism of the beating of hiPSC-CM is not well understood. I_{f} current is presumed to be responsible for the chronotropic effects in hiPSC cardiomyocytes and is carried by both $\text{Na}^{+}/\text{K}^{+}$ antiport channel across the sarcolemma. During diastole, the channel is activated on hyperpolarisation with an inwardly directed current and is characterised by low single-channel conductance and activation kinetics. Intracellularly, the I_{f} current is activated by cyclic adenosine monophosphate (cAMP), due to hyperpolarisation-activated cyclic nucleotide-gated family of ion channels (O'Regan and Gratzel, 1991) (Ludwig et al., 1998). Many studies have proven the role of I_{f} by pharmacological blocking (with Ivabradine) of I_{f} current in cardiomyocytes, leading to a negative chronotropic effect. Therefore, this current is considered as a Membrane Clock for generation of an impulse and rate of contraction in hiPSC-CM. B The Calcium and Membrane Clock mechanisms are established as pacemaker mechanism responsible for automaticity and chronotropy of the iPSC-EHTs (Sulfi and Timmis, 2006) (Mannhardt et al., 2016).

An important physiological phenomenon of Force-Frequency Response (FFR) is exhibited by all normal rodent and mammalian hearts. The generation of FFR is dependent on the maturation of the cardiomyocytes and is positive in a normally functioning heart while the

failing heart loses the ability to demonstrate it. The human embryonic heart starts beating at three weeks of gestation at a rate of 80bpm and this rate increases incrementally to 180 bpm at nine weeks of gestation (DuBose, Cunyus and Johnson, 1990) (Goenezen, Rennie and Rugonyi, 2013). After nine weeks of gestation till the time of birth, there is a median increase of 0.4bpm (Pildner von Steinburg et al., 2013). The estimated recorded frequency during this phase is between 4Hz – 8Hz. This increase in rate is critical in the development of the chambers and maturation of cardiomyocytes. When the replication of this model in vitro was carried out by stimulating the EHTs at 4 Hz, it showed a reduced calcium sensitivity, frequency-dependent acceleration of relaxation, and enhanced post-rest potentiation. At the cellular level, there was enhanced T-tubule formation, improved calcium storage, increased amounts of SERCA2a and RyR2 proteins and release capacity of the sarcoplasmic reticulum. The physiological chronotropic response of the myocardial tissue generates mechanical activity and hence a normal FFR.

Recently Godier-Furnémont et al. have shown a relationship between the rate of contraction and FFR (Godier-Furnémont et al., 2015). A modulation of chronotropic response is important not only in the embryonic state but to maintain a normal healthy myocardial activity. This pilot study was based on the hypothesis that constructs with fibroblasts of different origin will exhibit different chronotropic response. To study the baseline difference in the rate of contraction of these construct, no fixed electrical stimulation was used at any point.

Co-culture with fibroblasts were shown to enhance the differentiated phenotype of stem cell-derived cardiomyocytes; indicated by cell organisation, shape, dimensions, and sarcomere formation. Nevertheless, the influence of scaffold architecture and co-culture with human cardiac fibroblasts on maturity and construct functionality of EHTs needs further

investigation. The precise mechanism of cardiomyocytes and non-cardiomyocytes interaction in the context of these effects is unidentified: a paracrine and extracellular matrix-mediated effect of the fibroblasts on the iPSC-CM is a strong possibility but electrical coupling between fibroblasts and cardiomyocytes has been reported, and simple mechanical/structural support may play a part.

The potential role of adult human cardiac fibroblasts from different chambers of the human heart is used in this study to find out if one chamber is better than other on generating EHTs with better contractility. Following characterisation, cardiac fibroblasts isolated from all four chambers (from a patient with preserved biventricular function) are introduced into the EHTs. The impact of the introduction of fibroblasts from different cardiac chambers regarding contractility and sarcomere gene expression in EHT's is the prime focus.

1.17 HYPOTHESIS

I hypothesise that there is a significant variation between the characteristics of atrial and ventricular fibroblasts. This difference will be altered by the disease process, and these differences will affect the participation of fibroblasts in regenerative strategies especially the construction of EHT.

To investigate my hypothesis, I have further divided the hypothesis into two sub-hypotheses

1. My first sub-hypothesis is that all four chambers of the heart are geometrically, anatomically and physiologically different from each other and this variation is conveyed to the constituent cells. I am hypothesising that human cardiac fibroblasts from different chambers have different growth rate, pattern, proliferating ability and expression of cardiogenic transcription factors.
2. My second sub-hypothesis is that when these fibroblasts from different chambers are co-cultured with iPSC-CM then the rate of contraction, the force of contraction, secretory profile and gene expression of the engineered heart tissue will be different. If a difference between chambers is found, the underlying mechanism will be explored.

1.18 AIMS

The project consists of the following objectives.

1. To isolate, identify and quantify human cardiac fibroblasts in myocardial samples taken from all four cardiac chambers in the same patient with preserved biventricular function.
2. To investigate the rate of growth, the pattern of growth, proliferating ability of fibroblasts isolated from different chambers with a view to find a difference due to their chamber specificity.
3. To investigate the difference in expression of transcription factors among the chamber-specific fibroblasts.
4. To evaluate the effect of adult human cardiac fibroblasts from all four chambers on the contractile function of the iPSC-CM in engineered heart tissue. If a difference between chambers is found, the underlying mechanism will be explored.
5. To further investigate the paracrine effect of chamber-specific human cardiac fibroblasts (non-cardiomyocytes) on iPSC-CM in EHTs.
6. To identify any difference in the histology of EHTs developed with chamber specific fibroblasts.

CHAPTER 2: MATERIALS AND METHODS

NRES Committee London - Harrow

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Telephone: 01173421334

31 March 2015

Mr Prakash Punjabi
Imperial College Healthcare NHS Trust
Du Cane Road
London
W12 0HS

Dear Mr Punjabi

Study title: The characterisation of chamber and disease-specific fibroblasts from adult human heart and investigation of their potential contribution to myocardial regeneration.

REC reference: 15/LO/0098

Protocol number: N/A

IRAS project ID: 164456

Thank you for your email of 31 March 2015. I can confirm the REC has received the documents listed below and that these comply with the approval conditions detailed in our letter dated 27 March 2015

Documents received

The documents received were as follows:

Document	Version	Date
GP/consultant information sheets or letters [Discover III]	1.3	29 March 2015
Participant information sheet (PIS) [Participant Information Sheet (PIS)]	1.4	29 March 2015

Approved documents

The final list of approved documentation for the study is therefore as follows:

Document	Version	Date
Covering letter on headed paper		24 December 2014
Covering letter on headed paper		05 March 2015
GP/consultant information sheets or letters [Discover III]	1.3	29 March 2015
IRAS Checklist XML [Checklist_06012015]		06 January 2015
IRAS Checklist XML [Checklist_06032015]		06 March 2015
IRAS Checklist XML [Checklist_09032015]		09 March 2015
IRAS Checklist XML [Checklist_30032015]		30 March 2015
Letter from sponsor		23 December 2014
Letters of invitation to participant	1.2	18 February 2015
Participant consent form	1.2	18 February 2015
Participant information sheet (PIS) [Participant Information Sheet (PIS)]	1.4	29 March 2015

REC Application Form [REC_Form_06032015]		06 March 2015
Research protocol or project proposal	1.1	09 December 2014
Summary CV for Chief Investigator (CI)		09 December 2014
Summary CV for student		

You should ensure that the sponsor has a copy of the final documentation for the study. It is the sponsor's responsibility to ensure that the documentation is made available to R&D offices at all participating sites.

15/LO/0098 **Please quote this number on all correspondence**

Yours sincerely



Natasha Bridgeman
REC Assistant

E-mail: nrescommittee.london-harrow@nhs.net

Copy to: Miss Becky Ward, Joint Research Compliance Office, Imperial College Healthcare NHS Trust

23/12/2014

Mr Prakash Punjabi
Consultant Cardiothoracic Surgeon
Honorary Clinical Senior Lecturer
National Heart and Lung Institute
Imperial College London
DuCane Road
London, W12 0HS

Becky Ward
Research Governance Manager

Dear Mr Punjabi

RE: The characterisation of chamber and disease specific fibroblasts from adult human heart and investigation of their potential contribution to myocardial regeneration (The Discover III Study)

Joint Research Compliance Office Reference number: 14HH2440

This is to confirm that the above named research project utilises human participants, their organs, tissue and/or data as defined under the sponsorship requirements of the Research Governance Framework for Health and Social Care 2005, incorporating the Medicines for Human Use (Clinical Trials) Regulations 2004.

On behalf of Imperial College Healthcare NHS Trust, we undertake to act as the identified Research Sponsor for this project.

This letter confirms:

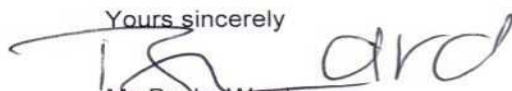
- The research proposal has been discussed, assessed and registered with the Joint Research Compliance Office of Imperial College Academic Health Science Centre, Imperial College Healthcare NHS Trust and provisional sponsor approval granted.
- The Chief Investigator has undergone a process of scientific critique commensurate with the scale of the project.
- Imperial College Healthcare NHS Trust will indemnify the project under standard NHS Hospital Indemnity for negligent or wrongful harm this will be provided under clinical negligence scheme for Trusts clinical risk management NHS Litigation Authority (NHSLA) for NHS Trusts in England.
- Resources and support are available to the research team to aid delivery of the research as proposed.
- Management, monitoring and reporting responsibilities for the research have been approved.
- Imperial College Healthcare NHS Trust will undertake and enforce those sponsor duties set out in the NHS Research Governance Framework for Health and Social Care.

Imperial College Healthcare NHS Trust Sponsorship is conditional on the project receiving applicable ethical and regulatory approval for all research related aspects of its conduct. It is also conditional on successful contract and agreement negotiations and sign off via the Joint Research Office of Imperial College Academic Health Science Centre, where relevant, and before the study commences.

A copy of the ethics approval letter **must** be sent to the Research Governance Manager prior to the study commencing.

Sponsorship is dependent on obtaining R&D Office approval for all NHS sites where the research is being conducted.

Yours sincerely



Ms Becky Ward
Research Governance Manager

2.2 MATERIALS

2.2.1 MATERIALS AND REAGENTS FOR ISOLATION OF HUMAN CARDIAC FIBROBLASTS

1. TRANSPORT MEDIUM FOR HARVESTED TISSUE

TRANSPORT MEDIUM FOR HARVESTED TISSUE – 120mls			
	Constituents	Final concentration	Supplier
1.	Dulbecco Modified Eagles Medium	41% (60ml)	GIBCO
2.	Cardioplegia solution	25% (15ml)	BAXTER
3.	Normal Saline solution	0.9% (45ml)	B BRAUN
4.	Penicillin streptomycin + Amphotericin	1% (1.2ml)	SIGMA

2. ISOLATION KIT

Dissection Kit		
	Instruments	Suppliers
1.	Disposable B/S Super snip Scissors	ROCIALLE
2.	Forceps Griprite Blue Sterile Single Wrapped	ROCIALLE
3.	120ml Single Wrapped Sterile Gallipot	ROCIALLE
4.	Iris Scissors Straight 11cm	ROCIALLE
5.	Petri dishes, polystyrene size 60 mm × 15 mm	SIGMA
6.	Disposable Sterile Scalpels	SWAN MORTON
7.	Ring tip, fine DeBakys forceps	SCANLAN
8.	Corning Square Tissue Culture Dish, Sterile	E&K Scientific
9.	Sterile gloves	Gammex

3. FIBROBLAST GROWTH MEDIUM (FGM)

CELL CULTURE MEDIUM – 500mls				
	Reagents	Final Concentration	Volume	Suppliers
1.	Dulbecco Modified Eagles Medium (low Glucose)	90%	440ml	GIBCO
2.	Foetal Bovine Serum	10%	50ml	GIBCO
3.	Human Insulin	1µg/ml	5ml	SIGMA
4.	Penicillin/ Streptomycin/ Amphotericin	1%	5 ml	SIGMA
5.	Fibroblast Growth Factor - 2	10 ng/ml	5ug	SIGMA
6.	L - glutamine	500µl	0.5ml	SIGMA
7.	Ascorbic acid	500µl	0.5ml	SIGMA

4. FIBROBLASTS ISOLATION REAGENTS – EXPLANT TECHNIQUE

FIRBOBLAST ISOLATION REAGENTS - EXPLANT TECHNIQUE			
	Reagents	Final Concentration	Suppliers
1.	Phosphate Buffer Saline		SIGMA
2.	Fibronectin	0.1%	SIGMA
3.	Trypsin - EDTA	0.05%	SIGMA
4.	Penicillin/ Streptomycin/ Amphotericin	1%	SIGMA
5.	Dulbecco Modified Eagles Medium		GIBCO

5. FIBROBLASTS ISOLATION REAGENTS – ENZYMATIC DIGESTION TECHNIQUE

FIRBOBLAST ISOLATION REAGENTS – ENZYMATIC DIGESTION TECHNIQUE			
	Reagents	Final Concentration	Suppliers
1.	Phosphate Buffer Saline		SIGMA
2.	Fibronectin	0.1%	SIGMA
3.	Trypsin - EDTA	0.05%	SIGMA
4.	Penicillin/ Streptomycin/ Amphotericin	1% & 2%	SIGMA
5.	Dulbecco Modified Eagles Medium		GIBCO
6.	Collagenase Type V	1mg/ml	SIGMA
7.	Proteinase	36mg/100ml	SIGMA

6. LOW CALCIUM SOLUTION

LOW CALCIUM SOLUTION			
	Reagents	Final Concentration	Suppliers
1.	Glucose	20mM	SIGMA
2.	Magnesium Sulphate (MgSO ₄)	5mM	BDH-GPR
3.	Pyruvate	5mM	SIGMA
4.	Potassium Chloride (KCl)	5.4mM	SIGMA
5.	Sodium Chloride (NaCl)	120mM	BDH- <u>AnalaR</u>
6.	Taurine	20mM	SIGMA
7.	1[4-(2-hydroxyethyl) – 1- piperazinyl] ethane Sulphonic acid	10mM	SIGMA
8.	Nitrilotriacetic acid (NTA)	10mM	
9.	ddH ₂ O	1 lit	

2.2.2 MATERIALS AND REAGENTS FOR IMMUNOSTAINING OF HUMAN CARDIAC FIBROBLASTS

1. REAGENTS USED FOR IMMUNOSTAINING

REAGENT FOR IMMUNOSTAINING			
	Reagents	Final Concentration	Suppliers
1.	Phosphate Buffer Saline	No dilution	SIGMA
2.	Paraformaldehyde	4%	SIGMA
3.	Triton X-100	0.5%	BioXtra
4.	Bovine Serum Albumin	3%	SIGMA
5.	Primary Antibodies	1:1000	ABCAM
6.	Secondary Antibodies	1:1000	ABCAM
7.	DAPI	1:2000	SIGMA
8.	Parmaflour – mounting medium	Neat	THERMO-SCI
9.	Chamber Slides		SIGMA

2.2.3 WESTERN BLOTTING SOLUTIONS AND GELS

1. REAGENTS USED FOR WESTERN BLOTTING

REAGENTS FOR WESTERN BLOTTING				
	Reagents	Final Concentration	Volume	Suppliers
1.	Phosphate Buffer Saline	No dilution		SIGMA
2.	RIPA-lysis buffer	10mls + 1 tab protease inhibitor	80-200ul	FISHER
3.	Bovine Serum Albumin	2mg/ml		FISHER
4.	Copper Sulphate (CuSO ₄)	1:50	1ml	
5.	Bicinchoninic Acid	50:1	49mls	
6.	NuPage Novex 10X Bis-Tris Gel			FISHER
7.	Bovine Serum Albumin	3% w/v		FISHER
8.	Dithiothreitol (DTT)			FISHER
9.	Lithium Dodecyl Sulfate (LDS)			FISHER
10.	Condensed milk	3%	50mls	
11.	Ponceau S	0.1%		SIGMA
12.	Acetic Acid	5%		
13.	ECL Solution (2 reagents)	1:1		FISHER

2. PAGE BUFFER

PAGE BUFFER FOR WESTERN BLOTTING				
	Reagents	Stock Solution	Final Concentration	Suppliers
10X SDS Page Buffer (2L)				
1.	Trisbase	60.6g		SIGMA
2.	Glycine	288.4g		SIGMA
3.	SDS	100ml		FISHER
4.	dH ₂ O	2L		
1X SDS Page Buffer				
1.	SDS Buffer	100ml	1%	FISHER
2.	dH ₂ O	900ml		

3. TRANSFER BUFFER

TRANSFER BUFFER FOR WESTERN BLOTTING				
	Reagents	Stock Solution	Final Concentration	Suppliers
10X Transfer Buffer (1L)				
1.	TrisBase	30.27g		SIGMA
2.	Glycine	144.0g		SIGMA
4.	dH ₂ O	1L		
1X Transfer Buffer (2L)				
1.	Methanol	400ml	1X	FISHER
2.	TrisBase (10X)	200ml		
3.	dH ₂ O	1400ml		

2.2.4 REAGENTS & CULTURE MEDIUM FOR CARDIOMYOCYTE DISSOCIATION & DIFFERENTIATION

1. CARDIOMYOCYTES DISSOCIATION REAGENTS

REAGENTS FOR CARDIOMYOCYTE DISSOCIATION			
	Reagents	Final Concentration	Suppliers
1.	Roswell Park Memorial Institute - RPMI	No dilution	GIBCO
2.	DNase-II (Type V)	24µg/ml	SIGMA
3.	Collagenase II	200U/ml	GIBCO
4.	HBSS	3%	GIBCO
5.	BTS-solution	30mM	GIBCO
6.	HEPES	1M	GIBCO
7.	Foetal Bovine Serum	90%	GIBCO
8.	Dimethyl Sulfoxide	10%	GIBCO
9.	Foetal Bovine Serum	10%	GIBCO

2. E8 CULTURE MEDIUM COMPONENTS – 1000mls

E8 CULTURE MEDIUM COMPONENTS – 1000mls					
	Reagents	Stock concentration	Final concentration	Final Volume	Supplier
1.	DMEM/F12 with L-glutamine + HEPES	Neat		1 litre	INVITROGEN
2.	Ascorbic Acid 2 - Phosphate	64mg/ml	64µg/ml	1ml	SIGMA
3.	Sodium Selenite	70µg/ml	14ng/ml	200µl	SIGMA
4.	Transferrin	50mg/ml	10µl/ml	214µl	SIGMA
5.	Insulin	4mg/ml	20µg/ml	5ml	INVITROGEN
6.	FGF-2	200ng/ml	100ng/ml	500µl	PEPROTECH
7.	TGF-β1	100ng/ml	2ng/ml	20µl	PEPROTECH
8.	Penicillin/ Streptomycin/ Amphotericin	Neat	0.5%	5ml	SIGMA

3. COLLAGENASE-II SOLUTION CONSTITUENTS

COLLAGENASE II SOLUTION			
	Reagents	Final Concentration	Suppliers
1.	HBSS without Ca ⁺⁺ /Mg ⁺⁺		GIBCO
2.	Collagenase	200U/ml	GIBCO
3.	HEPES	10mM	GIBCO
4.	ROCK inhibitor (Y-27632)	10µM	GIBCO
5.	BTS	30µM	GIBCO

2.2.5 REAGENTS & CULTURE MEDIUM FOR ENGINEERED HEART TISSUE

1. EHT CULTURE MEDIUM

EHT CULTURE MEDIUM – 500mls (SAMATA's PROTOCOL)				
	Reagents	Final Concentration	Volume	Suppliers
1.	Dulbecco Modified Eagles Medium (low Glucose)	90%	441.5ml	GIBCO
2.	Horse Serum	10%	50ml	GIBCO
3.	Human Insulin	1µl/ml	0.5ml	SIGMA
4.	Penicillin/ Streptomycin/ Amphotericin	1%	5 ml	SIGMA
5.	Aprotinin	0.1%	0.5ml	SIGMA
6.	Ascorbic acid		0.5ml	SIGMA
7.	Tranexamic Acid		2ml	SIGMA

2. REAGENTS FOR THE ENGINEERED HEART TISSUE HARVESTING

REAGENTS FOR THE ENGINEERED HEART TISSUE HARVESTING				
	Reagents	Stock Solution	Final Concentration	Suppliers
1.	Fibrinogen	200mg/ml		SIGMA
2.	Thrombin	100U/ml		BIOPUR
3.	Agarose		2%	INVITROGIN
4.	10X DMEM	135mg/ml	5.4%	GIBCO
5.	Chick Embryo Extract		2%	
6.	Insulin		0.1%	SIGMA
7.	Foetal Calf Serum		10%	GIBCO
8.	Glutamine	200mM	1%	SIGMA
9.	Tranexamic Acid	100mM	78.6mg/5ml	SIGMA
10.	Aprotinin	100mg/3ml	33/3mg/ml	SIGMA
11.	Ascorbic Acid	30mM	58.2mg/10ml	SIGMA
12.	Phosphate Buffer Saline	No dilution		SIGMA

3. REAGENTS FOR PROTEOMIC ANALYSIS

REAGENTS FOR PROTEOMIC ANALYSIS OF EHT BATHING MEDIUM				
	Reagents	Description	Final Concentration	Suppliers
1.	Human XL Cytokine Array	Nitrocellulose membrane, each containing 105 different antibodies	-	R&D
2.	Array Buffer 4	Buffered protein base + preservative	Neat	R&D
3.	Array Buffer 6	Buffered protein base + preservative	Neat	R&D
4.	Wash Buffer	Concentration solution of buffered surfactant with preservative	1:24	R&D
5.	Detection Antibody Cocktail	Biotinylated antibody cocktail	200µl	R&D
6.	Chemi Reagent 1	Stabilised H ₂ O ₂ with preservative	1 Part	R&D
7.	Chemi Reagent 2	Stabilised luminol with preservative	1 Part	R&D
8.	1X Streptavidin HRP	200µl of Streptavidin conjugate to horseradish -peroxide	1:2000	R&D
9.	4-Well Multidish	Clear 4-well rectangular multidish		R&D

2.3 METHODS

2.3.1 HUMAN CARDIAC FIBROBLAST ISOLATION

The isolation of human CF was employed from myocardial samples taken from all four cardiac chambers using the explant culture technique and enzymatic digestion technique. The harvested tissue samples were kept in cardioplegia solution mixed with DMEM + 10%FBS + 1% Penicillin/Streptomycin/ Amphotericin (Fibroblasts growth medium (FGM)) and were swiftly moved to the nearby ICTEM building, where the CFs were isolated and cultured with already established lab-based protocols. The sterile culture plates were coated with 1% Fibronectin and kept in 5% CO₂ incubator for 30min after which the fibronectin solution was removed, and the culture plates were left with FGM.

1. TISSUE EXPLANTATION TECHNIQUE

The collected tissue samples were washed 2-3 times with PBS and chopped into small pieces (2-3mm). The tissue was mildly digested with 0.05mg/ml trypsin for 3 mins. The trypsin solution was washed away with PBS 2-3 times. In the meantime, 1% Fibronectin coated 6cm petridishes (1-2ml per dish) were incubated for 30 mins. After 30 mins fibronectin was removed and petridishes were washed with PBS and 0.5ml of FGM (DMEM + 10%FBS + 1% Penicillin/Streptomycin/Amphotericin) was added to each petri dish. The explanted tissue was plated in these fibronectin coated petri dishes in rows maintaining 2-3cm distanced between the adjacent tissue samples. 2ml of FGM was added to individual petri dish for tissue sustenance until confluence was reached. During this stage, the FGM was changed every 2-3 days, and the culture plates were reviewed for tissue growth on day 5, 10 and 15. Once the confluence of fibroblasts was achieved (which usually took 2-3 weeks depending on the atrial or ventricular samples) they samples were passaged 2-3 times before the immunostaining.

2. TISSUE ENZYMATIC DIGESTION TECHNIQUE

The tissue sample was washed 2-3 times with PBS mixed with 2% antibiotics (penicillin/streptomycin/amphotericin) and was chopped into small pieces (2-3mm). The tissue sample was then collected in a Falcon tube and depending upon the size of sample 10-20ml of proteinase enzymatic solution (36mg/100mls) was added to it. The Falcon tube with tissue and proteinase enzymatic solution was agitated in a water bath for 20min and following that the supernatant, rich in cardiomyocytes and endothelial cells was removed. Following this, 10-15ml of collagenase V enzymatic solution (1mg/ml) was added to the Falcon tube, and the tube was agitated again in a water bath for 10min.

The supernatant was collected, and an equal volume of (1:1) of FGM was added to it. The digestion with collagenase enzyme was repeated 2-3 times, and the supernatants were collected together and spun for 5min at 1200 rpm. The pellet at the base was mixed with PBS and spun again for 5min, 2-3 times till the supernatant was clear. The pellet at the bottom of falcon tube (rich in fibroblasts) was mixed with FB growth medium and was plated. The plated fibroblasts were washed after 1-2h and then again after 12-24h. Depending upon the amount of debris, the isolated CFs were repeatedly washed in the first 48h followed by changing of the medium. The enzymatic digestion technique yielded CFs in 7-10 days before they can be passaged.

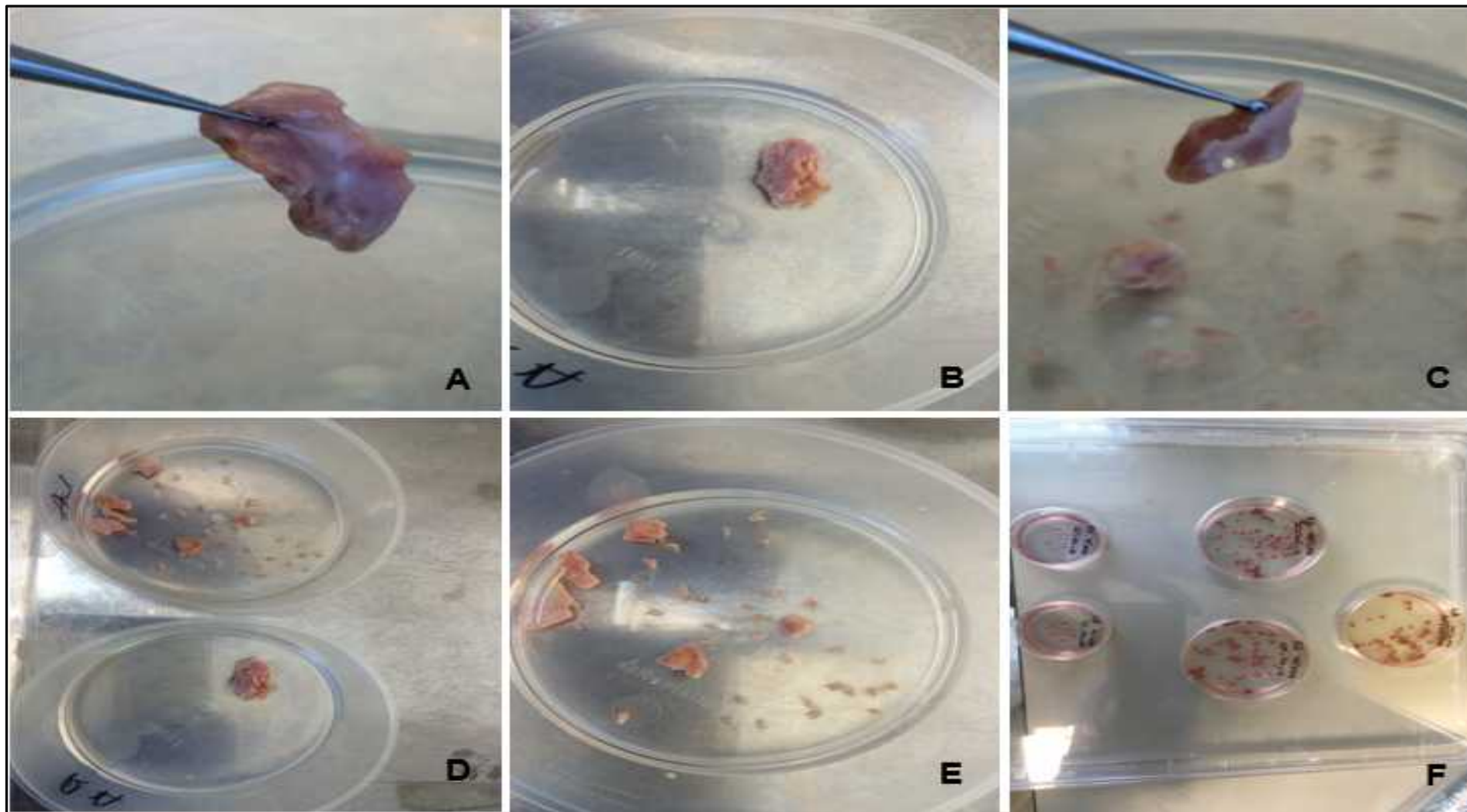


Fig. 2.1. PROCESSING OF HUMAN CARDIAC TISSUE AND ISOLATION OF CARDIAC FIBROBLASTS. The Initial steps of isolation are similar in both enzymatic and tissue explantation technique. (A, B) showed a 2 x 3 cm tissue sample from RA. (C, D, E) Tissue samples washed with PS containing PBS were divided into small pieces in explant technique. (F) Small tissue samples were plated in Fibronectin coated Petridishes.

2.3.2 HUMAN CARDIAC FIBROBLASTS GROWTH PATTERN ASSESSMENT

The rate and pattern of growth of human CFs plated from all four chambers were periodically assessed.

a) In the explant technique, the growth before P1 and after first 2 passages, i.e., P1 & P2 stage was assessed on day 5, 10 and 15. This technique yielded fibroblasts at a relatively slower pace than the enzymatic digestion.

b) In enzymatic digestion technique, the fibroblasts before first passage and at P1 & P2 stage were reviewed at day 3, 5, 7 and 10 as fibroblasts proliferate more rapidly. The cells can be reviewed at different intervals due to different specific reasons.

2.3.3 HUMAN CARDIAC FIBROBLAST COUNTING

1. COUNTING WITH HAEMOCYTOMETER

The number of fibroblasts was counted at different stages of growth. This was done at the time of passaging to the next stage. The optimal growth and confluency were checked under the microscope, and an assessment was made for the possible passage to next stage (either P1 or P2). After washing the cells, 0.05% Trypsin with EDTA was added, and FBs were kept in an incubator for 1-2 min. The plated FBs were shaken, tapped and reviewed under the microscope to ensure they were lifted from the bottom and then FGM is added to neutralise Trypsin (1:1). The entire solution with fibroblasts was aspirated and moved to a falcon tube, and then it is spun for 5 min at 1200rpm. The supernatant was discarded, and the pellet at the bottom was mixed with some fresh FGM. At this point, 10µl was aspirated from the solution and was mixed with 10µl of trypan blue dye. This staining method, also known as dye exclusion staining, uses a diazo dye that selectively penetrates cell membranes of dead cells, colouring them blue, whereas it is not absorbed by membranes of live cells, thus

excluding live cells from staining. After this 10µl was aspirated from this mixture and was loaded on a haemocytometer. Once the total cell count was obtained, cell concentration can be calculated from the following formula:

$$\text{Total cell/ml} = \text{Total cells count} \times \frac{\text{Dilution factor}}{\text{No of squares}} \times 10,000 \text{ cells/ml}$$

2. COUNTING WITH BRIGHT FIELD CELL COUNTER

The Luna-FL™ is a fluorescence automated cell counter. It is a quantum leap for automated cell counting and cell viability analysis. The Luna-FL™ automated cell counter gives you the sensitive and accurate live/dead cell counting results without limitation of sample types. The precision two-colour fluorescence microscope optics, high-resolution CCD imager, and the new software algorithm were carefully integrated to achieve excellent cell counting accuracy. Within a $5 \times 10^4 \sim 1 \times 10^7$ cells/ml concentration and $1 \sim 90 \mu\text{m}$ cell diameter range, Luna™ software accurately detects live/dead cells and discriminates cell debris. Individual cells in cell clusters are also successfully counted by Luna's new de-clustering algorithm.

It is difficult to count primary cells with the conventional cell counters such as Coulter counter or image-based automated cell counters utilising only bright field optics. This is partly because primary cells are often contaminated with the undesirable debris which can be misrecognized as cells with the conventional cell counters. Luna-FL™ is integrated with the dual fluorescence microscope optics to overcome this problem. Live/dead cells were stained with the green/red fluorescence dye (Acridine Orange / Propidium Iodide (AO/PI) cell viability kit and the fluorescence-labelled cell images were analysed with the accurate image analysis software.

2.3.4 IMMUNOCYTOCHEMISTRY

Fibroblast monolayers were used once confluence was achieved at the P1 stage and the cells were ready to passage to P2. Fibroblasts at the P2 stage were left in chamber slides or a 12 well plate for 24 h at least. CFs were fixed with 4% PFA for 10min and then washed twice with PBS. Following this 0.5%, Triton was added for 3min to create some pores in the walls of fibroblasts. Once Triton was removed, the CFs were washed twice with PBS and were blocked with 3% Bovine Serum Albumin for 30min. Having BSA removed, primary antibodies diluted in 1.5% Bovine Serum Albumin were added to the blocked fibroblasts for 60min. Following this, the specie-specific secondary antibodies prepared in (1:1000 dilution) were added for 60min. The fibroblasts were washed again twice with BPS and DAPI was added for 10min for staining of nuclei. After removal of DAPI, a mountant was added, and fibroblasts were covered carefully with a coverslip avoiding any bubble trapping between the coverslip and the mountant. The slides were left for drying out overnight and analysed using confocal microscopy.

Endogenous cardiac fibroblasts were categorised and quantified for each myocardial sample from each chamber as:

- a) DDR 2, THY-1, Periostin and Vimentin positive and
- b) α -SMA, vWF, α -cardiac actinin and cardiac MHC negative.

TABLE: 2.1. ANTIBODY DILUTIONS FOR PRIMARY ANTIBODIES

Primary Antibodies								
	<u>Primary Antibodies</u>	<u>Molecular Weight</u>	<u>Species</u>	<u>Incubation Time</u>	<u>Dilution for IF</u>	<u>Dilution for WB</u>	<u>Codes</u>	<u>Company</u>
1.	Anti-DDR-2	97 kDa	Mouse	1h[RT] – O/N [4°C]	1:1000	1:1000	Ab63337	Abcam
2.	Anti-THY-1	18 kDa	Mouse	1h[RT] – O/N [4°C]	1:100	1:1000	Ab23894	Abcam
3.	Anti-Vimentin	54 kDa	Chicken	1h[RT] – O/N [4°C]	1:1000	1:1000	Ab24525	Abcam
4.	Anti-Periostin	100 kDa	Rabbit	1h[RT] – O/N [4°C]	1:200	1:1000	Ab14041	Abcam
5.	Anti-α-SMA	37 kDa	Mouse	1h[RT] – O/N [4°C]	1:200	1:200	Ab7187	Abcam
6.	Anti-α-Actinin	100 kDa	Rat	1h[RT] – O/N [4°C]	1:2000	1:500*	Ab7811	Sigma
7.	Anti-vWF	**	Rabbit	1h[RT] – O/N [4°C]	1:1000	1:1000	Ab6994	Abcam
8.	Anti-MHC	223 kDa	Mouse	1h[RT] – O/N [4°C]	1:1000	1:1000	Ab50967	Abcam
9.	Anti-CD-31	83 kDa	Rabbit	1h[RT] – O/N [4°C]	1:3000	1:1000	Ab76533	Abcam

* For Western Blotting, α -Actinin can be diluted in 1:300.

** The molecular weight of Anti-vWF antibody is 500-2000 kDa.

2.3.5 WESTERN BLOTTING:

1. PROTEIN-ISOLATION (FROM CELLS IN MONOLAYER), USING RIPA-LYSIS BUFFER.

The fibroblast monolayer at the P2 stage was examined under a microscope to confirm the confluence pattern. The cells were washed with 1X PBS for 2-3 times, and 0.05% trypsin was added to the flask. The flask containing the fibroblast monolayer was then placed in an incubator with 5% CO₂ at 37°C for 3-5 minutes. The cells were aspirated, and an equal volume of DMEM was added to neutralise trypsin. The residual fibroblasts which were densely adhered to the bottom of the flask are scrapped off with cell scraper and added to the main volume of cells in the falcon tube. The cells were then spun at 1800 RPM for 10mins. The supernatant was discarded, and the pellet was washed with PBS 2-3 times until the supernatant was clear. Depending upon the size of pellet 80-200µl (usually 80µl is enough for a T-25 flask) of RIPA-lysis buffer containing phosphatases and proteases was added. The falcon tube was vortexed for 1 minute to break all the clumps. The Falcon tube containing fibroblasts in RIPA-lysis buffer was kept on ice for 10-20 minutes to prevent the activity of endogenous proteases. The fibroblasts can be either frozen or can be processed for protein quantification.

2. PROTEIN QUANTIFICATION, USING BCA ASSAY

A standard Bovine Serum Albumin (BSA) solution was prepared in a concentration of 2mg/ml for standard and calibration curves. 100µl of this solution was taken and diluted with 900µl of distilled water to obtain 1000µg/ml of working or stock BSA solution. A calibration curve was made in a duplicate manner:

- Stock solution = 1000µg/ml BSA.
- 75µl of previous solution + 75µl of distilled water = 500µg/ml BSA.

- 75 μ l of previous solution + 75 μ l of distilled water = 250 μ g/ml BSA.
- 75 μ l of previous solution + 75 μ l of distilled water = 125 μ g/ml BSA.
- 75 μ l of previous solution + 75 μ l of distilled water = 62.5 μ g/ml BSA.
- 75 μ l of previous solution + 75 μ l of distilled water = 31.25 μ g/ml BSA.
- 75 μ l of previous solution + 75 μ l of distilled water = 15.6 μ g/ml BSA.
- 75 μ l of previous solution + 75 μ l of distilled water = 7.8 μ g/ml BSA.
- 75 μ l of RIPA buffer = blank.

The protein sample was spun at 1200 RPM for 5min before use and supernatant were collected in a separate tube. This supernatant was rich in proteins. 2.5 μ l of protein sample was mixed with 22.5 μ l of RIPA buffer. A solution of Bicinchoninic acid and Copper Sulphate was prepared in a 1:50 ratio (49mls of BCA + 1ml of 4% Copper Sulphate) and 200 μ l of this solution was added to all wells. In addition to this 25 μ l of standard solution/protein sample/water was added to the wells already filled with 200 μ l of Copper Sulphate (CuSO₄) and BCA solution in duplicates. The proteins in the sample were quantified on a plate reader by measuring absorbance at 562 nm (A_{562}).

The results were divided into several tabs which include the raw data, corrected data, and concentration of the sample. The concentrations graph could be obtained by this as well. To get the actual concentration a mean of 2 readings was obtained.

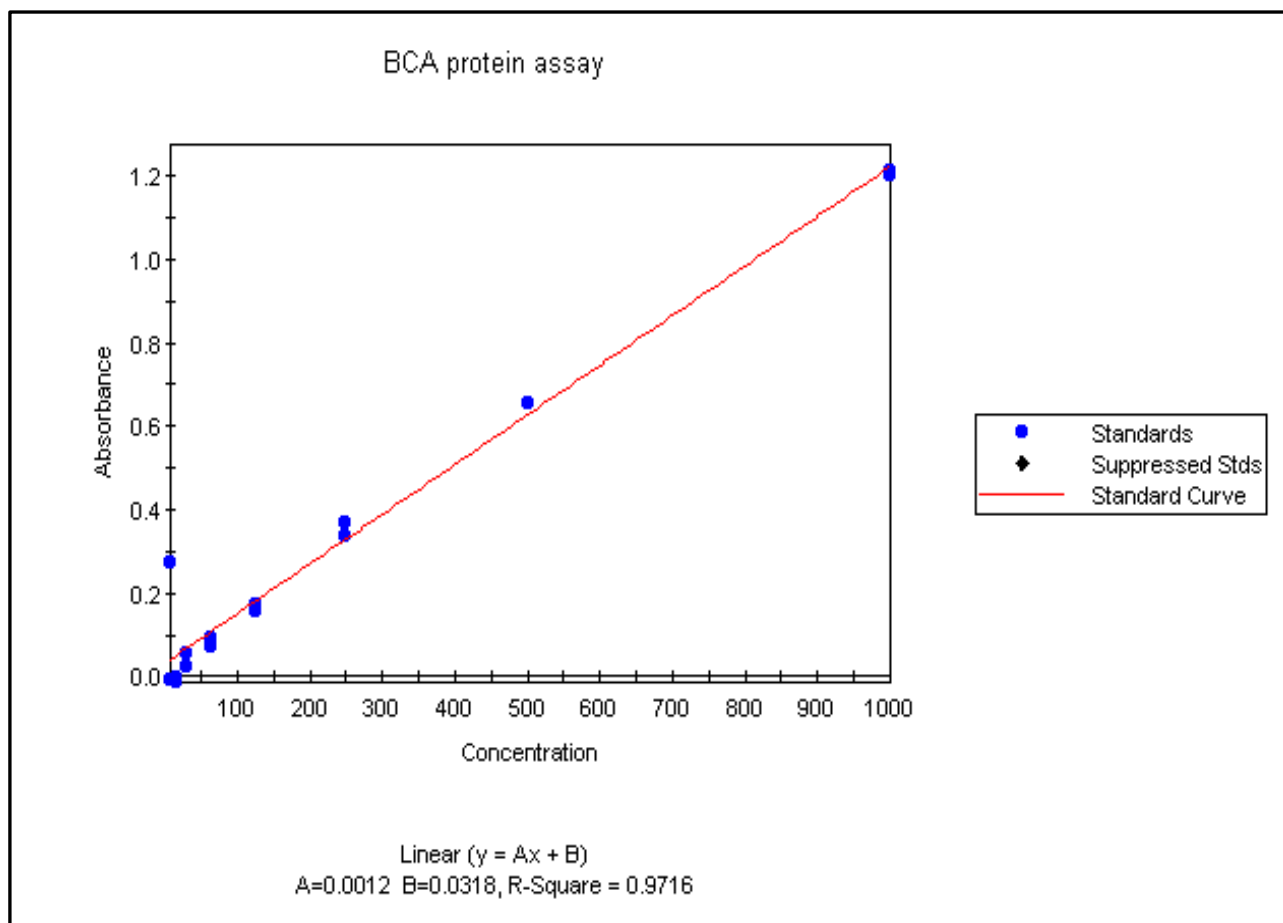


Fig. 2.2 BCA PROTEIN ASSAY

3. WESTERN BLOTTING USING LIFE TECHNOLOGY NuPAGE Novex 10%Bis-Tris GELS AND X-CELLS SURELOCK MINI-CELL

• SAMPLE AND MARKER PREPARATION

The samples and markers were prepared for loading in the gel to a total volume of 25µl per well. The loading volume of protein can be titrated to a concentration of 15µg, 20µg or 30µg. 6.25µl of LDS Sample buffer (NuPAGE) was used per well in 4X concentration. 2.5µl of 10 times concentrated DTT (NuPAGE) was added to each well, as a reducing agent. Distilled water was supplemented to achieve a total volume of 25µl per well. The multiples of protein volume, LDS buffer, DTT and water were titrated as per some wells used.

- **GEL PREPARATION**

A running buffer solution was prepared by diluting 20X MOPS SDS to 1x in distilled water, and a total amount of 800mls was prepared for one XCell Surelock Mini-Cell. The gel was prepared by gently removing the comb at the top end of the gel. One gel contains 15 wells, each having a thickness of 1.5mm. The gel was rinsed with running buffer to get rid of any air bubbles trapped in the wells.

- **LOADING OF GEL WITH SAMPLES AND MARKERS:**

The loading mixture containing sample protein, DTT, LDS buffer and distilled water was heated in a water bath at 70°C for 10 min. The markers for the first well was prepared by adding 7µl of a marker protein (high molecular weight, full range Rainbow Marker), 6.25µl of LDS Sample Buffer, 2.5µl of DTT and 10µl of distilled water followed by heating at 70°C for 10min. The samples were spun briefly before loading to the gel. Once loaded the white strip at the bottom of gel chamber was removed and the gel was transferred to XCell Surelock Mini-Cell. Running buffer was filled in the inner and outer chambers. The gel was run for 1hr at 115mA and 200 V.

- **BLOTTING**

The transfer buffer solution was prepared by adding 75% distilled water (v/v), 20% (v/v) of 10% methanol and 5% (v/v) of transfer buffer stock solution. (diluted from 20X to 1X) Six sponges were soaked in the transfer buffer solution and air bubbles were squeezed out meticulously. Watman paper and nitrocellulose membrane were also soaked in the transferred buffer solution just before its loading into the system. The sealed-off gel chamber was opened gently, avoiding damage to the gel and ensuring minimal manual handling of gel loaded proteins. The protein loading chambers right at the top of the gel were carefully

scraped off, and so was the bottom ridge of the gel. The order of system composition was as follows:

***3 sponges → 2 Watman papers → Gel → Nitrocellulose membrane → 2
Watman papers → 3 sponges***

- **PONCEAU STAINING**

The membrane was taken out of the system and was marked straight away. The protein face was kept facing upwards. The membrane was stained with Ponceau solution (0.1% Ponceau S (w/v) in 5% acetic acid) for 5 minutes. The bands of protein were observed to check for the adequate loading, running and blotting and any gaps in the loading due to air bubbles were noted.

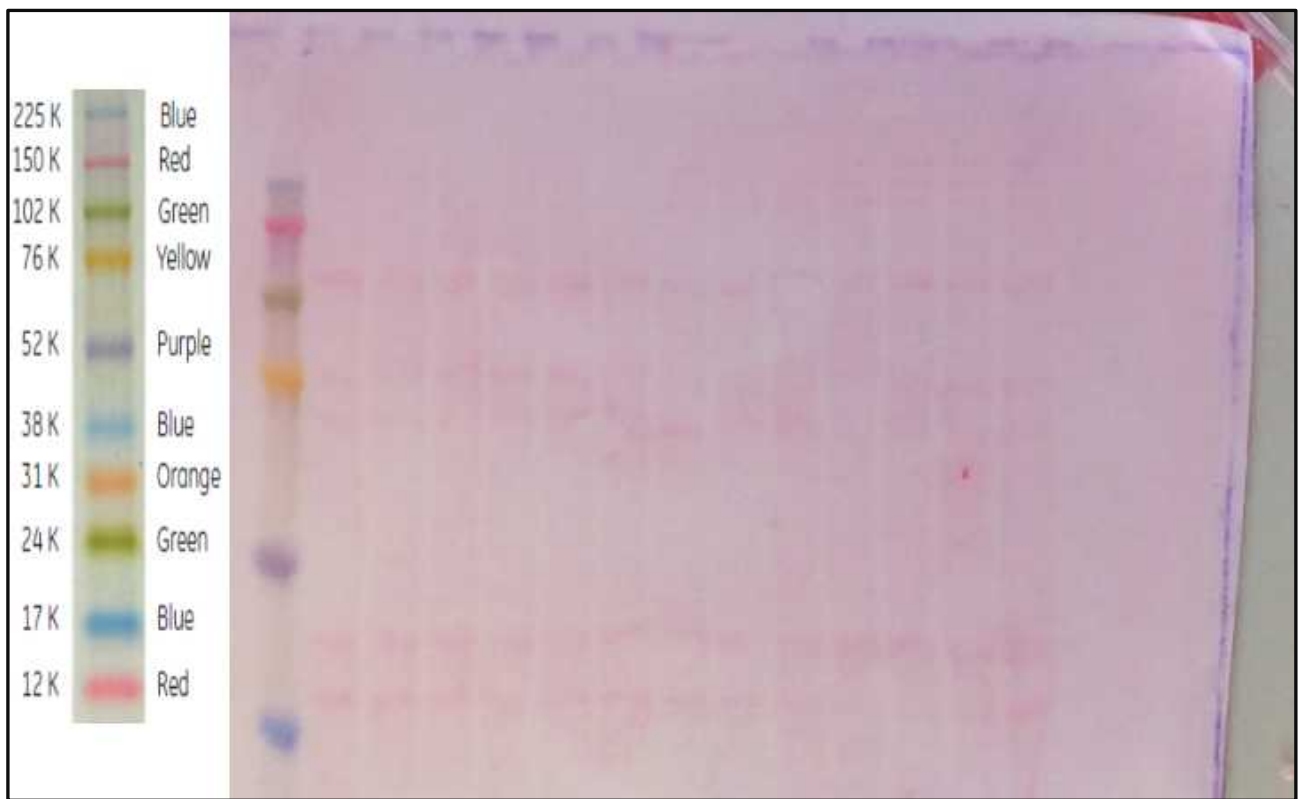


Fig. 2.3. PONCEAU STAIN SHOWING PROTEIN LOADING OF NITROCELLULOSE MEMBRANE.

- **BLOCKING**

The membrane was washed with PBS-Tween (PBS-T was prepared by adding 1ml of Tween to 1000ml of PBS). The membrane was blocked for 1hr with blocking solution comprising of freshly prepared skimmed milk diluted in PBS-T (3% w/v, i.e., 3gm of fat-free or skimmed milk dissolved in 100ml of PBS-T). The membrane was labelled and was cut from top to the bottom in the form of strips and was placed in a plastic bag.

- **STAINING WITH PRIMARY ANTIBODIES**

Primary antibodies were diluted in blocking solution as per data sheet, and 1ml was added to cover the membrane strip followed by incubation at 4°C for 1hr. The protein loaded strips were washed three times with 0.1% PBS-T at room temperature. Then secondary antibodies in a dilution of 1:1000 were added, and the strips are re-incubated at 4°C for 1hr. The membrane stripes were again washed three times with 0.1% PBS-T. The nitrocellulose membrane strip (loaded with proteins and stained) were then placed in a plastic container with 0.1% PBS-T and placed in a shaker for 30 – 60min.

The strips were then taken out and placed on a clean glass surface. An ECL solution was prepared with Thermo Scientific 37071, SuperSignal West Dura Kit (2 reagents mixed in 1:1; SuperSignal West Dura Kit Peroxide solution and SuperSignal West Dura Luminol Enhance Solution). Each membrane strip was covered drop by drop with 1ml of this solution in a way that the entire strip was soaked with a reagent for 5 min. The membranes were then placed in a plastic bag, and the air was removed.

4. DETECTION

The membrane strips sealed in a plastic bag were then placed in the BioRAD imaging system. Multiple scans were performed using chemiluminescence and once under

colourimetric. The detected bands representing predicted molecular weight were compared with the bands in the first strip loaded with the rainbow sample.

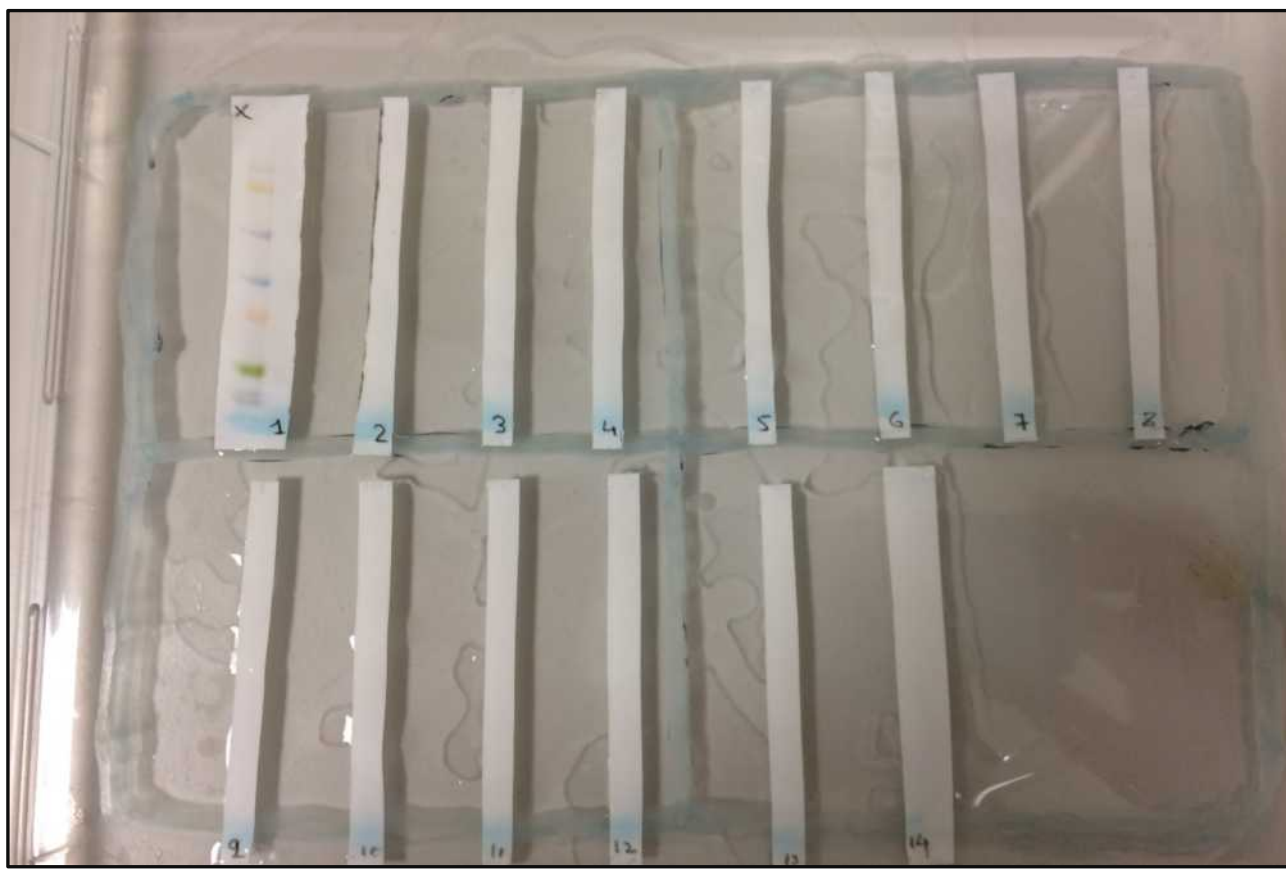


Fig. 2.4. PROTEIN LOADED & STAINED DUPLICATED NITROCELLULOSE STRIPS SEALED IN A BAG FOR CHEMILUMINESCENCE IMAGING.

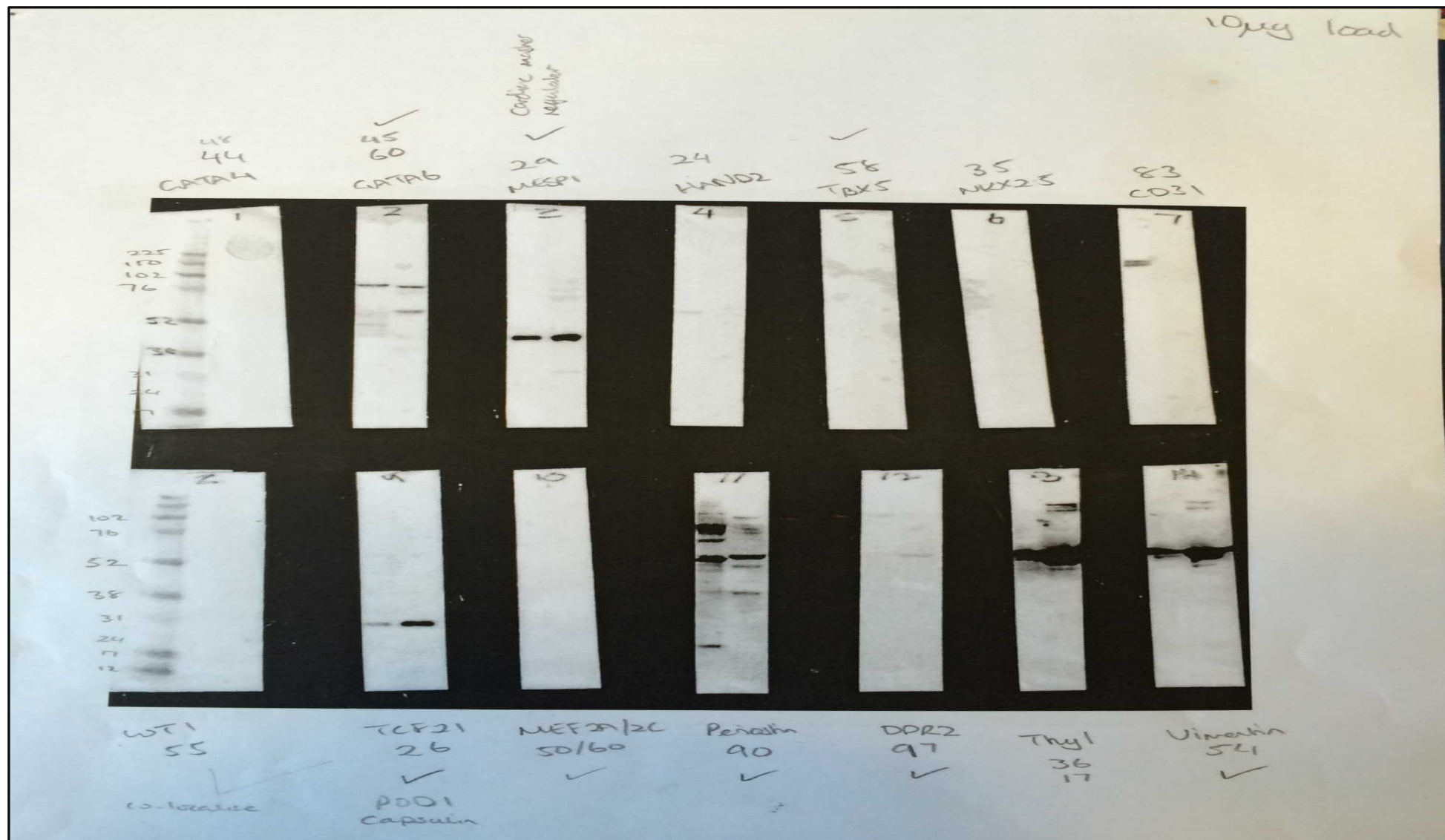


Fig. 2.5. CHEMILUMINESCENCE IMAGING FOR DETECTION OF PROTEIN BANDS REPRESENTING THE MOLECULAR WEIGHT OF PRIMARY ANTIBODIES AND TRANSCRIPTION FACTORS.

TABLE: 2.2. ANTIBODY DILUTIONS USED FOR TRANSCRIPTION FACTORS

Transcription Factors								
	<u>Transcription Factor</u>	<u>Molecular Weight</u>	<u>Species</u>	<u>Incubation Time</u>	<u>Dilution for IF</u>	<u>Dilution for WB</u>	<u>Codes</u>	<u>Company</u>
1.	Anti-GATA - 4	44 kDa	Rabbit	1h[RT] – O/N [4°C]	1:1000	1:500	Ab84593	Abcam
2.	Anti-GATA - 5	44 kDa	Rabbit	1h[RT] – O/N [4°C]	1:1000	1:500	Ab48820	Abcam
3.	Anti-GATA - 6	60 kDa	Rabbit	1h[RT] – O/N [4°C]	1:1000	1:500	Ab175927	Abcam
4.	Anti-TBX 2	75 kDa	Mouse	1h[RT] – O/N [4°C]	1:1000	1:500	Ab140345	Abcam
5.	Anti-TBX 5	58 kDa	Rabbit	1h[RT] – O/N [4°C]	1:1000	1:500	Ab137833	Abcam
6.	Anti-TBX 20	33 kDa	Rabbit	1h[RT] – O/N [4°C]	1:1000	1:500	Ab16683	Abcam
7.	Anti-Mesp-1	24-29 kDa	Mouse	1h[RT] – O/N [4°C]	1:1000	1:500	Ab77013	Abcam
8.	Anti-Mef 2A/2C	50-60 kDa	Rabbit	1h[RT] – O/N [4°C]	1:1000	1:500	Ab197070	Abcam
9.	Anti-Myo D1	45 kDa	Rabbit	1h[RT] – O/N [4°C]	1:1000	1:500	Ab64159	Abcam
10	Anti-TCF - 21	26 kDa	Rabbit	1h[RT] – O/N [4°C]	1:1000	1:500	Ab182134	Abcam
11.	Anti-HAND – 2	2000 kDa	Rabbit	1h[RT] – O/N [4°C]	1:1000	1:500	Ab60037	Abcam
12	Anti-WT -1	55 kDa	Rabbit	1h[RT] – O/N [4°C]	1:1000	1:500	Ab89901	Abcam
13	Anti-Nkx 2.5	35 kDa	Rabbit	1h[RT] – O/N [4°C]	1:1000	1:500	Ab91196	Abcam

* Anti-Hand-2 antibody is mentioned in the Abcam literature for not giving any bands for western blotting (molecular weight = 2000 kDa) but IF results were good.

TABLE: 2.3. ANTIBODY DILUTIONS FOR SECONDARY ANTIBODIES

Secondary Antibodies

Immunofluorescence

* All antibodies were diluted at 1:1000. All secondary antibodies Alexa Fluor antibodies obtained from Abcam and Invitrogen.

Goat Anti-Mouse	488
Goat Anti-Mouse	647
Goat Anti-Chicken	488
Donkey Anti-Rat antibody	488
Donkey Anti-Rabbit	546
Donkey Anti-Rabbit	488
Goat Anti-Rabbit	647
Goat Anti-Rat	594

Western Blotting

Donkey Anti-Rabbit-FITC	Ab6798	1:1000
Donkey Anti-Mouse IgG-HRP	Ab6820	1:1000
Donkey Anti-Rat IgG-HRP	Ab102265	1:1000

2.3.6 IMMUNOFLUORESCENCE MICROSCOPY:

Chamber specific human cardiac fibroblasts were plated in a chamber slide (comprised of 8 chambers) and were kept in an incubator for 48 hours at the P2 stage. Cells were visualised under a microscope for growth and adherence to the chamber slide. The FGM was discarded, and cells were washed twice with PBS. CFs were fixed with 4% PFA for 10 min and then washed twice with PBS. 0.5% Triton was added for 3 min to create some pores in the membranous walls of fibroblasts. The cardiac fibroblasts were washed twice with PBS and blocked with 3% BSA for 30 min.

In the meantime, transcription factors were prepared in the blocking solution (1.5% BSA) at a dilution given in the Table was added to the blocked fibroblasts for 60 min at room temperature. Subsequently, the chamber slides were washed 3 X in PBS. The species-specific secondary antibodies were prepared at a 1: 1000 dilution and were added to the chamber slides for 60 min. The chamber slides were rewashed, and DAPI (Molekula) was added at 1:1000 for 10 min. Following this, the chambers were removed carefully from the underlying slide and were allowed to dry out for 5-10 minutes. All coverslips were washed with PBS and dried out before mounting on the glass slides. A film of mountant was added, and the slides were covered with the coverslips. The slides were allowed to dry out overnight and were viewed under a confocal microscope.

2.3.7 CARDIOMYOCYTE DISSOCIATION PROTOCOL

Collagenase II solution (200U/ml) was prepared as described previously. (Collagenase II dissolved in Ca^{++} free HBSS) As no stock solution can be prepared therefore the solution was prepared fresh each time in the desired amount as per requirement. The cells were washed twice with pre-warmed Ca^{++} free HBSS. The amount of Collagenase II solution added was directed by the size of the well or flask used. (1.5ml/ 2.5ml/ 7ml/ 14ml of collagenase solution was used per 6-well/ T25/ T75/ T175 flasks respectively). As we used 6 wells plate hence 1.5ml of collagenase solution was used per well. The cells were incubated for 3.5h at 37°C. As per recommended protocol, the cells were checked once or twice during this period to determine the optimal incubation time. Acquisition of a monolayer of cells was the desired result of the incubation period, and if the cells were not found in a monolayer, they were incubated again for an additional 30min. After 3.5h the cells were triturated up to 10 times with a 10 ml stripette at a medium speed and were placed in a falcon tube. The same recommended volume of warm blocking buffer (RPMI + DNase, 1:500) was added to each well/flask, washed up to 5 times and was added to the falcon tube with cells suspended in it.

The Falcon tube with cells and blocking buffer was centrifuged for 15min at 800rpm/min (100G). Once centrifugation was stopped the pellet at the base of falcon tube was examined. The supernatant blocking buffer was aspirated carefully to avoid losing any cardiomyocytes. The final 1-2ml with the pellet was carefully pipetted, and the basal medium was added to it depending on the size of the pellet. (5ml for a small pellet, 10ml for a medium one and 15ml for a large pellet) The cardiomyocytes suspended in the basal medium were gently triturated again using a 10ml stripette. The cells were counted with the Luna cell counter. Depending on the volume of cell mixture, the cardiomyocytes were used, plated or frozen.

1. FREEZING OF CARDIOMYOCYTES

Cardiomyocytes were frozen with the cryopreservation mixture (20%DMSO in FBS). 0.5ml of cryopreservation mixture was added to the individual vial. Cardiomyocytes were treated with ROCK inhibitor for 2h at 37°C in an incubator with 5%CO₂. After 2h, the cardiomyocytes were removed from the incubator, and medium (RPMI + B27) was aspirated. 1ml of Tryple was added per well of the 6-well plate. (the volume of Tryple was adjusted as per surface area of the well or flasks) The cardiomyocytes were placed in the same incubation settings (5%CO₂ + 37°C) again for 8mins. After 8min the cells were removed from the incubator and using a p1000 the cells were gently pipetted up and down to dislodge them from the bottom of the 6-well plate.

Following this, 4ml of the basal medium was added to each well of 6-well plate to inactivate TrypLE by dilution. The entire cell mixture was then transferred to a conical 15ml universal tube and centrifuged at 240G for 4min. The supernatant was aspirated, and the pellet was resuspended in 0.5ml of FBS/well of a 6-well plate. This 0.5ml of cell mixture was transferred then to the vial already containing 0.5ml of cryopreservation mixture. The lids of the vials were secured tightly and transferred to the cryopreservation box (aka Mr Frosty) at room temperature. This box was then stored at -80°C overnight. The following day the box was transferred to liquid nitrogen and database of the liquid nitrogen storage was updated with the date and type of cells stored.

2. MAKING OF MATRIGEL PLATES

The growth factor reduced Matrigel mixture (Corning, 356237) was prepared by adding Matrigel to DMEM-F12 in 1:30 ratio. (300µl of Matrigel in 50mls of DMEM F-12). 2mls of this mixture was added to each well in a 6-well plate and was incubated for 30min at 37°C. For longer incubation period the 6-well plate was incubated at 4°C.

3. THAWING OF CARDIOMYOCYTES

The storing vial was removed from the liquid nitrogen and was quickly placed in a water bath at 37°C for 1min. Thawed cells were then transferred to a 15ml conical universal tube already containing 10mls of the medium. The tube with cell mixture was then centrifuged for 4mins at 240G. A Matrigel-coated 6-well plate was already prepared as mentioned earlier. The supernatant was aspirated, and cardiomyocytes were seeded and suspended in a 6-well plate with 2ml of the E8 medium in each well (approximately 3 million cells were added to each well). 10 μ M of ROCK inhibitor was added to each well for first 24hr only. The E8 medium was changed every day. The cardiomyocytes were allowed to grow to 75-90% confluence was attained.

4. SPLITTING OF CARDIOMYOCYTE

6-well Matrigel plates were prepared as described earlier. The E8 medium was removed from the well. 1ml of TrypLE was added to each well and cells were incubated at 37°C for 8mins. Following this, TrypLE was removed and inactivated by adding 4ml of E8 medium to the cardiomyocytes. The cells were triturated gently 4 X and transferred to a 15ml falcon tube. The cell mixture was then spun at 240G (1000 rpm) for 4min at 18°C. The supernatant was removed, and 3mls of E8 with RP27 was added followed by gentle trituration. Staining with 100mM filter is optional and was performed as and when required to obtain single cells in a 50mls falcon tube. Cell count was then performed by adding 10 μ l of cell mixture to 10 μ l of Trypan Blue with 2 μ l of LunaTM FI. Usual cell population in each well of a 6 well plate was 3-4million. The beating of the cardiomyocytes could take 2-3 days in healthy and 2-3 weeks for the diseased lines. The cells should not be split beyond d30-35 as after maturation (post d35) the cells are more difficult to attach to the base of the wells.

5. CARDIOMYOCYTE DIFFERENTIATION & METABOLIC SELECTION

Between d0 – d13, the cardiomyocytes were examined for growth, morphology, and confluence following the seeding. If the cell plated in the wells showed a bumpy layer or a thick coat on top of them, this signified the presence of ECM along with the cardiomyocytes. To prevent this phenomenon, the cardiomyocytes were treated with CHIR99021 (S292410, Selleck) first 2-3 splits for every new line before scaling up. (CHIR was used in concentration 4 μ M, 6 μ M, and 8 μ M) Cardiomyocytes were split and re-plated once optimal confluence was attained.

Feeding medium which included RPMI with B27 was changed after every 48 hours. CHIR99021 can be added to the medium as it is stable at the storage temperature of 4°C. Unlike other cell, cardiomyocytes rely on oxidative phosphorylation and not glycolysis. During the differentiation of the cardiomyocytes, the cells relying on glycolysis die and only cardiomyocytes survive. The only exception to this rule was the cardiomyocytes derived from the patient who has some inherent defect of oxidative phosphorylation in cardiomyocytes. The medium used for feeding the cells at this stage contained RPMI basal medium with low glucose and supplemented with B27. The medium was warmed only to the room temperature before use.

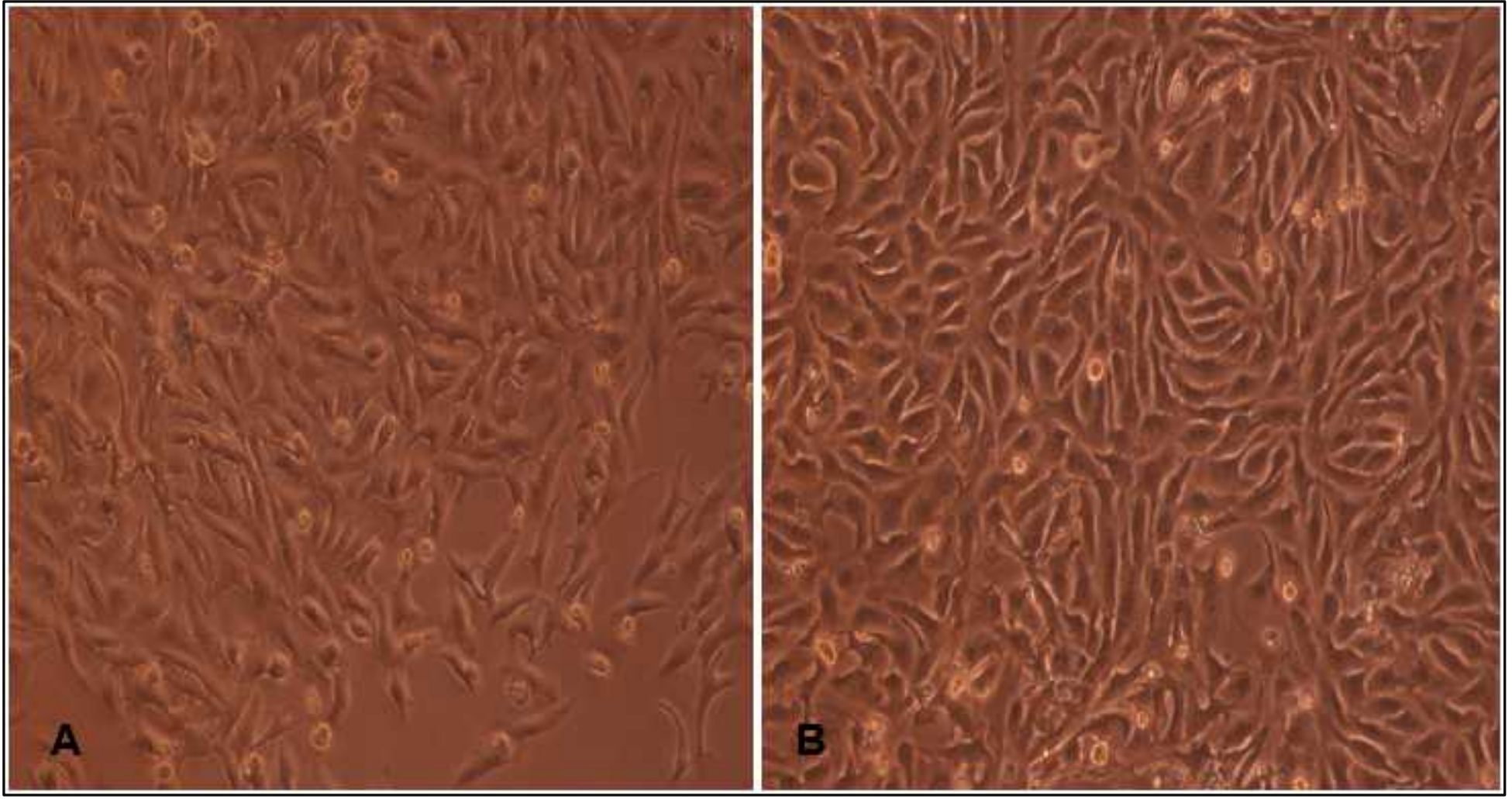


Fig. 2.6. DIFFERENTIATION OF CARDIOMYOCYTES (IMR).

Bright field microscopic images (20X) of unstained cardiomyocytes at d7 (A) and day 12(B). Maturation and differentiation of Rod-shaped cardiomyocytes (IMR) used for generation of EHTs are shown.

2.3.8 GENERATION OF EHTs

1. HUMAN CARDIAC FIBROBLASTS USED FOR EHT CONSTRUCTS

For the pilot data, the adult human atrial fibroblasts were isolated and harvested from the patients undergoing routine Coronary Artery Bypass Grafting and Aortic Valve replacement. The ventricular samples were taken from the native heart of the transplant recipients, predominantly with dilated cardiomyopathy. The human foetal fibroblasts used were at the P15 stage. Late Passage fibroblasts were of ventricular origin and were at P6.

However, for the main study, the human atrial and ventricular samples were taken from all four chambers from the same heart, in a patient population with normal LV function.

For the comparison group, human foetal fibroblasts were used from SIGMA to maintain the same passaging stage, i.e., P2. The late passage fibroblasts were of ventricular origin. They were harvested from the native hearts of the transplant recipients and were at the P6 stage.

2. HUMAN CARDIOMYOCYTES USED FOR EHT CONSTRUCTS

The cardiomyocytes used were of 2 different origins. The cardiomyocytes used in the pilot study were **CDI foetal cardiomyocytes**. In the main study, **IMR cardiomyocytes** were of **pulmonary origin (induced from human pulmonary fibroblasts)**. These IMR cardiomyocytes were replicated in our lab and used on day 18 of differentiation.

The engineered heart tissues were constructed as per Samata's Protocol (Schaaf et al., 2011) (Breckwoldt et al., 2017).

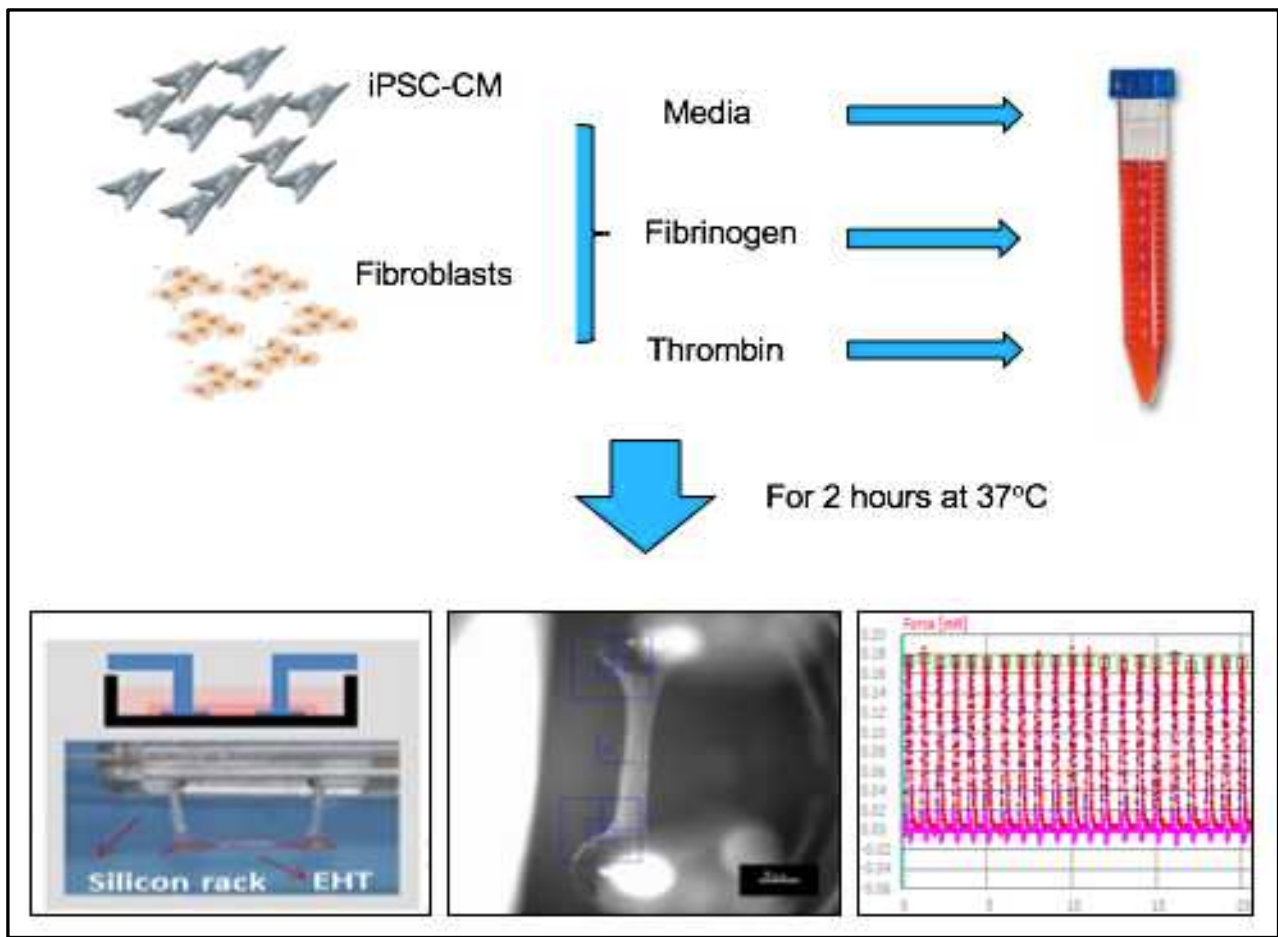


Fig. 2.7. CONSTITUENTS OF ENGINEERED HEART TISSUE. Adapted from Owen T and Harding S.

Engineered Heart Tissue was made by combining 95-95% hiPSC-CM (made under a separate project from pulmonary fibroblasts or purchased from CDI) with fibroblasts in varying proportions, within a fibrin matrix. In experiments to date, each EHT contained 5×10^5 hiPSC-CM and 1.5×10^5 fibroblasts. EHTs are made as formerly described by (Hansen 2010) but with human fibrinogen and thrombin (Evicel Human Fibrin Sealant). 1.6ml of 2% liquid agarose in PBS was placed in each well of 24-well plate and casting moulds were made by the addition of Teflon spacers. The agarose solidified around Teflon spacers in 10-15min. The master mix was prepared on ice, and the fibrinogen was pipetted 15 times to ensure it is the solution.

Engineered Heart Tissue (EHTs)

Fibrin-based mini engineered heart tissue (EHT)

- EHTs are three-dimensional, force-generating, reconstituted heart tissue.
- Generation under standardized conditions.

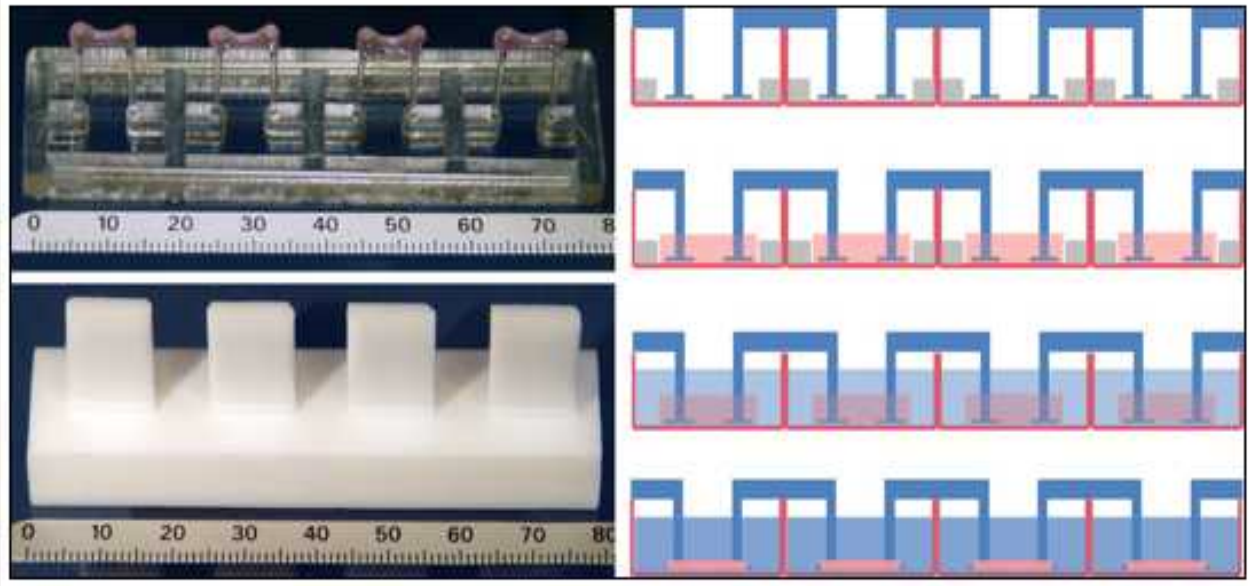


Fig. 2.8. GENERATION OF ENGINEERED HEART TISSUE. Engineered Heart Tissues attached to the silicon poles. Agarose moulds were used in the 24 well plate, and silicon racks were suspended in it. Pre-construct EHT mixture was added to the well so the construct can attach to the silicon posts. Within an hour the constructs were attached to the poles and can be transferred to the 24 well plate. **Adapted from Eschenhagen T et al. 2010.**

For both CDI (pilot data set) and IMR (main study data set) cardiomyocytes, thrombin stock from Evicel (800-1200 U/ml) is diluted 1:10 (in 60% PBS and 40% water) and aliquots of 3ul are placed into PCR tubes for mixing. A master mix of cells 4.1×10^6 , fibrinogen (Sigma 200mg/ml) and 2 x DMEM is made up in varying quantities based on the number of EHTs. The master mix is mixed with one thrombin aliquot and pipetted into agarose casting moulds quickly. The master mix is resuspended after 4-8 EHTs. The 24-well plate with fibrin gels was placed in the incubator for 90min. (37°C, 7%CO₂, 40%O₂, 100%humidity) After this, the fibrin gels was hydrated with 300ul EHT media (DMEM, 10% horse serum + 1% penicillin/streptomycin + 0.1% human insulin + 0.1% aprotinin) per well and were incubated

for a further 30min. For human EHTs 7µl undiluted Evicel thrombin is placed directly into casting moulds, with different fibrinogen concentrations and the same method was carried out. EHTs were transferred carefully to a 24-well plate with 1.5ml warm EHT media in each well. The tissue condenses and solidifies around these posts, and contracts spontaneously between them after 1-2 weeks.

2 OPTIMISATION OF THE RECORDING ENVIRONMENT BEFORE RECORDING OF KINETICS OF EHTs

The rate of contraction of EHTs was compared between the selected group once the constructs start to beat within 2-4 days of construction. The contractile function of EHTs is altered by changes in the surrounding temperature, humidity, change of the bathing medium and judder caused during the transfer of constructs from one place to another. The optimisation of these factors is very important before the recording of kinetics. The change and temperature of the bathing medium have the foremost impact on the kinetics and in most cases immediately after changing the medium either the construct stops beating altogether, or they start contracting slowly and pick up their intrinsic pace of beating after 1-2hrs. Based on this observation the kinetics were recorded 2-3hrs after a change of medium. Since the EHT bathing medium was stored at 0-4°C between the use, therefore the medium was left for 30mins in a pre-warmed water bath to optimise its temperature to 37°C. Before the recording of the kinetics of EHTs, the constructs are placed in the optimised surrounding in CMTV recording system and left for 10-15mins so that the constructs can acclimatise to the new environment and the factors affecting the kinesis were minimised. The comparative measurement of all constructs was done in the same setting and are recorded electronically.

3 VIDEO-OPTICAL RECORDING AND MEASUREMENT OF THE KINETICS OF EHTs

The EHTs after construction were left in their specific nourishing medium, start to beat within a few days. The EHTs attain the peak level of contraction after a few days and then gradually lose the inotropic and chronotropic response and eventually die. The periodic measurement of the kinetics of the Engineered Heart Tissue is carried out by using a bespoke automated system designed by Hamburg Group. The ingeniously designed system comprises of a well-illuminated box calibrated at 37°C and is flooded with 5%CO₂. The humidity is maintained at 33%. It maintained the temperature of the EHT constructs at 37°C during the measurements of kinetics. Pre-labelled 24-well-plates with EHT constructs (0-21 days) bathing in the medium was wedged on a fixed platform.

The glass roof of the incubator allowed a clear view for a movable customized software-controlled camera placed over the top. This camera can move along all three axes to focus and capture the EHTs images in individual wells selected at the beginning of the session. Based on the number of deflections in a defined time, post geometry and elastic modulus of the silicone; the rate, force, fractional shortening, contraction and relaxation time of individual EHT was calculated.

An automated figure recognition algorithm was used to evaluate the contractile parameters of spontaneously beating EHTs. The measurement of kinetics was tabulated in the final report as images, graphics and calculations. The scale used for the measurement is incorporated into the images as well.

Video-optic recording of kinetics of the Engineered Heart Tissue

- Multiplexed contraction assays using EHTs
- Automated evaluation of kinetics of contraction at different time points.

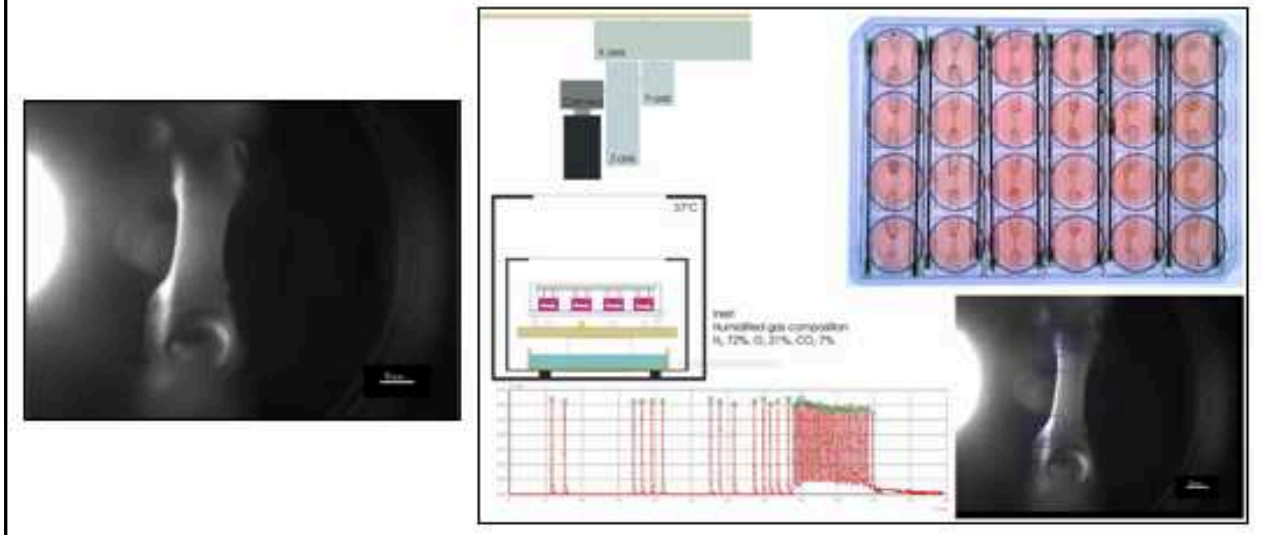


Fig. 2.9. MEASUREMENT OF THE KINETICS OF ENGINEERED HEART TISSUES. Video-optic kinetic recording of the Engineered Heart Tissue was done at a pre-defined time while EHTs are attached to the silicon posts and are bathing in the EHT medium. **Adapted from Eschenhagen, T et al. 2010.**

The optical system was primed for at least 10-15 minutes prior to recording to maintain the optimal temperature. The EHTs were allowed to adapt to the visual system before the kinetic footages were started. The wells were selected, and required essential parameters were adjusted. The rate and force of contraction of each EHT in each well were measured for 60sec. 2 visual image replicates were taken for each well. One visual frame was employed for derivative calculation. The maximum sum force threshold is adjusted to 0.030mN. The defined parameter for the maximum peak distance was 10sec. The parameter of interest calculated by the optical system included beats/min, Sum Force, T1&T2 (10%, 20%, 50%), Resting length in mm and fractional shortening. T1 (10%, 20% and 50%) refers to the time of contraction of EHT from rest to 10%, 20% and 50% of contraction till it reaches to peak of contraction, On the other hand T2 (50%, 20% and 10%) represents 50%, 80% and 90%

of relaxation from the peak of contraction until it comes back to a fully relaxed resting state. A calculated mean with standard deviation of all these parameters was generated at the end of every recording.

The EHTs can be electrically stimulated with sterile electrodes placed designed to fit into the silicon rack without damaging the EHTs or by introducing vasoactive agents and the measurement of the parameters mentioned above can be repeated. The data tabulation and analysis were done with the help of PRISM Version 7 software.

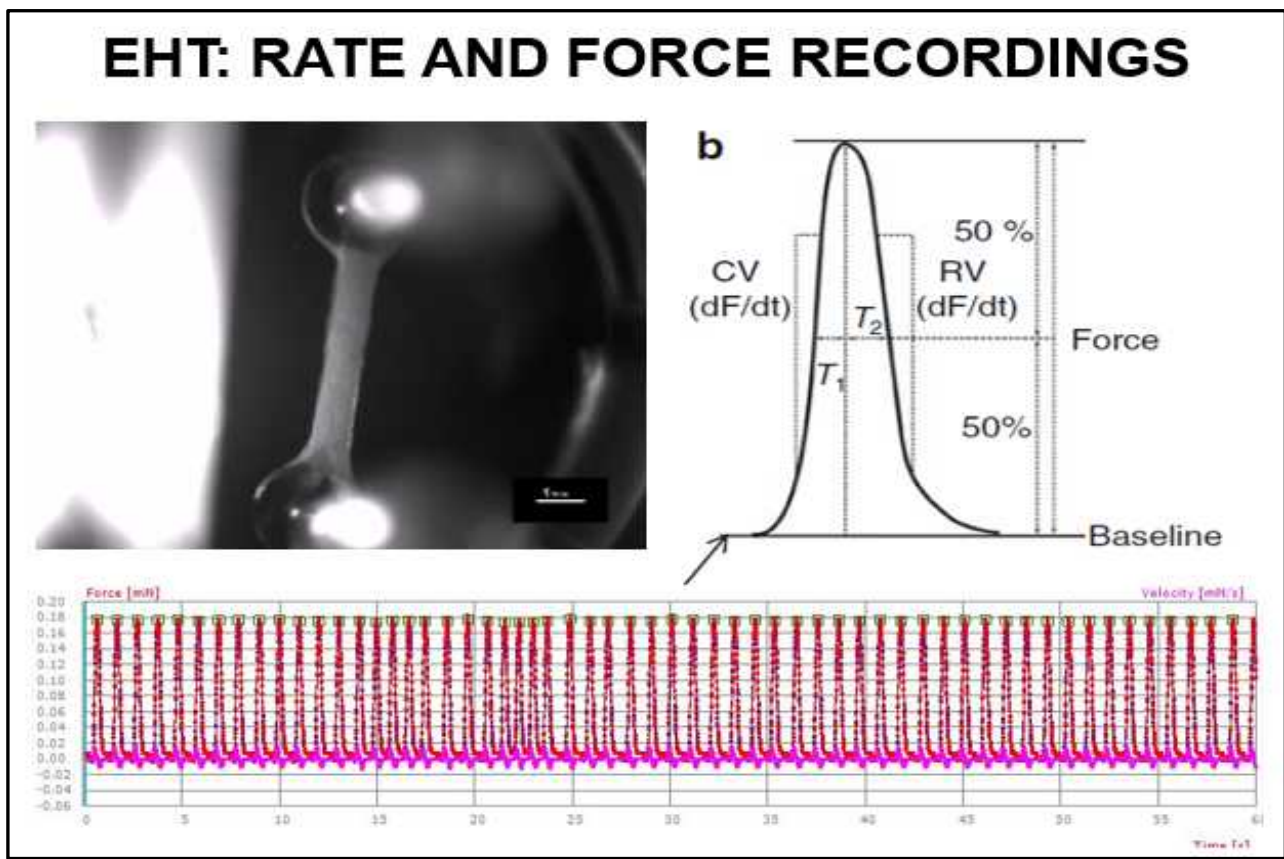


Fig. 2.10. RECORDING OF CONTRACTILE PARAMETER OF ENGINEERED HEART TISSUE. Adapted from Prof. Harding & Owen T.

2.3.9 PROTEOMIC PROFILE ANALYSIS OF EHT MEDIUM

Proteomic profile analysis of the medium bathing the engineered heart tissue was carried out at pre-determined intervals. Each EHT was suspended in a 24 well plate in 2mls of EHT medium. As per protocol, the EHTs bathing medium was changed after every 48 hours under sterile condition. The discarded EHT medium at the time was collected from the individual wells, labelled and stored at -20°C. The proteomic analysis of this EHT medium was done on day 2, 8 and 16 and compared among the groups. The days were selected as the EHT constructs start beating on day 2-4 on average and then reach their maximum kinetics on day 16. After this, they start to slow down and eventually by day 21 they are either too slow for any kinetic force measurements or they die completely.



Fig. 2.11. NITROCELLULOSE MEMBRANE IS CONTAINING CYTOKINE ANTIBODY ARRAY.

R&D Proteomic Profiler Human XL Cytokine Antibody Array kit was used (ARY022B) was used. It contained 4 nitrocellulose membranes in each pack, containing 102 proteins different capture antibodies imprinted in duplicates and can detect multiple cytokines,

chemokines, growth factors and other soluble proteins in the cell culture supernatant. The entire kit comprises of cytokine membrane, Array buffer 4 & 6, wash buffer, detection antibody cocktail, Streptavidin-HRP, chemi-reagent 1& 2 and 4 well multi-dish. 2 ml of buffer 6 was added to the array membrane in the multi-dish, and blur dots disappear gradually from the membrane array.

The array membrane in multi-dish was incubated for 1hr on the rocking platform shaker. After removing from the rocking platform, buffer 6 was removed, and 1ml of sample mixed with 0.5ml of Buffer 6 was added to the membrane in the multi-dish and was incubated overnight on the rocking platform at 2-8°C. Once removed from the rocking platform the residual sample and Buffer 6 mixture was removed. The membrane was washed three times with wash Buffer solution (895003; 40ml of 25X wash buffer added to 960ml of distilled water). Having washed 30µl of detection antibody cocktail (Human XL Cytokine Detection Antibody Cocktail is prepared by adding 200µl of distilled water to the powder) mixed with 1.5ml of a mixture of buffer 4&6 was added to the array membrane and was incubated for 1hr. Following this, the membrane was rewashed 3 times for 10min with wash buffer solution. Streptavidin-HRP (893019) was prepared in a dilution of 1:2000 with Array Buffer 6 immediately before use. 2 ml of 1X Streptavidin-HRP was added to the array membrane and was incubated on the rocking platform for 30min. Again, the membrane was rewashed 3 times for 10min with wash buffer solution. Chemi reagent 1 (894287) and Chemi reagent 2 (894288) were mixed in 1:1 within 15 minutes of use and 1 ml was added to the array membrane wrapped in a plastic sheet protector. The spread of chemi-reagent mixture over the membrane is ensured precautions are taken to remove any bubbles from the plastic bag. The plastic bag with array membrane cover with reagents is incubated for 1 min. The excess chemi-reagent mixture was removed, and array membrane was examined under the X-ray film for 1-10minutes. Multiples exposure times were used.

2.3.10 CHEMI-DOC PROTEOMIC EXPRESSION CALCULATION

The Protein expression of the nitrocellulose membranes was analysed as mean pixel densities with the help of BIO-RAD Image Lab™ Software Version 6.0. The software is a non-touch-screen imaging system application for acquisition and analysis of wide variety of gel and blots images. Image Lab 6.0 is used for data analysis of images generated on the ChemiDoc MP, ChemiDoc, ChemiDoc XRS+, GelDoc XR+, and GelDoc EZ along with the GS-900 calibrated densitometer. The expression of proteins was captured as duplicates calibrated against the reference dots and were expressed as mean pixel densities. A mean pixel density of two consecutive spots was calculated for the final reading.

2.3.11 HISTOLOGY OF THE EHTs

Once reached to the mark of 21 days period, the EHTs either lose all their kinetics or die. They were detached from the poles of silicon racks and washed with sterile PBS. The EHTs are then placed in OCT for few minutes in a small plastic well. The OCT well-containing tissue was dipped in a plastic container containing Isopentane. The plastic container with Isopentane was surrounded by dry ice to prevent the Swiss Cheese effect. The samples were labelled in foils and were stored at -80°C.

Cryosectioning of the OCT embedded EHTs was done, and 7µm slices were made. The sections were stained with eosin and haematoxylin for the general microscopic view. Masson trichome was used to stain the collagen in the ECM. Sections were further stained for immunofluorescence as described in chapter 2 with markers for fibroblasts and cardiomyocytes. Vimentin and DDR-2 were used for marking the fibroblasts and MHC, α -Actinin and connexin-43 were used to mark the cardiomyocytes. Nuclei were stained with DAPI.

2.3.12 STATISTICAL ANALYSIS OF DATA

Statistical analysis of data for this entire research project was carried out with the help PRISM Version 7 software (GraphPad). The number of replicate groups, different fibroblast groups, vials of CDI or tissue samples in case of human tissue is indicated by 'n' as described in the figure legend and text. All data sets were tested for normality using the Komogorov-Smirnov test. If it passed the normality test (i.e. the data has a Gaussian distribution) then the data was tested by parametric tests. Wherever parametric tests were not applicable, non-parametric equivalents were used. Results in the text are presented as the arithmetic mean $\pm$ one standard deviation or mean. All graphs are presented as mean with the error bars showing either standard error of the mean (SEM) or standard deviation (SD). Analyses of changes over increasing time for two groups were carried out using a t-test or the non-parametric equivalent test for comparison between two categories. Unpaired t-test was used for comparison of effect of fibroblast growth factor (FGF) on the myofibroblastic transformation of fibroblasts. And for the analysis of more than two categories, One-way Analysis of Variance (One-way ANOVA) with post-tests using Brown Forsythe test, Bartlett test and Tukey's multiple comparison tests were used. A Two-way Analysis of Variance (Two-way ANOVA) and post-tests using the Bonferonni correction was used for rate and force of contraction of EHTs in different groups.

Any post-hoc test carried out is described in the relevant sections. Statistical analyses were considered significant if p was ≤ 0.05 (*), $p \leq 0.01$ (**) or $p \leq 0.001$ (***). Non-significant results are referred to as ns. All data with multiple categories were analysed using these tests including rate of growth of fibroblasts, effect of FGF, rate and force of contraction of EHTs and maximum rate and force of contraction exhibited by thee EHTs.

2.4 CONCEPTION AND EXPERIMENTAL RECITAL OF PROJECT

The basic idea to perform characterisation of human all four-chamber fibroblasts was conceived by Mr. Prakash Punjabi. I developed it further and mapped the details of phenotypic characters of growth, markers for identification and transcription factors expression. Professor Harding advised me to investigate the role of these chamber specific fibroblasts in EHTs. I further added on the proteomic expression of the cardiomyocytes and non-cardiomyocytes in EHTs. I wrote the entire project with the experimental scopes by myself and got a prestigious BHF grant for it.

I performed all the experiments involved in this project by myself. I learnt the fibroblast harvesting techniques in lab for my pilot data, based on a murine protocol. The murine protocol was not fully applicable to the human myocardial samples; hence I modified it and eventually perfected the technique. My final human cardiac fibroblast growth protocol was later on used by other groups in the lab. I have modified the fibroblast growth medium used for the fibroblast growth and demonstrated the effect of addition of FGF in the medium leading to marked decrease in myofibroblastic transformation. I learnt the techniques of western blotting for validation of antibodies and immunostaining of fibroblasts and performed all the experiments on my own. For EHT constructs, I sought the help of my colleague Tom Owen only for the construction of EHTs. I learnt the use of video-optical system for measurement of kinetics of EHTs and measured all the kinetics for the project. For proteomic analysis, I used the new technique of chemi-doc analysis. This was used in the lab for the first time. I perfected the technique and later on my protocol was used by my other colleagues in the lab. I learnt the used of confocal microscope, bright field microscope and software's like Image-Lab and ImageJ. Prof. Harding taught me and guided for the statistical analysis of data with PRISM software. All these techniques mentioned above are optimised and reproducible.

CHAPTER 3: CHARACTERISATION OF HUMAN CARDIAC FIBROBLASTS

CHAPTER 3: CHARACTERISATION OF HUMAN CARDIAC FIBROBLASTS

3.1 OVERVIEW

This chapter focuses on the characterisation of the phenotype of human cardiac fibroblasts of different origins. Two sets of data representing the pilot study samples and the main study samples (all four chamber FBs) will be presented. The fibroblasts isolated and harvested from the atrial and ventricular myocardial tissue biopsy samples were studied for their rate and pattern of growth at different passages. The effect of varying enrichment mediums on the rate of growth was also studied.

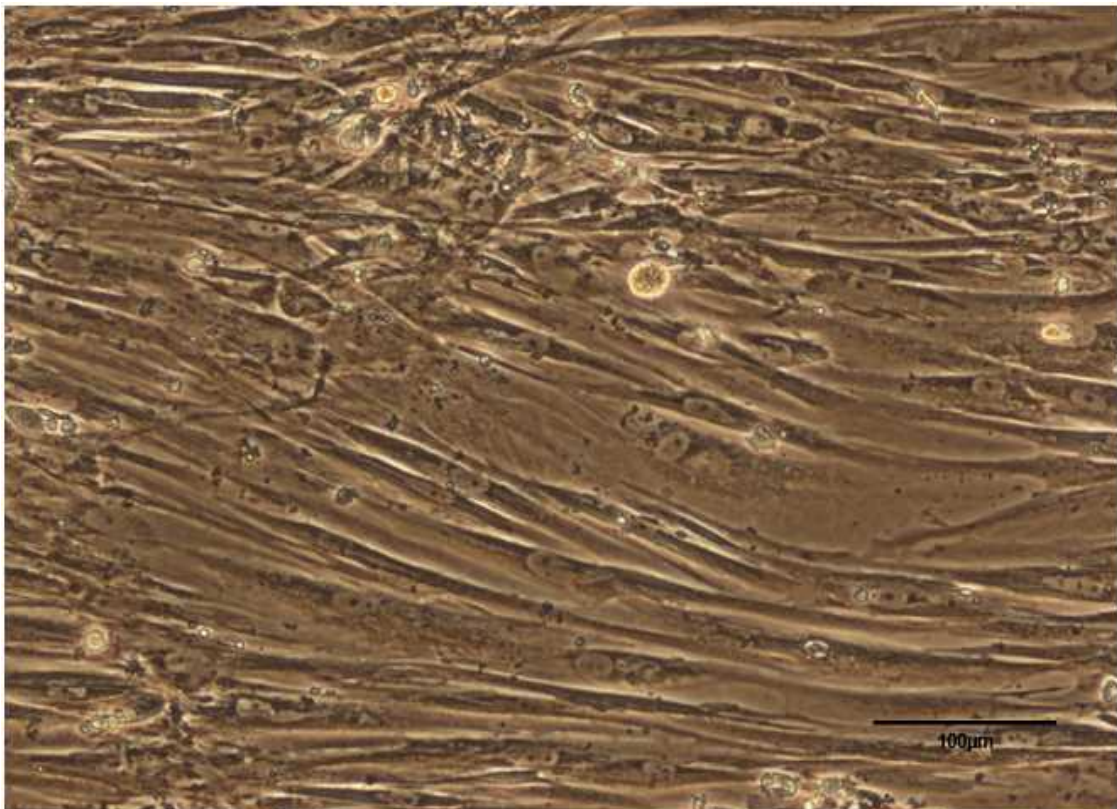


Fig. 3.1. GROWTH OF FIBROBLASTS IN A PETRIDISH.

Bright field Imaging of Fibroblasts growing in a petridish in FGM.

Scale bar: 100µm

In the pilot data, the endothelial cells were differentiated from fibroblasts. The fibroblasts and endothelial cells were harvested using enzymatic digestion technique from the same

RA sample and 2 different sets were made. Once plated, the cells were reviewed under bright field microscope on a daily basis and their different morphological features were noted (i.e. fibroblasts were large, elongated and spindle shaped in contrast to the small, rounded and discoid endothelial cells). Both endothelial cells and fibroblasts were grown in their specific mediums (FGM and ECGM (endothelial cell growth medium)) simultaneously. The cardiomyocytes in both samples were unable to replicate and died within 48 hours. In the first set, I used FGM for both FBs and ECs and it was noted that FGM was not sufficient enough to keep the endothelial cell alive after 4-5 days, but fibroblasts kept on proliferating steadily. In set 2, I used ECGM (LONZA, CC-5036 medium with CC-4542 supplement) for both cell types. With ECGM, the endothelial cells were proliferating and replicating optimally but the fibroblasts showed a massive myofibroblastic transformation after day 5 and majority of the cells died by day-8. These cells were stained with fibroblasts and endothelial cell specific positive and negative markers to confirm their respective true colonies (Appendix A; Figure A1.21 and Figure A1.22). The crossing over of mediums for fibroblasts and endothelial cells could not support the growth of these two cell populations after a few days. With the tissue explantation technique, only the fibroblasts grew out of the explanted tissue sample and this technique provided an unadulterated population of fibroblasts without any other resident cell population.

Further confirmation of cells isolated as fibroblasts was carried out by staining each sample with a pre-defined set of positive (DDR-2, THY-1, Vimentin and Periostin) and negative fibroblast markers (α -SMA, α -cardiac actinin, vWF and cardiac MHC). The endothelial cells were positive for vWF whilst fibroblasts were negative for it. Following the identification of a pure fibroblast population, the cells from all four chambers were analysed individually for expression of transcription factors and are discussed later in this chapter. The variation in the expression of TFs was compared among all four-chambers.

3.2 INTRODUCTION

Cardiac fibroblasts have been studied and characterised in murine and canine models previously. They are a highly pleomorphic population of non-cardiomyocytes and play a dynamic role in homeostasis and during the disease process. The interaction of cardiomyocytes and non-cardiomyocytes in maintaining the structural and functional integrity of cardiac tissues is pivotal and is a subject of interest in many recent studies. The biological characteristics of non-cardiomyocytes are identified in other species but their precise role is yet to be explored and defined.

Human cardiac fibroblasts have been investigated in many other studies but this is confined to an overview of markers and protein expression. Although they have been characterised extensively in the murine model and human non-cardiac models, a little is known about chamber-specific human cardiac fibroblasts. All chambers of the human heart are architecturally and functionally different from each other. The variation of physiological pressures is governed by the structural pattern and biochemical profile at cellular level. This difference might be translated to the cell characteristics of these chambers.

In this study, human cardiac fibroblasts from all four chambers in the same patients are studied for the first time. The morphological characteristics, growth pattern and secretory profile of fibroblasts from different chambers in the same patient will help to understand their precise role. The cardiac fibroblasts from all four chambers characterised by predicted markers and the expression of the transcription factors are investigated.

3.3 METHODOLOGY

For pilot data, the adult human atrial fibroblast population was isolated and harvested from tissue samples from the patients undergoing routine Coronary Artery Bypass Grafting and Valve Replacement surgery. The ventricular samples of the pilot data were taken from the native heart of the transplant recipients, predominantly with dilated cardiomyopathy (DCM) with the exception of three samples where the underlying pathology was ischaemic cardiomyopathy (ICM). However, for the main study, the human atrial and ventricular samples were taken from all four chambers from the same heart in a patient population with normal LV function.

The fibroblasts were isolated with both explantation and enzymatic digestion technique for the pilot data, but for the main study, fibroblasts from all four chambers via myocardial biopsy samples were isolated with explantation technique only.

The fibroblasts were examined under bright field microscopy for rate and pattern of growth at pre-defined intervals. They were further identified with a group of positive (Vimentin, DDR-1, Thy-1, and Periostin) and negative markers (α -SMA, α -Actinin, vWF and MHC) to distinguish them from the other cellular constituents of human myocardium. The additional characterisation was performed with a set of transcription factors as mentioned in chapter 2. The expression of transcription factors was compared between the chambers.

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3.4. EXPERIMENTAL DETAILS AND DESIGN:

3.4.1 STUDY DESIGN

This study was a single-centre, case-control study aimed to identify and characterise human cardiac fibroblasts from all four chambers in normal left ventricle function group. The design of the study is as following

- 4 chamber biopsies.
- Isolation of cardiac fibroblast.
- Characterisation of cardiac fibroblasts with immunocytochemistry.
- Transcription factors expression of chamber-specific fibroblasts.
- Incorporation of cardiac fibroblasts isolated from all four chambers into EHTs to investigate the interaction between non-cardiomyocytes and cardiomyocytes.
- Analysis of proteins secreted into the bathing medium for EHTs.

The research objectives were addressed by the characterisation of the human cardiac fibroblasts isolated from all four chambers of the heart in patients with normal myocardial function undergoing coronary artery bypass grafting (CABG) or valve repair/replacement surgery. The similar proposed investigation with impaired myocardial function group was not carried out due to the time factor. Both normal and abnormal myocardial function groups had 20 patients each. Transthoracic echocardiography (TTE) was performed as part of the standard pre-operative assessment care plan in all in-hospital and elective patients undergoing cardiac surgical intervention. No additional procedures or interventions were performed on study participants in normal LV function group. (However, in the patient with impaired myocardial function group confirmed by echocardiographic findings (Ejection Fraction < 30), a CMR for further evaluation of the infarcted, hibernation and normal myocardial segments before surgical procedure was planned). Qualified and trained technical staff in their respective departments performed the TTE examination. An accredited cardiologist reported the results respectively. No changes were made in the routine management of the patients or operative techniques because of participation in this study. Consent from these patients was obtained under the Brompton Biomedical Research Unit Protocols.

3.4.2 STUDY POPULATION

The following eligibility criterion was designed for the study participants.

Inclusion criteria	Exclusion criteria
Inclusion criteria to normal ventricular (LV) group (EF > 50): • Patients undergoing coronary artery bypass grafting (CABG) or aortic/mitral valve repair/replacement (AVR/MVR). • In-hospital urgent patients and outpatients who are undergoing TTE for other clinical indications and are known to have normal LV function and any valve pathology as evidenced by previous transthoracic echocardiography. • Inclusion criteria to abnormal ventricular (LV) group (EF < 30): • Patients listed for CABG or aortic/mitral valve repair/replacement operations with abnormal LV function as indicated by previous clinical investigations and assessments. A repeat pre-operative transthoracic echocardiography (TTE) will be performed in this group to confirm previous finding as part of standard preoperative care followed by CMR.	• Age < 18 years or > 85 years • Critical preoperative status with multi organ dysfunction. • Emergency Cardiac Surgical Intervention. • Cancer or history of cancer in the last five years • Viral hepatitis B and C and human immunodeficiency virus (HIV) infection. • Congenital heart or connective tissue disease. • Hepatic dysfunction (Bilirubin > 20 mmol/L, Prothrombin > 2.0 ratio). • Pulmonary disease (FEV1 < 40% predicted) • Renal failure (with a GFR < 30 ml/min/1.73m2) • HbA1C > 8.5 • Participation in another interventional study • Unable to given informed consent or unwilling to participate in research. • Definite contraindications for CMR monitoring (Pacemaker, defibrillator, metallic foreign bodies, bullet or gunshot pellets, cerebral aneurysm clips, cochlear implants, magnetic dental implants etc. • Unable to take adequate biopsies due to technical difficulties at the time of procedure. (Massive bleeding, excessive fibrotic tissues encasing heart, adhesions etc.)

Fig. 3.2. CRITERION FOR PARTICIPATION IN THE STUDY

3.4.3 SCHEME OF ANALYSIS OF HUMAN CARDIAC FIBROBLASTS

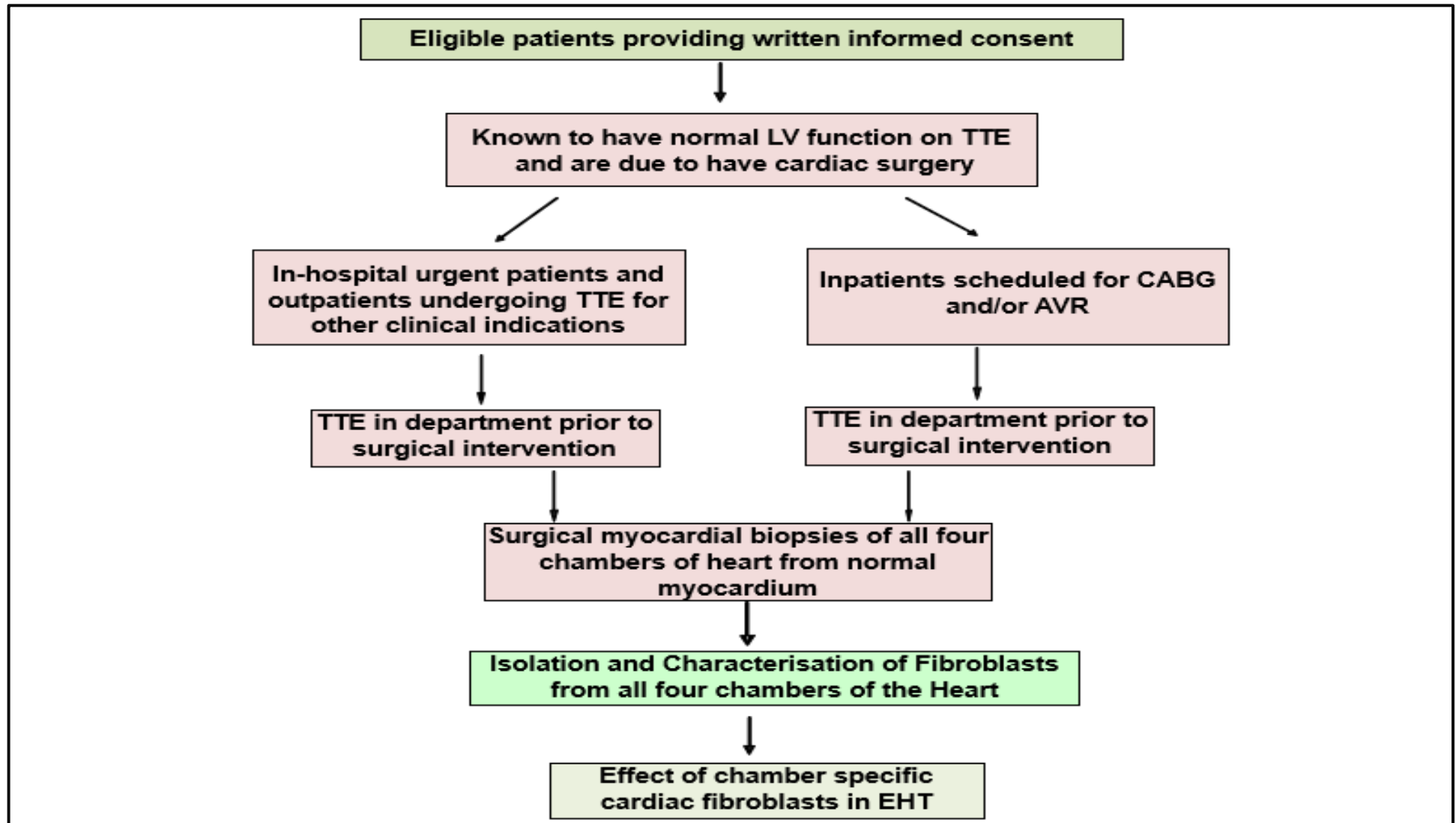


Fig 3.3. SCHEME OF ANALYSIS OF HUMAN CARDIAC FIBROBLASTS

3.5 RESULTS

3.5.1 FIBROBLASTS OF PILOT DATA

For pilot data set, 56 human myocardial samples were collected from different chambers in 45 patients with normal and abnormal ventricular groups to master the techniques of the fibroblast isolation. The fibroblasts were isolated and cultured with enzymatic digestion and explant technique followed by their immunostaining. Human cardiac biopsies were taken from different chambers in patients of diverse age groups (20 - 82 years of age) with different underlying pathologies. The left ventricular samples of pilot data were taken from the native heart of transplant recipient with established abnormal ventricular function (EF = 20%). The data collected from these samples revealed that the human cardiac fibroblasts from different chambers showed different patterns and rate of growth.

The first 12 samples collected between October 2014 – December 2014 were infected over the Christmas period and are highlighted with dark green coloured rows. Though the growth pattern assessment of the fibroblasts samples was noted down at specific intervals in these samples, the accurate cell count at different stages could not be carried out due to infection. The other 44 samples yielded fibroblasts with a measurable pattern and rate of growth.

TABLE. 3.1. MYOCARDIAL BIOPSY SAMPLES FOR PILOT DATA.

	Age	Sex	Date of Surgery	Procedure	Tissue Sample	Tissue Processing	Initial Fibroblast Morphology	Time to P1
1.	68	M	20/10/14	CABG + AF Ablation	Left & Right atrial Appendage	20/10/14	Good growing cells	03/11/14 (13 days)
2.	61	M	22/10/14	Transplant - ICM	Left Ventricle	22/10/14	FB growing slow in colonies	20/11/14 (28 days)
3.	72	M	27/10/14	CABG	Right atrial Appendage	27/10/14	Good growing cells	15/11/14 (18 days)
4.	71	M	30/10/14	CABG	Right atrial Appendage	30/10/14	Good growing cells	13/11/14 (13 days)
5.	48	M	05/11/14	M-AVR	Right atrial Appendage	05/11/14	Good growing cells	18/11/14 (13 days)
6.	80	F	10/11/14	MVR + CABG + AF Ablation	Left & Right atrial Appendage	10/11/14	Good growing fibroblasts	25/11/14 (15 days)
7.	58	M	14/11/14	Transplant - ICM	Left Ventricle + Right Ventricle	14/11/14	Slow growing Fibroblasts	07/12/14 (23 days)
8.	63	F	17/11/14	T-AVR	Right atrial Appendage	17/11/14	Good growing fibroblasts	01/12/14 (13 days)
9.	74	M	24/11/14	CABG	Right atrial Appendage	24/11/14	Good growing fibroblasts	10/12/14 (16 days)
10.	61	M	08/12/14	MV Repair + CABG	Right atrial Appendage	08/12/14	Good growing fibroblasts	22/12/14 (14 days)
11.	66	M	11/12/14	MVR	Mitral valve + Right Atrial appendage	11/12/14	Good growing RA fibroblasts + slow growth of MV fibroblasts	Infected
12.	62	M	19/12/14	CABG	Right atrial Appendage	19/12/14	Good growing fibroblasts	Infected
13.	77	F	05/01/15	AVR	Right atrial Appendage	05/01/15	Good growing fibroblasts	28/01/15 (13 days)
14.	64	M	05/01/14	CABG	Left & Right atrial Appendage	05/01/15	Good growing fibroblasts	31/01/15 (15 days)
15.	52	M	08/01/15	CABG	Right atrial Appendage	08/01/15	Good growing fibroblasts	22/01/15 (14 days)
16.	77	F	12/01/15	MV & TV Repair + LAAO + AF Ablation	Left & Right atrial Appendage	12/01/15	Good growing fibroblasts LA > RA	25/01/15 (13 days)
17.	42	M	12/01/15	Transplant - DCM	Left Ventricle + Right Ventricle	12/01/15	Good growing fibroblasts	13/02/15 (30 days)
18.	76	M	19/01/15	T-AVR	Right atrial Appendage	19/01/15	Good growing fibroblasts	02/02/15 (14 days)
19.	64	M	21/01/15	MV Repair	Left & Right atrial Appendage	21/01/15	Good growing fibroblasts LA > RA	05/02/15 (15 Days)
20.	64	M	26/01/15	T-MVR + T-AVR	Right atrial Appendage	26/01/15	Good growing fibroblasts	10/02/15 (15 days)
21.	70	F	02/02/15	MV Repair	Right atrial Appendage	02/02/15	Good growing fibroblasts	16/02/15 (14 days)
22.	61	M	11/02/15	Septal Myectomy	LV Septum	11/02/15	Good growing cells	16/03/15 (30 days)
23.	54	F	14/02/15	Transplant - DCM	Left Ventricle	14/02/15	Good growing cells	16/03/15 (28 days)

	Age	Sex	Date of Surgery	Procedure	Tissue Sample	Tissue Processing	Initial Fibroblast Morphology	Time to P1
24.	67	M	14/02/15	CABG	Right atrial Appendage	14/02/15	Good growing fibroblasts	01/03/15 (15 days)
25.	70	M	26/02/14	T-AVR	Right atrial Appendage	26/02/15	Slow growing Fibroblasts	13/03/15 (15 days)
26.	50	M	03/03/15	Transplant - DCM	Left Ventricle + Right Ventricle	03/03/15	Slow growing Fibroblasts	03/04/15 (30 days)
27.	78	M	17/03/15	CABG	Right atrial Appendage	17/03/15	Good growing fibroblasts	31/03/15 (16 days)
28.	42	M	27/03/15	Transplant – DCM	Left Ventricle	27/03/15	No cells growth at 3 weeks - Jelly like ECM around explants- started growing	23/06/15 (88 days)
29.	56	M	17/04/15	Transplant – DCM	Left Ventricle	17/04/15	No cells growth at 3 weeks - Jelly like ECM around explants - started growing	No cell growth
30.	50	M	21/04/15	Transplant – DCM	Left Ventricle	21/04/14	Very slow growing fibroblasts - Cells started growing after 5 weeks.	23/06/15 (58 days)
31.	80	M	22/04/15	CABG	Right atrial Appendage	22/04/15	Good growing fibroblasts	08/05/15 (16 days)
32.	75	M	27/04/15	T-AVR	Right atrial Appendage	27/04/15	Slow growing Fibroblasts	13/05/15 (15 days)
33.	82	F	10/05/15	CABG	Right atrial Appendage	10/05/15	Good growing fibroblasts	01/06/15 (20 days)
34.	69	M	14/05/15	CABG	Right atrial Appendage	12/05/15	Good growing fibroblasts	03/06/15 (20 days)
35.	51	M	12/06/15	Transplant - DCM	Left Ventricle	12/06/15	Good growing fibroblasts	05/09/15 (23 days)
36.	58	F	19/08/15	SIRCA Trial	Left Ventricle	19/08/15	Very Slow growing fibroblasts	30/09/15 (41 days)
37.	47	M	28/08/15	Transplant - DCM	Left Ventricle	28/08/15	Good growing fibroblasts	20/09/15 (23 days)
38.	65	M	31/08/15	MVR	Left & Right atrial appendage	31/08/15	Good growing fibroblasts	17/09/15 (17 days)
39.	24	M	04/09/15	Transplant - DCM	Left Ventricle	04/09/15	Slow growing Fibroblasts	29/09/15 (25 days)
40.	77	M	07/09/15	T-AVR	Right atrial Appendage	07/09/15	Good growing fibroblasts	26/09/15 (18 days)
41.	69	F	07/09/15	MV- Repair	Left & right atrial Appendage	07/09/15	Good growing fibroblasts	23/09/15 (15 days)
42.	44	F	08/09/15	Transplant - DCM	Left Ventricle	08/09/15	Good growing fibroblasts	25/09/15 (20 days)
43.	44	M	09/09/15	Transplant - DCM	Left Ventricle	09/09/15	Good growing fibroblasts	01/10/15 (21 days)
44.	61	M	14/09/15	Transplant- ICM	Left Ventricle	14/09/15	Good growing fibroblasts	05/10/15 (20 days)
45.	59	M	28/09/15	T-AVR	Right atrial Appendage	28/09/15	Moderately growing fibroblasts	17/10/15 (19 days)

	Cell count at onset of P1	Fibroblast Morphology at P1 stage	Time to P2	Cells count at onset of P2
1.	LA=95 X 10 ⁵ , RA = 90 x 10 ⁵	LA sample growing fast than RA. Good growth in sheets	25/11/14 (23 days)	Infected
2.		Scattered colonies - very slow to grow and non-confluent	15/12/14 (25 days)	Infected
3.		Slow growth at P1 - some cells are becoming more globular	05/12/14 (20 days)	Infected
4.		Slow growing cells in 1 x T 75 – other T75 is better than that.	05/12/14 (23 days)	Infected
5.		Good cells growth. All 3 T50 flasks are growing well.	08/12/14 (25 days)	Infected
6.		Good growth at LA sample. RA sample is FB are half attached and half lifted up from the flask	15/12/14 (20 days)	Infected
7.		Slow growing pattern with scattered FB - some cells are just growing in size. RV is better than LV	23/12/14 (19 days)	Infected
8.		Good cells growth. All T50 flasks are growing well.	23/12/14 (22 days)	Infected
9.		Good cells growth. 2 x T-50 flasks are growing well.	Infected	Infected
10.		Can't Assess the cell growth	Infected	Infected
11.	Not counted	Can't Assess the cell growth	Infected	Infected
12.	Not counted	Can't Assess the cell growth	Infected	Infected
13.	50 x 10 ⁵	Good cells growth. All 3 T50 flasks are growing well.	03/03/15 (34 days)	120 x 10 ⁴
14.	LA = 90 x 10 ⁴ , RA = 80 x 10 ⁵	Good cells growth. 2 x T50 flasks are growing well.	27/02/15 (28 days)	LA = 75 x 10 ⁴ , RA = 70 x 10 ⁵
15.	105 x 10 ⁵	Good cells growth. 2 x T50 flasks are growing well.	05/02/15 (14 days)	85 x 10 ⁵
16.	LA = 60 x 10 ⁵ , RA = 40 x 10 ⁵	Good growth at LA sample. RA sample is FB are piling up in the middle of the flask	20/02/15 (26 days)	LA = 95 x 10 ⁵ , RA = 80 x 10 ⁵
17.	LV = 110 x 10 ³ , RV = 190 x 10 ³	Cells are slow to grow and scattered colonies. Piling up seen in some. Large gigantic myofibroblast like cell are there.	20/03/15 (35 days)	LV = 125 x 10 ³ , RV = 160 x 10 ⁴
18.	70 X 10 ⁵	Cells are growing well. Black granules are spread all over 2 T50 flasks	20/02/15 (18 days)	95 x 10 ⁵
19.	LA = 70 x 10 ⁵ , RA = 50 x 10 ⁵	Cell of LA are growing better than RA. RA has scattered cell all over the flask. Colonies are dense in the middle and large cells at peripheries.	03/03/15 (26 days)	LA = 80 x 10 ⁵ , RA = 90 x 10 ⁴
20.	110 x 10 ⁴	Cells are growing well. One T50 got infected but the other one is good.	22/02/15 (12 days)	130 x 10 ⁴
21.	95 x 10 ⁵	Cells are relatively slow to grow in comparison with the other samples.	05/03/15 (17 days)	105 x 10 ⁵
22.	150 x 10 ³	Slow cell growth with very scattered colonies. After first week growth started picking up	01/04/15 (16 days)	110 x 10 ⁴
23.	360 x 10 ³	Cells are growing with good speed. T75 flask is well covered	02/04/15 (18 days)	325 x 10 ³
24.	75 x 10 ⁴	Good FB growth in one T75. in other 2 cells are still floating. Flasks were coated with fibronectin??	25/03/15 (24 days)	120 x 10 ⁴

	Cell count at onset of P1	Fibroblast Morphology at P1 stage	Time to P2	Cells count at onset of P2
25.	105×10^5	Good cells growth. 2 xT50 flasks are growing well. Black granules all over the cells in one flask.	25/03/15 (12 days)	75×10^5
26.	LV = 190×10^3 , RV = 120×10^4	Right ventricle is better but not comparable to LA/RA. LV cells were extremely slow to grow.	08/04/15 (36 days)	LV = 230×10^3 , RV = 170×10^4
27.	240×10^4	Good FB growth - 3 xT50 flasks are growing well.	13/04/15 (13 days)	190×10^4
28.	LV = 290×10^4	Good cells at last. All 25 6cm petridishes are growing well.	20/07/15 (28 days)	LV = 250×10^4
29.	LV = 350×10^3	Still in the process at P1 - on going	No growth – tissue discarded at P1.	
30.	LV = 210×10^3	Very confluent FB and are covering the bottom of all 4 T75	30/07/15 (37 days)	230×10^3
31.	185×10^4	Good growth of cells – Fibroblasts - myofibroblasts in 2 flasks	08/06/15 (30 days)	250×10^4
32.	80×10^5	Some cells are growing good in colonies and other are a bit scattered and looks like myofibroblasts	03/06/15 (21 days)	110×10^5
33.	45×10^3	Good growth - Black sand dust like deposit all over the T50 flask	15/06/15 (14 days)	120×10^4
34.	90×10^5	Normal cell growth - FB are settling well and attached to bottom of the flask	20/06/15 (17 days)	105×10^5
35.	LV = 230×10^4	Slow to grow in first 10 days at P1 stage. Later half growth was good	25/09/15 (20 days)	180×10^4
36.	LV = 45×10^3	Slow growing fibroblasts up to P1 maturation. P2 maturation was comparatively better	02/11/15 (33 days)	60×10^4
37.	LV = 110×10^4	Scattered colonies at P0 stage, Better confluence at P1 & P2 stage.	19/10/15 (29 days)	150×10^4
38.	RA = 130×10^5 LA = 150×10^5	Good growth rate and confluence pattern. At P2 2 T-75 with LA fibroblasts are growing better than the third one.	10/07/15 (20 days)	RA = 95×10^5 LA = 125×10^5
39.	LV = 70×10^5	Good growth and confluence pattern at all stages (P0, P1, P2)	26/10/15 (27 days)	190×10^4
40.	RA = 210×10^4	Tissue was very fatty and has to be washed repeated at P0. Good P2 growth	17/10/15 (21 days)	180×10^5
41.	LA = 170×10^5 , RA = 50×10^4	LA sample was twice the size of the RA sample and this effected the yield as well. Rate of growth of LA sample is better than the RA. At P2, rate of growth of RA sample was good	12/10/15 (day19)	LA = 135×10^5 , RA = 105×10^5
42.	97×10^5	Good growing fibroblasts in individual colonies but slow to develop confluence pattern	15/10/15 (20 days)	125×10^5
43.	85×10^5	Good growing fibroblasts forming a gradual sheet of cell with good confluence.	20/10/15 (19 days)	97×10^5
44.	105×10^5	Good growth at P1 - normal morphological fibroblast with gradual confluence. At P2 stage, growth rate was very slow, and cells died on day		
45.	RA = 145×10^5	Slower rate of growth at all stages in comparison with previous RA samples. However, the fibroblasts count was comparable.	11/11/15 (25 days)	RA = 110×10^5

Table 3.1. MYOCARDIAL BIOPSY SAMPLES FOR PILOT DATA. The tabulated data set enlisted the myocardial biopsy samples collected from different patients with varying pathologies undergoing different procedures. The samples were processed via explant and enzymatic digestion techniques followed by rate and pattern of growth assessment at pre-set time intervals.

	RIGHT ATRIUM	LEFT ATRIUM	LEFT VENTRICLE	RIGHT VENTRICLE
1	YES	YES		
2			YES	
3		YES		
4		YES		
5		YES		
6	YES	YES		
7			YES	YES
8		YES		
9		YES		
10		YES		
11		YES	YES	
12		YES		
13		YES		
14	YES	YES		
15		YES		
16	YES	YES	YES	YES
17				
18		YES		
19	YES	YES		
20		YES		
21		YES		
22			YES	
23			YES	
24		YES		
25		YES		
26			YES	YES
27		YES		
28			YES	
29			YES	
30			YES	
31		YES		
32		YES		
33		YES		
34		YES		
35			YES	
36			YES	
37			YES	
38	YES	YES		
39			YES	
40		YES		
41	YES	YES		
42			YES	
43			YES	
44			YES	
45		YES		
TOTAL	7	29	17	3

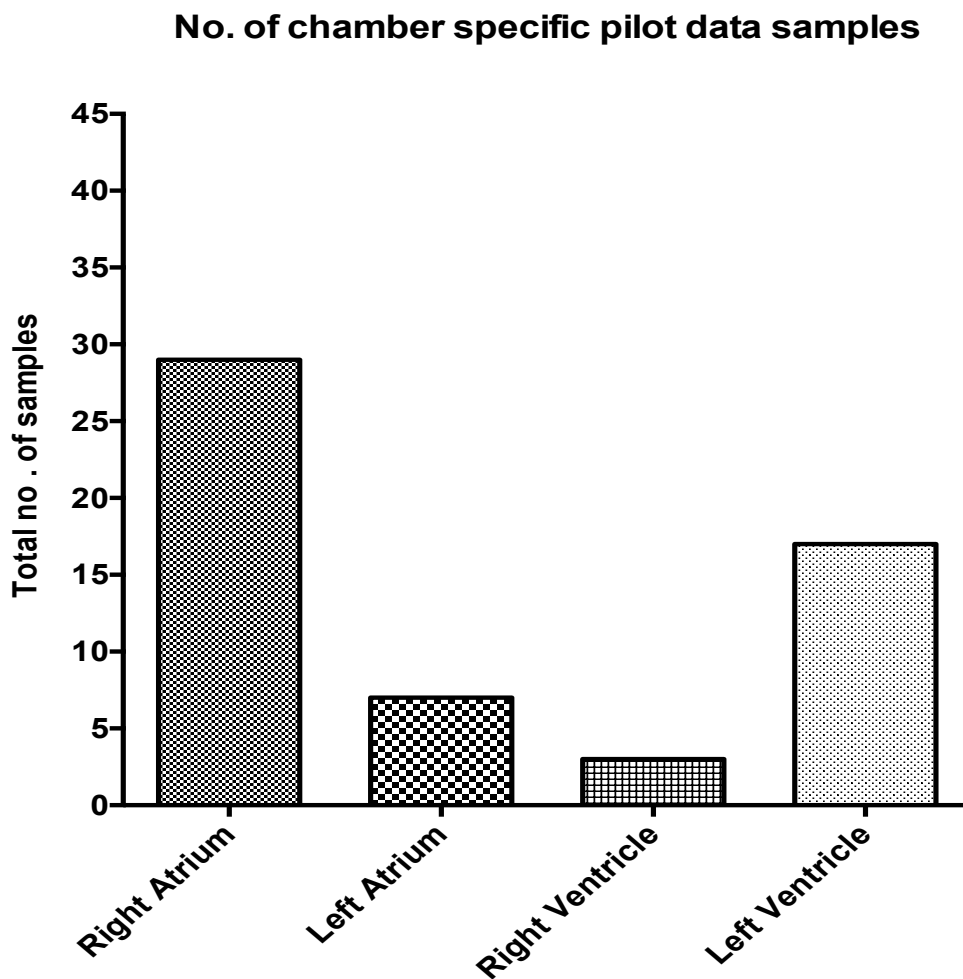


Table 3.2. MYOCARDIAL BIOPSIES FROM INDIVIDUAL PATIENT POPULATION AND CARDAIC CHAMBERS

3.5.2 GROWTH RATE OF HUMAN CARDIAC FIBROBLASTS:

Preliminary data from 56 tissue samples from 45 patients showed that

- a) From the time of explantation of tissue till the P1 stage, the fastest CF growth rate was observed in left atrial samples followed by right ventricle and right atrium samples with almost similar rates. The fibroblasts from left ventricle showed slowest growth and multiplication rate. The confluence pattern in 6-cm diameter petridishes was achieved after 32 days of explantation in LV fibroblasts. (Table. 3.3) All the ventricular samples in the pilot data were from failing hearts (dilated & ischaemic cardiomyopathy). The sample size had a strong bearing on the rate and tenure of growth (large sample i.e. more fibroblasts grow rapidly in colonies reaching a stage amenable for the next passage, and vice versa).
- b) The time interval required to achieve the optimal growth and confluency at P0, P1 & P2 stage in the atrial samples was 19 ± 2 days and almost 22 ± 2 days for the ventricular samples. (Table. 3.3) Although the right ventricular samples were taken from the failing heart of transplant recipient, the rate of growth was comparable to the atrial samples from the normal ventricular function patients.

Chambers	No. of Samples = 56	Time for P0 Confluence (Mean) - days	Time for P1 Confluence (Mean) - days	Time for P2 Confluence (Mean) – days
Left Ventricle	17	32	27	26
Right Ventricle	3	19	17	18
Left Atrium	7	14	19	17
Right Atrium	29	15	21	22

Table 3.3: GROWTH RATE OF HUMAN CARDIAC FIBROBLASTS: The rate of growth of cardiac fibroblasts, isolated from all four chambers from the time of explantation until they are ready for their first passage; are cited in terms of number of days. The time interval required for CF isolated from different chambers at P1 and P2 stage.

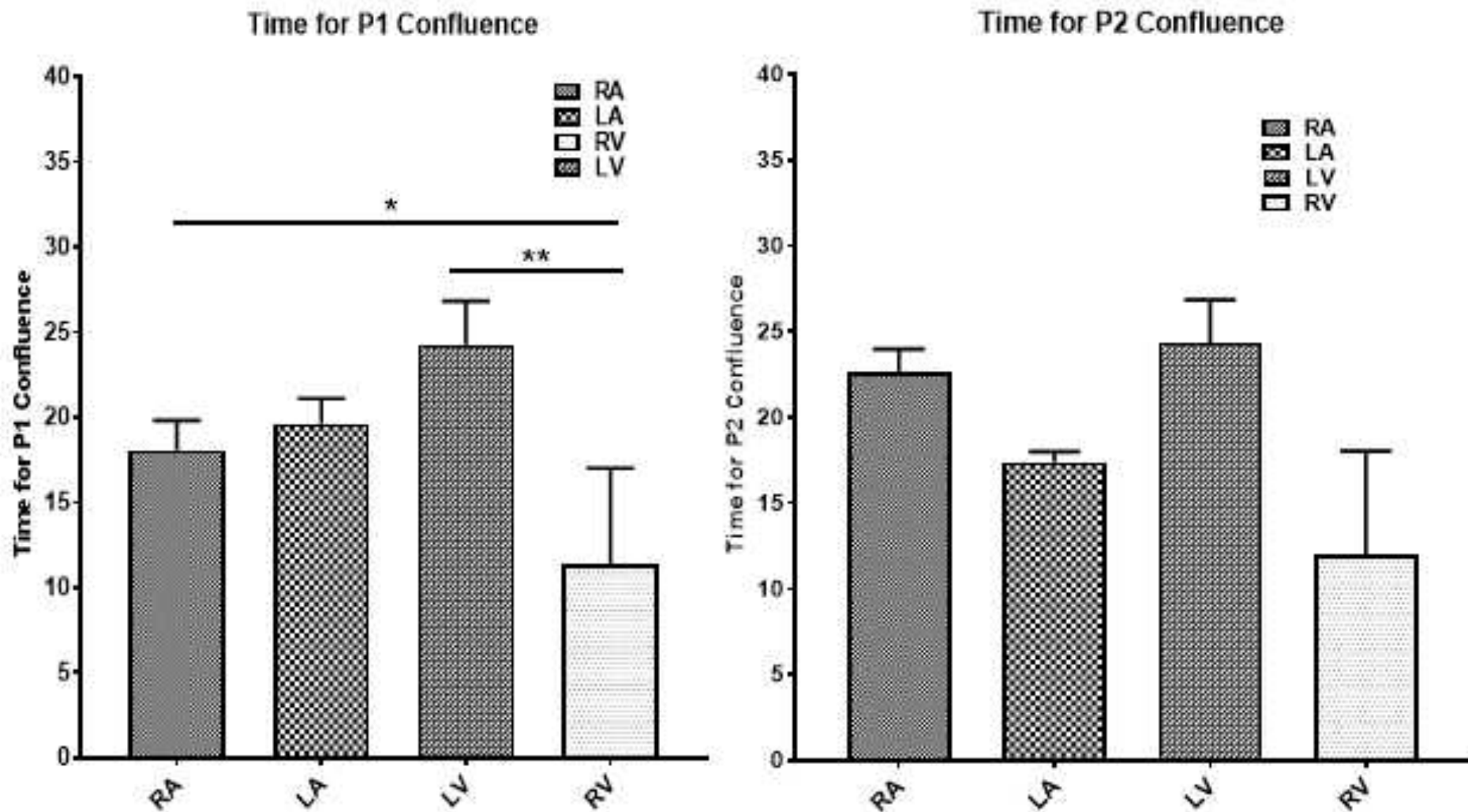


Fig. 3.4. GROWTH RATE OF CHAMBER-SPECIFIC FIBROBLAST FROM PILOT DATA. The time for P1 confluence between four chamber biopsy sample was statistically significant between LV and RV. $p = 0.05$ (**) and between RA and RV $p = 0.05$ (*). The time interval for P2 confluence between the chamber-specific fibroblasts was not statistically significant. **Error Bars: Mean +/- SEM**

3.5.3 GROWTH PATTERNS OF HUMAN CARDIAC FIBROBLASTS

The explanted tissue was plated on fibronectin coated petridishes and fibroblasts were allowed to grow out of the tissues. The tissue explantation technique was deemed to be better than enzymatic digestion technique, in terms of getting a true population of fibroblasts; as adult human cardiomyocytes have no replication potential and endothelial cells did not grow with the FGM used for enrichment of fibroblasts. The rate of infection was much less with explantation technique in comparison with enzymatic digestion technique as it is easier to keep the field sterile.

The growth of these fibroblasts was noted and serially monitored on days 5, 7, 10 and 15. The atrial and ventricular samples were compared on the same timelines regarding their growth pattern at different stages. Depending upon requirement, the tissue growth rate and pattern the explants were reviewed intermittently apart from the mentioned timeline. In atrial samples, the growth pattern assessment at these pre-set intervals in a 6cm Petridishes before P1 revealed that CFs were almost always able to achieve a uniform monolayer all over the floor of the petridish with occasional patches or islands in between colonies, which are devoid of the cell. (Figure 3.6. A-F)

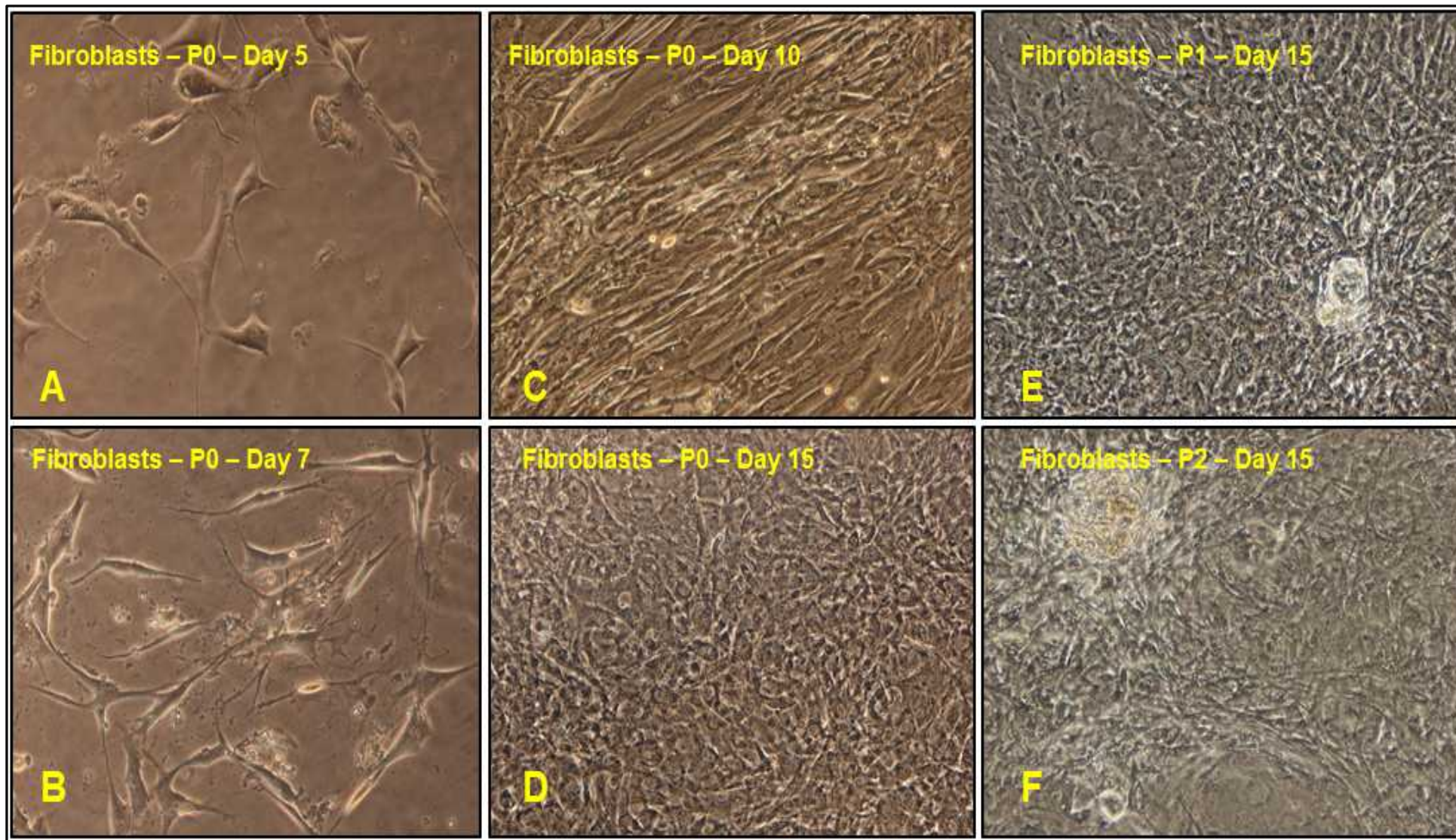


Fig. 3.5. GROWTH RATE AND PATTERN OF HUMAN CARDIAC FIBROBLASTS. Bright field microscopic images (10x) assessment of generalised growth pattern of human atrial fibroblasts at P0 stage on day 5, 7, 10 and 15. (Fig. A, B, C, D) After passage to next stage, the same growth and confluence pattern is observed at P1 and P2 stage (Fig. E & F)

In pilot data, the ventricular samples before P1 usually showed scattered colonies, with only a few growing and merging with each other. However, in almost half of the samples, there were islands and patches devoid of any cells in between the colonies. (Fig. 3.7B) The cells were passaged to P1 and P2 when approximately 75% of the area of petridish was covered with colonies.

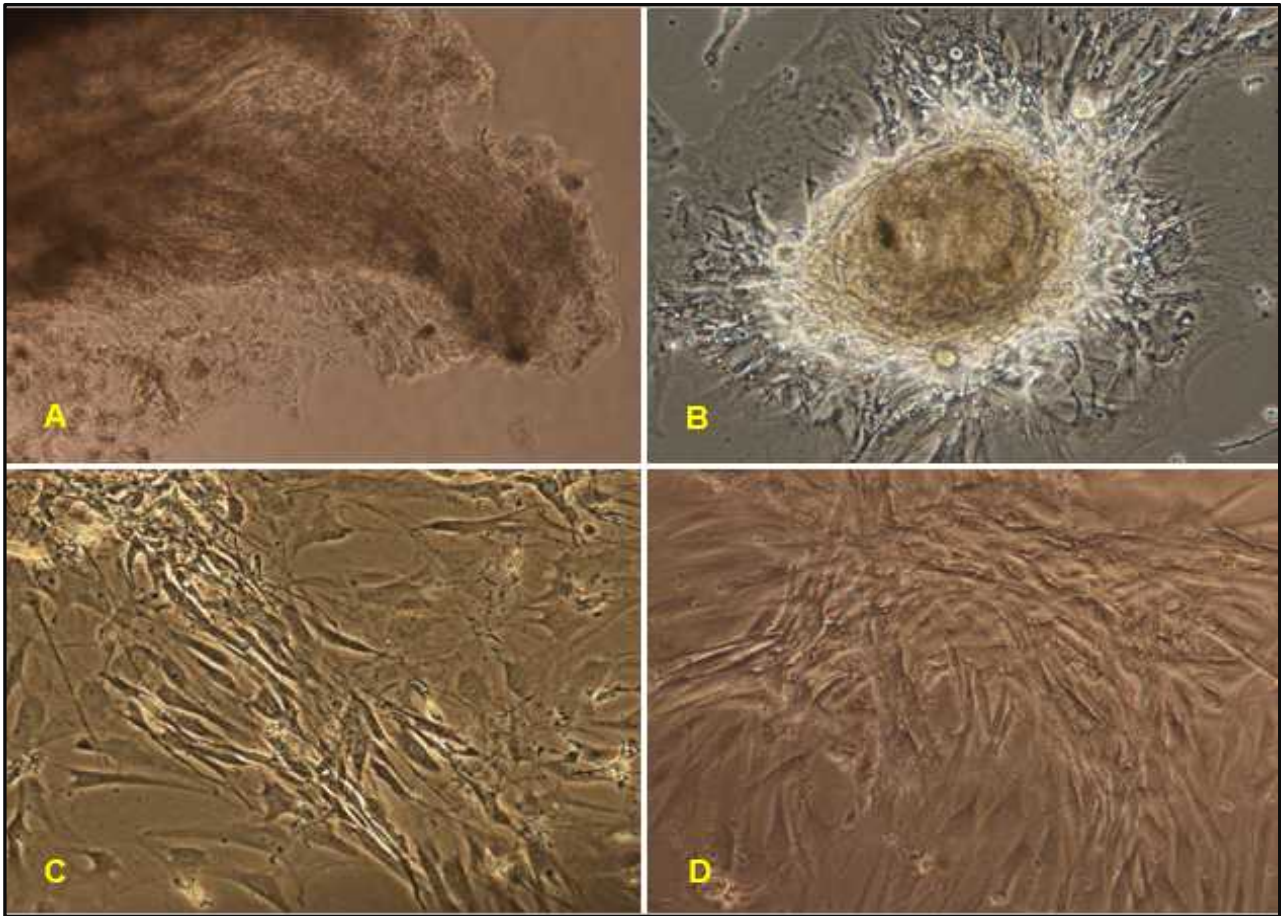


Fig. 3.6. FIBROBLASTS SHOWING ABNORMAL GROWTH PATTERN IN COLONIES. No. of fibroblast growth in the colony with a jelly-like tissue surrounding the colony was noted. (A) Fibroblasts derived from atrial and ventricular sample (B, C, and D) showing piling of cells in the center of the colonies at P1 stage preventing the formation of normal fibroblast monolayer.

Some ventricular tissue samples did not show any signs of growth. The colony front showed, only jelly-like extracellular Matrix (ECM) with no cellular component. (Fig. 2.7A) Since all the ventricular samples were collected from patients with dilated cardiomyopathy (with or without VADS) and patients with rheumatic mitral valve disease, they might differ in

characteristics, phenotype and differentiation potential in comparison with normal ventricular fibroblasts. In a few exceptional situations, cells had to be passaged to the next stage earlier, though the area of the petridish covered with fibroblasts monolayer were suboptimal (less than the desired 75% of total area) and was the confluency pattern of colonies. (Fig. 3.7. C-D). In these samples the fibroblasts growth was erratic, forming a pile in the middle of the colonies rather than expanding horizontally as a flat sheet of cells on the floor of petridish.

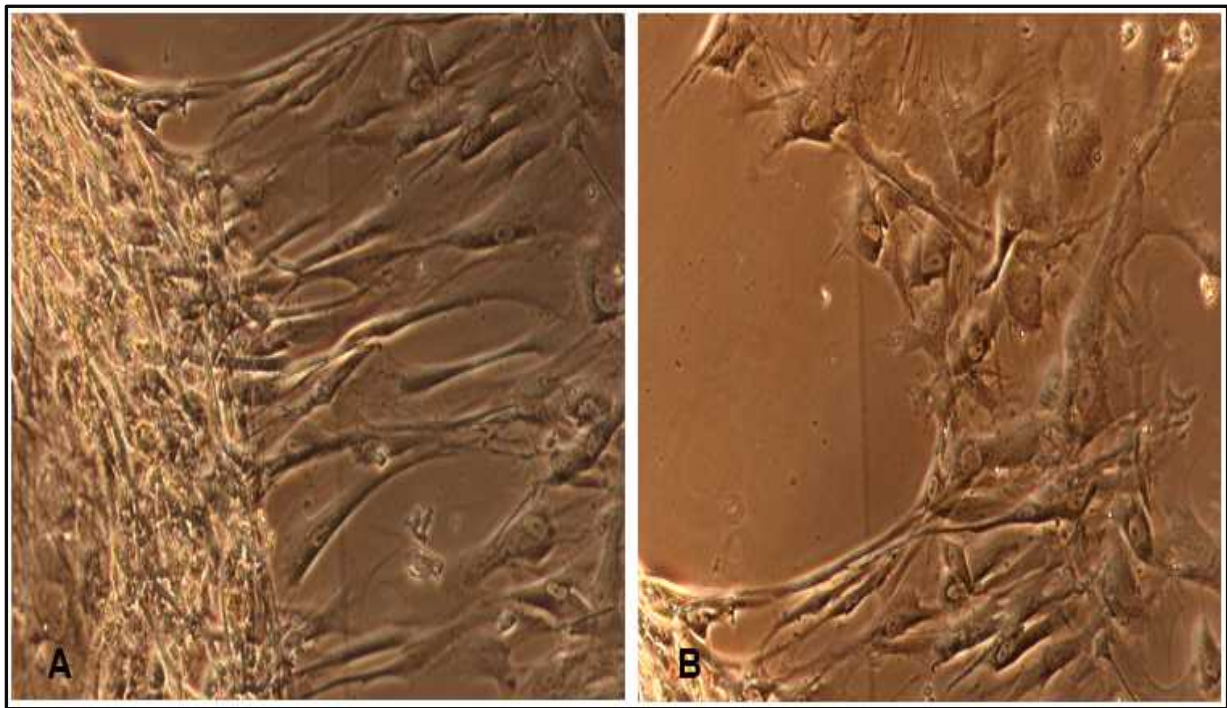


Fig. 3.7. DISPARITY OF GROWTH FRONT OF FIBROBLASTS COLONIES. Within the same colony, different growth fronts were observed. (A) active growth front of a colony against the passive growth front (B)

A disparity in the growth front of the colonies was observed as well. Within the same colony of growing fibroblasts, one side was more active than the others. The cells were migrating and making linking bridges to the other colonies in half (Fig. 3.8A) of the sphere while the other half was dormant (Fig. 3.8B).

3.5.4 CARDIAC FIBROBLASTS COUNT AT DIFFERENT STAGES

The fibroblast count at different stages was undertaken to assess the rate of growth in tissue samples from separate cardiac chambers. The maximum number of fibroblasts yielded was seen in the left atrium followed by the right atrium. Amongst the ventricles, the rate of growth and yield of fibroblasts from RV samples was much better in comparison with the LV samples. However, only one of the 3 RV samples passed the early passage stage; therefore, a more precise and definite pattern of growth and yield of fibroblasts per grams of tissue needs further evaluation. So far, the best results (primarily obtained from the explant technique) up to 2 passages are listed in Table 3.4. It is noted that that left atrial fibroblasts grow more rapidly and yield a substantial number of fibroblasts per gram of tissue as compared to other chambers (Figure 3.8). The number of fibroblasts at the time of first passage ranged between 4×10^5 and 1×10^7 .

Chambers	No. of FBs at P0 confluence	No. of FBs at P1 confluence	No. of FBs at P2 confluence
Left Ventricle	25×10^5	21×10^5	50×10^5
Right Ventricle	86×10^4	16×10^5	46×10^5
Left Atrium	82×10^5	130×10^5	113×10^5
Right Atrium	68×10^5	80×10^5	96×10^5

Table 3.4: CARDIAC FIBROBLASTS COUNT AT DIFFERENT STAGES - PILOT DATA. Cell count of cardiac fibroblasts isolated from all four chambers from the time of explantation until their first passage and count at the time of achieving full confluence at P1 and P2 stage.

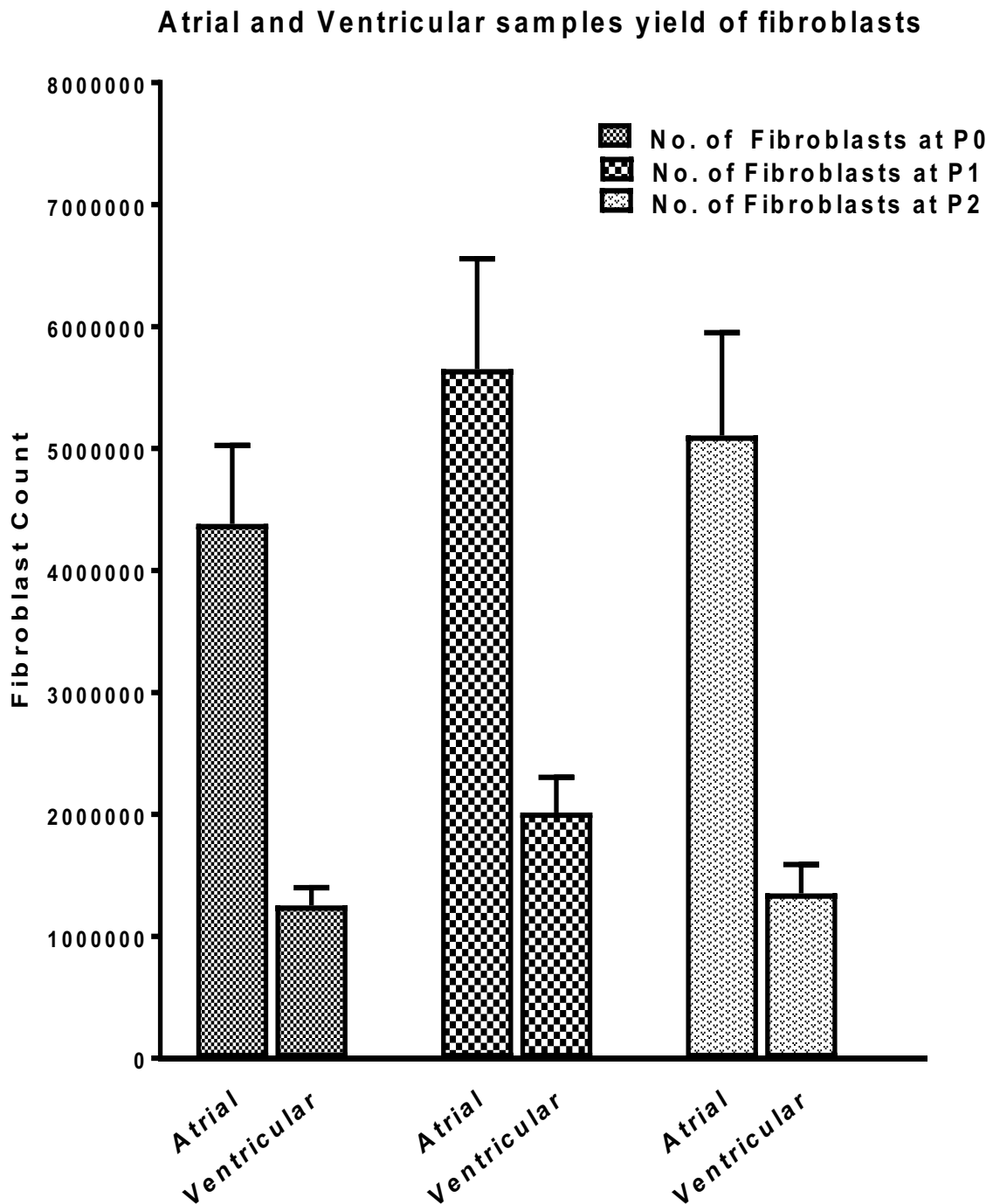


Fig 3.8. FIBROBLAST YIELD AT DIFFERENT STAGES OF GROWTH – PILOT DATA. The atrial and ventricular fibroblasts harvested through different passages with 2g of tissue for each sample. At each passage, the number of fibroblasts harvested from atrial samples were more than ventricular samples.

Atrial samples (n=5), Ventricular samples (n=5)

Error bars: Mean +/- SEM.

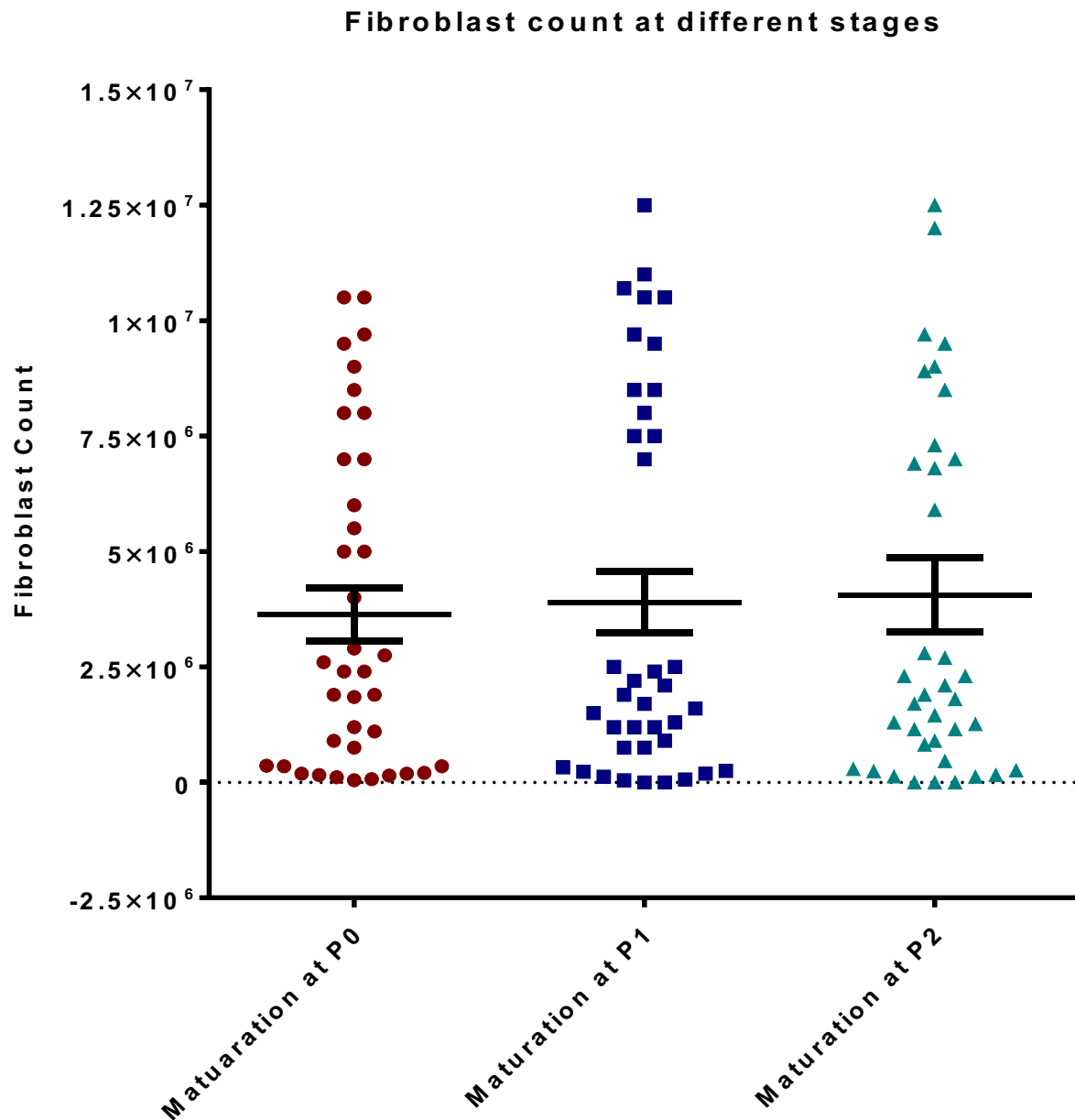


Fig 3.9. FIBROBLAST COUNT AT DIFFERENT STAGES OF GROWTH – PILOT DATA. Fibroblasts count at different stages of maturation (P0, P1 & P2) of combined atrial and ventricular samples is depicted in the plot. The rate of growth of same sample at different stages eludes to the fact that fibroblast yield at different passages of maturation is variable. Different samples show different rates of growth at different stages. Samples with higher cellular yield maintain the trend at various passages and vice versa.

Total fibroblast samples, $n = 45$.

Error bars: Mean $\pm$ SEM.

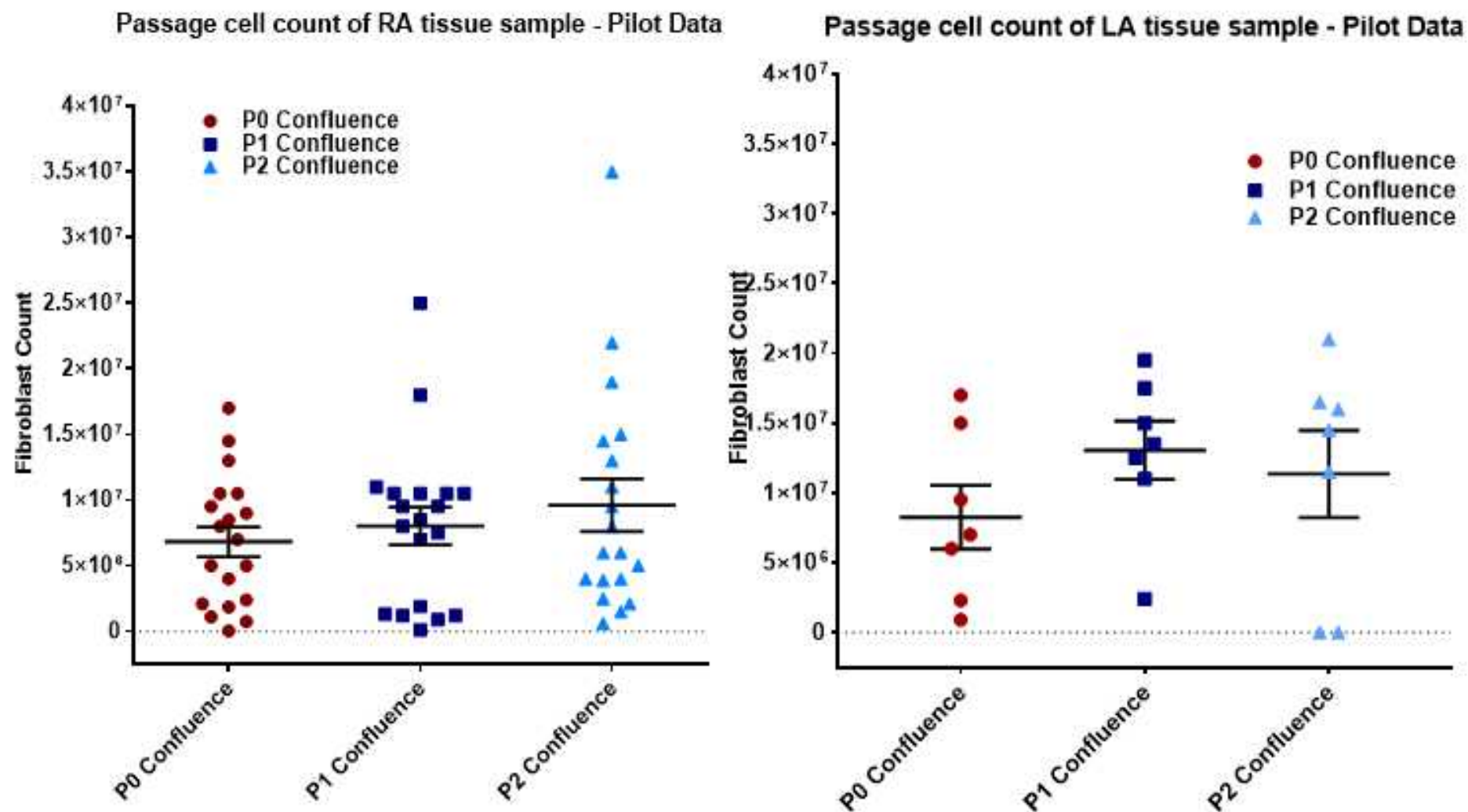


Fig 3.10. FIBROBLAST COUNT OF LEFT AND RIGHT ATRIAL TISSUE AT DIFFERENT STAGES OF GROWTH. The fibroblast count at different passages of LA samples were better than the RA samples. Although RA fibroblasts count at different passages remain more or less the same but LA fibroblasts showed more growth at P1 & P2 stage. (Right atrial fibroblasts, n = 19, Left atrial fibroblasts, n = 7).
Error bars: Mean +/- SEM.

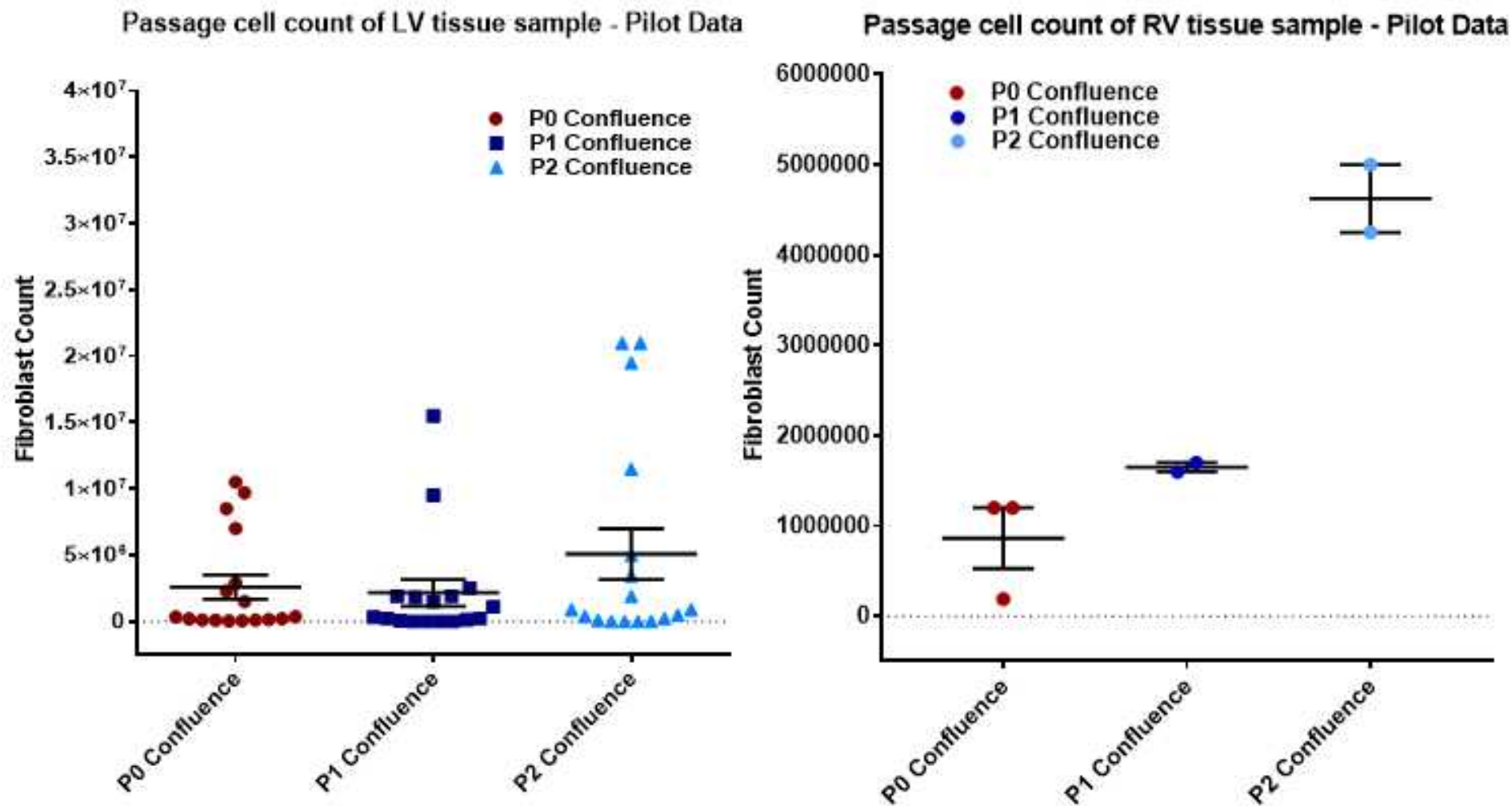


Fig 3.11. FIBROBLAST COUNT OF LEFT AND RIGHT VENTRICULAR TISSUE AT DIFFERENT STAGES OF GROWTH. The fibroblast count at different passage stages for LV samples were comparable and consistent. However, for RV samples, the rate of growth was noted to be better at P1 and P2 passage in comparison with P0 passage. (Right ventricular fibroblasts, n = 3, Left ventricular fibroblasts, n = 17). **Error bars: Mean +/- SEM.**

3.5.5. EFFECT OF FIBROBLAST GROWTH FACTOR ON GROWING COLONIES

In order to optimise the rate of fibroblast growth, different additives are used in the FGM. The basic Fibroblast Growth Factor (FGF, also called FGF-2) was added to FGM to augment the fibroblast growth and proliferation. In-vivo, it is an integral component of the basement membrane and subendothelial extracellular matrix of blood vessels, and is released secondary to tissue injury. In humans, the FGF is secreted by lipocytes, and serum levels of FGF are directly related to body mass index. It has an angiogenic effect and is known to enhance preosteoclast activity. The action is mediated via FGFR-1 receptor activation which in turn leads to activation of phosphoinositide 3-kinase (Kühn et al., 2012).

FGF is extensively used in human stem cells culture media, as it is known to enhance the capacity of a cell to remain undifferentiated. The precise mechanism for this action is still unidentified. However, some studies have shown that it induces Gremlin protein which in turn inhibits the initiation of differentiation by bone morphogenetic proteins (Pereira C et al., 2000).

In my project, the addition of FGF to FGM reinforced the notion of Pereira et al. It was useful in maintaining the fibroblast characteristics of the defined population and arrested their myofibroblastic transformation (Fig 3.13, 3.14). Lack of FGF in the fibroblast growth medium led to cellular change in the myofibroblasts at P1 and P2 stages in the atrial samples, and even before passage to P1 in the ventricular samples (confirmed with immunostaining).

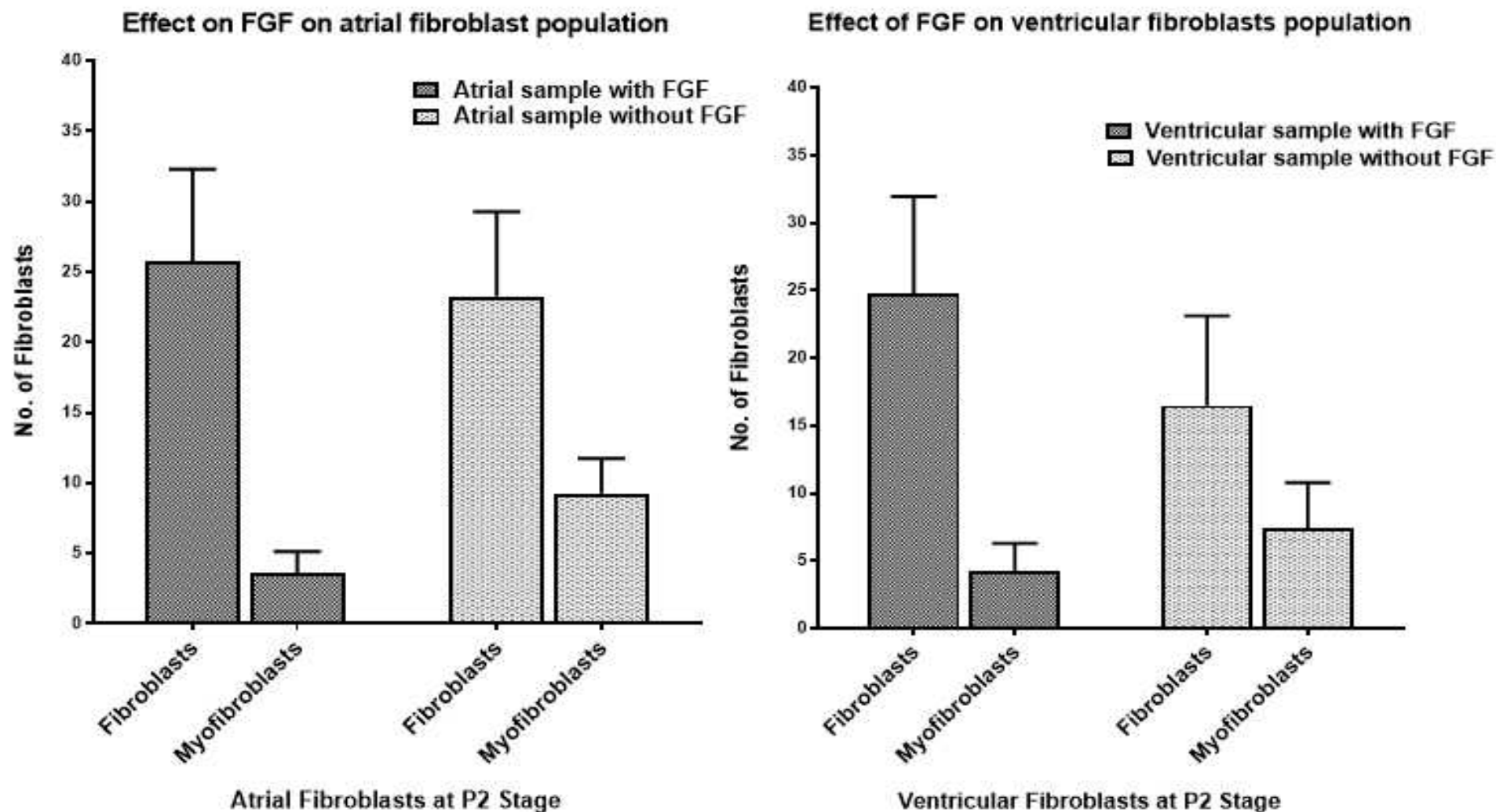
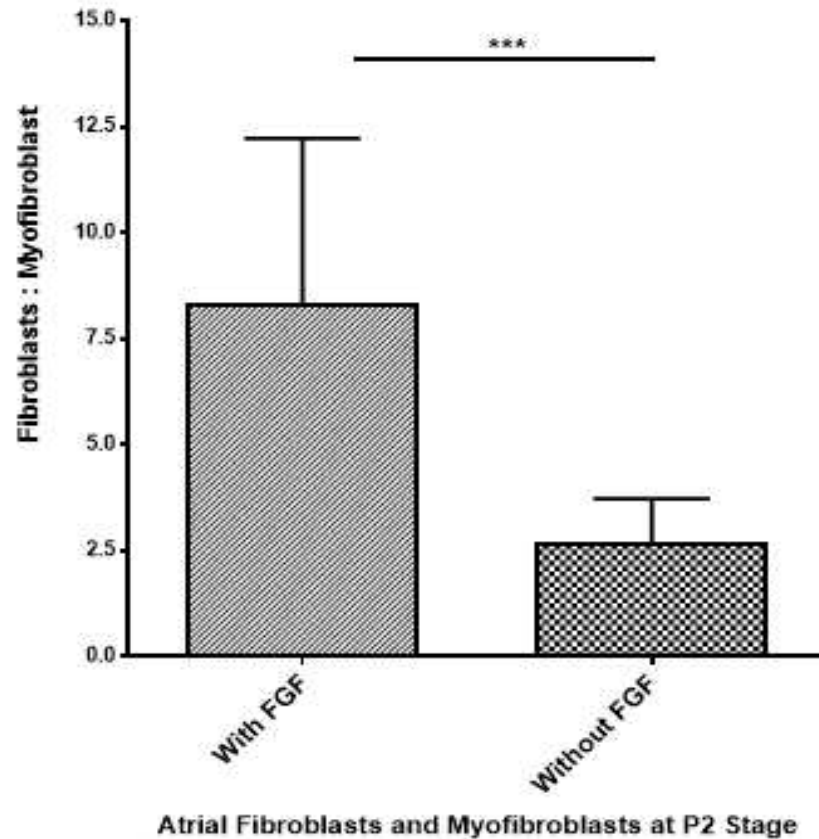


Fig. 3.12. FGF AND MYOFIBROBLASTIC TRANSFORMATION IN ATRIAL AND VENTRICULAR FIBROBLASTS. Addition of FGF to the fibroblast growth medium is associated with decreased myofibroblastic transformation in atrial and ventricular fibroblasts at the P2 stage. (n = 8, different samples from different patient), **Error bars: Mean with SD.**

Ratio of Atrial Fibroblasts to Myofibroblasts with/without FGF



Ratio of Ventricular Fibroblasts to Myofibroblasts with/without FGF

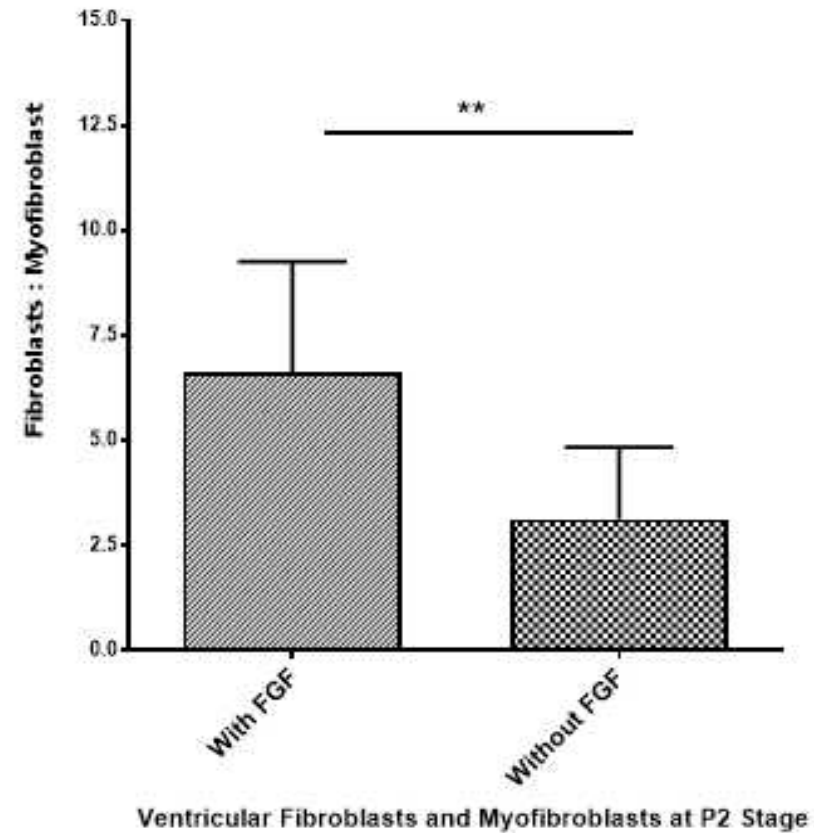


Fig. 3.13. EFFECT OF FIBROBLAST GROWTH FACTOR ON MYOFIBROBLASTIC TRANSFORMATION IN ATRIAL AND VENTRICULAR FIBROBLASTS AT P2 STAGE. Addition of FGF to the culture medium led to significantly decreased myofibroblastic transformation in both atrial and ventricular tissue samples. The cell count was done under the confocal microscope following staining. The ratio of fibroblasts to myofibroblasts is depicted in the atrial and ventricular samples. n = 8, different samples from different patient groups; Unpaired T-test; ***p < 0.001; **p < 0.01; Error Bars: Mean with SD.

3.5.6 DISTINCTIVE GROWTH FEATURES OF HUMAN CARDIAC FIBROBLASTS

The fibroblasts grew from explanted tissues in colonies and developed small projections on the growth front of the colonies. These filamentous projections connected them to the adjacent colonies. The colonies containing fibroblasts which developed projections grew more rapidly than colonies without.

The nanotubules cytoskeleton contains F-actin and microtubules and provides a structural pathway for intercellular cargo transportation. It has been shown that cardiomyocytes and non-cardiomyocytes share their intracytoplasmic vesicles and mitochondria through these nanotubules (He et al., 2010). The anatomical intercellular communication occurs through synapses and gap junctions, but this communication is limited to proximity within a radius of a few micrometres. Small molecules (up to 1.2 KDa) can move across this junction (Gangaraju Vamsi K. Lin Haifan, 2009). Contrary to this, the nanotubules can transfer intracellular organelles such as mitochondria, endosomes, and lysosomes at a distance (Koyanagi et al., 2005) (Plotnikov et al., 2008) (He et al., 2011).

Similar nanotubular connections were also observed between fibroblasts. Connections were observed not only in between the adjacent fibroblasts, but also between colonies. It was noted that the growth of colonies was preceded by a delicate network of nanotubules (Fig. 3.15). The growth front of colonies with an extensive microtubular network was quicker than fronts lacking it. The formation of the network followed the weaving of cells into this structurally connecting and functionally supportive meshwork.

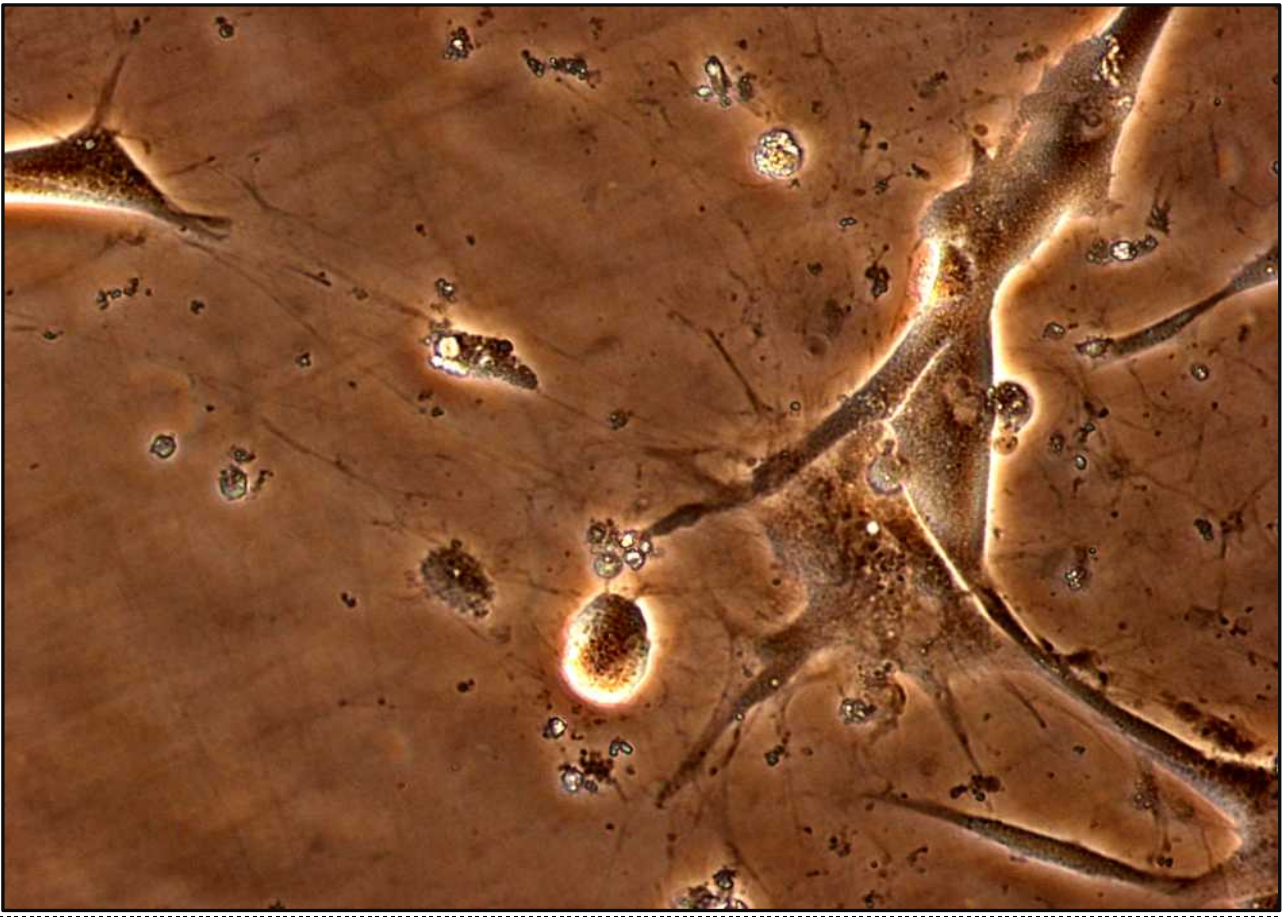


Fig. 3.14. NANOTUBULES CONNECTING THE ADJACENT FIBROBLASTS. Bright field microscopic (20x) image of human atrial fibroblast at P1. Nanotubular network between adjacent fibroblasts can be seen clearly.

The microtubular network exhibited variability among different samples. Connections developed best in foetal fibroblasts, followed by atrial and then ventricular samples. The human foetal fibroblasts showed nanotubules growth from the first few days of each passage and further developed extensively over the course of confluence. The fastest growth of the foetal fibroblasts may be related to the fact that the intercellular connections are better than adult fibroblasts. (Fig. 3.16) The least amount of tubular network was noted in DCM fibroblasts and communication between the colonies was almost negligible. This can be related to the fact that DCM fibroblasts are slow to grow in comparison to other groups due to lack of intercellular communications.

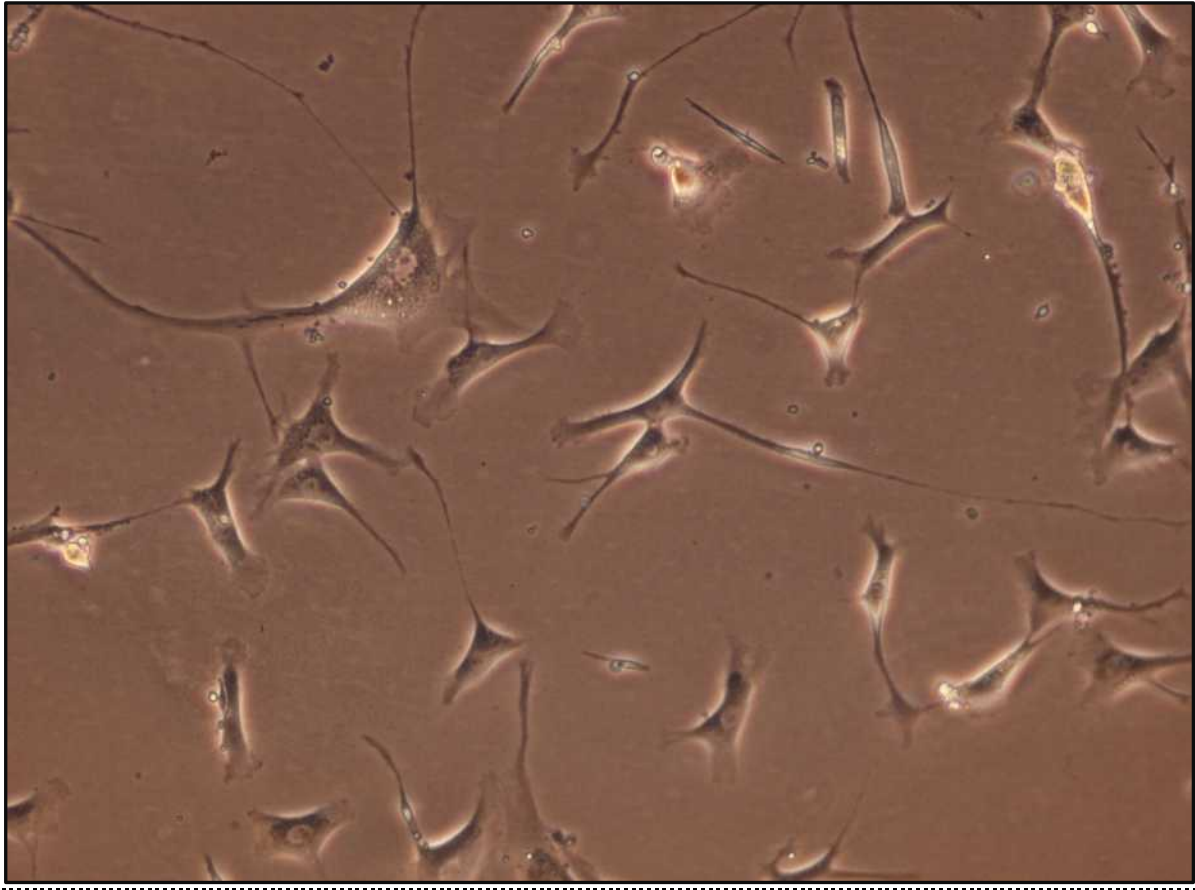


Fig. 3.15. HUMAN FOETAL FIBROBLASTS SHOWING INTERCELLULAR NANOTUBULAR NETWORK. Bright field microscopy (10x) shows extensive nanotubular network can be seen between the adjacent individual fibroblasts on day-3 of P1.

The atrial fibroblasts at P1 with the conspicuous tubular network on bright field microscopic images were stained with THY-1, Vimentin and viewed under a confocal microscope. Nanotubules or filopodia were noticed between the adjacent fibroblasts and between colonies.

An attempt was made to label the mitochondria in live cells with Mito-Tracker Green (MitoGreen) before immunostaining, but no mitochondria were stained, and no organellar movement was observed between the cells across the tubular bridges. This will be an exciting avenue to explore in future studies.

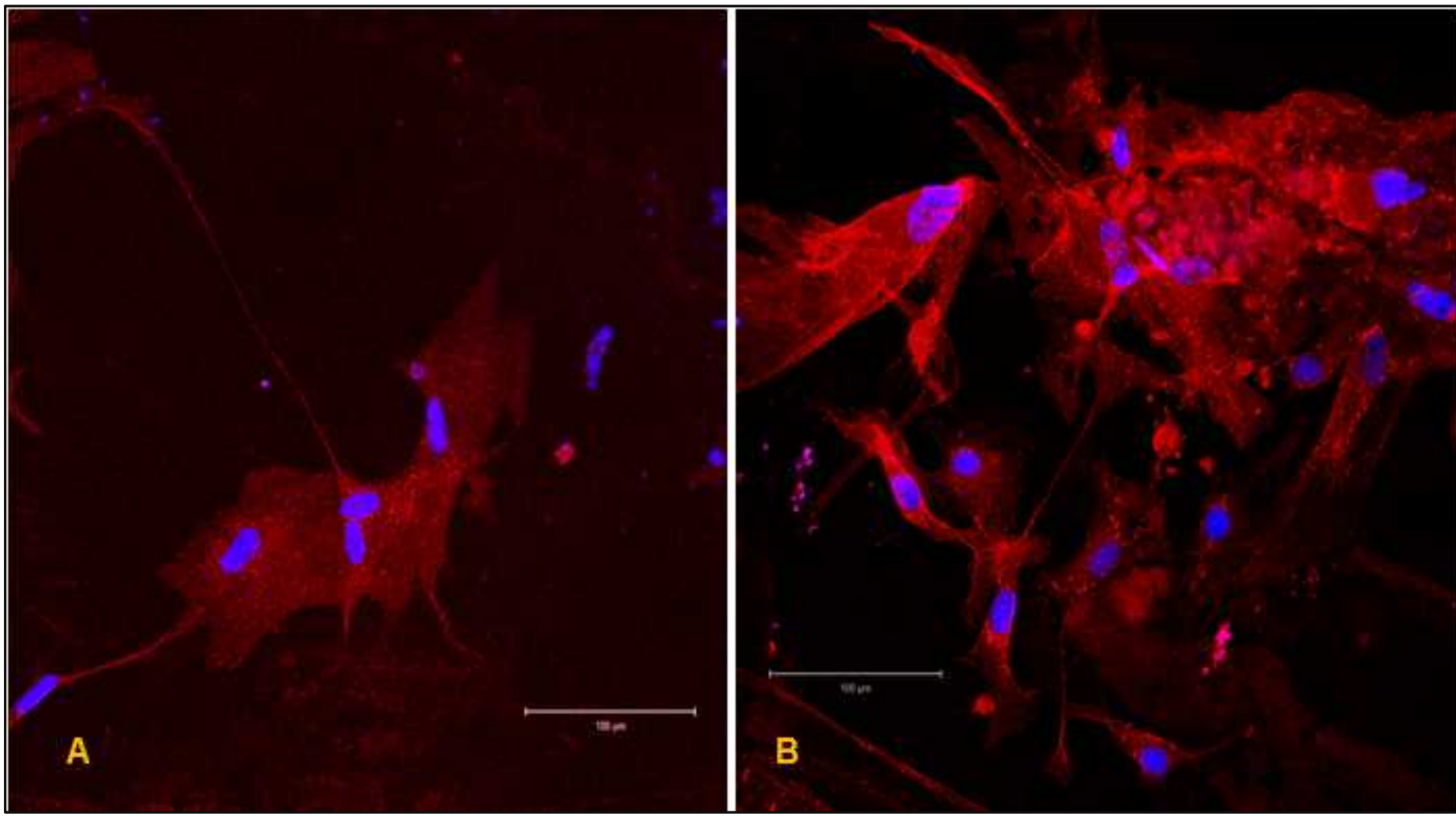


Fig. 3.16. IMMUNOSTAINING OF ATRIAL FIBROBLASTS WITH THY-1 SHOWING NANOTUBULES BETWEEN ADJACENT FIBROBLASTS AND COLONIES. Confocal images (20x, 1024 resolution) of Human atrial fibroblasts at P1, (day 9) stained with THY-1. Tubular connection between individual fibroblasts (A) and the fibroblast colonies (B) can be seen. Nuclei were stained with DAPI. **Scale bar: 100µm.**

3.5.7 AMPHOTERICIN GRANULAR DEPOSITION

Amphotericin is an effective fungicidal azole. It has a molecular weight of 923.488 g/mol. It was added as an antimicrobial component to FGM. The addition of the azole to FGM led to a substantial decrease in the incidence of fungal infection in the culture plates. Owing to its massive molecular weight, it can lead to granular deposits mimicking bacterial contamination in the culture dishes. The granules were of variable sizes and were usually residing on top of the growing fibroblast monolayer. It was noted in a few Petri dishes, but the light field microscopy showed no evidence of bacterial or fungal contamination. Following this additive, concentration of amphotericin was titrated with better outcomes.

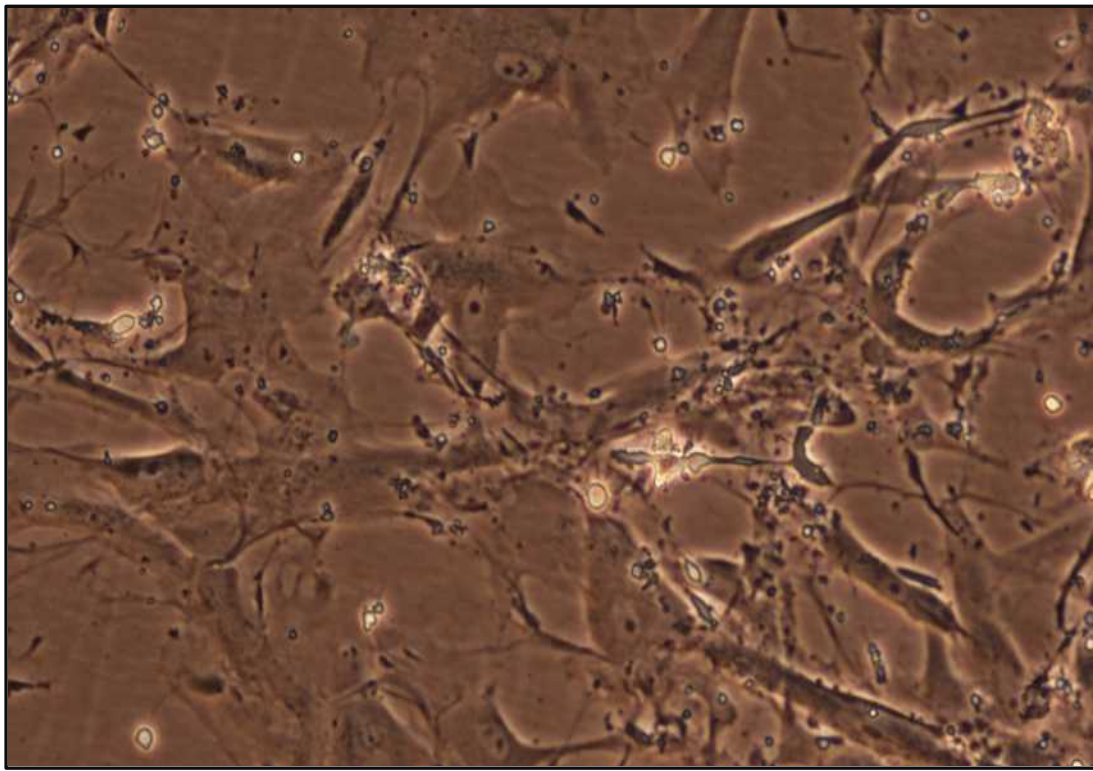


Fig. 3.17 AMPHOTERICIN GRANULES COVERING THE GROWING FIBROBLASTS MIMICKING BACTERIAL CONTAMINATION. Bright field microscopy (20x) of the image of a fibroblast colony with amphotericin granules.

3.5.8 TRANSFORMATION OF HUMAN CARDIAC FIBROBLASTS INTO MYOFIBROBLASTS

Having passaged to P1 and P2 stage, some ventricular fibroblasts underwent a sudden morphological transformation with loss of projections, development of intracellular contractile filaments, becoming large hypertrophic cells with loss of division potential. These hypertrophic cells exhibited the morphological features consistent with myofibroblasts, which was later confirmed with immunostaining. In samples with cells representing characteristics of myofibroblasts, the rate of growth after transformation ceased significantly and cell hypertrophy dominated hyperplasia. After myofibroblastic transformation; either cells died after a few days or were intolerant of any further passage. Some samples were more susceptible to myofibroblastic transformation than others; particularly LV samples from DCM showed predilection in comparison with others. (Figure 3.19. A-E)

Other factors that contributed towards the myofibroblast transformation were; keeping the fibroblasts in the same medium for more than 4 days; change in the batches of foetal bovine serum; change in the composition of fibroblast growth medium; increased distance between the adjacent colonies of fibroblasts; scanty fibroblasts in the colonies; and keeping the fibroblasts for a long time in the petridish once the monolayer was formed. A key factor contributing towards prevention of the myofibroblastic transformation was FGF. When FGF was not added to the fibroblast growth medium, the myofibroblastic transformation was noticeable in both the atrial and ventricular fibroblasts, even at P1 and P2 stage. However, the addition of FGF did not have any significant impact on halting the progression of fibroblasts to myofibroblast beyond P4/P5 stage. Another noteworthy factor which contributed towards this transformation was the time interval between explantation of the tissue until it was processed and plated in the FGM medium. The DCM sample received

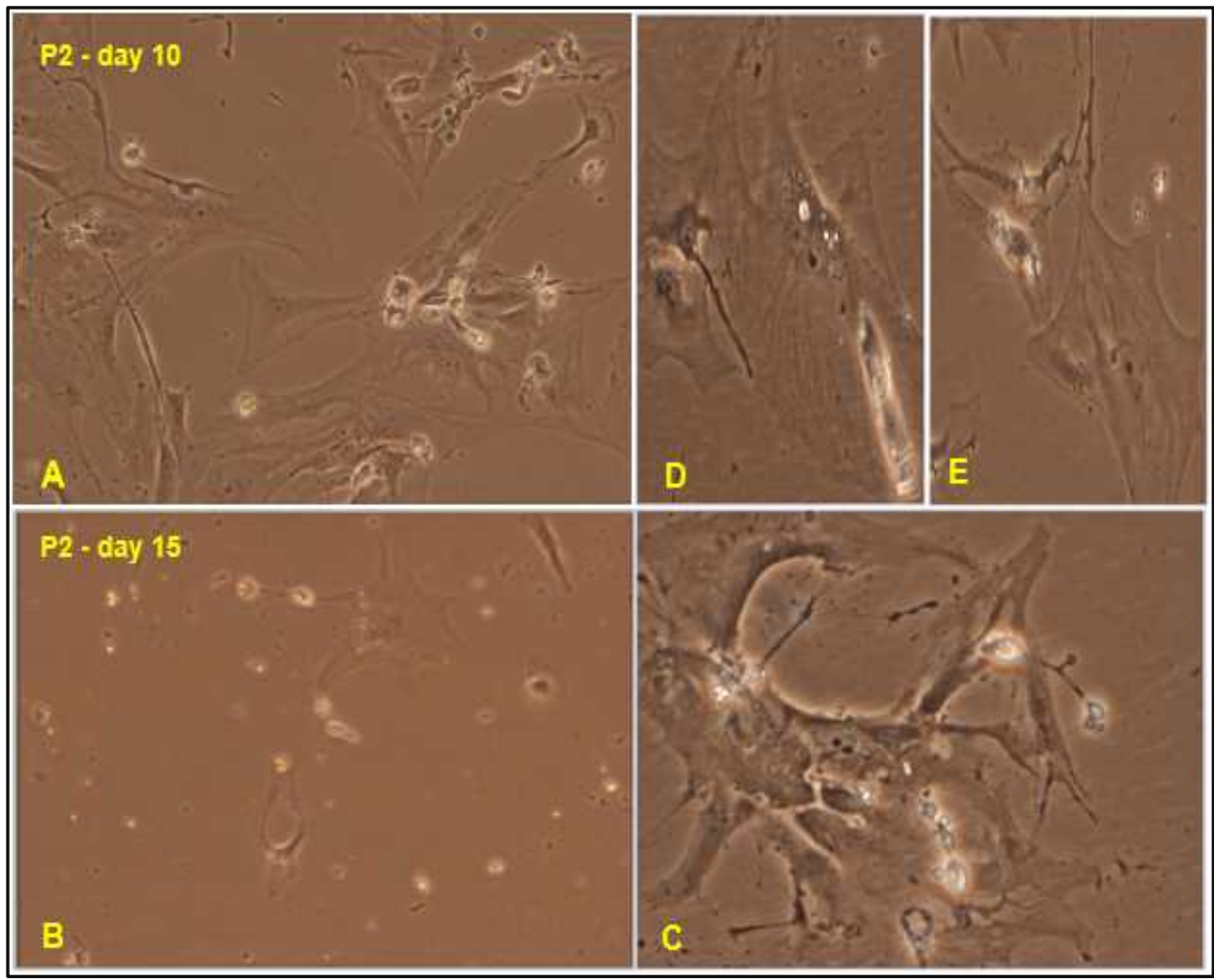


Fig. 3.18. MYOFIBROBLASTIC TRANSFORMATION OF FIBROBLASTS AT P2 STAGE. Bright field microscopic images of human left ventricular CFs at P2 stage showing arrested growth with increased distance between adjacent colonies of cells, loss of projections, (A) became hypertrophied (D) with of contractile elements within the cells (E), followed by cell death (B) within the next 3-5 days.

from the native heart of the transplant recipients after 4-6 hours of harvesting were more prone to myofibroblast transformation. The transformation was seen as early as P0 & P1. This phenomenon was accentuated by lack of cardioplegia (4:1 ratio) in the transfer solution used for cardiac tissues. Two samples of DCM hearts, received after 5 hours of harvesting from the transplant recipient did not show any growth. The HOCM samples showed a marked myofibroblast transformation at P2 and P3 stage (no effect detected at P1 stage) with no further growth. Having had a myofibroblastic transformation, all the FBs died within a few days.

3.6 FIBROBLASTS FROM ALL FOUR CHAMBERS

3.6.1 DEMOGRAPHICS OF PATIENTS UNDERGOING ALL FOUR CHAMBER BIOPSIES

Based on the scheme of the analysis, all four chamber biopsies were taken from 20 patients. The age range for the group was between 43 -79 years (Mean age = 63 years). Twenty percent of the patient population in the study (4/20) were female, and the rest (80%) were male. Almost 50% of the patient participating in the study had a previous MI, but biventricular function was preserved. One of the participants (3/2016) had a pre-operative echocardiogram which established good biventricular function, but the intro-operative trans-oesophageal echocardiography (TOE) demonstrated moderately preserved left ventricular function with an EF of 45%. By the time this finding was made, four-chamber biopsy samples had already been taken, and therefore were processed as per routine. All patients were checked for Hepatitis B, Hepatitis C, and HIV. None of the participants were diagnosed with any positive viral markers.

Coronary Artery Bypass Grafting was the focal procedure performed (13/20) on the patient population in the study. Three patients underwent Aortic Valve Replacement, and 2 underwent combined AVR and CABG. Only two patients had mitral valve surgery with atrial fibrillation (AF) ablation. All the patient in the study had an established history of hypertension and the majority of them were diagnosed with hypercholesterolaemia. Nearly 25% of the participants were diabetic. Almost half of them had a history of smoking as well as a family history of ischaemic heart disease (Table. 3.5). The participating patients were consented at least 12hrs before the surgical intervention. None of the participants declined after signing the consent form. However, three patients consented, but no biopsies were taken due to the intra-operative clinical indication. The patients were informed about the change in the plan, and the clinical reason for this was explained.

These three patients are not mentioned in Table. 3.5.

The weight of left and right atrial biopsy samples was measured immediately after harvesting. Due to the small size, it was difficult to measure the ventricular samples accurately. All the samples were transferred directly to the cold transport medium in a falcon tube placed in the ice. The biopsy samples were processed within an hour of harvesting as per established protocol (mentioned in Chapter No.2). For the pilot data, the majority of the samples were processed with the explantation technique, and only five samples were processed with enzymatic digestion technique. However, for the main limb of the study, all the samples were processed with explantation technique.

The bathing medium (FGM) was changed every 72hrs. The growth of fibroblasts was assessed periodically on day 5, 7, 10 and 15 for the assessment of growth and confluence. Once confluence was achieved, the samples were passaged to the next stage. After P1 confluence, the fibroblasts were put in the chamber slides and were left in the incubator for 24 hours. This allowed the fibroblasts to adhere to the chamber slide and they would not be lifted off the bottom of slides during washing. The fibroblasts were further investigated with immunostaining and expression of transcription factors. Due to small size, two samples (4/2016, 11/2016) did not grow well and there was insufficient material for immunostaining. The small ventricular tissue samples made the desired growth rate a difficult task. Only 8 ventricular samples grew up to the P2 stage. Amongst the atrial samples, 17 grew to the P2 stage and were investigated. The best growing biopsy samples were 7/2016, 15/2016 and 17/2016. Sample no. 7/2016 was not used for immunostaining as fibroblasts were used in the construction of EHTs. None of the other ventricular samples generated fibroblast enough to generate EHTs (Each EHT construct requires 1.5×10^5 fibroblasts)

	ID	Study ID	Age	Gender	CCS status	NYHA status	Previous MI	HTN	Dyslipidaemia	Diabetes	Smoking	Family hx of IHD	Operation	LV function	RV function
1	MD	01/2016	68	M	III	II-III	No	Yes	Yes	No	No	Yes	CABG	Good	Good
2	GS	02/2016	66	M	IV	II	Yes	Yes	Yes	No	Yes	Yes	CABG	Good	Good
3	PD	03/2016	57	M	II-III	III	Yes	Yes	Yes	Yes	Ex	Yes	CABG	Moderate	Good
4	JH	04/2016	65	M	III	II	No	Yes	Yes	No	Ex	Yes	CABG	Good	Good
5	BB	05/2016	77	M	I	II-III	Yes	Yes	Yes	No	No	Yes	CABG	Good	Good
6	SM	06/2016	71	F	III-IV	II - III	Yes	Yes	Yes	No	Ex	No	CABG	Good	Good
7	EW	07/2016	78	F	I	III	No	Yes	Yes	No	No	No	AVR	Good	Good
8	GM	08/2016	48	M	II	IV	No	Yes	Yes	Yes	No	No	MVR + AF Ablation	Good	Good
9	SL	09/2016	69	M	III	II - III	No	Yes	No	No	Ex	Yes	AVR + CABG	Good	Good
10	KD	10/2016	72	F	III-IV	II	Yes	Yes	Yes	No	Ex	Yes	CABG	Good	Good
11	SJ	11/2016	63	M	III	I	No	Yes	Yes	No	No	No	AVR	Good	Good
12	IS	12/2016	63	M	II - III	I-II	Yes	Yes	Yes	Yes	No	Yes	CABG	Good	Good
13	FM	13/2016	48	M	III-IV	II	Yes	Yes	Yes	No	Yes	Yes	CABG	Good	Good
14	AH	14/2016	55	M	II	I-II	No	Yes	Yes	No	Ex	Yes	CABG	Good	Good
15	MB	15/2016	52	M	IV	II-III	No	Yes	No	No	Ex	No	AVR	Good	Good
16	BT	16/2016	69	M	III	II	Yes	Yes	Yes	No	No	No	CABG	Good	Good
17	KG	17/2016	44	F	II	III-IV	No	Yes	Yes	No	No	No	MVR + AF Ablation	Good	Good
18	DC	18/2016	79	M	0	III	No	Yes	Yes	Yes	No	No	AVR + CABG	Good	Good
19	JC	19/2016	43	M	I	III	Yes	Yes	Yes	No	EX	No	CABG	Good	Good
20	BDM	20/2016	68	M	III	II	Yes	Yes	Yes	No	No	Yes	CABG	Good	Good

TABLE. 3.5. DEMOGRAPHIC AND RELEVANT CLINICAL DATA OF PATIENTS RECRUITED FOR ALL FOUR CHAMBER BIOPSIES:

The table concisely illustrates the demographic data, symptomatic status, cardiac risk factors, previous myocardial infarction, underlying pathologies effecting the cardiac functions and biventricular function of the heart for the patients recruited for 4 chamber biopsies for the main project. The project involved patients with good biventricular function only but patient no. 3 was included in the study as he was diagnosed to have moderate left ventricular function intraoperatively after biopsy samples were taken and processed.

3.6.2 SAMPLE SIZE OF ALL FOUR CHAMBER BIOPSY SAMPLES

The sample size of all four chamber biopsies were quite variable (Fig. 3.21, A & B). A number of factors affected the size of biopsy samples, including the structure of the atrial appendages, amount of fat around the atrial appendage, thickness of the ventricular wall and the operating surgeon harvesting the tissue. The ventricular samples were taken using a tru-cut biopsy needle, providing a strip of tissue 3-4mm wide and 10-15mm in length. In patients with thin walled right ventricles, the biopsy size and sample were compromised (Fig 3.20). The amount of fat surrounding the cardiac chambers also decreased the explantable myocardial tissue in the atrial samples. In some samples, (Fig. 3.21B) although the size of the atrial biopsies was sufficient, the main bulk of the tissue was fat with a very small proportion of myocardial tissue. The fat was infiltrating into the layers of the myocardium. The scarcity of the ventricular tissue in these samples led to poor growth of the tissue.

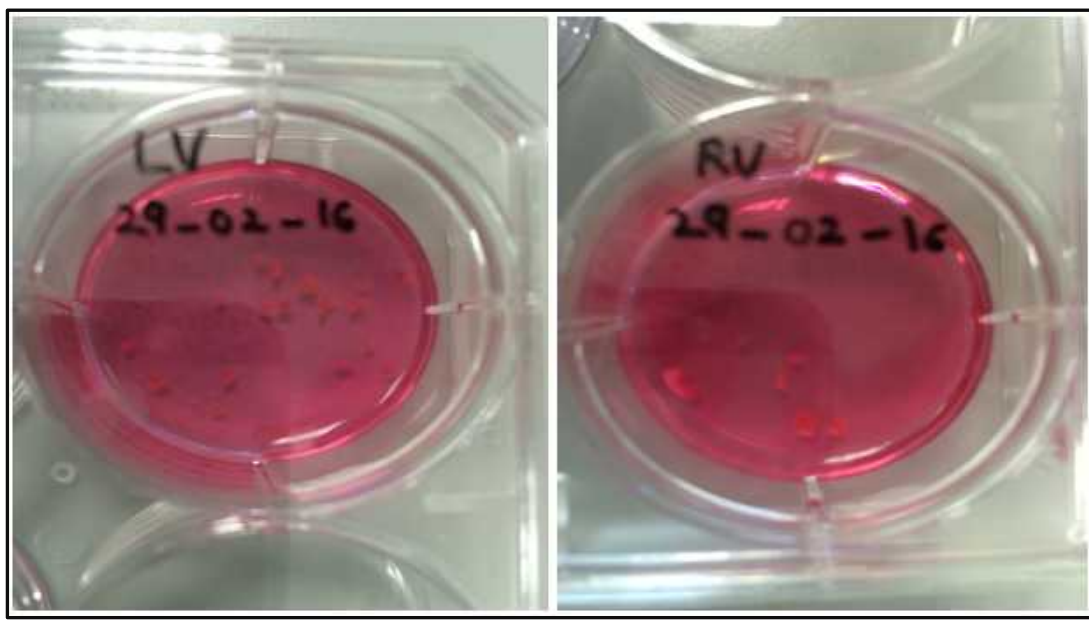


Fig. 3.19. SMALL SAMPLE SIZE OF VENTRICULAR BIOPSIES. The small size of the ventricular sample can be seen in RV sample.

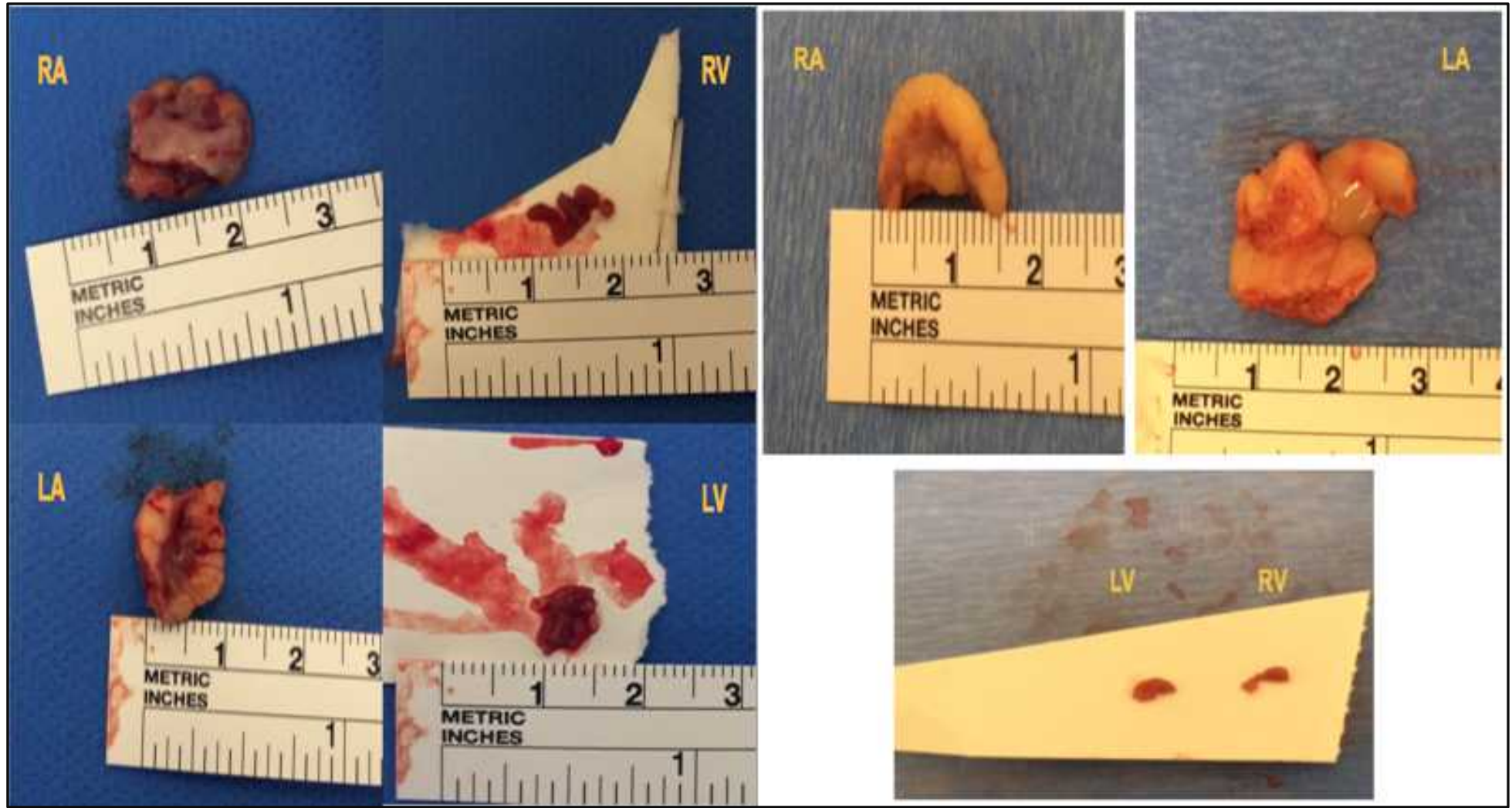


Fig. 3.20. VARIABILITY OF SAMPLE SIZE FOR ALL FOUR CHAMBER BIOPSIES. Different sizes of all four chamber biopsies are shown. Sample (A) shows all four-chamber biopsy sample with a good amount of explantable tissue. Sample (B) shows left and right atrial biopsies with scanty myocardial tissue and predominantly fibrofatty tissue. The ventricular biopsies of both samples have remarkably different size as well.

3.6.3 WEIGHT OF THE MYOCARDIAL BIOPSIES

The weight of myocardial biopsies were measured for all samples in the main study. The atrial appendages harvested in a few patients were very small (Table. 3.4: sample no. 3/2014, 4/2016, 6/2016, 9/2016 and 10/2016), but despite the small myocardial weight, it was possible to get good growing fibroblasts in half of them.

The average weight of the right atrial biopsy samples was 0.68g and left atrial samples 0.65g. The biventricular biopsy samples weighed 0.10g each. Due to small ventricular biopsy sample weight, it was difficult to obtain adequate fibroblasts growth suitable for the EHTs. This factor has limited the no. of EHTs generated with different samples, as only one sample (7/2016) generated enough ventricular fibroblasts to meet the criterion for EHT construction.

TABLE. 3.6. WEIGHT OF ALL FOUR CHAMBERS BIOPSY SAMPLES (GRAMS)

	RIGHT ATRIUM	LEFT ATRIUM	RIGHT VENTRICLE	LEFT VENTRICLE
1	0.66	1.00	0.09	0.20
2	0.58	0.69	0.08	0.12
3	0.32	0.19	0.07	0.09
4	0.24	0.25	0.08	0.10
5	0.78	0.81	0.09	0.10
6	0.21	0.34	0.06	0.08
7	0.98	0.96	0.18	0.15
8	0.48	0.53	0.05	0.07
9	0.38	0.44	0.10	0.07
10	0.41	0.20	0.06	0.08
11	0.56	0.47	0.08	0.08
12	0.72	0.75	0.08	0.1
13	0.81	0.69	0.11	0.09
14	0.78	0.81	0.12	0.10
15	1.20	0.95	0.20	0.13
16	0.96	0.74	0.12	0.11
17	0.83	1.15	0.15	0.08
18	1.10	0.59	0.11	0.10
19	0.71	0.82	0.08	0.12
20	0.92	0.68	0.09	0.10
Mean	0.68 gm	0.65 gm	0.10 gm	0.10 gm

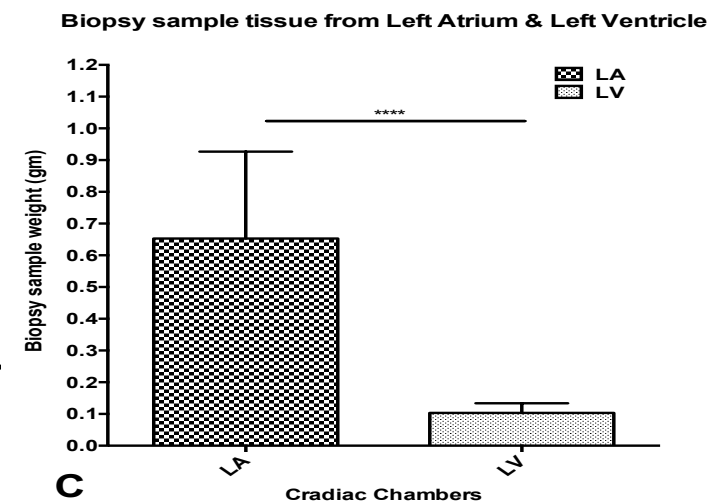
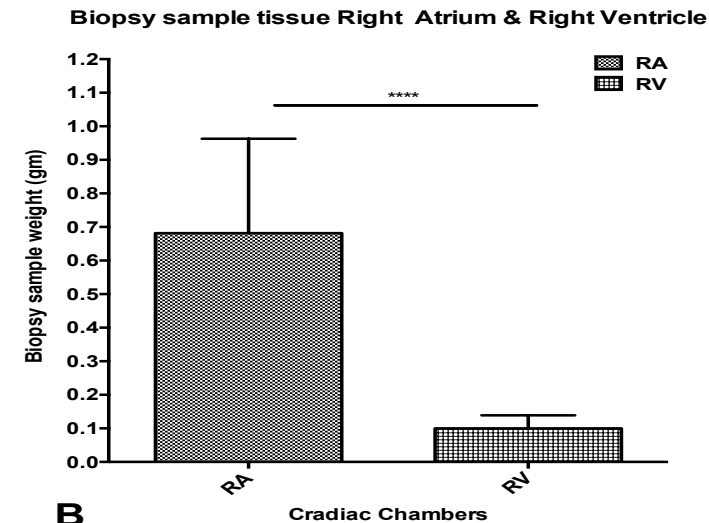
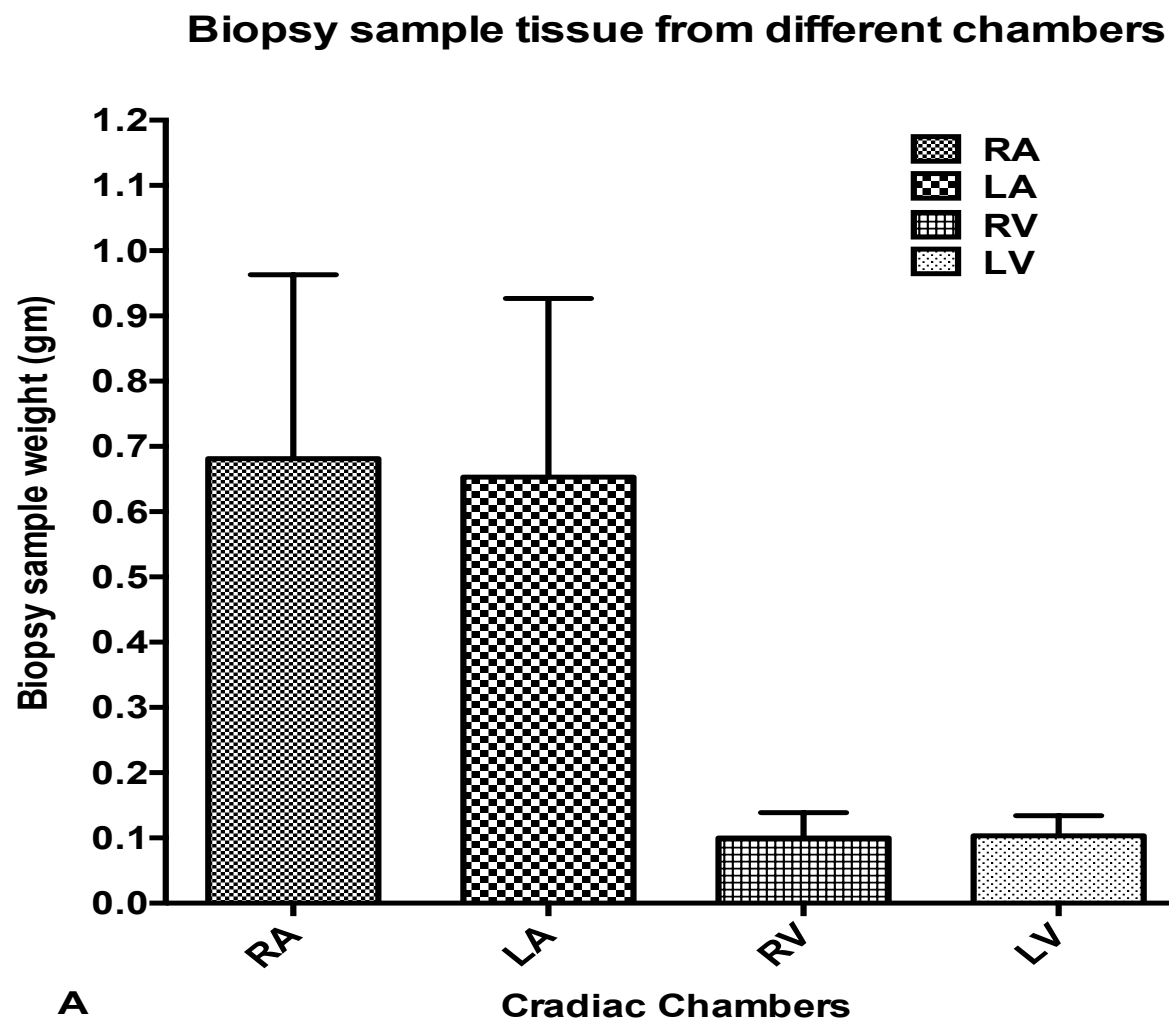


Fig. 3.21. WEIGHT OF BIOPSY SAMPLES FROM HUMAN ALL FOUR CARDIC CHAMBERS. The bi-atrial samples were larger in comparison with the biventricular samples. The weight of RA sample against the RV sample & LA against LV samples is statistically significant. One-way ANOVA with Tukey's correction of all four chamber biopsies show: RA/RV, **p-value= < 0.001(****)**; LA/LV, **p-value = < 0.001(****)**. Error Bar: Mean $\pm$ SEM

3.7 IMMUNOSTAINING OF HUMAN CARDIAC FIBROBLASTS

3.7.1 OPTIMIZATION OF ANTIBODY DILUTION FOR IMMUNOCYTOCHEMISTRY:

The antibodies for immunohistochemistry were diluted in different concentration for optimisation. Vimentin and α -SMA, which were already used in other ongoing projects in the lab, were an exception to this. The other predicted positive and negative markers were all validated and optimised. The antibodies were diluted in different concentrations as 1/2000, 1/1000 and 1/500, etc. to find the best possible concentration to achieve the finest image yield. Figure 3.22 (A-D) reveals the antibody dilution in different concentrations employed for DDR-2 and Periostin, and the impact of different dilution on image yield. Anti-DDR-2 & Periostin antibody was prepared in different concentrations and the results of DDR-2 in 1/2000 concentration (A) were not as optimal in comparison with 1/1000 (B). Likewise, for the anti-Periostin antibody, the concentration of 1/200 (C) revealed better images than 1/100 dilution(D).

Similarly, the THY-1 and negative predicted markers such as α -Actinin, MHC, and vWF were optimised for concentration.

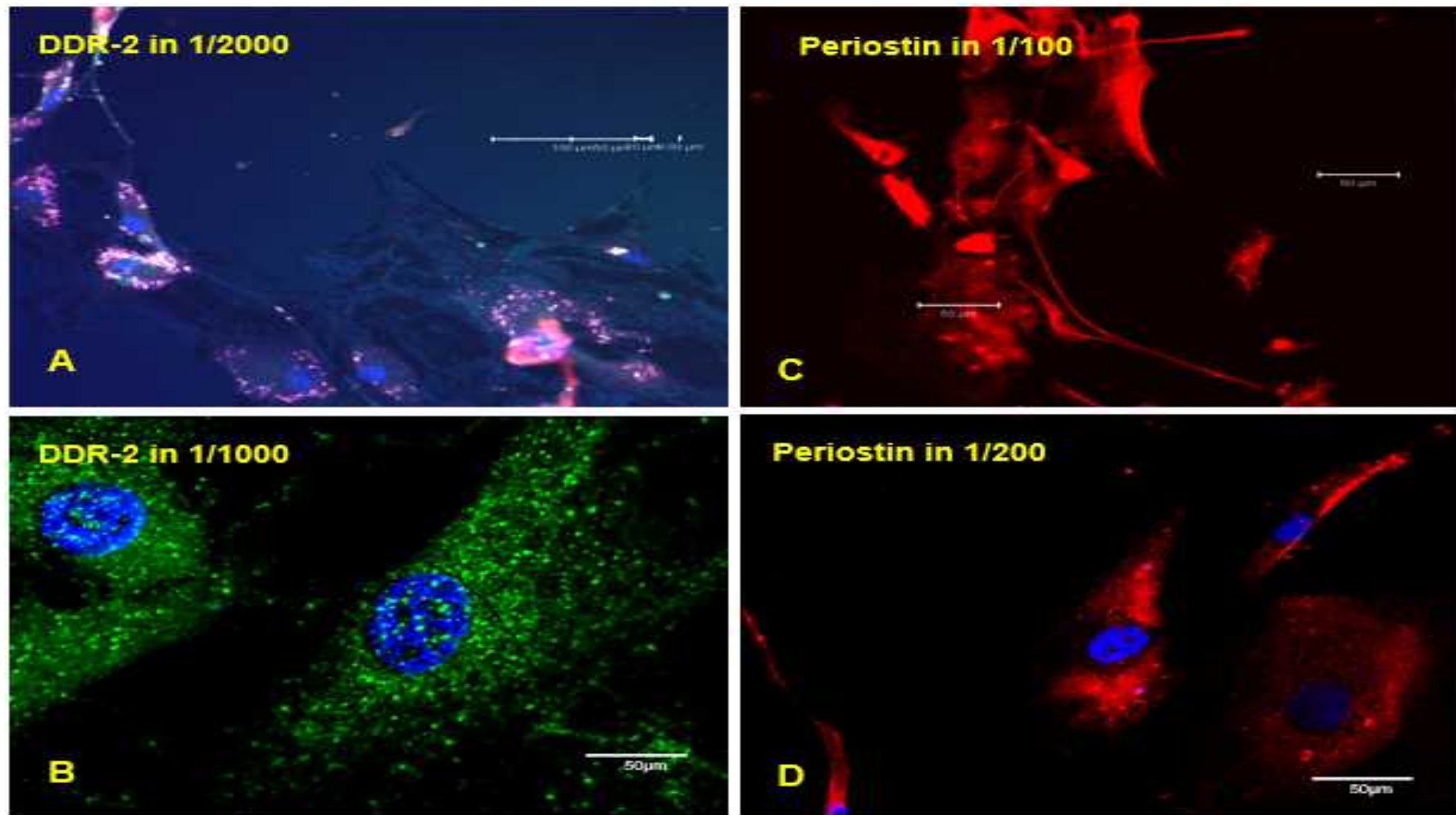


Fig. 3.22. OPTIMISATION OF ANTIBODY CONCENTRATION FOR IMMUNOHISTOCHEMISTRY. Confocal images (20X magnification, 1024 resolution) of Fibroblasts stained with DDR-2 in 1:2000 (A) and 1:1000 (B) concentration. Periostin was diluted in 1:100 (C) and 1:200 (D) concentration. **Scale bars: 50µm.**

3.7.2 LOCALISATION OF HUMAN CARDIAC FIBROBLASTS

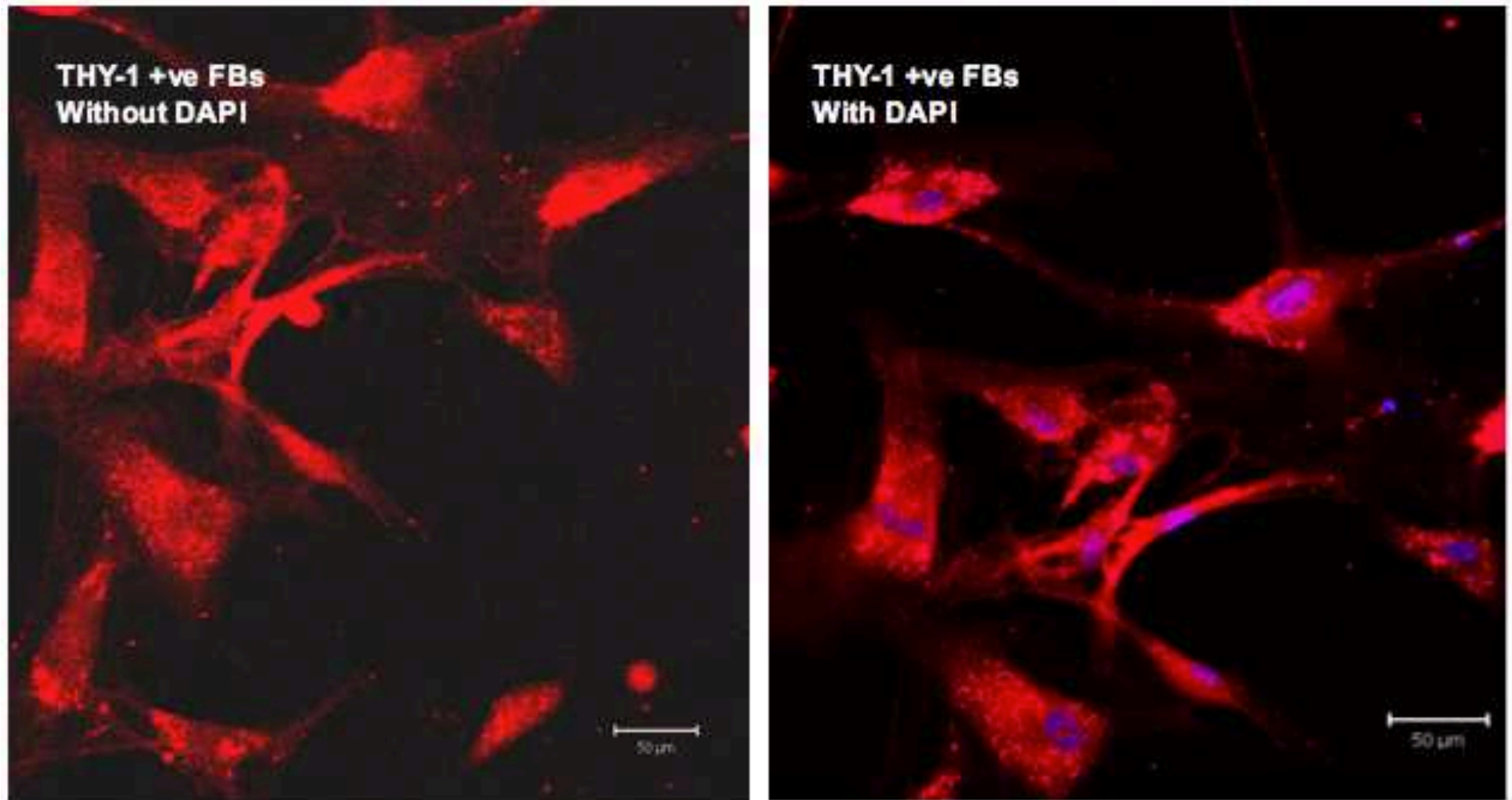


Fig. 3.23. THY-1 LOCALISATION IN HUMAN ATRIAL FIBROBLASTS. Confocal imaging (20X magnification, 1024 resolution) of fibroblasts isolated from human right atrial tissue stained with THY-1. A cluster of fibroblasts can be seen stained with THY-1. Nuclei are unstained in left sided image and stained with DAPI in the image on right side. **Scale bars: 50µm.**

3.7.3 POSITIVE PREDICTIVE MARKERS FOR IDENTIFICATION OF HUMAN CARDIAC FIBROBLASTS

Since no single specific cardiac fibroblast marker is identified so far, to distinguish CFs from the rest of the human cardiac cell population, a combination of four positive predicting factors (DDR-2, THY-1, Periostin, Vimentin) were used. Although, none of these markers have an absolute specificity to confirm the true fibroblasts population in isolation, a combination of all four factors can provide a better probability for confirmation.

Discoidin Domain-containing Receptor-2 (DDR-2) is a tyrosine kinase receptor. This receptor protein is coded by the DDR-2 gene in humans. The receptor located on the cell membrane shows a tripartite structure with an extracellular, transmembrane, and intracellular domain. The ligand for DDR-2 is fibrillar collagen which mediates fibroblast proliferation and migration. The attached ligand induces oligomerisation of the receptors and subsequently causes intracellular phosphorylation of cytosolic target associated with other target molecules (Fu HL et al., 2013). Its 10kDa major transcript is highly expressed by heart and lungs and is relatively less expressed by the liver, kidney, pancreas, skeletal muscle, placenta and brain. It also contributes towards cutaneous wound healing (Leitinger B, 2011). Expression of DDR-2 has an association with several diseases including cancer and fibrosis. The deficiency of DDR-2 leads to craniofacial deformities, premature calcification, irregular metaphysis, long bone dwarfism, pectus excavatum, brachydactyly and platyspondyly spine.

Vimentin is a type III intermediate filament protein coded by VIM gene and is expressed by all prokaryotic, eukaryotic and mesenchymal cells. Vimentin is an important constituent of the cellular cytoskeleton along with actin and tubulin. Like all other intermediate filaments, vimentin monomer has a central α -helical domain, capped on each end by non-helical amino

(head) and carboxyl (tail) domains (Fusch E et al 1994). Individual monomers are co-translationally expressed to form a coiled dimer, which is the basic subunit of vimentin assembly (Chang L et al. 2006). Vimentin is attached to the nucleus and cytoplasmic organelles either centrally or terminally and helps to maintain cell shape, integrity of cytoplasm by anchoring the cytoskeletal organelles in the cytosol and stabilising cytoskeletal interactions (Katsumoto T et al., 1990). It is used extensively as a marker of cells of mesenchymal origin. Recently methylation of vimentin gene DNA has been identified as a biomarker for the upper and lower GIT tract malignancies (Moinova, H, 2012). It can be used as a prognostic indicator in hormone positive breast carcinoma (Ulirsch, J, 2013). Down-regulation of vimentin gene is associated with papillary cell carcinoma of thyroid.

THY-1 (primarily discovered as thymocyte antigen) is a glycosylated cell surface protein with an immunoglobulin domain. It is coded by THY-1 gene located on chromosome no. 11 in humans, but is found in all vertebrates and even some invertebrate species. This was the first T-cell marker discovered and led to the discovery of many other markers. THY-1 is expressed by a variety of cells including lymphoid, neuronal, endothelial, myocardial, keratinocyte, epidermal, bone marrow, foetal liver-derived haematopoietic, and cardiac fibroblast. It plays an important role in T-cell activation, necrosis and apoptosis, tumour suppression, cognition, retinal development, axonal growth regulation and modulation of fibrosis. It also displays an essential role in adhesion, extravasation and migration of cells.

Periostin is an extracellular matrix protein that is coded in humans by POSTN gene. It was initially identified in the cells of mesenchymal origin and is associated with EMT transition in carcinogenic cells. The mesenchymal cells in the developing heart have high expression of periostin and it plays a critical role in the developing heart valves and degenerative valvular heart disease. In a healthy heart, periostin is located in the subendothelial layer of the valves

but the levels are highly expressed in inflammatory and rheumatic heart valve diseases. It increases the secretion of matrix metalloproteinase from valve interstitial cells, endothelial cells and macrophages. It is hypothesised that Periostin plays a crucial part in degenerative valvular disease by inducing angiogenesis and secretion of metalloproteinases (Hakuno D et al. 2012). Moreover, it plays a vital role in tissue regeneration, healing, asthma and carcinoma (Conway SJ et al. 2014) (Corren J et al. 2011) (Zhou W et al. 2015) (Mikheev AM et al. 2014).

The confocal images following the immunostaining of CFs have shown that the projected cell population has a granular appearance, long multiple tapering projections, lack of contractile filaments and focal digitations. All these features were consistent with cardiac fibroblast morphology. (Fig. 3.24). Immunostaining and confocal imaging were performed on fibroblasts from all four chambers at P2 stage. All CFs were Vimentin, DDR-1, Thy-1 and Periostin positive. Comparison between CFs from all four chambers demonstrated a similar pattern of immunostaining. Both atrial and ventricular fibroblasts were positive for all four markers (Fig. 3.24) and negative for α -SMA, α -Actinin, vWF and MHC (Fig. 3.25), confirming their fibroblast phenotype. The immunostaining performed on samples of all four-chamber biopsies further confirmed the cell population to be a true representation of fibroblasts.

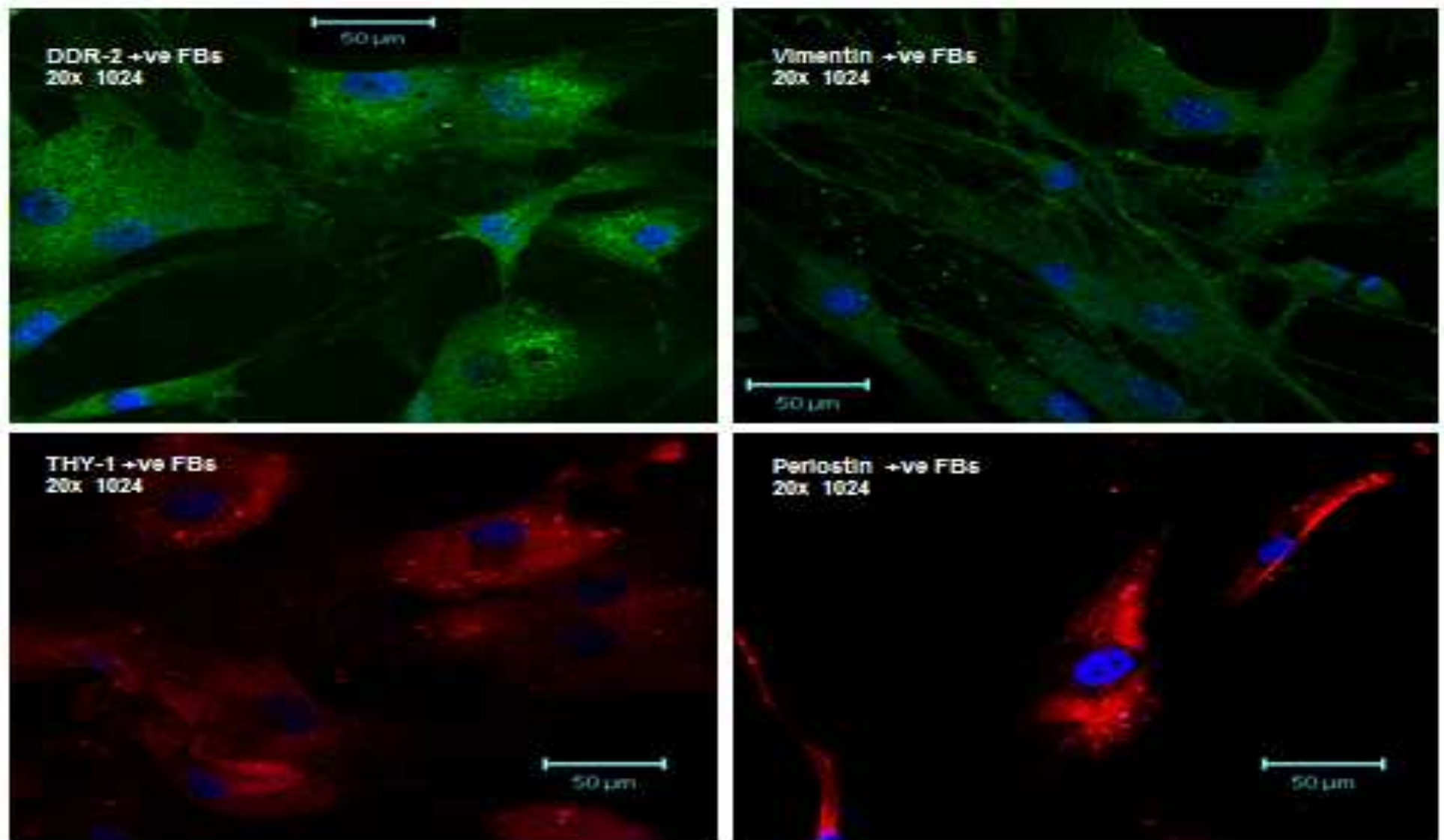


Fig. 3.24. HUMAN VENTRICULAR FIBROBLASTS STAINED WITH PREDICTED POSITIVE MARKERS. Confocal images (20X, 1024 resolution) of stained fibroblasts. To confirm the cells isolated from the human cardiac tissue as fibroblasts, staining was carried out with DDR-2, THY-1, Vimentin and Periostin. Nuclei were stained with DAPI. **Scale bars = 50µm.**

3.7.4 NEGATIVE PREDICTIVE MARKERS FOR IDENTIFICATION OF HUMAN CARDIAC FIBROBLASTS

Besides CFs, the cellular composition of the adult human heart comprises cardiomyocytes, endothelial cells and smooth muscle cells. Following confirmation of the CFs with four predicted positive factors it was imperative to distinguish them from rest of the cellular composition of heart. Based on this, four negative predicted markers specific for cell population other than FBs were selected to confirm pure colonies of fibroblasts.

α SMA is a protein coded in humans by ACTA-2 gene. This protein is found in smooth muscle and vascular smooth muscle cells. Human aortic smooth muscle actin is one of the six known isoforms. α SMA is an essential component of the contractile apparatus of cell. Actin is involved in cell structure, motility and integrity. Mutation in the coding gene results in thoracic aortic disease, coronary artery disease, stroke and systemic smooth muscle dysfunction syndrome. α SMA is commonly used as a myofibroblast transformation marker. Under normal physiological conditions cardiac FBs do not show α SMA staining.

α -Actinin is a protein coded by ACTN-1 gene in humans. It belongs to a diverse group of cytoskeleton proteins - the spectrin gene superfamily. α -Actinin is an actin-binding protein with multiple roles displayed in different cell types. The cytoskeletal isoform in non-muscle cells is found along microfilament bundles and adherens-type junctions, where it binds actin to the membrane. In contrast, the smooth muscle, skeletal and cardiac isoforms are confined to the Z-disc and comparable dense bodies, where they help to anchor the myofibrillar actin filaments.

vWF is a plasma-based multimeric glycoprotein with a vital haemostatic role. It is produced in Weibel-Palade bodies in endothelial cell, subendothelial cells of connective tissue, and

alpha granules of platelets. It facilitates the adhesion of platelets to exposed collagen at the site of vascular injury by forming a bridge between platelet surface receptors and vascular collagen. It also acts as a chaperone to deliver factor VIII to the site of injury, stabilising its heterodimeric structure, preventing its degradation and premature plasma clearance. It is released in response to injury to vascular endothelial cells and subsequent activation of platelets.

MHC is from a superfamily of motor proteins present invariably in all eukaryotic cells. Myosin-II is the myosin type responsible for contractile function of striated and cardiac muscles. Human cardiac myosin has two heavy chains and four light chains. Each heavy chain has a N-Terminal head domain and C-Terminal tail domain. Two heavy chains are intertwined to each other to form a head, neck and tail region. The light chains are located near the head and neck region of the heavy chains. The interaction of myosin and actin filament constitutes a sarcomere. The head region binds the actin filament and uses ATP hydrolysis to initiate the contraction. The neck domain acts as a lever to transmit the force to the rest of the myosin and tail domain interact with other myosin subunits. Except cardiomyocytes, all other human cardiac cellular components lack MHC.

The immunostaining of FB populations from all four chamber biopsy samples with predicted negative markers confirmed the hypothesis that true FB populations should be negative for non-FB markers. (Fig 3.25) Having defined a true population of fibroblasts using positive and negative markers, nuclei counting was done to confirm the number of fibroblasts which remained stained or unstained respectively. Under confocal microscopy, five different views of the stained or unstained fibroblast population were counted. The total number of cells were calculated by counting the nuclei, then the no. of stained cells (cells without a stained nucleus were not taking into account).

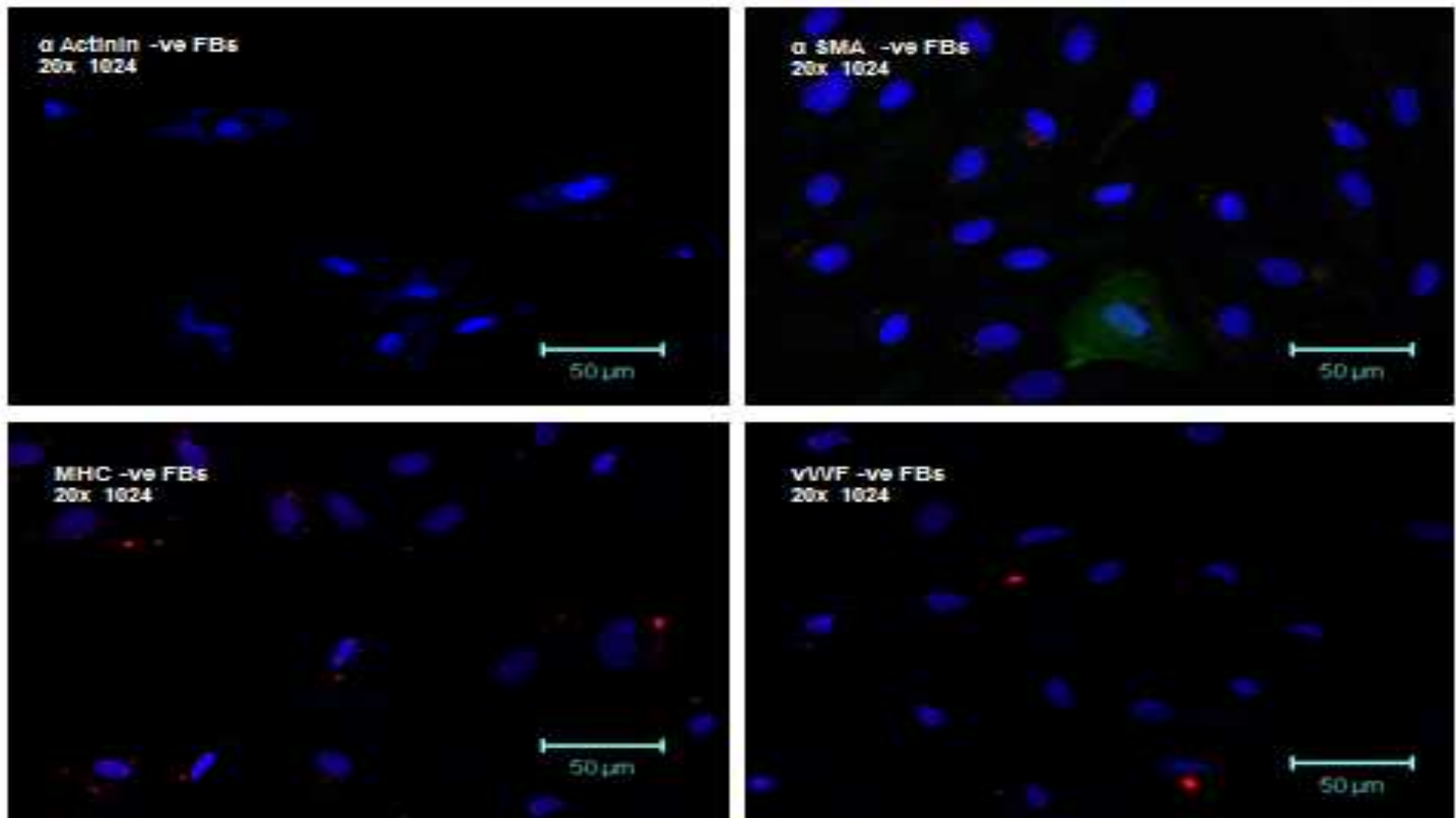


Fig. 3.25. HUMAN VENTRICULAR FIBROBLASTS STAINED WITH PREDICTED NEGATIVE MARKERS. Confocal images (20x, 1024 resolution) of fibroblasts stained with predicted negative markers. To confirm the cells isolated from the human cardiac tissue were fibroblasts, staining was carried out with α -Actinin, α SMA, MHC, and vWF. A few scattered α -SMA positive cell were seen in the field. Fibroblasts were negative for the markers. Nuclei stained with DAPI. **Scale bars = 50 μm.**

For negative markers, the total nuclei found in the field at five different points on the same slide were counted. The number of stained cells were deducted from the population to calculate the unstained cells. The cells with only nuclei stained with DAPI and the rest of the cells unstained represented an actual population of negative marking cells.

In this way, 10 samples were checked for positive and negative markers. The data was analysed, and it revealed that nearly all cells were stained with the positive predicted antibodies. Most CFs with the predicted negative antibodies remained unstained (Fig.3.26).

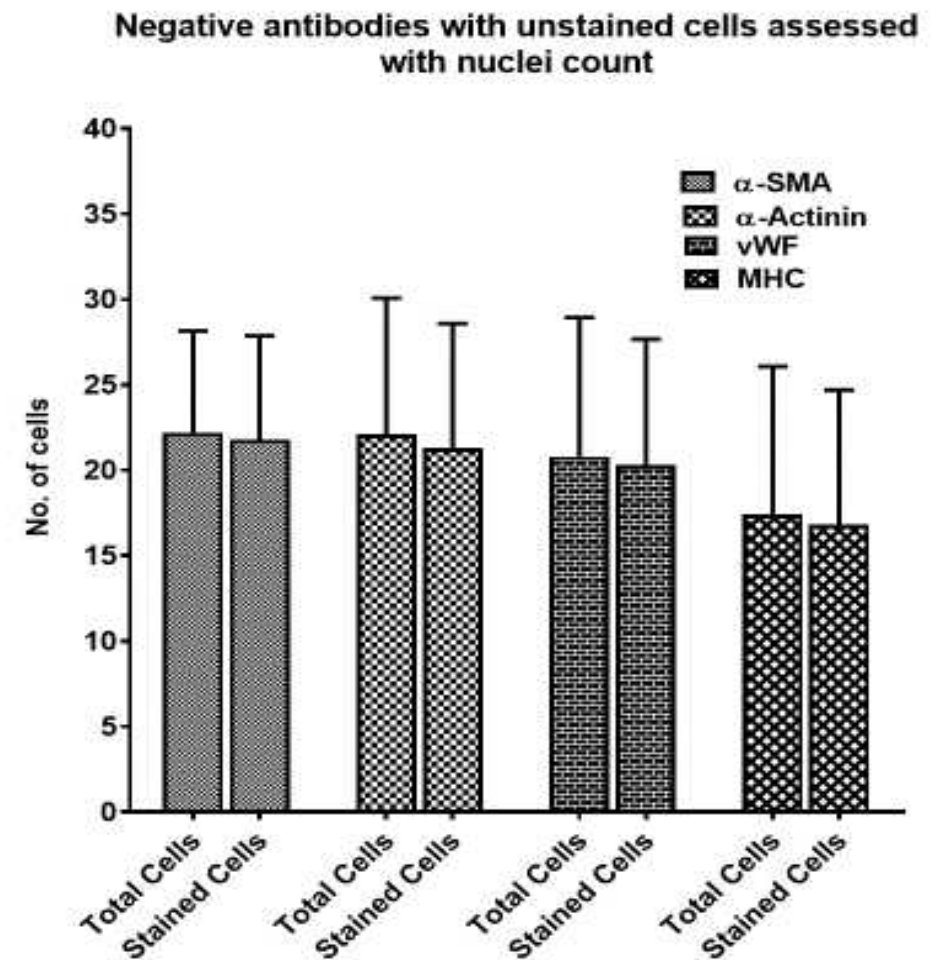
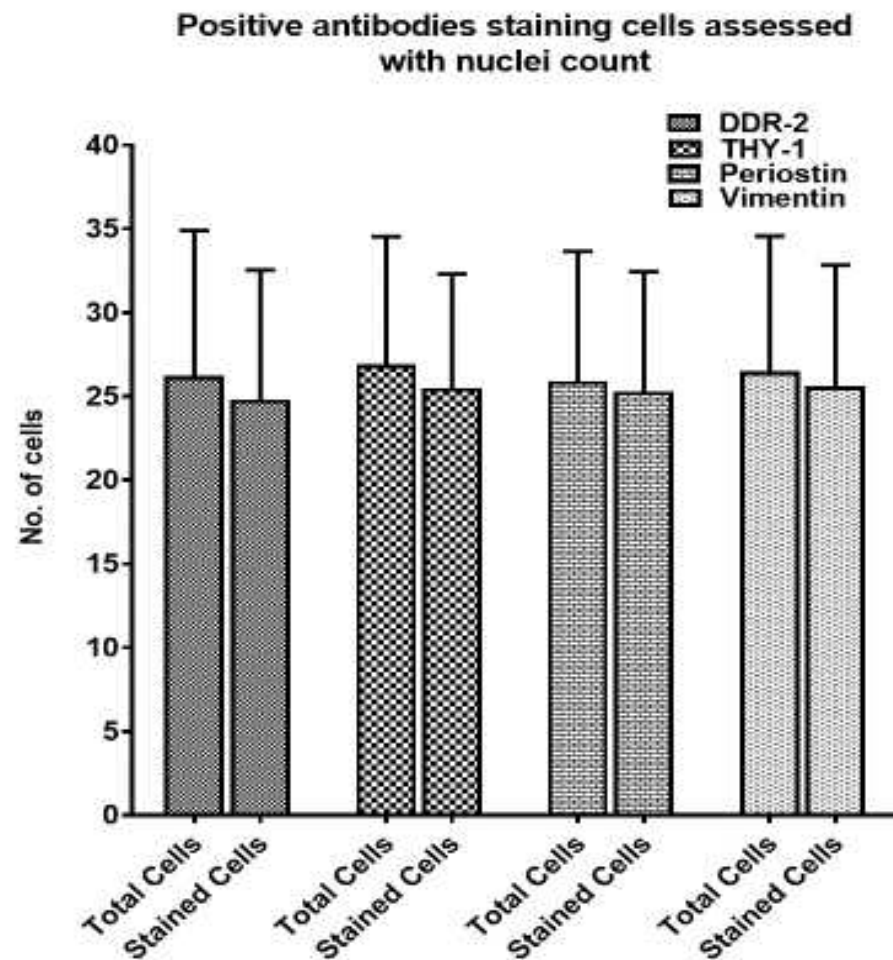


Fig. 3.26. CARDIAC FIBROBLASTS FROM ATRIAL AND VENTRICULAR SAMPLES STAINED WITH POSITIVE AND NEGATIVE PREDICTED MARKING ANTIBODIES. The stained and unstained fibroblasts with positive and negative antibodies were counted with their nuclei count and plotted against the total cell count. In positive antibodies group almost, all cells were stained with the antibodies and similarly in negative antibody group majority of the cells were unstained. n = 10, different samples from atria and ventricles taken from the different patient; **Error bars = Mean with SD.**

3.8 TRANSCRIPTION FACTORS

It had been shown recently that transcription factors also show a predilection for cardiac chambers. In murine models, the expression of the transcription factors is comparable in the atria and the ventricles. No significant difference was noted between the left and right-sided chambers (Furtado et al., 2014). (Fig 3.27)

CHAMBER	LEFT	RIGTH
ATRIUM	GATA 4	GATA 4
	GATA 6	GATA 6
	WT-1	WT-1
	TBX-5	TBX-5
		GATA 5
VENTRICLE	GATA 4	GATA 4
	GATA 5	GATA 5
	GATA 6	GATA 6
	TBX-2	TBX-2
	TBX-20	TBX-20
	Hand-2	Hand-2
	TCF-21	TCF-21
	MYO-D1	MYO-D1
	MEF 2A/2C	MEF 2A/2C
	MESP-1	MESP-1
	NKX 2.5	NKX 2.5

Fig. 3.27. TRANSCRIPTION FACTOR EXPRESSION OF DIFFERENT CARDIAC CHAMBERS IN A “MURINE MODEL” (Furtado et al., 2014). The transcription factors expressed by different chambers of the heart were based on a murine model published as above. Characterisation of murine atrio-ventricular and human atrial chamber fibroblasts with transcription factor expression was attempted in this study was not very systematic. However, the publication eluded to a noticeable difference in the expression of transcription factors. Fig. 3.27. is a systematic tabulated version of finding narrated in the publication which makes it easier to understand the difference in chamber-based TF expression. The publication was used as a baseline to establish the expression of human all four chamber-specific fibroblast expression in this project.

In this murine model, the expression of GATA-5 was unique to the right atrium. NKX 2.5 is expressed in the left atrium, but not the right. GATA-4 and GATA-6 are expressed by both atria and ventricles. WT-1 is expressed by both atria but neither ventricle, and TBX-20 by both ventricles and neither atria. Factors such as MESP-1, MEF-2A/2C, and MYO-D1 are specific to ventricles only and are not represented by the atria (Furtado et al., 2014).

The expression of transcription factors was investigated mostly in the murine model, but based on the concept, in this thesis, the transcription factor expression of fibroblasts from different cardiac chambers was studied.

To define a system of representation of Transcription Factors, a scheme was generated. Following immunostaining, the cells were counted in a field with the number of nuclei. Following this, the stained cells with nuclei were counted. (Fig 3.28)

	STAINED FIBROBLASTS	REPRESENTATION
1.	< 20% stained	-
2.	50% stained	+
3.	70% stained	++
4.	> 90% stained	+++

Fig. 3.28. SCHEME OF PRESENTATION OF THE TRANSCRIPTION FACTOR EXPRESSION DATA.

3.8.1 TRANSCRIPTION FACTORS USED FOR ANALYSIS

1. GATA-4

GATA-4, a transcription factor of GATA Family is an important protein and is required by all mammalian cardiac cells for development and survival of the embryo. It acts on the GATA motifs of many promoter genes. In the adult mammalian heart, GATA-4 is linked with cardiac morphogenesis, survival of cardiomyocytes and normal functioning of cardiac tissues (Harms, Dunlop and Schutt, 2009). During cardiac development, GATA-4 is essential for atrioventricular formation and function, and EMT of endothelial cells into atrioventricular cushions (Rivera-Feliciano, 2006).

GATA-4 is expressed by both embryonic and adult cardiomyocytes and is a transcriptional factor regulator for many cardiac genes in normal physiological conditions, as well as pathological conditions such as hypertrophy. In addition to this, GATA-4 plays an integral part in the development of foetal gonadal tissue and controls the early development stage of the pancreas and liver (Zhou, He and Pu, 2012) (Perrino and Rockman, 2006). Thus, any mutation or inactivation of GATA-4 can lead to septal formation defects, abnormal ventricular folding and hypoplasia of ventricular myocardium leading to congenital heart disease (McCulley and Black, 2013).

In eukaryotic cells, GATA-4 is regulated through the autophagy-lysosome pathway. In the process of cellular senescence, ATM and ATR inhibit p62, which is an autophagy adaptor responsible for selective autophagy of GATA-4. Inhibition of p62 leads to increased GATA4 levels, resulting in NF- κ B activation and subsequent SASP induction (Cao and Li, 2015).

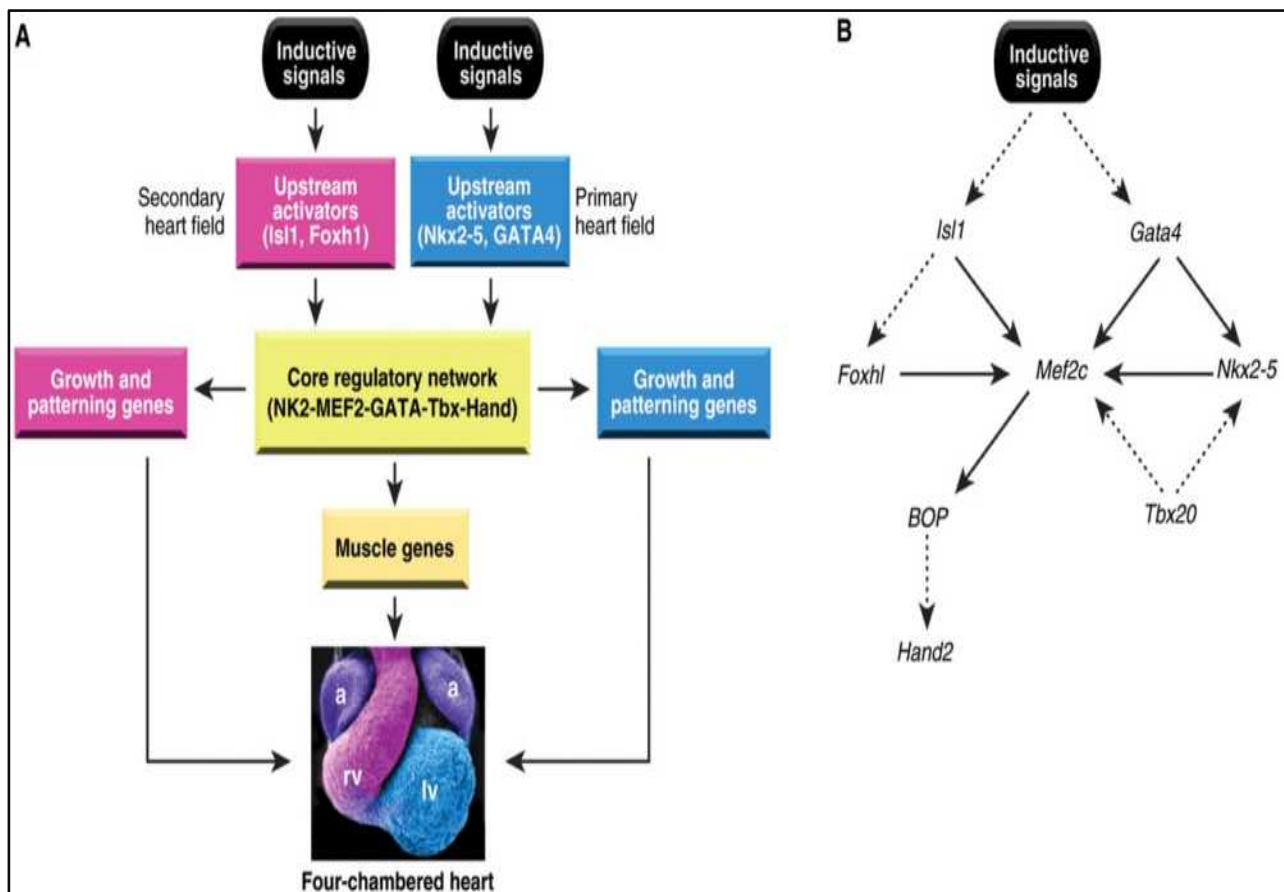


Fig. 3.29. SCHEMATIC DIAGRAM OF TRANSCRIPTIONAL NETWORKS INVOLVED IN MAMMALIAN HEART DEVELOPMENT. (A) Inductive signals activate a set of upstream regulatory genes, encoding transcription factors, in the primary and secondary heart fields. The products of these genes activate the genes in the core cardiac network (NK2-MEF2-GATA-Tbx-Hand). (B) Regulatory interactions among cardiac transcription factors in the secondary heart field. Solid lines indicate direct transcriptional connections, and dotted lines indicate connections not yet shown to be direct. (Adapted from Romer AS. *Science*. 1967; 158:1629)

2. GATA-5

GATA-5 is a transcription factor protein and in humans this is coded by the GATA-5 gene that contains two GATA type zinc fingers. During the early embryonic phase, GATA-5 helps to keep an ample supply of precursor cells and regulates other genes for final differentiation of cardiomyocytes (Zhang et al., 2015). With advancing development, GATA-5 shows more specification towards the ventricles. However, over expression of GATA-5 can lead to ectopic foci which in turn causes arrhythmias (tachycardia, bradycardia, ventricular fibrillation) in adult life (Reiter et al., 1999).

3. GATA-6

GATA-6 is a TF protein encoded by GATA-6 gene and preferentially binds the consensus binding sequence (Sakai et al., 1998). GATA-6 had been linked to the production of LIF which blocks epiblast differentiation and promotes proliferation of endodermal embryonic stem cell (Morgani and Brickman, 2015). A strong association is noted in the development, late stage differentiation of pulmonary alveolar epithelium and aquaporin-5 promotor activation. Any disruption in GATA-6 during intra-uterine period leads to stunted distal epithelial growth, which in turn affects development of distal-proximal bronchioles (Keijzer et al., 2001) (Yang et al., 2002). The mutation in the coding gene leads to defective TF protein synthesis and is linked with pancreatic agenesis, pulmonary hypoplasia and congenital heart defects. GATA-6 plays a dynamic role in the endodermal differentiation of the organ tissue (Chao et al., 2015).

4. TBX-2

In humans, T-Box transcription factor 2 (TBX-2) is coded by the TBX2 gene located on chromosome 17q21-22. T-box genes belong to one family and share a common DNA binding domain (Law et al., 1995). TBX-2 and TBX-3 are the only members of the family that are transcriptional repressor. They are closely related to embryonic development, foetal growth, and have implications in tumorigenesis. TBX-2 is linked with many signalling pathways such as FGF, Wnt, TGF β and BMP, which are associated with organogenesis and cellular proliferation during foetal development (Lu et al., 2010). TBX-2 performs an important role in the development of the limb, forebrain and atrioventricular tissues of the heart. In the murine knock out models, the role of TBX-2 in mediated via BMP signalling pathway for the development of atrioventricular cushions, nodal phenotype and atrioventricular canal is quite marked. The coordinated role of TBX-2 and TBX-3 in the formation of trunco-conal cushions of the heart is manifested by triggering a forward loop

with BMP2 (Singh et al., 2012) (Farin et al., 2013). The signalling for the atrioventricular canal involves the atrial natriuretic factor gene. ANF gene plays a key role in the chamber formation in the developing myocardium. TBX-2 in conjunction with NKX-2.5 forms a complex on the ANF gene to suppress its promoter activity. This inhibits the gene expression in the AV canal formation during chamber differentiation (Habets et al., 2002). In certain subset of myocardial cells, TBX-2 tend to suppress the proliferation and differentiation (Aanhaanen et al., 2009).

Duplication or deletion of TBX-2 gene or protein can lead to many different congenital abnormalities ranging from microcephaly, skeletal muscle abnormalities to VSDs. TBX-2 overexpression is noted to be associated with the lung, breast, bone, pancreas, and melanoma (Nandana et al., 2017).

5. TBX-5

TBX-5, a transcriptional factor protein is coded by the TBX-5 gene located on the long arm by chromosome number 12. The TBX-5 gene is involved in the development of the forelimb, cardiac chambers, myocardial conduction pathways, inter-atrial and inter-ventricular septum. Mutations in the TBX-5 protein/gene is linked with atrioventricular septal defects, disorders of conduction system of the heart, and skeletal abnormalities of the limbs. Mutations in TBX-5 gene is associated with Holt-Oram syndrome (Rodriguez-Esteban *et al.*, 1999).

6. TBX-20

TBX-20 transcription proteins are encoded by the gene of the same name. In murine, human and fruit fly models, this TF is associated with yolk sac vasculature remodelling, migration of motor neurons of rhombencephalon, early development of cardiac tube and adult heart

function. It plays an integral role in the growth of fetuses and any mutation in the gene leads to congenital heart disease. TBX-20 has shown to interact and potentiate the functioning of NKX-2.5 and GATA-4 (Stennard, 2005) (Akasaka et al., 2009). It regulates the other genes such as TBX-2 and N-myc1 modifying the proliferation of cardiomyocytes and their architectural organisation. (Fig 3.30)

In adult murine models, heterozygous knocking out of the TBX-20 gene has shown to cause thin walled dilated ventricles with severe contractile abnormalities (Stennard, 2005). Homozygous knocking out led to arrhythmias, bradycardia, abnormal AV conduction and dilated cardiomyopathy leading to death (Shen et al., 2011).

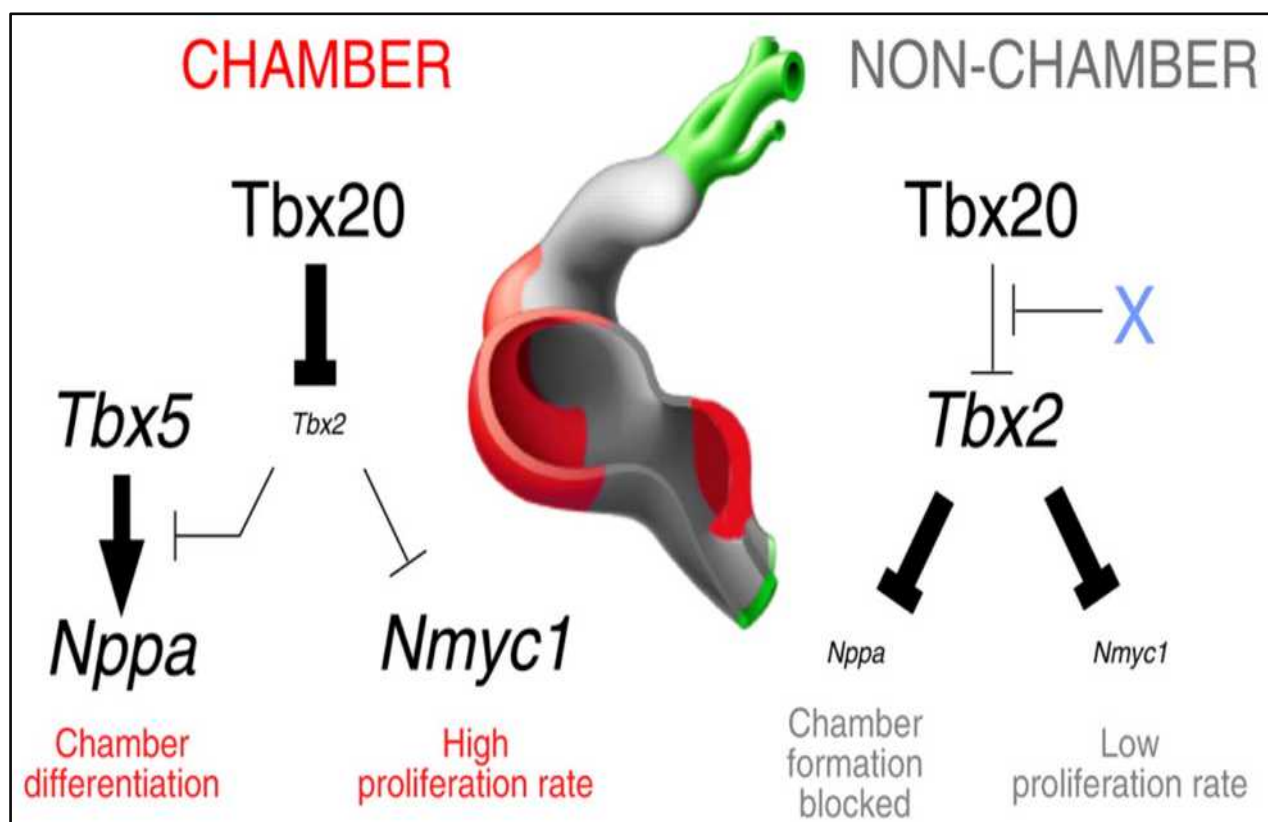


Fig. 3.30. MECHANISM OF ACTION OF TBX-20 ON DEVELOPING CHAMBER SPECIFIC MYOCARDIUM. The interaction of TBX20 with Tbx5 and Tbx2 leads to expression of Nppa and Nmyc1 genes. This blocking of this pathway leads to chamber and non-chamber specific deformities. The optimal and sequential co-expression of all three factors (Tbx20, Tbx5 and Tbx2) is important for the normal development of heart (**Adapted from Abrahams, Parker, and Prince 2010**).

7. WT-1

WT-1 (Wilms Tumour Protein) is a protein coded by WT-1 gene on chromosome 11p. WT-1 is a tumour suppressor gene and the inactivation of gene can lead to sporadic and hereditary forms of renal tumours (Buckler et al., 1991). Mutations of WT-1 in conjunction with other genes is noticed in malignancies such as acute myeloid leukaemia (Rampal et al., 2014). More recently, WT-1 has shown to be present in the nuclei of certain malignant cells (Mesothelioma, Ovarian Serous Carcinoma) and can be used as a tumour marker on immunohistostaining to distinguish these tumours from others. Because of cytoplasmic protein cross reactivity, it cannot be used as a marker for diagnostic cytoplasmic pathological staining. WT-1 is being used in different clinical trials as a target for vaccine for acquired immune response.

8. HAND-2

Heart and neural crest derivative-expressed protein 2 (HAND-2) is a basic helix-loop-family of TF protein coded by HAND-2 gene. It is symmetrically distributed in ventricles and plays a major role in the development of the heart especially the right ventricle and aortic arch with supra-aortic trunk vessels. Studies have shown a key impact of mutations and lack of interactions between HAND-2, GATA-4 and NKX2.5 leading to congenital heart disease and Tetralogy of Fallot (Lu et al., 2016).

In murine and avian models, HAND-2 is believed to contribute embryonic implantation, formation of the oral cavity and development of the enteric neurons respectively. Hand-2 is found to be a valuable biomarker in uterine pre-malignant lesions and endometrial cancers (Jones et al., 2013).

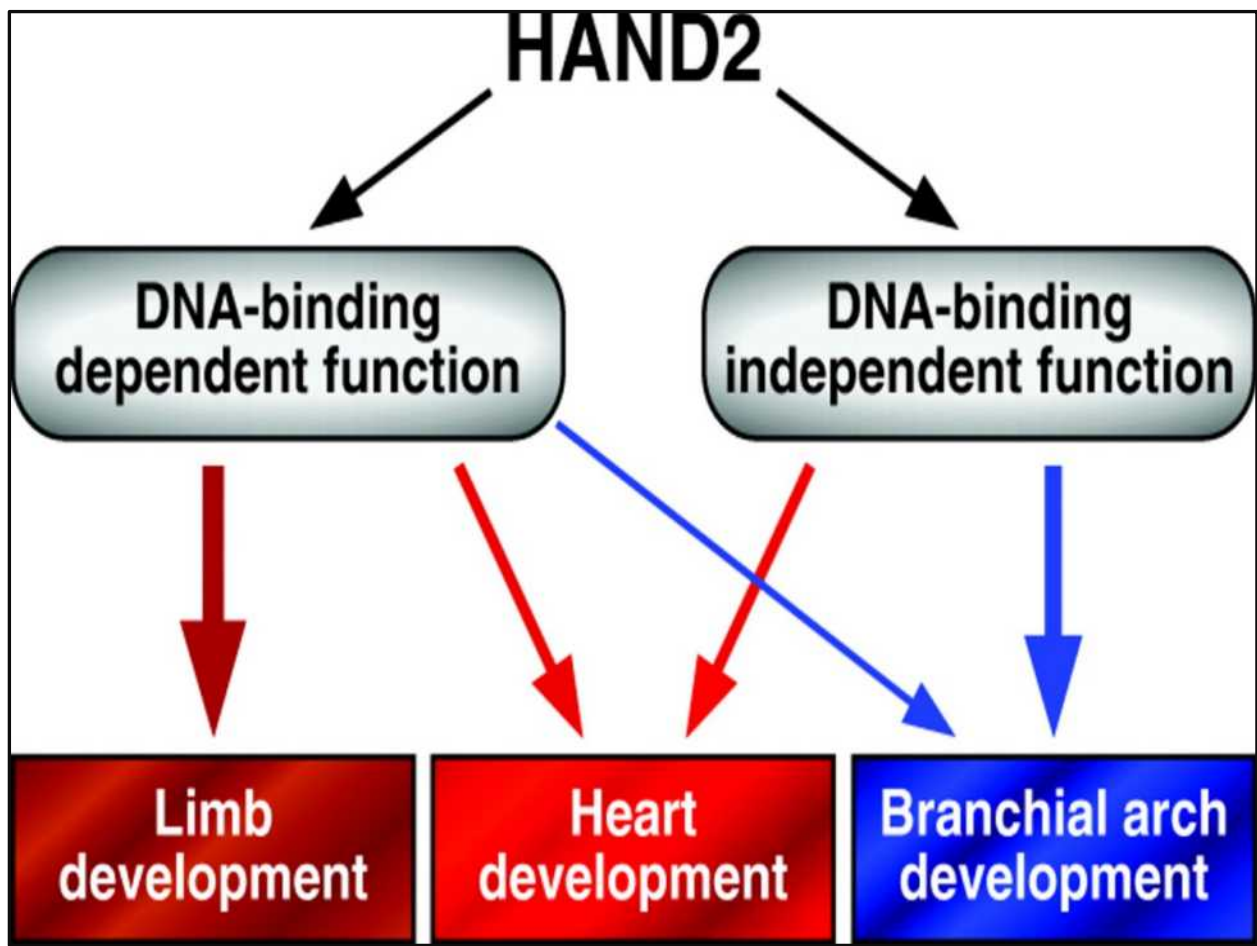


Fig. 3.31. SCHEMATIC MODEL OF HAND-2 FUNCTIONS DURING DEVELOPMENT. Adapted from (Liu et al., 2009)

9. TCF-21

Transcription Factor 21 is a protein coded by a gene located on chromosome 6. It is also called Epicardin, Capsulin and POD-1. Although TCF-21 is expressed by many tissues, it is significantly upregulated by the placenta and lungs. The presence of TCF-21 is regarded as a crucial factor for cardiac, pulmonary, renal and splenic development. (Quaggin et al., 1999; Lu et al., 2000; Acharya et al., 2012b). In mice TCF-21 is linked with epithelial to mesenchymal transition of cardiac fibroblasts. It is regarded as a tumour suppressor factor in human as its expression is deregulated in many carcinomas. Additionally, an association between single nucleotide polymorphism (SNP) of TCF-21 and coronary artery disease in human has been noted (Mega et al., 2015).

10. MEF 2A/2C

Myocyte Enhancing Factor 2 was first isolated from myotubes as a muscle-enriched DNA binding activity but later on was found to be present in most of the tissue of the body. In mammals, the family of MEF-2 comprises four separate gene products referred to as MEF2A – MEF2D, and are widely distributed across the vertebrates. All four member of the MEF2 transcription factor family are involved in a number of regulatory functions in skeletal, immune, neuronal and cardiac tissues (Molkentin and Olson, 1996). MEF-2A, preferably binds to the transcriptional regulatory DNA binding site of cardiac and skeletal muscles and has been postulated to contribute towards inducible gene expression in response to stress stimuli and mitogens. MEF2A DNA binding site are shown to be upregulated in hypertrophied and myopathic hearts (Xu et al., 2006).

In intrauterine life, MEF2C is associated with ventricular muscle development; as in MEF2C knocked out murine models, the ventricular myocardial growth is attenuated and the mice died in the immediate post-natal period (Jiang, Z & Kravchenko, 1997) (Lin et al., 1997). Both MEF2A/2C are required in the postnatal period for optimal functioning of the heart; as in the models depleted of the factors, the mice suddenly died in the perinatal period. The histological samples of the heart revealed right ventricular dilatation, myofibrillar disorganisation and anatomical mitochondrial abnormalities (Naya et al., 2002).

11. MESP-1

Mesodermal Posterior Basic Helix-Loop-Helix 1 Homolog is a transcription factor protein coded by MESP-1 gene located on chromosome 7. MESP-1 is expressed by the early mesoderm which is destined to the fate of cranial-cardiac mesoderm. MESP-1 is associated with optimal morphological growth of the cardiac chambers as the knock out model shows abnormal growth of cardiac chamber and cardia bifida (Saga et al., 1999).

12. MyoD-1

Muscle Differentiation protein 1 is a bHLH transcription protein and belong to the family of myogenic regulatory factors. Myo-D is established as a major myogenic controller and it acts via a DNA binding motif called as E-box mediated protein phosphorylation. It can induce chromatin remodelling and interacts with a variety of muscular gene promoters leading to myoblast proliferation. In terminally differentiated myofibroblasts, it causes cell cycle arrest in conjunction with tumour suppressor gene. During the developmental phase, Myo-D converts the mesoderm to the skeletal muscles and later on regulates the continued state and repair of striated muscles. Elevated levels of Myo-D are detected in senescent skeletal muscles (Hughes et al., 1997). It is involved in many intra cellular signalling pathways such as Wnt etc. MyoD-1, and is still an area of interest with many unknown mechanisms.

13. NKX2.5

It is a homeobox transcription factor protein coded by the NKX2.5 gene. It plays an important role in tissue differentiation, whilst maintaining the temporo-spatial patterns of development. In humans, NKX2.5 is essential for the anatomical and functional development of the heart. In the early phase of cardiogenesis, NKX2.5 is expressed by mesoderm throughout the atrial and ventricular chambers. During this phase, all the cardiac precursor cells (cardiac crescent of splanchnic mesoderm) are aggregated along the ventral midline of the developing embryonic heart and knocking out of NKX2.5 gene not only stops the cardiac tube formation but also disrupts the looping morphogenesis. Any mutation in NKX2.5 gene is associated with septal and conduction defects. In the post-natal period, NKX2.5 helps in transactivation of numerous cardiac genes involved in generation of cardiac hypertrophy and cardioprotective response of cytotoxic stress (Akazawa and Komuro, 2003). It is postulated that NKX2.5 helps cardiomyocytes to cope with the cytotoxic stress during chemotherapy. There is an established pathway of interaction between NKX2.5, GATA4 and TBX5.

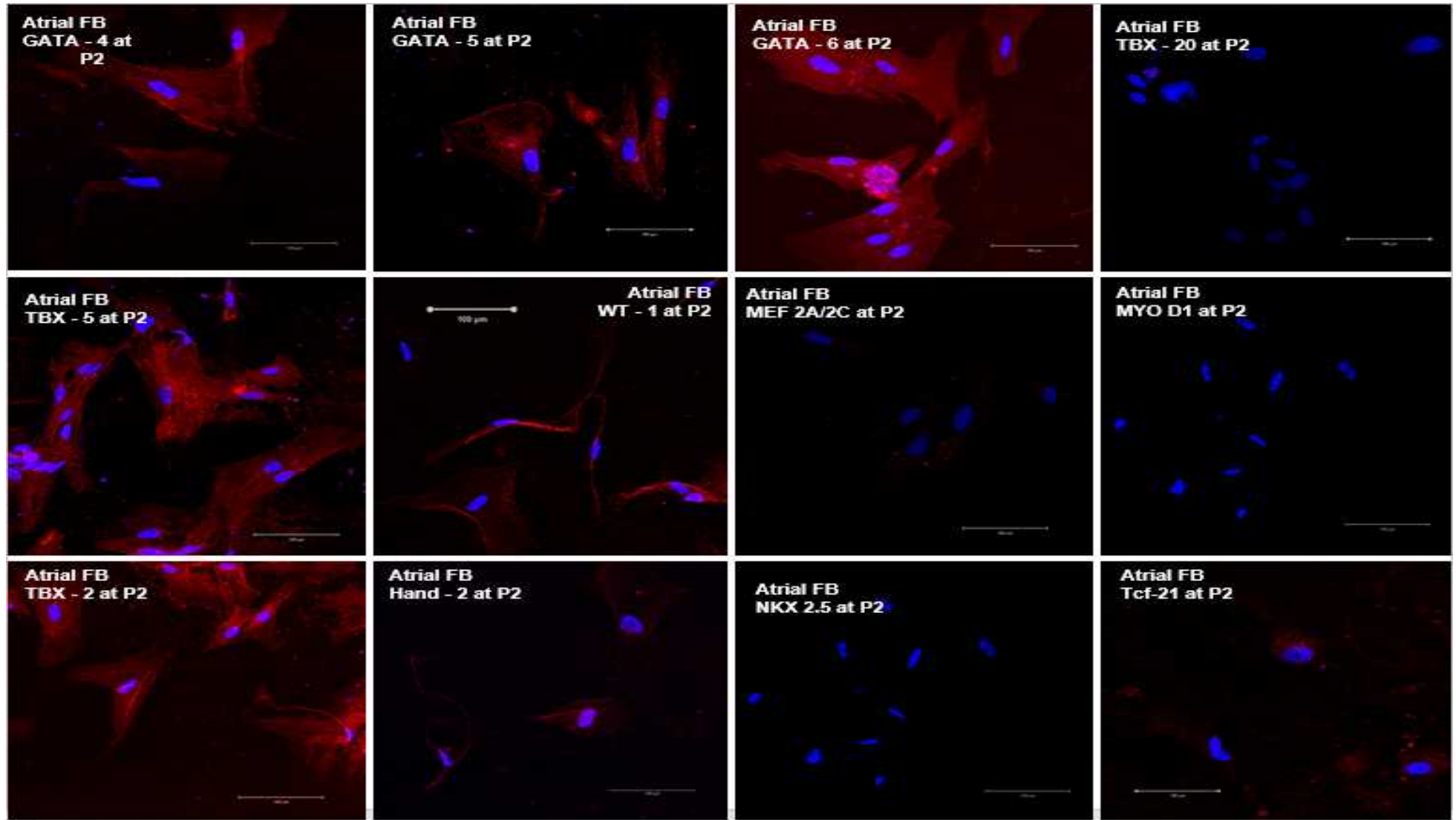


Fig. 3.32. TRANSCRIPTION FACTORS EXPRESSED BY ATRIAL FIBROBLASTS. Confocal images of human ventricular fibroblasts (20X magnification – 1024 resolution) at P2 stage. The fibroblasts were stained with individual transcription factors while nuclei were stained with DAPI. **Scale bar = 100µm.**

3.8.2 TRANSCRIPTION FACTORS EXPRESSED BY HUMAN ATRIAL FIBROBLASTS

A total number of 20 bi-atrial samples were harvested from the patients with normal LV function group. Three samples did not show any growth and confluence pattern (two at P0 stage + one at P1 stage). The rest of the 17 atrial samples grew satisfactorily to the P2 stage and hence were analysed for fibroblast markers and transcription factor expression.

Transcription factor (TF) expression of left atrial and right atrial fibroblasts were comparable to each other. Atrial fibroblasts strongly expressed GATA-4, GATA-5 and GATA-6. However, GATA-4 was better defined in the LA fibroblasts. In murine species, GATA-5 is expressed only by the RA fibroblasts. (Fig 3.29) Conversely, in human atrial fibroblasts, GATA-5 is expressed by both atria. Similarly, TBX-2 and TBX-5 were equally represented by both atrial fibroblasts. TBX-20 was absent in both atrial fibroblasts samples. As expected, atrial specific TFs like WT-1 and Hand-2 were strongly expressed by both atria. TCF-21 was noted to be present in atrial fibroblasts and its expression was more pronounced in LA than RA. (Table 3.7, 3.8)

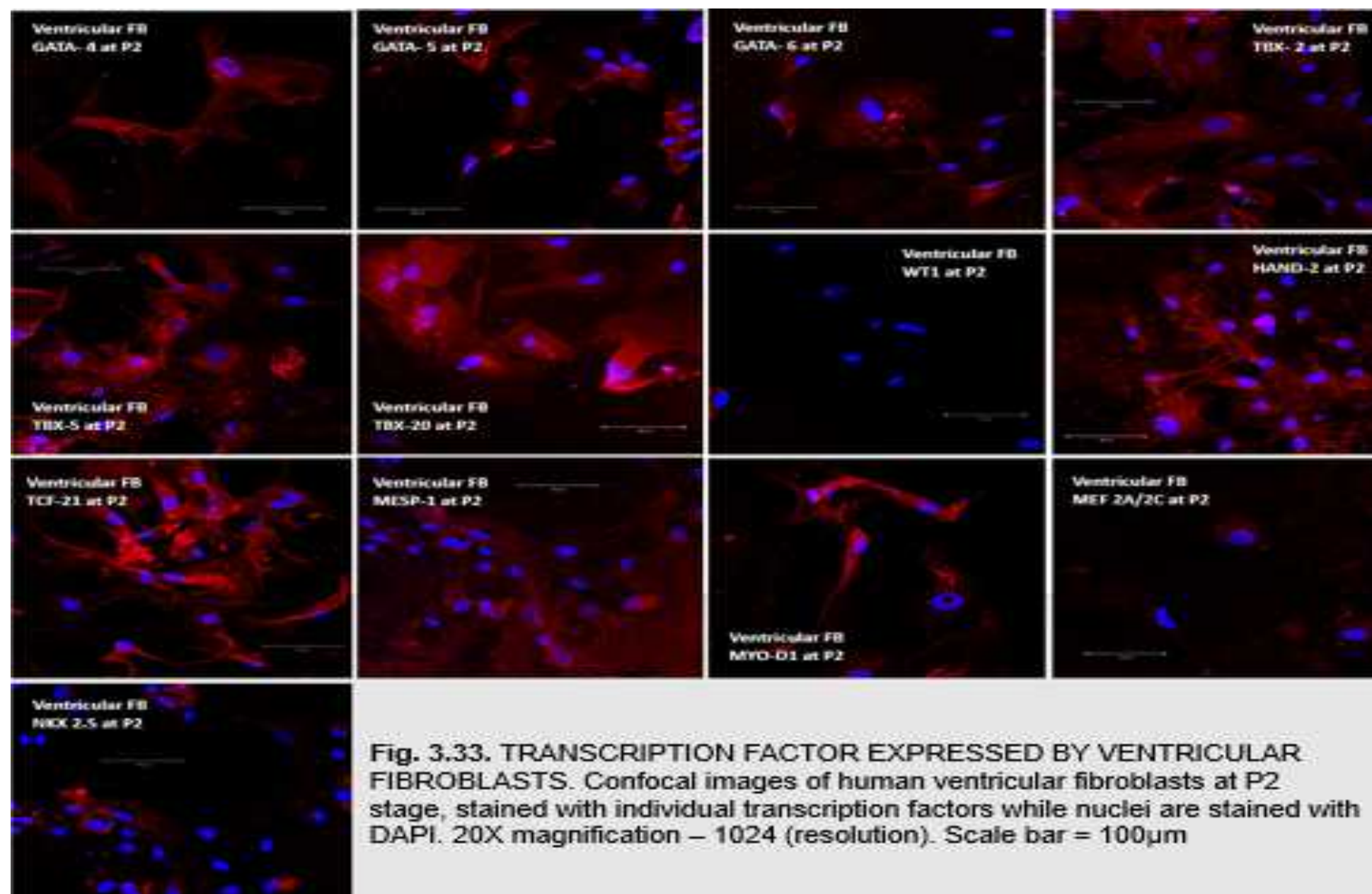
The TFs with predicted ventricular predilection (based on murine model) like MESP-1, MYO-D1, MEF2A/2C were absent in both atria. NKX2.5 is strongly expressed in murine atrial fibroblasts but was negative in both human atrial fibroblasts.

	TRANSCRIPTION FACTOR EXPRESSION OF RIHGT ATRIAL FIBROBLASTS (n = 17)																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
GATA 4	+	+	+	+	++	+	-	+	++	+	++	-	+	+	+	+	+
GATA 5	++	+	+	+	+	++	+	+	+	+	++	-	+	++	+	++	+
GATA 6	++	++	++	++	+++	+++	++	++	++	++	++	-	++	++	+++	++	++
TBX-2	+	+	+	+	+	+	+	+	++	+	+	+	+	+	+	+	+
TBX-5	+	+++	++	+++	++	+	+++	+	++	+++	+++	+	++	+	+++	+++	+++
WT-1	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
TBX-20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hand-2	+	+	+	+	+	+	++	++	++	+	++	-	+	+	+	+	+
TCF-21	+	+	+	+	+	++	+	+	+	+	+	+	+	+	+	+	+
MESP-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MYO-D1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MEF 2A/2C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NKX 2.5	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-

TABLE. 3.7. TRANSCRIPTION FACTOR EXPRESSION OF RIGHT ATRIAL FIBROBLASTS. The right atrial FBs showed strong expression of predicted atrial TFs. TFs with likely predilection for ventricles were all negative. TBX-5 was expressed in human atrial FBs (absent in the murine atrial FBs). NKX2.5 expressed in murine atrial FBs was absent in the human atrial FBs. The number of FBs stained were counted with their nuclei in 3 different field of views and were mapped in this table as per scheme mentioned in Fig. 3.26. (n = 17).

	TRANSCRIPTION FACTOR EXPRESSION OF LEFT ATRIAL FIBROBLASTS (n = 17)																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
GATA 4	+	+++	++	+++	+++	+++	++	++	++	++	++	+++	++	+++	++	++	+++
GATA 5	++	+	+	++	+	++	+	+	+	+	++	-	++	++	+	++	+
GATA 6	++	++	+++	++	+++	+++	+++	+++	+++	+++	++	+++	++	++	+++	++	++
TBX-2	+	+	+	+	+	+	+	+	++	+	+	+	+	+	+	+	+
TBX-5	+	+++	++	+++	++	++	+++	++	++	+++	+++	++	++	++	+++	+++	+++
WT-1	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+
TBX-20	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hand-2	+	++	++	++	+	+	+	++	++	+	++	++	++	+	+	+	+
TCF-21	+	+	+	+	+	+	+	+	+	++	+	++	++	+	++	++	+
MESP-1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MYO-D1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
MEF 2A/2C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
NKX 2.5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

TABLE. 3.8. TRANSCRIPTION FACTOR EXPRESSION OF LEFT ATRIAL FIBROBLASTS. The left atrial FBs showed strong expression of predicted atrial TFs. TFs with predicted affinity for ventricular FBs were all negative. GATA-4 and GATA-6 were better expressed in LA-FBs. TBX-5 was expressed in the human atrial FBs. NKX2.5 was absent in left atrial FBs. The number of FBs stained were counted with their nuclei and were mapped in this table as per scheme mentioned in Fig. 3.26. (n = 17).



3.8.3 TRANSCRIPTION FACTORS EXPRESSED BY HUMAN VENTRICULAR FIBROBLASTS

Corresponding to the atrial samples, a total of 20 ventricular biopsy samples were taken from the patients with normal LV function. These samples were small in size, and owing to this, only 8 grew to the P2 stage. Only 7 samples were stained for transcription factor expression. The ventricular sample with the best rate of growth was used for EHT generation, and no immunostaining or TF analysis was performed on this sample.

Ventricular fibroblasts were stained with the same set of transcription factors as atrial fibroblasts. Both LV and RV fibroblasts expressed GATA-4, GATA-5, and GATA-6. Similarly, TBX-2, TBX-5 were expressed by both left and right ventricles. TBX-20, which was absent in the atrial fibroblasts was strongly positive in the ventricular fibroblasts. The TFs with atrial partiality in the murine model (i.e., WT1 and Hand-2) were noted to be present in human atrial FBs. The human ventricular fibroblasts showed a different pattern of distribution of WT-1 and Hand-2. WT-1 was absent in both ventricular FBs. Conversely, HAND-2 was expressed by both fibroblasts in humans.

The TFs with proposed ventricular predilection (e.g. TCF-21, MESP-1, MYO-D1, MEF2A/2C) were strongly expressed in FBs of both ventricles. NKX2.5, which was absent in the human atrial samples, was also found in both ventricles (Table 3.9, 3.10).

	TRANSCRIPTION FACTOR EXPRESSION OF RIGHT VENTRICULAR FIBROBLASTS (n = 8)							
	1	2	3	4	5	6	7	8
GATA 4	+	+	+	+	+	+	+	+
GATA 5	+	+	++	+	+	+	+	++
GATA 6	+	+	+	++	+	++	++	+
TBX-2	+	+	+	+	+	+	+	+
TBX-5	++	+++	+++	+++	+++	+++	+++	+++
WT-1	-	-	-	-	-	-	-	-
TBX-20	++	++	+++	++	+++	+++	++	+++
Hand-2	+	++	++	++	+	++	+	++
TCF-21	+++	+	+++	++	++	++	++	+++
MESP-1	++	+++	+++	++++	++++	+++	+++	++++
MYO-D1	+++	+++	+++	+++	++++	+++	+++	++++
MEF 2A/2C	++	++++	+++	+++	++++	++++	++++	++++
NKX 2.5	+	+	+	++	+	+	++	+

TABLE. 3.9. TRANSCRIPTION FACTOR EXPRESSION OF RIGHT VENTRICULAR FIBROBLASTS. The predicted atrial TFs were weakly expressed by the ventricular FBs. TFs with ventricular predilection in murine models were strongly expressed by the human ventricular FBs. TBX-5 was strongly expressed by the RV-FBs. NKX-2.5 expressed in ventricular FBs was absent in atrial FBs. The number of FBs stained were counted with their nuclei in 2 separate fields under confocal microscope and are mapped in this table as per scheme mentioned in Fig. 3.26. (n = 8).

	TRANSCRIPTION FACTOR EXPRESSION OF LEFT VENTRICULAR FIBROBLASTS (n = 8)							
	1	2	3	4	5	6	7	8
GATA 4	+	+	+	+	+	+	+	+
GATA 5	++	+++	+++	++++	++++	+++	+++	++++
GATA 6	+	+	+	++	+	++	++	+
TBX-2	+	+	+	+	+	+	+	+
TBX-5	++	+++	+++	+++	+++	+++	+++	+++
WT-1	-	-	-	-	-	-	-	-
TBX-20	+	+	+	+	+	+	+	+
Hand-2	+	++	++	++	+	++	+	++
TCF-21	+++	+	+++	++	++	++	++	+++
MESP-1	++	+++	+++	++++	++++	+++	++++	++++
MYO-D1	++	++	++	+++	++	++	++	++
MEF 2A/2C	++	++++	+++	++++	++++	++++	+++	++++
NKX 2.5	+	++	+++	++	+++	+++	++	+++

TABLE. 3.10. TRANSCRIPTION FACTOR EXPRESSION OF LEFT VENTRICULAR FIBROBLASTS. GATA-5 and NKX-2.5 was strongly expressed by LV-FBs in comparison with RV-FBs. The rest of the TFs expression was comparable between the two ventricles. The number of FBs stained were counted with their nuclei in 2 different fields under confocal microscope and are mapped in this table as per scheme mentioned in Fig. 3.26. (n = 8).

3.9 DISCUSSION

3.9.1 SUMMARY

With a few minor modifications, both tissue explant and enzymatic digestion technique protocols were successful in isolating CFs. The amount of tissue required for the optimal growth and desired yield of fibroblast population is much larger with the enzymatic digestion technique compared to the explantation technique. The fibroblast population obtained with enzymatic digestion required multiple meticulous washings in the first 24 hours whilst the cells were attaching to the bottom of the growing petridishes. The settling cells tend to get washed away each time. Moreover, the population of FBs is at higher risk of bacterial contamination than using explant technique. For the first 72 hours a significant population of cardiomyocytes was seen in the culture medium but they all died after day 4. A noteworthy endothelial cell population was also seen during this period but again died after 4-5 days, as the culture medium for FBs does not nurture and support the sensitive and fragile cardiac endothelial cell growth. After day 5, a true population of fibroblasts is achieved. The maximum rate of bacterial contamination occurs during the first 48-72 hours. Any infection after this period is usually a fungal contamination. Furthermore, the rate of contamination was significantly reduced after using an antibiotic regimen of penicillin and amphotericin. For the first 4 days, a culture medium containing 2% antibiotics solution was used rather than 1%. After day 4, the nourishing culture medium was switched to the standard (with 1% penicillin-streptomycin + amphotericin). Fibroblast growth and turnover for the next passage was much quicker with this technique.

With the explant technique, the FBs were slow in growth rate but it yielded better and purer colonies of fibroblasts. The rate of infection in this technique was low, and there was considerably less loss of cell population at each passage. Moreover, as multiple washes were not required in the first 72 hours, it was less traumatic for the harvested cell population.

Repeated dilution of enrichment medium was prevented by this method. Fibroblasts from a smaller tissue sample can be harvested with this technique.

RV fibroblasts had a better growth potential and they tend to retain their true morphological features as compared to the other chambers. The growth pattern was followed in the order of LA > RA > LV fibroblasts.

Expression of TFs in fibroblast population was comparable in both atria (RA and LA) as in both ventricles (LV and RV), but atrial and ventricular groups showed systematic differences. The TF expression of CFs showed many similarities to cardiomyocyte progenitors. Different transcription factors were expressed by different cardiac cell population. The TF expression of each type of cells have an impact on the other cellular populations. The precise mechanism of effect of each of these factors on the cell, still needs further investigation.

More recently, the impact of different cardiac TFs was studied on the cardiac cell population. It was identified that human c-Kit positive cardiac progenitor cell population of four different lineage depicted a variable differentiation potential in response to same set of transcription factors. The individual response of these TFs on the cell population of different lineage is notable. When introduced separately, GATA-4 and TBX-5 induced a subset of myocyte markers. GATA-4 alone has shown to significantly induce smooth muscle cells and fibroblast markers. The gene expression changes brought by GATA-4 were also accompanied by morphological changes. In contrast, MEF2C and NKX2.5 were more or less unsuccessful in initiating cardiac gene expression in CPC's. When these CPC's were exposed to a cocktail of different combinations of TFs they failed to act synergistically. It was concluded that GATA-4 alone can induce gene expression in different cell lineage and is a favourable TFs with a potential use in stem cell therapy (Al-Maqtari et al., 2017).

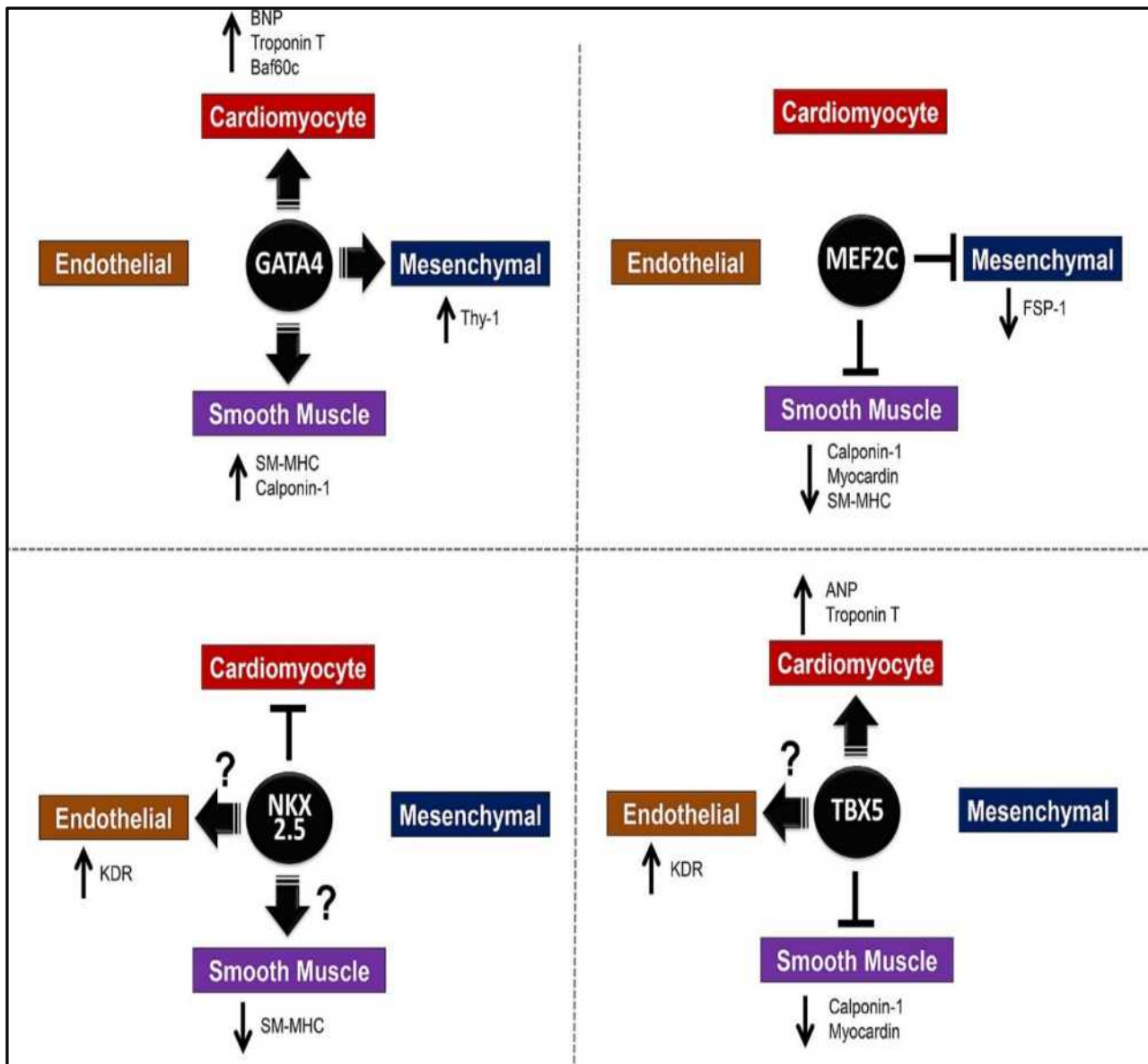


Fig. 3.34. ILLUSTRATION SHOWING THE EFFECTS of GATA4, MEF2C, NKX2.5 & TBX5 OVEREXPRESSION ON CARDIOVASCULAR DIFFERENTIATION POTENTIAL OF HUMAN CPCs. Large arrows indicate promoting action towards the lineage. T-shaped bar indicates inhibitory action towards the lineage. Arrows that are next to genes indicate either increase (upward) or decrease (downward) in the gene expression. Question marks indicate the questionable or inconclusive nature of the association. **Adapted from (Al-Maqtari et al., 2017)**

A similar pattern of expression of GATA-4 was observed among all four cardiac chamber fibroblasts. The atrial expression of GATA 4 was more marked with LA than RA fibroblasts. The ventricular expression of GATA-4 was weaker in comparison to the atrial expression. GATA-5 expression was more striking in ventricles than atrial FBs, which again was consistent with already published data. It is shown that with maturation of growing heart, GATA-5 shows more specification for the ventricles and is recognised to play a pivotal role

in myocardial hypertrophy when it is up regulated (Reiter et al., 1999) (Zhang et al., 2015). A similar pattern was observed in the FBs of the ventricular origin in this study, where GATA-5 showed an enhanced expression in the left ventricular FBs. GATA-6 was expressed by both atrial and ventricular FBs. However, expression is more noticeable in atria than ventricles.

T-BOX genes related transcription factors including TBX-2, TBX-5 and TBX-20 also showed a variable expression between the upper and lower chambers of the human heart. TBX-2 was further marked in atria. In adult life, over expression of this protein is related to increased incidence of carcinogenesis. It acts in conjunction with NKX2.5 to suppress the ANF gene promoter activity, and proliferation and differentiation of myocardial cells in murine models (Habets et al., 2002; Aanhaanen et al., 2009). In human atrial chambers, NKX2.5 is not expressed hence the synergistic action of the tumour suppressing activity is deemed to be ineffective.

TBX-5 was strongly expressed by human cardiac FBs in both atria and ventricles. Recently in another study, human cardiomyocytes and endothelial cell progenitor have shown a high expression of TBX-5 (Al-Maqtari et al., 2017). The expression of TBX-5 by fibroblasts from all four-chambers has endorsed the results of this previous observation. TBX-20 is exclusively present in ventricles and absent in atrial FBs. Since it potentiates the effect of NKX2.5 and GATA-4, its presence in ventricle FBs is more meaningful in comparison with atrial FBs which lack its expression (Stennard, 2005; Akasaka et al., 2009).

In the human heart, WT-1 was exclusively present in atrial FBs and was absent in the ventricle FBs. This finding was consistent with the murine model where the chamber related distribution exhibited the same pattern (Furtado et al., 2014). Hand-2 was weakly expressed

by the atrial FBs but the ventricular FBs displayed a much stronger expression. Previously published data suggested that HAND-2 was strongly expressed by ventricles and plays a vital role in the development of RV and aortic arch. HAND-2 showed a strong interaction with GATA-4 and NKX2.5, which were co-expressed in ventricular fibroblasts similar to this study finding (Lu et al., 2016).

TCF-21 was found to be equally distributed in fibroblasts of all four-chambers. This finding conflicted with Furtado's murine model data in which TCF-21 was only distributed to the ventricular FBs. The manifestation of other TFs such as Mef2A/2C, Mesp-1, MyoD-1 was consistent with the murine models and showed a predilection for the ventricular FBs and were absent in atrial FBs. MEF2C acts on the mesenchymal and smooth muscles cell progenitors to induce the protein secretions. It has no characteristic effect on endothelial and cardiomyocyte precursors. NKX2.5 exhibited by the ventricular FBs, was absent in atrial fibroblasts. Its synergistic effect with other co-existing TFs in ventricles advocates its efficacy more as a ventricular based TF.

CHAPTER 4: ENGINEERED HEART TISSUE

CHAPTER 4: ENGINEERED HEART TISSUE

4.1 OVERVIEW

This chapter describes the work carried out using human cardiac fibroblasts from different chambers to construct the Engineered Heart Tissues. The first aim was to build the EHTs using human cardiac fibroblasts. Subsequently, a comparison is made between the kinetics generated by these EHTs with fibroblasts of distinct origin.

The first section of this chapter describes the construction of the EHTs by using human cardiac fibroblasts of different origin. (Pilot data) Periodic measurement of chronotropy and inotropy of EHT constructs was done. A comparison of their rate and force of contraction was made serially right from the day of construction until three weeks. The second section of the chapter describes the investigation and comparative analysis of kinetics between the EHTs with chamber-specific fibroblasts in an auxotonic model. A further comparison was made with the EHTs with fibroblasts of different origin.

**BRIGHT FIELD IMAGE OF EHT
CONSTRUCT WITH HUMAN
CARDIAC FIBROBLASTS &
IPSC-CM. IMAGE FROM
VIDEO 1(BEATING OF EHTS).
SCALE BAR = 1mm.**

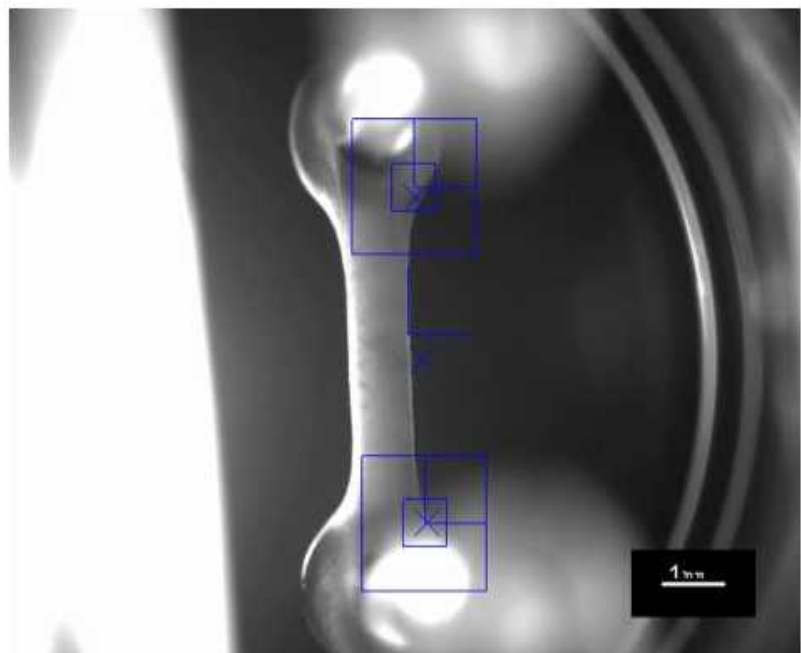


Fig 4.1. Engineered Heart Tissue construct with human cardiac fibroblast co-cultured with IPSC-CM. The constructs are attached to the silicon posts at each end and their rate and force of contraction is measured using a bespoke designed machine.

4.2 INTRODUCTION

The concept of construction of EHTs was introduced by Eschenhagen et al. who cultured the cardiomyocytes derived from an embryonic chick ventricle with a collagen matrix solution for the generation of spontaneously beating, three-dimensional models of heart tissues (Eschenhagen et al. 1997). The dissociated cardiomyocytes laden hydrogel-like matrices were attached to silicon posts to maintain a defined preload. Cardiomyocytes dispersed in the surrounding hydrogel not only remodel and align along the line of force but also show growth in size to form a cardiac syncytium. These matrices exhibited anatomical physiological and histological features analogous to the myocardial syncytium. Furthermore, it was possible to measure the force and rate of contraction displayed by this model (Eschenhagen et al. 2012, Hirt et al., 2014) (Zimmermann et al., 2002). The individual EHT constructs can be fused to generate larger constructs and these construct's exhibit force of contraction comparable to the human myocardial tissue (Zimmermann et al., 2006). The construction methodology and the kinetics depicted by the EHTs have improved ever since. Lately, Kensah et al. have established a model of the generation of EHTs with force of contraction only 5-fold less than that of the normal human myocardium (Kensah et al., 2013).

In our lab (BHF Regeneration Medical Centre of Imperial College) the methodology of EHTs was developed and tested. The preliminary data was collected from EHT constructs with cardiomyocytes only, and later fibroblasts were added to the constructs alongside cardiomyocytes (Fig. 1.8. Adapted from on-going project of Prof. Harding, Owen T). Initially, murine ventricular fibroblasts were used to study the effect of fibroblasts on iPSC-derived cardiomyocytes. This was followed by human foetal fibroblasts, adult atrial fibroblasts and ventricular fibroblasts. It was shown that addition of the fibroblasts to the EHT construct led to enhanced inotropic effect, sarcomeric gene expression, calcium transient acceleration and exhibit further maturation of troponin and myosin. This data reiterated the hypothesis of

previous studies that fibroblasts improved the connections between the cardiomyocytes and hence improves the contractile force alongside the maturation of the tissue (Parrag, Zandstra and Woodhouse, 2012) (Liau et al. 2012).

In the adults, the human heart, not only do the chambers have different anatomy, but they are subjected to various pressures and biochemical environment. Moreover, the same disease pattern affecting different chambers leads to variable outcomes not only clinically, but the pathological features vary as well. The ventricular fibroblasts used in the pilot data were harvested from the native heart of the transplant's recipients. The myocardial tissue was extremely hypertrophied with extensive fibrosis all through the muscle layers. The hypertrophy and fibrosis had a bearing on the cellular characteristics of the native fibroblast's population. It was presumed that the addition of these fibroblasts to the EHTs could affect their morphology and physiological characteristics. This necessitated the study to investigate the role and impact of fibroblasts from the normally functioning heart in EHTs.

In this study, for the first time, the human cardiac fibroblasts from the different chamber of heart, from the same patient were added to the EHTs to compare their effects. However, there were some limitations as the tissue samples obtained from the ventricular biopsies were very small and only a few samples could generate enough fibroblast counts at the P2 stage so that it can be used to construct EHTs. The results obtained from these chamber specific fibroblasts co-cultured with iPSC-CM were still able to bring forward some valuable information with regards to any chamber-specific behaviour of the fibroblasts.

The other limiting factor was the difficulty in obtaining and isolating cardiomyocytes from the adult human heart and using them for these constructs. Thus, human iPSC-CM were primarily used to generate the human EHTs. Human iPSC-CM were used previously in

different studies to construct EHTs that exhibit morphological and functional characteristics mirroring human myocardial tissue (Tulloch et al. 2011) (Schaaf et al. 2011) (Kensah et al., 2013) (Thanvandiran et al. 2013) (Nunes et al. 2013). Although iPSC-CM are extensively used in the many studies their morphological feature, functional characteristics, phenotype, gene expression and calcium transients were more comparable to foetal than adult cardiomyocytes (Gai et al., 2009) (Itzhaki et al., 2011) (Ma et al., 2011) (Rao et al., 2013).

The first section of this chapter describes the difference between the EHTs harvested from the atrial and ventricular tissue sample taken from different patients with distinct underlying pathologies. (Pilot Data) A comparison was made between the EHTs constructed from atrial and ventricular fibroblasts with the EHTs constructed from foetal and late passage fibroblasts. The iPSC-derived cardiomyocytes are referred to CDI or IMRs. Later in the chapter, a comprehensive anatomical and physiological comparison of chamber-specific fibroblasts (from the same heart) based EHT construct is made. These chamber specific EHTs are further compared with late passage fibroblasts, dilated cardiomyopathy fibroblasts, foetal fibroblasts and cardiomyocyte-only containing EHTs.

4.3 METHODOLOGY

EHTs were constructed as per Samata's protocol (Schaaf et al., 2011) (Breckwoldt et al., 2017). The composition and reagents employed to develop EHT medium for bathing EHT constructs were also as per protocol. Each construct contained a standardised and pre-tested ratio of cardiomyocytes and fibroblasts mixed with fibrin and thrombin.

$$\text{Cellular Composition of EHTs} = \frac{(\text{Cardiomyocytes}) 5 \times 10^5 + (\text{Fibroblasts}) 1 \times 10^5}{(\text{Per Construct})}$$

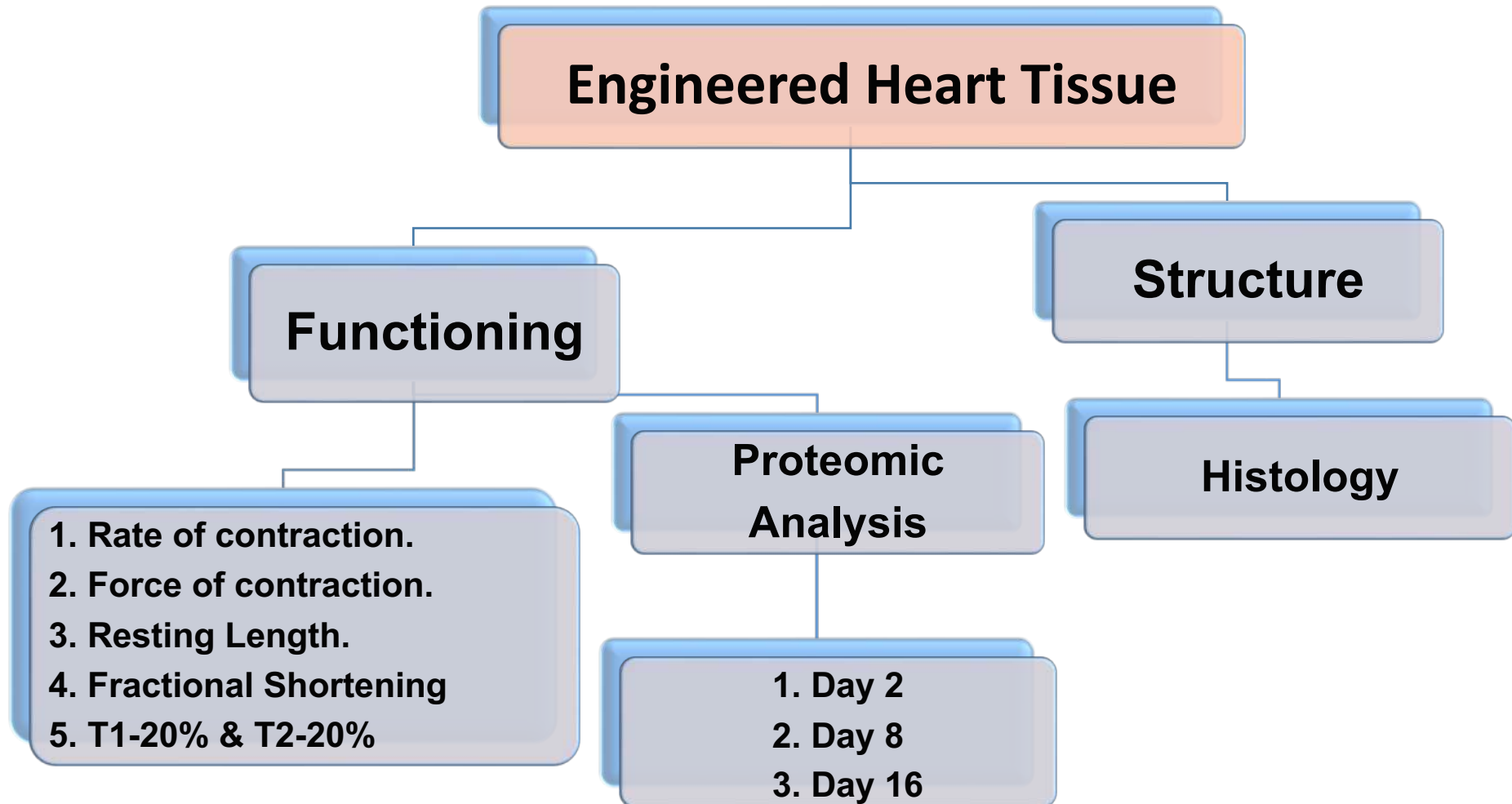
To maintain a consistency in the experiment, all EHTs in a series were constructed on the same day. The incubation setting, bathing medium, change of bathing medium, the temperature of the bathing medium and the environmental conditions during the kinetics recordings of EHTs were uniform and standardised as well (as mentioned in Chapter 2).

To sustain the standardisation, the bathing medium was prepared enbloc and this was changed at a regular time interval, i.e. every 48 hours. The recordings of the kinetics were done simultaneously, 2-3 hours after changing the medium. The temperature was optimised at the time of recordings to 37°C with pre-warming the recording chamber, maintaining 33% humidity and 5% CO₂ was flooded as well.

The video-optic recordings were continuously monitored during the recording period for accuracy. In case of an error in the recording, the specific well was identified and marked for a duplicate kinetics recording at the end of the study

4.4 SCHEME OF ANALYSIS OF EHTs

The Engineered Heart Tissues were analysed based on the following scheme.



4.5 KINETICS OF ENGINEERED HEART TISSUE - PILOT DATA ANALYSIS

4.5.1 FIBROBLAST POPULATION OF PILOT DATA

The selection of this group analysis was based on the previous experiments carried out in our lab (Fig. 1.8. Adapted from ongoing lab work of Prof. Harding, Owen, T) that had elicited a difference in the functional maturation parameters of the EHTs. It was shown that the kinetics of EHTs were improved when fibroblasts were added to the constructs containing cardiomyocytes. Different origin and stages of the passage of fibroblasts altered the functioning of constructs in vitro. The previous models had compared the murine, canine and human foetal fibroblasts population. Their data led to the selection of fibroblast population for this pilot study in which all the fibroblasts were of human origin. The human atrial and ventricular fibroblasts were at P2 stage. The human foetal fibroblasts were at P15 stage while the late passage fibroblasts of ventricular origin were at P6 stage.

The following four human cardiac fibroblast groups were selected for the Engineered Heart Tissue constructs.

1. EHTs with Atrial Fibroblasts.
2. EHTs with Ventricular Fibroblasts.
3. EHTs with Foetal Fibroblasts.
4. EHTs with Late Passage Fibroblasts.

4.5.2 EHT CONSTRUCTS FOR PILOT STUDY

The EHTs were constructed and bathed in the medium as per protocol described in Chapter 2. The EHTs started to contract after a few days. Occasionally, the EHTs detached from the poles, once they began to contract or the rate and force of contraction became stronger over the course of time. Comparison of kinetics was based on the EHTs, consistently contracting over the course of the evaluation period. The kinetics of the EHTs with ceased contractions or those detached from the poles were uncouned in the final analysis.

A total number of 10 EHTs containing atrial, ventricular and foetal fibroblasts were constructed. The number of EHTs from each group that exhibited contractility are plotted in the graph (Fig. 4.3). In the atrial and ventricular group, 9 out of 10 EHT constructs started beating. In foetal fibroblast based EHT group, 8 out of 10 EHTs were beating. In late passage group, all 5 EHTs started to beat initially, but on day 1 after the construction, one of the constructs detached from the silicon poles at one end and therefore, was excluded from the study. Having separated from one pole, the constructs do show contractility with a uniform beating pattern on the graph, but the force of contraction cannot be measured. It was not possible to reconnect the detached end of the EHT back to the pole as constructs shrink immediately and break in the middle (narrowest point of EHT) when they were stretched to bring them closer to the detached pole.

An attempt was made to electrically stimulate the non-contractile EHTs (2 from foetal fibroblasts and one each from the atrial and ventricular group) for three consecutive days, but there was no response, hence they were discarded on day 9 and were uncouned in the final calculations.

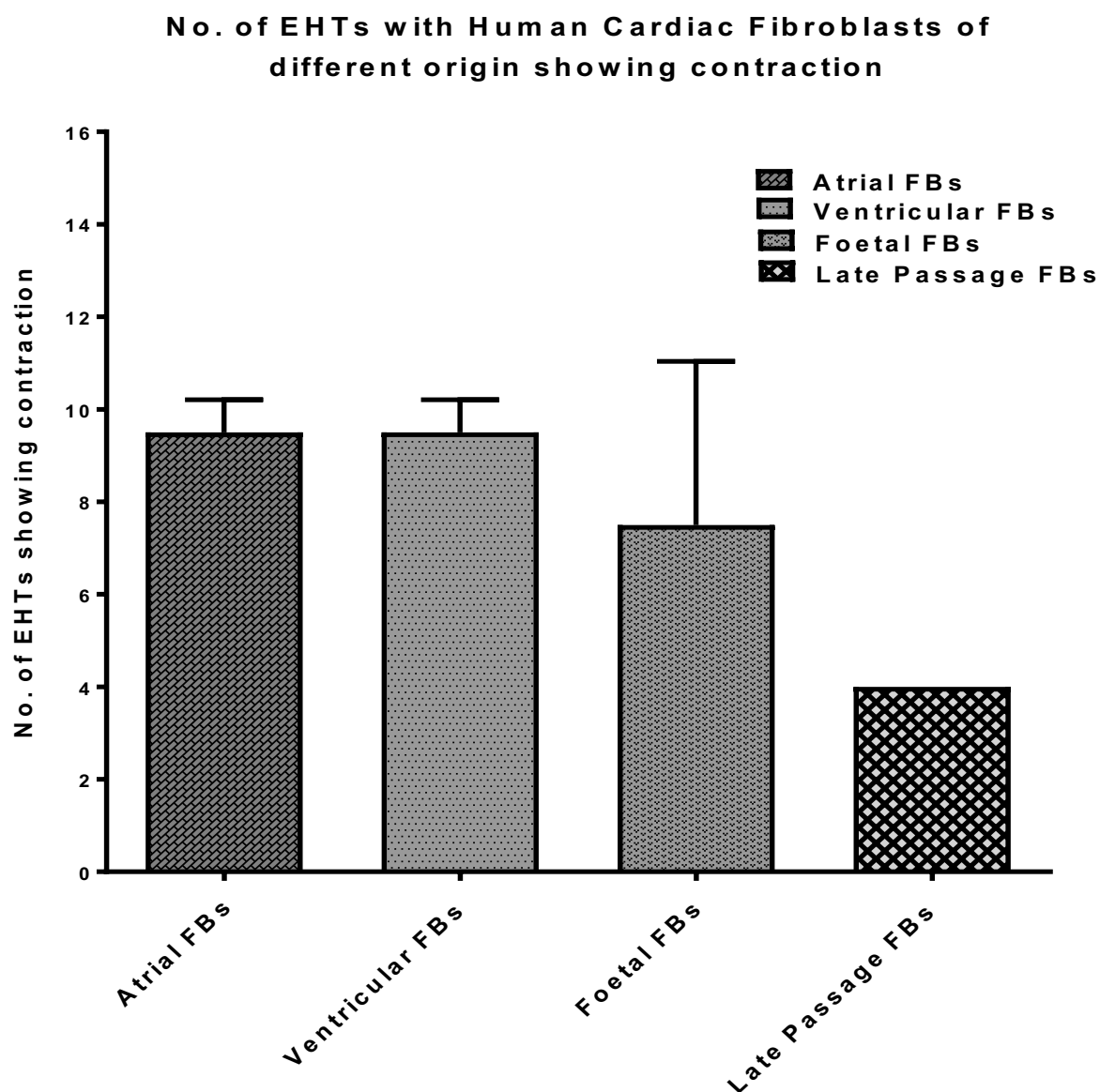


Fig. 4.2. NUMBER OF EHT CONSTRUCTS WITH HUMAN CARDIAC FIBROBLASTS OF DIFFERENT ORIGIN SHOWING CONTRACTION.

Atrial fibroblasts (n=9), Foetal fibroblasts (n=8), Ventricular fibroblasts (n=9) and Late passage fibroblasts (n=4).

One-way ANOVA showed no statistically significant difference between the groups.

Error bars: Mean +/- SEM

A comparative plot of the EHT constructs with different fibroblast population is shown. EHTs with consistent contractile function throughout observation period were considered at the time of final calculation. Non-contractile and detached EHTs were unaccounted.

4.5.3 CHRONOTROPIC RESPONSE OF EHTs WITH FIBROBLASTS OF DIFFERENT ORIGIN – PILOT DATA

The rate of contraction of EHTs was compared between the selected groups, once the constructs start to beat within 2-4 days of construction. Among different groups, the rate of contraction was variable on different days. Like the native cardiac tissue, the rate of contraction of EHTs displayed a direct impact on the force of contraction as the mechanical activity is preceded by the electrical activity. The rate of contraction has a huge impact on the physiological maturation of EHTs. It had been shown that improved and unremitting rate of contraction leads to better tissue maturation. Therefore, subjecting the EHT constructs to fixed pacing showed not only 7-fold improved inotropic response as compared to un-paced constructs but also induced better sarcomeric gene expression (Radisic et al., 2004).

An important physiological phenomenon of Force-Frequency Response (FFR) is exhibited by all normal mammalian hearts. The generation of FFR is dependent on the maturation of the cardiomyocytes and is positive in a normally functioning heart while the failing heart loses the ability to demonstrate it. The human embryonic heart starts beating at three weeks of gestation at a rate of 80bpm and this rate increases incrementally to 180 bpm at nine weeks of gestation (DuBose, Cunyus and Johnson, 1990) (Goenezen, Rennie and Rugonyi, 2013). After nine weeks of gestation until the time of birth, there is a median increase of 0.4bpm (Pildner von Steinburg et al., 2013). The estimated recorded frequency during this phase is between 4Hz – 8Hz. This increase in rate is critical in the development of the chambers and maturation of cardiomyocytes. When the replication of this model in vitro was carried out by stimulating the EHTs at 4Hz, it showed a reduced calcium sensitivity, frequency-dependent acceleration of relaxation, and enhanced post-rest potentiation. At the cellular level, there was enhanced t-tubule formation, improved calcium storage, increased amounts of SERCA2a and RyR2 proteins and release capacity of the sarcoplasmic

reticulum. The physiological chronotropic response of the myocardial tissue generates mechanical activity and hence a normal FFR.

Recently Godier-Furnémont et al. have shown a relationship between the rate of contraction and FFR (Godier-Furnémont et al., 2015). A modulation of chronotropic response is important not only in the embryonic state but to maintain a normal healthy myocardial activity. This pilot study was based on the hypothesis that constructs with fibroblasts of different origin will exhibit different chronotropic response. To study the baseline difference in the rate of contraction of these construct, no fixed electrical stimulation was used at any point.

The chronotropic response of EHTs with fibroblasts of different origin was compared. The atrial fibroblasts were a mixed population of left, and right atrial FBs. Six EHTs were from the RA fibroblasts while four atrial EHT were with from LA fibroblasts. All these fibroblasts were at P4/P5 passage. The ventricular fibroblasts were from the native heart of DCM patient receiving a cardiac transplant and were at the P1 stage. The human foetal fibroblasts were from the successive passaging of initially obtained foetal fibroblasts from Sigma and were at P15. The Late passage fibroblasts of left ventricular origin were at P5. The cardiomyocytes were of CDI cells.

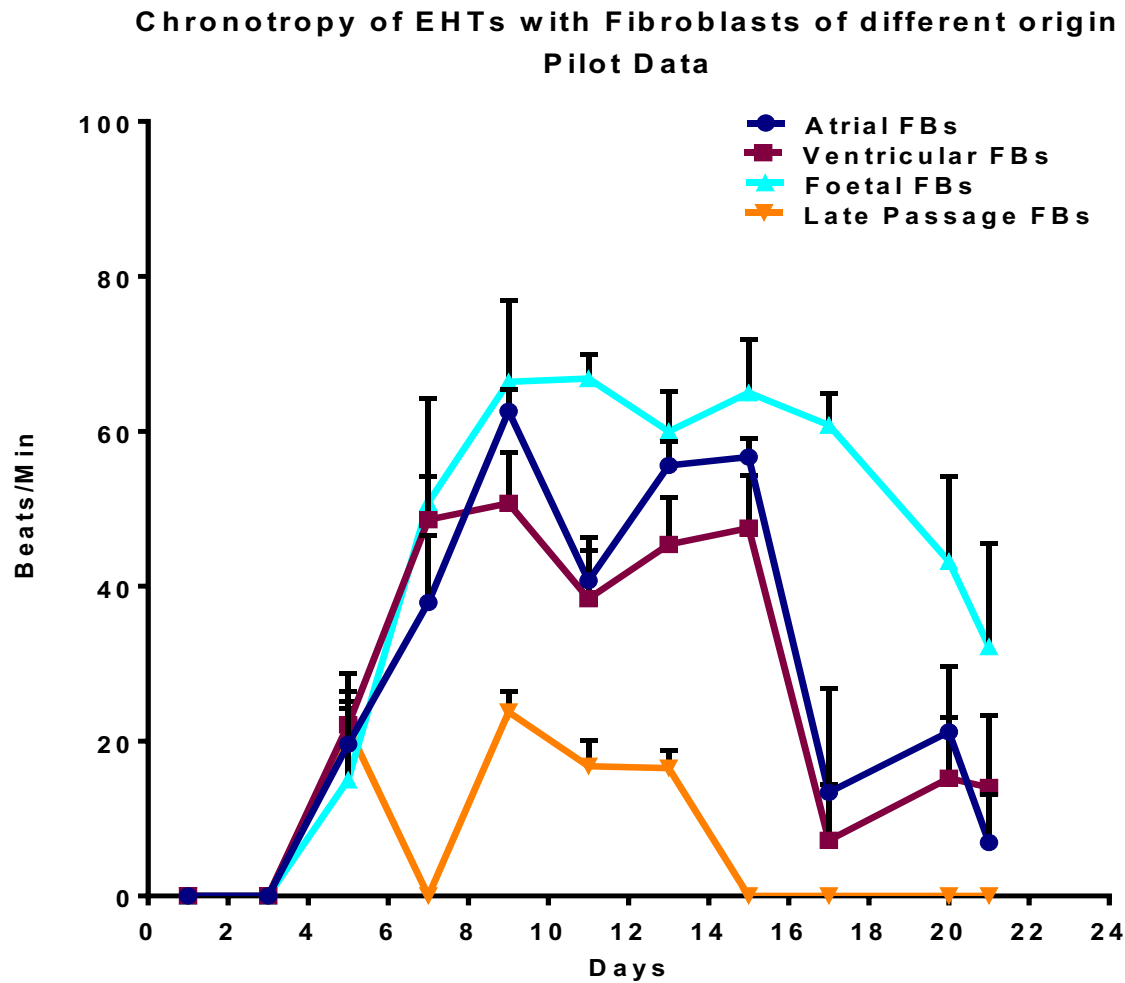


Fig. 4.3. RATE OF CONTRACTION OF EHTs WITH HUMAN CARDIAC FIBROBLASTS OF DIFFERENT ORIGIN. (PILOT DATA)

No. of EHT constructs for each fibroblast group were: atrial fibroblasts (n=9), foetal fibroblasts (n=8), Ventricular fibroblasts (n=9) and late passage fibroblasts (n=4).

Error Bars: MEAN +/- SEM

All groups of EHTs started contracting on day three, but the rate of contracting was variable between the groups. In atrial, ventricular and foetal fibroblast EHTs, the rate of contraction was increasing proportionately until day 8, and after that, the atrial and ventricular groups were showing matching chronotropy, but the foetal fibroblast containing EHTs were beating more vigorously than the atrial and ventricular fibroblast based constructs.

The rate of contraction was maintained until day 15, and afterwards, the rate of contraction started to decline. This decline was steeper and pronounced in EHTs with fibroblasts of atrial and ventricular origin than the foetal fibroblasts laden EHTs in which the curve was smoother and steadier during the same period. By day 21, all constructs were barely showing an unrecordable flickering movement. A MEAN + SEM of each group was plotted on the graph at each time point. (Fig. 4.4) A systematic statistical comparison of the groups using two-way ANOVA with Bonferroni correction showed no statistically significant difference in the rate of contraction of the atrial, ventricular and the foetal fibroblasts based EHTs at any given day (Fig. 4.5, Fig. 4.6). The late passage fibroblast group EHTs although started to contract on the same day as the other groups but the rate was much slower and never matched other groups all through the recording period. There was no contractile activity on day 7, but this was selected again on the very next day. All EHTs in this group stopped contracting on day 15 (Fig. 4.4). Nevertheless, the comparison of ventricular and foetal fibroblasts containing EHTs with the late passage fibroblast based EHTs have shown a statistically significant difference (Fig. 4.6, 4.7). Between day 5 to day 21, the chronotropic response generated by human foetal fibroblasts containing EHTs is three times more than the late passage FBs containing EHTs.

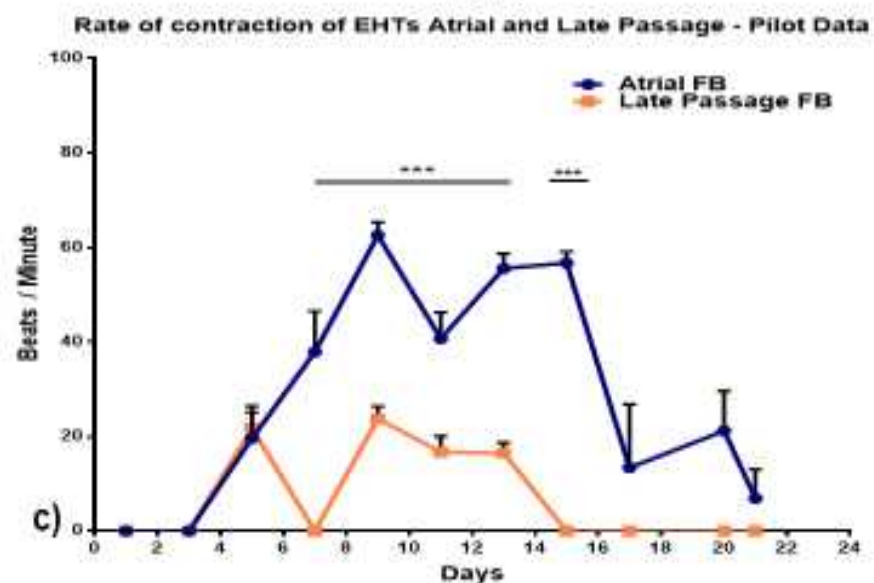
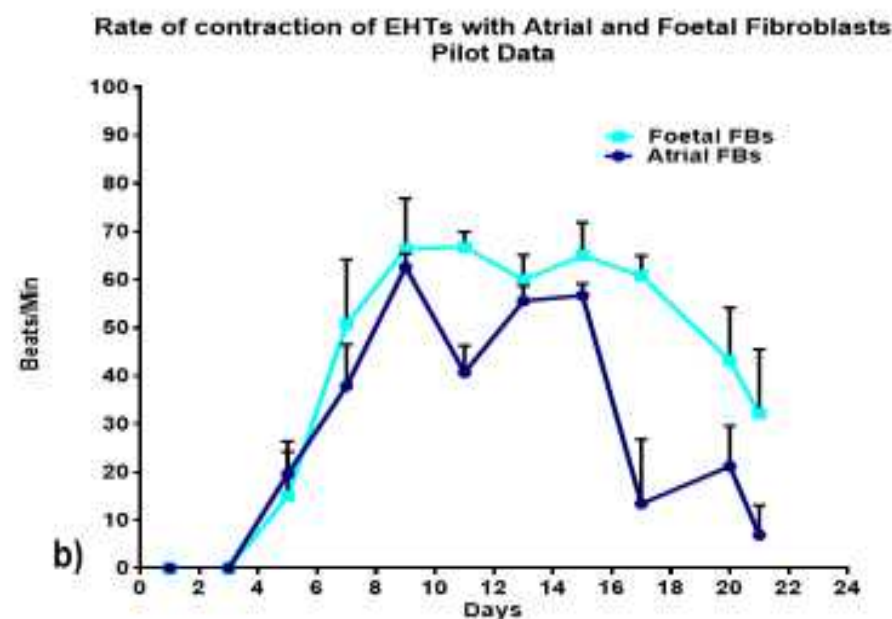
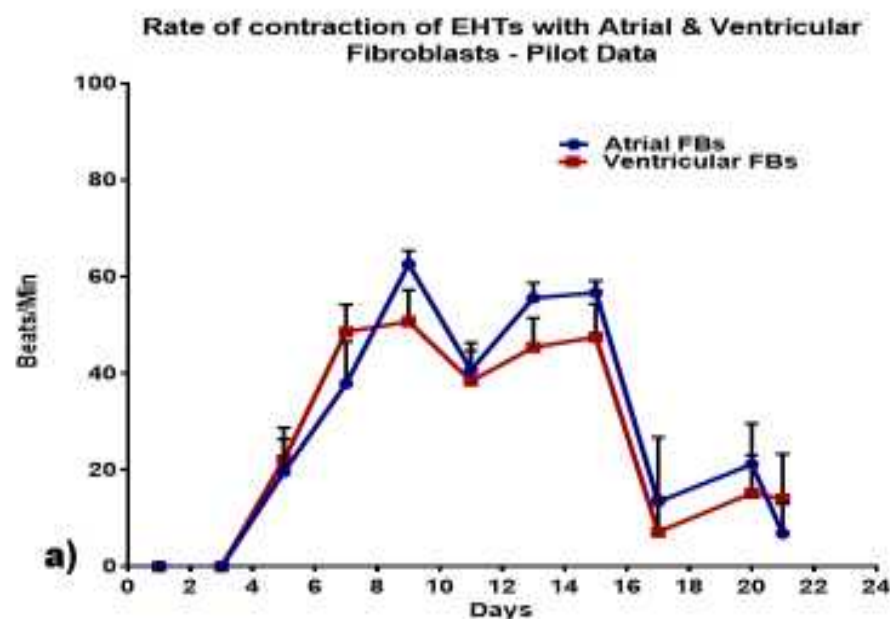


Fig. 4.4. COMPARISON OF RATE OF CONTRACTION OF EHTs WITH ATRIAL FIBROBLASTS AGAINST EHTs WITH VENTRICULAR, FOETAL AND LATE PASSAGE FIBROBLASTS.

- a) The rate of contraction of EHTs with atrial and LV fibroblasts ($n=10$), show no statistical difference. $P = 0.020208$.
- b) EHTs with atrial and foetal fibroblasts. ($n=10$) $P = 0.020208$.
- c) Chronotropy of EHTs with Atrial ($n=10$) & Late Passage Fibroblasts ($n=4$) depict some difference on day 8 – day12 and then at day 15. $P = 0.000462$

2-way RM ANOVA with Bonferroni correction was used for analysis.

Error bars: MEAN $\pm$ SEM

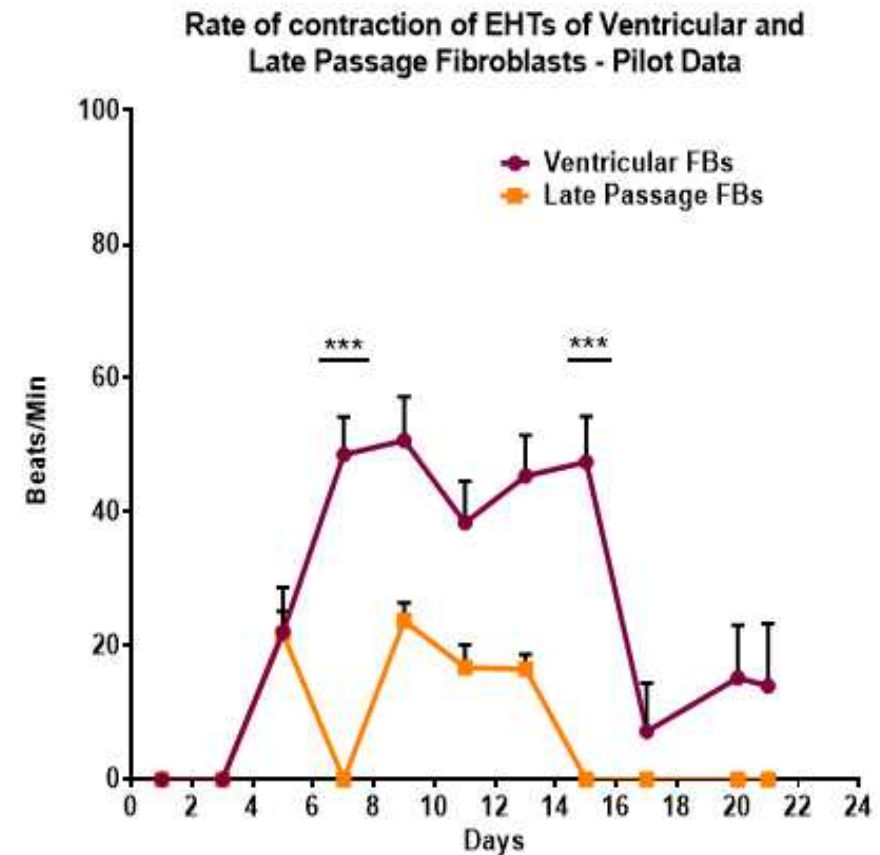
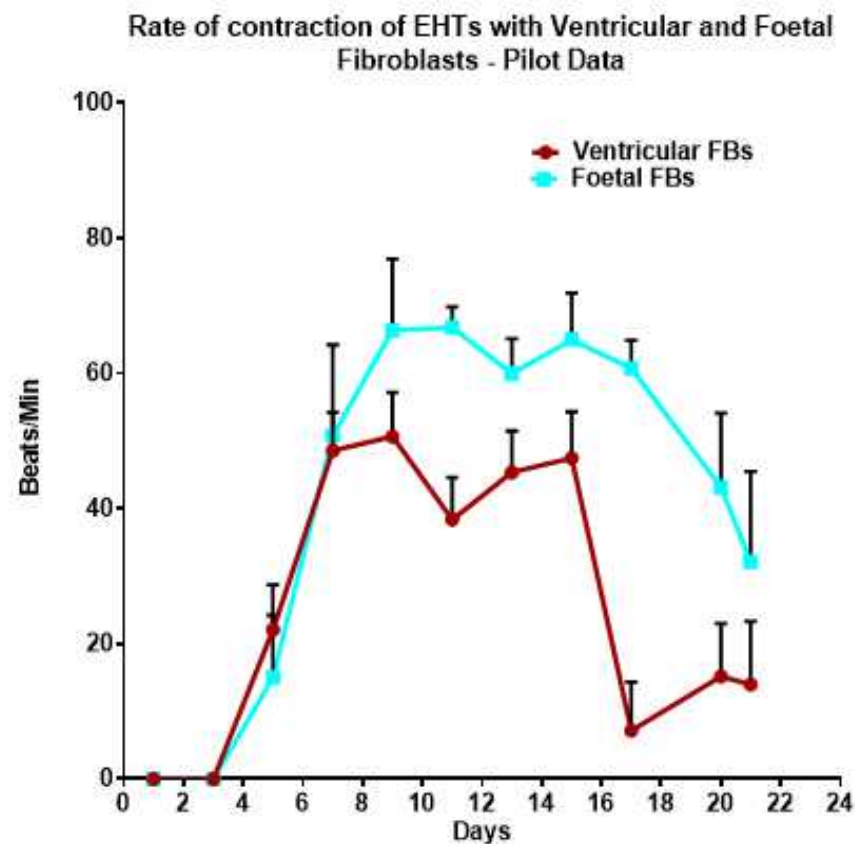


Fig. 4.5. RATE OF CONTRACTION OF EHTs WITH HUMAN VENTRICULAR FIBROBLASTS PLOTTED AGAINST EHTs WITH HUMAN FOETAL AND LATE PASSAGE FIBROBLASTS. (PILOT DATA)

No. of EHT construct for the individual fibroblast groups were: HFF (n= 8), Ventricular (n=9) and Late Passage (n=4).

EHTs with Ventricular and Foetal fibroblasts. **P = 0.5284.**

EHTs with Ventricular and Late Passage fibroblasts. **P = 0.3248.**

2-way RM ANOVA with Bonferroni correction was used for analysis.

Error bars: MEAN +/- SEM

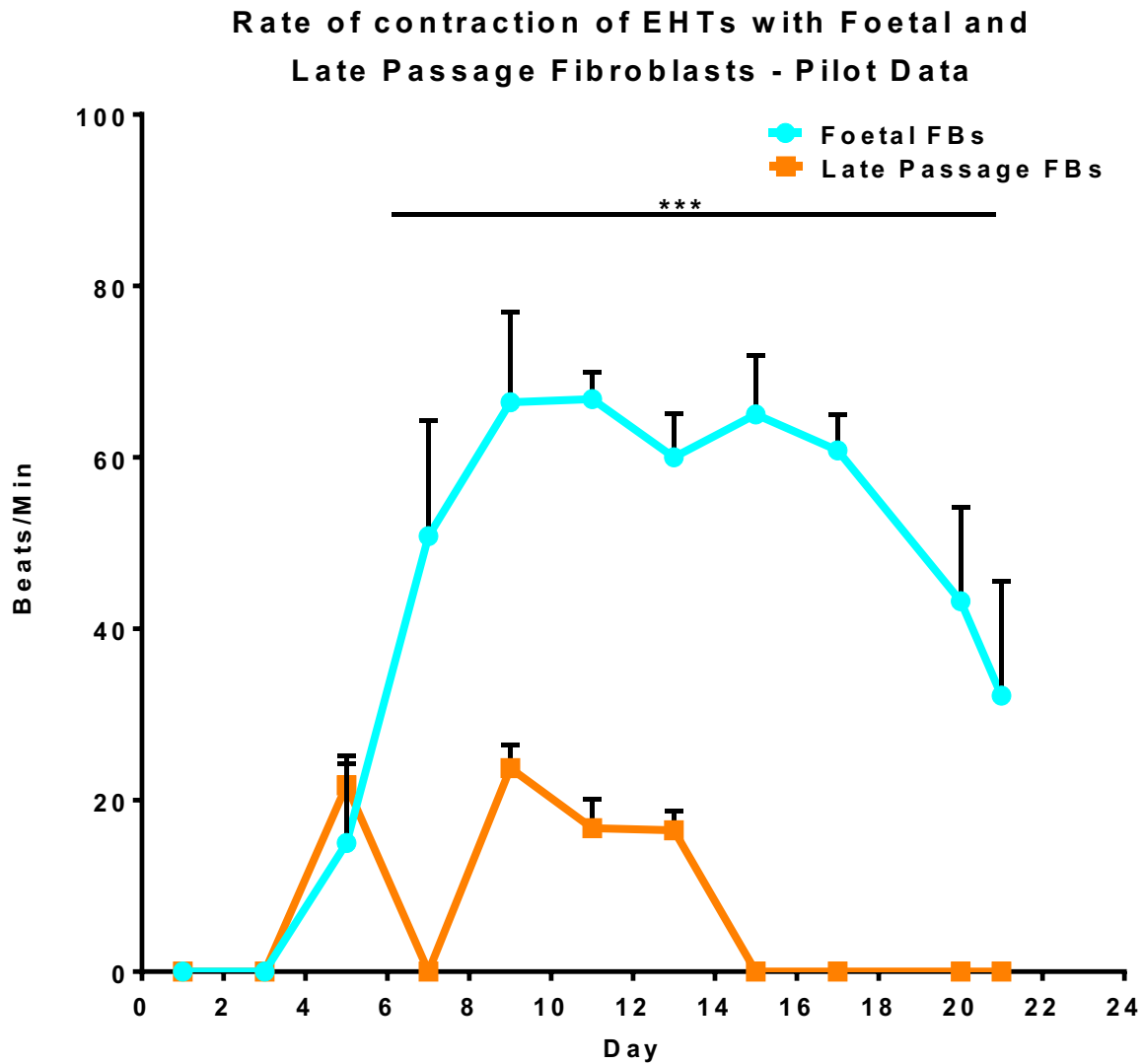


Fig. 4.6. RATE OF CONTRACTION OF EHTS WITH HUMAN FOETAL AND LATE PASSAGE FIBROBLASTS. (PILOT DATA)

No. of EHT constructs for the individual fibroblast groups were: HFF (n= 8) and Late Passage (n=4).

EHTs with Foetal and Late Passage fibroblasts. $p = 0.000251$ (***)

2-way RM ANOVA with Bonferroni correction was used for analysis.

Error bars: MEAN +/- SEM

4.5.4 INOTROPIC RESPONSE OF EHTs WITH FIBROBLASTS OF DIFFERENT ORIGIN – PILOT DATA

The electrical activity of the heart translates into the mechanical force of contraction and within a physiological range, an increase in the rate of contraction increases the force of contraction of myocardial tissue.

The inotropic response of myocardial tissue is governed by force-preload relationship (Frank-Starling law) and the FFR (Bowditch Phenomenon) (Liaw and Zimmermann, 2016). Frank-Starling mechanism explains a directly proportional relationship between the initial length of the myocardial fibres (LVEDP) cardiac output, and within the physiological limits, an increase in LVEDP increases the CO (Weil et al., 1998) (Asnes et al., 2006). The force-frequency relationship also known as Treppe effect, is an increase in the twitch tension in response to the stimulation frequencies. Both phenomena are a hallmark of healthy tissue, and in failing heart, they are blunted or lost altogether (Kobirumaki-Shimozawa et al., 2014).

The force of contraction of the EHTs can be measured with the help of various established models. Three different models are used to explain the force-frequency paradigm, and they include isometric, isotonic and auxotonic contraction models (Zimmermann, 2013). Different protocols are used to make 3D constructs in each model, and a comparison of all three have shown that a flexible loading is physiologically superior to the other two models. However, it is technically challenging to adapt the load to the intrinsic contractile properties of the 3D EHTs to enable auxotonic contraction within the confines of the Frank-Starling law (Zimmermann et al., 2006) (Liaw and Zimmermann, 2016).

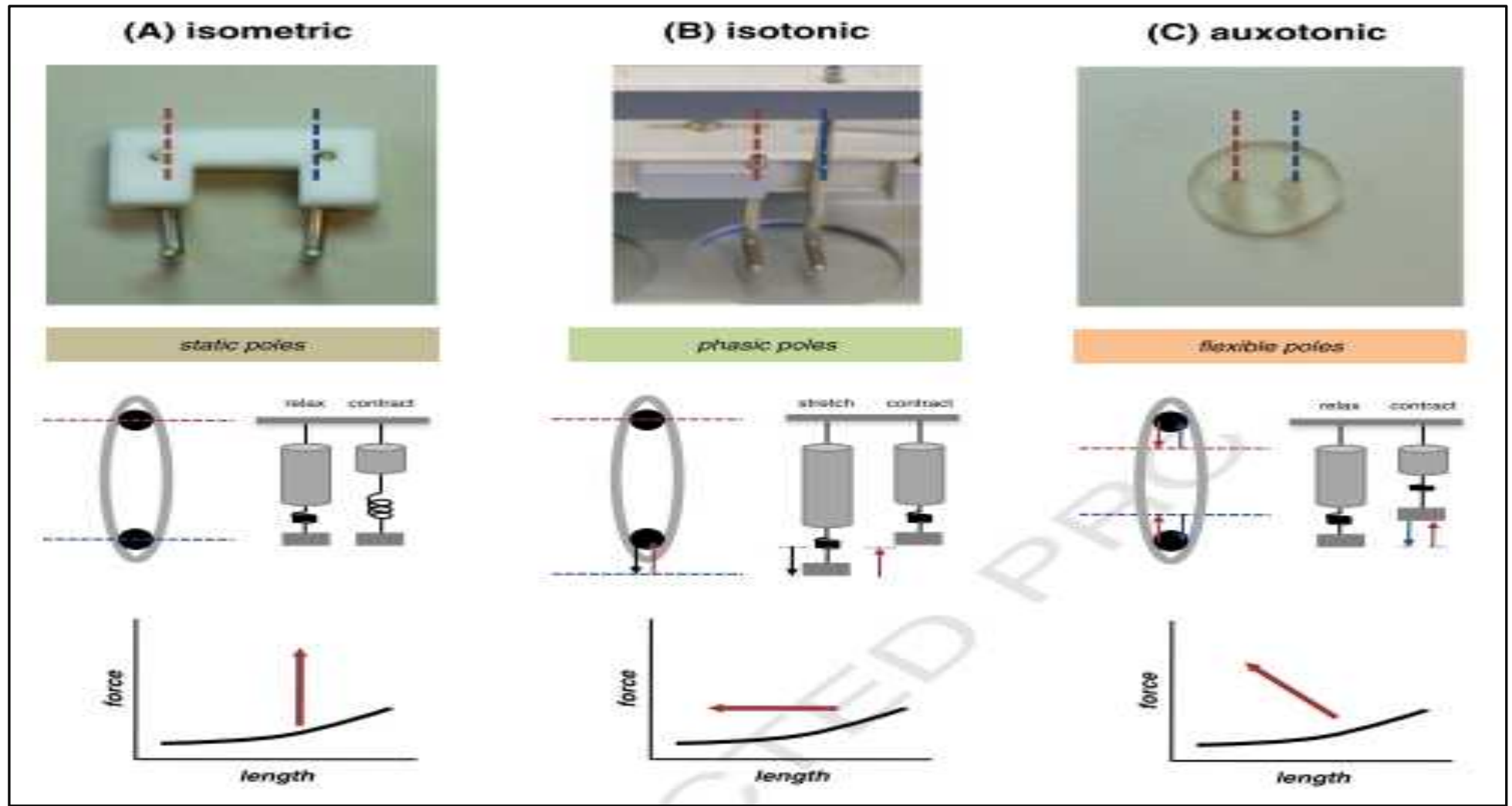


Fig. 4.7. DIFFERENT LOADING PARADIGMS TESTED IN EHTs. (A) Isometric contractions without a change in tissue length can be achieved by suspending EHTs on static holders; (B) isotonic contractions under a phasic reset of EHT length; (C) auxotonic contractions enabled by the suspension of EHT on flexible poles. Deflection of poles is a function of contractile force developed by the EHM.
Adapted from Zimmermann et al., 2015.

In this pilot study, the auxotonic model of loading was used. The EHTs were suspended from the flexible poles of the silicon racks, and the elastic nature of these poles helped to mimic the contraction of a human cardiac syncytium. The force of contraction was measured over the course of time using an optical system capable of counting each deflection of the pole.

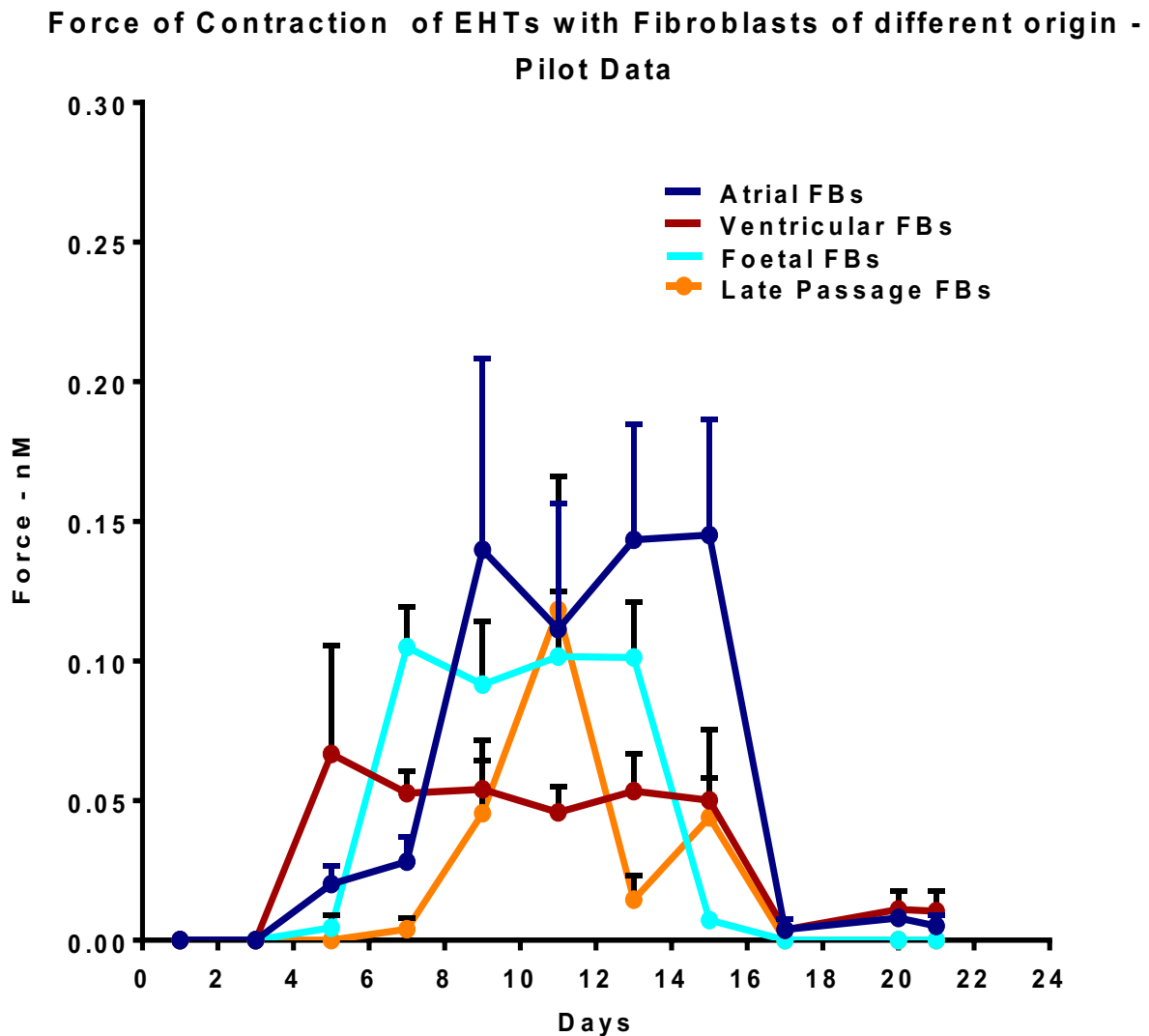


Fig. 4.8. FORCE OF CONTRACTION OF EHTs WITH HUMAN CARDIAC FIBROBLASTS OF DIFFERENT ORIGIN. PILOT DATA

No. of EHT constructs for individual fibroblast group: Atrial fibroblasts (n=9), Foetal fibroblasts (n=8), Ventricular fibroblasts (n=9) and Late passage fibroblasts (n=4).

Error Bars: MEAN +/- SEM

The force of contraction of EHTs with fibroblasts from various groups was measured simultaneously over a period of 60s. Although all constructs started to beat on day 3 in atrial, foetal and late fibroblast based EHT groups, the force of contraction was not recordable. It took an additional two days to pick up the recordable momentum (Day 5). The ventricular fibroblasts containing EHTs were the first to elicit a better recordable force of contraction on day 4. However, no progressive enhancement in the FOC was noted in this group over the course of the next two weeks. In comparison, the FOC of atrial and foetal fibroblasts based EHTs was slow to develop but was stronger with atrial fibroblasts group superseding the foetal fibroblasts group (Fig. 4.9). The FOC of all groups decreased dramatically on day 17, and not much FOC was observed in all EHT groups till day 21.

Although, the chronotropic response of foetal group EHTs was more pronounced than atrial group, the corresponding inotropic response was not marked. Contrary to this the atrial fibroblast group with less vigorous chronotropic response showed a better inotropic response (Fig. 4.4, Fig. 4.5, Fig. 4.9). The foetal fibroblasts loaded EHTs lost their FOC on day 15 before all other groups, and it was observed that the length of constructs decreased as compared to other groups. The late passage fibroblasts were slow to display the FOC until day 7. The FOC gradually picked up between day 9 to day 13 and following that was a sudden steep decline in the force of contraction almost similar to the ventricular fibroblast based EHTs. The FOC decreased significantly in all the groups after day 17. However, a barely minimal (flickering of EHTs with no recordable force generation) contractile response was observed till day 21 (Fig. 4.9). A MEAN + SEM of each group is plotted on the graph at each time point (Fig. 4.9). A systematic statistical comparison of the groups using two way ANOVA with Bonferroni correction showed no statistically significant difference in the force of contraction of the atrial, ventricular, foetal and the late passage fibroblasts containing EHTs at any given day (Fig. 4.10, Fig. 4.11).

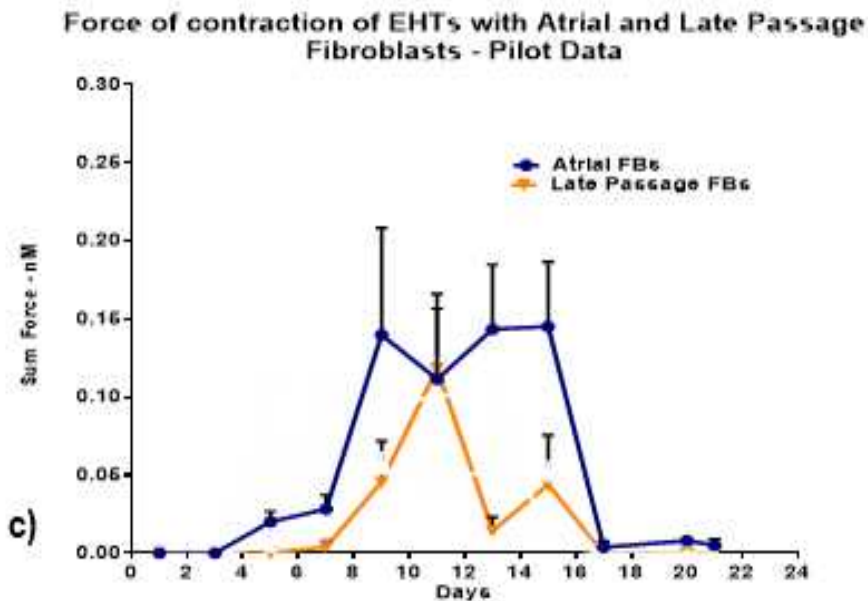
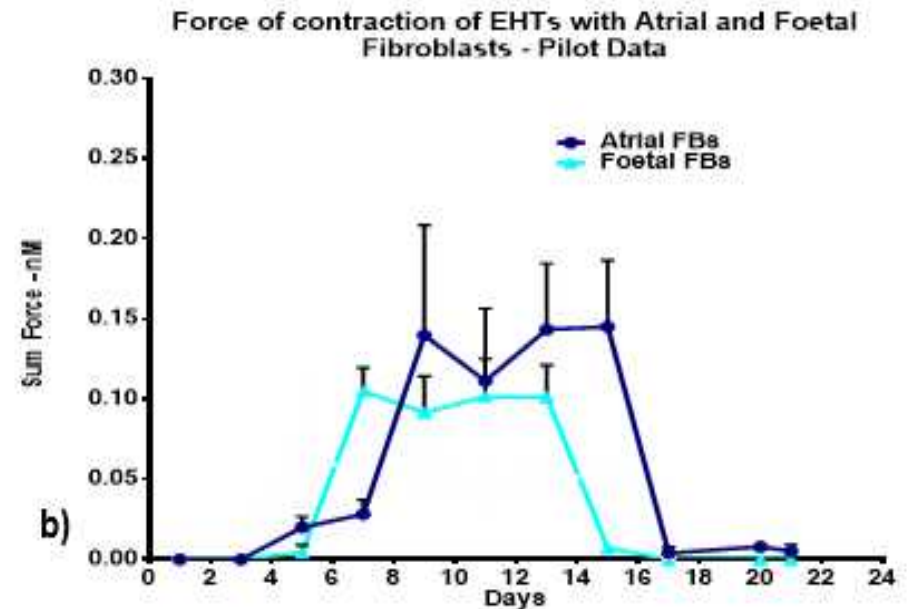
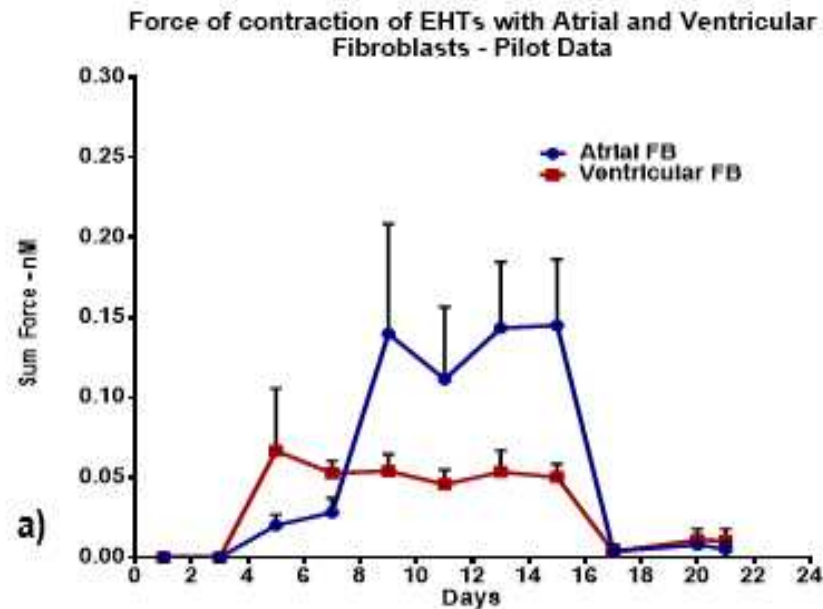


Fig. 4.9. FORCE OF CONTRACTION OF ATRIAL FIBROBLAST BASED EHTS AND OTHER GROUPS. (PILOT DATA)

No. of EHT construct for the individual fibroblast groups were: HFF (n= 8), Ventricular & Atrial (n=9) and Late Passage (n=4).

a). EHTs with Atrial & Ventricular fibroblasts. **P = 0.1283**

b). EHTs with Atrial and foetal fibroblasts. **P = 0.0783.**

c). EHTs with Atrial & Late Passage fibroblasts. **P = 0.1240**

2-way RM ANOVA with Bonferroni correction was used for analysis.

Error bars: MEAN +/- SEM

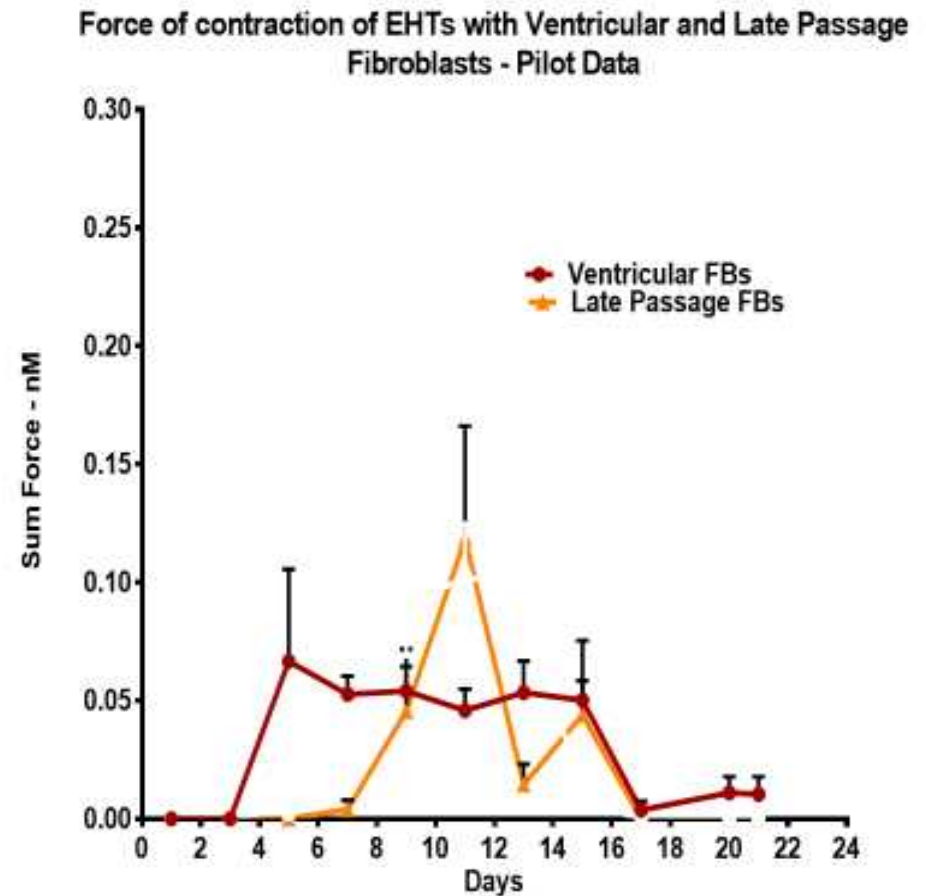
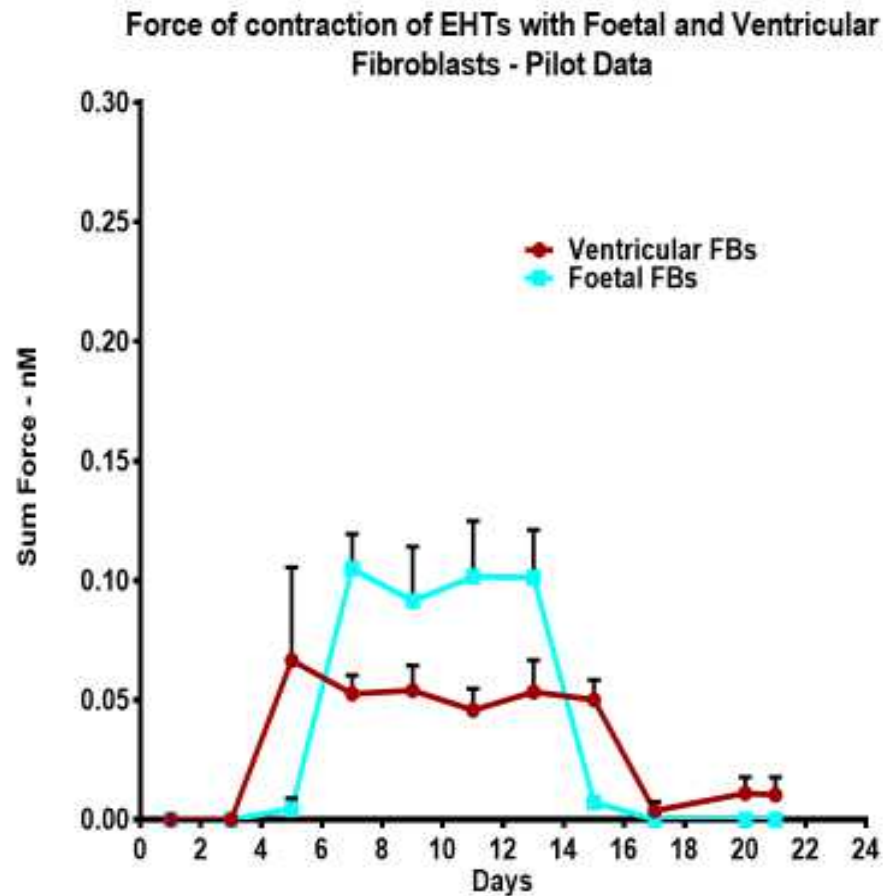


Figure. 4.10. FORCE OF CONTRACTION OF EHTs WITH HUMAN VENTRICULAR, FOETAL AND LATE PASSAGE FIBROBLASTS.

No. of EHT constructs for the individual fibroblast groups: Ventricle (n=9), foetal (n=8) and Late Passage (n=4).

EHTs with Ventricular and Foetal fibroblasts. **P = 0. 5284**

EHTs with Ventricular and Foetal fibroblasts. **P = 0. 3248**

2-way RM ANOVA was used for analysis.

Error bars: MEAN +/- SEM

4.5.5. T1-20% AND T2-20% OF EHTs WITH FIBROBLASTS OF DIFFERENT ORIGIN

T1-20% is a measure of time of contraction of the construct from 20% of contraction to the peak, and T2-20% is a measure of time of relaxation of an EHT construct from peak of contraction to the 80% relaxation.

The comparative plot of T1-20% of EHTs with fibroblasts of different origin reveals that atrial and ventricular fibroblasts show enhanced T1-20% in comparison with the foetal and late passage fibroblasts group. The contractile response of these two groups remained comparable through the course of the recording period. The foetal fibroblasts group of EHTs showed a slow uptake in the first seven days, but then a consistent trend was maintained for the next two weeks and this was better in comparison with the atrial and ventricular group. The constructs with later passage have a more sluggish T1-20% than the other three groups throughout the course, and the response was ceased off after day 16 (Fig. 4.11).

T2-20% comparative analysis showed the phase of better relaxation for atrial and ventricular fibroblasts in comparison with the foetal and late passage fibroblasts group. Foetal fibroblasts containing EHT shows a quick contraction and relaxation and this might be attributed to the faster heart rate than the adult. Beyond a certain point, increased heart rate is inversely proportional to fractional shortening of cardiac syncytium and hence reduces the inotropic response. Therefore, a rapid T1-20% response is followed by relatively faster T2-20%. The late passage fibroblasts group EHTS showed an impaired contractile and relaxation phase (Fig. 4.12).

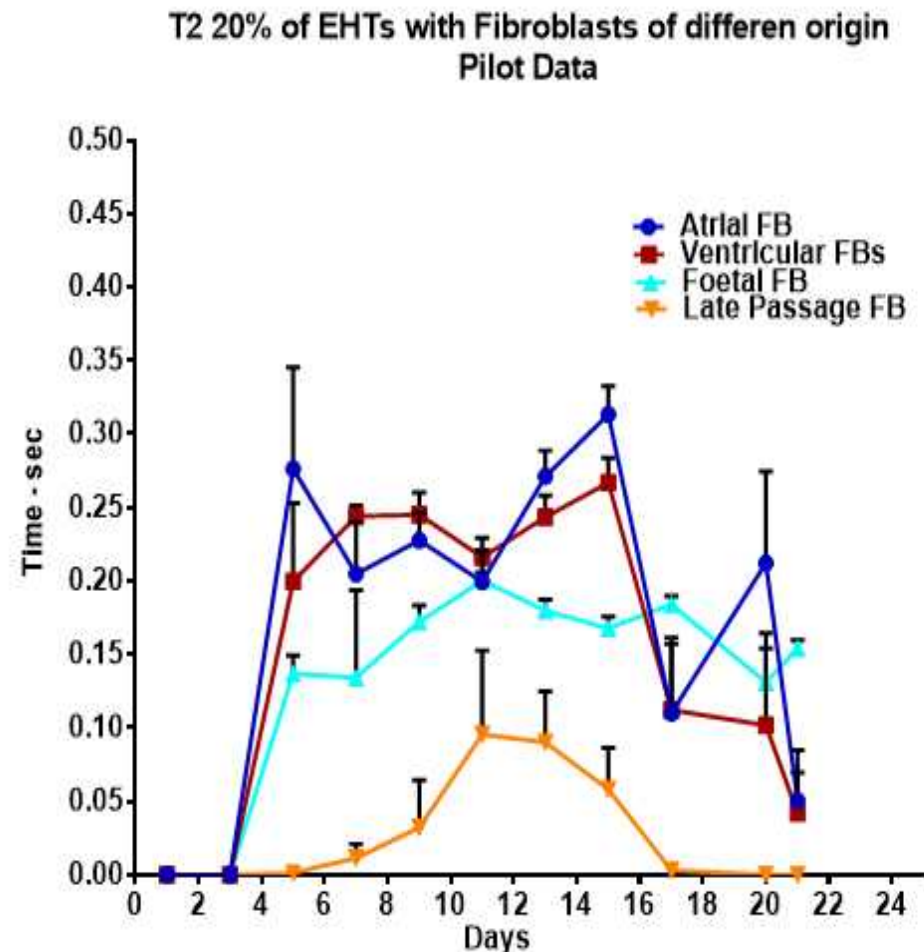
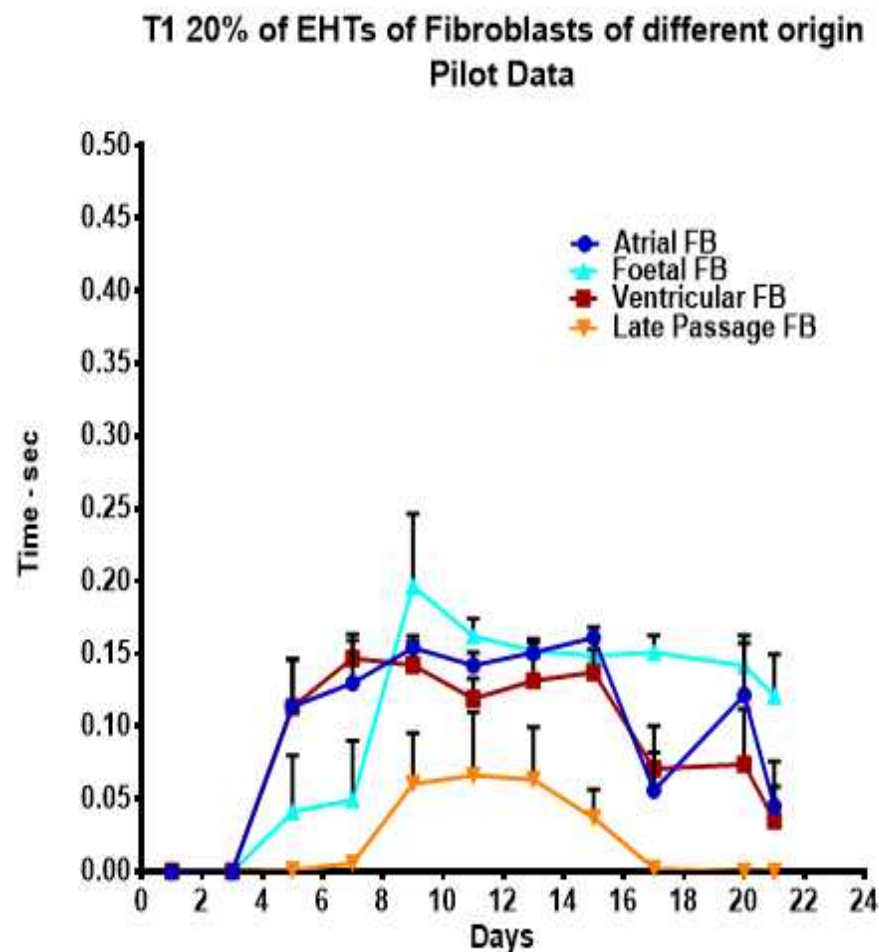


Figure. 4.11. T1-20% & T2-20 OF EHTs WITH ATRIAL, VENTRICULAR, FOETAL AND LATE PASSAGE FIBROBLASTS

No. of EHT constructs for the individual fibroblast groups: Atrial (n=9), Ventricle (n=9), HF (n=8) and Late Passage (n=4).

No statistically significant difference was noted between the groups.

2-way RM ANOVA was used for analysis.

Error Bars: Mean +/- SEM

4.6 KINETIC OF ENGINEERED HEART TISSUE - ANALYSIS FROM ALL FOUR CHAMBERS FIBROBLASTS & COMPARISON WITH OTHER GROUPS

4.6.1 EHTs WITH FIBROBLASTS OF DIFFERENT ORIGIN

Once the analysis of pilot data was finished, a more elaborative comparison of the EHTs with fibroblasts from all four chambers was made (Group 1-4 below). Another four groups were chosen for the comparative analysis (Group 5-8 below). For four chamber samples, fibroblasts were harvested from all four chambers from the patient with the normal LV function. The human foetal fibroblasts at P2 stage were from SIGMA. The DCM fibroblasts used at P2 stage were of ventricular origin. For fibroblast only group, right atrial fibroblasts at P2 stage were used. To maintain the uniformity of analysis all fibroblasts group included in the study (except late passage fibroblasts) were at the P2 stage of differentiation. The IMR-CMs (cardiomyocytes were iPSCs derived by induction of human pulmonary fibroblasts) were at day-18 of differentiation stage. The late passage fibroblasts at P6 stage were of ventricular origin (harvested from the native heart of transplant recipients). The cell count in the constructs was optimised.

The Engineered Heart Tissue were constructed with fibroblasts of different origin:

1. EHTs with Left Atrial Fibroblasts.
2. EHTs with Right Atrial Fibroblasts.
3. EHTs with Left Ventricular Fibroblasts.
4. EHTs with Right Ventricular Fibroblasts.
5. EHTs with Human Foetal Fibroblasts.
6. EHTs with DCM ventricular Fibroblasts.
7. EHTs with only Cardiomyocytes.
8. EHTs with only Fibroblasts.

4.6.2 CELL COUNT OF CONSTITUENT CELLS OF EHTs

The generation of an optimal EHT is constituent cell count dependent. Each EHT construct required 5×10^5 hiPSC-CMs and 1.5×10^5 fibroblasts. The EHTs are constructed based on the assumption that all the constituent cells are living cells. In the pilot data, the cells were counted with a haemocytometer, but a precise differentiation of the living and dead cells was not possible. Therefore, for the chamber specific and comparative groups data analysis the fibroblasts were counted with the help of Luna Bright Field Cell Counter. The cells are stained with the Trypan blue and analysed in the counter. A comprehensive analysis of the total number, viability, average size and dilution factor correction was available. The addition of a precisely calculated viable cell population in the EHTs was possible, and the non-viable population was adjusted in the final calculation for the number of cells used.

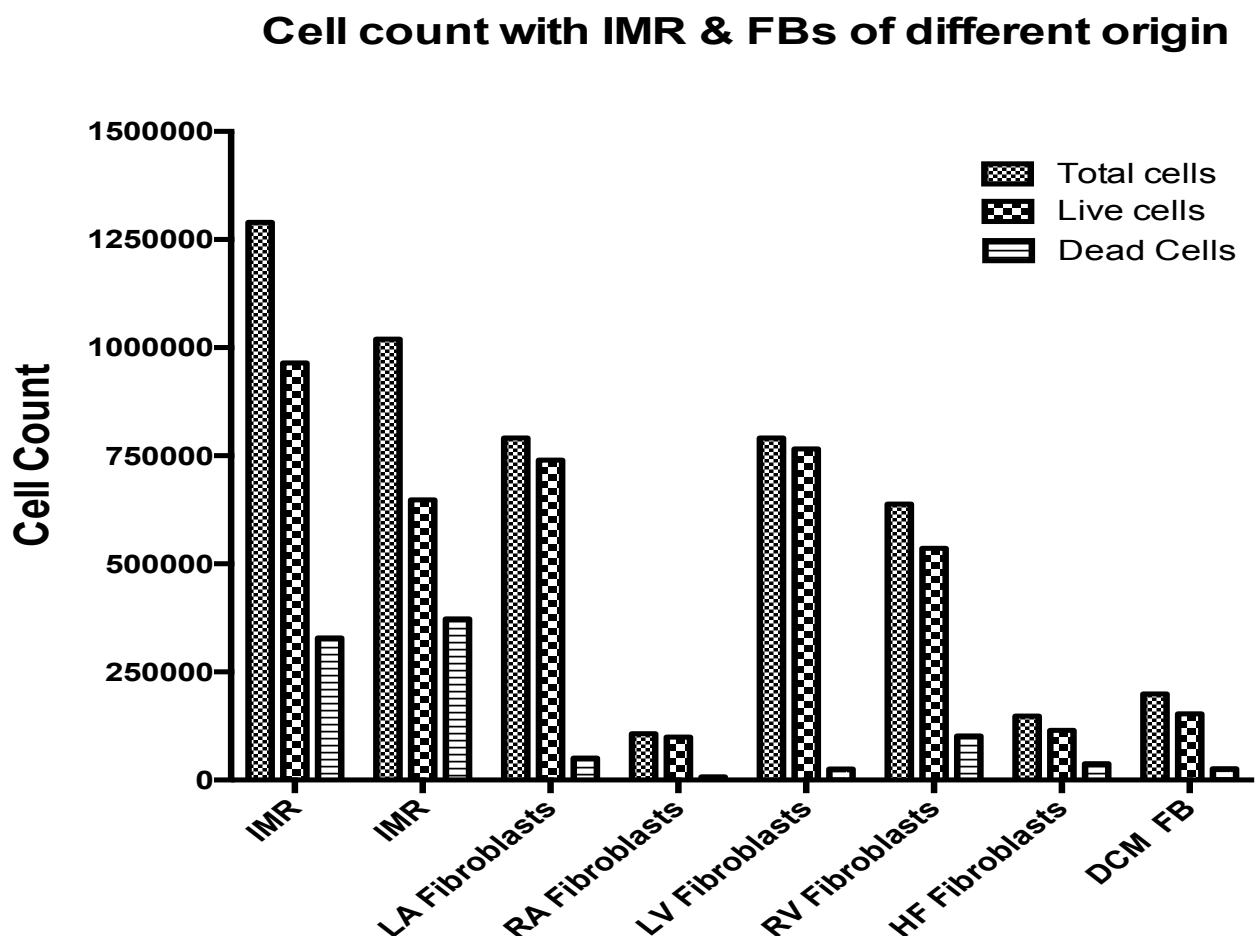


Fig. 4.12. CELL COUNT OF THE CONSTITUENT CELLS OF EHTs

4.6.3. SURFACE AREA OF CONSTITUENT CELL POPULATION OF EHTs

The average area of the cells was counted with bright field cell counter. The average area of the IMR-CMs was between 12-15 μ m. Human foetal fibroblasts revealed a diameter of 23 μ m. The left atrial, right atrial and right ventricular fibroblasts were sharing comparable average area between 25-27 μ m. The size of the left ventricular fibroblasts was about 35 μ m. The most significant diameter of fibroblasts was that of DCM group which was around 40 μ m.

Area of IMR & Fibroblasts

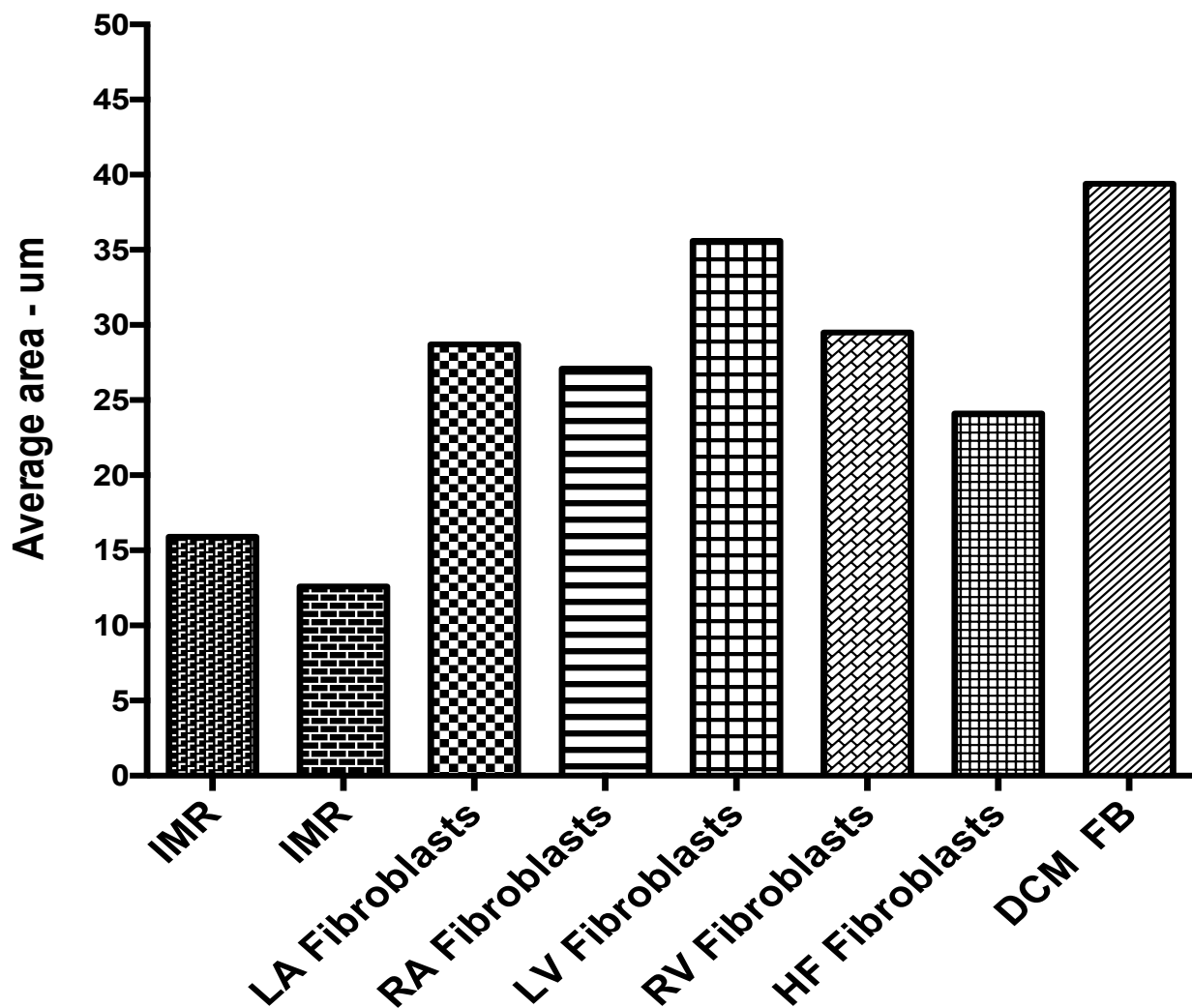


Fig. 4.13. AVERAGE AREA OF THE CONSTITUENT CELLS OF EHTs

4.6.4 CHRONOTROPIC RESPONSE OF EHTs – FINAL DATA

1. CHRONOTROPIC RESPONSE OF EHTs CULTURED WITH FIBROBLASTS FROM ALL FOUR CHAMBERS

The human cardiomyocytes were of IMR origin and were at day-18 of differentiation. 2 different batches of IMR cells were used. The fibroblasts were harvested from all four chambers in the patient with the normal LV function. The four chambers fibroblasts used in this study were from sample no. 7 of all the four chamber biopsies taken from the patients with the normal ventricular group. Due to a very small ventricular tissue biopsy sample size, it was difficult to get enough fibroblasts at the P2 stage for a generation of EHTs. All fibroblast groups were at the P2 stage. Serial recordings of the rate of contraction were taken over three weeks.

The rate of contraction of EHTs generated with fibroblasts of different origin was comparable. Different studies had shown that introduction of fibroblasts in the EHT constructs led to many-fold improved FOC but the rate was affected to a lesser extent. This is demonstrated by the fact that EHTs without fibroblasts (CM only EHTs) are demonstrating nearly the same rate of contraction as EHTs with fibroblasts (Fig. 4.15). The comparison of the rate of contraction of all four chamber fibroblasts based EHTs, demonstrated that the rate of contraction of RV fibroblasts based EHTs superseded other groups. (Fig. 4.16) The trend was followed by EHTs with LA, RA and LV fibroblast. In comparison with the pilot study group where EHT groups started contracting on day three onwards, (Fig. 4.5) the EHTs from all four chamber fibroblasts started to beat on day 1 (i.e. 48hrs earlier than the pilot study). The EHTs with LA fibroblasts started beating on day 2 (Fig. 4.16).

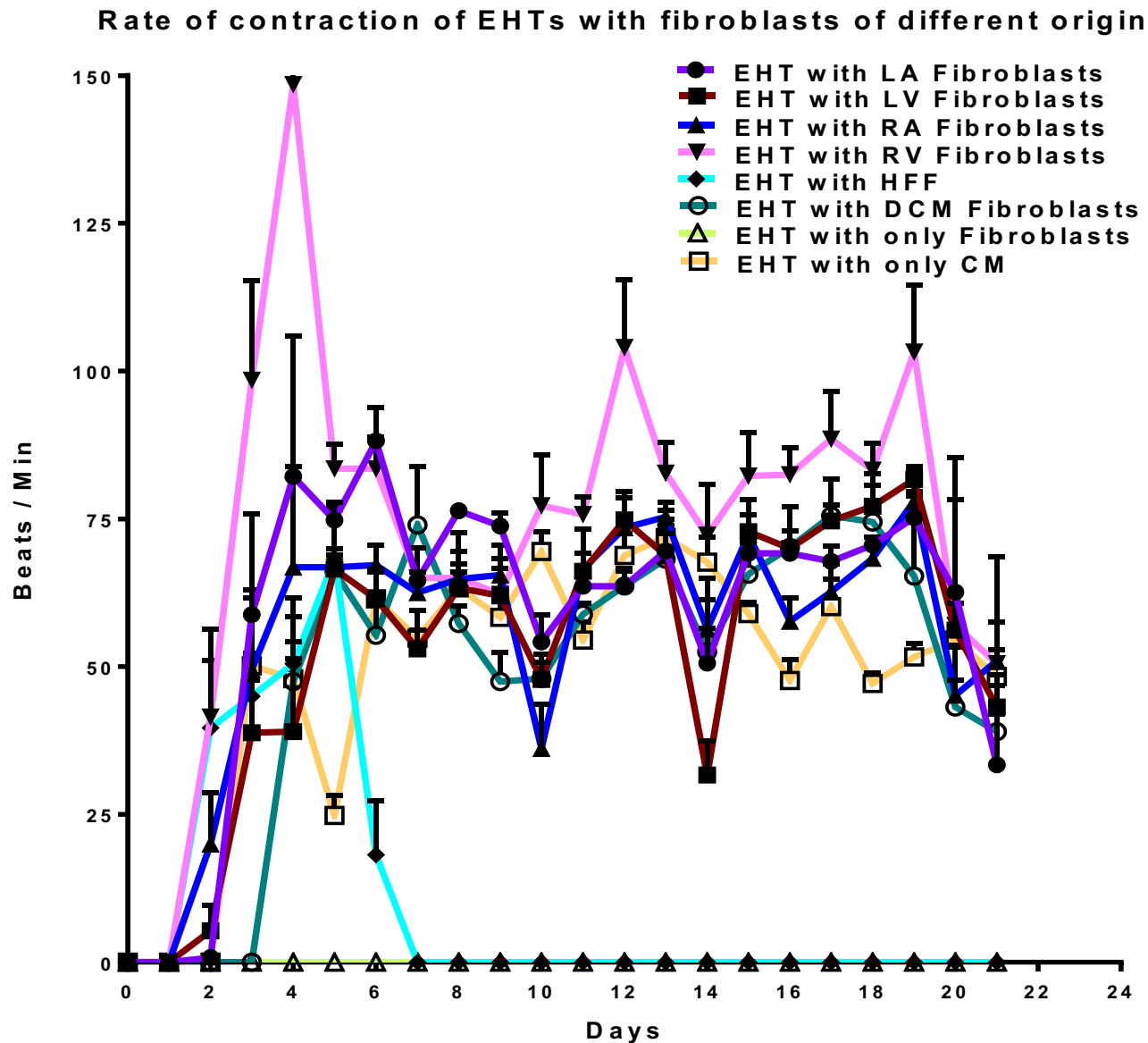


Fig. 4.14. RATE OF CONTRACTION OF EHTs WITH FIBROBLASTS OF DIFFERENT ORIGIN.

All four-chamber fibroblast laden EHTs are compared with the constructs containing other groups as human foetal fibroblasts and dilated cardiomyopathy fibroblasts. The two other groups are EHTs with cardiomyocytes only and fibroblasts only.

No. of EHT constructs for each fibroblast group: LA fibroblasts (n=5), RA & LV fibroblasts (n=6), RV fibroblasts (n=4), HFF fibroblasts, DCM cardiomyocyte only group (n=6) and fibroblast only group (n=3).

Error Bars: MEAN +/- SEM

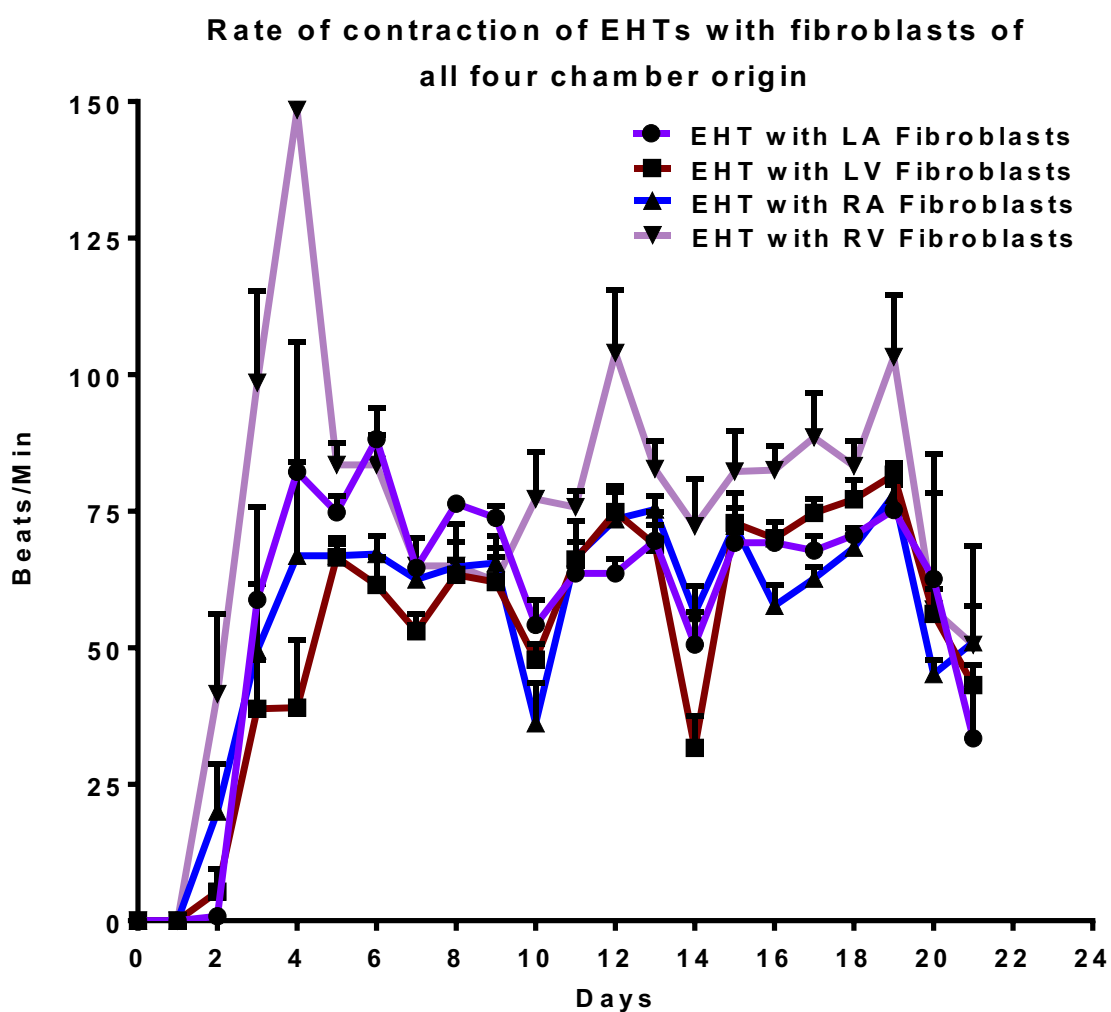


Fig. 4.15. RATE OF CONTRACTION OF EHTs WITH FIBROBLASTS FROM ALL FOUR CHAMBERS.

No. of EHT construct for the individual fibroblast groups were: LA (n= 5), RA (n=6), LV (n=6) and RV (n=4).

Error bars: MEAN +/- SEM

The rate of contraction of LA, LV and RA fibroblast containing EHTs remained essentially between 50-80bpm. The RV fibroblast containing EHTs showed a rate of contraction oscillating between 60-150bpm. The rate of contraction of the atrial fibroblasts was almost similar to each other at all time points and through the contractile period. However, the comparison of the ventricular fibroblasts based EHTs demonstrated the rate of contraction of the RV fibroblast containing EHTs was better than the LV fibroblasts containing EHTs. (P-value = 0.0002) (Fig. 4.17).

The comparison of the left-sided chambers fibroblasts (LA & LV) containing EHTs did not show a significant difference between the chronotropy. However, the comparison of right-sided fibroblasts containing EHTs brought forth the fact that RV fibroblasts laden EHTs exhibit a more vigorous chronotropic response (P-value = 0.0032) (Fig. 4.18).

The comparison of the rate of contraction of LV fibroblasts based EHTs and DCM fibroblasts loaded EHTs does not reveal any significant difference in the rate of contraction (Fig.4.22). This observation may elude to the fact that despite a significant difference between the force of contraction of a normal and failing heart, the rate of contraction of the cardiomyocytes is not affected.

The comparison of the chronotropic effect of EHTs with LV fibroblasts in normal ventricular function patients with EHTs laden with dilated DCM-LV fibroblasts does not show any major difference in chronotropic effect at any time point. The LV function for DCM patient was poor with EF < 20%.

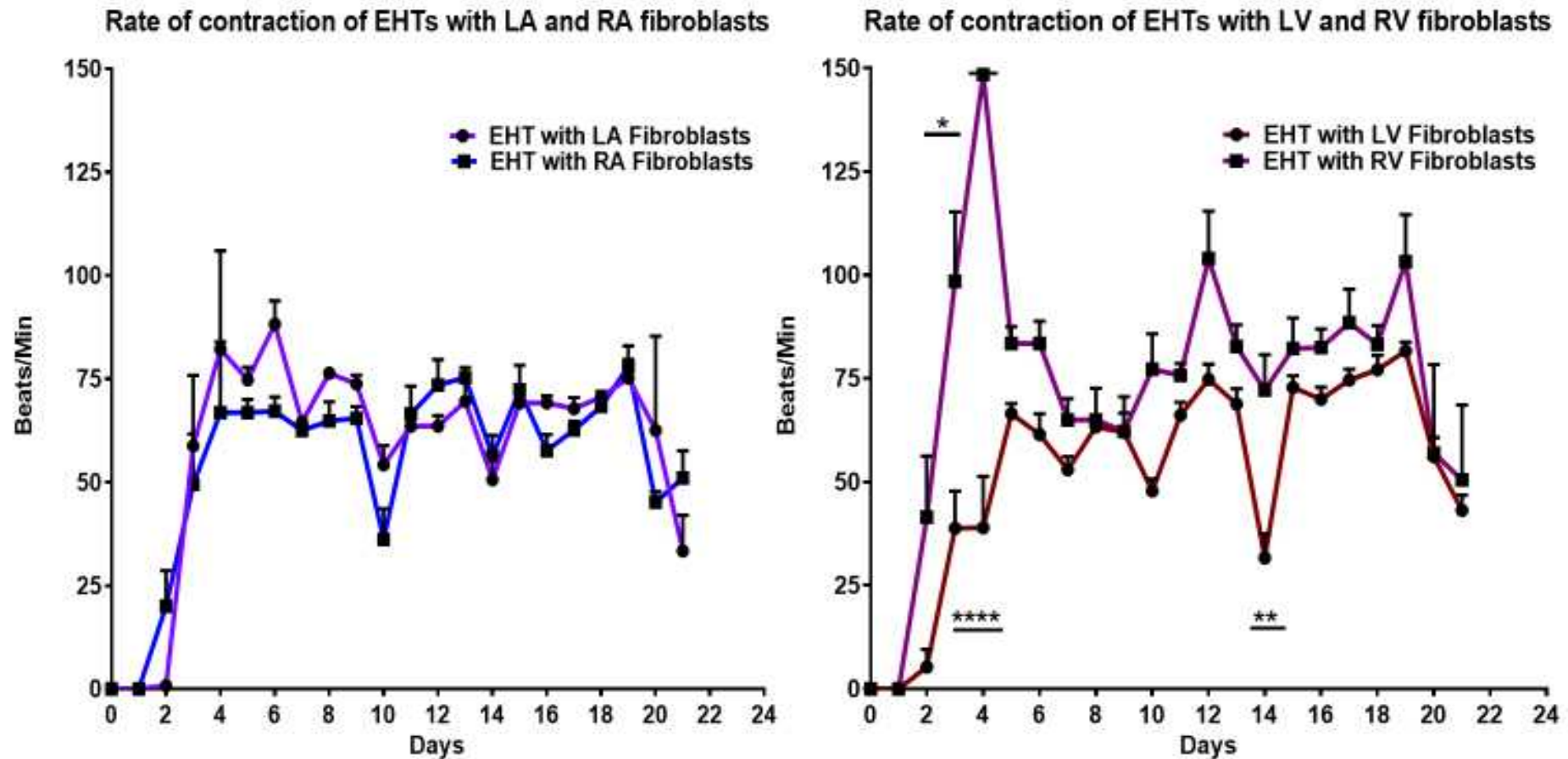


Fig. 4.16. RATE OF CONTRACTION OF EHTs WITH BI-TRIAL AND BI-VENTRICULAR FIBROBLASTS.

No. of EHT construct for the individual fibroblast groups: LA (n= 5), RA (n=6), LV (n=6) and RV (n=4).

EHTs with LA & RA fibroblasts. **P = 0.4491**

EHTs with the LV & RV fibroblast. Statistically significant difference of chronotropic response on day 2 (*), day 3 & 4 (****) and day 13 (**). **P = 0.0002**. 2-way RM ANOVA with Bonferroni correction was used for analysis.

Error bars: MEAN +/- SEM

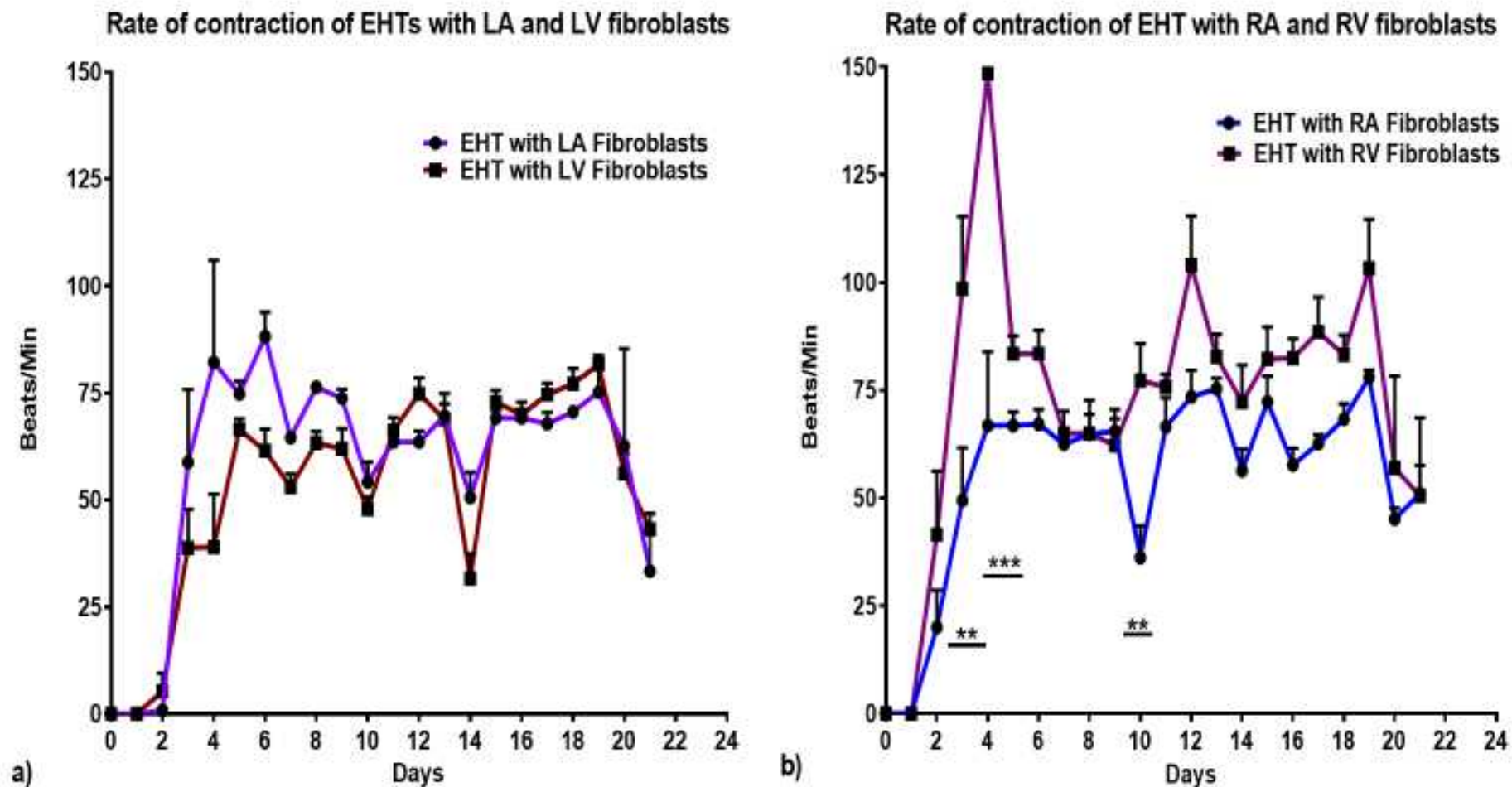


Fig. 4.17. RATE OF CONTRACTION OF EHTs BETWEEN LEFT AND RIGHT SIDED CHAMBERS.

No. of EHT construct for the individual fibroblast groups: LA (n= 5), RA (n=6), LV (n=6) and RV (n=4).

EHTs with LA and LV fibroblasts. **p = 0.0630**

EHTs with RA and RV fibroblasts. Enhanced chronotropic effect of RV fibroblasts on day 3 (**), day 4 (***) & day 10 (**). **P = 0.0032**

2-way RM ANOVA with Bonferroni correction was used for analysis.

Error bars: MEAN +/- SEM

Rate of contraction of EHTs with LV & DCM fibroblasts

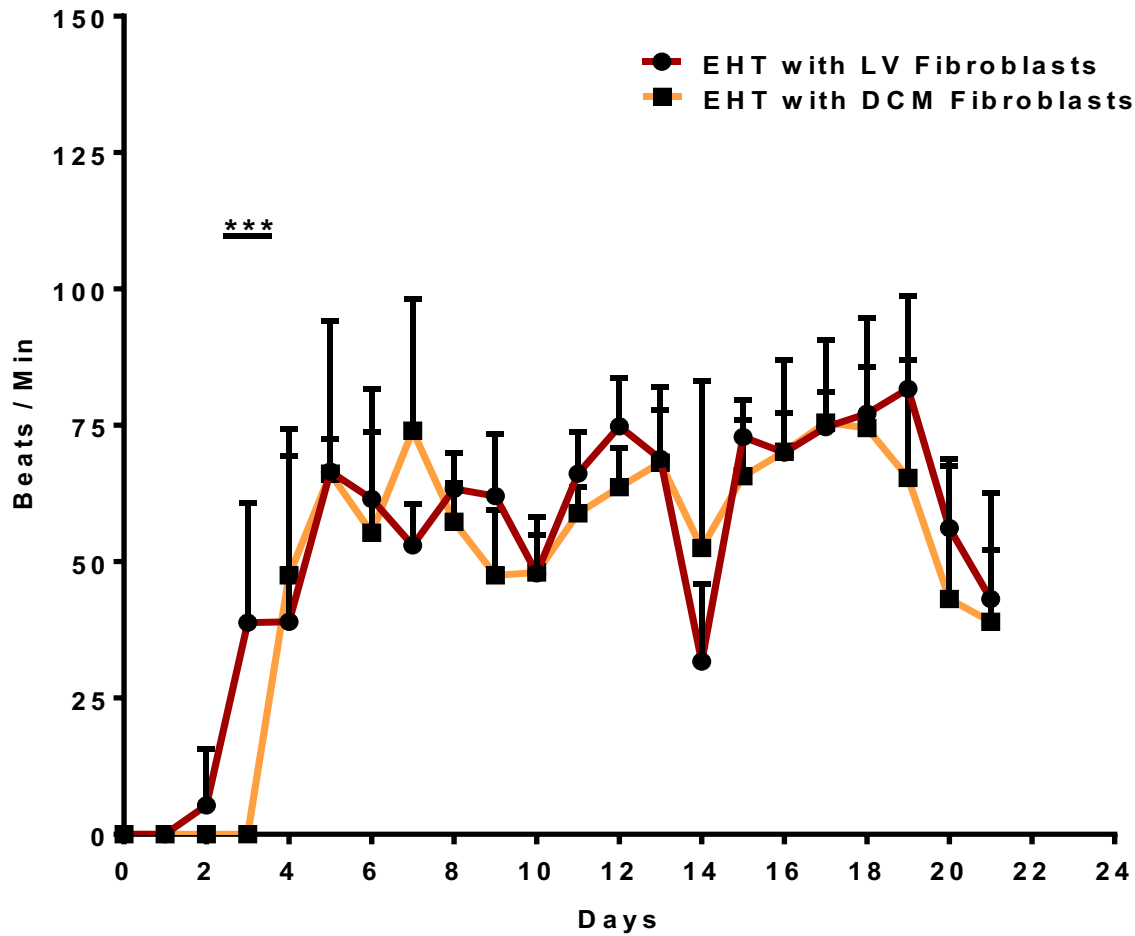


Fig. 4.18. RATE OF CONTRACTION OF EHTs WITH LV AND DCM FIBROBLASTS.

No. of EHT construct for the individual fibroblast groups: DCM (n=6), LV (n=6)

EHTs with DCM and LV fibroblasts only showed a difference of chronotropic effect only on day 3 (***). No statistically significant difference in ROC over the rest of the period. **P = 0.148034**

2-way RM ANOVA with Bonferroni correction was used for analysis.

Error bars: MEAN +/- SEM

2. CHRONOTROPIC RESPONSE OF EHTs CULTURED WITH FIBROBLASTS FROM OTHER GROUPS

The fibroblasts for DCM group were harvested from the native left ventricular myocardial tissue of transplant recipient. The human foetal fibroblasts were purchased from Sigma to maintain the same passage state through the groups. The cardiomyocytes for CMs only EHTs were of IMR origin. For fibroblasts only group the FBs were of RA origin.

The ROC in comparing groups fibroblasts containing EHTs with DCM, foetal fibroblasts and CM only was comparable. The EHTs constructed with only FBs and without any CMs never showed any beating during the observation period. The rate of contraction of foetal fibroblast contains EHTs picked up with the same pace as others from day 1 to day 5. After day 5, there was a sudden significant drop in the rate of contraction with complete cessation of ROC on day 7. No further beatings were recorded in any construct during the next two weeks (Fig. 4.21). A marked shortening of the size of these EHTs was noted, and the silicon poles moved closer to each (Fig. 4.20).

The CMs and DCM containing EHTs started to contract on day 2 & 3 but respectively, and for the rest of the 3-week period their rate of contractions was comparable (50-75bpm). The average beats/minute demonstrated by these two groups was almost the same as all four chambers group (Fig. 4.15, Fig. 4.21). The comparison of ROC of EHTs with foetal fibroblasts against CM only and DCM fibroblasts is significantly different (P -value = 0.0001). This was accentuated by the lack of contraction shown by the HF-EHTS after day 7 (Fig. 4.20). A comparison of FBs only EHTs cannot be made with any other group due to their lack of contractile response.

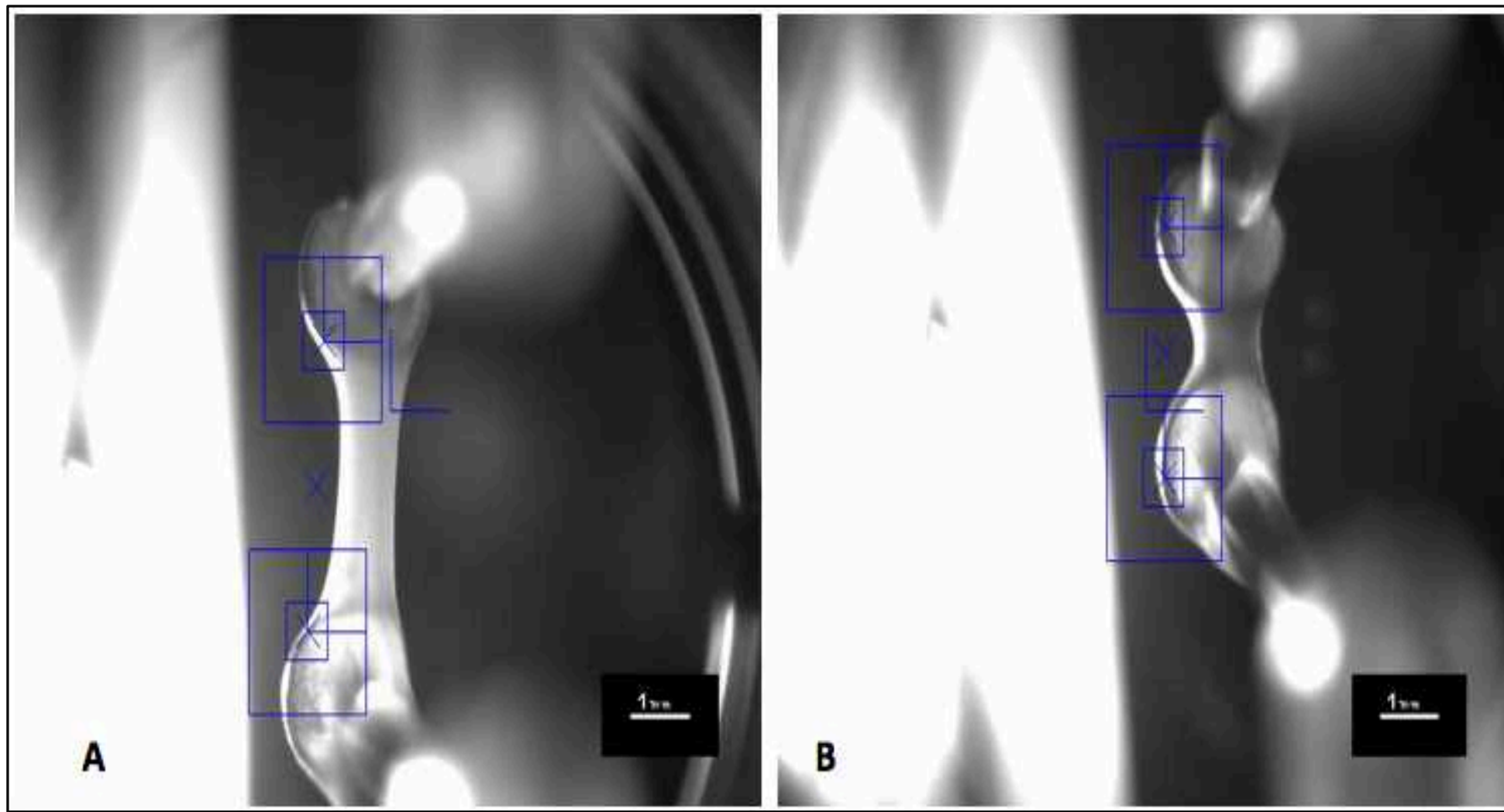


Fig. 4.19. SHORTENING OF THE LENGTH OF FOETAL FIBROBLASTS CONTAINING EHTs. (A) Foetal fibroblasts on day-3 showing the usual length of EHT construct attached to silicon posts can be seen. (B) Marked shortening in the length of EHT on day-7. The silicon poles can be seen pulled towards the middle. No contractile response detected after day-7.

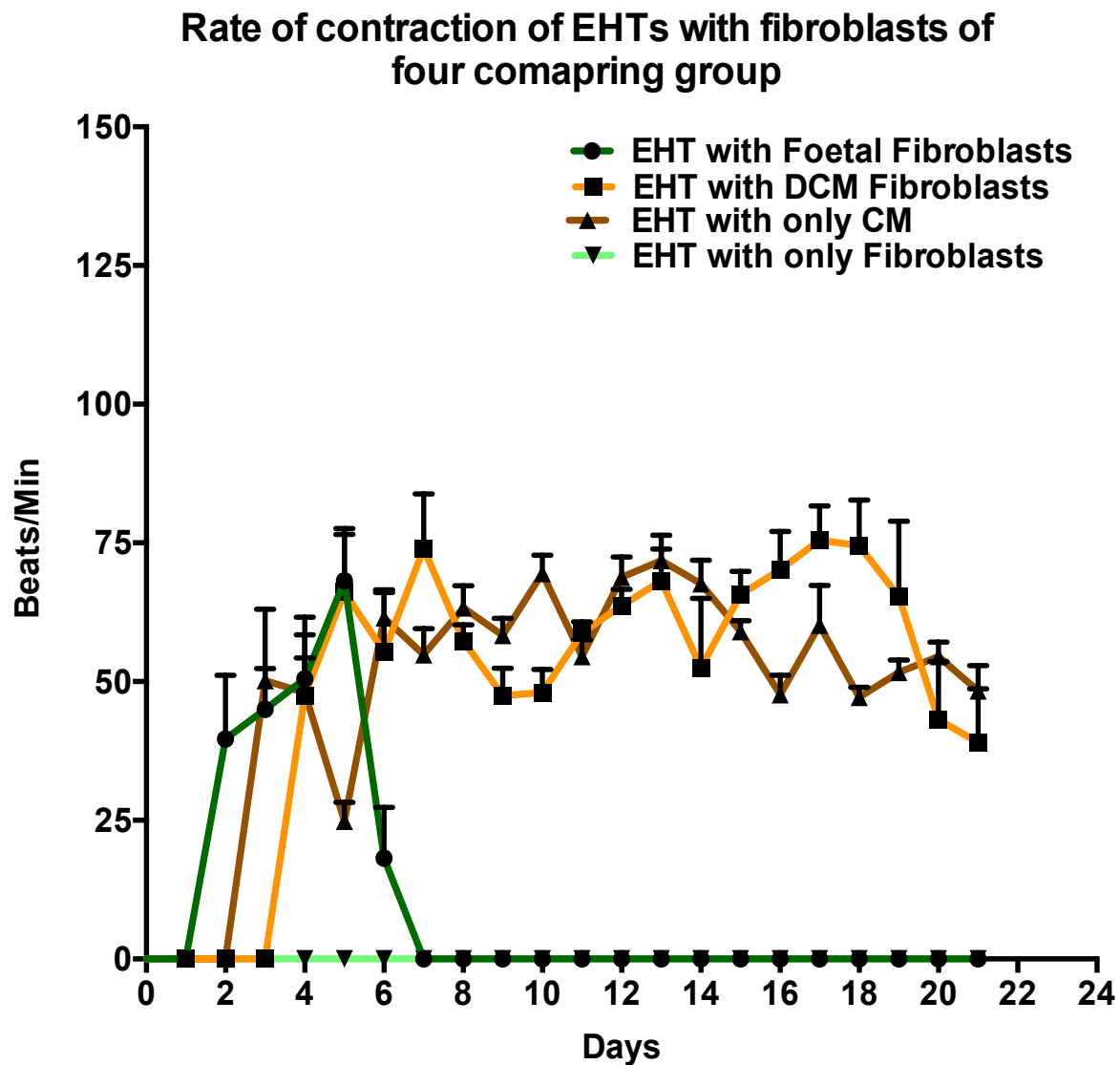


Fig. 4.20. RATE OF CONTRACTION OF EHTs WITH FIBROBLASTS POPULATION FROM FOUR COMPARING GROUPS.

No. of EHT construct for the individual fibroblast groups: FF (n= 6), DCM (n=6), CMs only (n=6) and FBs only (n=3).

EHT with DCM and CM-only Fibroblasts. **P-value = 0.1262**

EHT with DCM and Foetal Fibroblasts. **P-value = 0.0001 (****)**

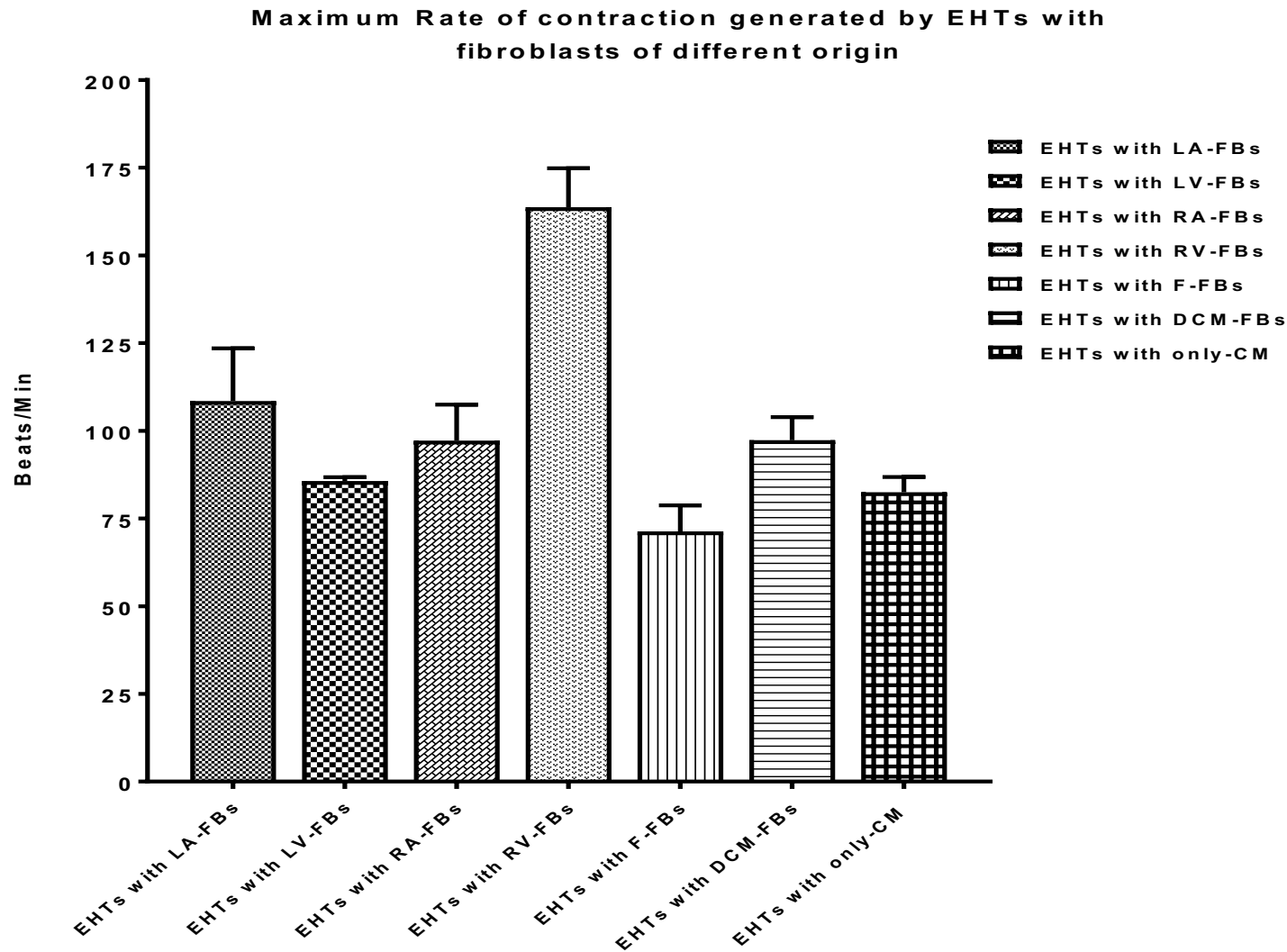
EHT with CM-only and Foetal Fibroblasts. **P-value= 0.0001 (****)**

Error bars: MEAN +/- SEM

3. MAXIMUM CHRONOTROPIC RESPONSE OF EHTs CULTURED WITH FIBROBLASTS OF DIFFERENT ORIGIN

The maximum chronotropic response of EHTs with fibroblasts of different origin was compared. The rate of contraction of RV fibroblasts superseded all other groups, and this was followed by the LA, RA and DCM fibroblasts based EHTs. The maximum chronotropic response generated by LV and CM-only based EHTs was parallel to each other. Overall, the comparison of the mean of maximum chronotropy was significantly different between the groups. One-way ANOVA of peak rate of contraction showed a noteworthy variance of peak rate of contraction (P value = 0.0001) (Fig. 4.22). This variance was further confirmed with Bartlett's test (P value = < 0.0049).

The mean peak rate of contraction showed a further accentuated response among all four chamber fibroblasts based EHTs, with one-way ANOVA showing a significant difference. P value = <0.0003. Tukey's multiple comparisons further highlighted the EHT groups with a difference of peak rate of contraction (Fig. 4.23) A similar difference between the peak ROC was noted when biventricular fibroblasts from normal ventricular function group were compared with the DCM fibroblasts laden EHTs (Fig 4.24)



No. of EHT constructs for each fibroblast group:
 LA fibroblasts (n=5),
 RA & LV fibroblasts (n=6),
 RV fibroblasts (n=4),
 Foetal fibroblasts (FF), DCM, cardiomyocyte-only group (n=6) and fibroblast-only group (n=3).

Error bars: Mean $\pm$ SEM

One-way ANOVA of peak force generated by EHTs.

A significant difference between the means of peak force between groups.
P value = 0.0001 (**).**

Brown Forsythe test.
P value = 0.2554 (ns).

Bartlett's test:
P value = < 0.0049 ()**

Fig. 4.21. MAXIMUM RATE OF CONTRACTION GENERATED BY FIBROBLASTS OF DIFFERENT ORIGIN

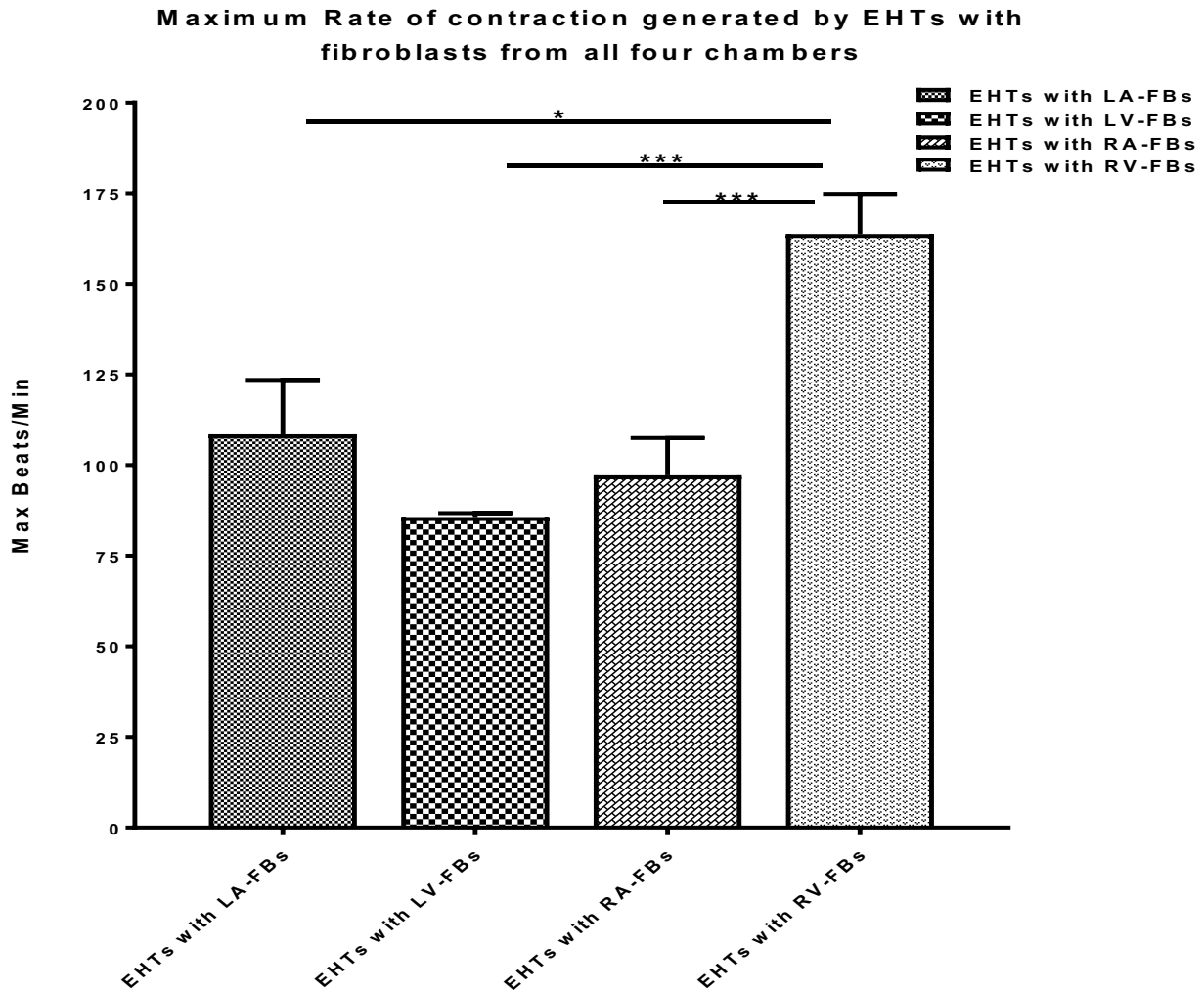


Fig. 4.22. MAXIMUM RATE OF CONTRACTION GENERATED BY EHTs WITH FIBROBLASTS OF ALL FOUR CHAMBERS ORIGIN.

No. of EHT constructs for the individual fibroblast groups were: LA (n= 5), RA (n=6), LV (n=6) and RV (n=4).

Error Bars: Mean +/- SEM

One-way ANOVA of peak rate of contraction generated by EHTs

A significant difference between the mean of peak rate of contraction. **P value = <0.0003 (***)**.

Brown Forsythe test. **P value = 0.1546 (ns)**.

Bartlett's test: **P value = 0.0015 (**)**

Tukey's Multiple comparison test of EHTs from different chambers:

LA vs RA. **P value = 0.8448 (ns)**

LA vs LV. **P value = 0.3807 (ns)**

LA vs RV. **P value = 0.0106 (*)**

LV vs RA. **P value = 0.7900 (ns)**

LV vs RV. **P value = 0.0002 (***)**

RA vs RV. **P value = 0.0010 (***)**

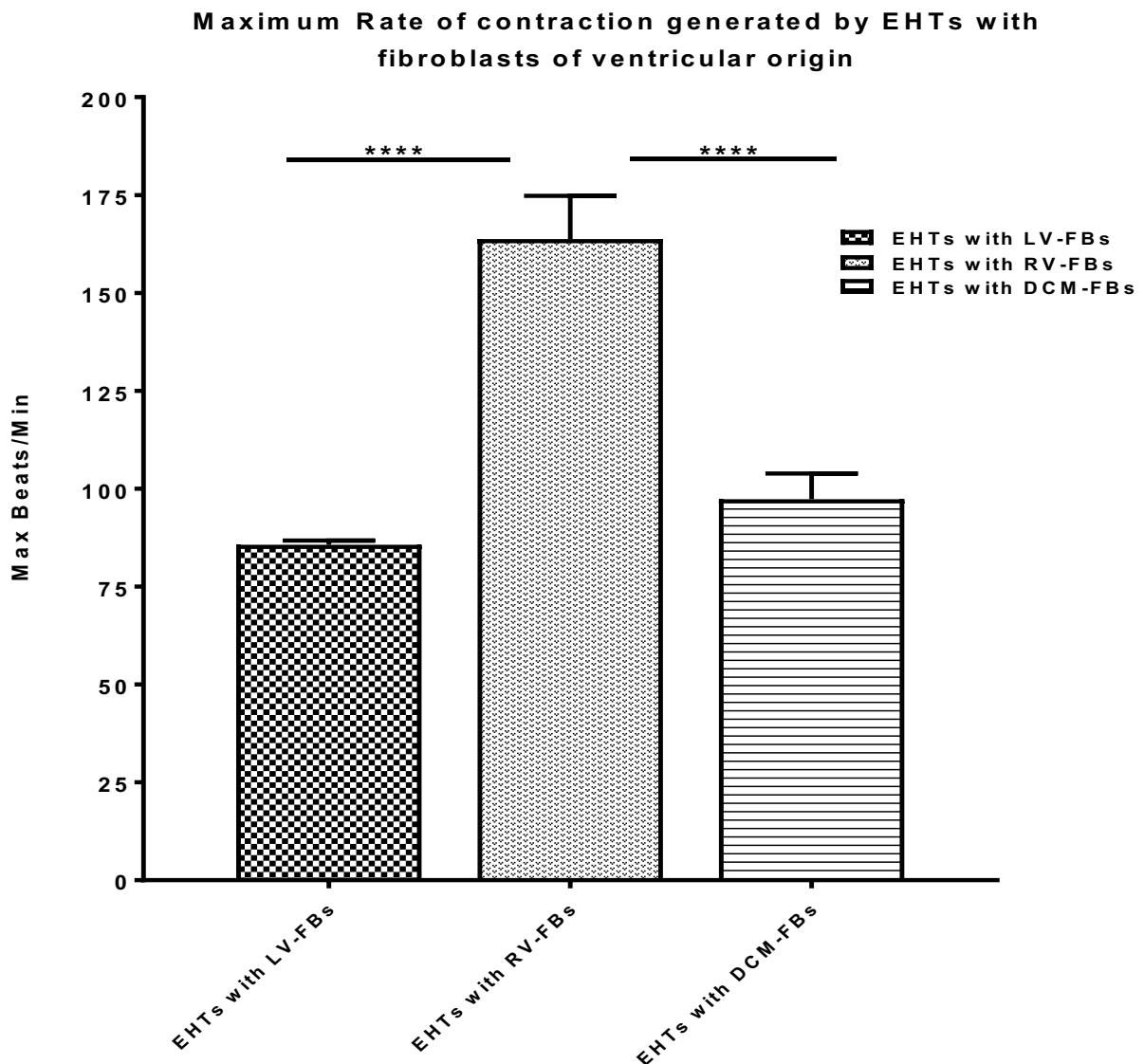


Fig. 4.23. MAXIMUM RATE OF CONTRACTION GENERATED BY EHTs WITH FIBROBLASTS OF VENTRICULAR ORIGIN.

No. of EHT construct for the individual fibroblast groups: LV (n= 6), RV (n= 4) and DCM (n=6).

Error bars: Mean +/- SEM

One-way ANOVA of peak rate of contraction generated by ventricular EHTs.

A significant difference between the mean of peak rate of contraction. **P value = <0.0001 (***).**

Brown Forsythe test. **P value = 0.1175 (ns).**

Bartlett's test: **P value = 0.0021 (**)**

Tukey's Multiple comparison test of EHTs from different chambers:

LA vs RV. **P value = <0.0001 (****)**

LV vs DCM. **P value = 0.3782 (ns)**

RV vs DCM. **P value = <0.0001 (****)**

4.6.5 INOTROPIC RESPONSE OF EHTs – FINAL DATA

1. INOTROPIC RESPONSE OF EHTs CULTURED WITH FIBROBLASTS FROM ALL FOUR CHAMBERS

The force of contraction of EHT is solely dependent on the cardiomyocytes in the construct, demonstrated by the fact that fibroblast only EHT which was lacking in cardiomyocyte population did not show any contraction throughout the observation period. Consequently, the cardiomyocytes are the source of initiation and propagation of the impulse leading to force of contraction. On a cellular level, this is carried out by a fine collaboration between electrical stimulation, calcium handling and contractility of the myofilament apparatus of sarcomeres.

The auxotonic model of EHT imparts inotropic properties similar to murine and human physiological myocardial tissue contraction. The human iPSC-EHT exhibit a positive Starling force-length relationship, frequency-dependent acceleration of relaxation (FDAR) and post-rest potentiation (PRP) but are unable to show a positive FFR (Mannhardt et al., 2016). The inotropic response of an EHT is dependent on t-tubule and sarcoplasmic reticulum spatial configuration. The incomplete development of sarcoplasmic reticulum can lead to a diminished PRP response which is a component of the inotropic response of the EHTs. The arrangement of t-tubules and the sarcoplasmic reticulum is not optimal in iPSC-CM, and this can lead to a poor FFR.

There was a comparable inotropic response of EHTs from all four chamber fibroblasts. No significant difference was noted in the FOC of left and right atrial fibroblast based EHTs. Corresponding to the better chronotropic response shown by the RV fibroblast based EHTs, the inotropic response was also better than other groups (Fig. 4.25) (Fig. 4.26). The FOC of contraction of LA and RA fibroblasts based EHTs did not show any significant difference (P-

value = 0.1573). A similar trend was noted in the comparison of LV and RV fibroblast group (P-value = 0.22464) (Fig. 4.27). The comparison of left-sided chambers (LA and LV) loaded EHTs showed a greater difference in the force of contraction (P-value = 0.001199). On the right side, the difference in inotropic response of RA and RV was also more pronounced (P-value = 0.0015) (Fig. 4.28). The relative difference of the FOC of LA and RV between day 4 and day 10 was significant and more than any other chambers (P-value = < 0.0060) (Fig. 4.29). It was hypothesised that there would be a difference in the FOC of LV and DCM fibroblasts based EHTs. However, the inotropic response between the LV and DCM fibroblasts based EHT groups showed no significant recordable difference (P-value = 0.22464) (Fig. 4.30). This can be attributed to the fact that EHTs cannot exhibit the native physiological isometric loading condition and their FFR is impaired.

Force of contraction of EHTs with fibroblasts of different origin

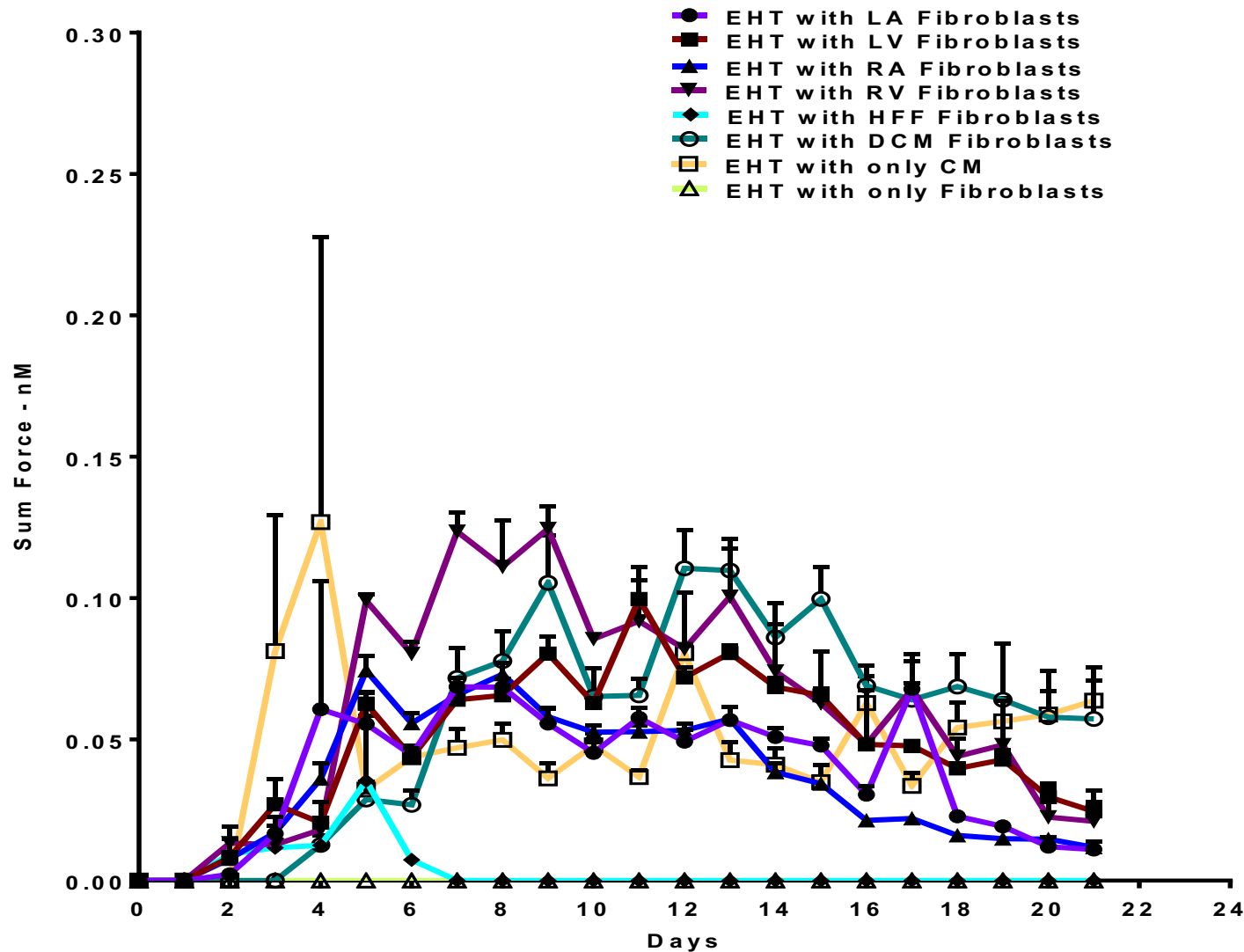


Fig. 4.24. FORCE OF CONTRACTION OF EHTs WITH FIBROBLASTS OF DIFFERENT ORIGIN.

All fibroblasts were at P2 stage of differentiation and IMR cardiomyocytes were at day 18 of differentiation.

No. of EHT constructs for each fibroblast group are: LA fibroblasts (n=5), RA & LV fibroblasts (n=6), RV fibroblasts (n=4), FF fibroblasts, DCM cardiomyocyte only group (n=6) and fibroblast only group (n=3).

Error Bars: MEAN +/- SEM

Force of contraction of EHTs with all four chamber fibroblasts

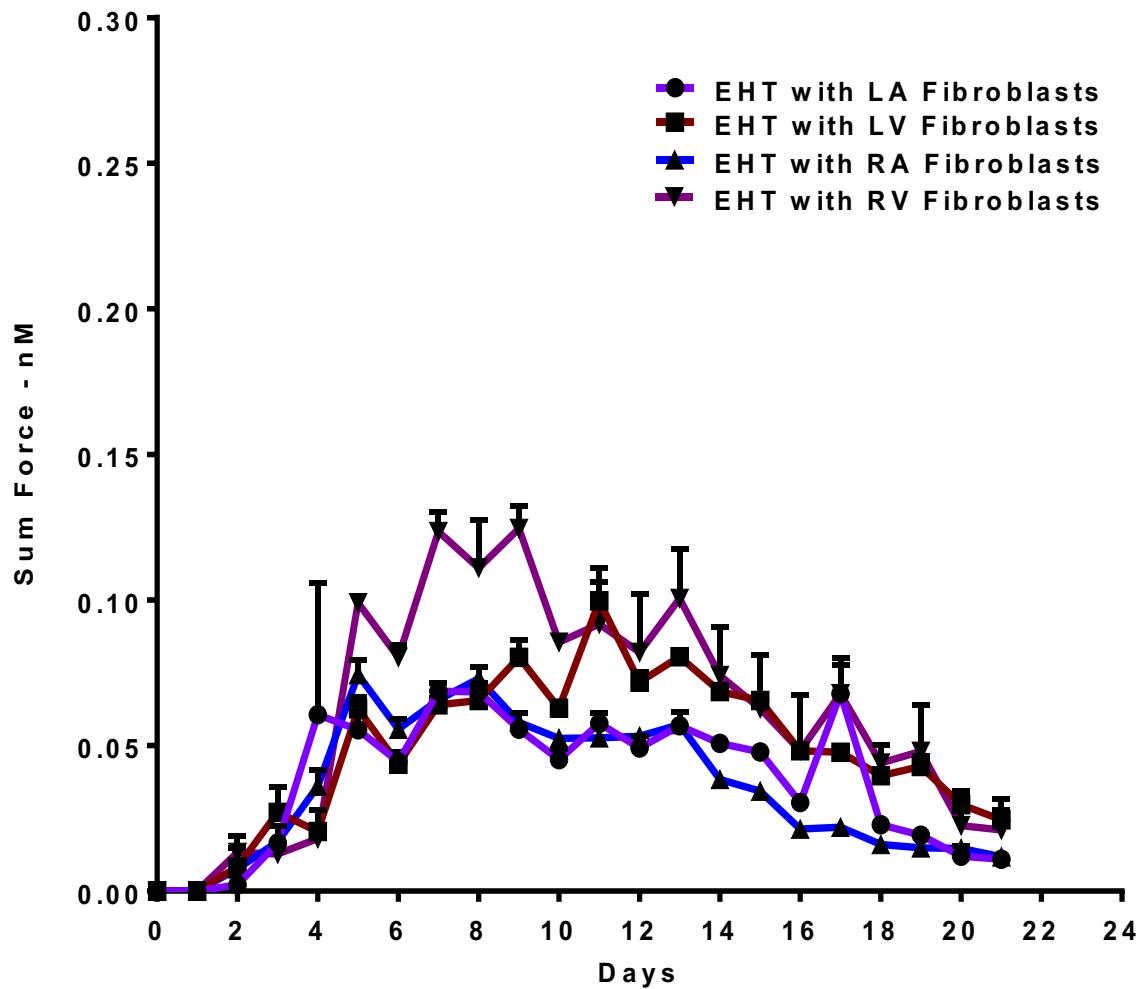


Figure. 4.25. FORCE OF CONTRACTION OF EHTs WITH FIBROBLASTS POPULATION FROM ALL FOUR CHAMBERS.

No. of EHT constructs for the individual fibroblast groups were: LA (n= 5), RA (n=6), LV (n=6) and RV (n=4).

EHT constructs with fibroblasts from all four chambers in the patient with normal LV biventricular function. The FOC of RV fibroblasts based EHTs is superseding other groups in the middle period. This followed by EHTs with LV and the atrial based EHTs are almost similar.

Error bars: MEAN +/- SEM

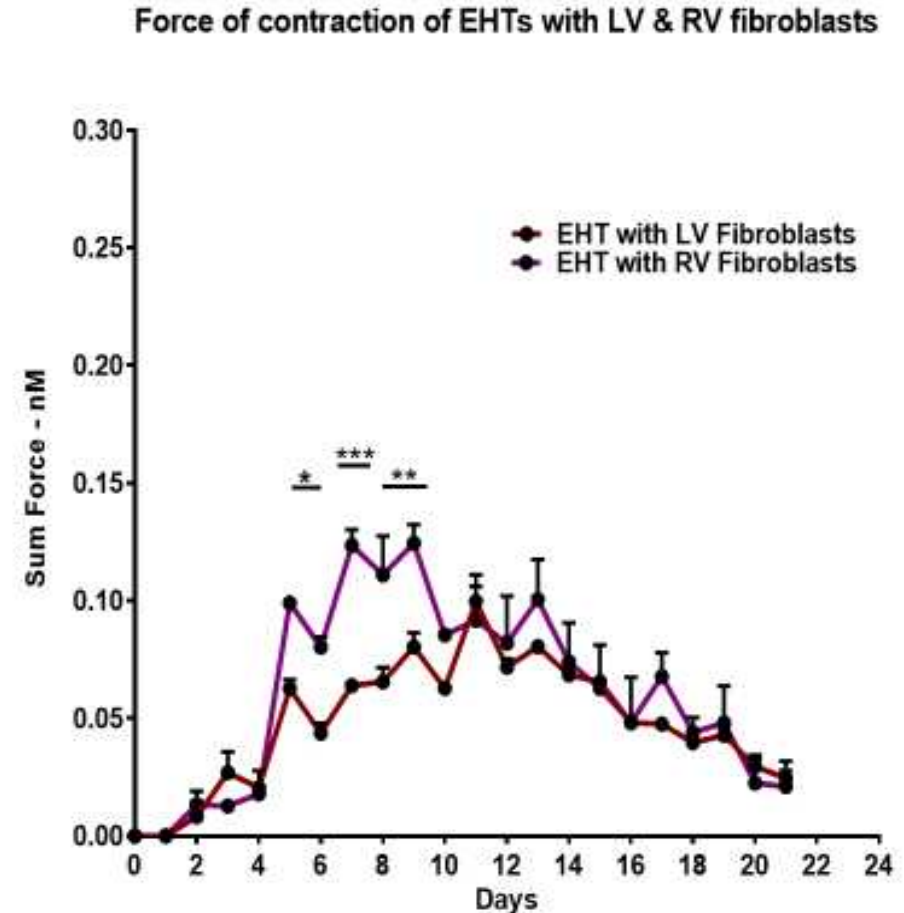
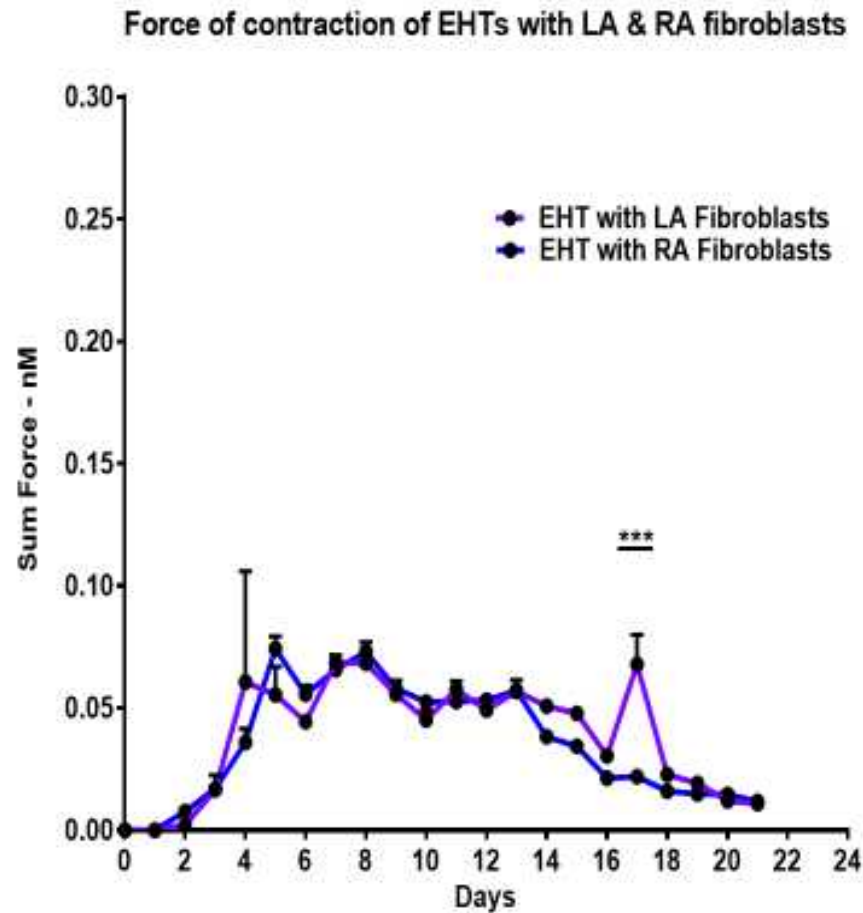


Figure. 4.26. FORCE OF CONTRACTION OF EHTs WITH ATRIAL AND VENTRICULAR FIBROBLASTS.

No. of EHT construct for the individual fibroblast groups were: LA (n= 5), RA (n=6), LV (n=6) and RV (n=4).

EHTs with Left and Right Atrial fibroblasts. **P = 0.1573**

EHTs with Left and Right Ventricular fibroblasts. **P = 0.0476 (*)** Between day 4 to day 9, [(day 5 (*), day 7(***), day 9(**))]

FOC of RV based EHTs was better but statistically insignificant than LV fibroblasts based EHTs.

2-way RM ANOVA with Bonferroni correction was used for analysis. **Error bars: MEAN +/- SEM**

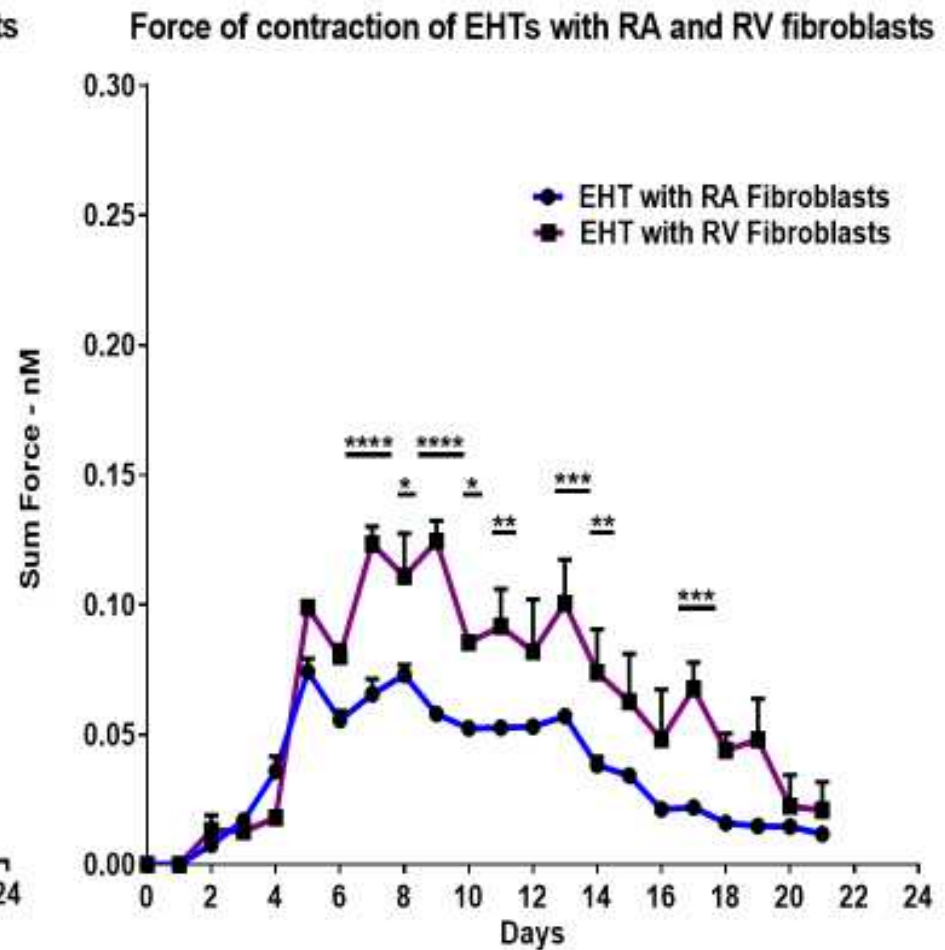
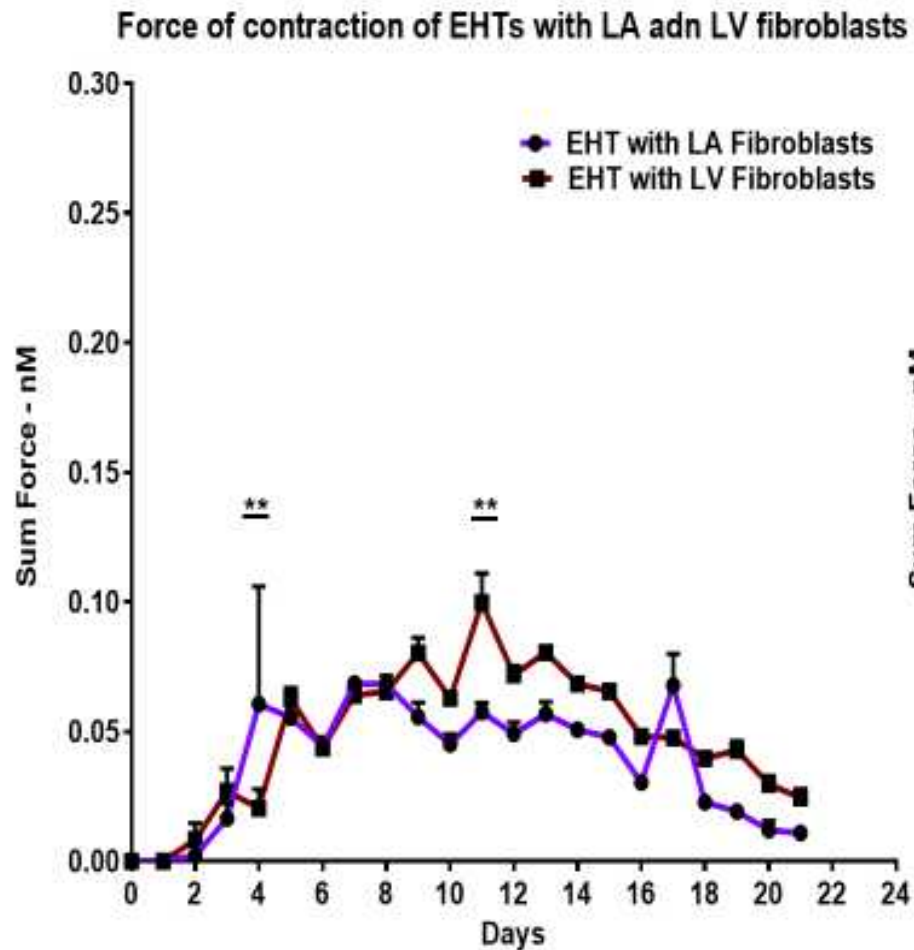


Figure. 4.27. FORCE OF CONTRACTION OF EHTs WITH LEFT AND RIGHT SIDED CHAMBER FIBROBLASTS.

No. of EHT construct for the individual fibroblast groups were: LA (n= 5), RA (n=6), LV (n=6) and RV (n=4).

EHTs with LA and LV fibroblasts. **P = 0.0012 (**)**

EHTs with the RA and RV fibroblasts. (day 6 - day18) **P = 0.0015 (**)**

2-way RM ANOVA with Bonferroni correction was used for analysis. **Error bars: MEAN +/- SEM**

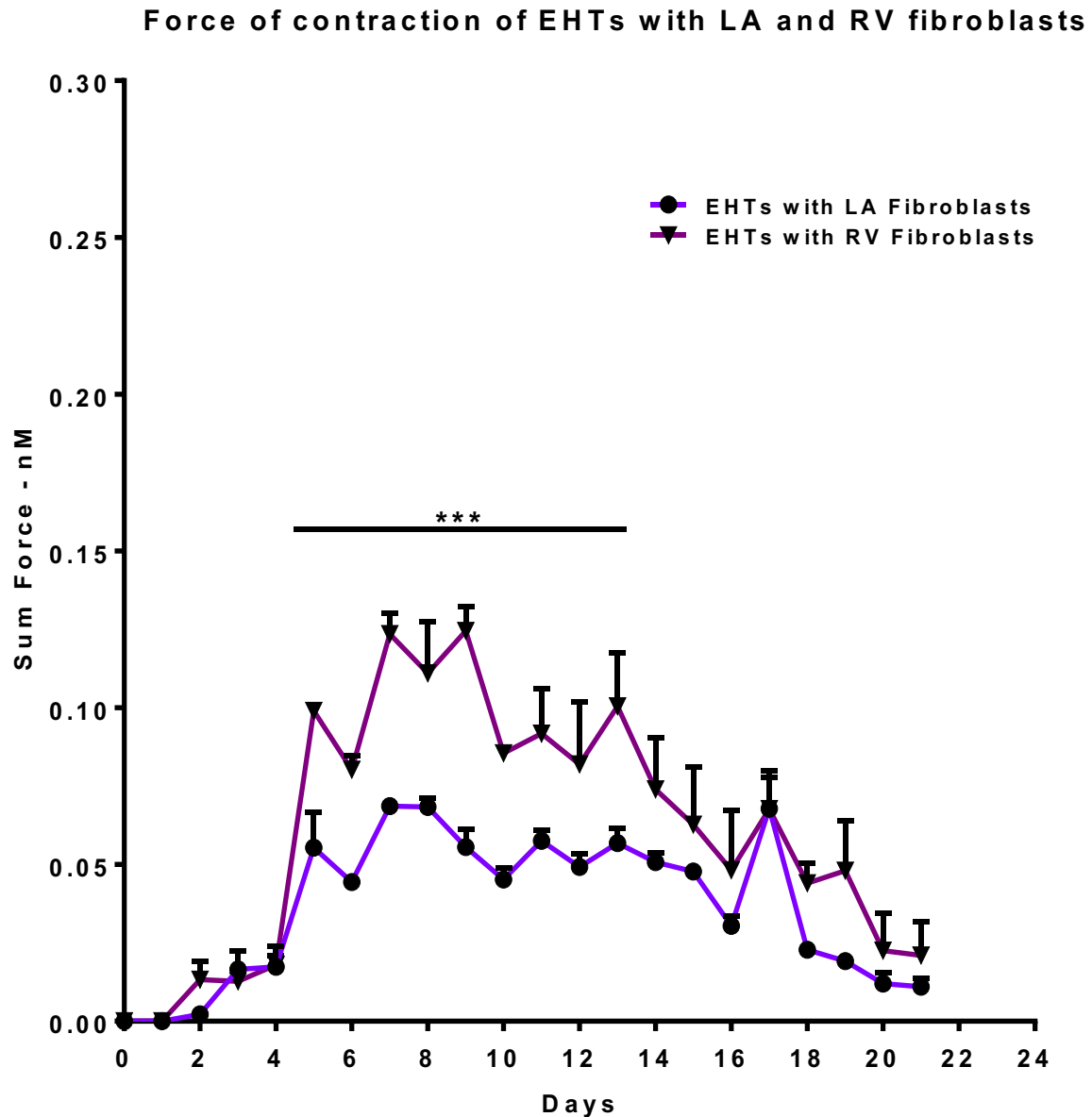


Figure. 4.28. FORCE OF CONTRACTION OF EHTs WITH LEFT ATRIAL AND RIGHT VENTRICULAR FIBROBLASTS.

No. of EHT constructs for the individual fibroblast groups: LA (n=5), RV (n=4.)
 Significant difference observed in the FOC of two groups between day 4 – day 11.
P = 0.0060

2-way RM ANOVA with Bonferroni correction was used for analysis.
Error bars: MEAN +/- SEM

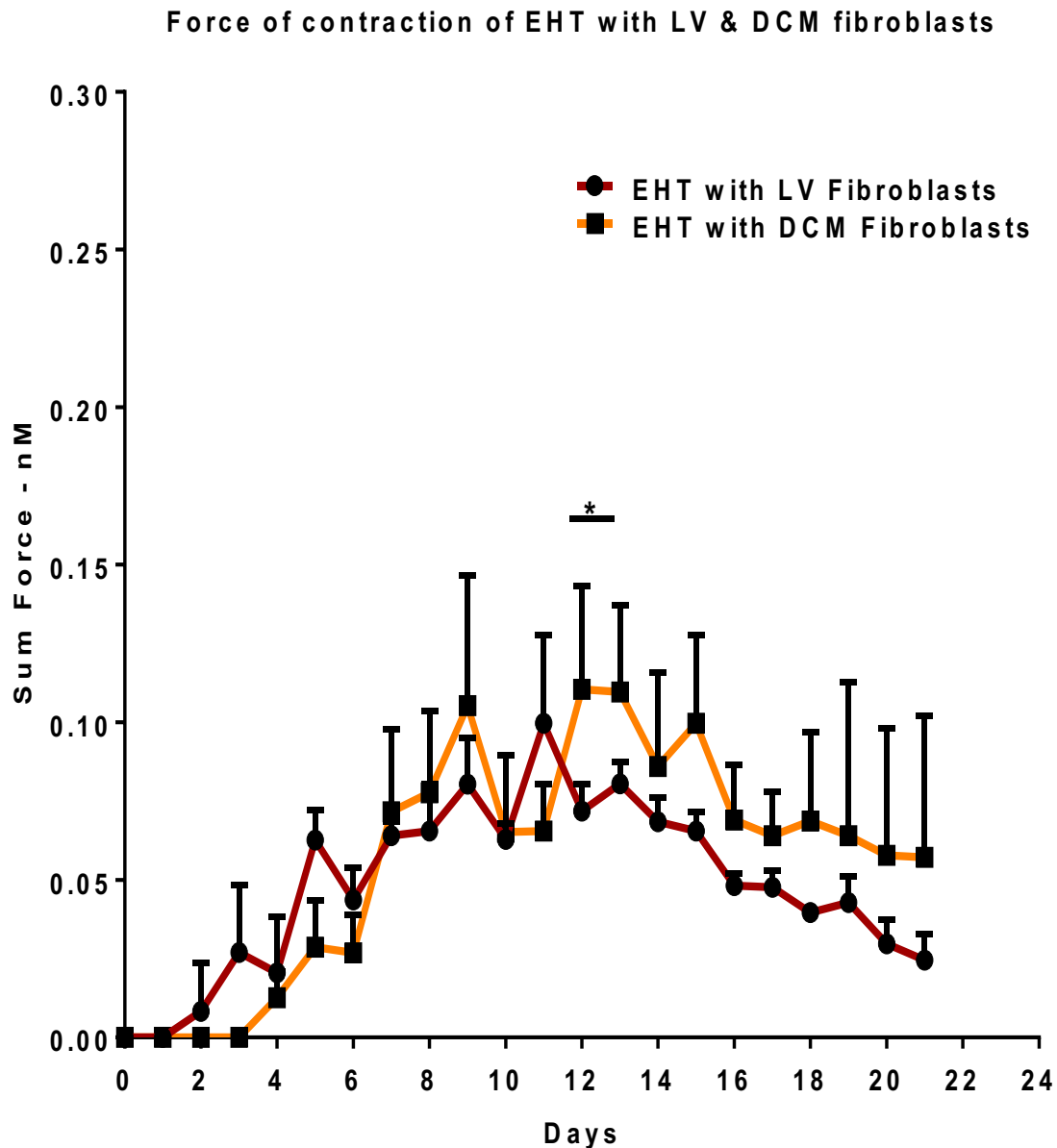


Figure. 4.29. FORCE OF CONTRACTION OF EHTs WITH PRESERVED LV AND DCM (POOR VENTRICULAR) FIBROBLASTS

No. of EHT construct for the individual fibroblast groups: DCM (n=6), LV (n=6.) EF in the normal ventricular group was 60% and EF in the DCM group was less than 20%

No statistically significant difference observed in the FOC of two groups. **P = 0.22464**

2-way RM ANOVA with Bonferroni correction was used for analysis.

Error bars: MEAN +/- SEM

2. INOTROPIC RESPONSE OF EHTs CULTURED WITH FIBROBLASTS FROM COMPARING GROUPS

The CM-only EHTs started contracting on day 2, and the FOC contraction peaked in the first four days followed by a steady FOC all through the observation period. The DCM fibroblasts containing EHTs started to contract on day 3, and the uptake of FOC was slow. However, after day six the FOC achieved a steady pace which was maintained throughout the next 14 days. In comparison with the CM-only EHTs, the FOC of contraction of (DCM fibroblasts containing EHTs) these EHTs after the first week was stronger and sustained. Nevertheless, the two-way ANOVA did not show a significant difference between the two groups (P value = 0.320876) (Fig. 4.31).

The FOC of the FF based EHTs was weaker and less sustained than groups above. The FF containing EHTs started to contract after day 4, and then flexible poles of the silicon racks were pulled to the middle with shortening of EHTs. This contracture became progressively pronounced, and the EHTs decreased to one-third of the initial length within a week. Due to this massive contracture, no contraction was noted after day 7. This corresponds to the phenomenon observed during the cardiac surgical intervention in the paediatric population where during the redo surgeries the previous suture line is barely visible, and even the chambers of the heart that are previously quite dilated undergo a marked regression in size within 2-3 years (provided the primary pathology is dealt with). While comparing the FF containing EHTs inotropic response with other groups, there is a significant difference in the FOC as there was no contractile response in the foetal group after 7 days (Fig. 4.31).

Force of contraction of EHTs with fibroblasts of four comparing group

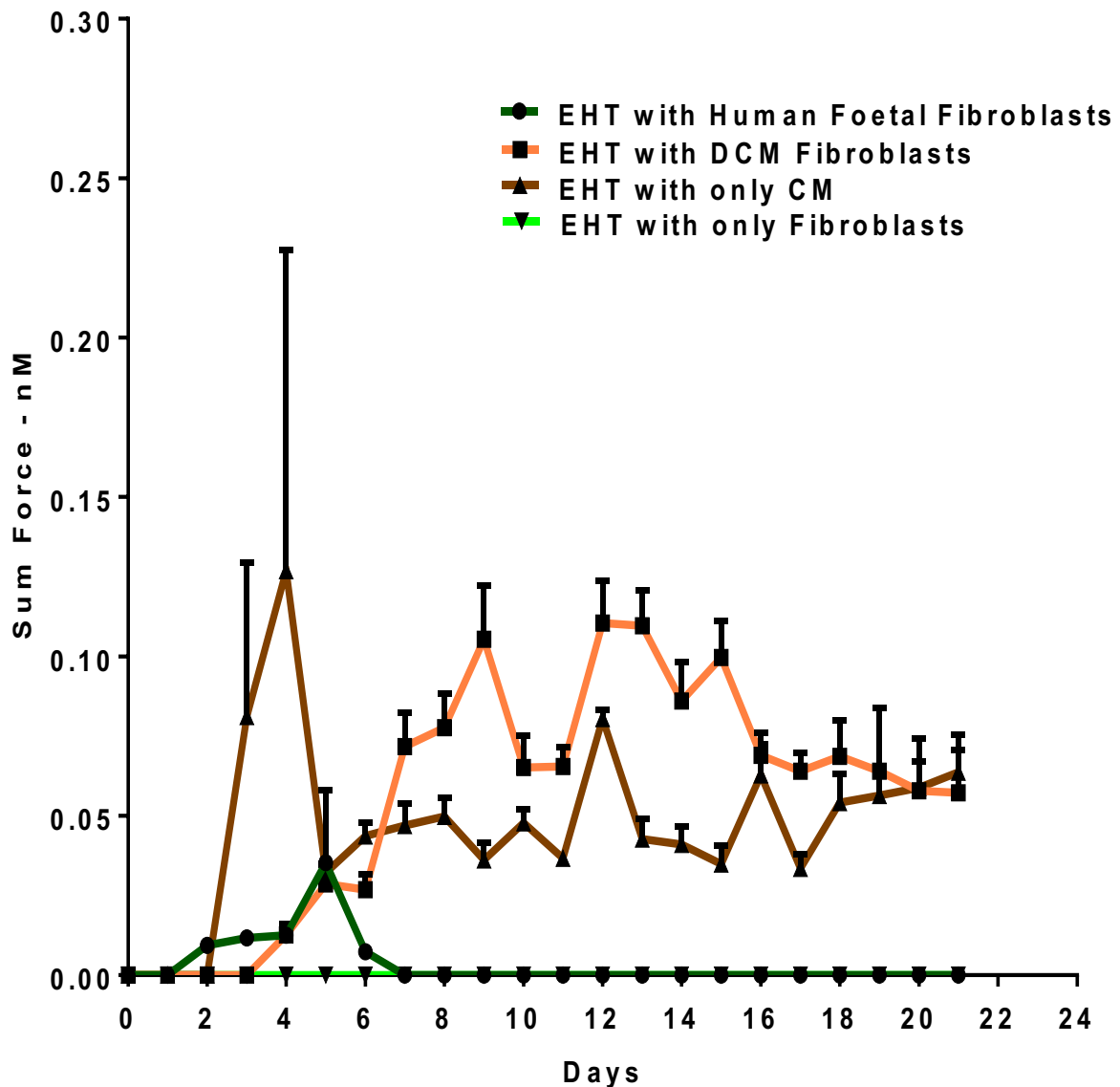


Figure. 4.30. FORCE OF CONTRACTION OF EHTS WITH FIBROBLASTS POPULATION FROM THE COMPARING GROUPS.

No. of EHT constructs for the individual fibroblast groups: FF (n= 6), DCM (n=6), CMs only (n=6) and FBs only (n=3).

EHT with DCM and CM fibroblasts. **P value = 0.320876 (ns)**

2-way RM ANOVA with Bonferroni correction was used for analysis.

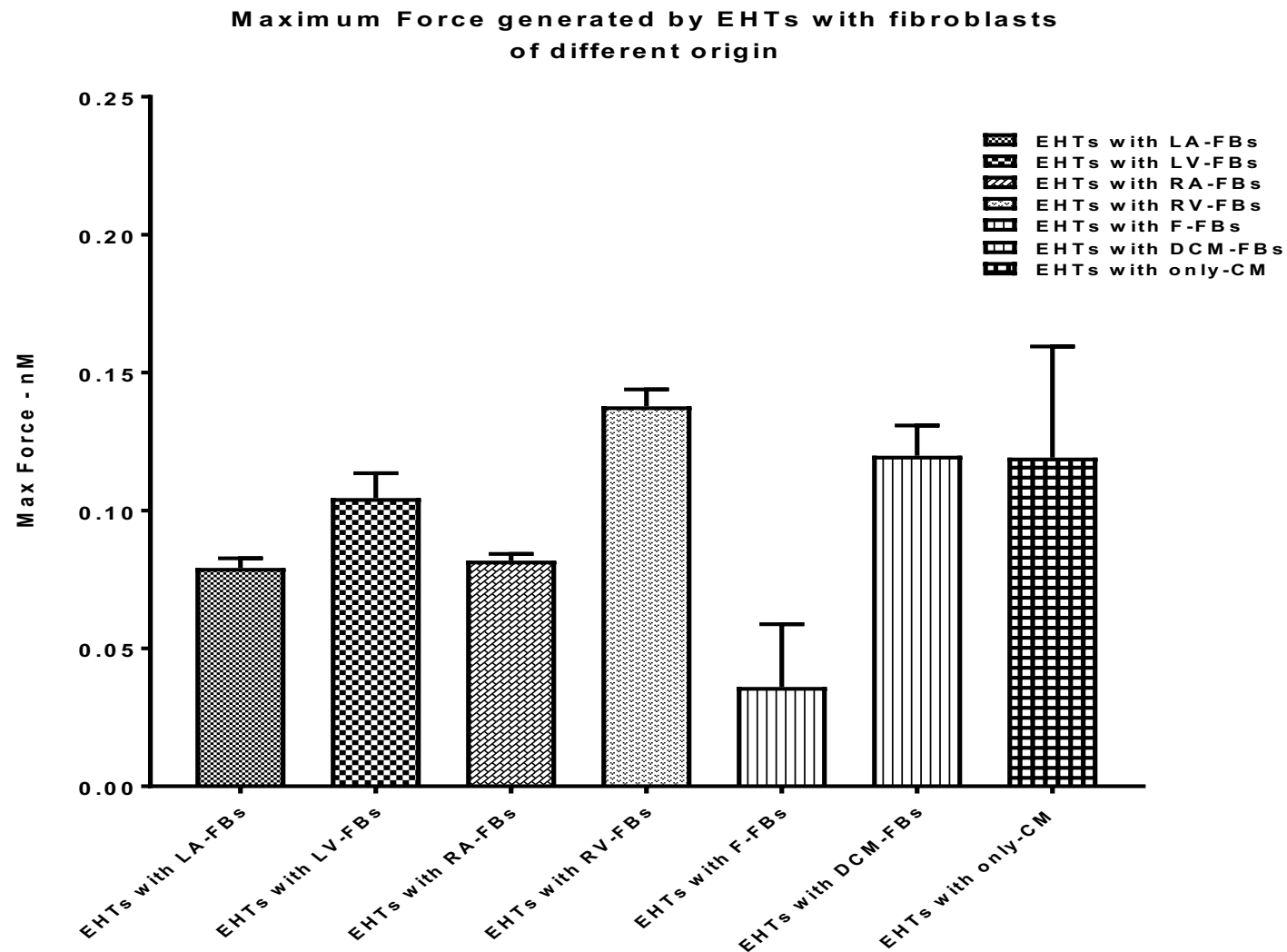
Error bars: MEAN +/- SEM

The fibroblast-only EHTs did not contract at all. Since the fibroblasts lack the fundamental pacemaker mechanisms incorporated into the cardiomyocyte they were unable to show contractility. The shape of the EHTs with fibroblast was almost the same as the other groups, but they showed no contraction and the overall length decreased over the course of the observation period. However, when fibroblasts were added to the EHT constructs in addition to the cardiomyocytes, they provided better architectural support, improved the gap junctions and promoted better architectural organisation of t-tubules and sarcolemma. This led to a more organised cardiac syncytium and hence a better force of contraction.

3. MAXIMUM INOTROPIC RESPONSE OF EHTs CULTURED WITH FIBROBLASTS OF DIFFERENT ORIGIN

The peak force of contraction generated by the EHTs with fibroblasts from all groups was compared. A significant difference was noted in the mean peak FOC across the groups. (P-value = 0.0240). This was further tested for group variance with the help of Bartlett's test, and P-value was = <0.0001 (Fig. 4.32).

When EHTs with all four chamber fibroblasts were compared, it showed that peak FOC was significantly different among the chambers. The right ventricular fibroblasts generated the maximum FOC of contraction. The comparison of peak FOC of RV-FBs based EHTs with the rest of the three chamber fibroblasts based EHTs was stronger (P-value = <0.0001). Tukey's multiple comparisons further elaborated the difference between the groups (Fig 4.33).



No. of EHT constructs for each fibroblast group:
 LA fibroblasts (n=5),
 RA & LV fibroblasts (n=6),
 RV fibroblasts (n=4),
 FF fibroblasts, DCM,
 cardiomyocyte-only group
 (n=6) and
 fibroblast-only group (n=3).

Error bars: MEAN +/- SEM

One-way ANOVA of peak
 force generated by EHTs.

Significant difference
 between the mean of peak
 force.

P-value = 0.0240 (*)

Brown Forsythe test:

P-value = 0.7633

Bartlett's test:

P-value = < 0.0001 (**)**

Fig. 4.31. MAXIMUM FORCE OF CONTRACTION GENERATED BY EHTs WITH FIBROBLASTS OF DIFFERENT ORIGIN

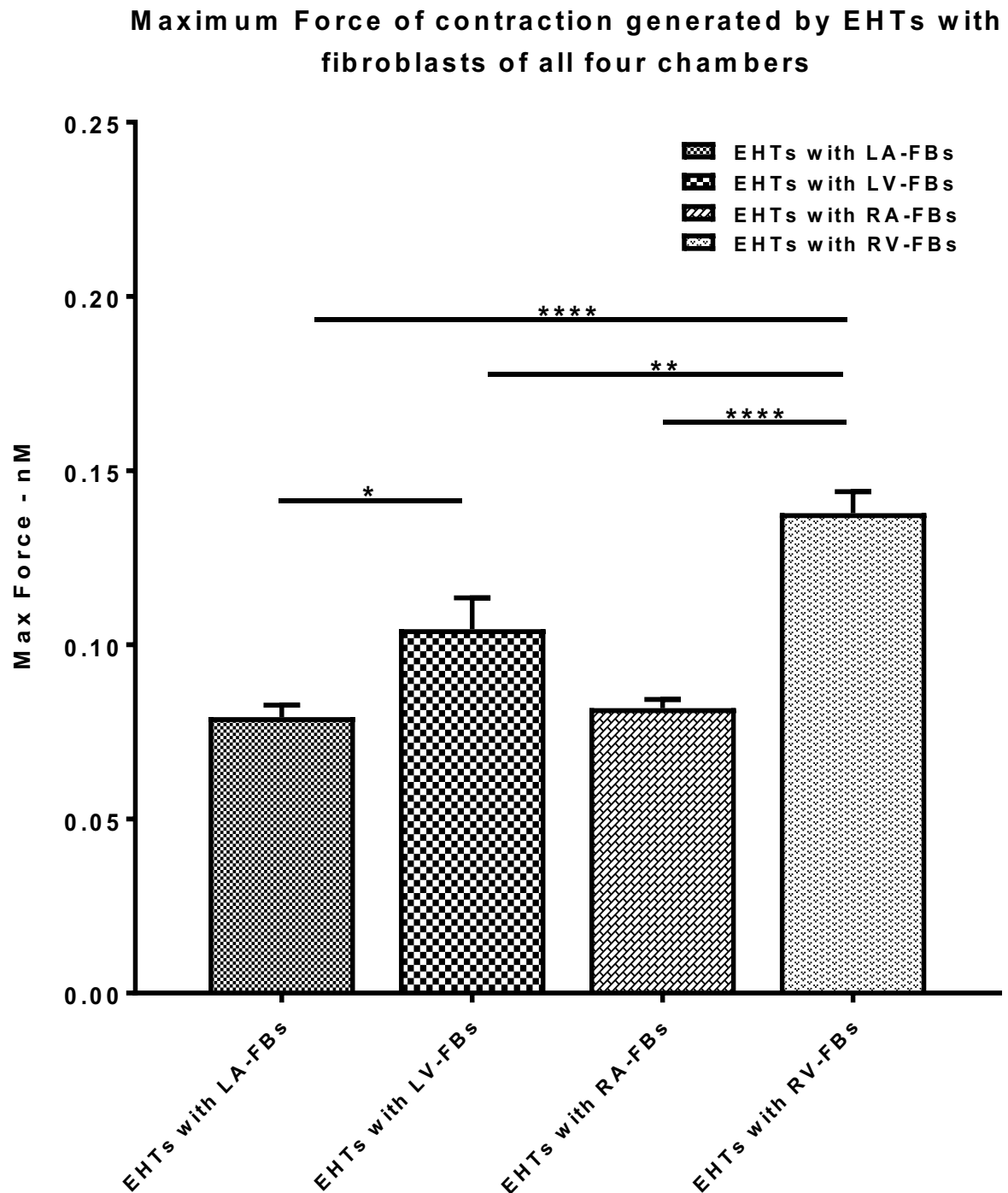


Fig. 4.32. MAXIMUM FORCE GENERATED BY EHTS WITH FIBROBLASTS FROM ALL FOUR CHAMBER ORIGIN.

No. of EHT constructs for the individual fibroblast groups were: LA (n= 5), RA (n=6), LV (n=6) and RV (n=4).

Error bars: MEAN +/- SEM

One-way ANOVA of peak force generated by EHTs.

Significant difference between the mean of peak force.

P-value = <0.0001 (**).**

Brown Forsythe test. **P-value = 0.3496.**

Bartlett's test: **P-value = < 0.0380 (*)**

Tukey's Multiple comparison test of EHTs from different chambers:

LA vs RA. **P-value = 0.0365 (ns)**

LA vs LV. **P-value = 0.0365 (*)**

LA vs RV. **P-value = <0.0001 (****)**

LV vs RA. **P-value = 0.0521 (ns)**

LV vs RV. **P-value = 0.0087 (**)**

RA vs RV. **P-value = <0.0001 (****)**

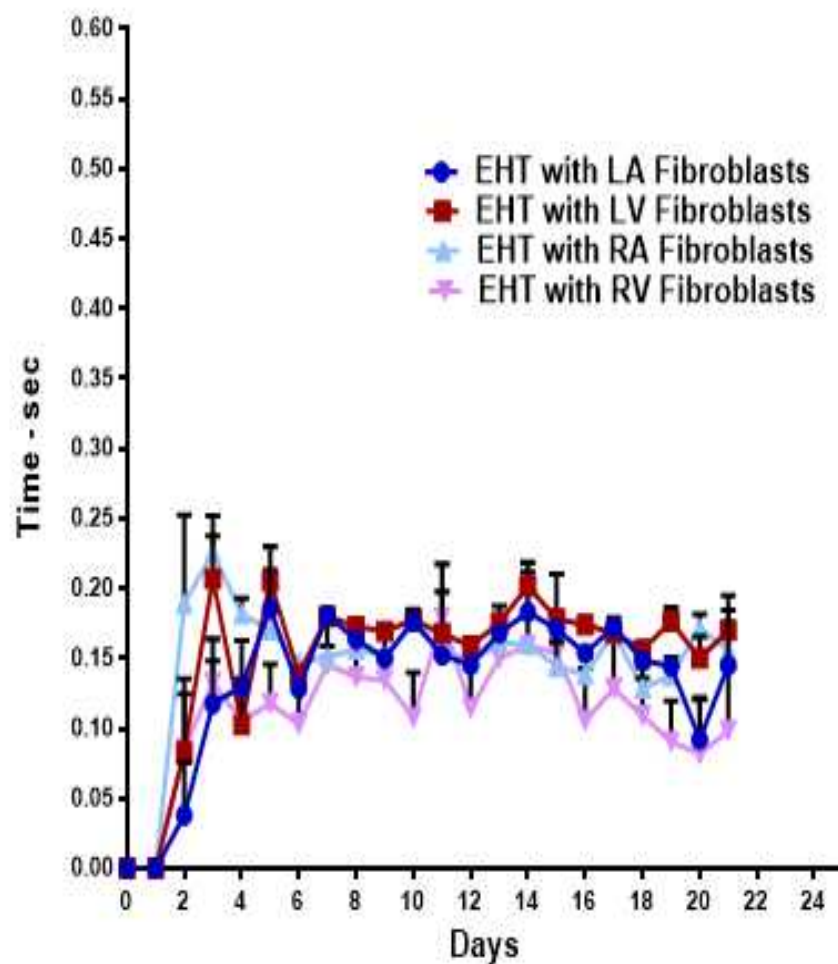
4.6.6 T1-20% & T2-20% OF EHTs

1. T1-20% & T2-20% of EHTs CULTURED WITH FIBROBLASTS FROM ALL FOUR CHAMBERS AND COMPARING GROUPS

The T1-20% of all four-chamber specific fibroblast containing EHTs showed the same trend spanning the entire period of study. The T2-20% in the same group shows better relaxation of the right atrial fibroblast as compared to the other groups. Similarly comparing the two ventricular groups, EHTs with left ventricle fibroblasts had better T2-20% than the RV group EHTs. Towards the start and the end of the study, the difference in the T2-20% was more distinct than in the middle period where the relaxation phase is more comparable among all the groups. Especially towards the end of the study the T2-20% in the right atrial and left ventricular fibroblasts based EHTs is more accentuated in comparison with the left atrium and left ventricle. However, the 2-way ANOVA of the period does not show a significant difference between the groups (Fig. 4.34).

In the comparison group, the T1-20% of the CM-only EHTs show better contractility than the other groups. The other main group, DCM fibroblast containing EHTs, show a consistently reduced T1-20% than the CM-only EHTs. The T2-20% of these groups depicted the same tendency. CM-only EHTs showed relatively better phasic relaxation compared to DCM containing EHTs, all along the contractile phase. However, the difference between the groups was not significant (Fig. 4.35).

T1 20% of EHTs with chamber specific fibroblasts



T2 20% of EHTs with chamber specific fibroblasts

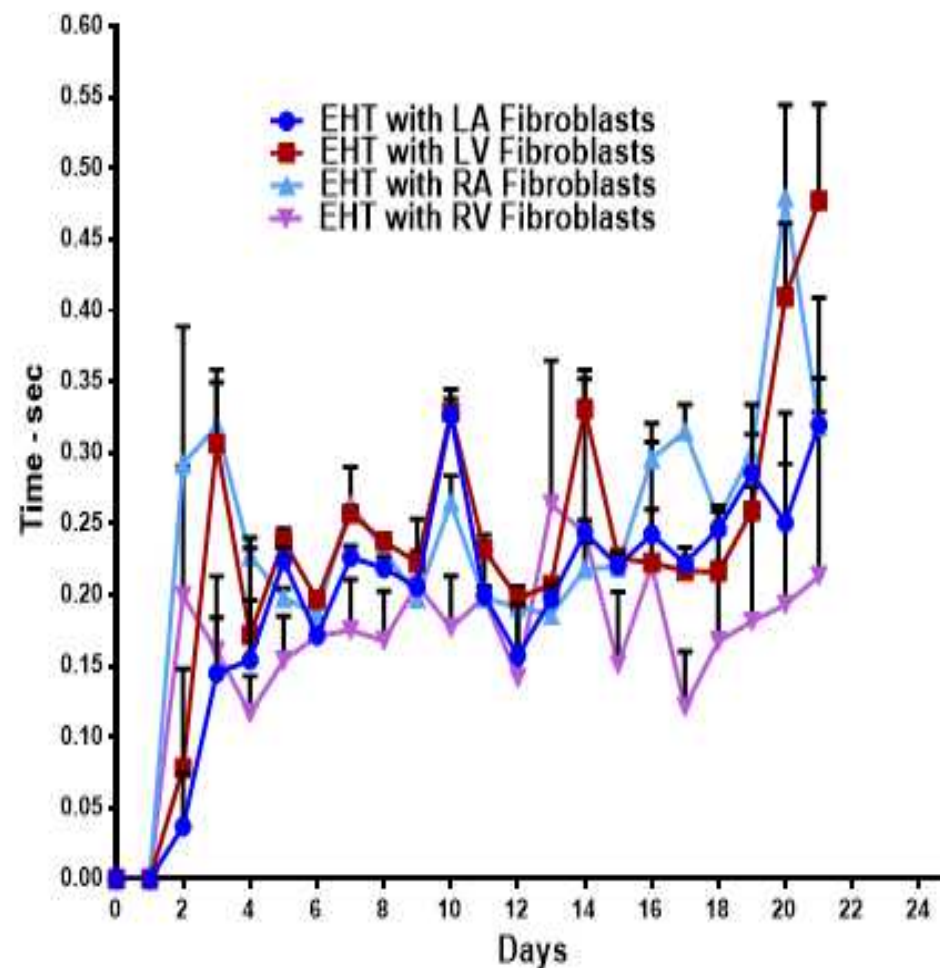


Fig. 4.33. T1-20% & T2-20 of EHTs WITH ALL FOUR CHAMBERS FIBROBLASTS

No. of EHT constructs for the individual fibroblast groups: LA (n= 5), RA (n=6), LV (n=6) and RV (n=4).

No significant difference between the groups.

2-way RM ANOVA was used for analysis. **Error Bars: Mean +/- SEM**

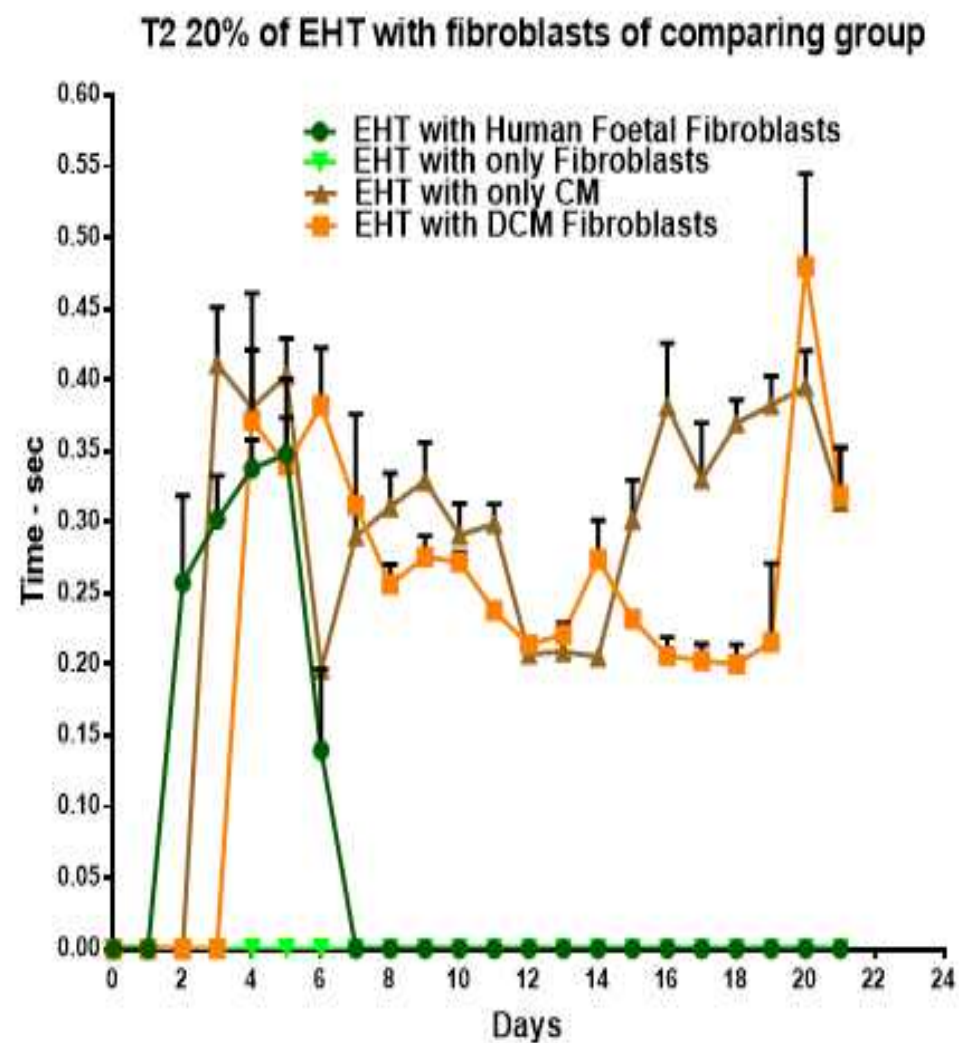
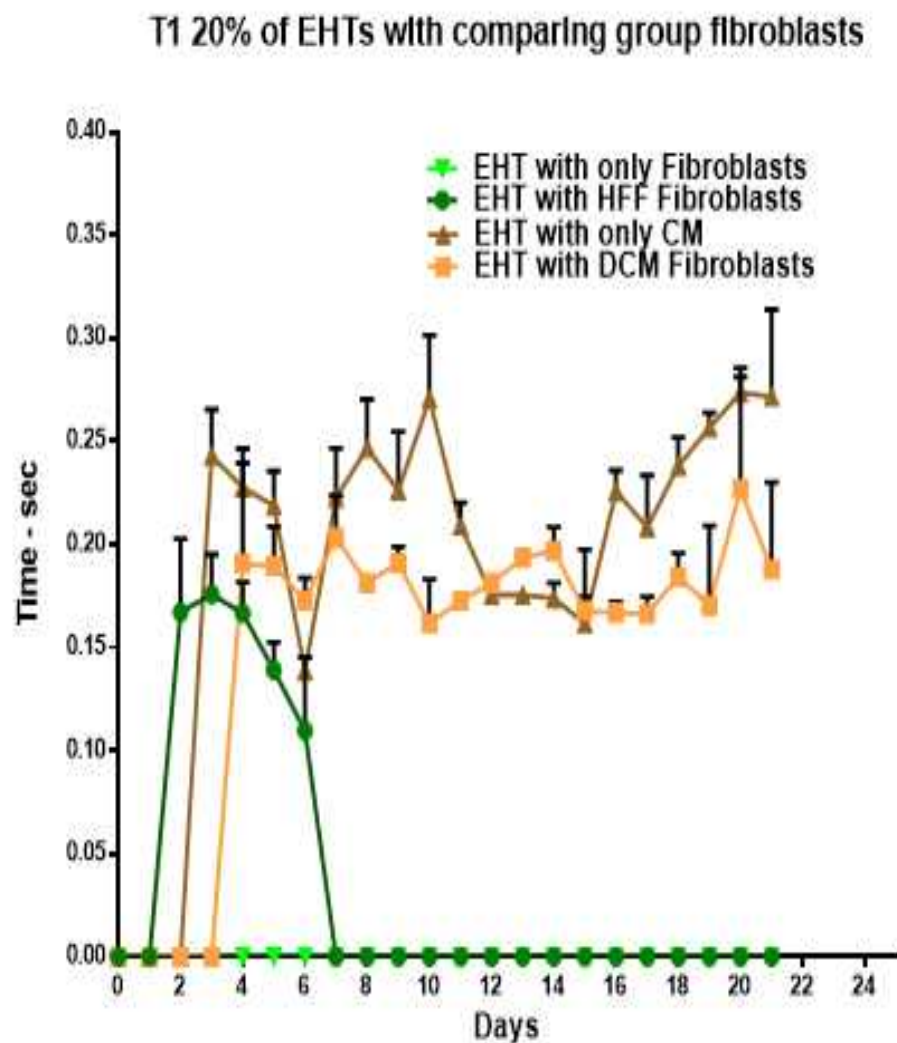


Fig. 4.34. T1-20% & T2-20 OF EHTs OF COMPARING GROUP FIBROBLASTS.

No. of EHT constructs for the individual fibroblast groups: FF (n= 6), DCM (n=6), CMs only (n=6) and FBs only (n=3).

2-way RM ANOVA was used for analysis. No significant difference between the groups.

Error Bars: Mean +/- SEM

4.6.7 RESTING LENGTH OF EHTs

1. RESTING LENGTH OF EHTS CULTURED WITH FIBROBLASTS FROM ALL FOUR CHAMBERS AND COMPARING GROUPS

In a healthy heart, the resting length of the myofilaments represents the end-diastolic dimension of the chambers. It corresponds to the preload against which the heart contracts. Within the confines of the normal ventricular function, the increased preload or the resting length of the fibre is directly proportional to the force of contraction. However, in a failing heart, the Frank-Starling's law loses its applicability, and a further increase in the preload worsens heart failure rather than increase in the contractile force (Kemp and Conte, 2012). Since EHTs represent the sarcomeric unit of the myocardium (attached to 2 poles), they demonstrate an enhanced FOC with an increase in the resting length.

The resting length of EHTs is affected by the increase in the rate of contractility. As the ROC increases, the diastolic relaxation gets compromised as does the resting length. This explained the observation that RV fibroblast based EHTs that showed the fastest rate of contraction had the smallest resting length in comparison with the other three chambers. In comparison group EHTs, the CM-only EHTs showed the largest resting length compared with other groups. This is attributed to the fact that the absence of fibroblasts leads to the less mature extracellular architectural organisation. The lack of cell to cell adhesive factor (due to fibroblasts) leads to an increase in the resting length of the EHTs. However, due to the enhanced resting length, the FOC of contraction depicted by CM-only based EHTs was stronger than other EHTs in the group. The FF based EHTs showed the smallest resting length as they developed contracture during the early phase of the study and hence showed no contractile response (Fig. 4.36).

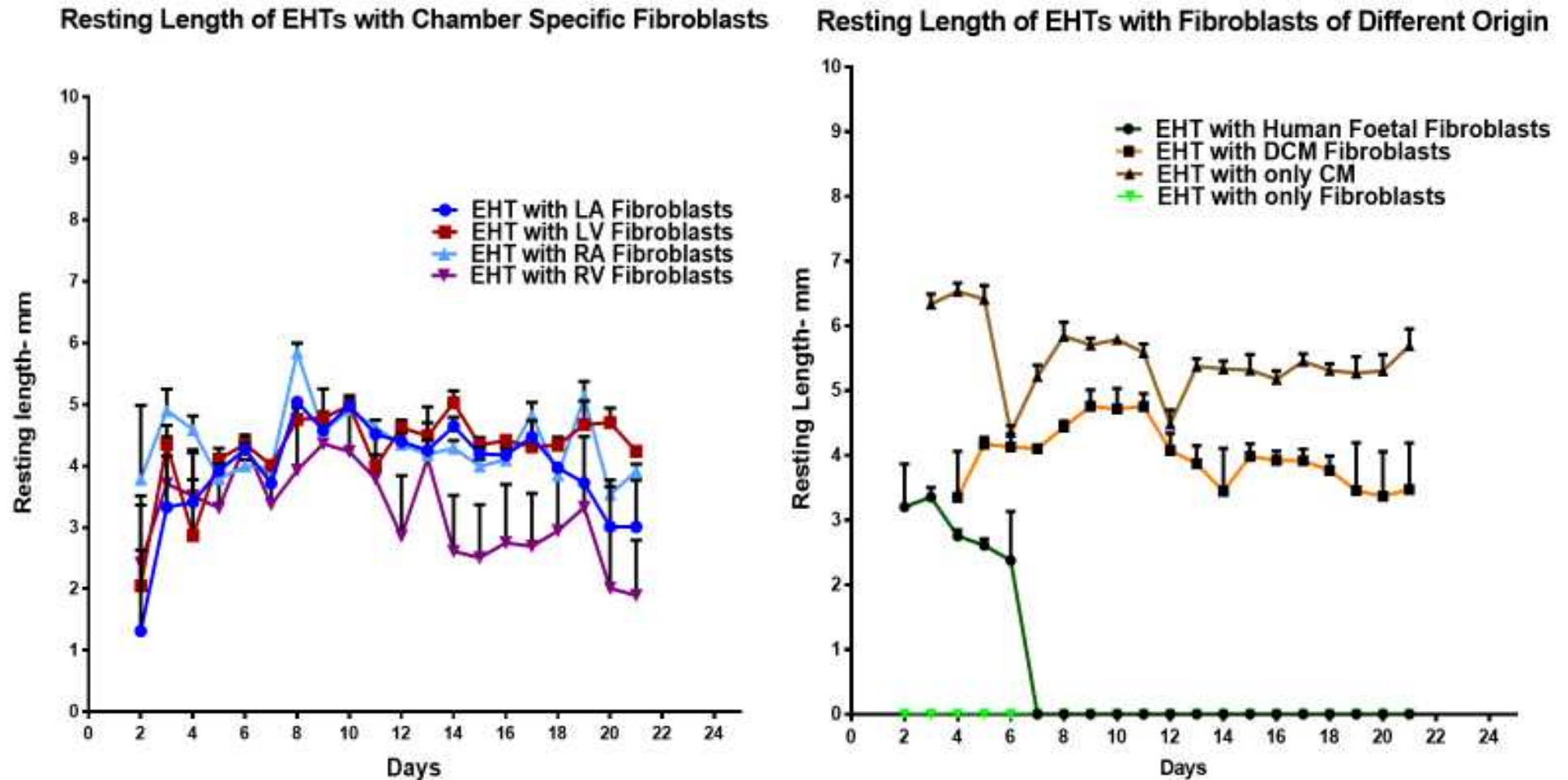


Fig. 4.35. RESTING LENGTH OF EHTS WITH ALL FIBROBLASTS GROUPS.

No. of EHT constructs for the individual fibroblast groups: LA (n= 5), RA (n=6), LV (n=6), RV (n=4), FF (n= 6), DCM (n=6), CMs only (n=6) and FBs only (n=3).

No significant difference between the groups.

2-way RM ANOVA was used for analysis. **Error Bars: Mean +/- SEM**

4.7 HISTOLOGY OF EHTs

Once the EHTs lost their kinetics, they were carefully detached from the poles of scaffoldings and were preserved in OCT and frozen. Prior to freezing, a few EHTs were examined under bright field microscope with 20X magnification. The cellular architecture comprising of a combination of cardiomyocytes and fibroblasts was reviewed with cardiomyocytes occupying the periphery of the EHTs and the fibroblasts were concentrated more towards the centre. These EHTs embedded and frozen in OCT were sliced and different stains were tried. No cellular components were stained with eosin and haematoxylin. The anatomical presence of the sliced EHTs was noted under the microscope but the cells could not be stained. We have attributed this to the unsuccessful protocol and methodology for the histological review and hence this had to be repeated in other projects in the lab.

4.8 DISCUSSION

4.8.1 SUMMARY

The work in this chapter is focused on the development of EHTs with fibroblast from all four-chambers from the same patient. The difference in the characteristics and kinetics of EHTs with chamber specific fibroblasts were compared. These chamber specific EHTs were further compared against the EHTs with fibroblasts of different origin (another group) but all of them were cultured at the same time. The unique feature of this study was that for the first time, chamber specific fibroblasts were used to generate EHTs in our lab. Moreover, a comparison of EHTs with human cardiac fibroblasts of different origin was not done before.

All fibroblasts used in this study were from an adult human heart with or without a preserved biventricular function. The FFs in the comparison group were the only non-adult population of cells. The CDI-CMs used in the pilot study and IMR-CMs used in the main group of experiments were structurally and functionally immature cardiomyocytes and are more like

the human foetal cardiomyocytes. The calcium transit durations of these cells matched that of the foetal cardiomyocytes. Therefore the experiment mimics more of a foetal heart model than an adult. In future, this model can be modified for an adult cell population (Cardiomyocytes & Fibroblasts). A standard protocol was used to develop EHTs based on a precise pre-calculated number of cells used for each construct.

The results showed that EHTs with all four-chamber fibroblasts exhibited some chamber specific variations of kinetics (chronotropy, inotropy, resting length, fractional shortening and T1-20% and T2-20%). The comparison of kinetics of left sided chambers (LA compared to LV) and right sided chamber kinetic changes (RA compared to RV) revealed prominent differences. The comparison among both atria (LA compared to RA) and both ventricles (LV compared to RV) FBs based EHTS did not show any significant differences. The comparison of these chamber specific fibroblast based EHTs against other group EHTs highlighted a significant variation in the kinetics. It reflected that age, size and passaging stage of the fibroblasts used for EHT constructs did affect the kinetic behaviour of EHTs. The fibroblasts harvested from the same environment (i.e. all four chambers within the same heart) showed a similar pattern of kinetics.

Lastly, the individual kinetic parameters are known to affect each other (chronotropy affecting inotropy and resting lengths), and this correlation was clear in the comparison as well.

CHAPTER 5: ANALYSIS OF PROTEINS SECRETION IN MEDIUM BATHING ENGINEERED HEART TISSUES

CHAPTER 5: ANALYSIS OF PROTEIN SECRETION IN MEDIUM BATHING EHTs

5.1 OVERVIEW

This chapter describes, investigation of the paracrine effect of the fibroblasts on the iPSC-CMs. Engineered heart tissues were kept in the specific medium, (EHT medium, described in chapter 2) which was changed periodically. A precise composition of EHT medium and maintenance of a near ideal micro-environment of individual EHTs, is of paramount importance for generation of their optimal kinetics. It was noted that trivial alterations such as changing the batch of horse serum in the EHT medium led to significant changes in kinetics.

Previously it was shown, that cardiomyocyte and non-cardiomyocytes interact with each other, not only via gap junctions but also through the paracrine communication. This paracrine interaction between the constituent cell population of EHTs is mediated via the medium bathing the constructs. Therefore, the protein analysis of the EHT medium can highlight the proposed variable protein expression of EHTs with fibroblasts of different origins.

To achieve this goal, initially the protein expression of freshly prepared EHT medium was evaluated. EHTs with fibroblasts of all four-chambers were submerged in the EHT medium for 48 hours (EHT medium was changed every 48 hours as part of the protocol). The discarded medium was collected and protein expression was assessed. The proteins expressed in the medium bathing all four-chamber based FBs were compared against the fresh EHT medium and variances of expression were noted. The protein expression of the CM-only and FB-only cardiomyocytes further categorised the proteins contributed by the cardiomyocytes and non-cardiomyocytes.

5.2 INTRODUCTION

Paracrine communication between cells is mediated via the extracellular signalling protein molecules such as growth factors, cytokines, and chemokines. The protein molecules released from the cells have a major role to play in the various biological processes such as cell growth, differentiation, inflammation, migration, gene expression and immunogenic response. In the majority of biological processes, the role and action of one type of excreted protein molecule (i.e. growth factor, cytokine, etc.) is dependent and regulated by either presence or absence of multiple other cytokines.

In the past, the cardiac fibroblasts have shown a potential to couple with the cardiomyocytes, express gap junctions and communication via paracrine signalling pathways (Nguyen, Kirkton and Bursac, 2016). The fibroblasts under physiological and pathological conditions (transformed to myofibroblasts) can modify size of myocyte, electrophysiological properties, excitation-contraction coupling and calcium transit times, both at cellular and tissue levels. Paracrine signalling plays a pivotal role in all these mechanisms (Long et al., 2016) (Kohl et al., 2016). Fibroblast-mediated soluble and insoluble paracrine factors were identified to promote the cardiac hypertrophy. So far, the accredited soluble paracrine factors leading to cardiac hypertrophy includes IL-6, IGF-1, endothelin-1, TGF- β and angiotensin-II. The mechanisms leading to cardiac hypertrophy are different from each other (Mayourian et al., 2017).

Recent studies have shown that bilayer membrane-bound nanovesicles called exosomes play an essential role in paracrine communication between cardiomyocytes and non-cardiomyocytes. The role played by these exosomes is cardioprotective, proangiogenic and antifibrotic but can also lead to cardiac hypertrophy (Annis, Mosher and Roberts, 2009). Cardiac fibroblasts have been recognised as a factor responsible for the release of

exosomal miRNA passenger strands, which causes hypertrophy in cardiomyocytes (Bang et al., 2014). Paracrine effect mediated by the cardiac fibroblasts leads to remodelling and downregulation of the ion channels. This causes an increased resting membrane potential, prolonged action potential, decreased upstroke velocity and reduced monolayer conduction velocity (Pedrotty et al., 2009). The precise mechanism for all these changes at the tissue and cellular level is still unidentified.

Since EHTs were constructed with a pre-calculated number of cardiomyocytes and non-cardiomyocytes, thus, the paracrine effect mediated by fibroblasts on cardiomyocyte was an interesting finding. In this study, only the soluble paracrine factors were studied.

5.3 METHODOLOGY

Proteomic profile analysis of the medium, bathing the individual engineered heart tissues was carried out at pre-determined intervals. The Proteomic Profiler Human XL Cytokine Antibody Array kit (ARY022B) was used. It contains 4 nitrocellulose membranes in a pack, and each membrane contained 102 different proteins imprinted in duplicates (capture and control antibodies). Every membrane can detect multiple cytokines, chemokines, growth factors and other soluble proteins. The membranes were exposed overnight to the extracellular proteins in the bathing medium. After rinsing the unbound proteins, the membranes were incubated with a cocktail of biotinylated detection antibodies. The subsequent exposure to Streptavidin-HRP and chemiluminescent detection agents produced a signal at each spot corresponding to the amount of protein bound. Subsequently, the individual spots were analysed against the control, and the mean pixel density was detected for expression of individual proteins. A comparison was made between all four chamber fibroblasts based EHTs.

The chemi-dots on individual membranes were analysed for signal generation and capture by Image Lab (version 5.3 & 6.0). The mean pixel density (MPD) of 2 spots for individual protein was measured and tabulated.

5.4 PROTEOMIC ANALYSIS OF FRESH EHT MEDIUM

Fresh EHT medium comprises of a mixture of constituents already mentioned in Chapter 2. A freshly prepared medium was kept in incubator (with same temperature, setting and specifications as defined for EHT incubation protocol) for 6 hours and subsequently was analysed for protein expression. The expression of proteins in this medium was independent of the proteins secreted by co-culture of cardiomyocytes and fibroblasts. This helped to identify and distinguish the proteins in the medium that were either expressed or their expression was enhanced/suppressed by bathing the EHTs.

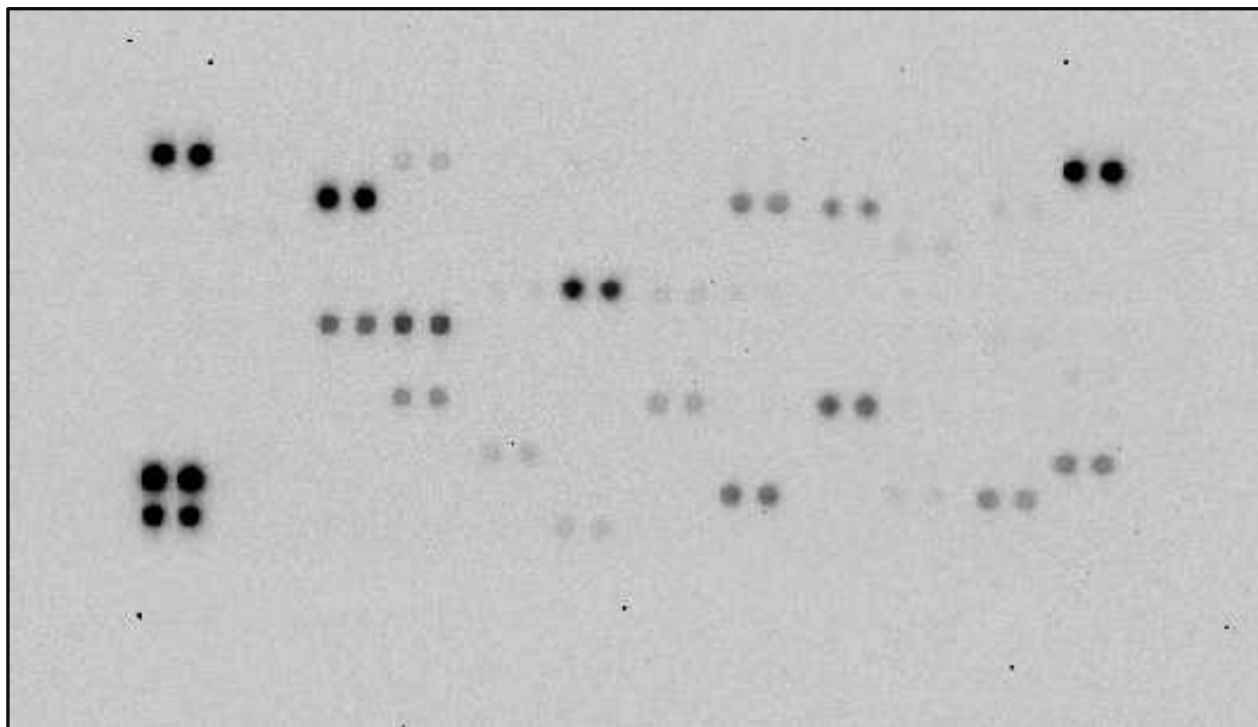


Fig. 5.1 CHEMI-DOT IMAGE OF INDIVIDUAL PROTEIN OBTAINED FOR FRESHLY PREPARED EHT MEDIUM.

The Intensity of signals depicts the Mean Pixel Density of individual protein spots.

Note: The top and bottom left corner spots are Reference Spots.

Proteins Expressed by Fresh EHT Medium			
	Proteins	Mean Pixel Density	Sign
1	Angiogenin	97856	++
2	Angiopoitin-2	41104	+
3	CHI3K1	1190320	+++++
4	Cystatin C	272432	++++
5	DKK-1	216064	++++
6	G-CSF	56704	++
7	IGFBP-2	689248	+++++
8	IGFBP-3	49472	+
9	IL-1ra	18912	-
10	IL-2	23360	+
11	IL-6	375792	++++
12	IL-8	494464	+++++
13	Kallikrein 3	28928	+
14	MCP-1	190144	+++
15	MIF	134992	+++
16	MIP-1 α , MIP-1 β	410480	+++++
17	Pentaxin	97120	++
18	SDF-1 α	262320	++++
19	Serpin E1	2354704	++++++
20	TGF- α	328048	++++
21	TNF- α	37488	+
22	μ -PAR	207888	++++
23	VCAM-1	73216	++

Table: 5.1. ANALYSIS OF SECRETION OF PROTEINS IN FRESHLY PREPARED MEDIUM FOR ENGINEERED HEART TISSUES.

The expression of proteins in the EHT medium showed a mixture of angiogenic factors, interleukins, mesodermal growth factors, extracellular membrane proteins, vascular membrane adhesion molecules and proteases inhibitors. The precise role of these individual proteins in the medium and their interaction with protein secreted by fibroblasts and cardiomyocytes still needs further experimental evaluation.

Angiogenin (ANG) is a 14-kDa extracellular plasma-based protein. It is secreted by a wide range of cells in human body and tumour cells. Its primary role is neo-angiogenesis; therefore, this allows the cell growth and formation of solid tumours. The interaction between

ANG, endothelial cells and smooth muscles leads to cell migration, invasion, proliferation, canalisation of the solid vascular structures and activation of actin in SMCs and ECs. This leads to the activation of proteolytic cascade. The activated proteases and plasmin in turn degrade the laminin and fibronectin layers of basement membrane. Moreover, it inhibits the degranulation of leucocytes. The fact that AGN is secreted by many other cells of the body, points to fact that there may be other unknown functions related to the protein which are yet to be discovered. Angiopoietin-2 is a circulating vascular growth factor secreted by endothelial and vascular smooth muscle cells in response to hypoxia and VEGF. Its expression is markedly enhanced at the site of microcirculatory capillary dysfunction and vascular remodelling.

CHI3L1 is a glycoprotein secreted by various types of cells such as SMCs, chondrocytes, macrophages, hepatic stellate cells and fibroblasts like synovial cells. The release of protein is noted in many conditions like asthma, inflammation, fibrosis, solid carcinomas and extra-T-cellular remodelling, but the precise role and mechanism of action is not known.

Cystatin-C is a non-glycosylated basic protein with the coding gene located on chromosome 20. It codes for the extracellular inhibitors of lysosomal and cysteine proteases. High concentrations are found in body fluids and it is secreted by all cells of the body. It can be used as a marker in renal impairment with better prognostic value than creatinine. Increased levels are associated with increased incidence of myocardial infarction, stroke, peripheral vascular disease and metabolic syndrome, while decreased levels can lead to rapidly developing diseases like atherosclerosis, aneurysm, lesions of aorta and pre-eclampsia.

Growth factors like IGFBP-2 and Interleukins 6 & 8 are expressed in abundance in the medium. Other proteins like MIF, MIP, SDF1 α , Serpin E1, TGF- α and μ PAR are strongly

expressed in the medium. None of these proteins has a specific predilection in the cardiac homeostasis, as they are not a part of cluster of proteins that are induced by the transcription factors. The various pathways induced by them as mentioned earlier in Chapter 3.

Protein expression in the EHT medium is a mixture of excitatory and inhibitory proteins. The acknowledged roles and function of these proteins is enlisted in the Table 5.2. The precise interaction of these proteins with other proteins secreted by the cardiomyocytes and fibroblasts is yet to be defined. Proteins, which are strongly expressed by EHT medium includes CH13L1, Cystatin C, DKK-1, IGFBP-2, IL-6, IL-8, MCP-1, MIF, MIP-1 α , MIP-1 β , SDF-1 α , Serpin E1, TGF- α , μ -PAR and VCAM-1. It is noteworthy, that all these proteins exhibit a balance between expression of excitatory and inhibitory proteins. All excitatory proteins are responsible for cellular proliferation and sustenance in different organs, but the inhibitory proteins play their role in the maintenance of a balance between cellular architectural and functional turnover. These proteins can play an excitatory and inhibitory role in different organ under different physiological and pathological conditions. Any alteration in the expression of protein sequence can alter the homeostasis and this can lead to a pathological condition. The role of these protein secretion is mainly elucidated in non-human models and their precise role in humans needs further exploration.

It is difficult to illustrate the role of these proteins and mimic the homeostatic condition in an in-vitro model; as in-vivo models secretion of proteins is very much dependant on multiple factor including temperature, acidosis, inflammation and infections etc. Moreover, the mechanism of action of proteins in different environments (i.e. in-vivo or in-vitro) are yet to be explored. The precise role of secreted proteins cannot be predicted in-vitro. The proteins that exhibit both excitatory and inhibitory role in different conditions have got predefined triggering factors in-vivo. Each protein triggers an individual pathway and cascade of factors

Proteins	Location	Secreted	Function	Role
Angiogenin	Extracellular	Almost all body cells	Angiogenesis, Increases expression of rRNA, Protein synthesis modulation, Activation of proteolytic cascade, Cell survival.	Excitatory
CH13L1	Extracellular	Macrophages, Chondrocytes, Fibroblast-like Synovial cells, Vascular smooth muscle cell and hepatic stellate cells.	Unclear. Expressed in Inflammation, Fibrosis, Remodelling, Solid carcinomas & Asthma	Excitatory
Cystatin C	Extracellular	All body tissue + fluids	Biomarker of renal function. <i>Lysosomal proteinase inhibitor. Cysteine protease.</i>	Inhibitory
DKK-1	Intracellular	Plasma cells	Embryonic development, <i>Wnt Pathway inhibitor</i> . Cranial development. Osteoblastogenesis inhibitors	Inhibitory
IGFBP-2	Intracellular Extracellular	Several tissue, Blood vessels, Skeleton	Modulation of IGF-1 actions Regulation of cell processes	Excitatory
IL-6	Extracellular	Osteoblasts, VSMC, Macrophages, Adipocytes,	Acute phase protein, Temperature modulation, Growth of B-cells, Myokine	Excitatory Inhibitory
IL-8	Intracellular	Epithelial cell, Smooth Muscle cells, endothelial cells, macrophages,	Neutrophil chemotactic factor, Neutrophilic recruitment, Neutrophilic degranulation, Angiogenesis, Oxidative burst	Excitatory
MCP-1		Endothelial cells, Monocytes, Macrophages, Dendritic cells.	Granuloma formation,	Excitatory
MIF	Extracellular	White blood cells,	Cell mediated immunity, Immunoregulation, Inflammation, Regulation of macrophage function, Integrin signalling pathway, Suppression of anti-inflammatory effect of glucocorticoids,	Excitatory Inhibitory
MIP-1α/MIP-1β	Extracellular	Macrophages, Dendritic cells, Lymphocytes	Chemokines, Proinflammatory effect, Chemotaxis, promotes haemostasis.	Excitatory
Pentaxin	Extracellular		Promotes agglutination, Bacterial capsular swelling and phagocytosis, Activation of classical complement pathway, Synaptic plasticity, Amyloidosis protein deposition, Progression of atherosclerosis.	Excitatory
SDF-1α	Intracellular	Lymphocytes, Brain, Thymus, Heart, Lung, Liver, Kidney, Spleen, Bone marrow	Chemotactic factor, Angiogenesis, Migration of neuron, Osteoclastogenesis,	Excitatory
Serpin E1	Extracellular Intracellular	Placenta, Adipose tissue, Endothelial cell,	tPA & uPA inhibitor. Blood vessel remodelling, Thrombosis, progression of fibrosis. Induces Amylin expression. Secretion of adipokines. Involvement in neuroinflammatory process.	Inhibitory
TGF-α	Intracellular	Macrophages Neurons, Keratinocytes	Epithelial cell development, Neural cell proliferation, Activate EGFR, Activate LHRH, Suppression of circadian rhythm, Negative dipsogenic effect.	Excitatory Inhibitory
μ-PAR	Intracellular	Platelets, Endothelial cells, Myocytes, Neurons	Thrombin signalling, Regulation of vascular tone and permeability, SMC regulation of vascular tone and permeability, Muscle growth, Bone cell differentiation and proliferation, Tyrosine phosphorylation.	Excitatory
VCAM-1	Intracellular	Endothelial cell	Adhesion of cells to vascular endothelium,	Excitatory

Table: 5.2. ROLE OF PROTEINS EXPRESSED IN FRESHLY PREPARED EHT MEDIUM

for executing their inhibitory or stimulating roles.

The factors expressed by freshly prepared EHT medium were a constant factor in the experiments performed on generation of EHTs. However, this constant expression helped us to find out the proteins expressed or suppressed by different EHTs. The increased expression of proteins in the medium led to conclusion that the same proteins were secreted by the constituent cell population. If the protein expression of freshly prepared medium was decreased then it was concluded that these factors were inhibited by other secreted proteins. The expression of proteins and factors was quantified by their mean pixel densities.

The unique feature of this study was that for the first time, the proteins expression of cellular and non-cellular constituent of EHTs was explored. This expression needs further systematic evaluation aided with multiple samples in a heart with good biventricular function compared against poor biventricular function. Since different pathological conditions effect cardiac tissues in different ways, therefore, EHTs from these cardiac tissues may have different protein expression.

5.5 PROTEOMIC ANALYSIS OF MEDIUM BATHING EHTs

The proteomic analysis of the medium bathing EHTs from fibroblasts of different origin was performed. The expression of protein load at each spot on the membrane was measured as pixel density. Each time point on the specific days, the protein load was a mean of two readings. The EHT medium was compared on day 2, 8 and 16 amongst all four chamber specific FBs based EHT groups. These days were selected based on the fact that most EHT groups started to contract on day 2, and maximum kinetics were attained around day 8-10. Day 16 was selected as kinetics showed a substantial decline after it (Figure 4.14, Figure. 4.24). Since all EHTs were constructed with fibroblasts from sample no. 7, therefore, this data represented a quantitative pilot study.

To further simplify the expression of proteins secreted by the chamber-specific FBs based EHTs, a system was devised to present the MPD at each point. The MPD less than 20000 was taken as negative. From 20000 and above, the representation of MPD was plotted as following.

	MEAN PIXEL DENSITY	SIGN		MEAN PIXEL DENSITY	COLOUR
1.	0 - 20000	-	1.	0-20000	
2.	20000 - 50000	+	2.	20000-500000	
3.	50000 - 100000	++	3.	50000-200000	
4.	100000 - 200000	+++	4.	200000-4000000	
5.	200000 - 400000	++++	5.	400000-1000000	
6.	More than 400000	+++++	6.	more than 1000000	

Fig. 5.2. SCHEME OF PRESENTATION OF DATA FOR TRANSCRIPTION FACTOR EXPRESSION DATA. The table shows colour codes displayed on HEAT MAPS. The strong or weak expression of proteins in EHT medium is expressed as MPD.

5.6 PROTEOMIC ANALYSIS OF MEDIUM BATHING EHTS WITH FIBROBLASTS FROM ALL FOUR CHAMBERS

The comparison of proteins expressed by EHTs with fibroblasts of different origin revealed, that many of the factors expressed were an accentuated response of the factors already existing in the bathing medium. The proteomic analysis of the freshly prepared EHT medium had shown (Table 5.1) that a cluster of 23 proteins were already present in the medium. 17 proteins, out of a total of 23 proteins were strongly expressed (depicted by their mean pixel densities), whereas the other 6 proteins (Angiopoietin-2, G-CSF, IGFBP-3, IL-1 α , Kallikrein-3, Pentaxin, TNF- α) were weakly expressed.

Expression of proteins by EHTs with atrial FBs was more constant and marked. The comparison of bi-atrial FBs based EHTs showed more consistent and defined protein expression by LA-FBs based EHTs than any other group (RA or bi-ventricular FBs based EHTs). On day-2 and day-8, the RA-FBs based EHTs showed a comparable protein secretion profile to LA-FB based EHTs, but on day-16 the protein expression was significantly decreased. EHTs with LA-FBs based EHTs showed the better kinetic response among the atrial FBs based EHTs (Chapter 4), therefore it can be hypothesised that an enhanced protein expression manifested by this group was the reason for their enhanced kinetic profile. The decreased protein secretions had a marked impact on the kinetics on day-16 (depicted by RA-FBs based EHTs). This may be attributed to the inhibitory role of proteins expressed by the RA-FBs based EHTs which mask many factors inhibitory for the inotropic and chronotropic response of EHTs. The interaction of these proteins is complex and needs further investigation as it may highlight some intricate interaction between the intracellular and extracellular proteins. In bi-atrial FBs based EHTs, LA-FBs based EHTs can be better choice for the generating EHT patches for application in the myocardial regeneration models.

	Left Atrium			Right Atrium			Left Ventricle			Right Ventricle		
	Mean Pixel Density			Mean Pixel Density			Mean Pixel Density			Mean Pixel Density		
	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16
Adiponectin	70812	111696	73482	68745	98933	0	19006	67419	0	71320	98960	0
Aggrecan	97174	136280	76855	42190	32370	0	14605	41103	0	21234	38710	0
Angiogenin	523537	497288	211311	94789	455832	59480	143273	322812	11180	29450	10880	0
Angiopoietin-1	25018	0	19988	23761	0	0	18863	19116	0	17240	0	0
Angiopoietin-2	205541	286664	218148	199752	222680	16110	68854	250677	10260	115392	202960	26723
BAFF	32557	0	17831	13213	0	0	0	23607	0	9876	19390	0
BDNF	111286	209992	180718	102367	128839	12070	70570	186619	10570	127347	154060	14335
C5/C5a	24304	0	14383	10902	20368	0	0	19116	0	0	0	0
CD14	64373	47752	34042	54340	36071	0	8749	31266	0	13206	14550	0
CD30	15078	40248	0	17732	27521	0	0	22212	0	0	22350	0
CD40L	86703	64168	105668	109341	137180	0	49426	33579	8000	135891	106650	0
CHI3L1	4646936	2394152	1348629	2674389	1804274	355470	218920	85761	13220	1327653	72970	20472
Comp Factor D	93828	30565	52231	79256	20900	0	25701	0	0	12309	20230	0
CRP	62518	36848	79268	74902	40603	0	11355	39663	0	12783	26900	0
CRIPTO-1	17710	0	0	21504	22733	0	7722	13653	0	4328	3510	0
Cystatin C	94033	57624	105371	84537	54387	0	29887	68202	0	17443	35630	0
DKK-1	306565	49768	78983	241032	62614	0	85429	50616	0	206325	55090	0
DPPIV	2455103	1589104	178457	199215	1003124	52320	1890268	483273	18800	213998	48320	0
EGF	28637	0	26353	25412	24035	0	0	18324	0	0	0	0
EMMPRIN	191338	147880	132677	174883	124583	10280	102264	123606	34700	93467	110280	0
ENA -78	230017	162680	56515	135760	41743	0	38655	25749	0	13405	0	0
Endoglin	40980	185560	102989	32302	45068	0	70232	44532	0	78610	30950	0
Fas Ligand	48675	83360	72189	13375	0	0	17745	28544	0	169730	22210	10272
FGF - 2	85050	62008	73402	70460	45068	0	23406	39024	8380	31580	22800	12236
FGF-7	39232	0	0	0	13214	0	11830	0	0	10250	0	0
FGF-19	81667	85192	35720	66552	60496	0	35698	79830	0	19297	35770	0
Flt-3 Ligand	66615	0	30201	13290	0	0	22609	0	0	18440	0	0
G-CSF	22132	33760	41695	51302	46758	0	0	46692	0	12160	28420	0
GDF-15	341752	613296	234384	317390	407928	50590	127296	269010	39290	111660	20370	0
GM-CSF	107797	36768	37962	41358	0	0	11693	42903	0	95877	12090	0
GRO - α	101145	56000	37582	88250	46980	0	21209	37494	0	24954	35640	0
GH	14115	0	0	9256	0	0	0	0	0	0	0	0

0-20000		50000-200000		400000-1000000	
20000-500000		200000-4000000		more than 1000000	

HGF	140782	44416	68466	135422	50996	0	16203	55395	0	14680	30270	0
ICAM-1	76087	0	50502	73884	37829	0	13357	29016	0	10290	17570	0
IFN- γ	282810	326904	481453	267493	211926	28910	135479	229518	75680	154638	231210	85186
IGFBP-2	1846537	1083896	1180688	1820745	1123289	189400	484198	787896	438688	316773	389840	169461
IGFBP-3	406607	379720	223867	290845	257402	26300	110214	199521	29653	108766	19970	0
IL-1 α	260032	315112	288439	280328	239409	32520	100776	240768	26240	198378	218700	31787
IL-1 β	35707	0	33487	36694	0	0	0	19386	0	0	0	0
IL-1ra	67635	64320	52867	58940	42854	0	3698	52002	0	11558	47550	0
IL-2	45967	22208	25108	60203	31863	0	6188	33543	0	10902	22835	0
IL-3	19665	19680	0	0	0	0	0	17100	0	0	4030	0
IL-4	84960	53720	34830	20945	0	0	9204	35658	0	0	0	0
IL-5	76695	168072	109860	79751	66785	0	47469	68787	0	57480	84340	22828
IL-6	1230815	1065976	364900	423482	523972	76180	313059	161352	40420	44385	23320	0
IL-8	980278	1494424	816780	1020783	1031101	124230	300449	259218	50200	26455	13860	8521
IL-10	44827	16432	37860	47463	34827	0	18070	27171	5550	0	0	0
IL-11	42562	122624	82790	40208	88084	0	38759	63864	5050	33442	55310	13176
IL-12p70	91732	36008	52100	74300	30346	0	35776	32598	7670	29399	14630	0
IL-13	34720	15296	0	13200	0	0	6669	15066	0	7609	0	0
IL-15	40957	17768	30370	42717	13946	0	22873	25182	0	16772	10530	0
IL-16	49552	22008	35990	64307	25365	0	23003	30402	0	19200	22770	0
IL-17A	103987	56680	88090	145499	74527	0	37753	49203	0	37365	48080	0
IL-18BPa	26467	25597	32570	35850	42237	0	14976	18603	0	16428	0	0
IL-19	51495	39525	29210	30240	17828	0	24537	38124	0	27312	0	0
IL-22	54500	32200	32170	44239	21679	0	24986	43353	0	0	0	0
IL-23	34560	45136	30000	35525	30713	0	11667	23112	0	0	10030	0
IL-24	28350	48984	28850	35890	15257	0	10315	17694	0	62570	46000	0
IL-27	149955	138552	207370	109640	86688	15750	61080	113229	16170	71003	115860	18078
IL-31	40852	37096	24800	37882	21603	0	17231	26010	0	14213	0	0
IL-32 $\alpha/\beta/\gamma$	84600	77984	107850	65380	59660	0	38675	52056	10550	68340	83440	0
IL-33	37455	27520	29810	31366	20330	0	24336	19746	0	15930	0	0
IL-34	17767	17576	36490	14320	25403	0	24641	11511	0	18650	0	0
IP-10	47842	32416	38660	53476	34475	0	11719	27513	0	17862	0	0
I-TAC	53145	62600	78940	46544	53751	0	23140	34029	0	35757	44330	0
Kallikrein3	103230	153208	225970	120340	167038	13360	39169	113958	20580	56811	134800	0
Leptin	98317	89778	29210	32752	0	0	0	113916	0	42670	17390	0
LIF	57262	68016	32170	32002	0	0	18954	21582	0	88386	13090	0

0-20000		50000-200000		400000-1000000	
20000-500000		200000-4000000		more than 1000000	

Lipocalin 2	110490	83448	30000	76512	57855	0	50869	61902	21870	32500	43520	14953
MCP-1	2388577	1597112	28850	261449	1683799	272710	759622	820296	450760	88367	31020	8520
MCP-3	73545	69544	182370	72583	47462	0	32610	50508	12730	34870	41720	0
M-CSF	146017	126048	24800	116605	92596	0	51954	94581	18030	50496	66870	17546
MIF	1385917	938144	107850	870420	863217	51410	508156	668847	33770	75844	23830	0
MIG	77370	51552	29810	60009	35017	0	29932	36693	0	25784	29740	0
MIP-1 α , MIP-1 β	3335745	729582	36490	880448	164986	0	1466595	386280	0	46355	18230	0
MIP-3 α	131535	67784	38660	886080	47414	0	30602	55449	0	21925	38590	0
MIP-3 β	69112	51952	78940	73857	52420	0	26494	50670	0	19430	39210	0
MMP-9	80475	79168	225970	69483	62662	0	9431	52335	0	23004	39650	0
Myeloperoxidase	88710	156624	9770	21748	46122	0	19038	40669	0	59300	69550	0
Osteopontin	177240	277192	28180	123520	158412	42690	68243	187299	27560	98450	75956	15970
PDGF- AA	118905	58128	157730	96393	42341	0	26208	43191	0	35650	32410	0
PDGF- AA/BB	28620	21800	1290520	25905	0	0	0	7677	0	0	0	0
Pentaxin	625732	489096	81200	625784	330875	43140	201220	340137	55560	230410	256400	61890
PF4	42262	39512	159250	41744	0	0	0	28926	0	9332	20530	0
RAGE	47280	34064	252800	48312	0	0	0	18513	0	0	18460	0
RANTES	101092	44416	53340	98380	40365	0	6636	31329	0	17530	13990	0
RBP-4	97162	42480	48480	97200	43519	0	17049	26406	0	12555	19120	0
Relaxin-2	100290	96218	67290	97504	91466	0	28407	69939	0	59496	92500	0
Resistan	157815	415328	46890	175428	485887	215310	70633	442784	213730	63540	295990	294129
SDF-1 α	1023232	861744	44390	95680	673181	102930	335003	488177	86260	221250	74040	0
Serpin E1	7198180	6484448	6890380	5934722	4891379	1887910	2471696	6315255	3420206	310562	249960	188720
SHBG	70805	36720	41870	124335	82279	0	0	69048	13530	73560	68190	0
ST2	76455	37312	28180	44950	16076	0	13390	22437	0	23561	31830	0
TARC	86550	75272	78100	63487	42503	0	21294	48537	0	58358	63290	0
TFF3	22770	0	0	19870	0	0	0	0	0	0	0	0
TfR	112117	141536	126440	88350	111425	0	13936	125928	0	26460	88410	0
TGF- α	35145	31200	33160	27980	0	0	0	18189	0	41210	26840	0
Thrombospondin	1508962	933208	634560	105360	372124	68780	519467	375543	66900	74560	25790	0
TNF- α	25897	19280	0	15430	0	0	0	0	0	0	0	0
μ -PAR	142665	88608	69840	71009	37880	0	17615	28485	0	42740	54720	0
VEGF	314797	283832	206790	325824	288439	73140	15892	85527	10890	0	0	0
Vit-D BP	158512	277280	237560	81423	114133	9950	78810	163377	13990	93680	171303	0

Table: 5.3. HEAT MAP PRESENTATION OF SECRETION OF PROTEIN IN MEDIUM BATHING ENGINEERED HEART TISSUES. Data is expressed as mean pixel density. Fibroblasts from all four-chambers were co-cultured with cardiomyocytes of same origin. The heat map clearly showed a variability of expression of proteins among all four-chambers FBs based EHTs. The expression of proteins was stronger in the atrial FBs based EHTs in comparison with the ventricular FBs based EHTs. (n = 3 calculated for 3 different day).

Table. 5.4. COMPARISON OF SELECTION OF SECRETED PROTEIN AMONG CHAMBER-SPECIFIC FBs BASED EHTs.

		Left Atrium - MPD			Right Atrium - MPD			Left Ventricle - MPD			Right Ventricle - MPD		
		Day 2	Day 8	Day 16	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16
1	Angiogenin	+++++	+++++	++++	++	++	++	+++	++++	+	+	+	-
2	Angiopoietin-2	++++	++++	++++	+++	++++	+	++	++++	+	+++	++++	+
3	BDNF	+++	++++	+++	+++	+++	+	++	+++	+	+++	+++	+
4	CHI3L1	+++++	+++++	+++++	+++++	+++++	++++	++++	++	+	+++++	++	+
5	DKK-1	++++	++	++	++++	++	-	++	++	-	++++	++	-
6	DPPIV	+++++	+++++	++++	+++	+++++	++	+++++	+++++	+	++++	++	-
7	EMMPRIN	+++	+++	+++	+++	+++	+	+++	+++	+	++	+++	-
8	ENA-78	++++	+++	++	+++	++	-	+	+	-	+	-	-
9	GDF-15	++++	+++++	++++	++++	+++++	++	+++	++++	+	+++	+	-
10	IFN- γ	++++	++++	+++++	++++	++++	+	+++	++++	++	+++	++++	++
11	IGFBP-2	+++++	+++++	+++++	+++++	+++++	+++	+++++	+++++	+++++	++++	++++	+++
12	IGFBP-3	+++++	++++	++++	++++	++++	+	+++	+++	+	+++	+	-
13	IL-1 α	++++	++++	++++	+++++	++++	+	+++	++++	+	+++	++++	+
14	IL-6	+++	+++	++++	++++	+++++	++	++++	+++	++	++	+	-
15	IL- 8	++	+++	++	+++	+++	+++	++++	++++	++	+	+	-
16	IL - 27	+++	+++	++++	+++	++	+	++	+++	+	++	+++	+
17	Kallikrein3	+++	+++	++++	+++	+++	+	+	+++	+	++	+++	-
18	MCP-1	+++++	+++++	+	++++	+++++	++++	+++++	+++++	+++++	++	+	-
19	MIF	+++++	+++++	+++	+++++	+++++	++	+++++	+++++	++++	++	+	-
20	MIP-1 α , MIP-1 β	+++++	+++++	+	+++++	+++	-	+++++	++++	-	++	+	-
21	Osteopontin	++++	++++	+	+++	+++	++	++	+++	+	++	++	+
22	Pentaxin	+++++	+++++	++	+++++	++++	++	++++	++++	+++	++++	++++	++
23	Resistan	+++	+++++	++	+++	+++++	++++	++	+++++	++++	++	++++	++++
24	SDF - 1 α	+++++	+++++	++	++	+++++	+++	++++	+++++	++	++++	++	-
25	Serpin E1	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	++++	++++	+++
26	Thrombospondin	+++++	+++++	+++++	+++	++++	++	+++++	++++	++++	+++	+	-
28	VEGF	+++++	++++	++++	++++	++++	++	+	++	+	-	-	-
29	Vit - D BP	+++	++++	++++	++	+++	-	++	+++	+	++	+++	-

	Left Atrium			Right Atrium - MPD			Left Ventricle - MPD			Right Ventricle - MPD		
	Mean Pixel Density			Mean Pixel Density			Mean Pixel Density			Mean Pixel Density		
	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16
Adiponectin	+++++	+++++	++++	++	++	++	+++	++++	+	+	+	-
Aggrecan	++++	++++	++++	+++	++++	+	++	++++	+	+++	++++	+
Angiogenin	+++	++++	+++	+++	++++	+	++	+++	+	+++	+++	+
Angiopoietin-1	+++++	+++++	+++++	+++++	+++++	+++++	+++++	++	+	+++++	++	+
Angiopoietin-2	++++	++	++	++++	++	-	++	++	-	++++	++	-
BAFF	+++++	+++++	++++	+++	+++++	++	+++++	+++++	+	++++	++	-
BDNF	+++	+++	+++	+++	+++	+	+++	+++	+	++	+++	-
C5/C5a	++++	+++	++	+++	++	-	+	+	-	+	-	-
CD14	++++	+++++	++++	++++	+++++	++	+++	++++	+	+++	+	-
CD30	++++	++++	+++++	++++	++++	+	+++	++++	++	+++	++++	++
CD40L	+++++	+++++	+++++	+++++	+++++	+++	+++++	+++++	+++++	++++	++++	+++
CHI3L1	+++++	++++	++++	++++	++++	+	+++	+++	+	+++	+	-
Comp Factor D	++++	++++	++++	+++++	++++	+	+++	++++	+	+++	++++	+
CRP	+++	+++	++++	++++	+++++	++	++++	+++	++	++	+	-
CRIPTO-1	++	+++	++	+++	+++	+++	++++	++++	++	+	+	-
Cystatin C	+++	+++	++++	+++	++	+	++	+++	+	++	+++	+
DKK-1	+++	+++	++++	+++	+++	+	+	+++	+	++	+++	-
DPPIV	+++++	+++++	+	++++	+++++	++++	+++++	+++++	+++++	++	+	-
EGF	+++++	+++++	+++	+++++	+++++	++	+++++	+++++	++++	++	+	-
EMMPRIN	+++++	++++	+	+++++	+++	-	+++++	++++	-	++	+	-
ENA -78	++++	++++	+	+++	+++	++	++	+++	+	++	++	+
Endoglin	+++++	+++++	++	+++++	++++	++	++++	++++	+++	++++	++++	++
Fas Ligand	+++	+++++	++	+++	+++++	++++	++	+++++	++++	++	++++	++++
FGF - 2	+++++	+++++	++	++	+++++	+++	++++	+++++	++	++++	++	-
FGF-7	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	++++	++++	+++
FGF-19	+++++	+++++	+++++	+++	++++	++	+++++	++++	++++	+++	+	-
Flt-3 Ligand	+++++	++++	++++	++++	++++	++	+	++	+	-	-	-
G-CSF	+++	++++	++++	++	+++	-	++	+++	+	++	+++	-

Table. 5.5. HEAT MAP OF SELECTION OF SECRETED PROTEINS AMONG CHAMBER-SPECIFIC FBs BASED EHTs. Data is expressed as mean pixel density. The expression of proteins by atrial FBs based EHTs is comparable (LA & RA) and better than the ventricular fibroblasts based EHTs. In ventricular FBs based groups, the expression of proteins by LV-FBs based EHTs supersedes RV-FBs based EHTs. (n = 3 calculated for 3 different day).

The LV-FBs based EHTs showed an enhanced protein expression on all three days as compared to the response elicited by RV-FBs laden EHTs. The secretion profile of LV-FBs based EHTs on day 16 exhibited a precipitous decline in comparison with the previous 2 days (day-2, day-8). However, this response was still better than RV-FBs based EHTs on day 16. (Table. 5.3, 5.4, 5.5)

The protein expression of RV-FBs based EHTs on all three days (day 2, day 8 & day 16) was far less in comparison with the EHTs with other FBs groups (LA, RA and LV). In spite of this weak and meagre protein expression, the RV-FBs based EHTs depicted the best kinetic response (Chapter no. 4). This can lead to the conclusion, that the expression of inhibitory proteins was more profound in other groups. As the overall expression of proteins including the inhibitory proteins was scanty RV-FBs based EHTs, therefore they exhibited improved kinetics. It implies that RV-FBs based EHTs can be used for construction patches of EHTs for experiments in future for myocardial rejuvenation. A comparison of RV-FBs based EHTs and LA-FBs based EHTs will be an interesting finding in experimental myocardial regeneration.

The proteins secreted into the medium bathing EHTs bear a significant impact on the growth and kinetics of these contractile units. The soluble proteins in the medium interacted in a paracrine manner with the EHTs on the scaffolds. In addition to nourishment of the EHTs, these proteins have an impact on the secretory profile of these EHTs. When the bathing medium was analysed at set intervals, it depicted that most of the factors in the freshly prepared medium were augmented. It eludes to the fact that CMs and FBs secrete the same proteins as well and hence the expression of proteins in the medium were amplified. In the final four-chamber EHTs, a group of 16 proteins (Table 5.4 & 5.5) were already detected in the medium. Cystatin C was highly expressed in freshly prepared medium but was not expressed by the cellular constituents of EHTs and hence there was a weak expression in all

chamber-specific FBs based EHTs at any given day (Table. 5.4 & 5.5). This effect is more marked in the ventricular FBs based EHTs than atrial FBs based EHTs.

The factors which were highly expressed by chamber-specific FBs based EHTs on day 2, 8, and 16 (and were absent in the freshly prepared medium) included BDNF, DPP4, EMMPRIN, ENA-78, GDF-15, IFN- γ , IL-27, Osteopontin, Resistin, Thrombospondin, VEGF and Vit-DBP. BDNF is neurotropic growth factor located in brain, peripheral nerves and is associated with growth and differentiation of new neurons and synapses. DPP4 is an antigenic enzyme expressed by a large number of cells and regulates immune regulation, signal transduction and apoptosis. EMMPRIN (CD147) is an extracellular metalloprotein inducer. It is a determinant of ABO blood group system and retinal pigmented epithelium maintenance. GDF-15 is constantly expressed by most of the organs in low concentrations and is upregulated after injury. It plays a role in the regulation of inflammatory pathway and is involved in apoptosis. IFN- γ is a soluble cytokine required for the intrinsic and acquired immune response. Osteopontin and Thrombospondin are extracellular glycoproteins involved in maintenance of homeostasis and angiogenesis respectively. VEGF is involved in the vasculogenesis. Vit D-BP is a globulin with different function in different organ systems. All these factors are present in almost all tissues and are non-specific for the cardiac cellular population and their functional activities. In the expression of all these proteins, atrial FB based EHTs supersedes the ventricular FB based EHTs.

It is noteworthy that vascular growth factors such as, Angiogenin, Angiopoietin-2 and VEGF are better expressed by the atrial FBs based EHTs on all three days. Among the ventricular FBs based EHTs, their expression is relatively better on day-2 and day-8 but then fades away after that. VEGF was released by the EHTs as this was not present in the EHT medium. Hence, this can be the source of angiogenesis inducing factor in the EHT patches. The

expression of proteins already present in the EHT medium were difficult to quantify in terms of its accentuation whilst bathing the EHTs. However, the better expression of all the known proteins in Table 5.4 can be interpreted, that they are secreted in abundance by the cellular population of EHTs.

	Cardiomyocytes Only EHTs			Fibroblast Only EHTs		
	Mean Pixel Density			Mean Pixel Density		
	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16
Adiponectin	129180	101167	179750	106601	54703	27940
Aggrecan	433700	308770	601260	64405	37026	26950
Angiogenin	256546	205952	345653	162393	96679	58025
Angiopoietin-1	38241	32010	55726	29777	17127	12133
Angiopoietin-2	192507	144161	267476	191565	99858	59554
BAFF	94003	89870	164648	33825	11363	8723
BDNF	575116.5	448134	816838	130163	82236	50446
C5/C5a	129570	102080	187858	24420	9768	6336
CD14	66129	42614	89111	52459	21846	13574
CD30	53980.5	45760	82218	64515	35387	21384
CD40L	72523	57002	99011	82159	37257	31262
CHI3L1	4702038	3626612	6134304	141636	78793	43054
Comp Factor D	78518	49753	75966	23573	10813	9603
CRP	39479	31658	57816	46662	27192	16511
CRIPTO-1	19448	16555	28974	35695	19998	13277
Cystatin C	124740	100738	173756	87879	45727	27291
DKK-1	1429296	1060708	1780240	133342	72226	46893
DPPIV	471086	356928	639132	85943	45749	37521
EGF	61556	96580	85976	22319	14773	8965
EMMPRIN	74613	91993	158488	289586	160468	85063
ENA -78	44979	32098	59884	36619	25542	13761
Endoglin	246576	169114	257785	41118	26081	12716
Fas Ligand	34716	31185	43956	38632	26356	13904
FGF - 2	52129	37400	67243	41855	25982	11792
FGF-7	27060	35101	38764	27533	19569	9933
FGF-19	102740	72908	131193	77660	44088	22473
Flt-3 Ligand	31141	24013	32185	17853	13497	9922
G-CSF	24068	24640	39072	25949	16929	18282
GDF-15	469832	350306	425656	208604	115203	70598
GM-CSF	46541	35145	54582	28149	23408	17325
GRO - α	42394	28941	58685	15818	8217	7183
GH	22033	14223	32995.5	26268	9559	6974
HGF	82814	83688	216194	64537	36124	20625

0-20000		50000-200000		400000-1000000	
20000-500000		200000-4000000		more than 1000000	

ICAM-1	40700	43714	115005	25927	12562	8140
IFN- γ	156310	112442	208681	136961	82137	45639
IGFBP-2	2742498	2098712	3865952	2645654	1563375	891726
IGFBP-3	111232	78012	146514	70642	38984	25190
IL-1 α	103246	76923	142373	104665	63668	36080
IL-1 β	20240	16203	30140	22671	10318	5687
IL-1ra	30591	24497	49896	20207	11660	8085
IL-2	20471	23408	47322	28281	15906	9757
IL-3	20801	18326	32527	19657	10769	12254
IL-4	20350	18513	24618	12232	9416	9086
IL-5	184470	142560	265507	127138	70224	39864
IL-6	1583208	1211669	2032052	44088	27852	14168
IL-8	639034	485329	866822	74712	47014	27467
IL-10	26532	31702	35871	20119	16456	11385
IL-11	72765	135817	125543	167585	108966	61699
IL-12p70	42878	38280	50325	48312	27225	19690
IL-13	16544	14498	22561	13277	11165	6006
IL-15	18553	22969	33858	29689	16687	10362
IL-16	23452	21626	36311	22935	13057	10967
IL-17A	83872	65230	126555	101618	58421	32439
IL-18BP α	40224	52701	50127	37301	14014	7315
IL-19	50270	31834	70258	34056	16071	10439
IL-22	33638	31790	57904	27544	17457	9482
IL-23	35651	108449	108757	16467	10527	5863
IL-24	155276	37070	204292	40238	27060	19723
IL-27	43362	37895	63921	45188	26334	19580
IL-31	18964	14190	21165	19085	13970	8074
IL-32 $\alpha/\beta/\gamma$	29612	29106	53867	84568	50952	33407
IL-33	17996	15565	31009	21450	11066	10142
IL-34	26149.5	29590	65285	52690	30844	18205
IP-10	35024	26818	52536	22484	17237	11187
I-TAC	32054	30305	63811	39028	22088	13563
Kallikrein3	80894	97317	176904.5	145882	84469	50160
Leptin	27835	20988	36223	8734	3894	3421
LIF	25982	19987	36113	8239	5874	4268
Lipocalin 2	133892	96459	174361	119746	66803	39600
MCP-1	1725812	1481260	2576816	389785	210210	120835
MCP-3	65681	31866	81939	40084	24519	15048

0-20000		50000-200000		400000-1000000	
20000-500000		200000-4000000		more than 1000000	

M-CSF	98062	82467	143759	97504	62667	28754
MIF	83457	58905	107492	1157783	627088	359161
MIG	39710	22869	52822	29183	16280	16720
MIP-1 α , MIP-1 β	21009.5	13453	43637	226798	122364	71533
MIP-3 α	55858	29601	74316	41503	27258	17358
MIP-3 β	55011	62601	86273	48334	27918	18403
MMP-9	59465	39809	92719	69509	40590	26642
Myeloperoxidase	174240	171655	192792	666501	369567	134563
Osteopontin	64526	53592	110022	43461	22627	9141
PDGF- AA	79376	62568	116710	92400	47036	27533
PDGF- AA/BB	21065	16327	28831	10659	7964	3905
Pentaxin	972323	796565	1362713	181203	104709	59730
PF4	44737	32263	56650	20372	12716	7524
RAGE	23199	21802	40502	19690	14322	8657
RANTES	146498	181269	331540	74668	36949	16577
RBP-4	27335	20438	42782	21549	12023	7711
Relaxin-2	60743	41954	85910	53449	31515	17732
Resistan	1535590	1158366	2078208	987360	547569	300234
SDF-1 α	358329	276848	485980	567292	339306	181874
Serpin E1	6203681	4887861	7474335	2719816	2119623	1342330
SHBG	96151	61125	126423	44572	28171	16302
ST2	27533	17341	33726	11627	8228	5192
TARC	42504	29183	72809	35409	18337	12672
TFF3	53427	35915	91311	16533	9977	7744
TfR	89309	70477	145717	81983	70719	31548
TGF- α	38203	23452	54087	31427	16093	8349
Thrombospondin	1325148	972708	1732918	1151799	672573	382591
TNF- α	25861	16654	34408	21373	9680	5192
μ -PAR	125048	67727	180906	37356	24882	14674
VEGF	71841	64108	115060	645744	373340	201509
Vit-D BP	105952	81826	164104	61204	31119	19129

Table: 5.6. HEAT MAP REPRESENTATION PROTEINS SECRETED IN MEDIUM BATHING ENGINEERED HEART TISSUES CONTAINING FBs-ONLY AND CMs-ONLY. Data is expressed as mean pixel densities (n = 3 calculated for 3 different day). Expression of proteins by CM-only based EHTs showed, enhanced expression of proteins in comparison with FB-only based EHTs for all three day.

5.7. COMPARISON OF SECRETED PROTEINS AMONG CM-ONLY & FB-ONLY BASED EHTs AGAINST THE PROTEINS IN EHT MEDIUM

The comparison of protein excreted in medium bathing the CM-only and FB-only based EHTs have highlighted the factors and proteins released by cardiomyocytes and fibroblasts. These proteins were different from the protein already present in the EHT medium. A comparison of proteins present in fresh EHT medium was made against the proteins secreted by the cardiomyocyte only EHTs. A similar comparison is made between EHT medium and fibroblast only EHTs in Table 5.6 & 5.7.

It was clear that angiogenic factor were expressed by cardiomyocytes as they were not expressed by the EHT medium and fibroblast only EHTs. Angiogenin which is expressed just moderately in EHT medium was expressed strongly by cardiomyocytes but were not expressed by fibroblasts, as the expression was almost comparable to the EHT medium. Angiopoietin-2 was weakly in the EHT medium, but a strong expressed was noted in cardiomyocytes. Again, the fibroblasts exhibited a weak and comparable expression to the EHT medium.

Factors like CH13L1, Cystatin C and DKK-1 were only present in the EHT medium and were not expressed by the cardiomyocytes, but the expression remained sustained in the media in all three days. The proteins were rather inhibited in the FB-only EHTs as expression was weak to begin with and later on progressively diminished. This led to conclusion that although cardiomyocytes are not be secreting theses factor but equally, they did not inhibit them. Similarly, IGFBP-2 was expressed by EHT medium and was not secreted by the cardiomyocytes. The FB-only EHTs expressed it strongly and hence it was concluded that it not inhibited by both these groups of EHTs. IGFBP-3 was secreted by was cardiomyocyte as expression is moderate in CM-only EHTs, but FB-only EHTs have a faint and comparable

expression to the EHT medium.

Expression of interleukins like IL-6 and IL-8 was strongly exhibited by the EHT medium and this expression was maintained by cardiomyocytes. This eludes that cardiomyocytes are not excreting these interleukins but nonetheless they are not inhibiting it. However, the expression of these interleukins was strongly inhibited by fibroblasts. The expression of Kallikrein-3 in EHT medium was comparable to the CM-only based EHTs but FB-only EHTs showed an accentuated response. Similarly, MCP-1 was present in EHT medium and was not expressed by cardiomyocytes and fibroblasts. MIF, MIP-1 α and MIP-1 β were secreted and strongly expressed by FB-only EHTs in comparison with CM-only EHTs and fresh medium. Since, they are not secreted by cardiomyocytes therefore FB-only EHTs will have an inhibitory effect on the macrophages.

Pentaxin had a moderate expression in EHT medium and fibroblasts-only EHTs but was moderately expressed by cardiomyocytes. Hence, in EHTs, the expression was mainly contributed by the cardiomyocytes. SDF-1 α and Serpin-E1 were released by EHT medium and are expressed in the same manner in both group of EHTs and therefore are less likely to be secreted by cardiomyocyte and fibroblast only EHTs.

Proteins like TGF- α and μ -PAR, which were strongly expressed by EHT medium, were not present in the cardiomyocyte and fibroblast only EHTs. It was assumed that their expression is suppressed by fibroblasts as FB-only EHTs don't express these two factors at all (Table 5.6 & Table 5.7).

		Freshly Prepared EHT Medium	Cardiomyocytes Only EHTs			Fibroblast Only EHTs		
		Mean Pixel Density	Mean Pixel Density			Mean Pixel Density		
			Day 2	Day 8	Day 16	Day 2	Day 8	Day 16
1	Adiponectin		+++	+++	+++	+++	++	+
2	Aggrecan		+++++	++++	+++++	++	+	+
3	Angiogenin	++	++++	++++	++++	+++	++	++
4	Angiopoietin-2	+	+++	+++	++++	+++	++	++
5	BDNF		+++++	+++++	+++++	+++	++	++
6	C5/C5a		+++	+++	+++	+	-	-
7	CHI3L1	+++++	+++++	+++++	+++++	+++	++	+
8	Cystatin C	++++	+++	+++	+++	++	+	+
9	DKK-1	++++	+++++	+++++	+++++	+++	++	+
10	DPPIV		+++++	++++	+++++	++	+	+
11	EMMPRIN		++	++	+++	++++	+++	++
12	Endoglin		++++	+++	++++	+	+	-
13	FGF-19		+++	++	+++	++	+	+
14	GDF-15		+++++	++++	+++++	++++	+++	++
15	HGF		++	++	++++	++	+	+
16	IFN- γ		+++	+++	++++	+++	++	+
17	IGFBP-2	+++++	+++++	+++++	+++++	+++++	+++++	+++++
18	IGFBP-3	+	+++	++	+++	++	+	+
19	IL-1 α	-	+++	++	+++	+++	++	+
20	IL-5		+++	+++	++++	+++	++	+
21	IL-6	++++	+++++	+++++	+++++	+	+	-
22	IL-8	+++++	+++++	+++++	+++++	++	+	+
23	IL-11		++	+++	+++	+++	+++	++
24	IL-17A		++	++	+++	+++	++	+
25	Kallikrein3	+	++	++	+++	+++	++	++
26	Lipocalin 2		+++	++	+++	+++	++	+
27	MCP-1	+++	+++++	+++++	+++++	++++	++++	+++
28	MIF	+++	++	++	+++	+++++	+++++	+++++
29	MIP-1 α , MIP-1 β	+++++	+	-	+	++++	+++	++
30	Myeloperoxidase		+++	+++	+++	+++++	+++++	+++
31	Pentaxin	++	+++++	+++++	+++++	+++	+++	++
32	RANTES		+++	+++	++++	++	+	-
33	Resistan		+++++	+++++	+++++	+++++	+++++	+++++
34	SDF-1 α	++++	++++	++++	+++++	+++++	++++	+++
35	Serpin E1	+++++	+++++	+++++	+++++	+++++	+++++	+++++
36	Thrombospondin		+++++	+++++	+++++	+++++	+++++	+++++
37	VEGF		++	++	+++	+++++	++++	++++

Table. 5.7. COMPARISON OF PROTEINS SECRETED BY CARDIOMYOCYTES-ONLY AND FIBROBLASTS ONLY BASED EHTs WITH FRESHLY PREPARED EHT MEDIUM. Most of the proteins secreted in the bathing medium of EHTs were originating from cardiomyocytes. Expression of proteins from FB-only EHT was marked on day-2 but later on there was a precipitous decline in the expression on day-8 & day-16. (n = 3 calculated for 3 different day).

5.8. COMPARISON OF SECRETED PROTEINS AMONG CM-ONLY AND ALL FOUR CHAMBERS FIBROBLASTS BASED EHTs.

Adiponectin, Aggregan, Endoglin, FGF-19, HGF, IL-11, IL-17, Myeloperoxidases, Lipocalin and Rantes were secreted by CM-only EHTs. These proteins were absent in all four-chamber FBs based EHTs and fresh EHT medium. Hence, these factors were secreted by cardiomyocytes and inhibited by fibroblasts. Angiogenic factors like Angiogenin and Angiopoitin-2 were secreted predominantly by cardiomyocytes and were strongly expressed in left sided chambers (LA & LV, FBs based EHTs) during all three days of assessment but were weakly expressed in right sided FBs based EHTs. This can lead to conclusion that left sided FBs based EHTs could be better for promotion of angiogenesis in experimental designs in future. Complement factors such as C5 & C5a, secreted and expressed by cardiomyocytes, were absent in chamber-specific fibroblasts based EHTs and hence were completely inhibited by fibroblasts. Cystatin C, present in EHT medium is not inhibited by CM-only EHTs, as its expression persisted. But once fibroblasts were cocultured with cardiomyocytes, it got inhibited by fibroblasts and consequently was missing in all four-chambers fibroblast based EHTs (Table 5.8).

IL-6 (an inhibitory protein) and IL-8 (an excitatory protein) expressed by EHT medium, persisted continuously throughout the life span of three chamber-specific FBs based EHTs (i.e. LA-FBs, RA-FBs and LV-FBs based EHTs). The expression was suppressed by RV-FB based EHTs. The strong expression of MCP-1 in EHT medium was accentuated by cardiomyocytes. The expression was stronger in the left sided EHTs and was weakly expressed in right sided EHTs. Pentaxin expression in EHT medium and chamber-specific FBs based EHTs was similar for IL-1. SDF-1 α , Serpin-E1 and Thrombospondin. These proteins were secreted by cardiomyocytes and the expression remained comparable in LA, RA and LV FBs based EHTs but were inhibited by RV-FBs based EHTs (Table 5.8).

Table. 5.8. COMPARISON OF PROTEINS SECRETED BY CARDIOMYOCYTES-ONLY AND CHAMBER SPECIFIC FBs BASED EHTs.

		CM-Only EHTs			Left Atrium			Right Atrium		
		Mean Pixel Density			Mean Pixel Density			Mean Pixel Density		
		Day 2	Day 8	Day 2	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16
1	Adiponectin	+++	+++	+++						
2	Aggrecan	+++++	++++	+++++						
3	Angiogenin	++++	++++	++++	+++++	+++++	++++	++	++	++
4	Angiopoietin-2	+++	+++	++++	++++	++++	++++	++++	+	++++
5	BDNF	+++++	+++++	+++++	+++	++++	+++	+++	+	+++
6	C5/C5a	+++	+++	+++						
7	CHI3L1	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++
8	Cystatin C	+++	+++	+++						
9	DKK-1	+++++	+++++	+++++	++++	++	++	++	-	++
10	DPPIV	+++++	++++	+++++	+++++	+++++	++++	+++++	++	+++++
11	EMMPRIN	++	++	+++	+++	+++	+++	+++	+	+++
12	Endoglin	++++	+++	++++						
13	FGF-19	+++	++	+++						
14	GDF-15	+++++	++++	+++++	++++	+++++	++++	+++++	++	+++++
15	HGF	++	++	++++						
16	IFN- γ	+++	+++	++++	++++	++++	+++++	++++	+	++++
17	IGFBP-2	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++	+++++
18	IGFBP-3	+++	++	+++	+++++	++++	++++	++++	+	++++
19	IL-1 α	+++	++	+++	++++	++++	++++	++++	+	++++
20	IL-5	+++	+++	++++						
21	IL-6	+++++	+++++	+++++	+++	+++	++++	+++++	++	+++++
22	IL-8	+++++	+++++	+++++	++	+++	++	+++	+++	+++
23	IL-11	++	+++	+++						
24	IL-17A	++	++	+++						
25	Kallikrein3	++	++	+++	+++	+++	++++	+++	+	+++
26	Lipocalin 2	+++	++	+++						
27	MCP-1	+++++	+++++	+++++	+++++	+++++	+	+++++	+++++	+++++
28	MIF	++	++	+++	+++++	+++++	+++	+++++	++	+++++
29	MIP-1 α , MIP-1 β	+	-	+	+++++	+++++	+	+++	-	+++
30	Myeloperoxidase	+++	+++	+++						
31	Pentaxin	+++++	+++++	+++++	+++++	+++++	++	++++	++	++++
32	RANTES	+++	+++	++++						
33	Resistan	+++++	+++++	+++++	+++	+++++	++			
34	SDF-1 α	++++	++++	+++++	+++++	+++++	++	+++++	+++	+++++
35	Serpin E1	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++
36	Thrombospondin	+++++	+++++	+++++	+++++	+++++	+++++	++++	++	++++
37	VEGF	++	++	+++	+++++	++++	+++++	++++	++	++++
38	ENA-78				++++	+++	++	++	-	++
39	IL - 27				+++	+++	++++	++	+	++
40	Vit-DBP				+++	++++	++++	+++	-	+++
41	Osteopontin				+++++	++++	+	+++	++	+++

		CM-Only EHTs			Left Ventricle			Right Ventricle		
		Mean Pixel Density			Mean Pixel Density			Mean Pixel Density		
		Day 2	Day 8	Day 2	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16
1	Adiponectin	+++	+++	+++						
2	Aggrecan	++++	++++	++++						
3	Angiogenin	++++	++++	++++	+++	++++	+	+	+	-
4	Angiopoietin-2	+++	+++	++++	++	++++	+	+++	++++	+
5	BDNF	++++	++++	++++	++	+++	+	+++	+++	+
6	C5/C5a	+++	+++	+++						
7	CHI3L1	++++	++++	++++	++++	++	+	++++	++	+
8	Cystatin C	+++	+++	+++						
9	DKK-1	++++	++++	++++	++	++	-	++++	++	-
10	DPPIV	++++	+++	++++	++++	++++	+	++++	++	-
11	EMMPRIN	++	++	+++	+++	+++	+	++	+++	-
12	Endoglin	++++	+++	++++						
13	FGF-19	+++	++	+++						
14	GDF-15	++++	++++	++++	+++	++++	+	+++	+	-
15	HGF	++	++	++++						
16	IFN- γ	+++	+++	++++	+++	++++	++	+++	++++	++
17	IGFBP-2	++++	++++	++++	++++	++++	++++	++++	++++	+++
18	IGFBP-3	+++	++	+++	+++	+++	+	+++	+	-
19	IL-1 α	+++	++	+++	+++	++++	+	+++	++++	+
20	IL-5	+++	+++	++++						
21	IL-6	++++	++++	++++	++++	+++	++	++	+	-
22	IL-8	++++	++++	++++	++++	++++	++	+	+	-
23	IL-11	++	+++	+++						
24	IL-17A	++	++	+++						
25	Kallikrein3	++	++	+++	+	+++	+	++	+++	-
26	Lipocalin 2	+++	++	+++						
27	MCP-1	++++	++++	++++	++++	++++	++++	++	+	-
28	MIF	++	++	+++	++++	++++	++++	++	+	-
29	MIP-1 α , MIP-1 β	+	-	+	++++	++++	-	++	+	-
30	Myeloperoxidase	+++	+++	+++						
31	Pentaxin	++++	++++	++++	++++	++++	+++	++++	++++	++
32	RANTES	+++	+++	++++						
33	Resistan	++++	++++	++++	++	++++	++++	++	++++	++++
34	SDF-1 α	++++	++++	++++	++++	++++	++	++++	++	-
35	Serpin E1	++++	++++	++++	++++	++++	++++	++++	++++	+++
36	Thrombospondin	++++	++++	++++	++++	++++	++++	+++	+	-
37	VEGF	++	++	+++	+	++	+	-	-	-
38	ENA-78				+	+	-	+	-	-
39	IL - 27				++	+++	+	++	+++	+
40	Vit-DBP				++	+++	+	++	+++	-
41	Osteopontin							++	++	+

5.9. COMPARISON OF SECRETED PROTEINS AMONG FIBROBLASTS-ONLY EHTs AND ALL FOUR CHAMBERS FIBROBLASTS BASED EHTs.

The expression of proteins by FB-only EHTs was weak and inhibition of many proteins was more distinctive than in other EHT groups. Some proteins like IL-11, IL-17A were secreted by fibroblasts but are absent in the chamber specific EHTs altogether. This was possibly attributed to the fact that these proteins are inhibited by factors secreted by cardiomyocytes. Enzymes like myeloperoxidases were highly expressed by FBs but were inhibited by cardiomyocytes as well. Protein such as Resistin and Osteopontin were expressed by fibroblasts in abundance, but were inhibited by RA-FBs based EHTs only. This can be the possible reason that RV-FBs based EHTs revealed the best kinetics. Proteins present in EHT medium were uninhibited by fibroblasts and continued to persist in their expression in chamber-specific EHTs. While proteins secreted solely by cardiomyocytes were partially inhibited by fibroblasts more than the proteins expressed in EHT medium.

Although this data is semi-quantitative but has provided us with a valuable insight into the secretion profile of individual constituent cells. The excitatory and inhibitory effects of these proteins were postulated based on observation of recurrence of expression but their conclusive pathways and interactions have yet to be defined and proven. The paracrine effect of fibroblasts dictates the functioning of cardiomyocytes and vice versa. This is function is in addition to the architectural support that is provided by fibroblasts (Table 5.9).

Table. 5.9. COMPARISON OF PROTEINS SECRETED BY FIBROBLASTS-ONLY AND CHAMBER SPECIFIC FBs BASED EHTs.

		Fibroblast Only EHTs			Left Atrium			Right Atrium		
		Mean Pixel Density			Mean Pixel Density			Mean Pixel Density		
		Day 2	Day 8	Day 2	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16
1	Adiponectin	+++	++	+						
2	Aggrecan	++	+	+						
3	Angiogenin	+++	++	++	+++++	+++++	++++	++	++	++
4	Angiopoietin-2	+++	++	++	++++	++++	++++	+++	++++	+
5	BDNF	+++	++	++	+++	++++	+++	+++	+++	+
6	C5/C5a	+	-	-						
7	CHI3L1	+++	++	+	+++++	+++++	+++++	+++++	+++++	+++++
8	Cystatin C	++	+	+						
9	DKK-1	+++	++	+	++++	++	++	++++	++	-
10	DPPIV	++	+	+	+++++	+++++	++++	+++	+++++	++
11	EMMPRIN	++++	+++	++	+++	+++	+++	+++	+++	+
12	Endoglin	+	+	-						
13	FGF-19	++	+	+						
14	GDF-15	++++	+++	++	++++	+++++	++++	++++	+++++	++
15	HGF	++	+	+						
16	IFN- γ	+++	++	+	++++	++++	+++++	++++	++++	+
17	IGFBP-2	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++
18	IGFBP-3	++	+	+	+++++	++++	++++	++++	++++	+
19	IL-1 α	+++	++	+	++++	++++	++++	+++++	++++	+
20	IL-5	+++	++	+						
21	IL-6	+	+	-	+++	+++	++++	++++	+++++	++
22	IL-8	++	+	+	++	+++	++	+++	+++	+++
23	IL-11	+++	+++	++						
24	IL-17A	+++	++	+						
25	Kallikrein3	+++	++	++	+++	+++	++++	+++	+++	+
26	Lipocalin 2	+++	++	+						
27	MCP-1	++++	++++	+++	+++++	+++++	+	++++	+++++	++++
28	MIF	+++++	+++++	++++	+++++	+++++	+++	+++++	+++++	++
29	MIP-1 α , MIP-1 β	++++	+++	++	+++++	+++++	+	+++++	+++	-
30	Myeloperoxidase	+++++	++++	+++						
31	Pentaxin	+++	+++	++	+++++	+++++	++	+++++	++++	++
32	RANTES	++	+	-						
33	Resistan	+++++	+++++	++++	+++	+++++	++			
34	SDF-1 α	+++++	++++	+++	+++++	+++++	++	++	+++++	+++
35	Serpin E1	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++
36	Thrombospondin	+++++	+++++	++++	+++++	+++++	+++++	+++	++++	++
37	VEGF	+++++	++++	++++	+++++	++++	++++	++++	++++	++
38	ENA-78				++++	+++	++	+++	++	-
39	IL - 27				+++	+++	++++	+++	++	+
40	Vit-DBP				+++	++++	++++			
41	Osteopontin	+++	++	+	++++	++++	+			

		Fibroblast Only EHTs			Left Ventricle			Right Ventricle		
		Mean Pixel Density			Mean Pixel Density			Mean Pixel Density		
		Day 2	Day 8	Day 2	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16
1	Adiponectin	+++	++	+						
2	Aggreca	++	+	+						
3	Angiogenin	+++	++	++	+++	++++	+	+	+	-
4	Angiopoietin-2	+++	++	++	++	++++	+	+++	++++	+
5	BDNF	+++	++	++	++	+++	+	+++	+++	+
6	C5/C5a	+	-	-						
7	CHI3L1	+++	++	+	++++	++	+	++++	++	+
8	Cystatin C	++	+	+						
9	DKK-1	+++	++	+	++	++	-	++++	++	-
10	DPPIV	++	+	+	++++	++++	+	++++	++	-
11	EMMPRIN	++++	+++	++	+++	+++	+	++	+++	-
12	Endoglin	+	+	-						
13	FGF-19	++	+	+						
14	GDF-15	++++	+++	++	+++	++++	+	+++	+	-
15	HGF	++	+	+						
16	IFN- γ	+++	++	+	+++	++++	++	+++	++++	++
17	IGFBP-2	++++	++++	++++	++++	++++	++++	++++	++++	+++
18	IGFBP-3	++	+	+	+++	+++	+	+++	+	-
19	IL-1 α	+++	++	+	+++	++++	+	+++	++++	+
20	IL-5	+++	++	+						
21	IL-6	+	+	-	++++	+++	++	++	+	-
22	IL-8	++	+	+	++++	++++	++	+	+	-
23	IL-11	+++	+++	++						
24	IL-17A	+++	++	+						
25	Kallikrein3	+++	++	++	+	+++	+	++	+++	-
26	Lipocalin 2	+++	++	+						
27	MCP-1	++++	++++	+++	++++	++++	++++	++	+	-
28	MIF	++++	++++	++++	++++	++++	++++	++	+	-
29	MIP-1 α , MIP-1 β	++++	+++	++	++++	++++	-	++	+	-
30	Myeloperoxidase	++++	++++	+++						
31	Pentaxin	+++	+++	++	++++	++++	+++	++++	++++	++
32	RANTES	++	+	-						
33	Resistan	++++	++++	++++	++	++++	++++	++	++++	++++
34	SDF-1 α	++++	++++	+++	++++	++++	++	++++	++	-
35	Serpin E1	++++	++++	++++	++++	++++	++++	++++	++++	+++
36	Thrombospondin	++++	++++	++++	++++	++++	++++	+++	+	-
37	VEGF	++++	++++	++++	+	++	+	-	-	-
38	ENA-78				+	+	-	+	-	-
39	IL - 27				++	+++	+	++	+++	+
40	Vit-DBP				++	+++	+	++	+++	-
41	Osteopontin	+++	++	+	++	+++	+	++	++	+

5.10 SUMMARY

The expression of proteins by CM-only EHTs have shown that most of the proteins secreted in the bathing medium of EHTs were contributed by cardiomyocytes. The protein secretion in this group of EHTs were not only more in number as compared to the other groups (table 5.3), but the expression remained fairly constant over the course of days. It can be concluded from the profile that cardiomyocytes are the main cellular population that continue secreting and replenishing proteins in the entire life span of EHT constructs. Its paracrine effect on other cellular constituents need a further evaluation though and the exact pathways for the mechanism of action needs to be ascertained as well.

Although the expression of the proteins in the medium remains constant for the first two weeks but after day-16, its effects on the EHT kinetics is not as marked as expected. This can be attributed to the fact that after a few days, the protein receptors in EHT constructs undergo down regulation, and therefore the kinetics of the EHTs take a toll and start to decline. Another postulation can be that after 2 weeks the paracrine effect of proteins secreted by the cardiomyocytes on fibroblasts and vice-versa fades away. However, this hypothesis also needs further investigation.

A comparison of protein profile of CM-only EHTs and freshly prepared EHT medium marked a clear difference in the expression of the proteins (Table 5.7). When compared with FB-only EHTs, the proteins secreted by the fibroblasts into the bathing medium were sparse and their expression was weak and faded away over the course of time (Table 5.7 & 5.9). It appeared, that the expression of proteins was merely an accentuation of the factors already there in the nascent medium. There is a strong possibility that proteins secreted from fibroblasts were strongly inhibited by RV-FBs based EHTs (Table 5.9).

The expression of proteins from EHTs with all four-chamber fibroblasts showed that the protein expression of RA-FBs and LA-FBs based EHTs was similar. The protein expression of LV-FBs based EHTs showed significant differences on comparison with RV-FBs based EHTs. However, the comparison of atrial and ventricular groups showed systematic differences. The release of proteins declines day-16 onwards, particularly for ventricle groups.

Proteins released include angiogenic factors, cytokines, growth factors and homing factors. The overall expression of the soluble proteins is more pronounced by atrial FBs based EHTs than the ventricular FBs containing EHTs.

CHAPTER 6: GENERAL DISCUSSION

CHAPTER 6: GENERAL DISCUSSION

6.1 SUMMARY

The variance of characteristics and transcription factors expression among fibroblasts isolated from different cardiac chambers have been described in various murine studies, and comparison were made to human atrial or ventricular fibroblasts. The work presented in this dissertation aimed to characterise the chamber-specific behaviours of human cardiac fibroblasts and their different plausible roles. This study is unique in its conceptual layout and exploration of role of non-cardiomyocyte (fibroblasts) population in term of growth, proliferation, expression of transcription factors and their interaction with cardiomyocytes for generation of engineered heart tissues. This work was divided into two parts. The first part of chapter 3 has highlighted the rate and patterns of growth alongside other phenotypic characteristics of the human cardiac fibroblasts from different chambers in different patients (Pilot Data). In the main study, for the first time, the human cardiac fibroblasts from all four chambers were isolated and compared on the same pattern as pilot data samples. For this project, I did extensive exploration of harvesting techniques of human cardiac fibroblasts from different chambers and defined a true cell population. For the first time in a human model, the rate and pattern of growth of these fibroblasts was compared. Having confirmed the morphological features and growth patterns at different stages, these cells were further characterised with a set of positive and negative predicted markers (immunostaining) to distinguish them from other constituent cell populations of the human heart. Moreover, a comprehensive comparative analysis of expression of transcription factors among the chambers was studied. In the previous published series, comparisons were made between human atrial and murine atrioventricular fibroblasts but none of the studies have compared the physiological and morphological characteristic of human cardiac fibroblasts from same individual with good biventricular function. This is explored for the first time in the literature so far.

More recently, the transcription factor expression of fibroblasts has gained a lot of attention, but the main focus rested on murine and canine models. In this dissertation, for the first-time transcription factor expression of fibroblasts all four-chambers from the same adult human heart with defined biventricular function is explored. The study has compared the same set of transcription factors across all four-chambers to find out a significant difference of expression and to define a subset of prominent markers in humans. Again, this is a new addition to the ongoing research to find out a set of TFs suitable for further research and possible direct reprogramming in future. Lastly for the first time the fibroblasts from all four chambers were used to construct the engineered heart tissue to find out the best chamber that can contribute to better kinetics.

The second half of the study was focused on the functional and biochemical aspects of these chamber-specific fibroblasts. The chamber-specific fibroblasts were introduced in a precalculated numeral at the same growth stage to the Engineered Heart Tissue to find out any predicted differences in the kinetics of these constructs. (Note: All constituents of Engineered Heart Tissues including the constituent cell populations are measured and are added in a pre-calculated ratio: Chapter 2). In the past, it had been shown that foetal and adult fibroblasts containing EHTs displayed a variance of kinetic response. This dissertation had investigated the effect of chamber-specific fibroblasts on the kinetics of the EHTs. The chamber-specific fibroblasts in a co-culture with cardiomyocytes have shown difference of kinetics between the chambers. **This led to the identification of a chamber (Right Ventricle) yielding fibroblasts with better phenotypic and functional characteristics, and improved kinetics in EHTs.** These chamber-specific fibroblasts need further evaluation and can potentially be used in future for direct programming. However, further investigations are required before this can be proposed for a clinical trial. This model of EHT constructs with fibroblasts of different origin needs further paradigm testing.

Lastly, the protein expression of the chamber specific fibroblasts in the bathing medium was studied to understand the paracrine role and interaction of cardiomyocytes and non-cardiomyocytes. This is again performed for the first time as well. This study has shown that the protein expression of cardiomyocytes and fibroblasts is different. Moreover, the interaction in the medium revealed the interesting excitatory and inhibitory roles of these proteins. The study has brought forward a subset of proteins secreted by the EHTs with fibroblasts of different origin.

Both parts focused on developing a better understanding of the chamber-specific behaviour of the human cardiac fibroblasts and identification of a chamber with better phenotypic and kinetic characteristics which can be used later for other research projects.

6.1.1 OPTIMISATION OF HUMAN CARDIAC FIBROBLASTS ISOLATION TECHNIQUES

Two different harvesting techniques of CFs (previously used in the murine models) were used and found to be efficacious and reproducible in harvesting the human cardiac fibroblasts. The isolation of the human CFs from atrial or ventricular tissue samples was achieved with tissue explant and enzymatic digestion techniques in murine models previously. However, further improvement of the established protocols for isolation of human cardiac fibroblast was required for both techniques. In this study, **I optimised both techniques for harvesting the fibroblasts from all four-chambers of heart in a human model. Both these techniques mentioned in the Chapter 2 are efficacious and reproducible.** It was noted that, for a given amount of tissue, the enzymatic digestion technique yielded more fibroblasts, with rapid proliferation rate in comparison with the explant technique. However, the fibroblast obtained via the explant technique were free of debris and risk of infection was considerably lower in comparison with the enzymatic digestion technique. Using both techniques, in majority of the tissue samples, enough

fibroblasts were retrieved for immunostaining and transcription factor analysis. With enzymatic digestion technique, it was mandatory to wash fibroblasts for at least 3-4 times in the first 24 hours to avoid infection (when fibroblasts are not firmly adherent to the floor of the flask), therefore many a times the fibroblast population got washed away. The concentration of the antibiotics used in FGM for enzymatic digestion technique was doubled in comparison with explantation technique (2% vs 1%). This led to increased amphotericin granular deposits on the surface of fibroblasts in enzymatically digestion samples as compared to the explanted samples. Provided the sample size was adequate the fibroblast yield from the enzymatic digestion technique was as good as the explantation technique. Based on this observation from pilot data, the explantation technique was used for harvesting fibroblasts in the main study.

6.1.2 FACTORS EFFECTING ISOLATION & CULTURING OF HUMAN CARDIAC FIBROBLASTS

Several factors confounding factors were noted during the isolation and culturing of human cardiac fibroblasts in the culture system. In the pilot data, the amount of tissue obtained from living donors during the surgery was sometimes too small for the explant technique, and only a few fibroblasts were retrieved by employing the enzymatic digestion technique. The ventricular samples, mainly received from the native heart of the transplant recipient, were of good size. The time interval between harvesting of native cardiac tissue samples till it reached the lab for processing was noted to be a limiting factor for the fibroblast yield. Many samples were kept in a suboptimal condition in the operation theatre (i.e. without immediate bathing in cardioplegia solution) and hence were unable to deliver any fibroblasts even after 6-8 weeks of incubation period. **I noted at the beginning of my research project that the tissue samples from the native heart of the transplant recipients were being transferred (from Harefield Hospital to Hammersmith Campus) bathing only in cold Hartman's solution. Having had a discussion with my research groups, I prompted**

the use of cardioplegia (St. Thomas Cardioplegia solution in a ratio of 1:4) mixed with Hartman's for the transfer of tissue samples. This improved the viability of the tissue samples leading to a better yield of cells (fibroblasts, cardiomyocytes and endothelial cells) harvested from the samples. This new transfer medium protocol was uniformly accepted by all groups in the host laboratory. Also when the sample size was adequate, each sample had its own growth and multiplication rate, which varied across samples and chambers. Some samples (e.g. the LV sample was taken from hypertrophic obstructive cardiomyopathy patients) exhibited vulnerability to passage for the next stage, and the fibroblast populations died without any apparent reason. A similar pattern was observed from time to time in samples from other chambers but predominantly was reserved for ventricular samples. The risk of infection should never be underestimated and could halt the good growth of fibroblasts at any given stage. Therefore, various concentrations of antibiotics were used at different stages to overcome this issue. It was noted, that for a given amount of tissue, the number of fibroblasts retrieved from the atrial samples was three times more than the ventricular samples. However, it should be kept in mind that the ventricular samples received were from the transplant recipients and had an extensive area of infarction and scarring as well as fat encroaching the myocardium; in comparison to atrial samples that have no infarction or extensive fibrosis and are relatively disease-free.

In the main study group, although the atrial tissue sample size was adequate for most of the samples, the ventricular tissue sample size was very small (due to Tru-cut biopsy needle used for sampling). The average weight of the ventricular sample was 0.10g, and that of atrial biopsy sample was 0.66g. The fibroblasts were harvested with the explantation technique. Only 8 out of 20 ventricular samples could yield fibroblast to P2 stage. The other 12 ventricular samples either showed no growth or could not grow beyond P0 stage.

6.1.3 FACTORS AFFECTING RATE OF GROWTH OF HUMAN CARDIAC FIBROBLASTS

The rate of growth of fibroblasts was noted to be dependent on the proximity of cells and colonies. Based on the cell count, if the fibroblasts were transferred to a large flask, the growth was indolent with a tendency to myofibroblasts transformation. The confluence pattern in such samples was delayed (3-4 weeks), and scattered colonies grew sluggishly. This could be explained by the fact that fibroblasts exchange the growth factors and chemokines with each other not only via the medium around them but also through their microfilaments projecting and connecting to other cells. The rate of growth of fibroblasts is substantially affected by constituents of the medium and the additives. Some batches of foetal bovine serum (FBS) were found to be more efficient than others. Increasing the concentration of FBS from 10% to 20% was noted to be a useful adjunct for some samples and led to better and faster yield of fibroblasts.

6.1.4 EFFECT OF PASSAGE ON RATE OF GROWTH AND DIFFERENTIATION OF HUMAN CARDIAC FIBROBLASTS

Each passage has an undesirable effect on the growth rate, morphology and myofibroblastic transformation of the cardiac fibroblasts. The myofibroblast transformation was noted to be directly related to the passage stage. Although the addition of FGF has a significant impact on prevention of the conversion of the CFs to myofibroblasts, but during the late passage stages (i.e. beyond P4) even the addition of the FGF to FGM could not suppress and prevent the myofibroblastic transformation.

6.1.5 CHARACTERISATION OF CHAMBER SPECIFIC HUMAN CARDIAC FIBROBLASTS

Cardiac fibroblasts play a pivotal role in the post-myocardial infarction fibrosis and myocardial remodelling. Recently, it has been shown in murine models that despite the

same mesenchymal origin, fibroblasts within the same tissue display the functional diversity when exposed to distinctive environmental conditions. In response to injury, two discrete dermal lineages were found to be contributing uniquely towards the tissue repair and regeneration. This functional assortment can be attributed to the differentiation hierarchy or the origin from diverse lineages (Driskell et al., 2013). This alludes to the possibility of distinctive fibroblast lineage in other organs and species. It was hypothesised that due to structural and functional differences of the human cardiac chambers, the fibroblasts in these chambers might exhibit distinctive phenotypes and functional spectra. The fibroblasts populating the scarred area may also display phenotypic variance in comparison to the fibroblasts in the adjacent normal functional myocardium in the same chamber.

To characterise the fibroblasts from cardiac chambers in the pilot data, biopsies were taken from different chambers in different patients, and their growth rate and the pattern were studied serially. They were further characterised by a set of positive and negative predictors to distinguish them from other cardiac resident cell populations. In the main study group, the fibroblasts isolated from all four-chamber were harvested and defined as true population depicted by the same immunostaining pattern with a pre-defined set of markers. Once the identification was completed, the variance in the transcription factors expression was assessed. The expression of transcription factors was comparable between the left and right atrium as well as left and right ventricle. **However, when atrial TFs expression were compared with the ventricle TFs expression, a distinctive variance was clearly shown. This is similar to the previously published data based on the murine models in which atria and ventricles showed a difference in the transcription factor expression (Furtado et al., 2014).** However, in the mentioned published data, the atrial samples were of human origin and ventricular samples were taken from murine ventricles.

6.1.6 CHAMBER-SPECIFIC HUMAN CARDIAC FIBROBLASTS IN EHTs

Following confirmation and extensive characterisation of a true population of CFs, the chamber-specific fibroblasts were added to the EHTs. Conventionally, so far only human foetal fibroblasts were used as a co-culture to cardiomyocytes in the previous EHT models. The well-established protocol for EHTs was already refined and used in other parallel projects in the laboratory. However, the addition of the left ventricular fibroblasts led to EHTs with morphological features very different from the foetal-containing EHTs. The LV fibroblasts containing EHTs were bulkier and were lacking the classic hour-glass shape. They dislodged more frequently from the silicon posts during the early phase of maturation, probably due to excessive condensation of the construct. Although the fibroblasts count of foetal and ventricular fibroblasts added to EHTs was the same, however the pellet formed by the ventricular EHTs cells was much bigger in size when compared to the atrial fibroblasts pellet during isolation. This can be possibly attributed to the fact that ventricular fibroblasts added to EHTs were larger in size (on bright field microscopic view only but the dimensions/area of these ventricular FBs were not measured) in comparison to the foetal fibroblasts (observed during the cell counting phase).

In the main study, the cell count and the average area of the fibroblasts were measured with a cell counter. A noteworthy difference was seen between the atrial, ventricular and foetal fibroblasts. The left and right atrial fibroblasts although have a comparable average area (25-30 μ m) but the rate of growth and cell count at P2 stage of LA fibroblasts was 5 times more than RA fibroblasts. The average area of LV fibroblasts was 35 μ m in contrast to the RV fibroblasts which exhibited an average area of 30 μ m. The rate of growth of RV fibroblasts was more comparable with bi-atrial FBs than left ventricular FBs. The average area and cell count of LA and RV fibroblasts was similar and comparable. (Fig. 4.12, 4.13) This confirmed the observation finding of the pilot data of variance across the size of fibroblast population.

6.1.7 CDI AND IMR CARDIOMYOCYTES USED FOR GENERATION OF ENGINEERED HEART TISSUE

The construction of EHTs is dependent on a precise, calculated number of cardiomyocytes used for individual constructs. Human iPSC- cardiomyocytes labelled as CDI, were used for the pilot data. These cells were immature cardiomyocytes, and the morphology of these cells is very similar to the human foetal and neonatal rat cardiomyocytes than adult human cardiomyocytes. The CDI cardiomyocytes are often round, oval, triangular or star-shaped in contrast to rod-shaped adult cardiomyocytes. (Harding et al., 2007). These CDI-CM have been used in the past in various pharmacological experiments. The calcium transient studies and the sarcomeric gene expression of these cells are different from the adult cardiac myocyte population. In addition to this the mitochondrial morphology and density, sarcoplasmic reticular organisation, t-tubule arrangement, sarcomeric outline and intercellular connections are less developed as compared to the native adult cardiomyocytes. The cardiomyocytes in EHTs have mitochondria in abundance, but their cristae are immature and scanty. They show erratic A and I bands. The average length of sarcomere in a normal cardiac muscle is 2-2.5 μm , but in CDI-CM this is decreased to 1.6 $\pm$ 0.1 μm . (Gorelik et al., 2009) (Mannhardt et al., 2016). Since the FOC is directly proportional to the initial length of the fibre, therefore, these the EHTs will display less vigorous and compromised kinetics.

The CDI cells are available commercially with 90-95% purity (Naito et al., 2006) and some studies have claimed the purity is reaching up to 99% (Gorelik et al., 2009). The IMR- cardiomyocytes exhibit almost similar morphological and functional properties as CDI and also show purity in the same range. In this study, the CDI and IMR cardiomyocytes were used as they have been used and tested in our lab previously with good results.

The human heart starts beating during the third week of gestation and continues through its maturation and developmental stages till birth. The physiological role of the chambers of the heart is defined after birth but the maturation of the cardiomyocytes continues (Goenezen, Rennie and Rugonyi, 2013) (Ziman, Gómez-Viquez, Bloch, 2011). The developmental maturation of the human cardiomyocytes from the neonatal to the adult follow a defined course and timeline.

Different on-going projects in our lab have shown that phenotypic and morphological features of these CDI and IMR cardiomyocytes look like human foetal cardiomyocytes and morphological changes continues with the age of the cell population (Gorelik et al., 2009). With advancing age, the cell shape changes, and the cell starts to look like the adult cardiomyocytes. These observations are consistent with previous published data (Ziman, Gómez-Viquez, Bloch, 2011) (Mummery et al., 2012) (Batalov and Feinberg, 2015).

The IMR-cardiomyocytes (induced from human pulmonary fibroblasts) used for the EHT constructs were at day 18 of maturation. They were round to oval with an average area of 10-15 μ m. A normal healthy adult cardiomyocyte is rod-like or cylindrical with an approximated length of 100 μ m and 10-25 μ m in diameter. Assuming the cardiomyocyte as a cylindrical structure with an average diameter of 15 μ m, the calculated area of the cardiomyocyte was 706 μ m. It implies that the area of an average adult cardiomyocyte is 47 times large the IMR-CMs used in the study.

Structurally the CDI and IMR cardiomyocytes are immature and lack the spatially organised t-tubule, developed sarcoplasmic reticulum, mature actin and myosin filaments and Z grooves; which are a hallmark of the adult cardiomyocyte. In native human myocardium, the t-tubules are arranged so that there is a gap of 10-15nm from the SR and even a small

change in the architectural organisation leads to an altered calcium-induced calcium-release mechanism (CICR) (Gómez et al., 1997) (Lyon et al., 2009). The human foetal and murine neonatal cardiomyocytes also lack in t-tubules, Z groves and developed SR. These structures appear later as the structural development of the heart continues. In addition to this, the proteins like troponin-T, α -actinin, caveolin 3 and connexin 43 are either lacking or functionally immature in neonates and develop over the period after birth. These proteins have a direct impact on excitation-contraction coupling and inotropic response of the cardiomyocytes (Ziman, Gómez-Viquez, Bloch, 2011). Since CDI and IMR cardiomyocytes used in this study were immature cells, therefore, the inotropic response, fractional shortening and the resting length of the constructs cannot depict the unique chronotropic and inotropic response of the native adult cardiac syncytium.

6.1.8 RATIONALE OF USING PROTEOMIC PROFILE KIT

Human proteomic cytokine array kit (ARY002B) was preferred for the analysis of proteins secreted by cardiomyocytes and non-cardiomyocytes into the medium bathing the EHTs. The named array kit was selected, as it contains relatively large set number target of proteins (102 proteins in duplicates) and the protein analysis can be performed in a few hours. The literature review of the kit revealed that it has a cluster of proteins specified for detection of human fibroblasts and cardiomyocytes. Moreover, the array kit is cost effective, as it contains 4 nitrocellulose membranes in one pack. This methodology was preferred over other techniques such as Mass Spectrometry (MS), Enzyme-linked Immunosorbent Assay (ELISA), etc. as the set of proteins in ARY002B were pre-defined and specific for human cardiac cellular composition. MS can detect a large number of proteins (more than a thousand proteins) at one time but technically it is more laborious, time consuming and gives a raw data of large number of proteins, which has to be filtered extensively for the proteins of interest. Similarly, ELISA is widely used for quantitative assessment of proteins but again

an individual kit is required for each protein; which makes it is time consuming and costly especially when set number of protein expression needs to be checked in quite a few samples. For the first time, the protein expression in the medium bathing EHTs was attempted and a set of proteins were identified with some variable expression between the atrial and ventricular fibroblast based EHTs (as the cardiomyocytes were of the same origin in all groups of EHTs. **A remarkable finding of this study is the fact, that most of the protein were secreted by cardiomyocytes and only a few were contributed by the fibroblasts.** These findings can be further tested and confirmed with other protein identification techniques in future.

6.2 LIMITATIONA OF STUDY

6.2.1 SAMPLE SIZE OF HUMAN CARDIAC TISSUE

Although the human cardiac fibroblasts can be easily harvested and grown in the culture medium, the rate of growth of individual sample is variable and unpredictable. The size of sample dictates the fibroblast growth to a certain extent. With a good sample size, it is much easier to get an adequate number of fibroblasts at any specific passage. Since the number of fibroblasts required for each EHT construct is 1.5×10^5 , therefore a good sample size is a mandatory requirement for the EHTs. The ventricular samples from the four-chamber biopsy sample were small in size (Fig. 3.6). Only sample no. 7 generated enough ventricular fibroblasts suitable for EHT constructs. Therefore, comparative kinetics in all four-chamber fibroblasts based EHTs in this study is a quantitative data and is based on EHTs with fibroblasts from one sample. The uniformity of passage stage for fibroblast population was maintained for the comparing groups.

6.2.2 RATE OF GROWTH OF HUMAN CARDIAC FIBROBLASTS

Under the same culture environment and using the same harvesting technique, different samples of cardiac tissue shows different growth rate and generate a different number of fibroblasts. There is no scalable and measurable parameter to predict the rate of growth and proliferation of fibroblasts in the individual samples. A variable response was noted in the same sample at different passages. The addition of FGF had shown to improve the rate of growth of fibroblasts and myofibroblast transformation till P4.

6.2.3 QUNATIFICATION OF FIBROSIS AND HYPERTROPHY

In pilot data, the ventricular samples were weighed and it was noted that the phenotypic characters of the growing ventricular fibroblasts were different in comparison with the was fibroblasts from the normal ventricles. The total weight of the heart was not considered for

the pilot data samples. On naked eye examination of these samples, extensive fibrosis was noted with strands of fibrous tissue invading the myocardium. The fibroblasts showed a predilection for myofibroblastic transformation at P2 and P3 and were slow to grow. For the main experimental data, the fibroblasts were harvested from all four chambers in patients with normal ventricular function. Although the individual samples were weighed but the total weight of the heart was not measured (a pitfall of study design) as no pre-operative CMR was done for these patients. For any future experiments, the average weight of heart based on a CMR should be taken into consideration.

6.2.4 CELL CULTURE ENVIRONMENT

The biopsied human cardiac tissues are devoid of the perfect homeostatic condition present inside the heart and are subjected to less than ideal artificial culture environment. The simulated culture medium cannot offer the complex myocardial tissue ambience provided by a constellation of three-dimensional strain, hormonal and growth factors. The different mechanical environment may induce growth factor secretion triggering various autocrine and paracrine effects that can modulate the physiological status of cells. This may alter the precise phenotypic characteristics; accentuate the inherent phenotypic disparities in growth patterns of the CFs and the expression of the transcription factors exhibited by these cells (MacKenna, Summerour and Villarreal, 2000).

Moreover, it is difficult to maintain the same culture environment with the same level of growth factors all the time, as they tend to wash away at the time of changing the medium. In future, the ideal way of identifying the cellular physiological role will be to perform a single cell analysis; which is a better, rational and robust way of identifying the physiological and secretory profile of any cell.

6.2.5 CELLULAR MODELS FOR STUDY OF ADULT HUMAN HEART KINETICS

In this dissertation, the kinetic assessment of the adult human heart was carried out using a cellular model (EHTs). This model provides valuable information regarding the in-vitro interaction between cardiomyocyte (IMR/CDI) and non-cardiomyocyte from the human heart. However, it is important to understand this three-dimensional structure cannot replicate the precise kinetics of the adult human heart in-situ. The optimisation of this CDI and IMR based In-vitro model is in progress in our lab, and the result will be published soon (Owen, T et al). The current CDI cardiomyocytes kinetic model is representation of a foetal heart than an adult human heart.

6.2.6 IMMATURE CARDIOMYOCYTES

The construction of EHTs cannot replicate the adult myocardial tissue as cardiomyocytes used were having immature morphological and physiological features. The maturation of the cardiomyocytes is dependant on many paracrine factors secreted by the non-cardiomyocytes. The lack of these paracrine growth factors alters the maturation and differentiation of the cardiomyocytes. Moreover, in-vivo the cardiomyocyte differentiation is a result of various kinetic and neurohormonal signals over a period of 9 months and even during the early post-natal period. This cannot be replicated over the course of 18 days and that too without the optimal homeostatic environment. The complex neurohormonal signals is extremely difficult to generate in-vitro. The work carried out in this thesis assumes that the immature cardiomyocytes (i.e. CDI/IMR) behave in the same manner as the adult cardiomyocytes.

6.2.7 AUXOTONIC AND ISOMETRIC MODELS OF EHTs

The contraction of heart follows an auxotonic model under normal physiological conditions. However, under the pathological conditions, the heart undergoes hypertrophy and dilatation

resulting in a stiff ventricle. Hirt et al. have shown that by increasing the load on the EHTs by introducing metal braced induces a pathological cardiac hypertrophy phenotype. This indicates that isometric condition can predispose to a pathological phenotype (Hirt et al., 2012). The exact replication of these models is still difficult, but can be optimised as this influences the force kinetics. It has been shown in the rat ventricular CM-EHTs that the isometric model elicits 50% less force of contraction than the auxotonic model (Nishimura et al., 2004). The auxotonic model used for the EHTs construct can be valid for the foetal and the four chamber fibroblasts based EHTs. The DCM heart is a hypertrophied and is dilated with isometric loading pattern. Therefore, the fibroblasts harvested from such tissue should be evaluated by selecting an isometric model to evaluate the exact effect of kinetics.

6.2.8 INFECTION OF THE SAMPLES AND CULTURE MEDIUM

In the human body, a cascade of the immune system (cellular & humoral immunity) prevent the infection of the rapidly dividing cell and provides an inherent defence mechanism. The cells in the culture medium are deprived of these preventive mechanisms and therefore are more susceptible to infections especially fungal infection.

6.2.9 VIDEO-OPTICAL MEASUREMENT OF KINETICS

The kinetic recordings of the EHTs were carried out for a fixed period on a daily basis (1min/well/24hour). There was no measure to record the kinetics of the individual wells all along the contractile period. There can be a period when the contractile force generated by the EHTs is at its peak or its lowest, but there was no definite way to record any variation. The optical recording of the kinetics and data evaluation is based on the concept that EHTs are beating uniformly all through the day with no major peaks and troughs.

6.3 FUTURE DIRECTION

In this thesis, the all four-chamber biopsies were taken from the patient population with normal bi-ventricular function. However, in the pilot data, the biopsies were taken from the left ventricles of the native heart of the transplant recipients only. All four-chamber biopsy samples were not taken from the patients with poor ventricles. So, a precise comparison of all four chambers in the patient with the normal and abnormal ventricular function was not possible. In future multiple four chamber biopsies can be taken from the native heart of the transplant recipient and from the patient with the normal ventricular function to make a qualitative comparison. The foetal fibroblast population from all four-chambers can be studied in aborted foetuses.

The size of the tissue harvested from the human heart is a crucial factor in isolation of the fibroblasts. Optimisation of the tissue size based on the samples mentioned in this thesis will help to get an adequate biopsy sample in future projects. Moreover, the addition of FGF can further optimise for a better rate of growth of fibroblasts and prevention of myofibroblast transformation. In this thesis, an attempt was made to keep the FGM very basic with minimal additional growth enhancing factors. As developing foetal heart is exposed to an incremental surge of circulating maternal pituitary hormone and corticosteroids. Therefore, the additional supplementation of FGM with steroid and other pituitary hormones could be tried and tested in future studies.

CDI and IMR cardiomyocytes used in this study were immature cells with anatomical and physiological characteristics closer to foetal than adult cardiomyocytes. A delayed maturation phase CDI and IMR cardiomyocytes (day 25-33) can be used in future to find a difference in the kinetics of EHTs. Moreover, the cardiomyocytes harvested from the adult human heart, using enzymatic digestion technique can be used for construction EHTs. The

kinetics of these construct with mature adult cardiomyocytes can be compared with other groups. In addition to this, the human foetal cardiomyocytes can be obtained from the aborted foetuses for EHT constructs. The calcium transients and amplitude of foetal, adult, CDI and IMR cardiomyocytes can be compared to isolate the population of cell which is morphologically and functionally akin to adult cardiomyocytes. Expression levels of major sarcomeric and calcium handling proteins in the iPSC-CM can be compared between the mentioned groups.

More recently, single cell analysis has been developed as a new technique to identify the proteins secreted by an individual cell. This will be very helpful to find out the anatomical, physiological characteristics as well as accurate secretory profile and expression of transcription factors in individual cells.

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APPENDICES

APPENDIX A

APPENDIX A: GROWTH OF CHAMBER-SPECIFIC FIBROBLASTS.

APPENDIX A1: GROWTH PATTERNS OF FIBROBLASTS.

The growth of atrial and ventricular fibroblasts from all four-chambers biopsy, at maturation of P1 stage. The fibroblasts were then passaged to P2 stage in the chamber slides and were strained to confirm their true fibroblast origin with positive and negative markers followed by staining with the transcription factors. Out of 20 atrial samples, 17 samples grew to the P2 stage and in ventricular samples only 8 samples grew to the P2 stage. The growth pattern of all four chamber biopsy samples are illustrated in the figures.

	ATRIAL SAMPLES	VENTRICULAR SAMPLES
01/2016	Good growth	Good RV but moderate LV growth
02/2016	Moderate growth	Scanty growth RV < LV
03/2016	Good growth	Good growth
04/2016	No Growth	No Growth
05/2016	Moderate growth	Moderate growth LV > RV
06/2016	Moderate growth RA > LA	Scanty growth – no staining possible
07/2016	Best growing sample	Best growing sample
08/2016	Scanty Growth LA > RA	Scanty growth – Big intercolonial gaps
09/2016	Moderate growth	Moderate growth
10/2016	Moderate growth	Scanty growth
11/2016	No growth	No growth
12/2016	Good growth	Good growth
13/2016	Moderate growth. RA > LA	Scanty growth. RV > LV
14/2016	Good growth	Moderate LV growth, Scanty RV growth
15/2016	Good growth	Good growth
16/2016	Good growth	No growth
17/2016	No growth	No growth
18/2016	Good growth	No growth
19/2016	Good growth	No growth
20/2016	Good growth	No growth

The ventricular samples with scanty growth were not good enough for P2 passage and when were lifted up from the base of petridish at the end of P1 maturation these samples did not grow well in chamber slides for staining.

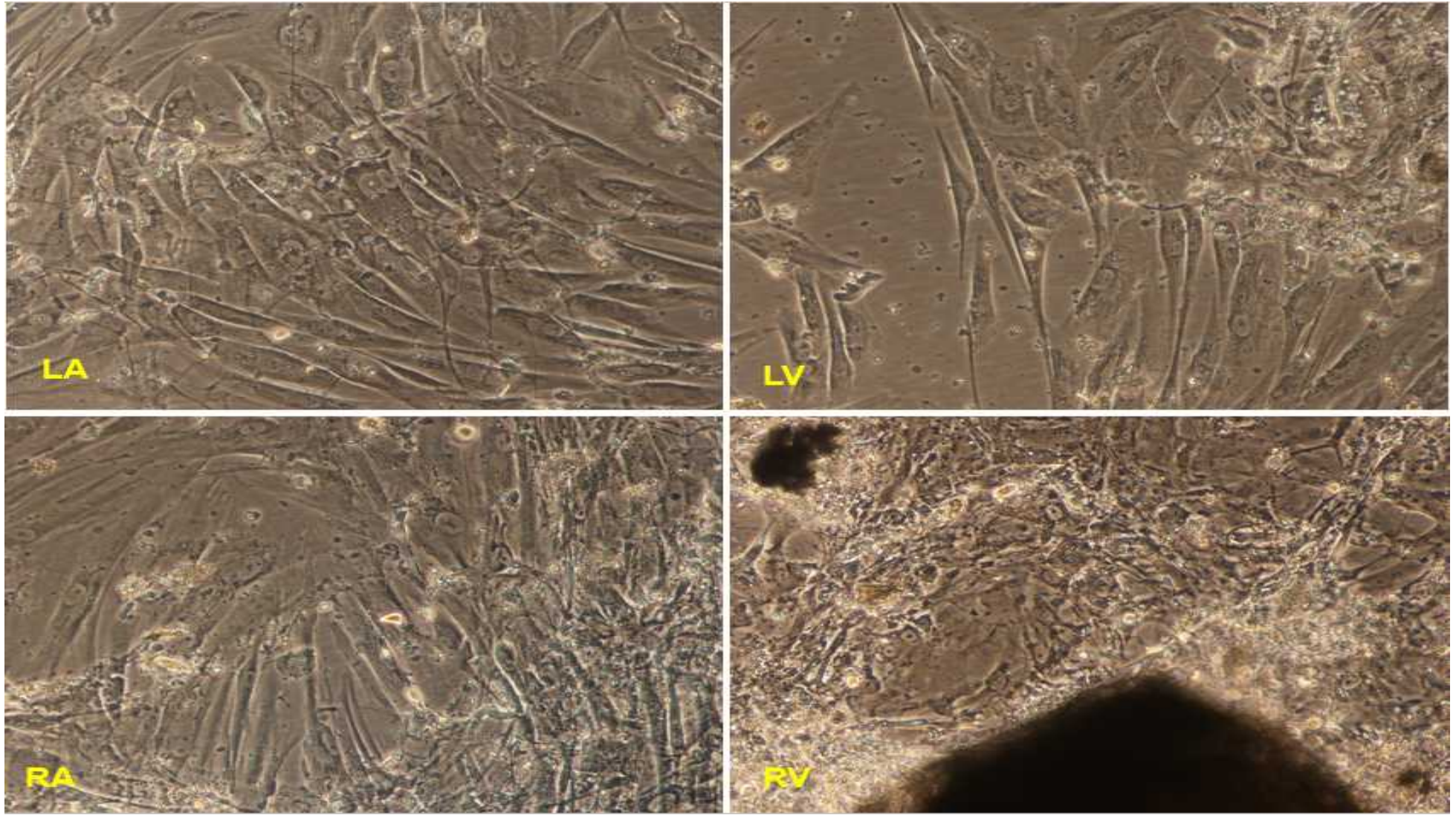


FIGURE A1.1: SAMPLE 1/2016. Bright field microscopic (10x). Growth of all four-chamber fibroblasts at maturation of P1 stages is depicted. The black shadow in the RV is a piece of tissue in the middle of the colony with emerging FBs from the edges. The black dots are the amphotericin granules precipitating at the bottom of the petridish.

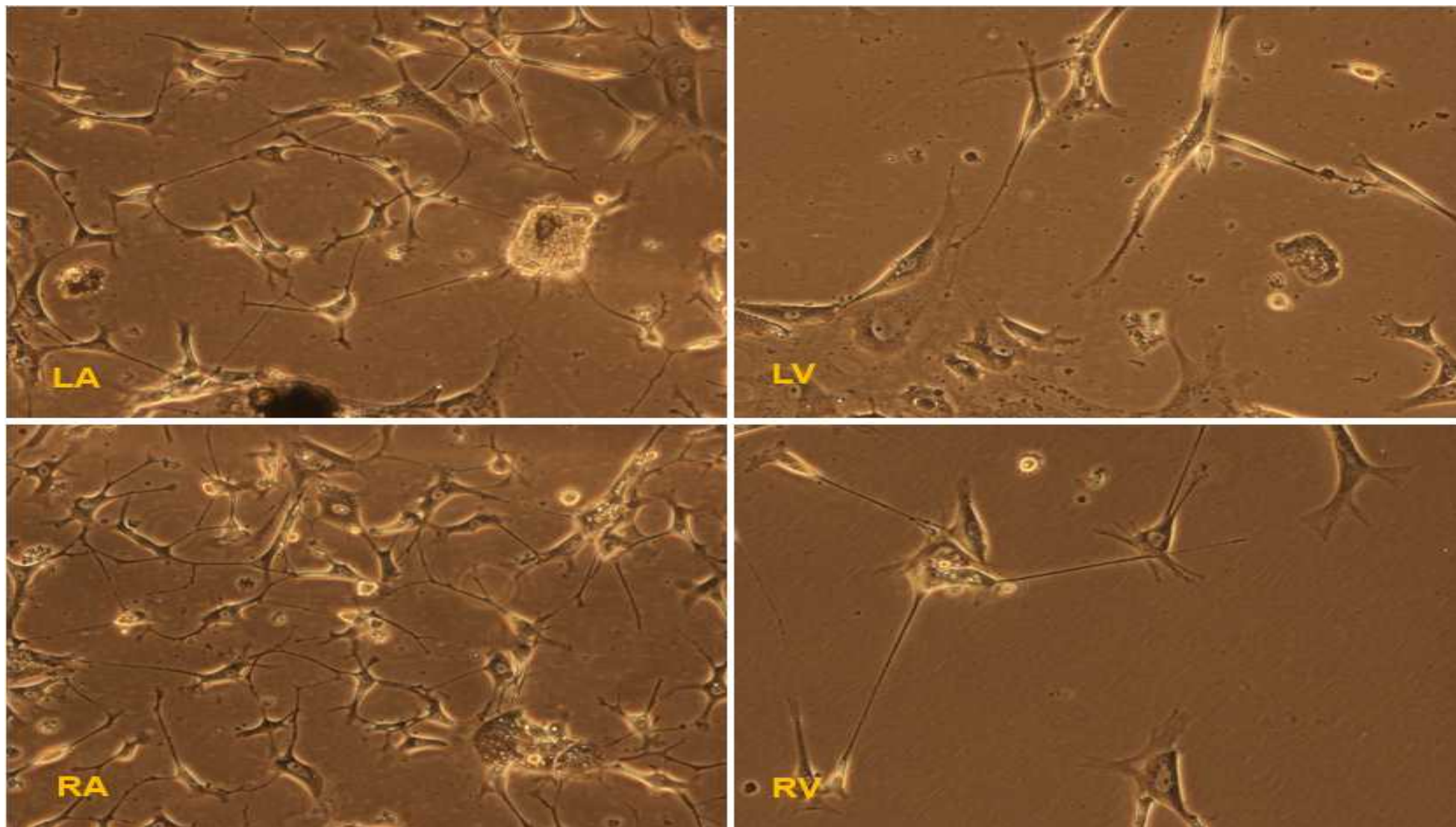


FIGURE A1.2: SAMPLE 2/2016: Bright field microscopic (10x). Moderate growth of atrial samples is depicted. The ventricular samples were slow to grow and individual fibroblasts were sparsely spaced. Gaps between the colonies were quite wide as well. Right ventricular samples were extremely slow to grow and myofibroblastic transformation was noted in both left and right ventricular samples.

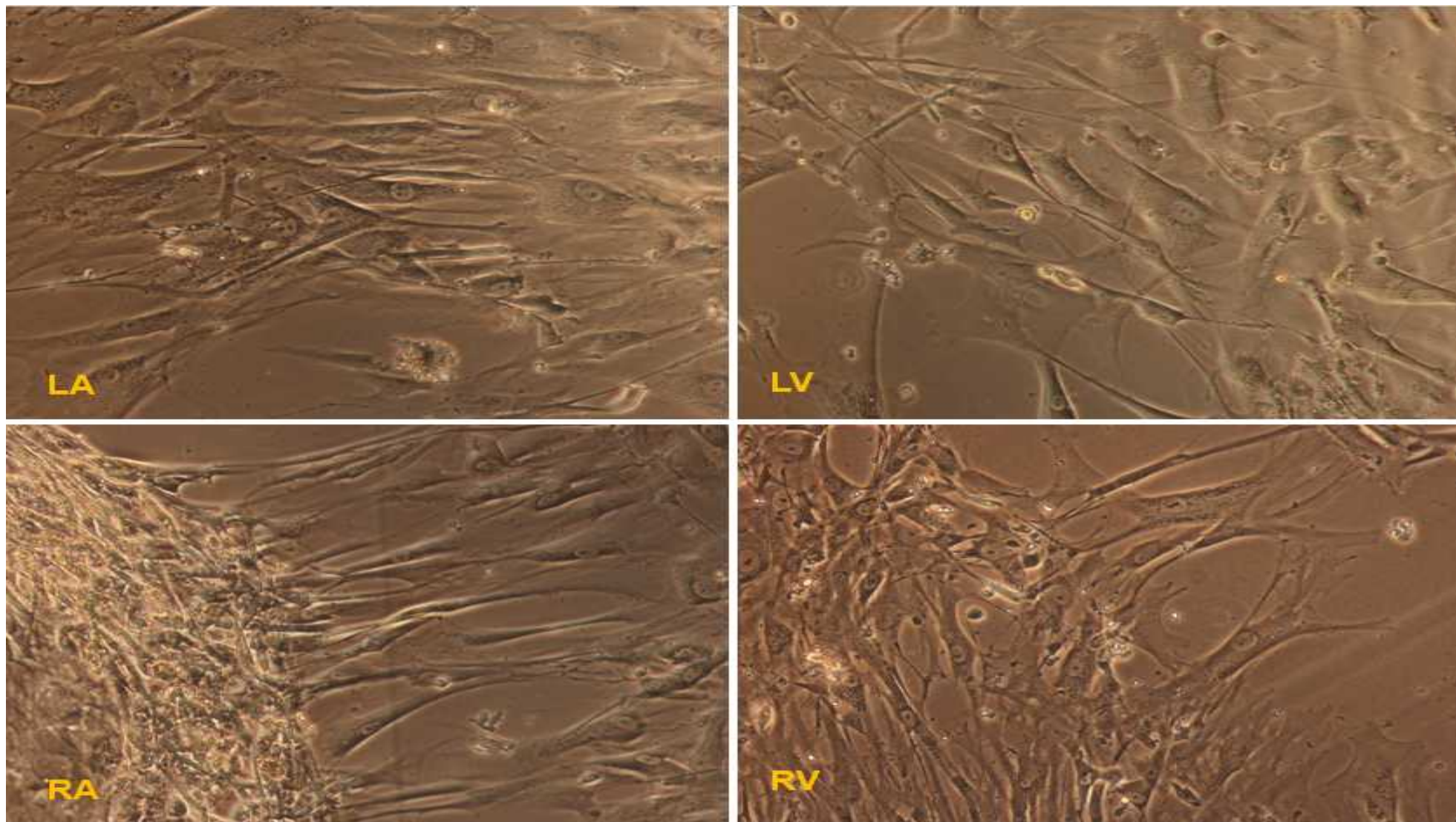


FIGURE A1.3: SAMPLE 3/2016. Bright field microscopic (10x). Good growth of atrial and ventricular fibroblasts. The growth of right atrium and right ventricle samples were better than the left atrial and left ventricle sample. Dense colonies of fibroblasts were noted in right sided samples and inter colonial fibroblasts communication was accentuated in this samples.

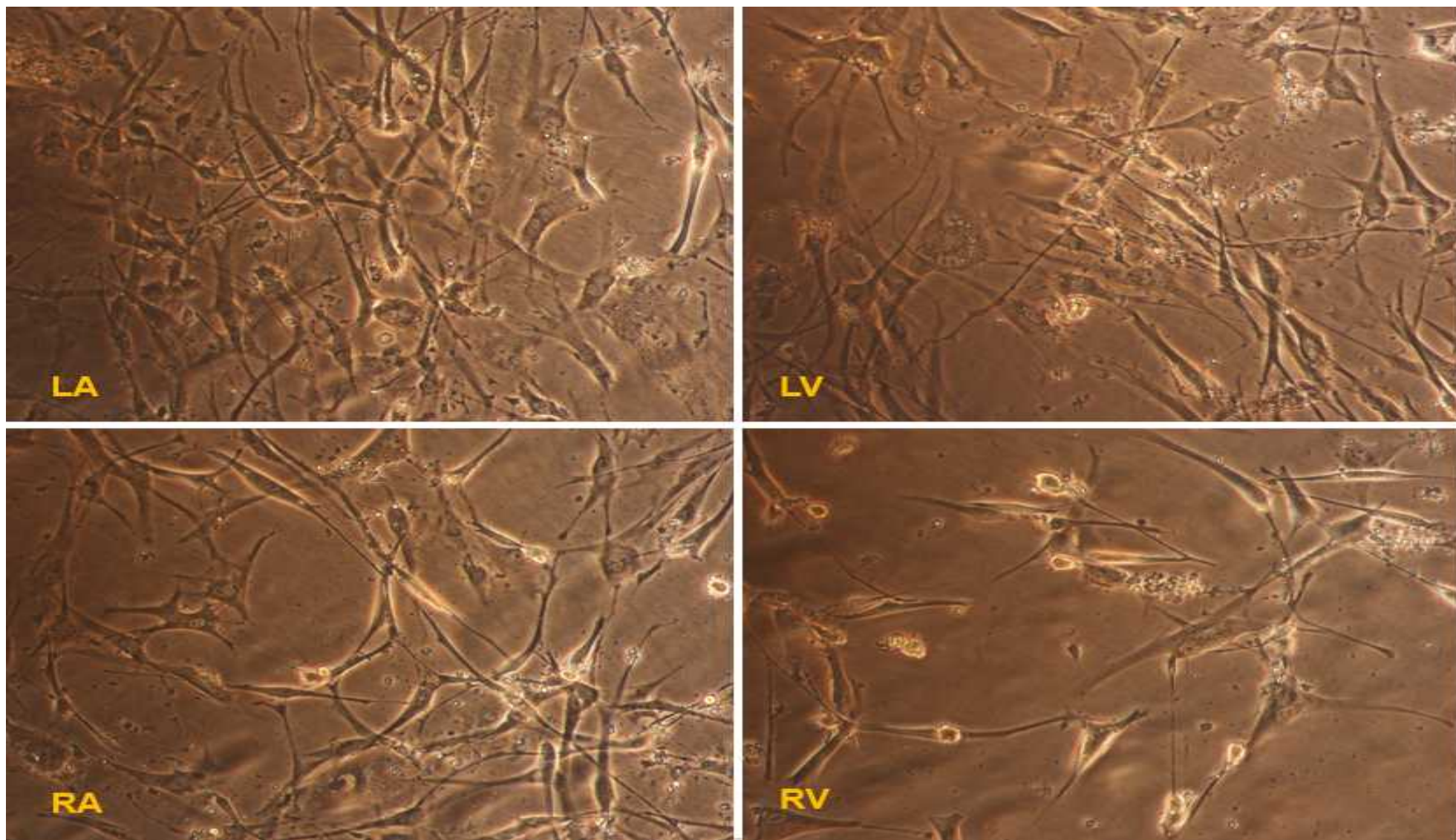


FIGURE A1.4: SAMPLE 5/2016: Bright field microscopic (10x). Moderate growth of all four chamber fibroblasts was noted. In left ventricular samples, the growth of fibroblasts was better than the right ventricular samples. The atrial samples showed parallel growth.

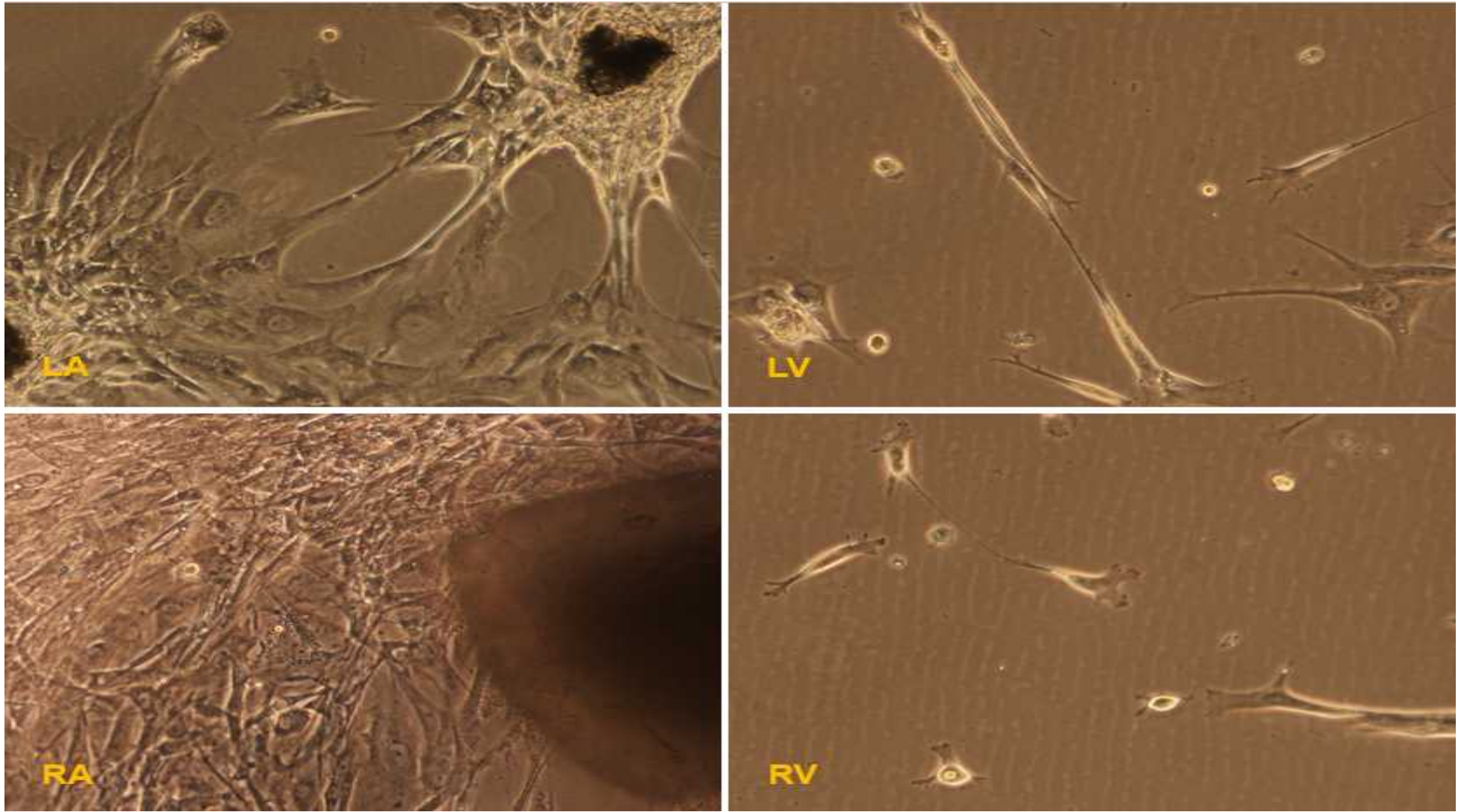


FIGURE A1.5: SAMPLE 6/2016: Bright field microscopic (10x). Growth of right atrial samples was the best out of all four chamber samples. Left atrial sample growth was moderate and there was scanty growth of both ventricular samples. Biventricular samples did not proceed to P2 stage.

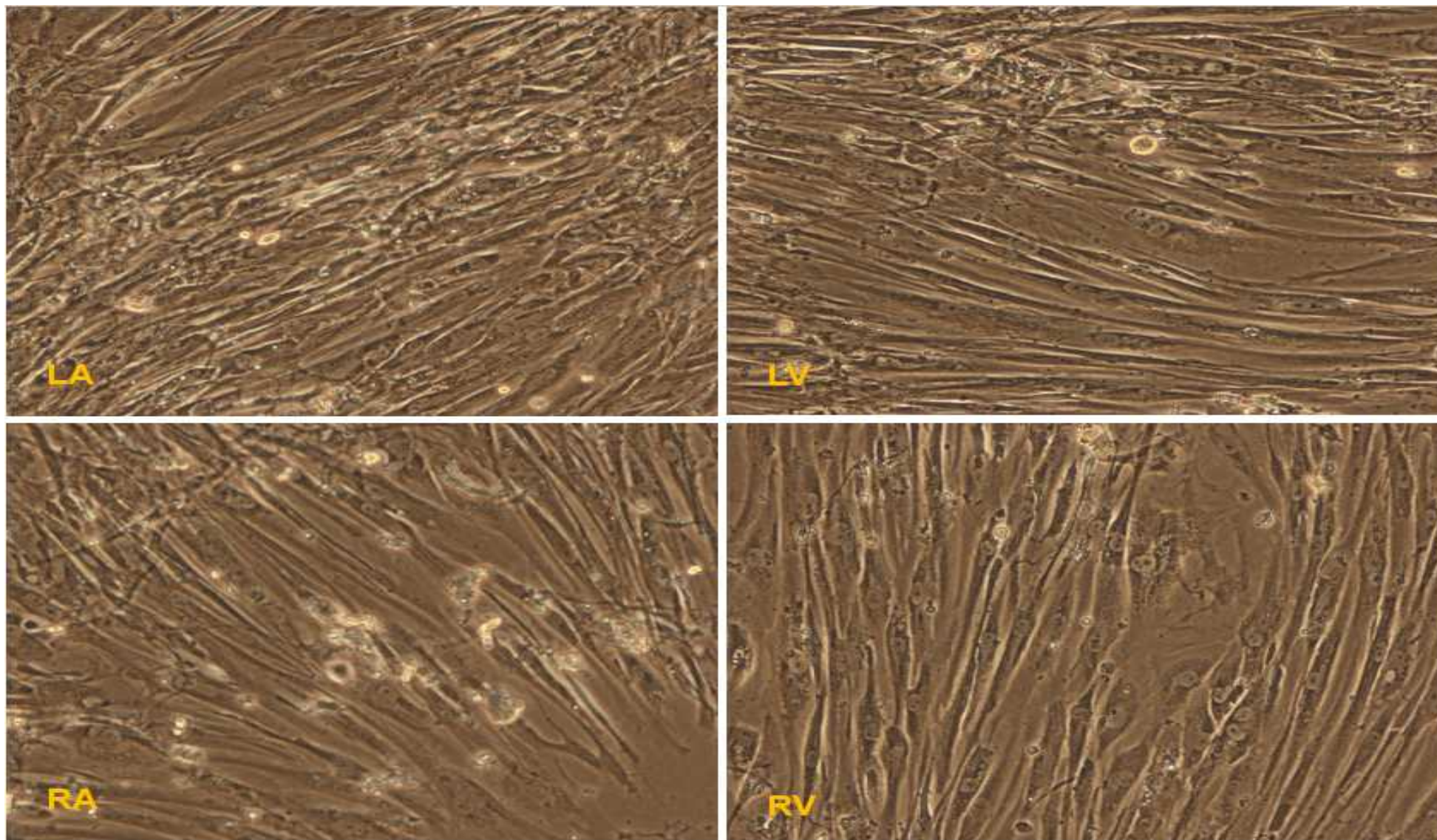


FIGURE A1.6: SAMPLE 7/2016: Bright field microscopic (10x). Best growth in all four chamber fibroblasts. Sample was not stained to avoid the cell loss in staining process but was entirely used for EHT preparation. Best sample from P0 – P2 stage.

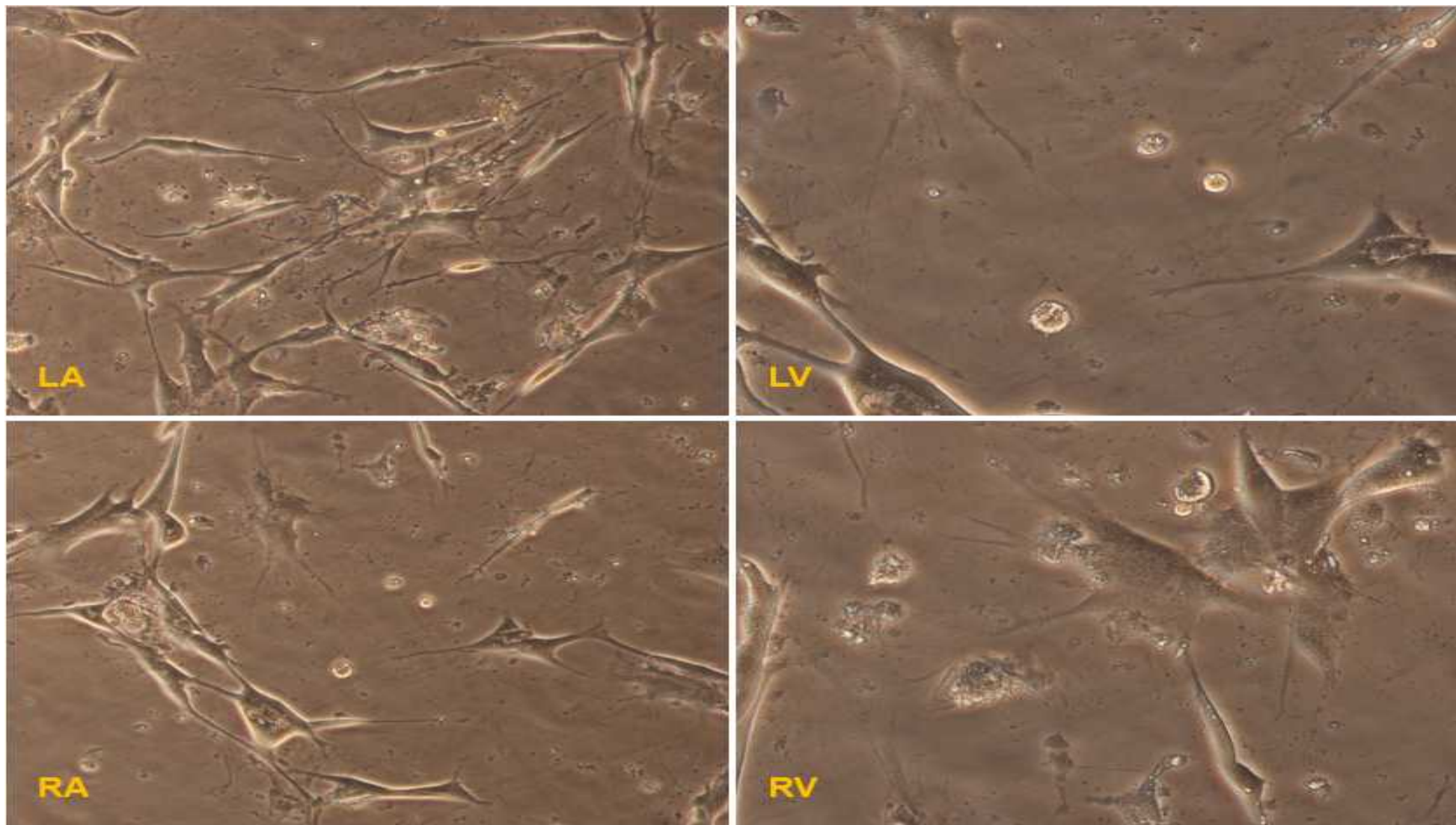


FIGURE A1.7: SAMPLE 8/2016: Bright field microscopic (10x for atrial sample & 20X for ventricular sample). Moderate growth of atrial sample. Ventricular sample showed very scanty growth. However, both atrial and ventricular samples showed more pronounced nanotubules. Atrial samples showed more extensive nanotubular network as compared to ventricular samples.

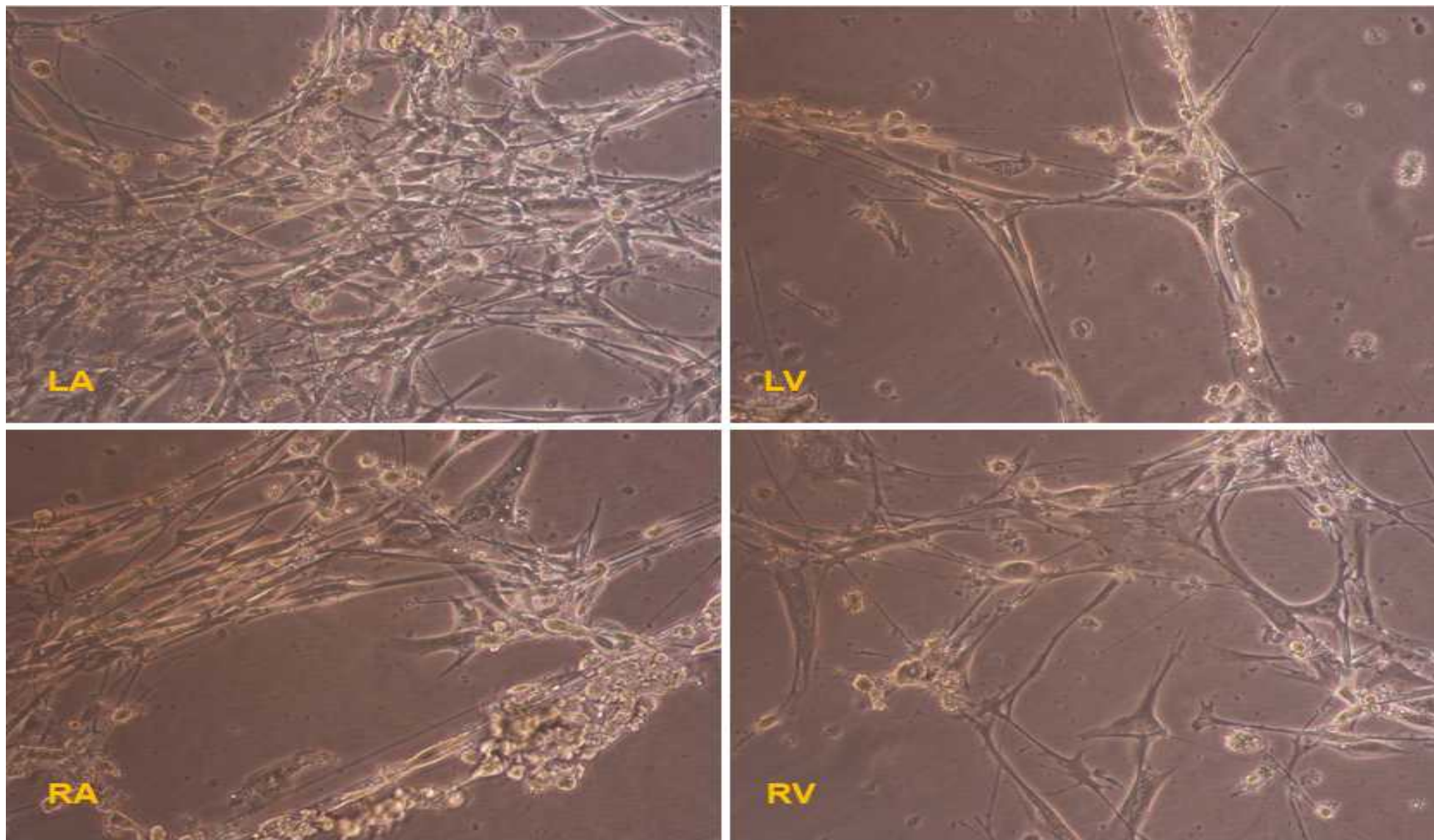


FIGURE A1.8: SAMPLE 9/2016: Bright field microscopic (10x). Moderate growth of all four chamber fibroblasts. Large gaps were present between the colonies.

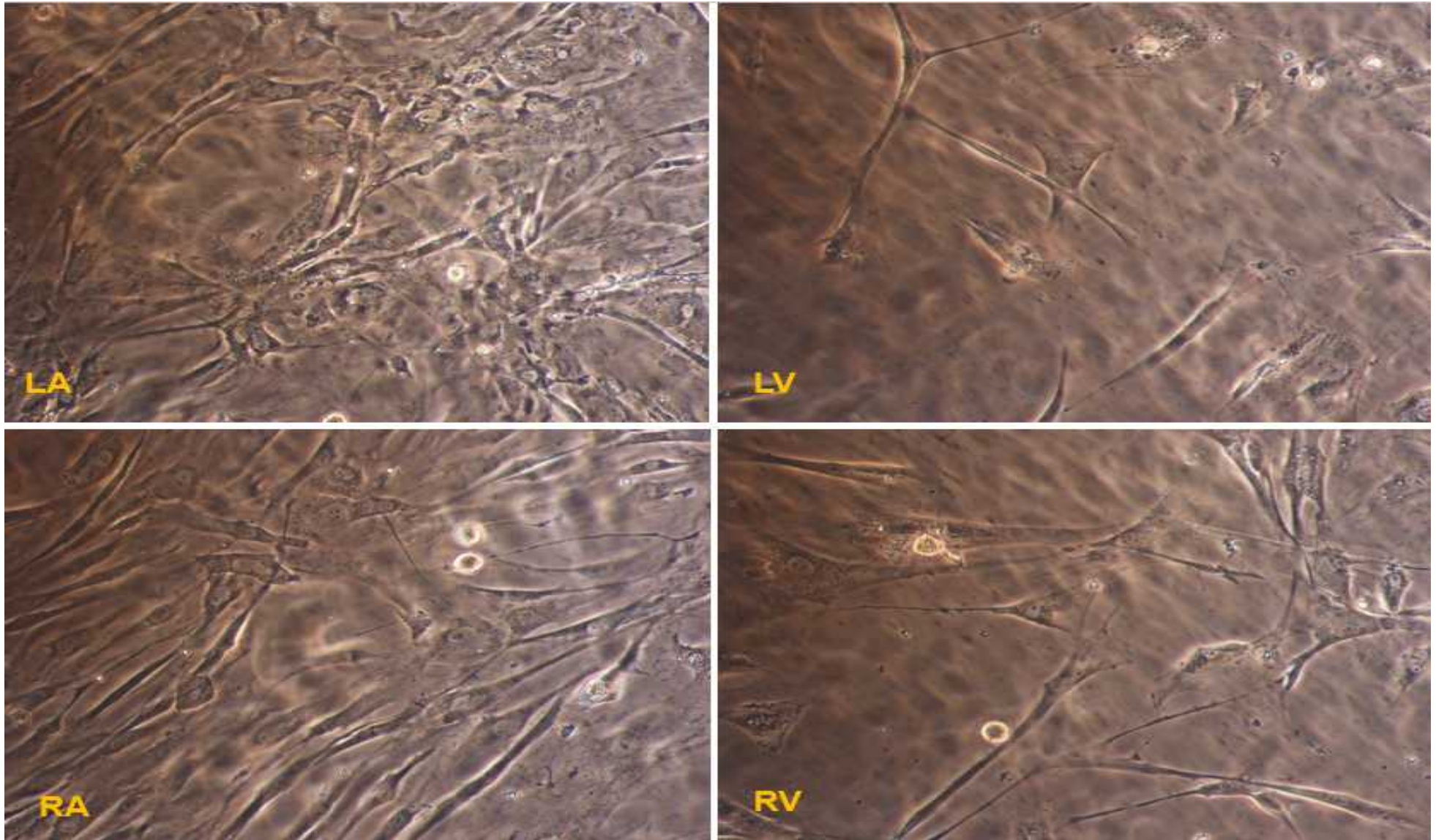


FIGURE A1.9: SAMPLE 10/2016: Bright field microscopic (10x). Moderate growth of atrial samples up to P2. Scanty growth of ventricular sample. Myofibroblastic transformation in left ventricular sample.

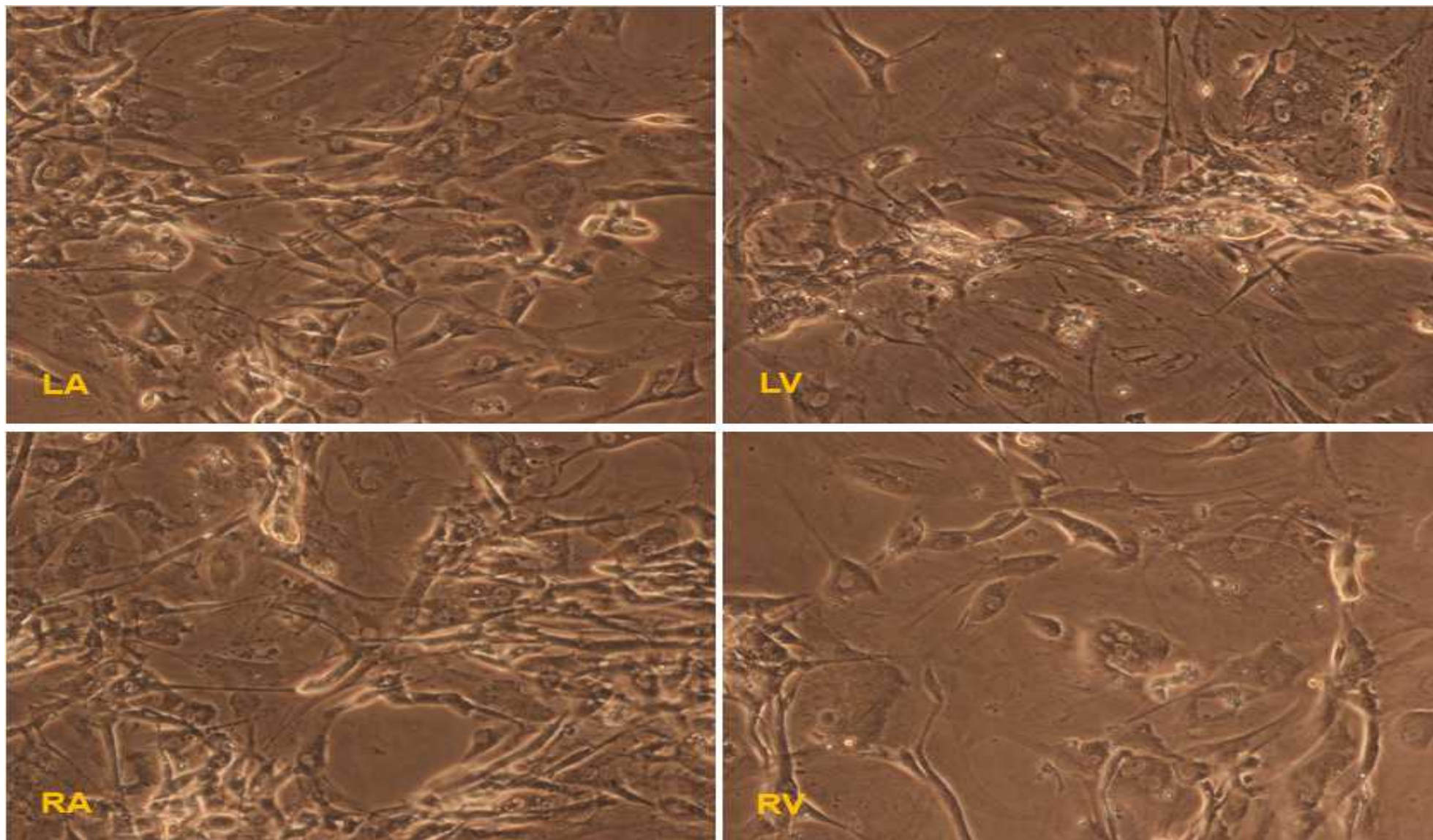


FIGURE A1.10: SAMPLE 12/2016: Bright field microscopic (10x). Good growth of atrial and ventricular fibroblast. However, in all four chambers colonies were over lapping each other.

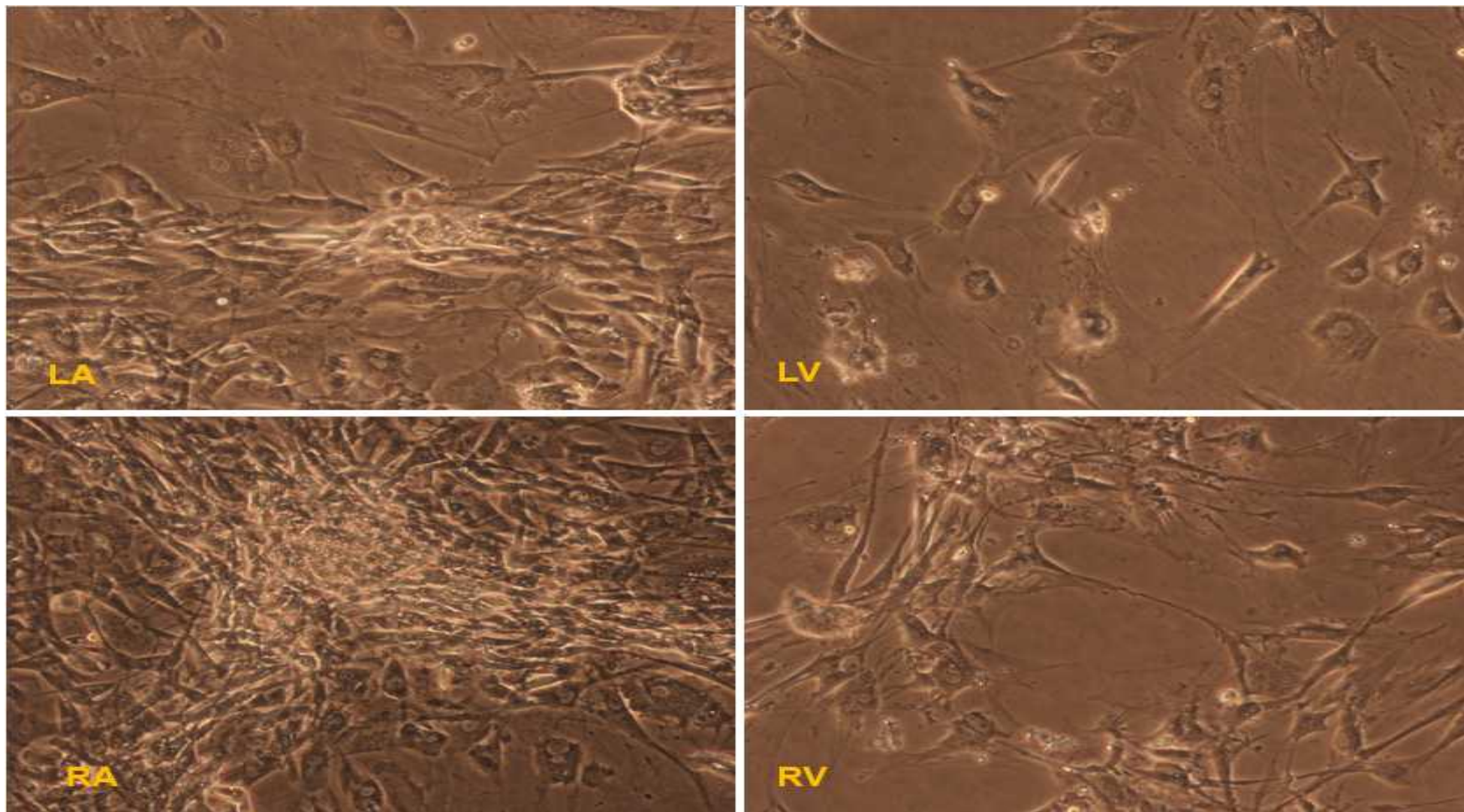


FIGURE A1.11: SAMPLE 13/2016: Bright field microscopic (10x). Moderate growth of left and right atrial samples. Right atrial samples were having a better growth profile than left atrial sample. Right ventricular sample showed scanty growth but still was better than the left ventricular sample. Myofibroblastic transformation of left ventricular sample was prominent.

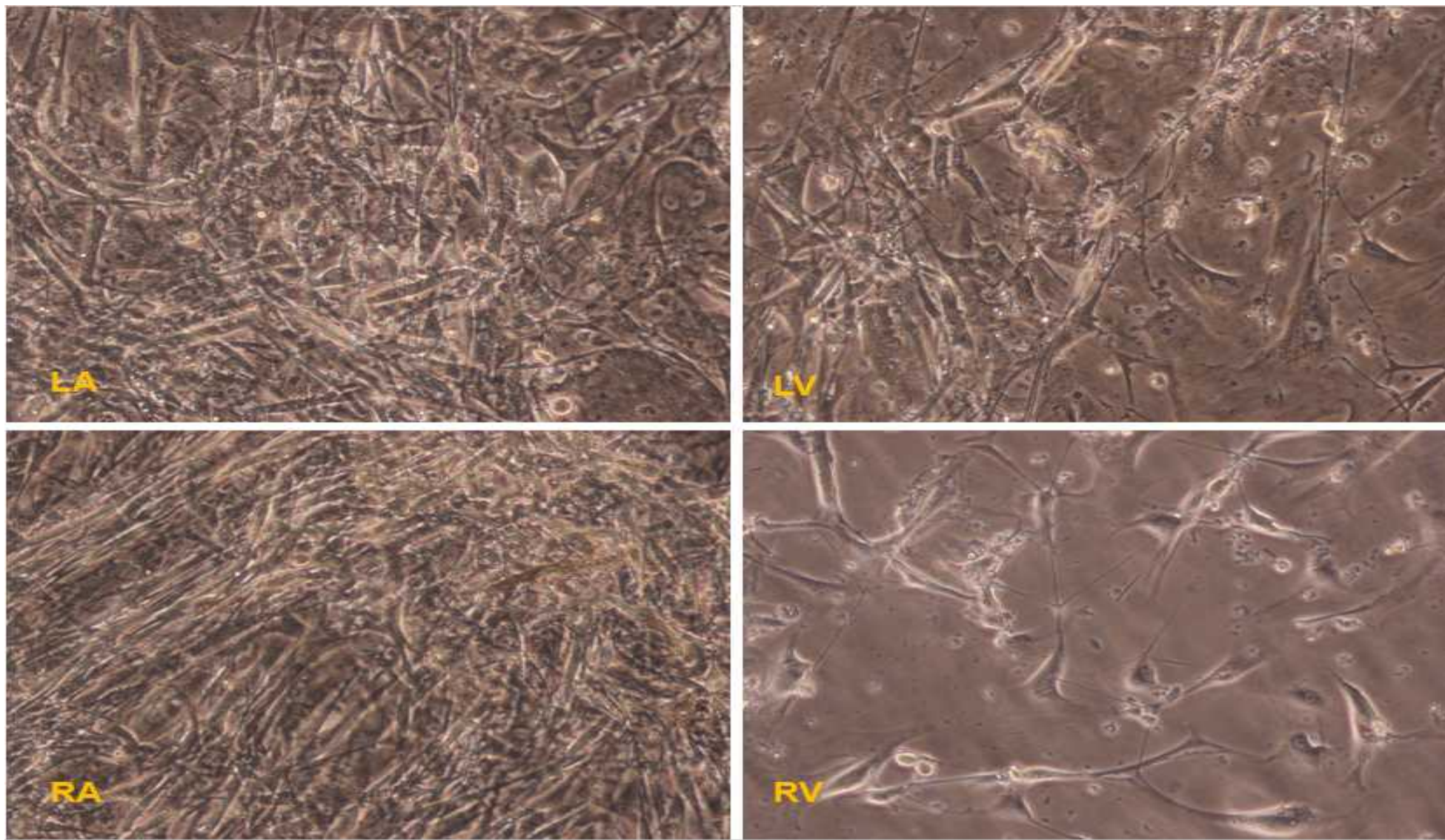


FIGURE A1.12: SAMPLE 14/2016: Bright field microscopic (10x). Good growth of both atrial samples. Among the ventricular samples, the left ventricular sample was growing better than right ventricular sample.

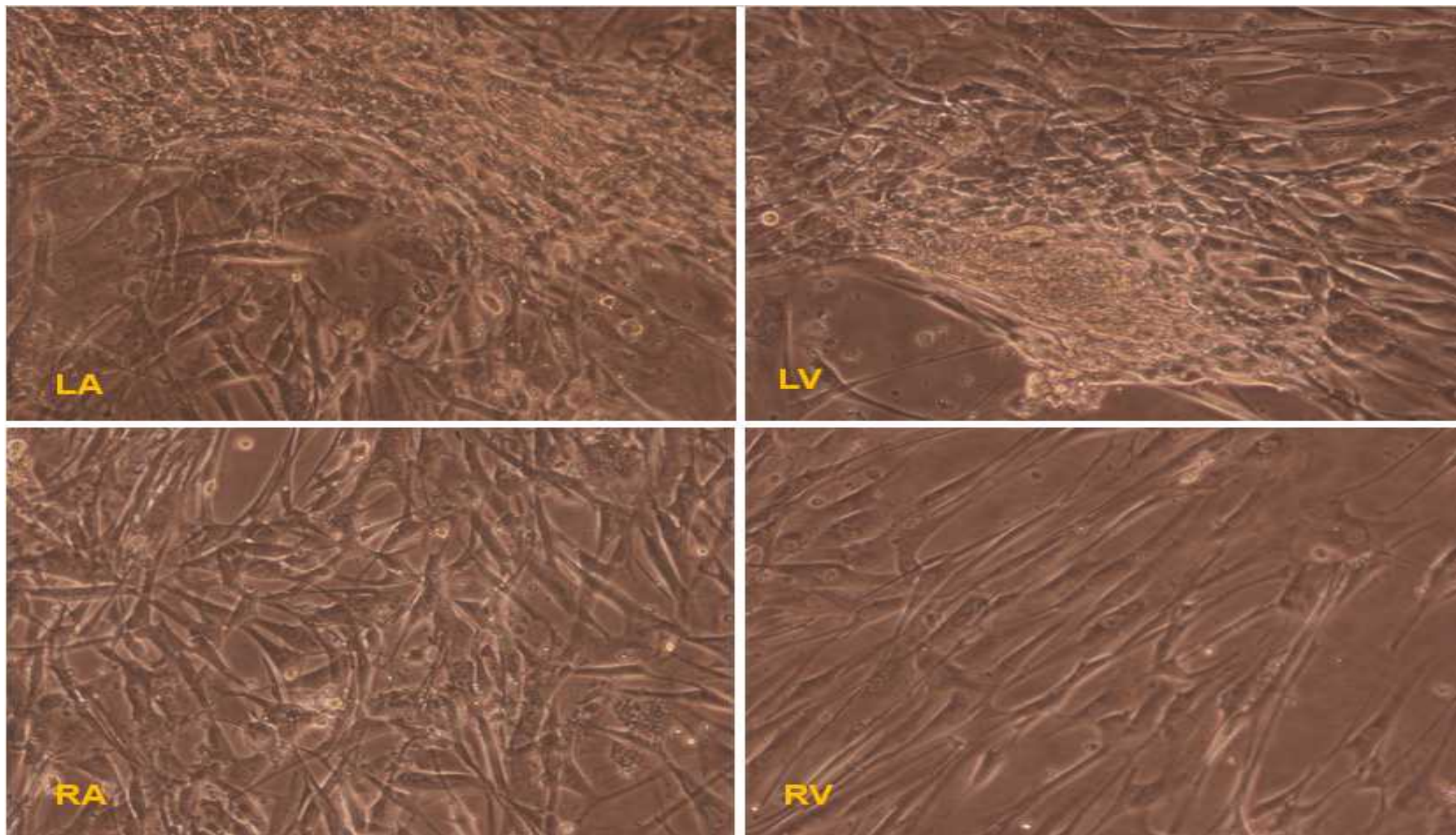


FIGURE A1.13: SAMPLE 15/2016: Bright field microscopic (10x). Good growing all four chamber fibroblasts. The colony formation of left ventricle was more prominent.

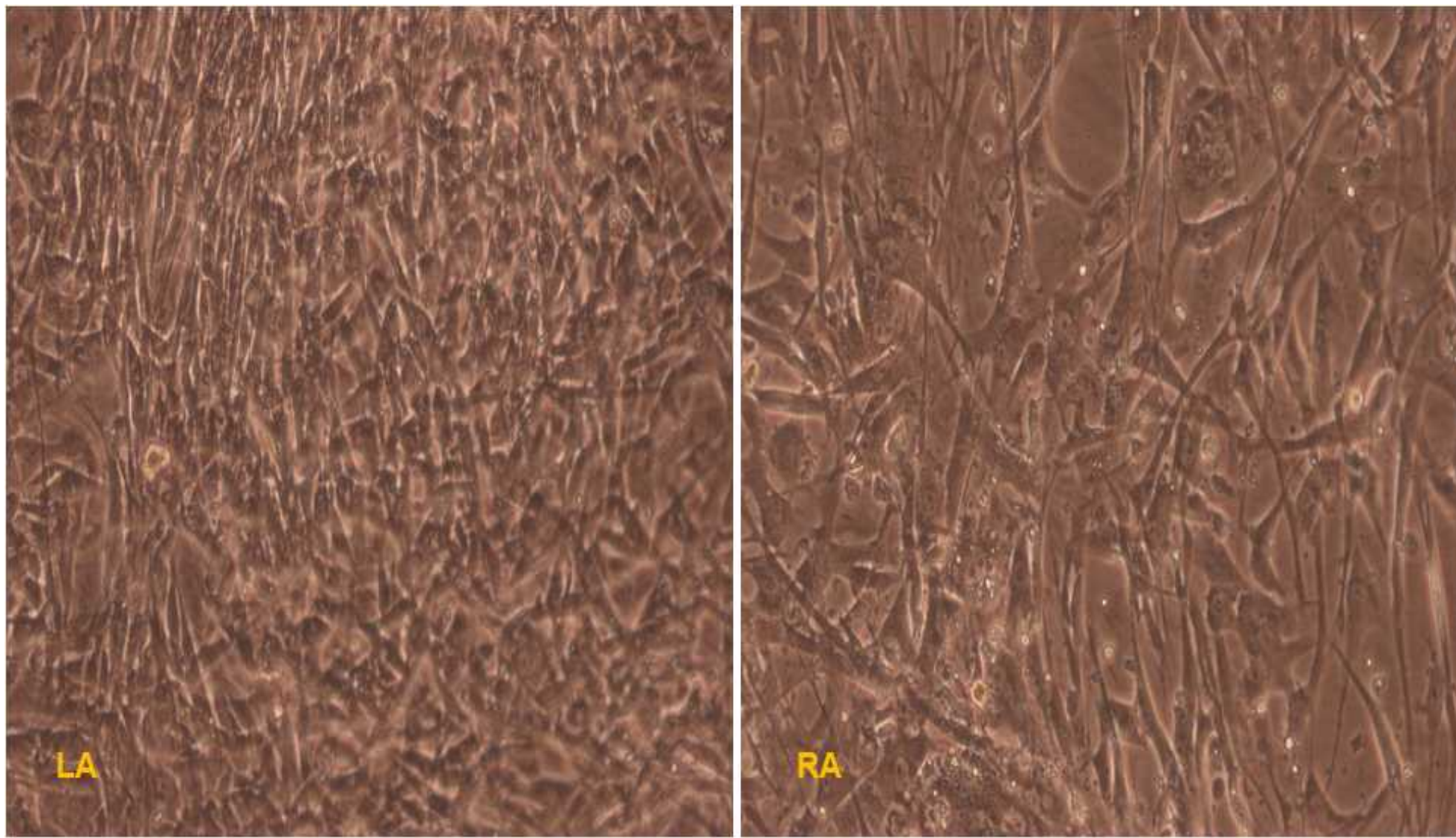


FIGURE A1.14: SAMPLE 16/2016: Bright field microscopic (10x). Good bi-atrial fibroblasts growth. No fibroblast growth in ventricular samples.

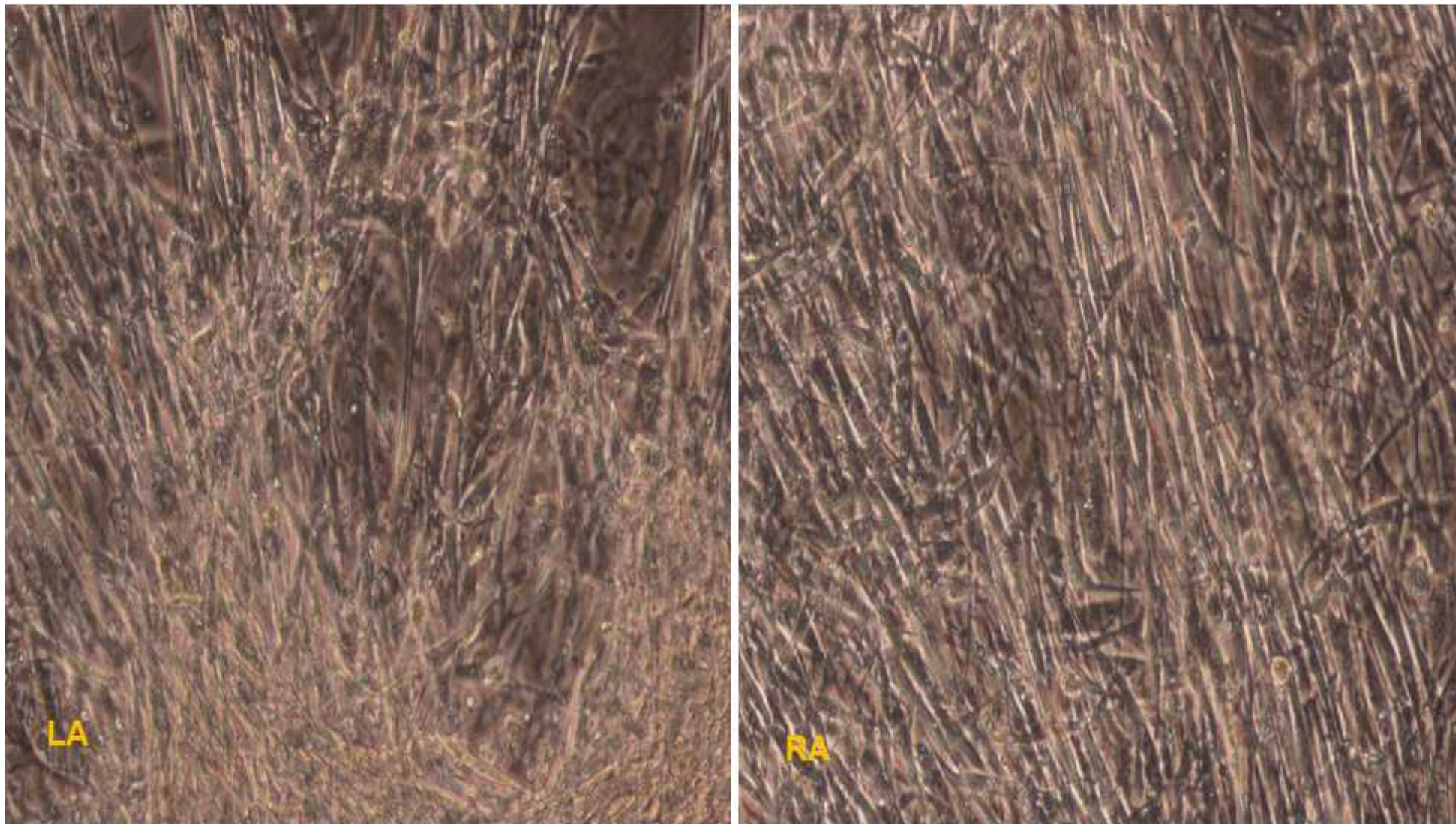


FIGURE A1.15: SAMPLE 18/2016: Bright field microscopic (10x). Good bi-atrial fibroblasts growth. No fibroblast growth in ventricular samples.

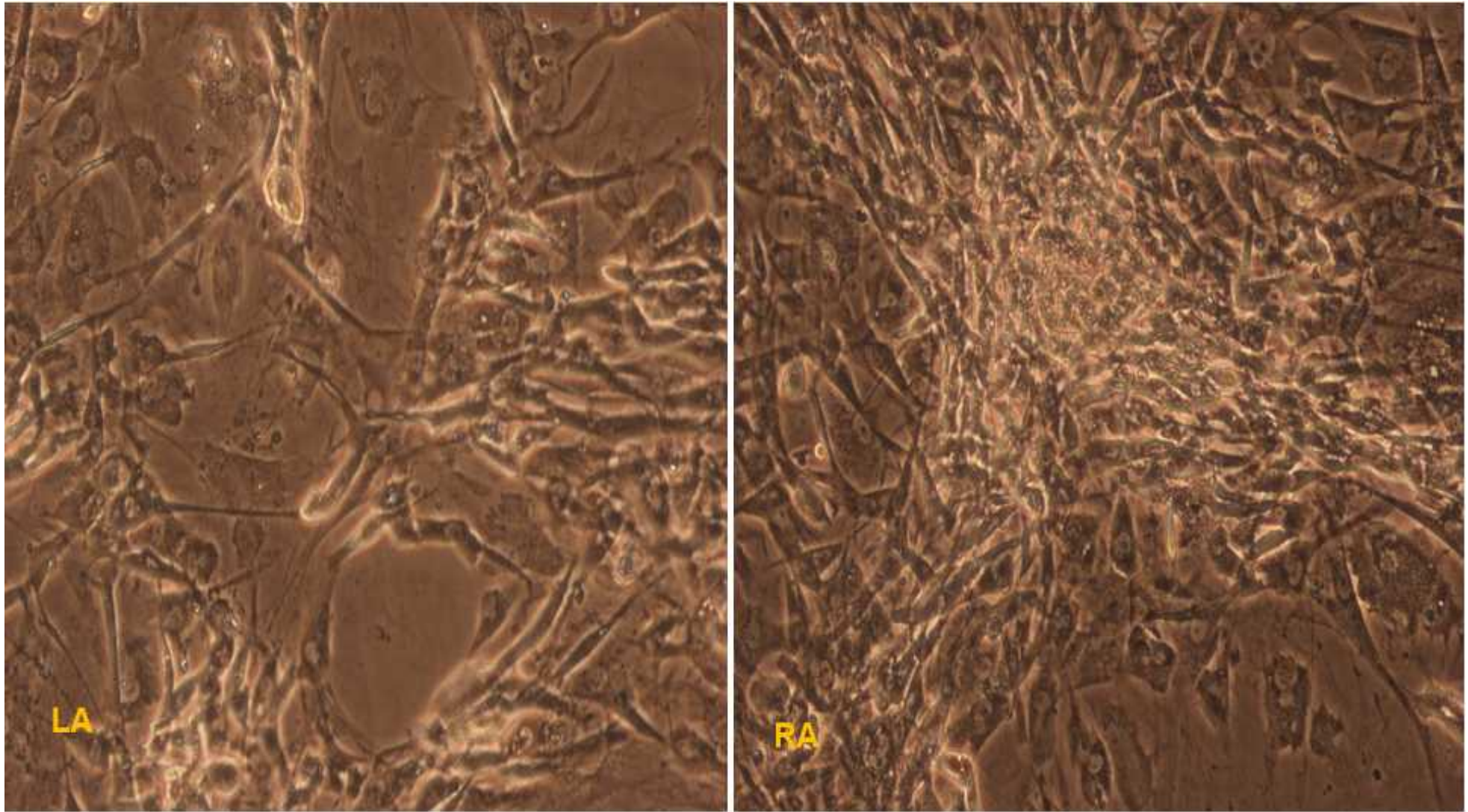


FIGURE A1.16: SAMPLE 19/2016: Bright field microscopic (10x). Moderate bi-atrial fibroblasts growth. No fibroblast growth in ventricular samples.

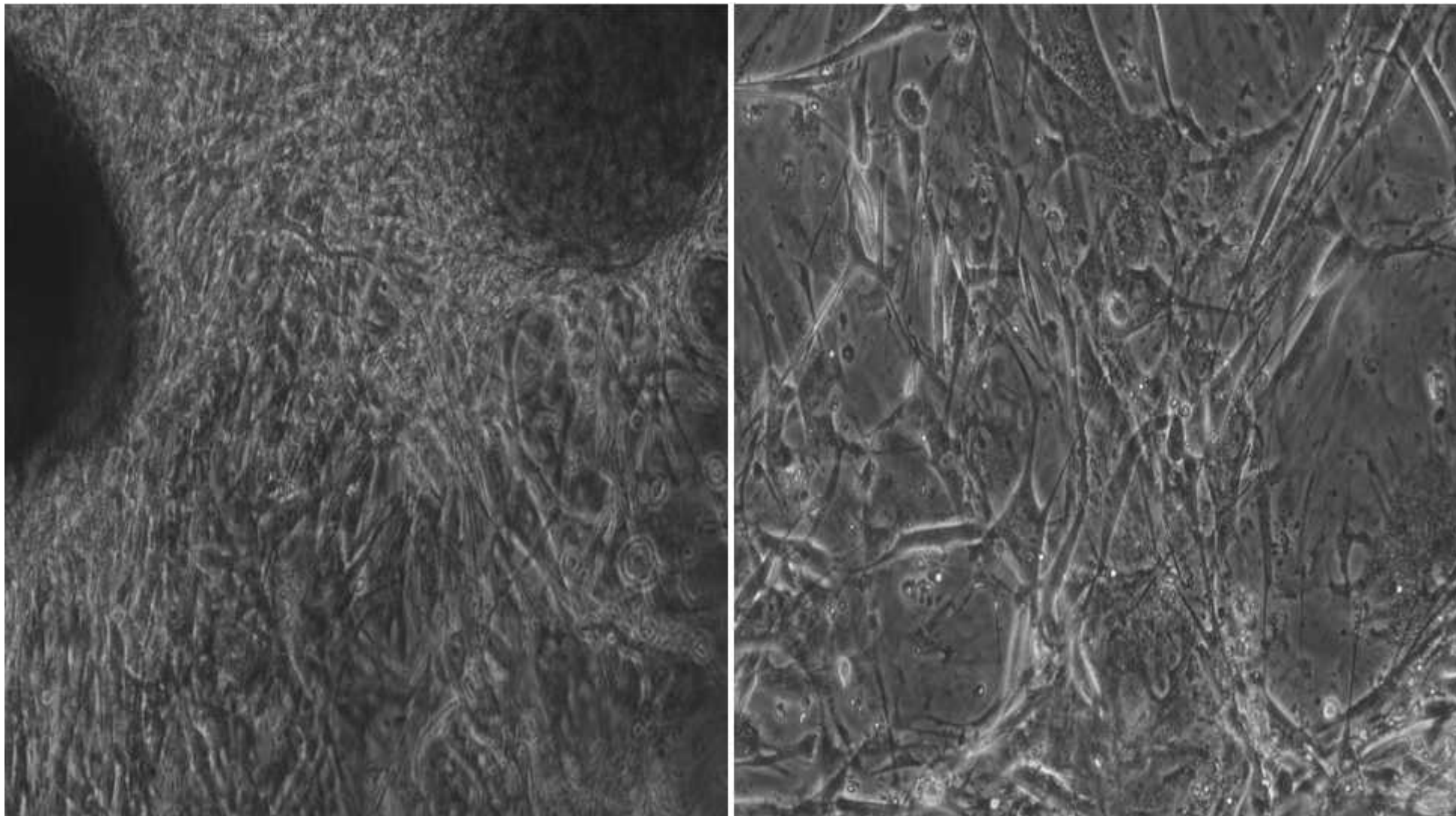


FIGURE A1.17: SAMPLE 20/2016: Bright field microscopic (10x). Good bi-atrial fibroblasts growth. No fibroblast growth in ventricular samples.

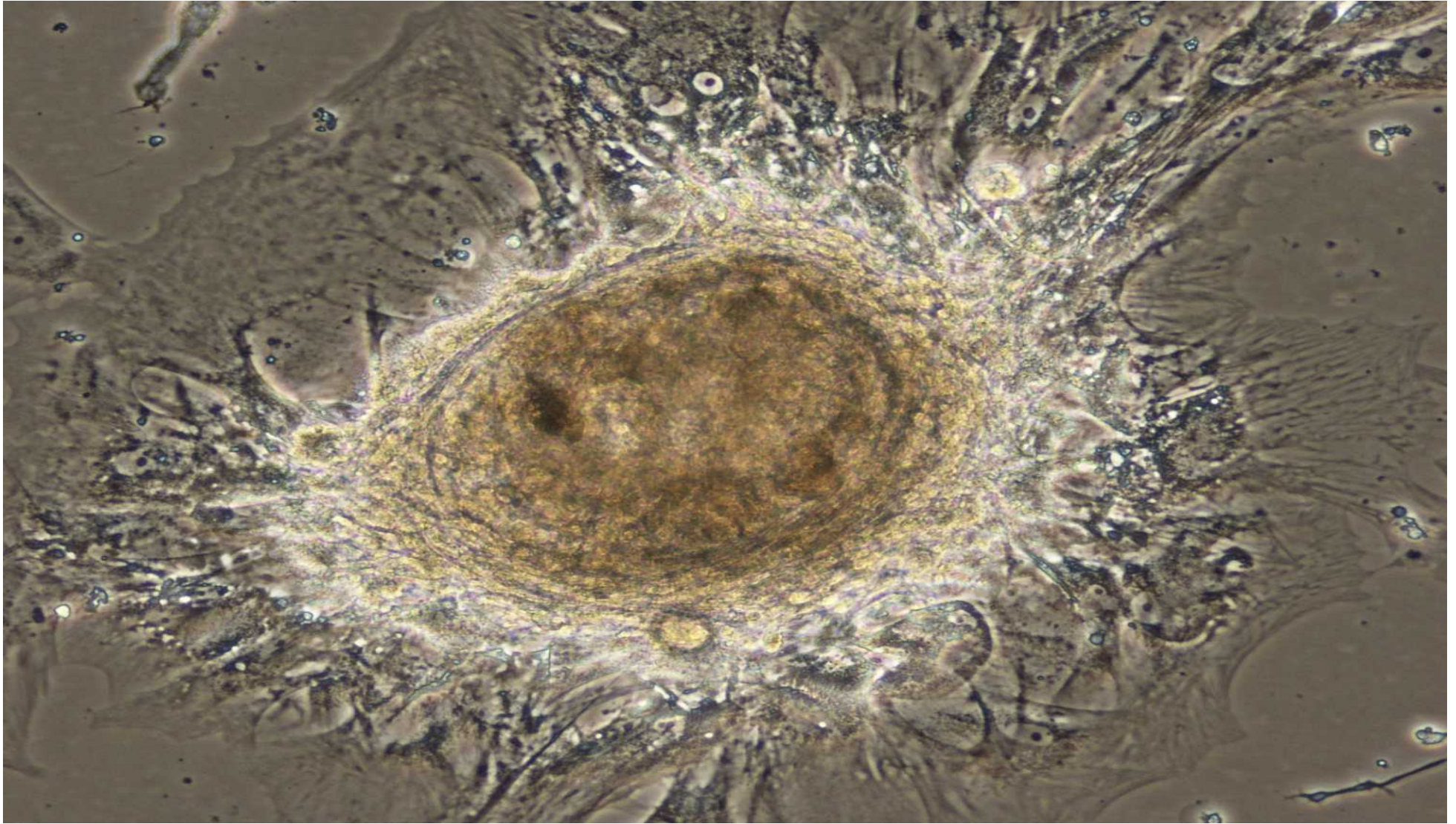


FIGURE A1.18: BRIGHT FIELD MICROSCOPIC (20X). Growth of fibroblast from the cardiac tissue. Early myofibroblastic transformation is noted in fibroblast emerging from the colony. Isolated colonies with no other colonies in the vicinity showed this pattern more often.

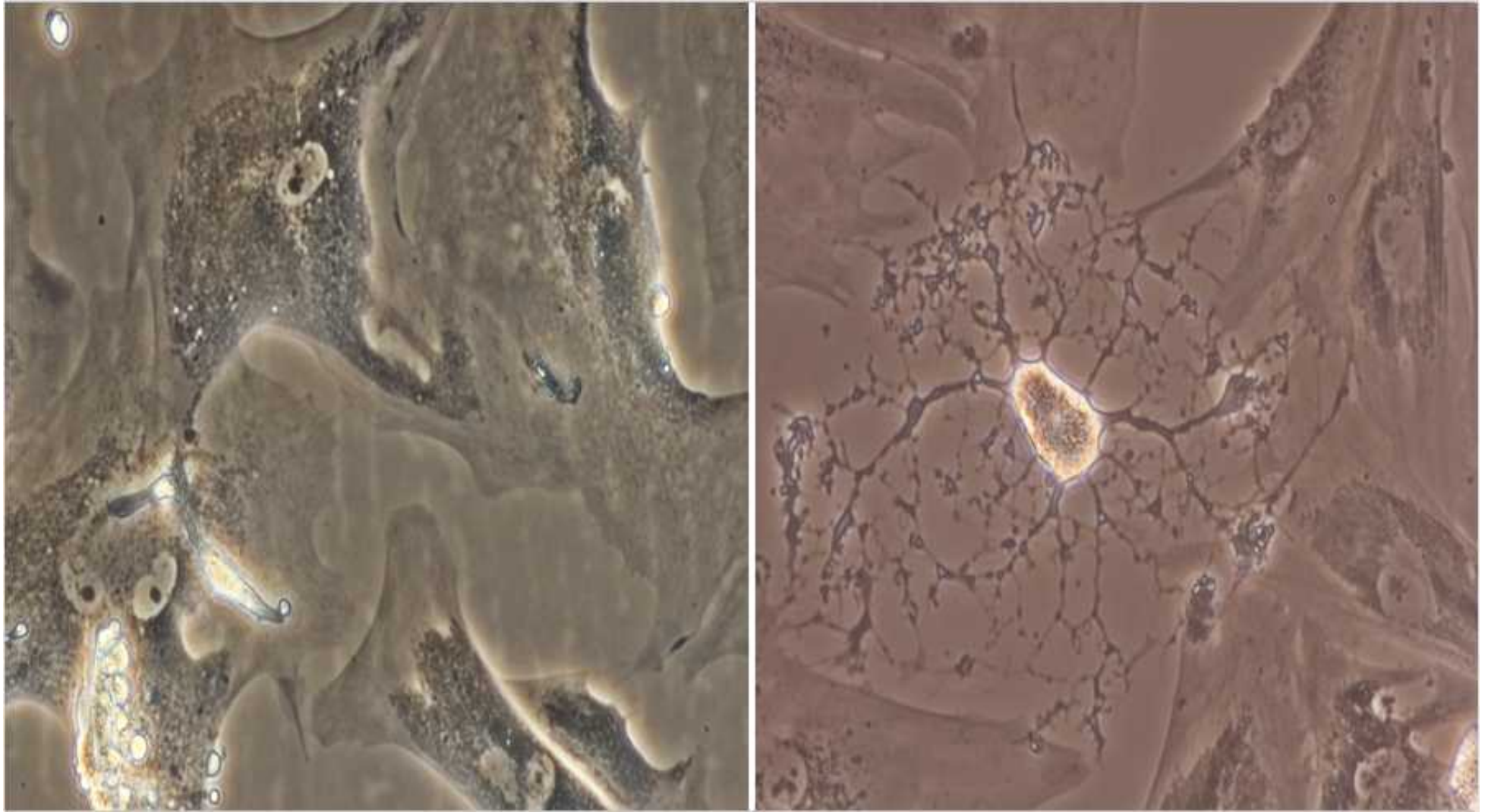


FIGURE A1.19: BRIGHT FIELD MICROSCOPIC (20X). Myofibroblastic transformation and senescence of fibroblasts at late passage stages (beyond P3 stage). Fibroblasts lose their projections, develop intracellular cytoskeleton and became hexagonal or globular and transforms into myofibroblasts. Later passage stages showed further defined cytoskeleton and eventually granular deposits and vacuole formation around the nuclei. Once perinuclear granular deposits are formed, cell disintegrate in next 48-72 hours.

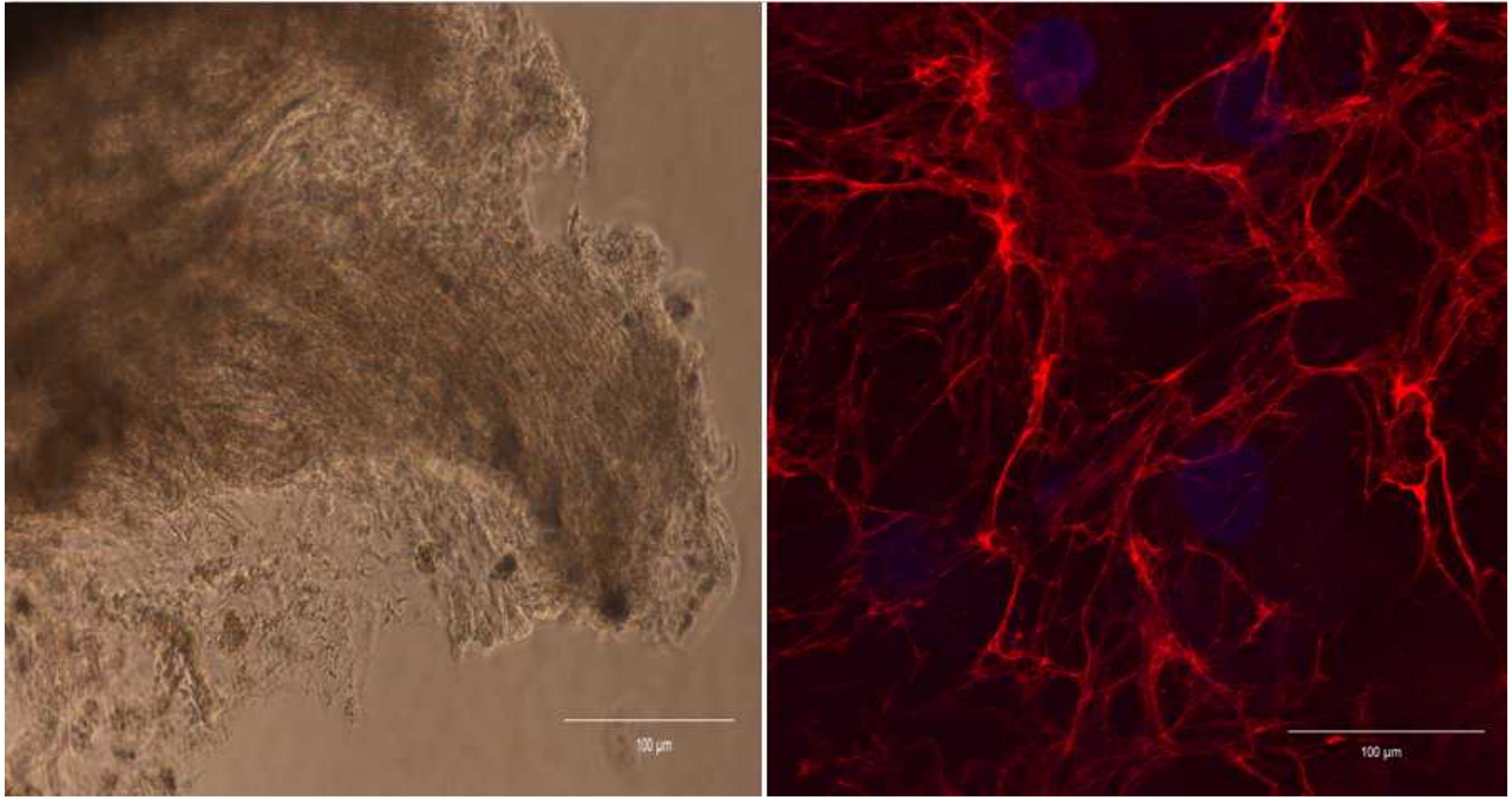


FIGURE A1.20: BRIGHT FIELD MICROSCOPIC (10X) (LEFT). CONFOCAL IMAGE (1024) OF CYTOSKELETON STAINED (RIGHT). Some samples did not show any fibroblast growth. These samples revealed cytoplasmic extensions projecting out of the harvested tissue and colony but there was either no or minimal cellular component to it. These cytoskeletal projections were shown in bright field microscopy (left) and with confocal image (right) on staining with Phalloidin. The cytoskeleton stained with Phalloidin was stained with DAPI as well to see any nuclei. Scanty nuclei were noted in a few samples such as one shown on the right-hand side.

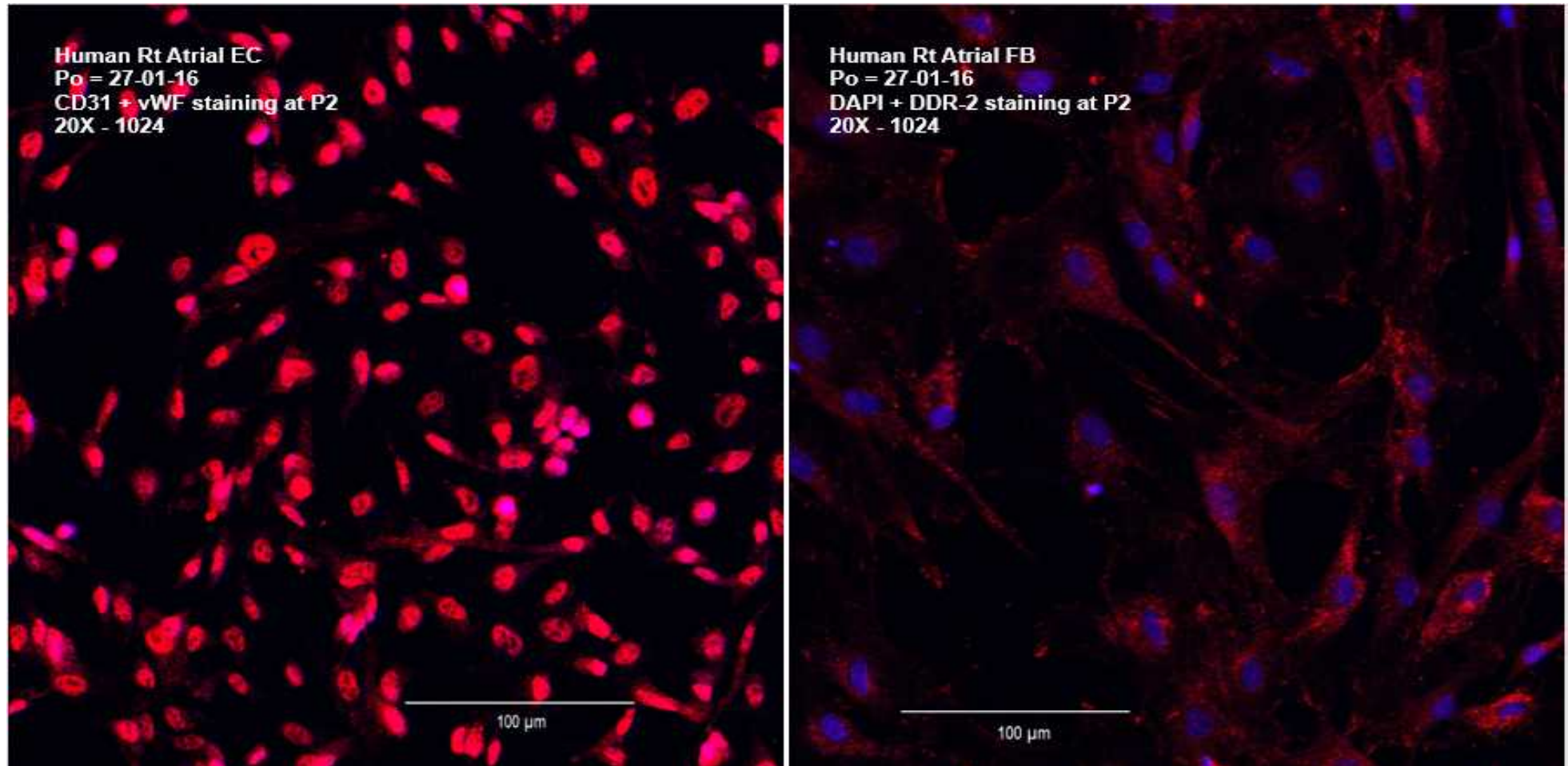


FIGURE A1.21: Confocal Image (1024 X) of Endothelial Cells and Fibroblasts stained with positive markers at P2. To differentiate the fibroblast population from endothelial cells both cell populations were harvested from the same sample on the same day and were incubated in their specific medium and vice versa. Human Rt atrial endothelial cells (left) incubated with endothelial specific medium (ECM Medium) showed a good growth pattern up to P2 stage, However, the endothelial cells incubated with FGM did not survive beyond day-5 at P0 stage. Similarly, fibroblasts growth with FGM was good at P2 stage. When ECM was added to fibroblasts, the fibroblasts showed less growth in comparison to the fibroblasts with FGM medium. The myofibroblastic transformation of fibroblasts with ECM medium was more pronounced. Fibroblasts (right) were stained with DDR-2 and nuclei are stained with DAPI.

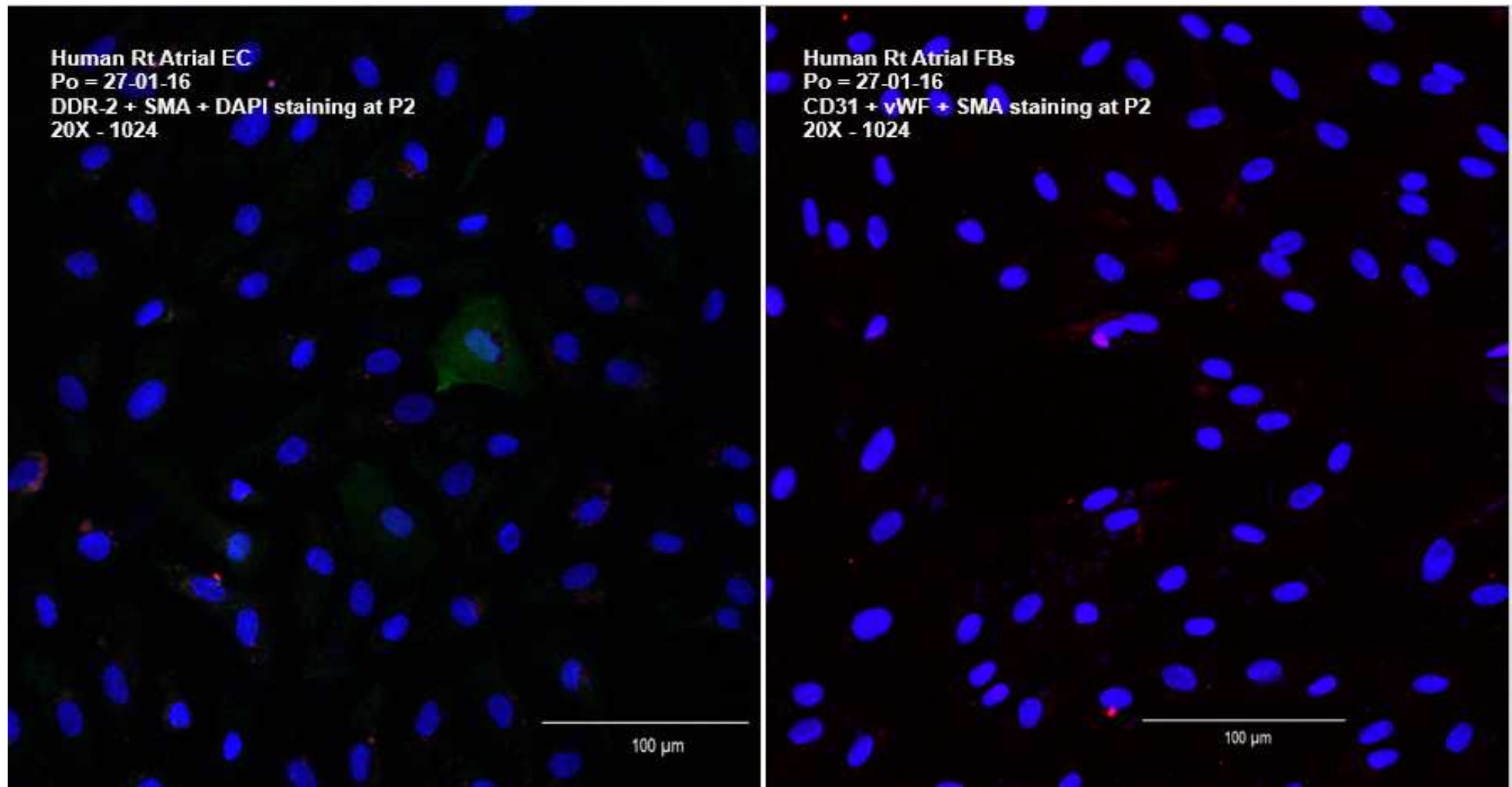


FIGURE A1.22: Confocal Image (1024 X) of Endothelial Cells and Fibroblasts stained with negative markers at P2. The endothelial cells at P2 stage were stained with DDR-2, SMA & DAPI. The confocal image (left) showed only one cell stained with SMA and all nuclei were stained with DAPI revealing a true endothelial cell population. When fibroblasts were stained with endothelial cell specific markers such as CD31, vWF and SMA and nuclei stained with DAPI they were negative for all three markers depicting a true fibroblast population. In the middle of the confocal image some faint staining was visible only after SMA was added to the cells.

APPENDIX B

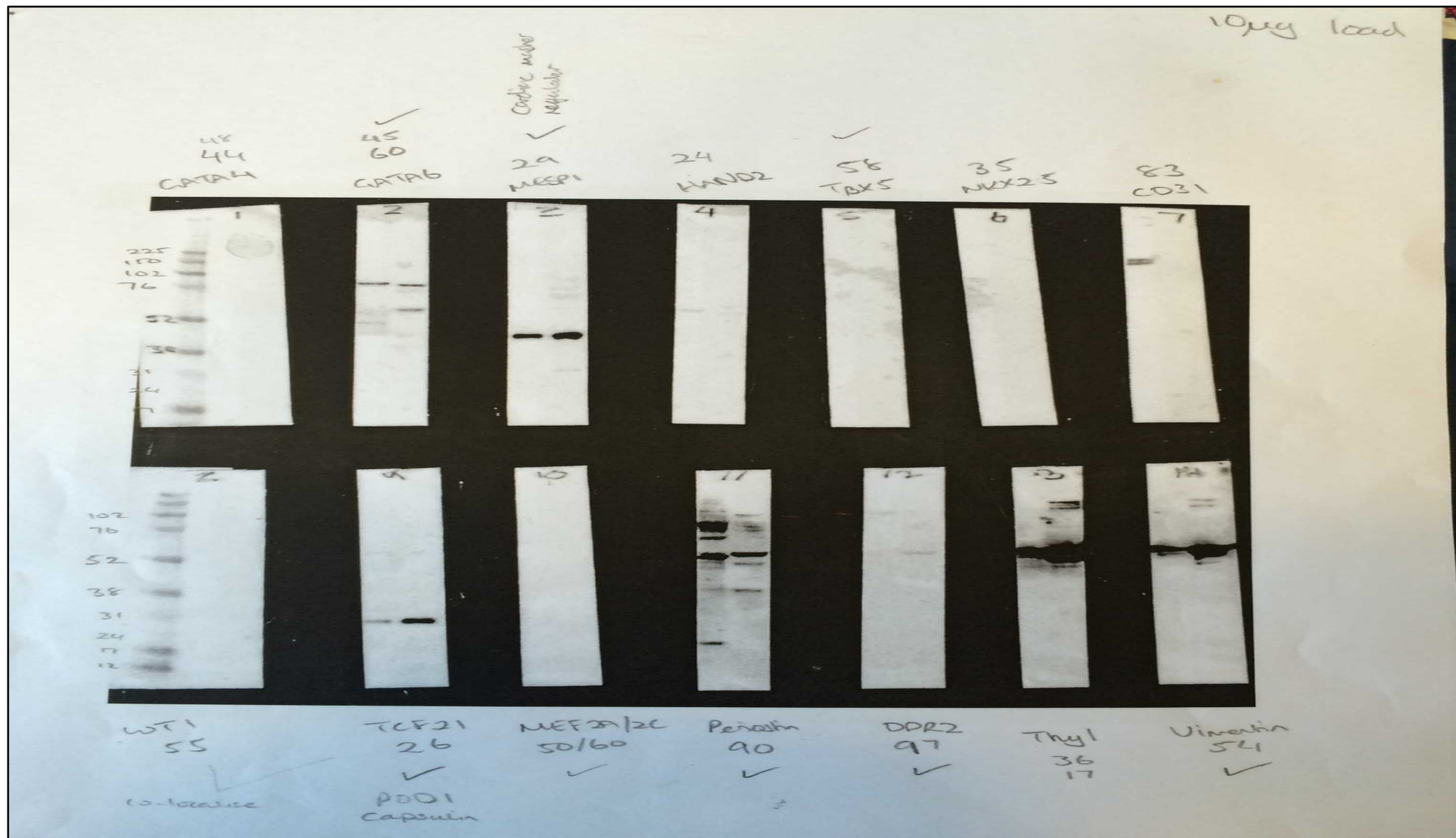


FIGURE B1.1: CHEMILUMINESCENCE IMAGING FOR DETECTION OF PROTEIN BANDS REPRESENTING THE MOLECULAR WEIGHT OF PRIMARY ANTIBODIES AND TRANSCRIPTION FACTORS.

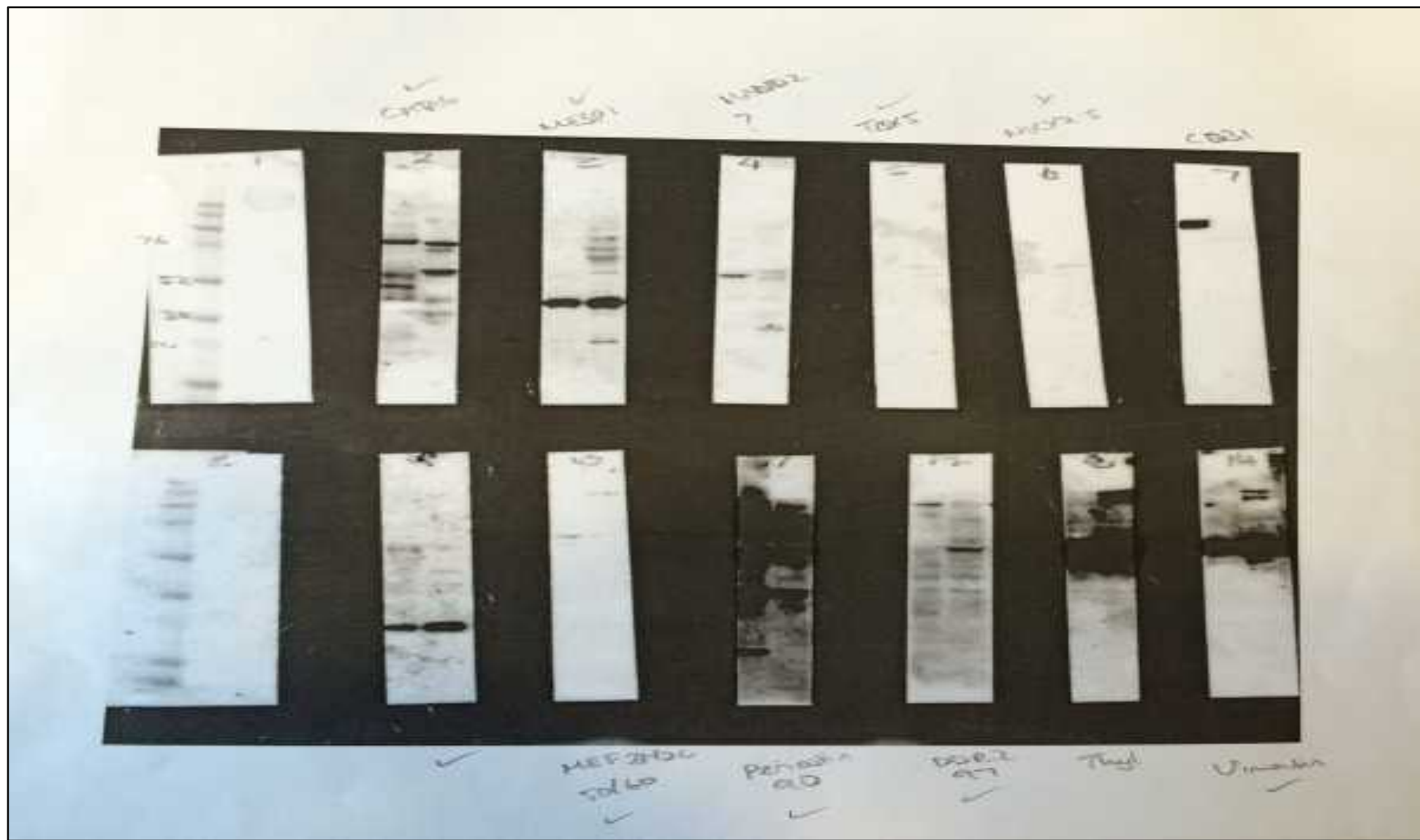
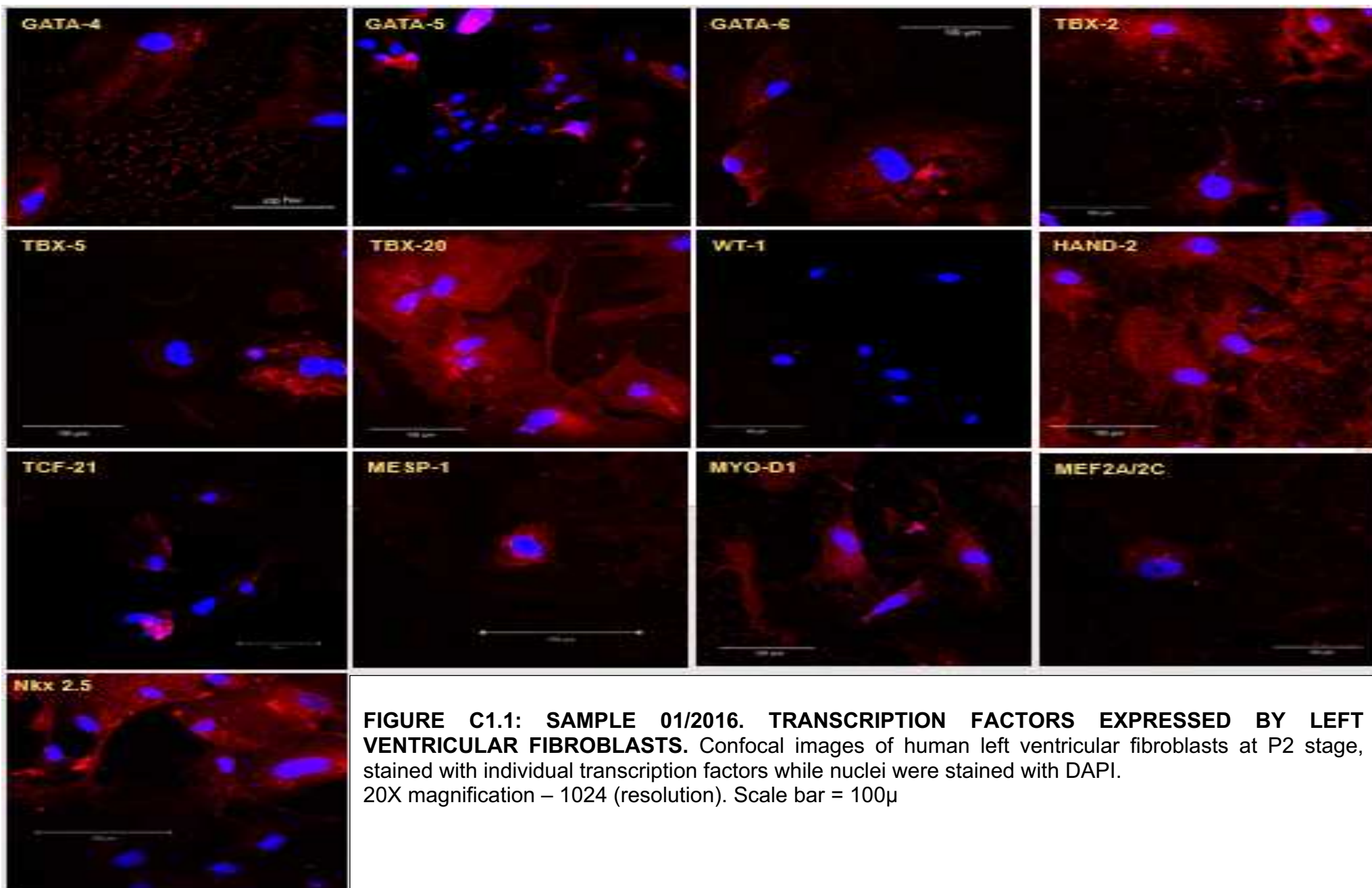
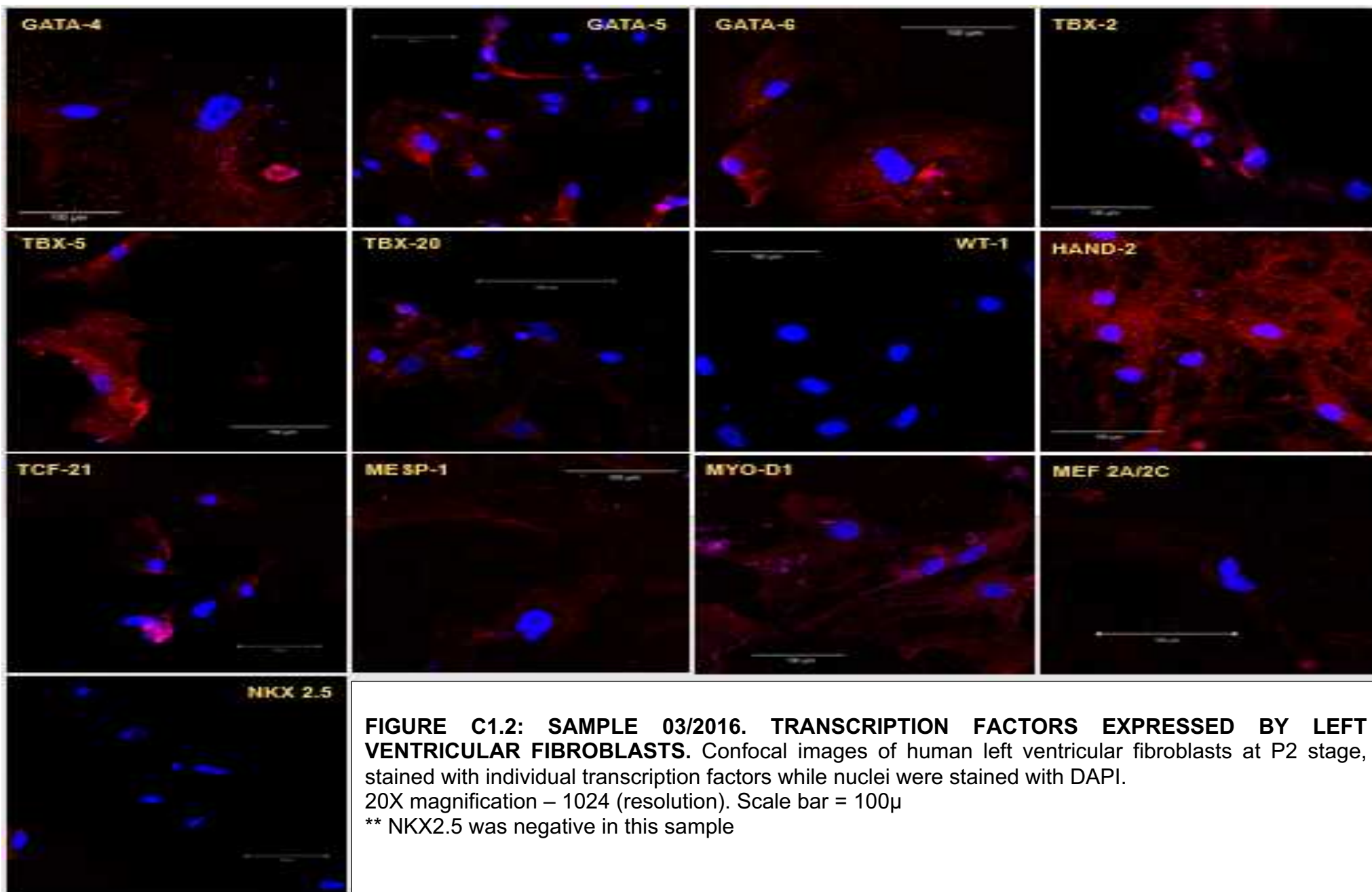
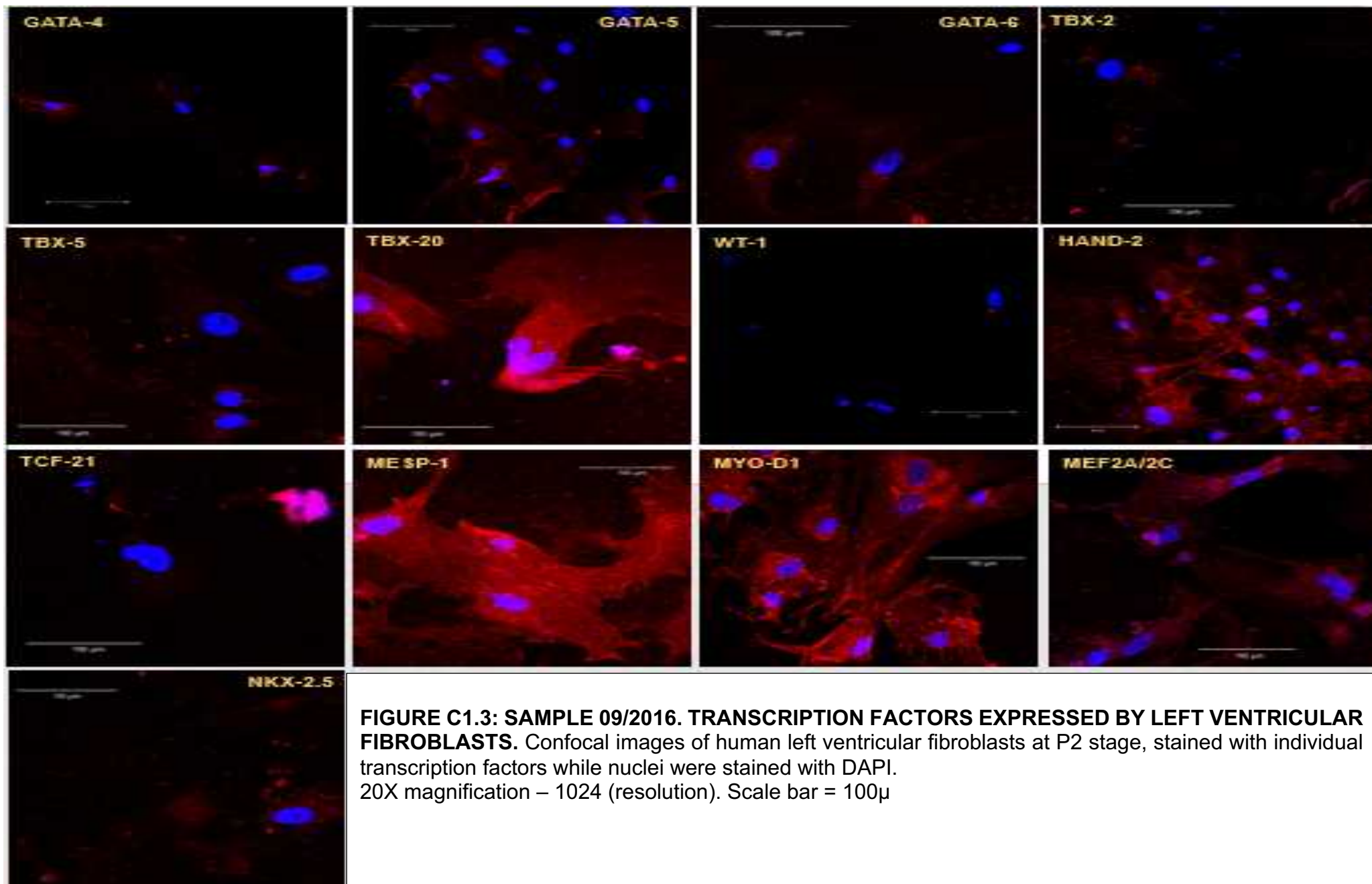


FIGURE B1.2: CHEMILUMINESCENCE IMAGING FOR DETECTION OF PROTEIN BANDS REPRESENTING THE MOLECULAR WEIGHT OF PRIMARY ANTIBODIES AND TRANSCRIPTION FACTORS.

APPENDIX C







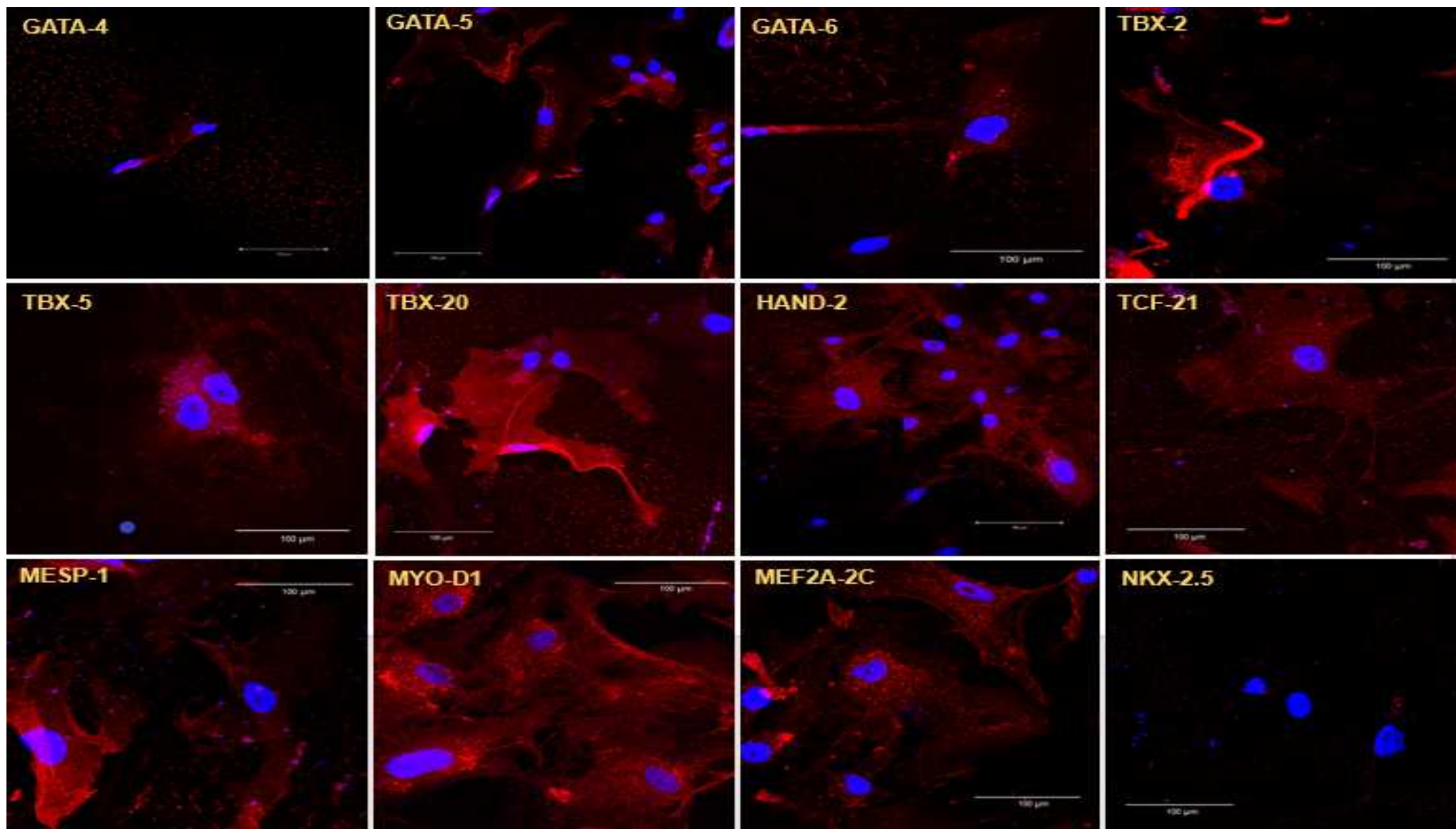


FIGURE C1.4: SAMPLE 12/2016. TRANSCRIPTION FACTORS EXPRESSED BY LEFT VENTRICULAR FIBROBLASTS. Confocal images of human left ventricular fibroblasts at P2 stage, stained with individual transcription factors while nuclei were stained with DAPI. 20X magnification – 1024 (resolution). Scale bar = 100 μ . NKX2.5 was faintly positive.

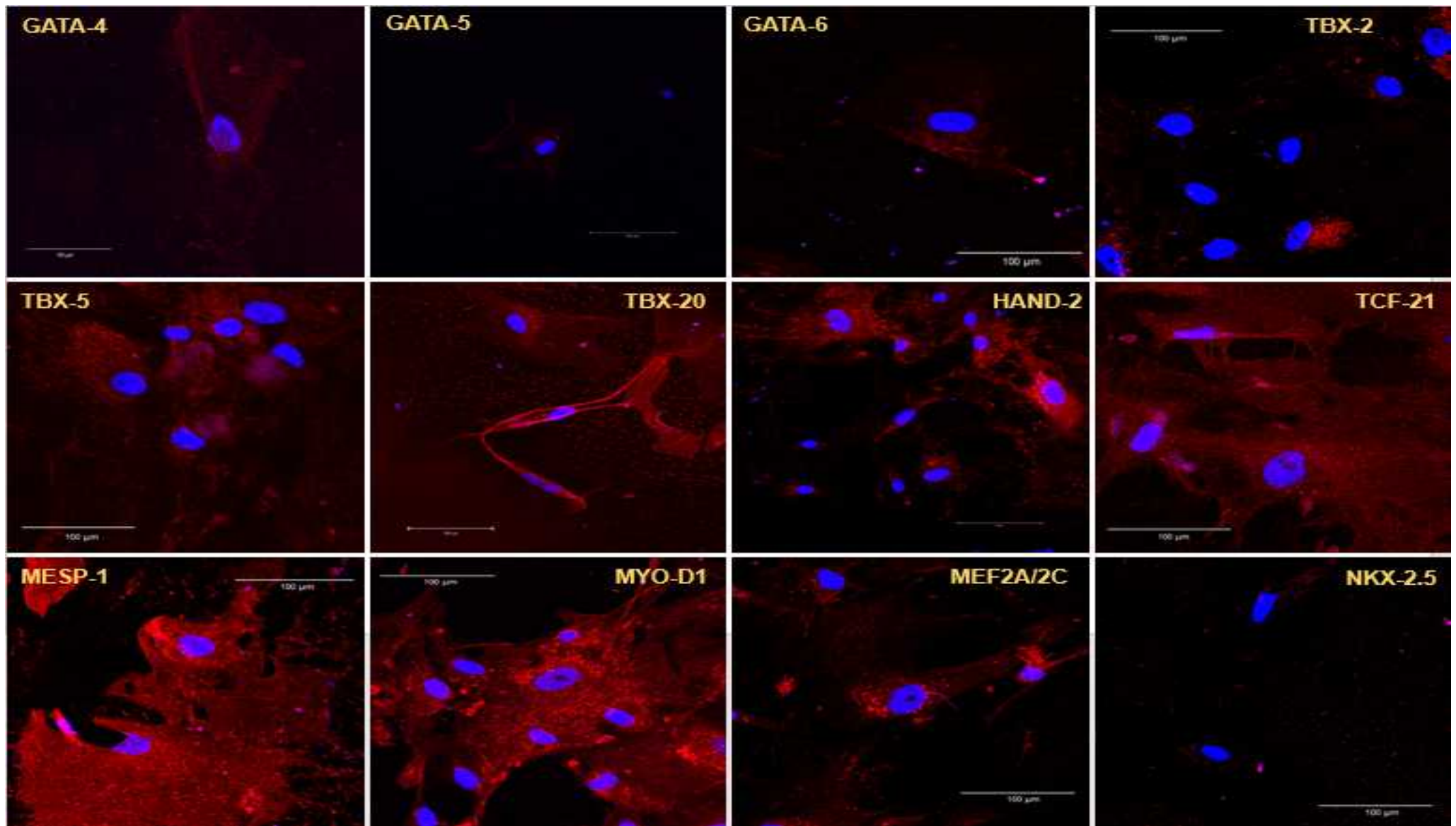
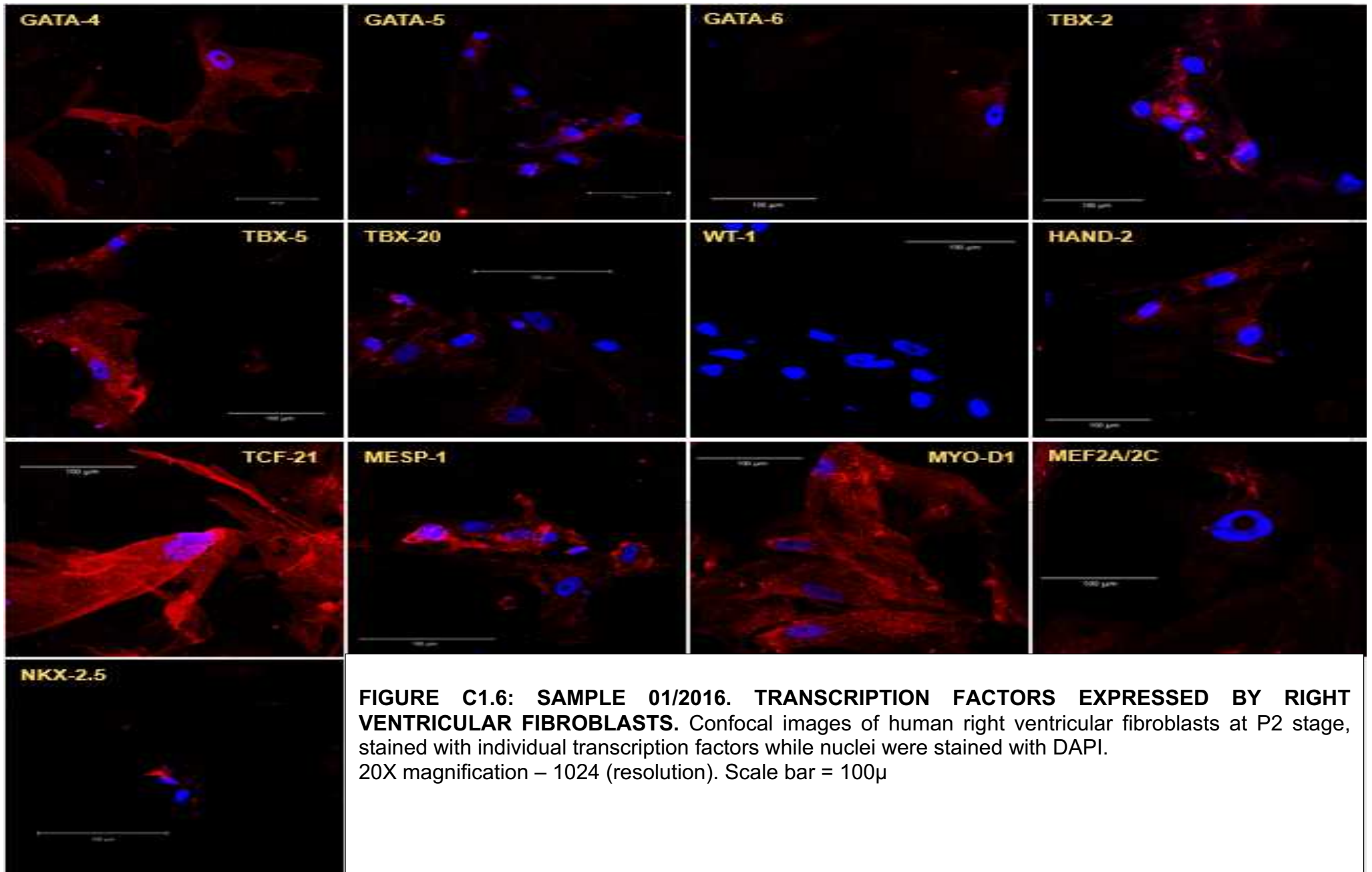
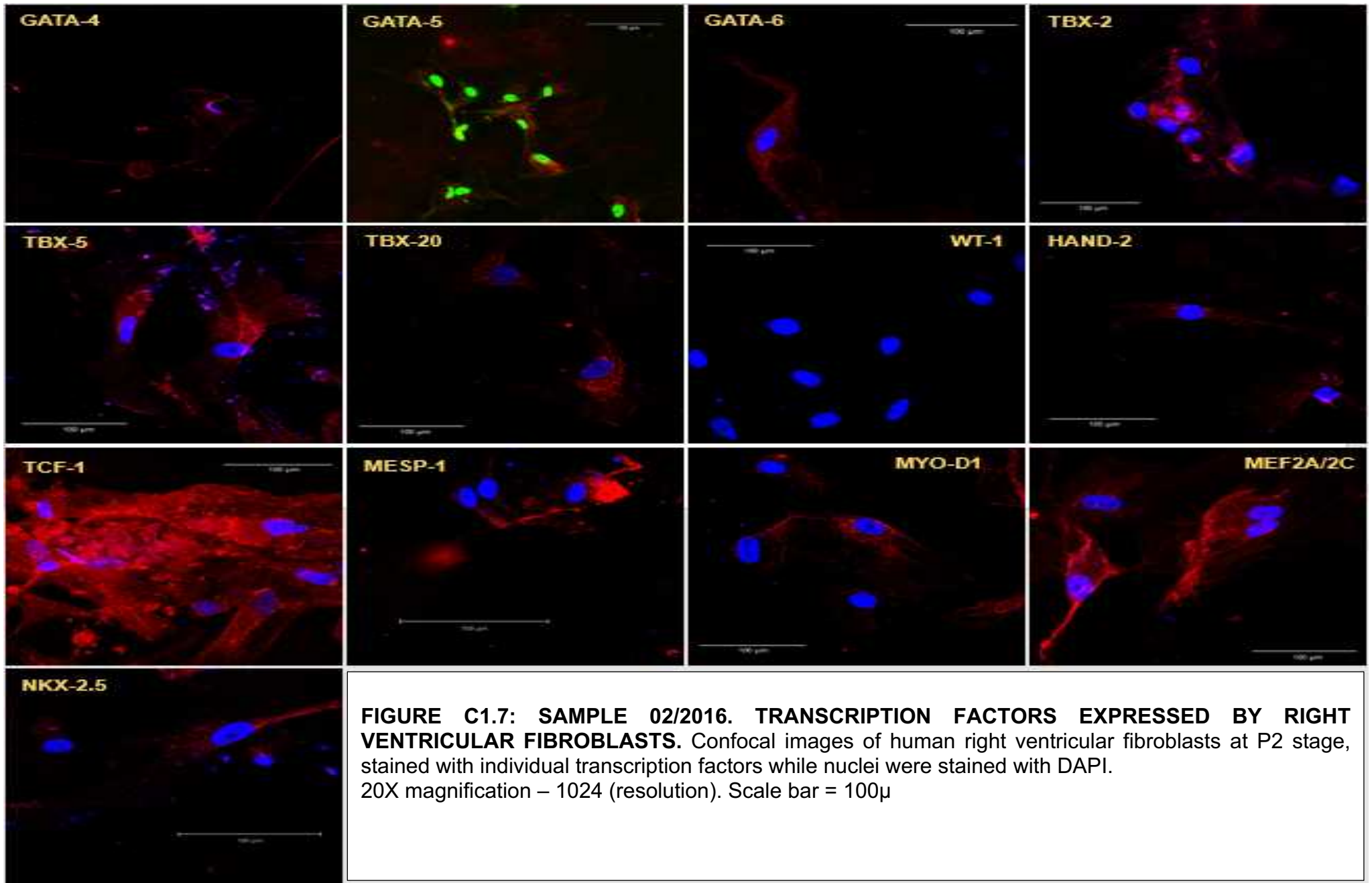


FIGURE C1.5: SAMPLE 15/2016. TRANSCRIPTION FACTORS EXPRESSED BY LEFT VENTRICULAR FIBROBLASTS. Confocal images of human left ventricular fibroblasts at P2 stage, stained with individual transcription factors while nuclei were stained with DAPI. 20X magnification – 1024 (resolution). Scale bar = 100 μ . NKX2.5 was faintly positive.





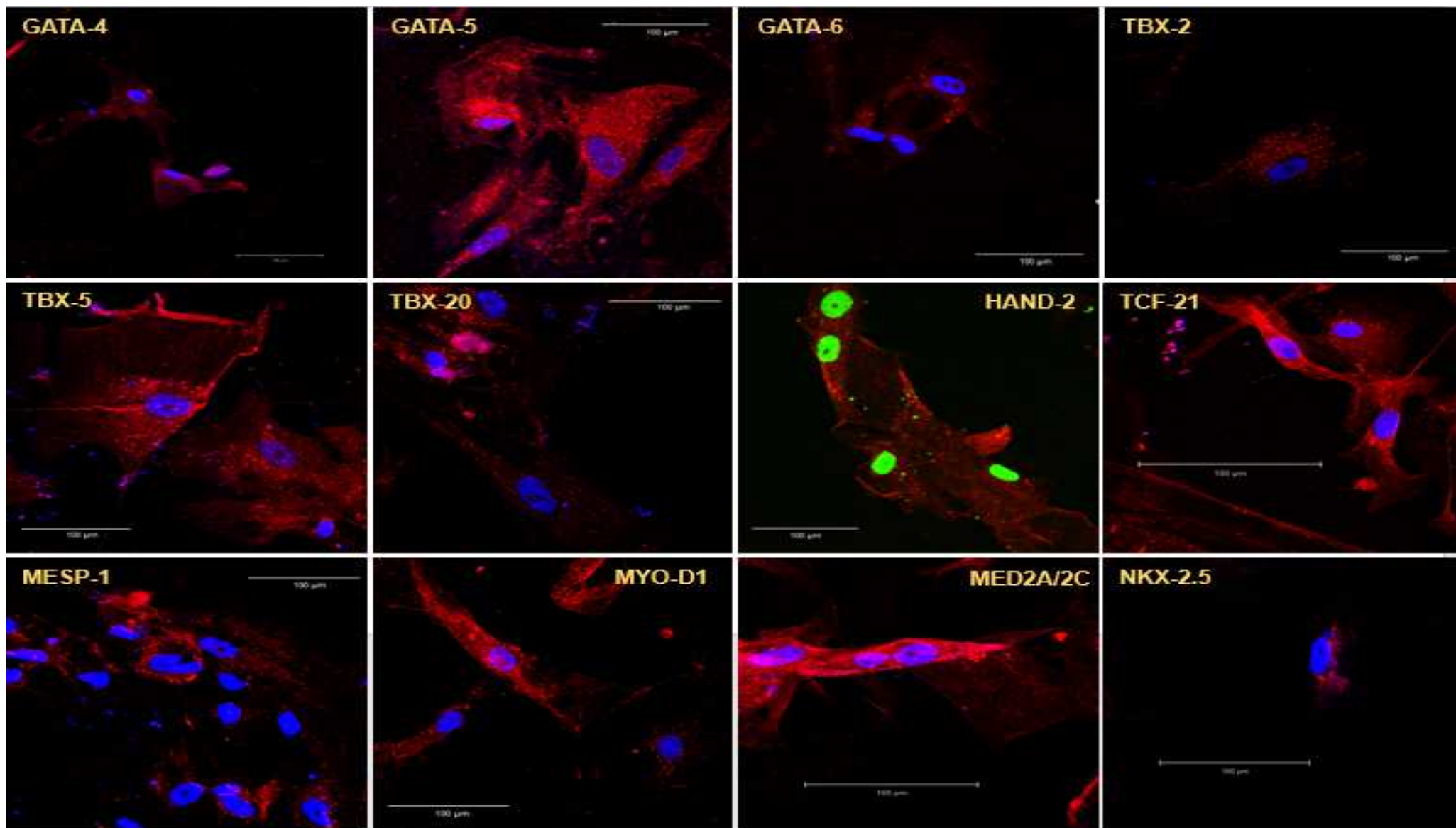


FIGURE C1.8: SAMPLE 09/2016. TRANSCRIPTION FACTORS EXPRESSED BY RIGHT VENTRICULAR FIBROBLASTS. Confocal images of human right ventricular fibroblasts at P2 stage, stained with individual transcription factors while nuclei were stained with DAPI. 20X magnification – 1024 (resolution). Scale bar = 100µ

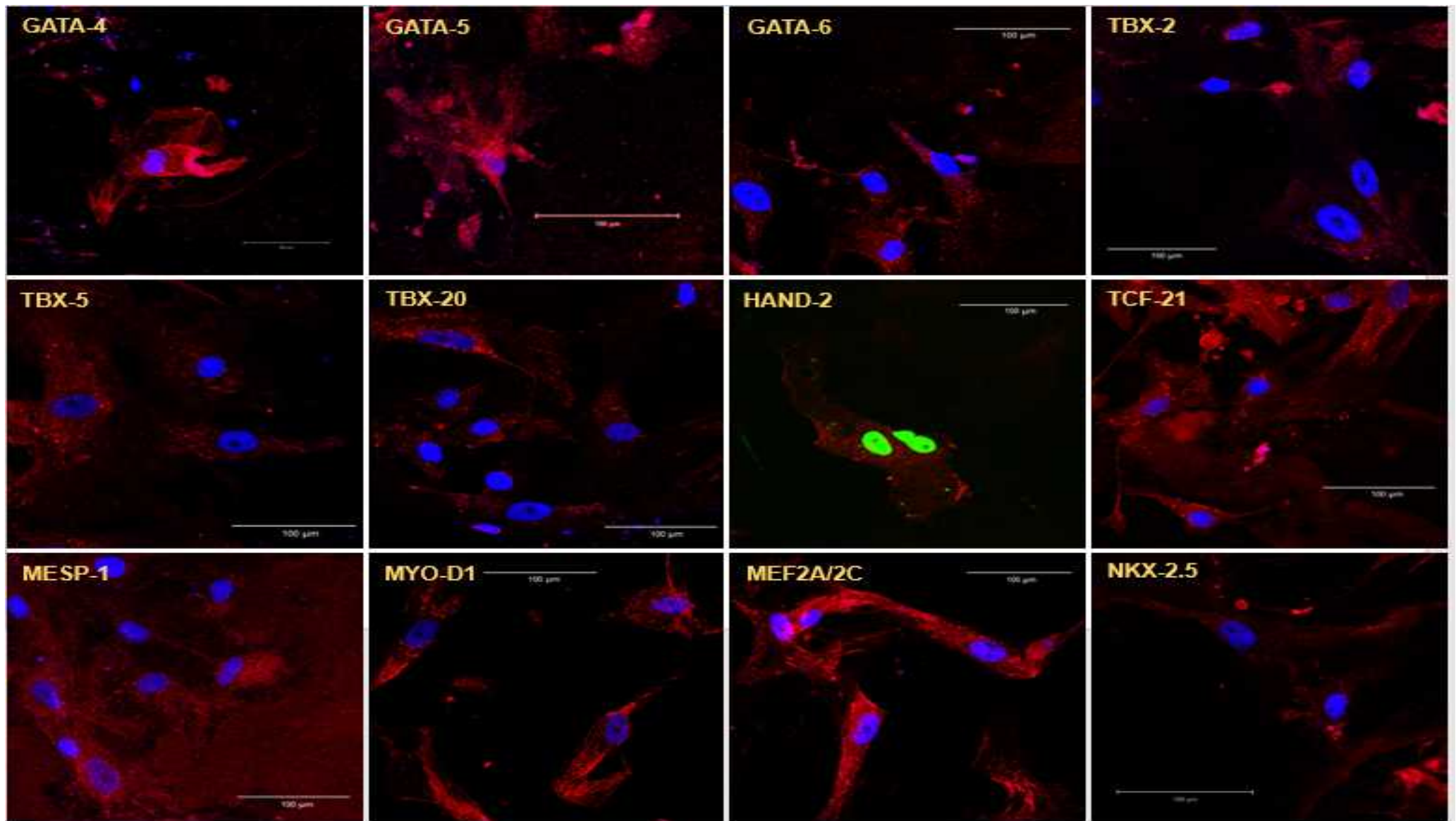


FIGURE C1.9: SAMPLE 12/2016. TRANSCRIPTION FACTORS EXPRESSED BY RIGHT VENTRICULAR FIBROBLASTS. Confocal images of human right ventricular fibroblasts at P2 stage, stained with individual transcription factors while nuclei were stained with DAPI. 20X magnification – 1024 (resolution). Scale bar = 100μ

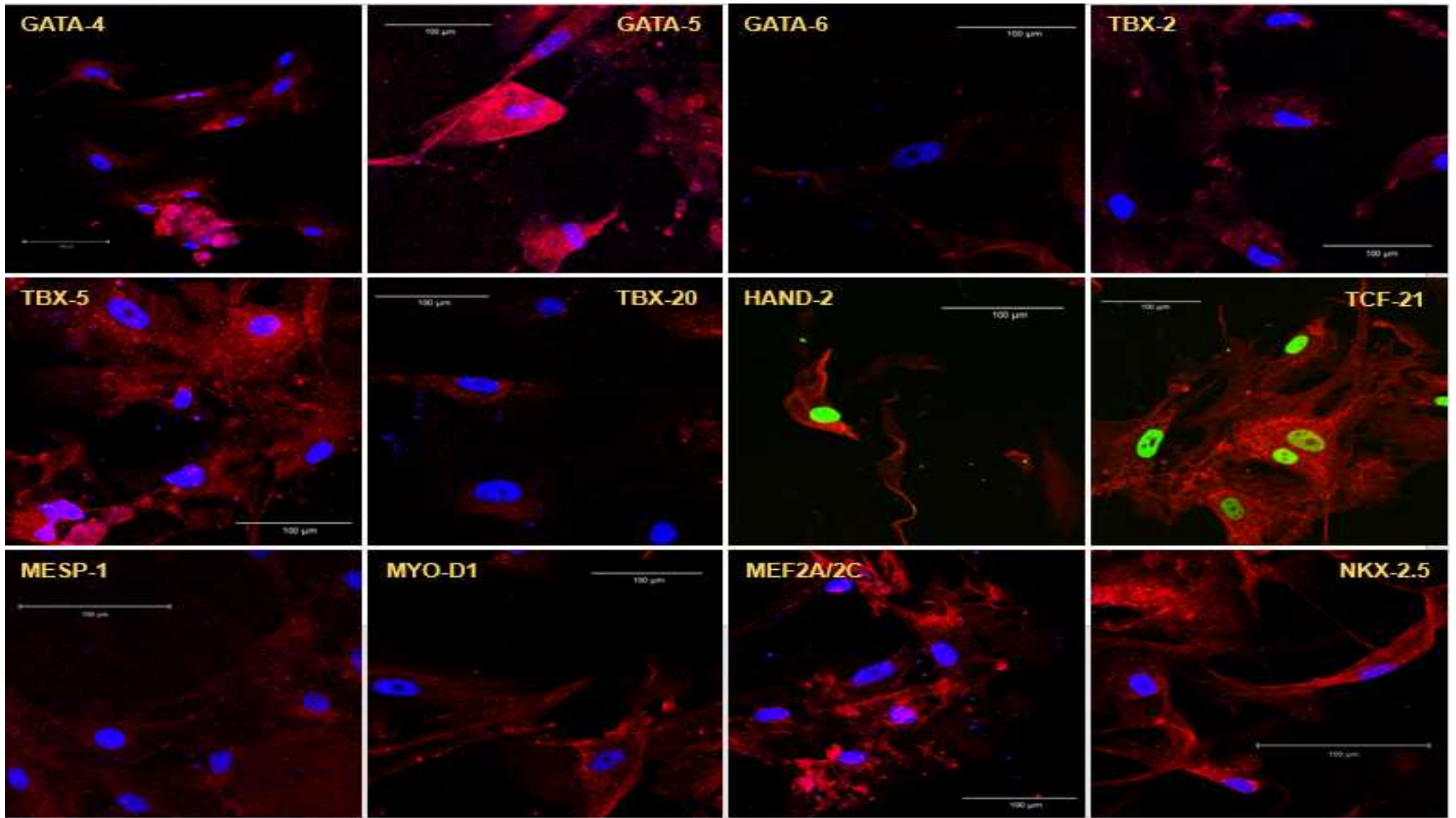
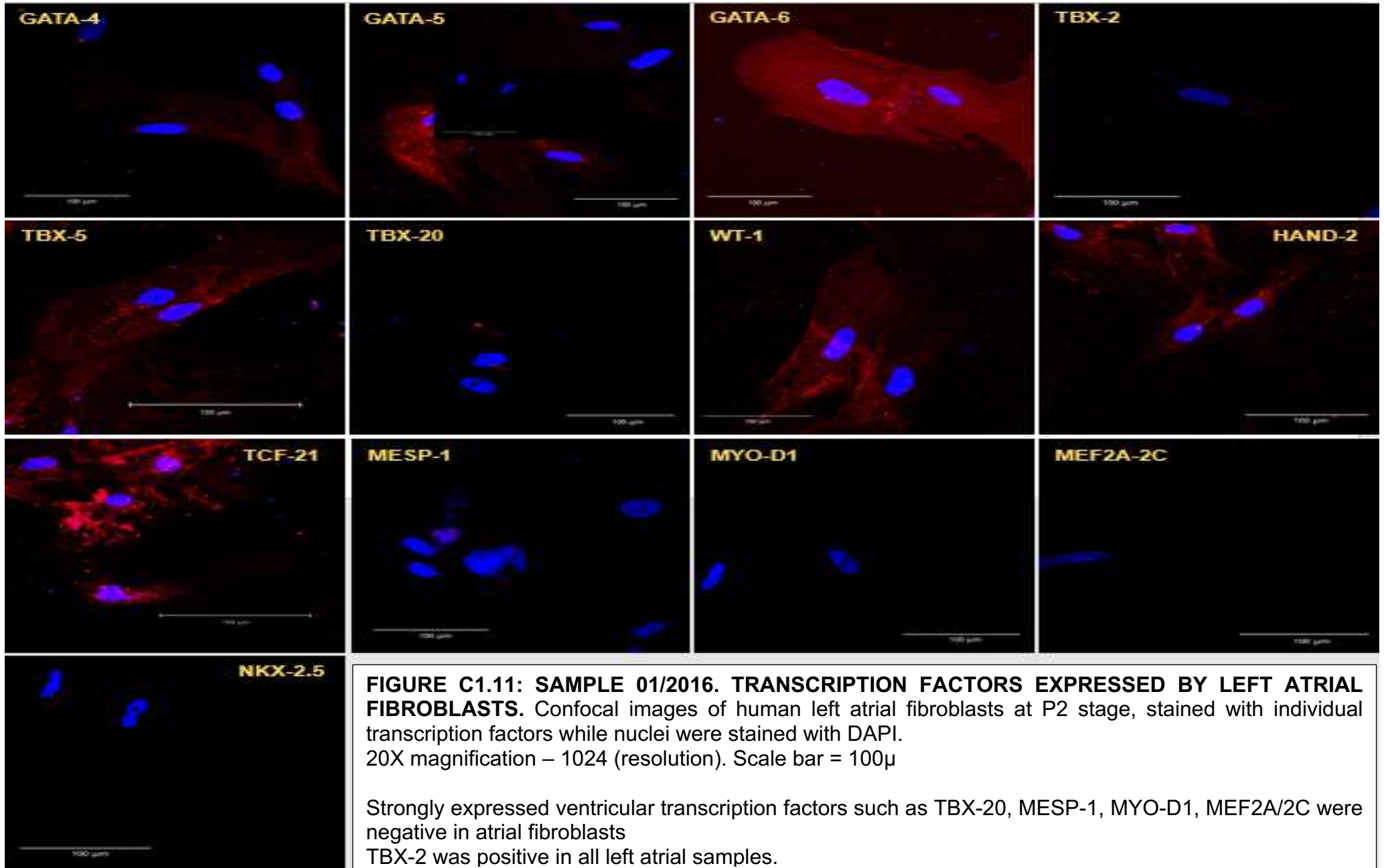
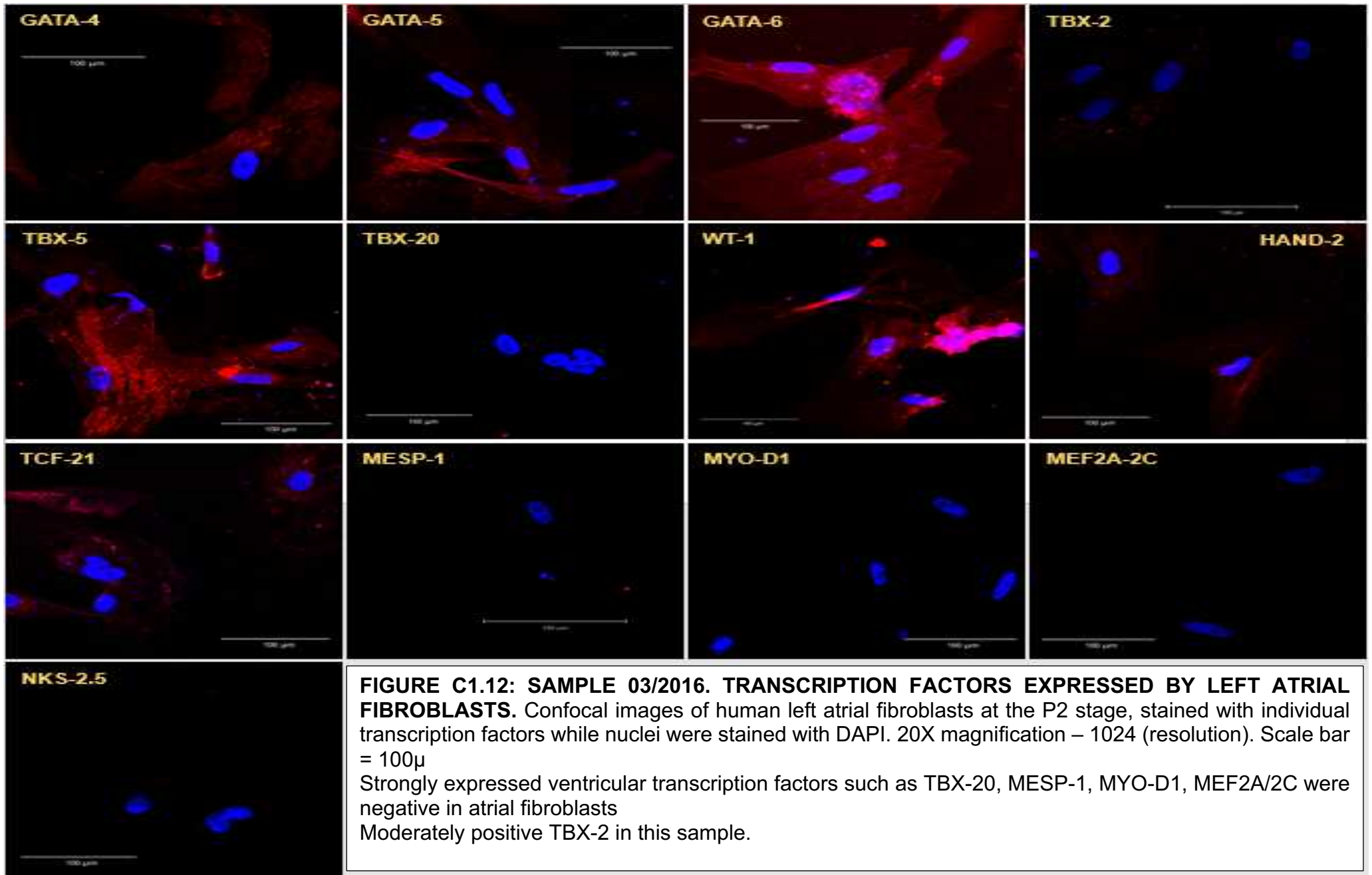
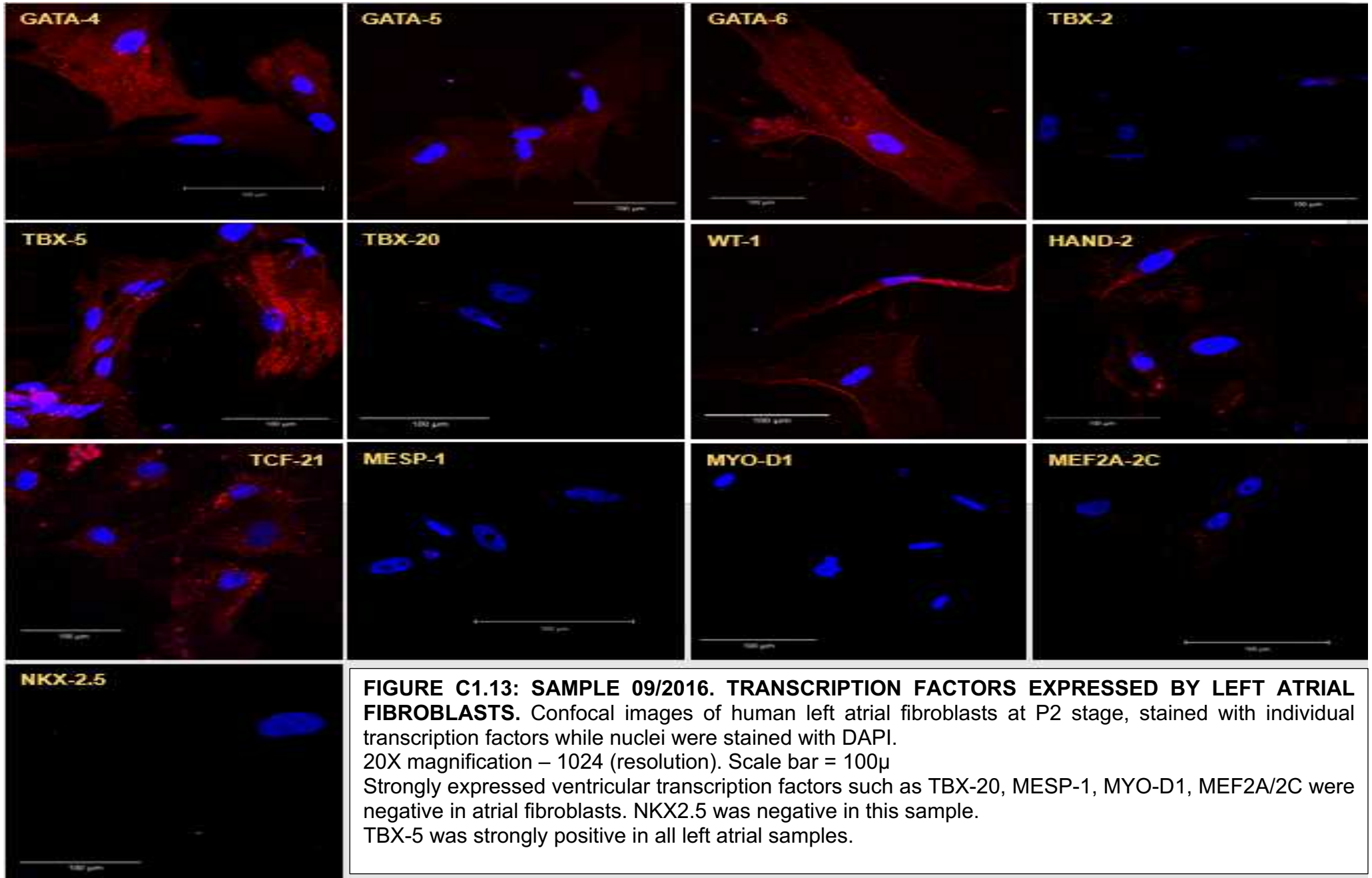


FIGURE C1.10: SAMPLE 15/2016. TRANSCRIPTION FACTORS EXPRESSED BY RIGHT VENTRICULAR FIBROBLASTS. Confocal images of human right ventricular fibroblasts at P2 stage, stained with individual transcription factors while nuclei were stained with DAPI. 20X magnification – 1024 (resolution). Scale bar = 100μ







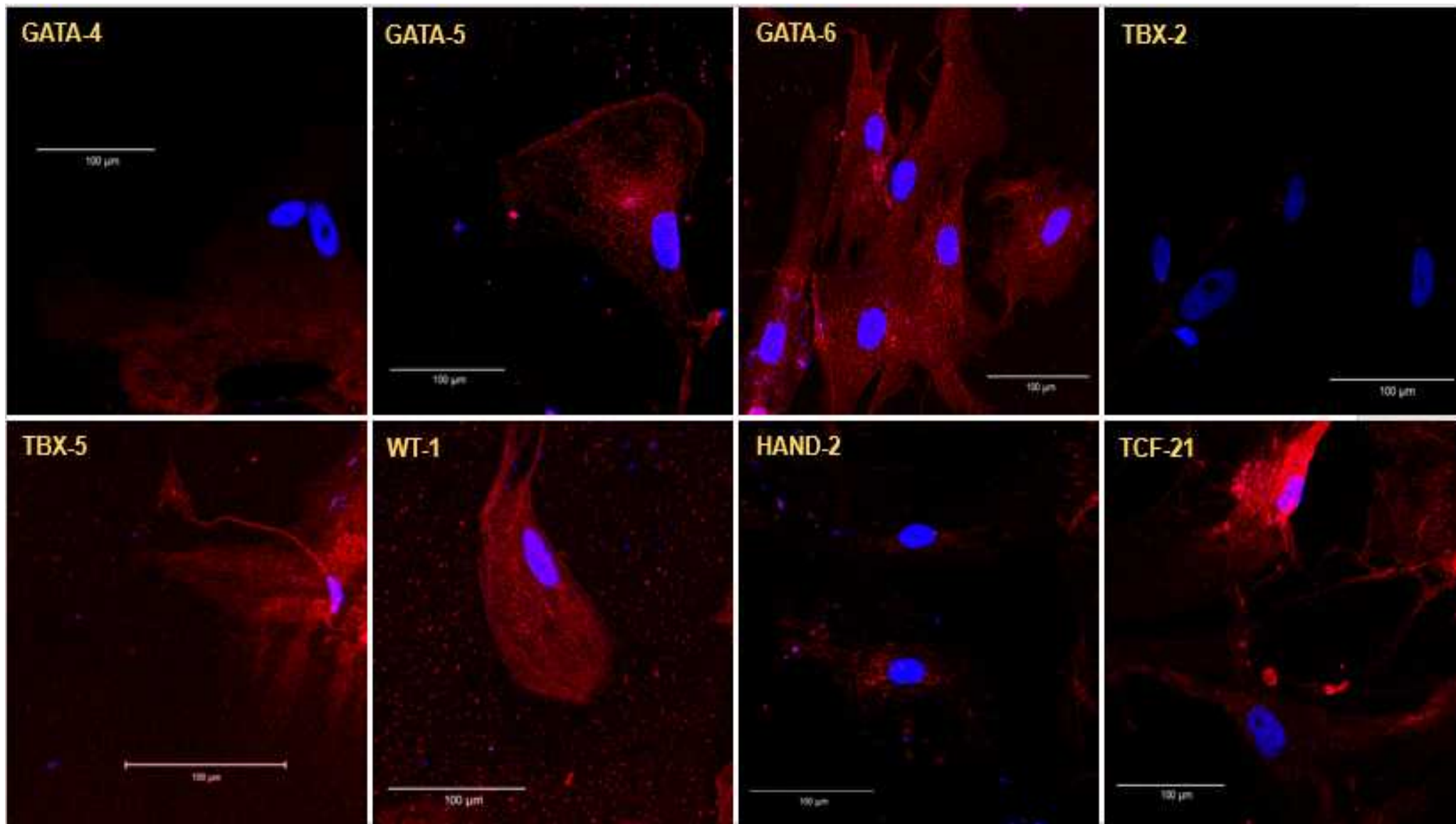


FIGURE C1.14: SAMPLE 12/2016. TRANSCRIPTION FACTORS EXPRESSED BY LEFT ATRIAL FIBROBLASTS. Confocal images of human left atrial fibroblasts at the P2 stage, stained with individual transcription factors while nuclei were stained with DAPI. 20X magnification – 1024 (resolution). Scale bar = 100μ

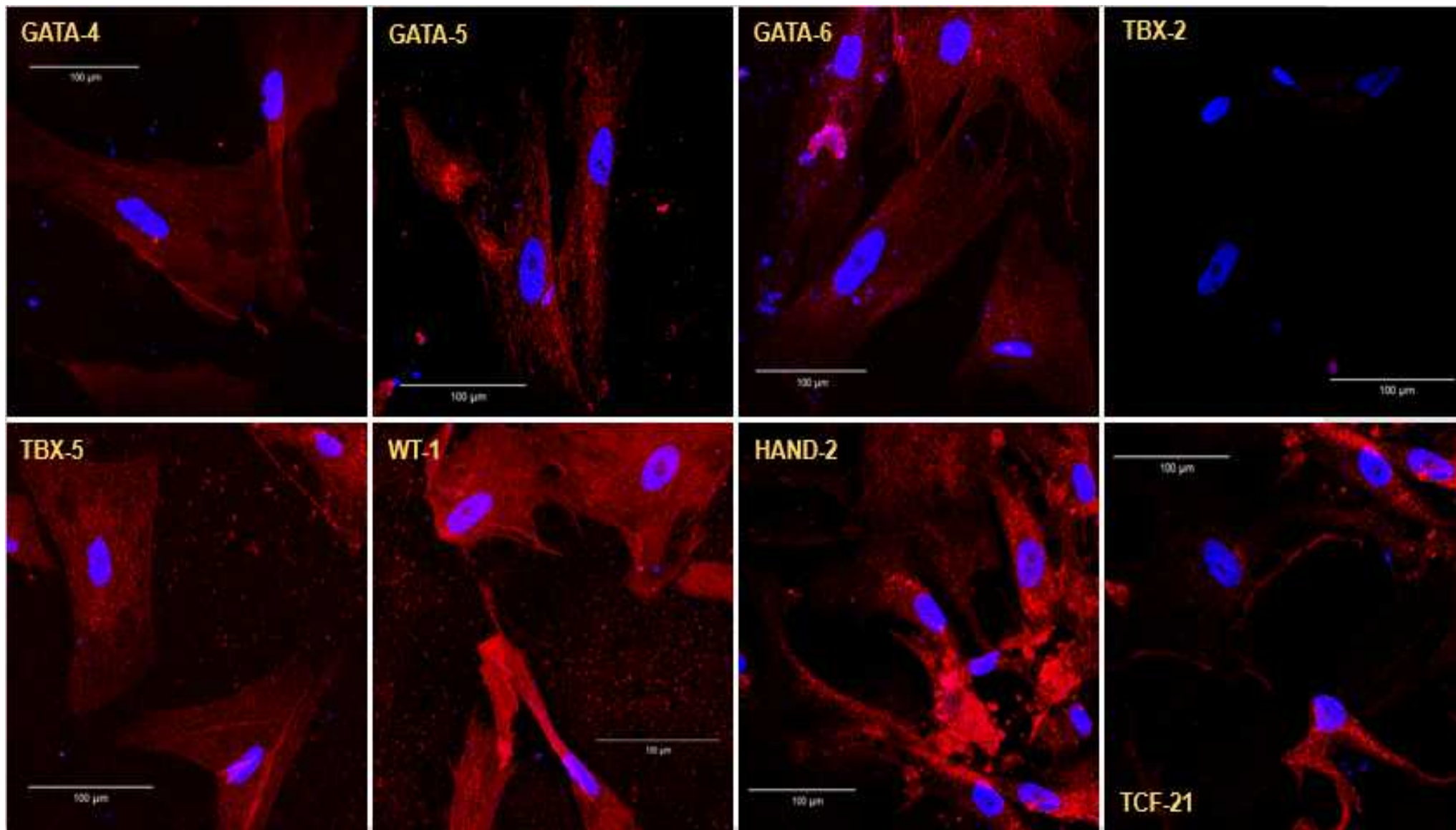


FIGURE C1.15: SAMPLE 15/2016. TRANSCRIPTION FACTORS EXPRESSED BY LEFT ATRIAL FIBROBLASTS. Confocal images of human left atrial fibroblasts at the P2 stage, stained with individual transcription factors while nuclei were stained with DAPI. 20X magnification – 1024 (resolution). Scale bar = 100μ

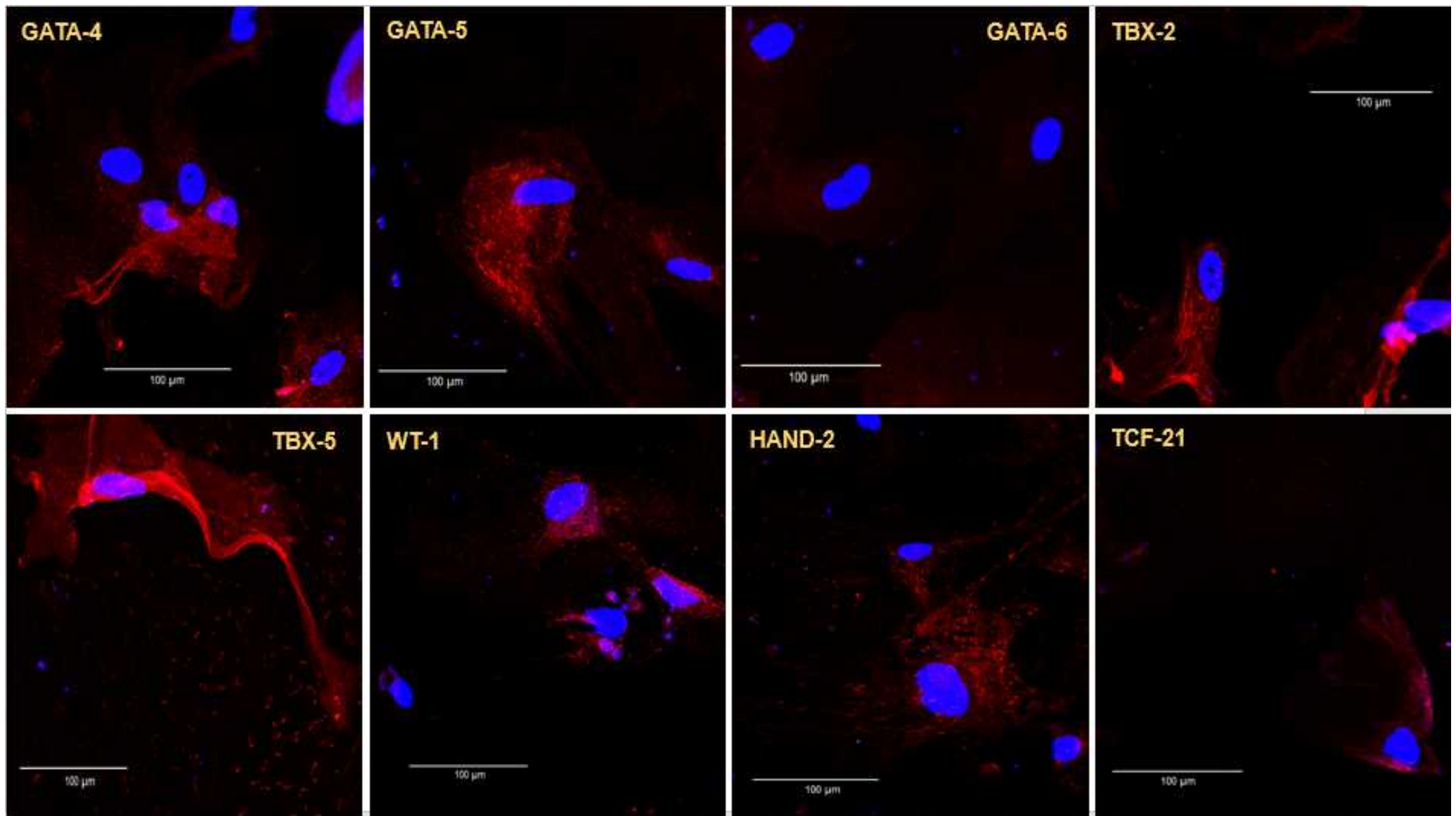


FIGURE C1.16: SAMPLE 01/2016. TRANSCRIPTION FACTORS EXPRESSED BY RIGHT ATRIAL FIBROBLASTS. Confocal images of human right atrial fibroblasts at the P2 stage, stained with individual transcription factors while nuclei were stained with DAPI. 20X magnification – 1024 (resolution). Scale bar = 100μ. Only positive factors are depicted for the right atrial samples.

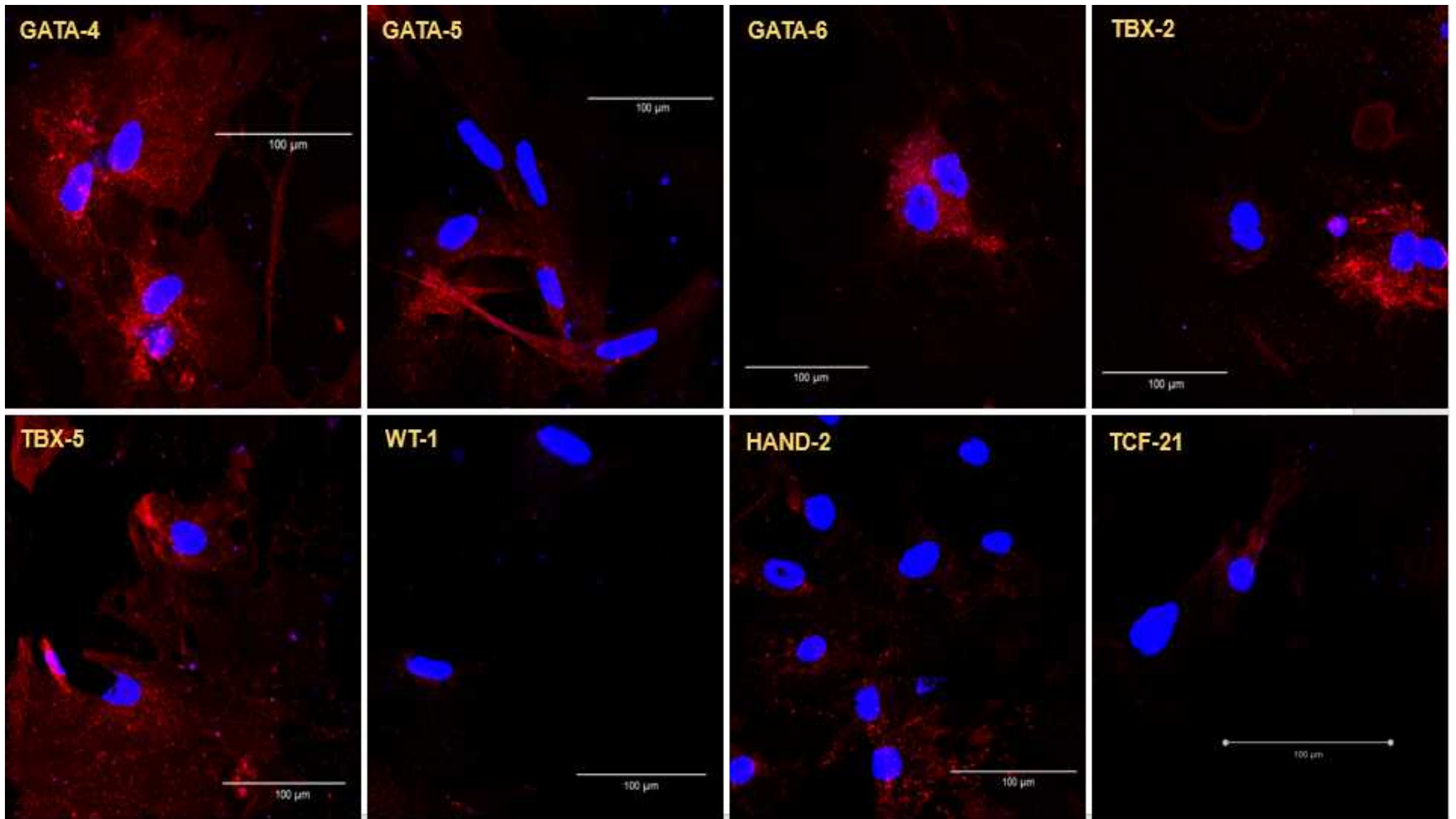


FIGURE C1.17: SAMPLE 03/2016. TRANSCRIPTION FACTORS EXPRESSED BY RIGHT ATRIAL FIBROBLASTS. Confocal images of human right atrial fibroblasts at the P2 stage, stained with individual transcription factors while nuclei were stained with DAPI. 20X magnification – 1024 (resolution). Scale bar = 100μ. TBX-2 was positive in right atrial sample. WT-1 was negative in this sample.

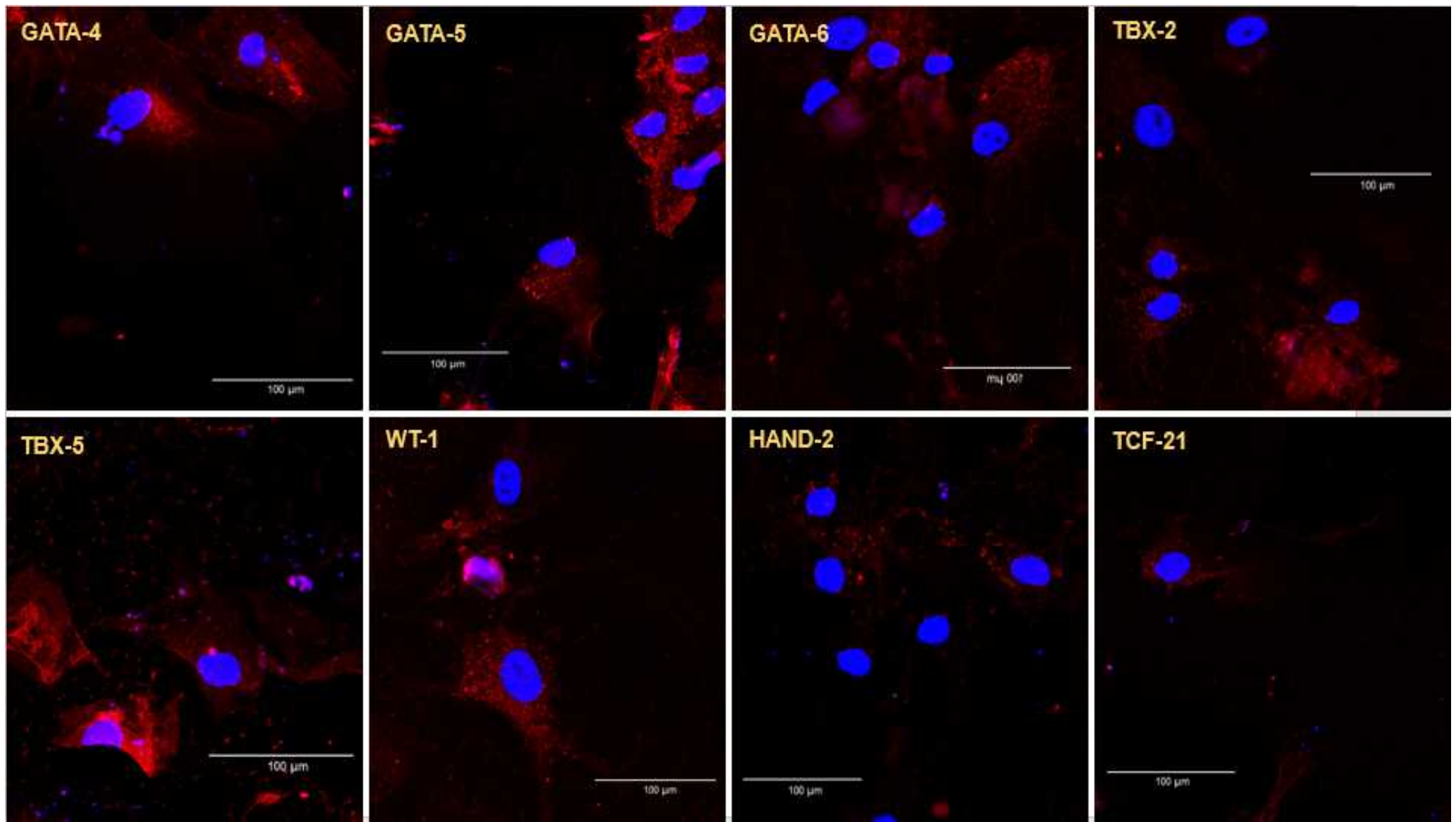


FIGURE A C1.18: SAMPLE 09/2016. TRANSCRIPTION FACTORS EXPRESSED BY RIGHT ATRIAL FIBROBLASTS. Confocal images of human right atrial fibroblasts at the P2 stage, stained with individual transcription factors while nuclei were stained with DAPI. 20X magnification – 1024 (resolution). Scale bar = 100μ.

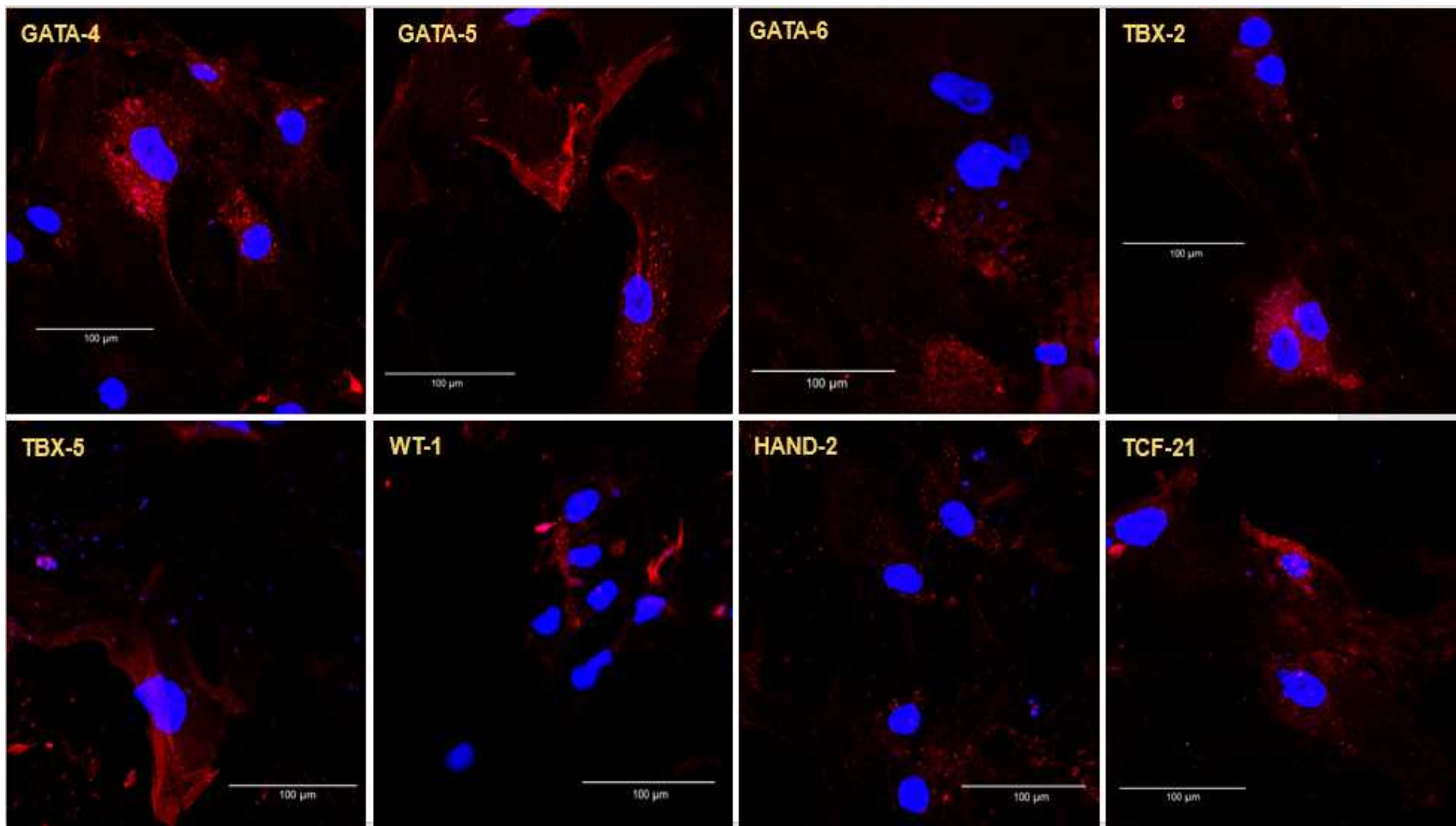


FIGURE C1.19: SAMPLE 12/2016. TRANSCRIPTION FACTORS EXPRESSED BY RIGHT ATRIAL FIBROBLASTS. Confocal images of human right atrial fibroblasts at the P2 stage, stained with individual transcription factors while nuclei were stained with DAPI. 20X magnification – 1024 (resolution). Scale bar = 100μ

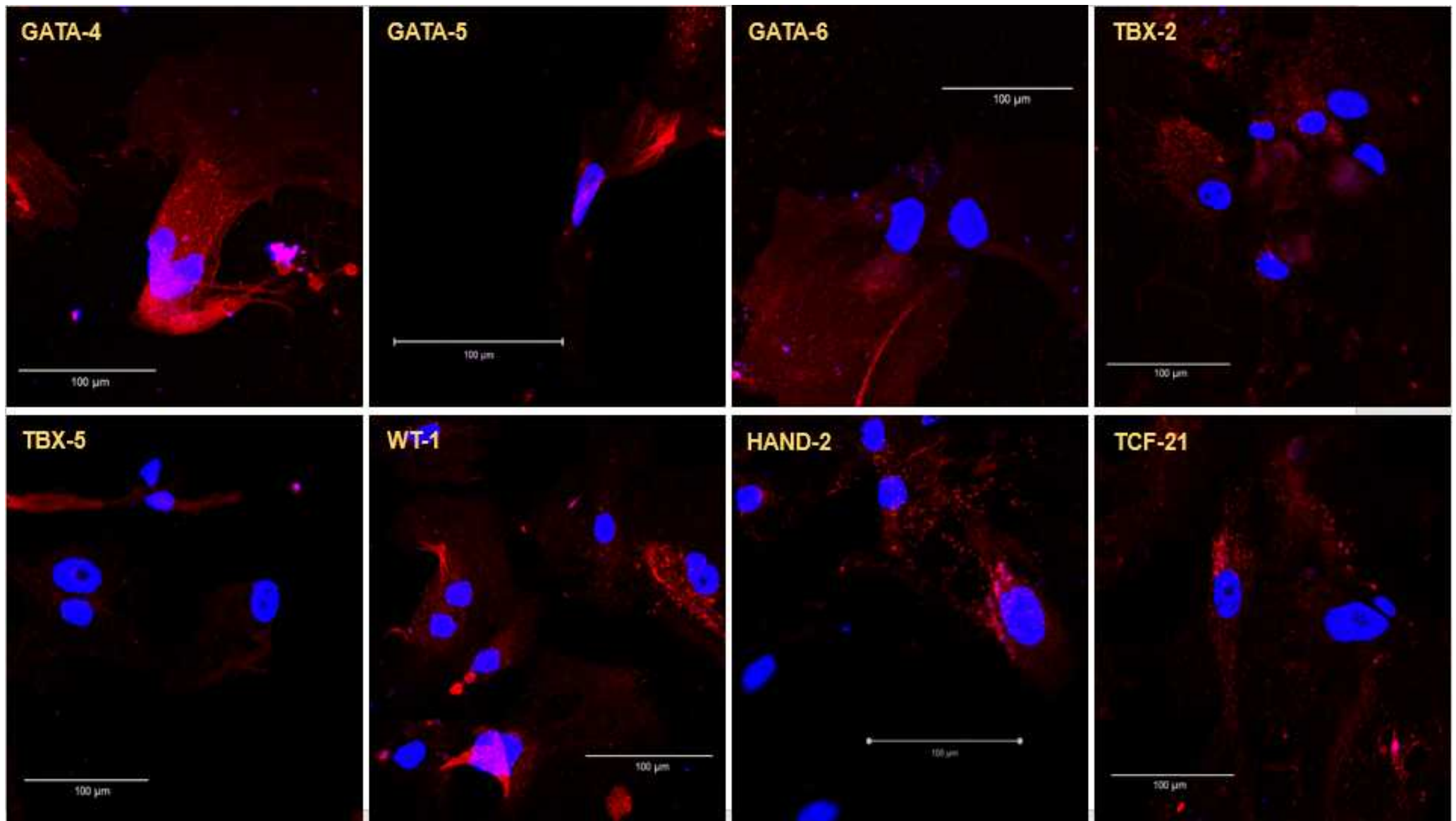


FIGURE C1.20: SAMPLE 15/2016. TRANSCRIPTION FACTORS EXPRESSED BY RIGHT ATRIAL FIBROBLASTS. Confocal images of human right atrial fibroblasts at the P2 stage, stained with individual transcription factors while nuclei were stained with DAPI. 20X magnification – 1024 (resolution). Scale bar = 100μ.

APPENDIX

CYTOKINES ARRAY MEMBRANES USED FOR DIFFERENT DAYS IN EHT SUPRENATANT

	Chambers	Membrane Serial no.	EHTs no
DAY-2			
1	LA	B536	B1
2	LV	B535	D3
3	RA	B537	A1
4	RV	B538	B6
DAY- 8			
1	LA	B505	B1
2	LV	B506	D3
3	RA	B507	A1
4	RV	B508	B6
DAY-16			
1	LA	B585	B1
2	LV	B586	D3
3	RA	B587	A1
4	RV	B588	B6

FIGURE D1.1: CYTOKINE MEMBRANES USED FOR PROTEOMIC ANALYSIS

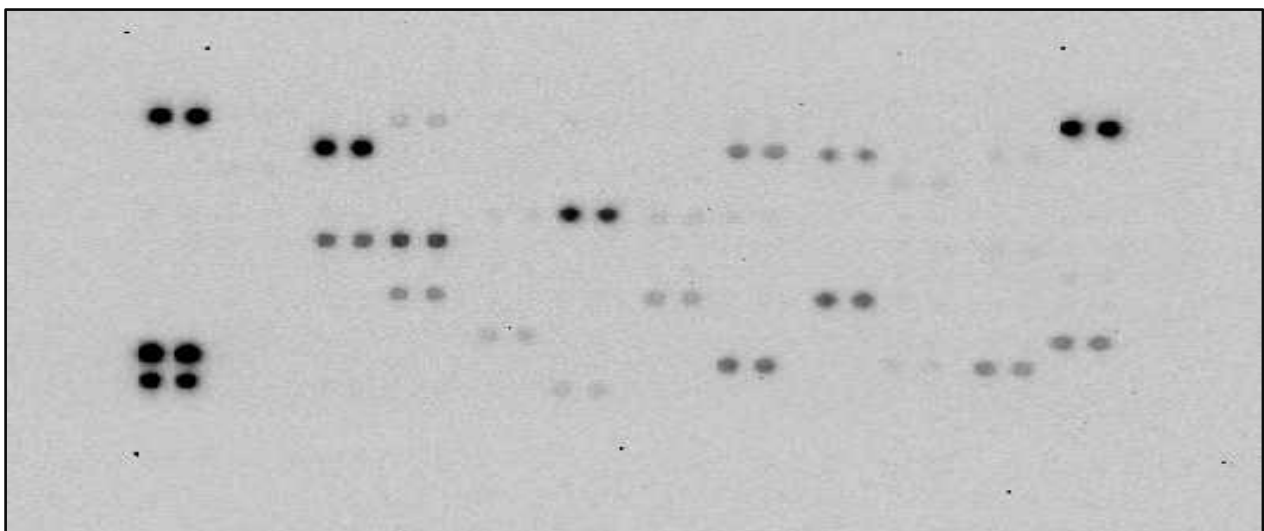
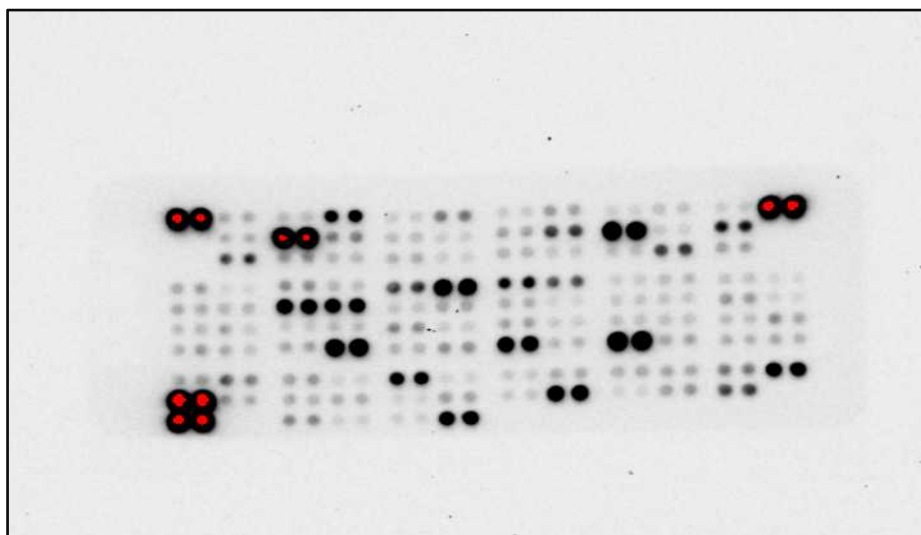
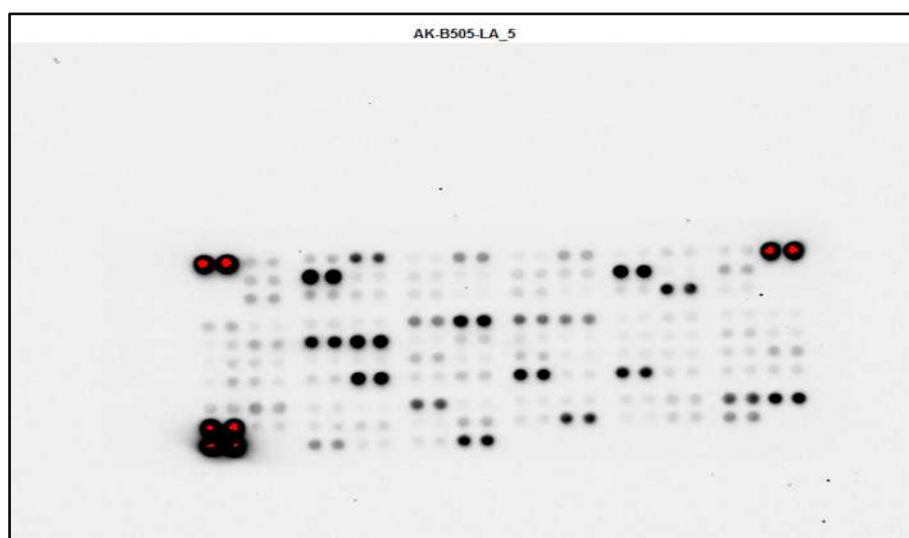


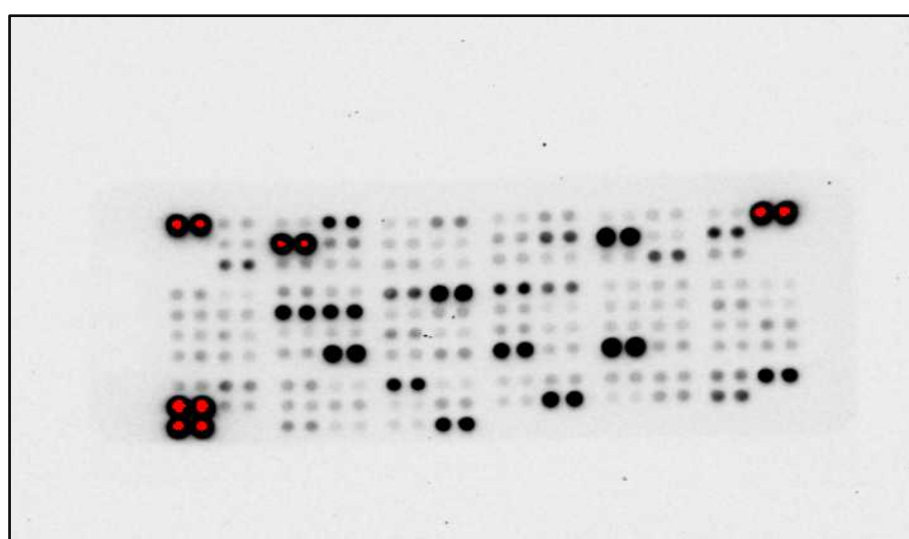
FIGURE D1.2: PROTEIN EXPRESSION OF FRESHLY PREPARED EHT MEDIUM



B536

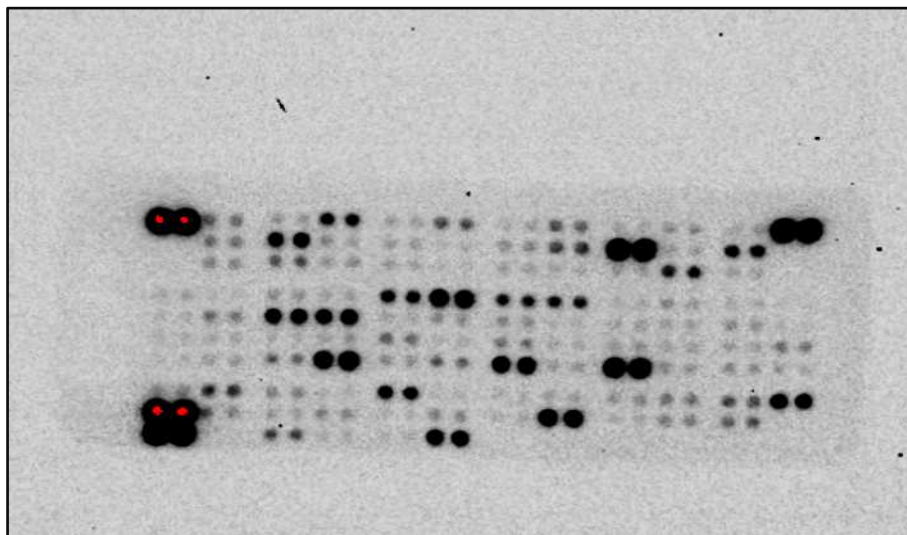


B505

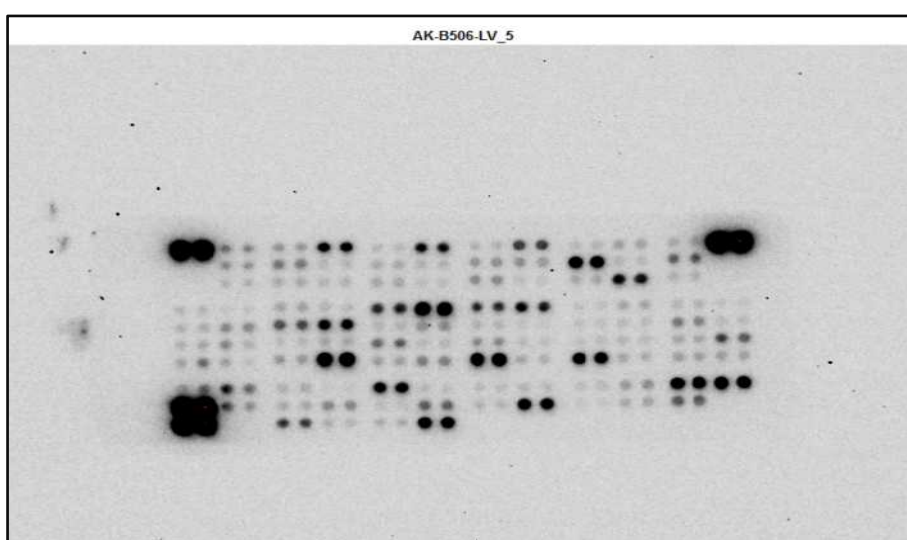


B586

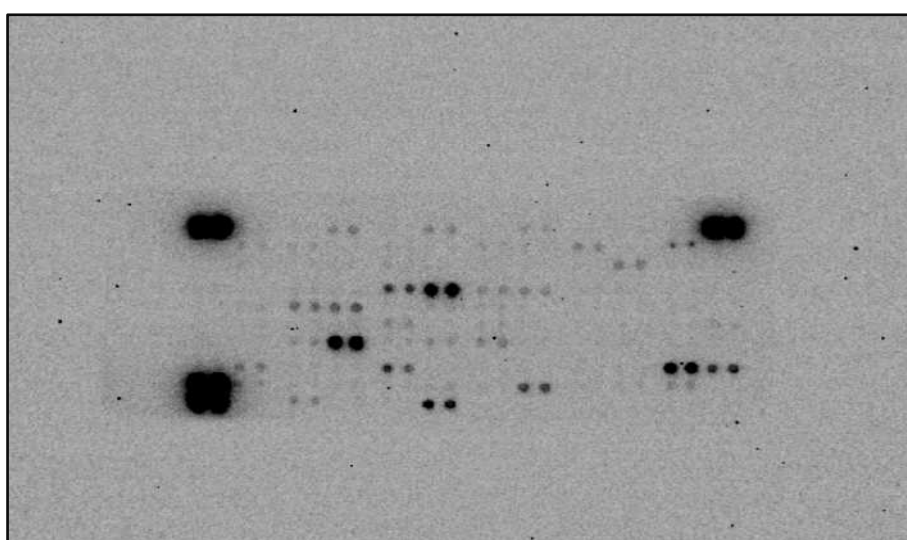
FIGURE D1.3: PROTEIN EXPRESSION OF LA-FBs BASED EHTs



B535

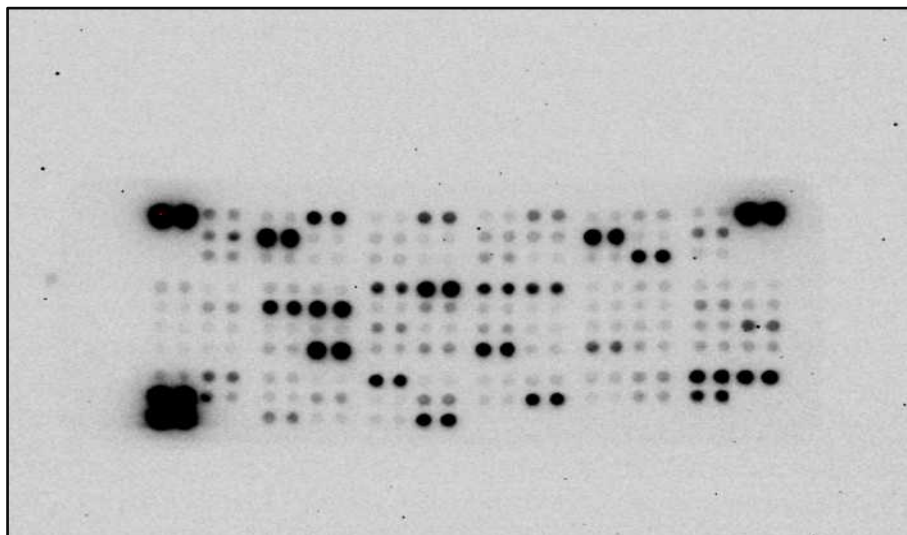


B506

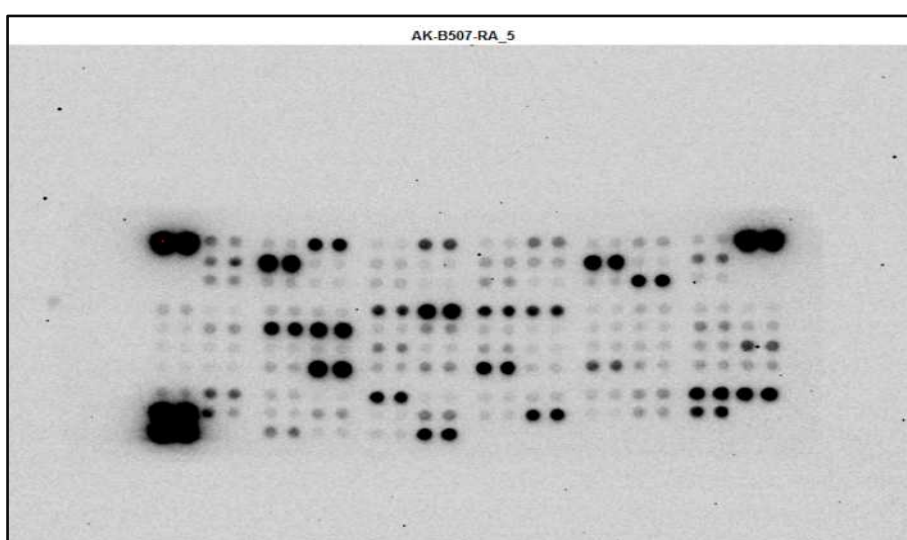


B586

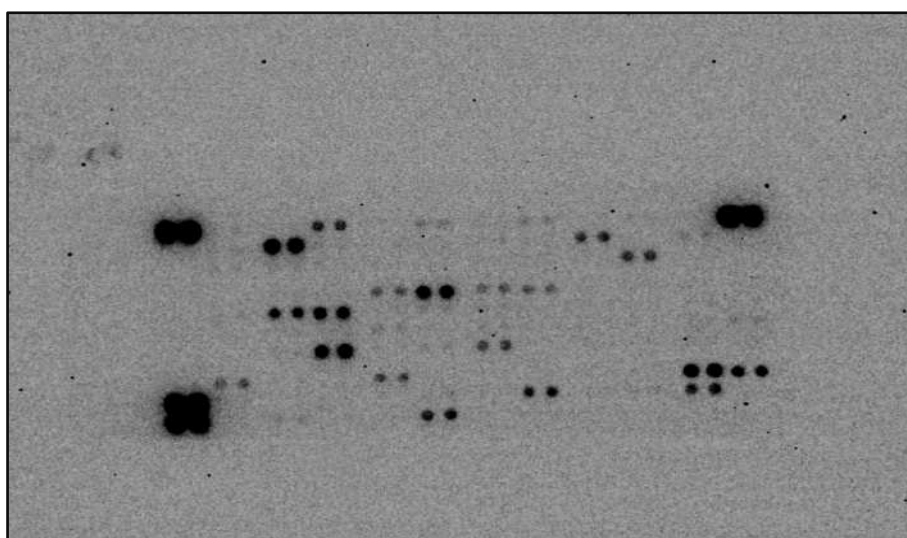
FIGURE D1.4: PROTEIN EXPRESSION OF LV-FBs BASED EHTs



B537

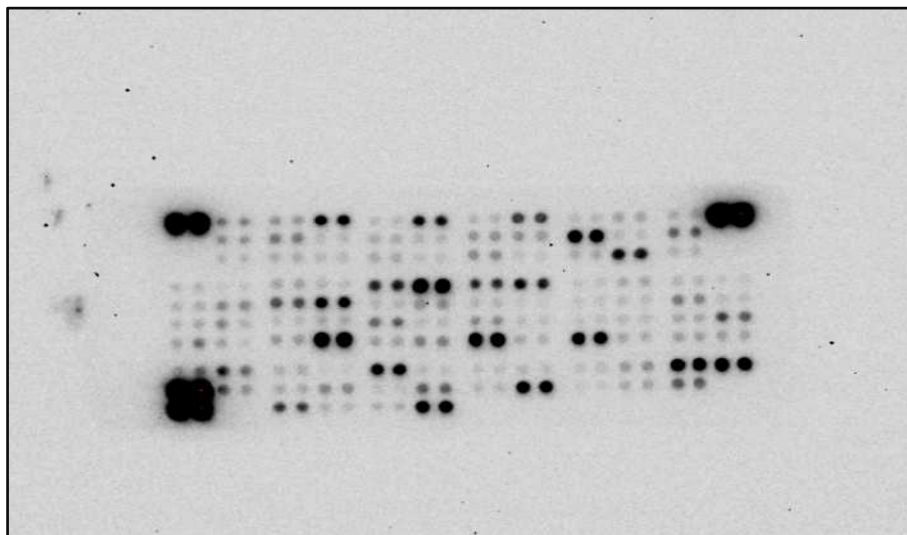


B507

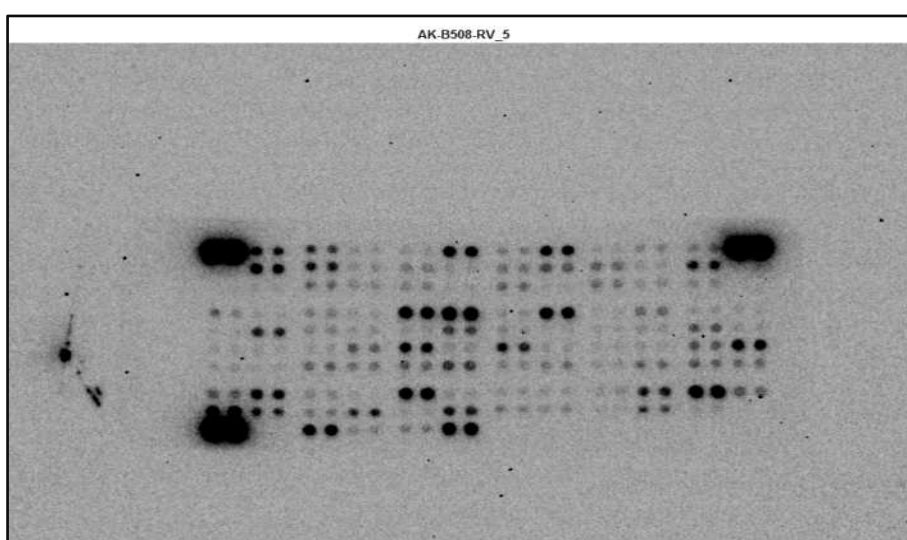


B587

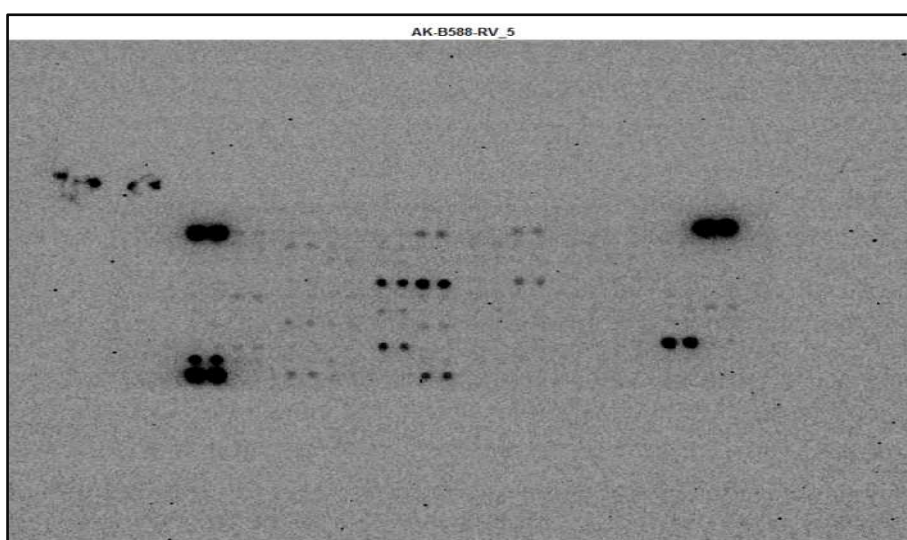
FIGURE D1.5: PROTEIN EXPRESSION OF RA-FBs BASED EHTs



B538



B508



B588

FIGURE D1.6: PROTEIN EXPRESSION OF RV-FBs BASED EHTs

		Left Atrium - MPD			Right Atrium - MPD			Left Ventricle -MPD			Right Ventricle - MPD		
		Day 2	Day 8	Day 16	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16
1	Adiponectin	70812	111696	73482	68745	98933.	0.	19006.	67419.	0.	71320.	98960.	0.
2	Aggrecan	97174	136280	76855	42190	32370.	0.	14605.	41103.	0.	21234.	38710.	0.
3	Angiogenin	523537	497288	211311	94789	455832.	59480.	143273.	322812.	11180.	29450.	10880.	0.
4	Angiopoietin-1	25018	0	19988	23761	0.	0.	18863.	19116.	0.	17240.	0.	0.
5	Angiopoietin-2	205541	286664	218148	199752	222680.	16110.	68854.	250677.	10260.	115392.	202960.	26723.
6	BAFF	32557	0	17831	13213	0.	0.	0.	23607.	0.	9876.	19390.	0.
7	BDNF	111286	209992	180718	102367	128839.	12070.	70570.	186619.	10570.	127347.	154060.	14335.
8	C5/C5a	24304	0	14383	10902	20368.	0.	0.	19116.	0.	0.	0.	0.
9	CD14	64373	47752	34042	54340	36071.	0.	8749.	31266.	0.	13206.	14550.	0.
10	CD30	15078	40248	0	17732.	27521.	0.	0.	22212.	0.	0.	22350.	0.
11	CD40L	86703	64168	105668	109341.	137180.	0.	49426.	33579.	8000.	135891.	106650	0.
12	CHI3L1	4646936	2394152	1348629	2674389.	1804274.	355470.	218920.	85761.	13220.	1327653.	72970.	20472.
13	Comp Factor D	93828	30565	52231	79256.	20900.	0.	25701.	0.	0.	12309.	20230.	0.
14	CRP	62518	36848	79268	74902.	40603.	0.	11355.	39663.	0.	12783.	26900.	0.
15	CRIPTO-1	17710	0	0	21504.	22733.	0.	7722.	13653.	0.	4328.	3510.	0.
16	Cystatin C	94033	57624	105371	84537.	54387.	0.	29887.	68202.	0.	17443.	35630.	0.
17	DKK-1	306565	49768	78983	241032.	62614.	0.	85429.	50616.	0.	206325.	55090.	0.
18	DPPIV	2455103	1589104	178457.	199215.	1003124.	52320.	1890258.	483273.	18800.	213998.	48320.	0.
29	EGF	28637	0	26353.	25412.	24035.	0.	0.	18324.	0.	0.	0.	0.
20	EMMPRIN	191338.	147880.	132677.	174883.	124583.	10280.	102264.	123606.	34700.	93467.	110280.	0.
21	ENA -78	230017.	162680.	56515.	135760.	41743.	0.	38655.	25749.	0.	13405.	0.	0.
22	Endoglin	40980.	185560.	102989.	32302.	45068.	0.	70232.	44532.	0.	78610.	30950.	0.
23	Fas Ligand	48675.	83360.	72189.	13375.	0.	0.	17745.	28544.	0.	169730.	22210.	10272.
24	FGF - 2	85050.	62008.	73402.	70460.	45068.	0.	23406.	39024.	8380.	31580.	22800.	12236.
25	FGF-7	39232.	0.	0.	0.	13214.	0.	11830.	0.	0.	10250.	0.	0.
26	FGF-19	81667.	85192.	35720.	66552.	60496.	0.	35698.	79830.	0.	19297.	35770.	0.
27	Flt-3 Ligand	66615.	0.	30201.	13290.	0.	0.	22509.	0.	0.	18440.	0.	0.
28	G-CSF	22132.	33760.	41695.	51302.	46758.	0.	0.	46692.	0.	12160.	28420.	0.
29	GDF-15	341752.	613296.	234384.	317390.	407928.	50590.	127296.	269010.	39290.	111660.	20370.	0.
30	GM-CSF	107797.	36768.	37962.	41358.	0.	0.	11693.	42903.	0.	95877.	12090.	0.
31	GRO - α	101145.	56000.	37582.	88250.	46980.	0.	21209.	37494.	0.	24954.	35640.	0.

32	Growth Hormone	14115.	0.	0.	9256.	0.	0.	0.	0.	0.	0.	0.	0.
33	HGF	140782.	44416.	68466.	135422.	50996.	0.	16203.	55395.	0.	14680.	30270.	0.
34	ICAM-1	76087.	0.	50502.	73884.	37829.	0.	13357.	29016.	0.	10290.	17570.	0.
35	IFN- α	282810.	326904.	481458.	267493.	211926.	28910.	135479.	229518.	75680.	154638.	231210.	85186.
36	IGFBP-2	1846537.	1083896.	1180688.	1820745.	1123289.	189400.	484198.	787896.	438680.	316773.	389840.	169461.
37	IGFBP-3	406807.	379720.	223867.	290845.	257402.	26300.	110214.	199521.	29653.	108766.	19970.	0.
38	IL-1 α	260032.	315112.	288439.	280328.	239409.	32520.	100776.	240768.	26240.	198378.	218700.	31787.
39	IL-1 β	35707.	0.	33487.	36694.	0.	0.	0.	19386.	0.	0.	0.	0.
40	IL-1ra	67635.	64320.	52867.	58940.	42854.	0.	3698.	52002.	0.	11558.	47550.	0.
41	IL-2	45967.	22208.	25108.	60203.	31863.	0.	6188.	33543.	0.	10902.	22835.	0.
42	IL-3	19665.	19680.	0.	0.	0.	0.	0.	17100.	0.	0.	4030.	0.
43	IL-4	84960.	53720.	34830.	20945.	0.	0.	9204.	35658.	0.	0.	0.	0.
44	IL-5	76695.	168072.	109860.	79751.	66785.	0.	47469.	68787.	0.	57480.	84340.	22828.
45	IL-6	1230615.	1065976.	364900.	423482.	523972.	76180.	313059.	161352.	40420.	44385.	23320.	0.
46	IL-8	960270.	1494424.	610780.	1020783.	1031101.	124230.	300449.	259218.	50200.	26455.	13860.	8521.
47	IL-10	44827.	16432.	37860.	47463.	34827.	0.	18070.	27171.	5550.	0.	0.	0.
48	IL-11	42562.	122624.	82790.	40208.	88084.	0.	38759.	63864.	5050.	33442.	55310.	13176.
49	IL-12p70	91732.	36008.	52100.	74300.	30346.	0.	35776.	32598.	7670.	29399.	14630.	0.
50	IL-13	34720.	15296.	0.	13200.	0.	0.	6669.	15066.	0.	7609.	0.	0.
51	IL-15	40957.	17768.	30370.	42717.	13946.	0.	22873.	25182.	0.	16772.	10530.	0.
52	IL-16	49552.	22008.	35990.	64307.	25365.	0.	23003.	30402.	0.	19200.	22770.	0.
53	IL-17A	103987.	56680.	88090.	145499.	74527.	0.	37753.	49203.	0.	37365.	48080.	0.
54	IL-18BPa	26467.	25597.	32570.	35850.	42237.	0.	14976.	18603.	0.	16428.	0.	0.
55	IL-19	51495.	39525.	29210.	30240.	17828.	0.	24537.	38124.	0.	27312.	0.	0.
56	IL-22	54500.	32200.	32170.	44239.	21679.	0.	24986.	43353.	0.	0.	0.	0.
57	IL-23	34560.	45136.	30000.	35525.	30713.	0.	11667.	23112.	0.	0.	10030.	0.
58	IL-24	28350.	48984.	28850.	35890.	15257.	0.	10315.	17694.	0.	62570.	46000.	0.
59	IL-27	149955.	138552.	207370.	109640.	86688.	15750.	61080.	113229.	16170.	71003.	115860.	18078.
60	IL-31	40852.	37096.	24800.	37882.	21603.	0.	17231.	26010.	0.	14213.	0.	0.
61	IL-32 $\alpha/\beta/\gamma$	84600.	77984.	107850.	65380.	59660.	0.	38675.	52056.	10550.	68340.	83440.	0.
62	IL-33	37455.	27520.	29810.	31366.	20330.	0.	24336.	19746.	0.	15930.	0.	0.
63	IL-34	17767.	17576.	36490.	14320.	25403.	0.	24641.	11511.	0.	18650.	0.	0.
64	IP-10	47842.	32416.	38660.	53476.	34475.	0.	11719.	27513.	0.	17862.	0.	0.

65	I -TAC	53145.	62600.	78940.	46544.	53751.	0.	23140.	34029.	0.	35757.	44330.	0.
66	Kallikrein3	103230.	153208.	225970.	120340.	167038.	13360.	39169.	113958.	20580.	56811.	134800.	0.
67	Leptin	98317.	89778.	29210.	32752.	0.	0.	0.	113916.	0.	42670.	17390.	0.
68	LIF	57262.	68016.	32170.	32002.	0.	0.	18954.	21582.	0.	88386.	13090.	0.
69	Lipocalin 2	110490.	83448.	30000.	76512.	57855.	0.	50869.	61902.	21870.	32500.	43520.	14953.
70	MCP - 1	2388577.	1597112.	28850.	261449.	1683799.	272710.	759622.	820296.	450760.	88367.	31020.	8520.
71	MCP - 3	73545.	69544.	182370.	72583.	47462.	0.	32610.	50508.	12730.	34870.	41720.	0.
72	M-CSF	146017.	126048.	24800.	116605.	92596.	0.	51954.	94581.	18030.	50496.	66870.	17546.
73	MIF	1385917.	938144.	107850.	870420.	563217.	51410.	506155.	668547.	33770.	75844.	23830.	0.
74	MIG	77370.	51552.	29810.	60009.	35017.	0.	29932.	36693.	0.	25784.	29740.	0.
75	MIP-1 α , MIP-1 β	3335745.	729552.	36490.	880446.	164986.	0.	1466595.	386280.	0.	46355.	18230.	0.
76	MIP-3 α	131535.	67784.	38660.	866060.	47414.	0.	30602.	55449.	0.	21925.	38590.	0.
77	MIP-3 β	69112.	51952.	78940.	73857.	52420.	0.	26494.	50670.	0.	19430.	39210.	0.
78	MMP-9	80475.	79168.	225970.	69483.	62662.	0.	9431.	52335.	0.	23004.	39650.	0.
79	Myeloperoxidase	88710.	156624.	9770.	21748.	46122.	0.	19038.	40669.	0.	59300.	69550.	0.
80	Osteopontin	177240.	277192.	28180.	123520.	158412.	42690.	68243.	187299.	27560.		75956.	15970.
81	PDGF - AA	118905.	58128.	157730.	96393.	42341.	0.	26208.	43191.	0.	35650.	32410.	0.
82	PDGF - AA/BB	28620.	21800.	1290520.	25905.	0.	0.	0.	7677.	0.	0.	0.	0.
83	Pentaxin	625732.	489096.	81200.	525754.	330875.	43140.	201220.	340137.	55560.	230410.	256400.	61890
84	PF4	42262.	39512.	159250.	41744.	0.	0.	0.	28926.	0.	9332.	20530	0
85	RAGE	47280.	34064.	252800.	48312.	0.	0.	0.	18513.	0.	0.	18460	0
86	RANTES	101092.	44416.	53340.	98380.	40365.	0.	6636.	31329.	0.	17530.	13990	0
87	RBP- 4	97162.	42480.	48480.	97200.	43519.	0.	17049.	26406.	0.	12555.	19120	0
88	Relaxin -2	100290.	96218.	67290.	97504.	91466.	0.	28407.	69939.	0.	59496.	92500	0
89	Resistan	157815.	415328.	46890.	175428.	485687.	215310.	70633.	442764.	213730.	63540.	295990	294129
90	SDF - 1 α	1023232.	861744.	44390.	95680.	673151.	102930.	335003.	489177.	86260.	221250.	74040	0
91	Serpin E1	7198180.	6484448.	6890380.	5934722.	4891379.	1887910.	2471696.	6315255.	3420206.		249960	188720
92	SHBG	70805.	36720.	41870.	124335.	82279.	0.	0.	69048.	13530.	73560.	68190	0
93	ST2	76455.	37312.	28180.	44950.	16076.	0.	13390.	22437.	0.	23561.	31830	0
94	TARC	86550.	75272.	78100.	63487.	42503.	0.	21294.	48537.	0.	58358.	63290	0
95	TFF3	22770.	0.	0.	19870.	0.	0.	0.	0.	0.	0.	0	0
96	TfR	112117.	141536.	126440.	88350.	111425.	0.	13936.	125928.	0.	26460.	88410	0
97	TGF- α	35145.	31200.	33160.	27980.	0.	0.	0.	18189.	0.	41210.	26840	0
98	Thrombospondin	1508962.	533208.	534560.	105360.	372124.	68780.	519467.	375543.	66900.		25790	0

99	TNF - α	25897.	19280.	0.	15430.	0.	0.	0.	0.	0.	0.	0	0
100	μ - PAR	142665.	88608.	69840.	71009.	37880.	0.	17615.	28485.	0.	42740.	54720	0
101	VEGF	314797.	283832.	206790.	325824.	288439.	73140.	15892.	85527.	10890.	0.	0	0
102	Vit - D BP	158512.	277280.	237560.	81423.	114133.	9950.	78810.	163377.	13990.	93680.	171303	0

FIGURE D1.7: ANALYSIS OF SECRETION OF PROTEIN IN MEDIUM BATHING ENGINEERED HEART TISSUE. The expression of proteins at each point is represented as mean pixel density. Each recording presents the mean of pixel density (MPD) of 2 consecutive reading. 102 protein duplicates are plotted against EHTs with fibroblasts from all four chambers on day-2, day-8 and day-16.

		Left Atrium - MPD			Right Atrium - MPD			Left Ventricle - MPD			Right Ventricle - MPD		
		Day 2	Day 8	Day 16	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16
1	Adiponectin	++	+++	++	+++	+++	-	+	++	-	++	++	-
2	Aggrecan	++	+++	++	++	+	+	+	++	-	+	+	-
3	Angiogenin	+++++	+++++	+++++	++	++	++	+++	+++++	+	+	+	-
4	Angiopoietin-1	+	-	+	+	-	-	+	+	-	+	-	-
5	Angiopoietin-2	++++	++++	++++	+++	++++	+	++	++++	+	+++	++++	+
6	BAFF	+	-	+	+	-	-	-	+	-	-	+	-
7	BDNF	+++	++++	+++	+++	+++	+	++	+++	+	+++	+++	+
8	C5/C5a	+	-	+	+	+	-	-	+	-	-	-	-
9	CD14	++	++	+	++	+	-	-	+	-	+	+	-
10	CD30	+	++	-	+	+	-	-	+	-	-	+	-
11	CD40L	++	++	+++	+++	+++	-	++	+	-	+++	+++	-
12	CHI3L1	+++++	+++++	+++++	+++++	+++++	++++	++++	++	+	+++++	++	+
13	Comp Factor D	+++	++	+++	+++	+	-	+	-	-	+	+	-
14	CRP	+++	+	++	++	++	-	+	+	-	+	+	-
15	CRIPTO-1	+	-	-	+	+	-	-	+	-	-	-	-
16	Cystatin C	++	++	+++	++	++	-	+	++	-	+	+	-
17	DKK-1	++++	++	++	++++	++	-	++	++	-	++++	++	-
18	DPPIV	+++++	+++++	+++++	+++	+++++	++	+++++	+++++	+	++++	++	-
29	EGF	+	-	+	+	+	-	-	+	-	-	-	-
20	EMMPRIN	+++	+++	+++	+++	+++	+	+++	+++	+	++	+++	-
21	ENA-78	++++	+++	++	+++	++	-	+	+	-	+	-	-
22	Endoglin	++	+++	+++	+	++	-	++	++	-	++	+	-
23	Fas Ligand	+++	++	++	+	-	-	+	+	-	+++	+	+
24	FGF-2	++	++	++	++	++	-	+	+	-	+	+	+
25	FGF-7	+	-	-	-	+	-	+	-	-	+	-	-
26	FGF-19	++	++	+	++	++	-	+	++	-	-	-	-
27	Flt-3 Ligand	++	-	+	+	-	-	+	-	-	+	-	-
28	G-CSF	+	+	++	+++	++	-	-	++	-	+	+	-
29	GDF-15	++++	+++++	++++	++++	+++++	++	+++	++++	+	+++	+	-
30	GM-CSF	+++	+	+	++	-	-	+	++	-	++	+	-
31	GRO- α	+++	++	+	++	++	-	+	+	-	+	+	-

32	Growth hormone	+	-	-	-	-	-	-	-	-	-	-	-
33	HGF	+++	++	++	+++	++	-	+	++	-	+	+	-
34	ICAM-1	++	-	++	++	+	-	+	+	-	+	+	-
35	IFN- γ	++++	++++	++++	++++	++++	+	+++	++++	++	+++	++++	++
36	IGFBP-2	+++++	+++++	+++++	+++++	+++++	+++	+++++	+++++	+++++	++++	++++	+++
37	IGFBP-3	+++++	++++	++++	++++	++++	+	+++	+++	+	+++	+	-
38	IL-1 α	++++	++++	++++	++++	++++	+	+++	++++	+	+++	++++	+
39	IL-1 β	+	-	+	+	-	-	-	+	-	-	-	-
40	IL-1ra	++	++	++	++	++	-	-	++	-	+	++	-
41	IL-2	++	+	+	++	+	-	-	+	-	+	+	-
42	IL-3	+	+	-	-	-	-	-	+	-	-	-	-
43	IL-4	++	++	+	+	-	-	-	+	-	-	-	-
44	IL-5	++	+++	+++	++	++	-	++	++	-	++	++	++
45	IL-6	+++	+++	++++	++++	++++	++	++++	+++	++	++	+	-
46	IL-8	++	+++	++	+++	+++	+++	++++	++++	++	+	+	-
47	IL-10	++	+	+	++	+	-	+	+	-	-	-	-
48	IL-11	++	+++	++	++	++	-	+	++	-	+	++	+
49	IL-12p70	++	+	++	++	+	-	+	+	-	+	+	-
50	IL-13	+	+	-	+	-	-	-	+	-	-	-	-
51	IL-15	++	+	+	++	+	-	+	+	-	+	+	-
52	IL-16	++	+	+	++	+	-	+	+	-	+	+	-
53	IL-17A	+++	++	++	+++	++	-	+	++	-	+	++	-
54	IL-18BP α	+	+	+	+	++	-	+	+	-	+	-	-
55	IL-19	++	+	+	+	+	-	+	+	-	+	-	-
56	IL-22	++	+	+	++	+	-	+	++	-	-	-	-
57	IL-23	+	++	+	+	+	-	+	+	-	-	+	-
58	IL-24	+	++	+	+	+	-	+	+	-	++	++	-
59	IL-27	+++	+++	++++	+++	++	+	++	+++	+	++	+++	+
60	IL-31	++	+	+	+	+	-	-	+	-	+	-	-
61	IL-32 $\alpha/\beta/\gamma$	++	++	+++	++	++	-	+	++	+	++	++	-
62	IL-33	+	+	+	+	+	-	+	+	-	+	-	-
63	IL-34	+	+	+	+	+	-	+	+	-	+	-	-
64	IP-10	++	+	+	++	+	-	+	+	-	+	-	-

65	I-TAC	++	++	++	++	++	-	+	+	-	+	++	-
66	Kallikrein3	+++	+++	++++	+++	+++	+	+	+++	+	++	+++	-
67	Leptin	++	++	+	+	-	-	-	+++	-	++	+	-
68	LIF	++	++	+	+	-	-	-	+	-	++	+++	-
69	Lipocalin 2	+++	++	+	++	++	-	++	++	+	+	++	+
70	MCP-1	+++++	+++++	+	++++	+++++	++++	+++++	+++++	+++++	++	+	-
71	MCP-3	++	++	+++	++	+++	-	+	++	+	+	++	-
72	M-CSF	+++	+++	+	+++	+++	-	++	++	+	++	++	+
73	MIF	+++++	+++++	+++	+++++	+++++	++	+++++	+++++	++++	++	+	-
74	MIG	++	++	+	++	+	-	+	+	-	+	+	-
75	MIP-1 α , MIP-1 β	+++++	+++++	+	+++++	+++	-	+++++	++++	-	++	+	-
76	MIP-3 α	+++	++	+	+++++	++	-	+	++	-	+	+	-
77	MIP-3 β	++	++	++	++	++	-	+	++	-	+	+	-
78	MMP-9	++	++	++++	++	++	-	-	++	-	+	+	-
79	Myeloperoxidase	++	+++	-	+	++	-	+	++	-	++	++	-
80	Osteopontin	++++	++++	+	+++	+++	++	++	+++	+	++	++	+
81	PDGF-AA	+++	++	+++	++	++	-	+	++	-	+	+	-
82	PDGF-AA/BB	+	+	+++	+	-	-	-	-	-	-	-	-
83	Pentaxin	+++++	+++++	++	+++++	+++++	++	++++	++++	+++	++++	++++	++
84	PF4	++	+	+++	++	-	-	-	+	-	-	+	-
85	RAGE	++	+	++++	+++	-	-	-	+	-	-	+	-
86	RANTES	+++	++	++	++	++	-	-	+	-	+	+	-
87	RBP-4	++	++	++	++	++	-	-	-	-	+	+	-
88	Relaxin-2	+++	++	++	++	++	-	+	++	-	++	++	-
89	Resistan	+++	+++++	++	+++	+++++	++++	++	+++++	++++	++	++++	++++
90	SDF-1 α	+++++	+++++	++	++	+++++	+++	++++	+++++	++	++++	++	-
91	Serpin E1	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	++++	++++	+++
92	SHBG	++	+	++	+++	++	-	-	++	+	++	++	-
93	ST2	++	+	+	++	+	-	-	+	-	+	+	-
94	TARC	++	++	++	++	++	-	+	++	-	++	++	-
95	TFF3	+	-	-	+	-	-	-	-	-	-	-	-
96	TfR	+++	+++	+++	++	+++	-	+	+++	-	+	+++	-
97	TGF- α	+	+	+	+	-	-	-	+	-	++	+	-

98	Thrombospondin	+++++	+++++	+++++	+++	++++	++	+++++	++++	++++	+++	+	-
99	TNF- α	+	+	-	+	-	-	-	-	-	-	-	-
100	μ -PAR	+++	++	++	++	+	-	+	+	-	++	++	-
101	VEGF	+++++	++++	++++	++++	++++	++	+	++	+	-	-	-
102	Vit-D BP	+++	++++	++++	++	+++	-	++	+++	+	++	+++	-

FIGURE D1.8: PRESENTATION OF SECRETION OF PROTEIN IN MEDIUM BATHING ENGINEERED HEART TISSUE. The MPD is presented from - to +++++ sign to represent the range. (Compiled data set)

		Left Atrium - MPD			Right Atrium - MPD			Left Ventricle - MPD			Right Ventricle - MPD		
		Day 2	Day 8	Day 16	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16
1	Angiogenin	+++++	+++++	++++	++	++	++	+++	++++	+	+	+	-
2	Angiopoietin-2	++++	++++	++++	+++	++++	+	++	++++	+	+++	++++	+
3	BDNF	+++	++++	+++	+++	+++	+	++	+++	+	+++	+++	+
4	CHI3L1	+++++	+++++	+++++	+++++	+++++	++++	++++	++	+	+++++	++	+
5	DKK-1	++++	++	++	++++	++	-	++	++	-	++++	++	-
6	DPPIV	+++++	+++++	++++	+++	+++++	++	+++++	+++++	+	++++	++	-
7	EMMPRIN	+++	+++	+++	+++	+++	+	+++	+++	+	++	+++	-
8	ENA-78	++++	+++	++	+++	++	-	+	+	-	+	-	-
9	GDF-15	++++	+++++	++++	++++	+++++	++	+++	++++	+	+++	+	-
10	IFN- γ	++++	++++	+++++	++++	++++	+	+++	++++	++	+++	++++	++
11	IGFBP-2	+++++	+++++	+++++	+++++	+++++	+++	+++++	+++++	+++++	++++	++++	+++
12	IGFBP-3	+++++	++++	++++	++++	++++	+	+++	+++	+	+++	+	-
13	IL-1 α	++++	++++	++++	+++++	++++	+	+++	++++	+	+++	++++	+
14	IL-6	+++	+++	++++	++++	+++++	++	++++	+++	++	++	+	-
15	IL-8	++	+++	++	+++	+++	+++	++++	++++	++	+	+	-
16	IL-27	+++	+++	++++	+++	++	+	++	+++	+	++	+++	+
17	Kallikrein3	+++	+++	++++	+++	+++	+	+	+++	+	++	+++	-
18	MCP-1	+++++	+++++	+	++++	+++++	++++	+++++	+++++	+++++	++	+	-
19	MIF	+++++	+++++	+++	+++++	+++++	++	+++++	+++++	++++	++	+	-
20	MIP-1 α , MIP-1 β	+++++	+++++	+	+++++	+++	-	+++++	++++	-	++	+	-
21	Osteopontin	++++	++++	+	+++	+++	++	++	+++	+	++	++	+
22	Pentaxin	+++++	+++++	++	+++++	++++	++	++++	++++	+++	++++	++++	++
23	Resistan	+++	+++++	++	+++	+++++	++++	++	+++++	++++	++	++++	++++
24	SDF-1 α	+++++	+++++	++	++	+++++	+++	++++	+++++	++	++++	++	-
25	Serpin E1	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	++++	++++	+++
26	Thrombospondin	+++++	+++++	+++++	+++	++++	++	+++++	++++	++++	+++	+	-
28	VEGF	+++++	++++	++++	++++	++++	++	+	++	+	-	-	-
29	Vit-D BP	+++	++++	++++	++	+++	-	++	+++	+	++	+++	-

FIGURE D1.9: COMPARISON OF WIDELY EXPRESSED SECRETED PROTEIN AMONG CHAMBER-SPECIFIC FBs BASED EHTs.

		Cardiomyocytes Only EHTs			Freshly Prepared EHT Medium
		Mean Pixel Density			Mean Pixel Density
		Day 2	Day 8	Day 16	
1	Adiponectin	+++	+++	+++	
2	Aggrecan	+++++	++++	+++++	
3	Angiogenin	++++	++++	++++	++
4	Angiopoietin-2	+++	+++	++++	+
5	BDNF	+++++	+++++	+++++	
6	C5/C5a	+++	+++	+++	
7	CHI3L1	+++++	+++++	+++++	+++++
8	Cystatin C	+++	+++	+++	++++
9	DKK-1	+++++	+++++	+++++	++++
10	DPPIV	+++++	++++	+++++	
11	EMMPRIN	++	++	+++	
12	Endoglin	++++	+++	++++	
13	FGF-19	+++	++	+++	
14	GDF-15	+++++	++++	+++++	
15	HGF	++	++	++++	
16	IFN- γ	+++	+++	++++	
17	IGFBP-2	+++++	+++++	+++++	+++++
18	IGFBP-3	+++	++	+++	+
19	IL-1 α	+++	++	+++	-
20	IL-5	+++	+++	++++	
21	IL-6	+++++	+++++	+++++	++++
22	IL-8	+++++	+++++	+++++	+++++
23	IL-11	++	+++	+++	
24	IL-17A	++	++	+++	
25	Kallikrein3	++	++	+++	+
26	Lipocalin 2	+++	++	+++	
27	MCP-1	+++++	+++++	+++++	+++
28	MIF	++	++	+++	+++
29	MIP-1 α , MIP-1 β	+	-	+	+++++
30	Myeloperoxidase	+++	+++	+++	
31	Pentaxin	+++++	+++++	+++++	++
32	RANTES	+++	+++	++++	
33	Resistan	+++++	+++++	+++++	
34	SDF-1 α	++++	++++	+++++	++++
35	Serpin E1	+++++	+++++	+++++	+++++
36	Thrombospondin	+++++	+++++	+++++	
37	VEGF	++	++	+++	

FIGURE D1.10: COMPARISON OF SECRETED PROTEIN AMONG CM-only FBs BASED EHTs AGAINST THE PROTEINS IN EHT MEDIUM

		Fibroblast Only EHTs			Freshly Prepared EHT Medium
		Mean Pixel Density			Mean Pixel Density
		Day 2	Day 8	Day 16	
1	Adiponectin	+++	++	+	
2	Aggrecan	++	+	+	
3	Angiogenin	+++	++	++	++
4	Angiopoietin-2	+++	++	++	+
5	BDNF	+++	++	++	
6	C5/C5a	+	-	-	
7	CHSL1	+++	++	+	+++++
8	Cystatin C	++	+	+	++++
9	DKK-1	+++	++	+	++++
10	DPPIV	++	+	+	
11	EMMPRIN	++++	+++	++	
12	Endoglin	+	+	-	
13	FGF-19	++	+	+	
14	GDF-15	++++	+++	++	
15	HGF	++	+	+	
16	IFN- γ	+++	++	+	
17	IGFBP-2	+++++	+++++	+++++	+++++
18	IGFBP-3	++	+	+	+
19	IL-1 α	+++	++	+	
20	IL-5	+++	++	+	
21	IL-6	+	+	-	++++
22	IL-8	++	+	+	+++++
23	IL-11	+++	+++	++	
24	IL-17A	+++	++	+	
25	Kallikrein3	+++	++	++	+
26	Lipocalin 2	+++	++	+	
27	MCP-1	++++	++++	+++	+++
28	MIF	+++++	+++++	+++++	
29	MIP-1 α , MIP-1 β	++++	+++	++	+++
30	Myeloperoxidase	+++++	++++	+++	
31	Pentaxin	+++	+++	++	++
32	RANTES	++	+	-	
33	Resistan	+++++	+++++	++++	
34	SDF-1 α	+++++	++++	+++	+++++
35	Serpin E1	+++++	+++++	+++++	+++++
36	Thrombospondin	+++++	+++++	++++	
37	VEGF	+++++	++++	++++	
38	G-CSF				++
39	IL-1ra				-
40	IL-2				+
41	TGF- α				++++
42	TNF- α				+
43	μ -PAR				++++
44	VCAM-1				++

FIGURE D1.11: COMPARISON OF SECRETED PROTEIN AMONG FB-only BASED EHTs AGAINST THE PROTEINS IN EHT MEDIUM

		Freshly Prepared EHT Medium	Cardiomyocytes Only EHTs			Fibroblast Only EHTs		
		Mean Pixel Density	Mean Pixel Density			Mean Pixel Density		
			Day 2	Day 8	Day 16	Day 2	Day 8	Day 16
1	Adiponectin		+++	+++	+++	+++	++	+
2	Aggrecan		+++++	++++	+++++	++	+	+
3	Angiogenin	++	++++	++++	++++	+++	++	++
4	Angiopoietin-2	+	+++	+++	++++	+++	++	++
5	BDNF		+++++	+++++	+++++	+++	++	++
6	C5/C5a		+++	+++	+++	+	-	-
7	CHI3L1	+++++	+++++	+++++	+++++	+++	++	+
8	Cystatin C	++++	+++	+++	+++	++	+	+
9	DKK-1	++++	+++++	+++++	+++++	+++	++	+
10	DPPIV		+++++	++++	+++++	++	+	+
11	EMMPRIN		++	++	+++	++++	+++	++
12	Endoglin		++++	+++	++++	+	+	-
13	FGF-19		+++	++	+++	++	+	+
14	GDF-15		+++++	++++	+++++	++++	+++	++
15	HGF		++	++	++++	++	+	+
16	IFN- γ		+++	+++	++++	+++	++	+
17	IGFBP-2	+++++	+++++	+++++	+++++	+++++	+++++	+++++
18	IGFBP-3	+	+++	++	+++	++	+	+
19	IL-1 α	-	+++	++	+++	+++	++	+
20	IL-5		+++	+++	++++	+++	++	+
21	IL-6	++++	+++++	+++++	+++++	+	+	-
22	IL-8	+++++	+++++	+++++	+++++	++	+	+
23	IL-11		++	+++	+++	+++	+++	++
24	IL-17A		++	++	+++	+++	++	+
25	Kallikrein3	+	++	++	+++	+++	++	++
26	Lipocalin 2		+++	++	+++	+++	++	+
27	MCP-1	+++	+++++	+++++	+++++	++++	++++	+++
28	MIF	+++	++	++	+++	+++++	+++++	+++++
29	MIP-1 α , MIP-1 β	+++++	+	-	+	++++	+++	++
30	Myeloperoxidase		+++	+++	+++	+++++	+++++	+++
31	Pentaxin	++	+++++	+++++	+++++	+++	+++	++
32	RANTES		+++	+++	++++	++	+	-
33	Resistan		+++++	+++++	+++++	+++++	+++++	++++
34	SDF-1 α	++++	++++	++++	+++++	+++++	++++	+++
35	Serpin E1	+++++	+++++	+++++	+++++	+++++	+++++	+++++
36	Thrombospondin		+++++	+++++	+++++	+++++	+++++	+++++
37	VEGF		++	++	+++	+++++	++++	++++

FIGURE D1.11: COMPARISON OF SECRETED PROTEIN AMONG CM-only & FB-only BASED EHTs AGAINST THE PROTEINS IN EHT MEDIUM

		CM-Only EHTs			Left Atrium			Right Atrium		
		Mean Pixel Density			Mean Pixel Density			Mean Pixel Density		
		Day 2	Day 8	Day 2	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16
1	Adiponectin	+++	+++	+++						
2	Aggrecan	+++++	++++	+++++						
3	Angiogenin	++++	++++	++++	+++++	+++++	++++	++	++	++
4	Angiopoietin-2	+++	+++	++++	++++	++++	++++	++++	+	++++
5	BDNF	+++++	+++++	+++++	+++	++++	+++	+++	+	+++
6	C5/C5a	+++	+++	+++						
7	CHI3L1	+++++	+++++	+++++	+++++	+++++	+++++	+++++	++++	+++++
8	Cystatin C	+++	+++	+++						
9	DKK-1	+++++	+++++	+++++	++++	++	++	++	-	++
10	DPPIV	+++++	++++	+++++	+++++	+++++	++++	+++++	++	+++++
11	EMMPRIN	++	++	+++	+++	+++	+++	+++	+	+++
12	Endoglin	++++	+++	++++						
13	FGF-19	+++	++	+++						
14	GDF-15	+++++	++++	+++++	++++	+++++	++++	+++++	++	+++++
15	HGF	++	++	++++						
16	IFN- γ	+++	+++	++++	++++	++++	+++++	++++	+	++++
17	IGFBP-2	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++	+++++
18	IGFBP-3	+++	++	+++	+++++	++++	++++	++++	+	++++
19	IL-1 α	+++	++	+++	++++	++++	++++	++++	+	++++
20	IL-5	+++	+++	++++						
21	IL-6	+++++	+++++	+++++	+++	+++	++++	+++++	++	+++++
22	IL-8	+++++	+++++	+++++	++	+++	++	+++	+++	+++
23	IL-11	++	+++	+++						
24	IL-17A	++	++	+++						
25	Kallikrein3	++	++	+++	+++	+++	++++	+++	+	+++
26	Lipocalin 2	+++	++	+++						
27	MCP-1	+++++	+++++	+++++	+++++	+++++	+	+++++	++++	+++++
28	MIF	++	++	+++	+++++	+++++	+++	+++++	++	+++++
29	MIP-1 α , MIP-1 β	+	-	+	+++++	+++++	+	+++	-	+++
30	Myeloperoxidase	+++	+++	+++						
31	Pentaxin	+++++	+++++	+++++	+++++	+++++	++	++++	++	++++
32	RANTES	+++	+++	++++						
33	Resistan	+++++	+++++	+++++	+++	+++++	++			
34	SDF-1 α	++++	++++	+++++	+++++	+++++	++	+++++	+++	+++++
35	Serpin E1	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++
36	Thrombospondin	+++++	+++++	+++++	+++++	+++++	+++++	++++	++	++++
37	VEGF	++	++	+++	+++++	++++	++++	++++	++	++++
38	ENA-78				++++	+++	++	++	-	++
39	IL - 27				+++	+++	++++	++	+	++
40	Vit-DBP				+++	++++	++++	+++	-	+++
41	Osteopontin				++++	++++	+	+++	++	+++

FIGURE D1.12: COMPARISON OF SECRETED PROTEIN AMONG CM- only & ATRIAL FBs BASED EHTs PROTEINS

		CM-Only EHTs			Left Ventricle			Right Ventricle		
		Mean Pixel Density			Mean Pixel Density			Mean Pixel Density		
		Day 2	Day 8	Day 2	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16
1	Adiponectin	+++	+++	+++						
2	Aggrecan	+++++	++++	+++++						
3	Angiogenin	++++	++++	++++	+++	++++	+	+	+	-
4	Angiopoietin-2	+++	+++	++++	++	++++	+	+++	++++	+
5	BDNF	+++++	+++++	+++++	++	+++	+	+++	+++	+
6	C5/C5a	+++	+++	+++						
7	CHI3L1	+++++	+++++	+++++	++++	++	+	+++++	++	+
8	Cystatin C	+++	+++	+++						
9	DKK-1	+++++	+++++	+++++	++	++	-	++++	++	-
10	DPPIV	+++++	++++	+++++	+++++	+++++	+	++++	++	-
11	EMMPRIN	++	++	+++	+++	+++	+	++	+++	-
12	Endoglin	++++	+++	++++						
13	FGF-19	+++	++	+++						
14	GDF-15	+++++	++++	+++++	+++	++++	+	+++	+	-
15	HGF	++	++	++++						
16	IFN- γ	+++	+++	++++	+++	++++	++	+++	++++	++
17	IGFBP-2	+++++	+++++	+++++	+++++	+++++	+++++	++++	++++	+++
18	IGFBP-3	+++	++	+++	+++	+++	+	+++	+	-
19	IL-1 α	+++	++	+++	+++	++++	+	+++	++++	+
20	IL-5	+++	+++	++++						
21	IL-6	+++++	+++++	+++++	++++	+++	++	++	+	-
22	IL-8	+++++	+++++	+++++	++++	++++	++	+	+	-
23	IL-11	++	+++	+++						
24	IL-17A	++	++	+++						
25	Kallikrein3	++	++	+++	+	+++	+	++	+++	-
26	Lipocalin 2	+++	++	+++						
27	MCP-1	+++++	+++++	+++++	+++++	+++++	+++++	++	+	-
28	MIF	++	++	+++	+++++	+++++	++++	++	+	-
29	MIP-1 α , MIP-1 β	+	-	+	+++++	++++	-	++	+	-
30	Myeloperoxidase	+++	+++	+++						
31	Pentaxin	+++++	+++++	+++++	++++	++++	+++	++++	++++	++
32	RANTES	+++	+++	++++						
33	Resistan	+++++	+++++	+++++	++	+++++	++++	++	++++	++++
34	SDF-1 α	++++	++++	+++++	++++	+++++	++	++++	++	-
35	Serpin E1	+++++	+++++	+++++	+++++	+++++	+++++	++++	++++	+++
36	Thrombospondin	+++++	+++++	+++++	+++++	++++	++++	+++	+	-
37	VEGF	++	++	+++	+	++	+	-	-	-
38	ENA-78				+	+	-	+	-	-
39	IL - 27				++	+++	+	++	+++	+
40	Vit-DBP				++	+++	+	++	+++	-
41	Osteopontin							++	++	+

FIGURE D1.13: COMPARISON OF SECRETED PROTEIN AMONG CM-only & VENTRIUCLAR FBs BASED EHTs PROTEINS

		Fibroblast Only EHTs			Left Atrium			Right Atrium		
		Mean Pixel Density			Mean Pixel Density			Mean Pixel Density		
		Day 2	Day 8	Day 2	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16
1	Adiponectin	+++	++	+						
2	Aggrecan	++	+	+						
3	Angiogenin	+++	++	++	+++++	+++++	++++	++	++	++
4	Angiopoietin-2	+++	++	++	++++	++++	++++	+++	++++	+
5	BDNF	+++	++	++	+++	++++	+++	+++	+++	+
6	C5/C5a	+	-	-						
7	CHI3L1	+++	++	+	+++++	+++++	+++++	+++++	+++++	+++++
8	Cystatin C	++	+	+						
9	DKK-1	+++	++	+	++++	++	++	++++	++	-
10	DPPIV	++	+	+	+++++	+++++	++++	+++	+++++	++
11	EMMPRIN	++++	+++	++	+++	+++	+++	+++	+++	+
12	Endoglin	+	+	-						
13	FGF-19	++	+	+						
14	GDF-15	++++	+++	++	++++	+++++	++++	++++	+++++	++
15	HGF	++	+	+						
16	IFN- γ	+++	++	+	++++	++++	+++++	++++	++++	+
17	IGFBP-2	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++
18	IGFBP-3	++	+	+	+++++	++++	++++	++++	++++	+
19	IL-1 α	+++	++	+	++++	++++	++++	+++++	++++	+
20	IL-5	+++	++	+						
21	IL-6	+	+	-	+++	+++	++++	++++	+++++	++
22	IL-8	++	+	+	++	+++	++	+++	+++	+++
23	IL-11	+++	+++	++						
24	IL-17A	+++	++	+						
25	Kallikrein3	+++	++	++	+++	+++	++++	+++	+++	+
26	Lipocalin 2	+++	++	+						
27	MCP-1	++++	++++	+++	+++++	+++++	+	++++	+++++	+++++
28	MIF	+++++	+++++	+++++	+++++	+++++	+++	+++++	+++++	++
29	MIP-1 α , MIP-1 β	++++	+++	++	+++++	+++++	+	+++++	+++	-
30	Myeloperoxidase	+++++	++++	+++						
31	Pentaxin	+++	+++	++	+++++	+++++	++	+++++	++++	++
32	RANTES	++	+	-						
33	Resistan	+++++	+++++	++++	+++	+++++	++			
34	SDF-1 α	+++++	++++	+++	+++++	+++++	++	++	+++++	+++
35	Serpin E1	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++	+++++
36	Thrombospondin	+++++	+++++	++++	+++++	+++++	+++++	+++	++++	++
37	VEGF	+++++	++++	++++	+++++	++++	++++	++++	++++	++
38	ENA-78				++++	+++	++	+++	++	-
39	IL - 27				+++	+++	++++	+++	++	+
40	Vit-DBP				+++	++++	++++			
41	Osteopontin	+++	++	+	++++	++++	+			

FIGURE D1.14: COMPARISON OF SECRETED PROTEIN AMONG FB-only & ATRIAL FBs BASED EHTs PROTEINS

		Fibroblast Only EHTs			Left Ventricle			Right Ventricle		
		Mean Pixel Density			Mean Pixel Density			Mean Pixel Density		
		Day 2	Day 8	Day 2	Day 2	Day 8	Day 16	Day 2	Day 8	Day 16
1	Adiponectin	+++	++	+						
2	Aggrecan	++	+	+						
3	Angiogenin	+++	++	++	+++	++++	+	+	+	-
4	Angiopoietin-2	+++	++	++	++	++++	+	+++	++++	+
5	BDNF	+++	++	++	++	+++	+	+++	+++	+
6	C5/C5a	+	-	-						
7	CHI3L1	+++	++	+	++++	++	+	++++	++	+
8	Cystatin C	++	+	+						
9	DKK-1	+++	++	+	++	++	-	++++	++	-
10	DPPIV	++	+	+	++++	++++	+	++++	++	-
11	EMMPRIN	++++	+++	++	+++	+++	+	++	+++	-
12	Endoglin	+	+	-						
13	FGF-19	++	+	+						
14	GDF-15	++++	+++	++	+++	++++	+	+++	+	-
15	HGF	++	+	+						
16	IFN- γ	+++	++	+	+++	++++	++	+++	++++	++
17	IGFBP-2	++++	++++	++++	++++	++++	++++	++++	++++	+++
18	IGFBP-3	++	+	+	+++	+++	+	+++	+	-
19	IL-1 α	+++	++	+	+++	++++	+	+++	++++	+
20	IL-5	+++	++	+						
21	IL-6	+	+	-	++++	+++	++	++	+	-
22	IL-8	++	+	+	++++	++++	++	+	+	-
23	IL-11	+++	+++	++						
24	IL-17A	+++	++	+						
25	Kallikrein3	+++	++	++	+	+++	+	++	+++	-
26	Lipocalin 2	+++	++	+						
27	MCP-1	++++	++++	+++	++++	++++	++++	++	+	-
28	MIF	++++	++++	++++	++++	++++	++++	++	+	-
29	MIP-1 α , MIP-1 β	++++	+++	++	++++	++++	-	++	+	-
30	Myeloperoxidase	++++	++++	+++						
31	Pentaxin	+++	+++	++	++++	++++	+++	++++	++++	++
32	RANTES	++	+	-						
33	Resistan	++++	++++	++++	++	++++	++++	++	++++	++++
34	SDF-1 α	++++	++++	+++	++++	++++	++	++++	++	-
35	Serpin E1	++++	++++	++++	++++	++++	++++	++++	++++	+++
36	Thrombospondin	++++	++++	++++	++++	++++	++++	+++	+	-
37	VEGF	++++	++++	++++	+	++	+	-	-	-
38	ENA-78				+	+	-	+	-	-
39	IL - 27				++	+++	+	++	+++	+
40	Vit-DBP				++	+++	+	++	+++	-
41	Osteopontin	+++	++	+	++	+++	+	++	++	+

FIGURE D1.15: COMPARISON OF SECRETED PROTEIN AMONG FB-only & VENTRIUCLAR FBs BASED EHTs PROTEINS