Begeleidingsformulier aanvraag dierproef DEC- UM

Versie 2006

Herziene versie

DECNR: 2011-078

Ontvangen: 31-05-2011

DEC datum goedkeuring#	Type aanvraag [2]
31-05-2011	Nieuw

VROM/GGONR[3]

LNV/CBDNR[4]

Hoofdproject	CARIM X	NUTRIM	Hersenen en gedrag	GROW	biomaterialen	Ander UM	Geen UM
Deelproject	2	1. 2. 3. 4.	1. 2. 3.	1. 2. 3.			

Financieel beheerder		Budgetnummer	3098.2.308N

Titel van het onderzoek:

Post(transcriptional) regulation of pathological cardiac remodeling: isolation of neonatal rat cardiomyocytes for in vitro analysis.

startdatum July 2011 einddatum [9] July 2015 *Duur van de proef [10]: 1 day (plan 4 yrs)*

	Naam	Tel (+ Tel privé enkel VO, VVO en VM)	E-mailadres	Bevoegdheid[5]	Cap. groep /afdeling
1.Verantwoordelijk onderzoeker (VO)				t Art.9	
2. Vervanger VO (VVO)				Art.9	
3. Verantwoordelijk medewerker (VM) GGO[7]					
4. overige uitvoerenden				Art.12	
PI				Art.9	

Diergroep	1	2	.	.	.	.	.
ctrl/exp/sham	Exp1	Exp2					
Diersoort	02	02					
Stam	Wistar	Wistar					
Construct / mutatie ?	n.a.	n.a.					
Herkomst (leverancier) *	01	01					
Aantal	192	2040					
Geslacht	F	M/F					
Dieren immuuncompetent ?	Ja	Ja	ja/nee[8]	ja/nee[8]	ja/nee[8]	ja/nee[8]	ja/nee[8]
Leeftijd/gewicht	pregnant	Neonatal (0-3 days)					
Doel van de proef *	37	37					
Belang van de proef *	01	01					
Toxicologisch onderzoek *	01	01					
Bijzondere technieken *	01	02					
Anesthesie *	01	01					
Pijnbestrijding *	01	01					
Mate ongerief *	01	01					
Toestand dier einde exp*	02	01					

* VHI-coderingen zie bijlage

1 Verantwoording

Aanvraag dierproef DEC-UM **(kaders zijn licht flexibel, maar het geheel is max. 5 pag. versie 2006)**

Titel: Post(transcriptional) regulation of pathological cardiac remodeling: Isolation of neonatal rat cardiomyocytes for in vitro analyses.

1. Doel van de proef.

Our research group, within the department of Cardiology, focuses on understanding the molecular and cellular background of pathological cardiac remodeling. Cardiac hypertrophy often progresses to ventricular dilatation and heart failure, a major cause of morbidity and mortality in the Western world. Clinical evidence demonstrates that sustained hypertrophy is a key risk factor in heart failure development and much effort is centred on the identification of the pathways leading to hypertrophy regarding drug development and heart failure therapy. Over the last years, calcineurin/NFAT signalling has been shown to play a central role in the development of pathological cardiac hypertrophy. Although many steps of this signalling pathway have been elucidated in the ventricular muscle, to date it remains unclear what the function of its target genes are in provoking heart failure. It is appreciated that many changes in the genetic make-up of the cell are not only governed at the transcriptional level (which genes are expressed) but we are progressively becoming aware that gene expression is also governed at the post-transcriptional level (how genes are translated to protein). Recently, a very powerful post-transcriptional mechanism has been uncovered involving small RNA molecules, microRNAs (miRs), that are able to fine-tune messenger RNA levels. MiRs are small non-coding RNAs (20-23 nucleotides) that negatively regulate the gene expression at the posttranscriptional level by base pairing to the 3' untranslated region (3' UTR) of target messenger RNA.

Previous gene and miR expression profiling studies in our lab have identified several miRs that may play an important role in pathological remodeling. MiR expression patterns have been identified in two different animal models of cardiac hypertrophy, the MHC-can Tg mouse and mice that have been subjected to transverse aortic constriction as a model for cardiac pressure overload. We have also compared murine hearts that were subjected to exercise and therefore developed cardiac physiological hypertrophic growth. Furthermore we made use of a bank of human cardiac biopsies (UMCU, Utrecht) to identify miRs that were differentially regulated specifically in the remodeled human heart. These studies have provided us a list of potentially interesting miRs that we propose to further investigate by means of the experiments (among others) described in this application. The genes and miRs that we are planning to first investigate are Beclin-2, Drak2, PPARδ, MCM7, Hand2, miR-25, miR-let7b, miR-1301, miR-376b and miR-216a.

Altogether, the goal of this research is to test, in vitro, the role of specific regulators cardiac hypertrophy. Before switching to an optimized in vivo setup (mouse models of cardiac disease) it is important and necessary to test our hypothesis in primary cultured cardiomyocytes (type of cells where the main events that we are studying take place).

2. Maatschappelijke relevantie en/of wetenschappelijk belang

In the western world, heart failure has the highest mortality and morbidity of all diseases, which puts great strain on our societies, both from an economical as well as from a medical point of view. Therefore, it is of enormous importance to gain better insight in the (molecular) mechanisms that drive myocardial pathogenesis in order to help discovering novel therapeutic treatments.

3. Alternatieven

At present there are no in vitro models (cell lines) of cardiac myocytes and/or cardiac fibroblasts.

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Before testing our hypothesis in vivo, it is important to perform specific tests in vitro. This will not only contribute for correction and optimization of the experimental setup but more importantly, it will help choosing the most suitable animal model while minimizing the number of animals needed in such in vivo study.

4. Ethische afweging

Heart failure is a leading cause of morbidity and mortality in the Western world. In the Netherlands, 45.000 people die each year because of cardiovascular diseases (approximately 120 per day). Many research efforts in the past decade have focused on the identification of the molecular pathways that mediate progressive cardiac remodeling. However, progress in developing new heart failure therapies has been impaired by an incomplete comprehension of the signaling events underlying cardiac hypertrophy and failure. Over the last several years, calcineurin has been the focus of intense research interest based on the discovery that it plays a central role in the principal hypertrophic signaling cascade. The scientific profit and potential clinical application justify the use of these animals to achieve the above mentioned study goals especially because human and cellular alternatives are not an option.

While studying the molecular processes behind the onset and development of heart failure we make general use of in vitro/cell models and in vivo/mouse models. However, at present there are no suitable cell models of cardiomyocytes that could be used for in vitro experiments and therefore, the use of primary cardiac cells is crucial for our research. The use of these cells is always carefully and efficiently planned so that we can statistically test as many conditions as possible. As mentioned already in point 3., combining different in vitro setups will allow a better planning of animal studies (suitable animal model and necessary conditions) and minimize the number of animals needed per experiment.
In contrast, the researcher is aware that the number of neonatal rats that is required for the isolation of the necessary amount of cardiomyocytes is very high. The animals are sacrificed by decapitation with scissors. This method, combined with the extensive experience of the researchers involved in the research minimizes the discomfort for the animals (score 02, in point 9a/c, 10a/b). Furthermore, the experiment will be conducted precisely as described in this DEC application.

Altogether, we think that the scientific and social relevance of our research and findings justify the number of animals and respective discomfort during the experiments.

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5. Wetenschappelijke onderbouwing

Heart failure is a disease with a high mortality and an increasing incidence and prevalence. Neonatal cardiac myocytes are terminally differentiated and thus unable to divide and are responsive to stress stimuli either by individual growth (hypertrophy) or by death (cell death or apoptosis).

Persistent pathological hypertrophy is the single most important risk factor for heart failure development in humans. Cardiac muscle hypertrophy is defined as a growth of the individual myofibers in size but not in number and is a response of the cardiac muscle to either altered mechanical loading conditions (e.g. resulting from valvular disease or hypertension) or decreased performance due to loss of contractile units (e.g. after myocardial infarction). Beyond just increased mass, the specific long-term transcriptional responses to increased load entail a myriad of quantitative and qualitative changes in cardiac gene expression that are reminiscent of fetal cardiac myocytes. Over the last several years, calcineurin/NFAT signaling has been the focus of intense research interest based on the discovery that it plays a central role in the principal hypertrophic signaling cascade. When studying target genes of the NFAT transcription factors in cardiac muscle, we have also discovered a subset of calcineurin/NFAT-responsive miRs that are coregulated with heart disease progression. In fact, our own previous study had shown that miRs have a crucial role in maintaining adult myocardial homeostasis [1].

Conventional gene arrays and more recently, microRNA arrays, have identified a set of genes that are potential direct targets of the Cn/NFAT signaling pathway. To study each of these genes in particular, one has to start with an in vitro approach. This implicates the use of primary neonatal cardiomyocytes. In the past we have submitted different DEC applications to study a specific gene [redacted]. All the studies performed resulted in valuable findings and subsequently, good scientific publications [2-4]. Recently a DEC application (2010-112) for a study that explores the role of the Hand2 transcription factor in the onset/development of cardiac hypertrophy has been approved. These studies altogether have provided us a list of potentially interesting genes and miRs that we propose to further investigate by means of the experiments described in this application. The genes and miRs that we are planning to first investigate are genes that seem to play a role in cardiac remodeling post-MI (Beclin-2, Drak2, miR-216a), genes that seem to play a role in cardiac hypertrophic remodeling (PPARδ, MCM7, Hand2 and miR-25) and genes that might be important in physiological cardiac remodeling (miR-let7b, miR-1301, miR-376b).

Altogether, the goal of this research is to test, in vitro, the role of specific regulators cardiac hypertrophy in order to achieve a better fundamental understanding of the molecular signals that drive the hypertrophic growth of the cardiac muscle and cell death processes during and after an ischemic lesion. These studies will provide better insight into the particulars of heart failure, and may have significant therapeutic consequences for clinical management of heart failure patients. The scientific history of our research group confirms the need and the suitability for the use of primary neonatal rat cardiomyocytes as a functional pre-screening anticipating eventual in vivo procedures.

In the course of the coming 4 years, every time we will start studying a new target gene/miR (not yet specifically indicated in this application) we will communicate to the [redacted] and the DEC by adding an addendum to this project and explaining the potential role of the "new gene/miR" in cardiac disease. Although we have included the number of animal necessary for new studies, no studies on genes that have not been yet referred in this application, will be initiated without previous approval from the DEC commission.

6. Wetenschappelijke beoordeling

This study has been read and approved **by a PI within the research group**

5 Proefdier

7. Proefdier keuze

7a. Soort, stam / herkomst / eindbestemming

We will use neonatal (0-3 days old) Wistar rats, from

7b. Sexe

Both male and female pups will be included in the study.

7.c. Aantallen

In DEC application (2010-112) we indicated the number of animals that is necessary for one single experiment, related to one single project (refer to point 8 for example of experimental setup). From experience we know that it is very difficult to calculate with accuracy the number of animals that will be necessary in total per research project. This is the reason why the number of animals requested in this application is based on previous experience and previous DEC applications Maastricht University). For this we have taken into account the fact that the number of projects remains constant every year (3), as well as the need for primary neonatal rat cardiomyocytes.

As mentioned above, the calculation was based in the number of animals requested in previous DEC protocols and DEC number 2010-112, where in average, 16 pregnant rats and an estimated number of 125-170 pups were requested, per single experiment / project / year. Optimization of transfection conditions by testing and determining the best working concentrations of precursors and LNA probes for this specific cell type have been considered in the calculations.

Based on the main projects being developed in our group, in this application we request animals for 3 projects, per year, for 4 consecutive years (from 2011 until 2015). This means 3*16 pregnant rats and therefore, 3*125-170 (48 pregnant rats, 375-510 pups) per year.

In conclusion we request for **4 years**: 4*48 = 192 pregnant rats and 4*375-510 = 1500-2040 pups; in a maximum total of **2232** animals.

Planning for 4 years (based upon above mentioned calculations)

jaar	Wistar rat (pregnant)	Wistar rat (pup)
2011	48	0-3 dagen - 375-510
2012	48	0-3 dagen - 375-510
2013	48	0-3 dagen - 375-510
2014	48	0-3 dagen - 375-510

6 Dierproef

8. Experiment

Example of one experimental setup per project (in italic the number of cells needed per condition):

	Ad-GFP			Ad-CnA			PE		
	proteins	RNA	imaging	proteins	RNA	imaging	proteins	RNA	imaging
-	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*
Pre-miR-X	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*
Pre-miR-Y	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*
Pre-miR-Z	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*
Scrambled precursor	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*
LNA-miR-X	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*
LNA-miR-Y	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*
LNA-miR-Z	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*
Scrambled LNA	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*	*1*10⁶*

Experiment:
Neonatal rat cardiomyocyte isolation will be performed as previously described [2-5]. In short, two- to three-days old rat pups will be removed from the mother's cage and will be sacrificed by decapitation. The heads, which are very ischemic-resistant, will be dropped in liquid nitrogen so that they will stop living instantly. We will open the chest, remove the hearts and cut it into pieces. The cells will be isolated using collagenase digestion, count and plated at 1*106 cells per 6cm culture disch. Cells will be treated with microRNA precursor molecules, LNA microRNA-inhibitor probes and then stimulated with Ad-CnA or PE (stimuli for cellular hypertrophic growth). All control cells will also be treated with Ad-GFP. The cells will be used for protein and RNA isolation, and also for immunostaining in order to assess cell surface and cardiomyocyte function.

9. Experimentele condities

9a. Anesthesie

n.a.

9b. Pijnbestrijding

n.a.

9c. Euthanasie en Humane eindpunten

The females are not going to be sacrificed; they will only be used for breeding. Rat pups will be decapitated and the heads, which are very ischemic-resistant, will be dropped in liquid nitrogen so that they will stop living instantly.

Zorg

10a. Ongerief
Estimated discomfort levels:

- removal of pups from mother's cage: estimated discomfort level 02
- decapitation of pups, head in liquid nitrogen, opening of chest, removal of heart: estimated discomfort level 02

10b. Welzijnsevaluatie
n.a.

11. Verzorging en huisvesting
The Animal Facility (CPV) of the Maastricht University will be responsible for the housing and caring of the animals during pregnancy. The rats will be housed in standard cages, with standard enrichment, in a soundproof room under conditions of controlled humidity, temperature and a 12h light/dark cycle (lights on from 7am to 7pm). Rats will be allowed free access to tap water and chow. The animal caretaker from CPV will inform article 9 person) when the rats are born and isolation can start.
Decapitation of the neonatal rats and isolation of cardiomyocytes will be performed in the and the molecular biology analysis will occur in the laboratory at the

12. Deskundigheid
(both art.9) will follow the complete procedure and perform the cell isolation. Researchers with article 9 have experience with handling the rats and performing all mentioned techniques. In case of emergency/questions on the condition of animals, we will contact a person with WOD art.12 and/or a person with WOD art.14.

13. Standard Operation Procedures (SOP)
All procedures described above will be in accordance to the SOPs established by the Maastricht University (*www.cpv.unimaas.nl*).
(0-3 dagen old) Wistar rats.

Relevante literatuur

1. Da Costa Martins PA, (...), De Windt LJ. Conditional Dicer deletion in the postnatal myocardium provokes spontaneous cardiac remodeling. *Circulation.* 18:1567-1576. (2008)

2. Van Oort RJ, (...), De Windt LJ. MEF2 activates a genetic program promoting chamber dilation and contractile dysfunction in calcineurin-induced heart failure. *Circulation.* 14:298-308. (2006)

3. Da Costa Martins PA, (...), De Windt LJ. MicroRNA-199b targets the nuclear kinase Dyrk1a in an auto-amplification loop promoting calcineurin/NFAT signalling. *Nat Cell Biol.* 12:1220-1227. 2010

4. El Azzouzi H, (...), De Windt LJ. PPAR gene profiling uncovers insulin-like growth factor (IGF-1) as a PPAR-alpha target gene in cardioprotection. *J Biol Chem.* Doi:10.1074/jbc.M111.220525. (2011)

5. De Windt LJ, (...), Molkentin JD. Calcineurin promotes protein kinase C and c-Jun NH2-terminal kinase activation in the heart. Cross-talk between cardiac hypertrophic signaling pathways. *J Biol Chem.* 275:13571-13579. (2000)

University Maastricht

Faculty of Health, Medicine and Life Sciences

Dierexperimenten Commissie

DEC

Aan:

, voorzitter
p/a Secretariaat DEC-UM
Postbus 616
NL-6200 MD Maastricht
Telefoon: 043

Uw referentie:

Onze referentie

Maastricht, 31-05-2011

Geachte Onderzoeker,

Uw projectaanvraag: "*Post (transcriptional) regulation of pathological cardiac remodeling: isolation of neonatal rat cardiomyocytes for in vitro analysis*", is op de DEC vergadering van 27 mei 2011 besproken.

De DEC heeft een aantal vragen en opmerkingen:

- De DEC verzoekt op het voorblad het privételefoonnummer van de vervangend verantwoordelijke onderzoeker te vermelden.
- De DEC verzoekt aan te geven door wie of welke commissie **dit DEC protocol** wetenschappelijk is beoordeeld en goedgekeurd (dit kan vanzelfsprekend NOOIT de VO of VVO zijn).

Conclusie:
Het project wordt aangehouden.

Gelieve eventuele vragen te beantwoorden in **een brief en indien noodzakelijk Uw project aan te passen en duidelijk de aanpassingen grijs te markeren.**
Uw project staat bij de DEC geregistreerd onder nummer **2011-078**, gelieve dit nummer in verdere correspondentie te vermelden.

Hoogachtend,

Voorzitter DEC-UM

Maastricht, 31-05-2011

Project 2011-078

Dear

Thank you for the evaluation of my DEC proposal.

Regarding the questions/comments, I have corrected the "voorblad" and also the proposal. Changes are marked in grey.

- in the voorblad the personal phone number of the VVO was added;

- in the proposal we changed the sentence regarding the commission/person who has evaluated the scientific background of the project to: "...This study has been read and approved by a PI within the research group (Department of

I hope I have informed you enough and that the changes made will be sufficient to get the project approved.

Maastricht University

Faculty of Health, Medicine and Life Sciences

Aan:

Ons kenmerk	*Doorkiesnummer*	*Maastricht*
	043-	31-05-2011

Project: *Post(transcriptional) regulation of pathological cardiac remodeling: isolation of neonatal rat cardiomyocytes for in vitro analysis.*

Verantwoordelijk onderzoeker (VO):

Hierbij delen wij U mede dat voornoemd project aan de ethische toetsingscriteria voor proefdiergebruik voldoet.
De DEC maakt geen bezwaar tegen uitvoering van dit project zoals aangevraagd en geeft een **positief advies**.

Projectnummer:	**2011-078**
Diersoort:	**rat**
Aantal dieren:	**2232**
Einddatum:	**31-05-2015**

Uw project staat bij de DEC en CPV geregistreerd onder bovenstaand nummer. Gelieve dieren, die voor dit project bestemd zijn, ook onder dit nummer aan te vragen.

Voorzitter DEC-UM

Vice-Voorzitter DEC-UM

DEC-UM
Voorzitter DEC-UM

p/a secretariaat DEC-UM

Secretariaat DEC-UM
T (043)

Bezoekadres

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