		Inventaris Wob-verzoek W17-11								
			wordt verstrekt				**weigeringsgronden**			
nr.		**document NTS 20171047**	**reeds openbaar**	**niet**	**geheel**	**deels**	**10.1.c**	**10.2.e**	**10.2.g**	**11.1**
1		Origineel aanvraagformulier				x		x		
2		NTS initieel			x					
3		Projectvoorstel initeel				x	x	x	x	
4		Figuur 1			x					
5		Figuur 2			x					
6		Bijlage beschrijving dierproeven 1				x	x	x	x	
7		Bijlage beschrijving dierproeven 2 initieel				x		x		
8		Bijlage beschrijving dierproeven 3 initieel				x		x		
9		Bijlage beschrijving dierproeven 4 initieel				x		x		
10		DEC-advies				x		x		
11		Ontvangstbevestiging				x		x		
12		Aanvullende informatie				x		x		
13		NTS aangepast	x							
14		Projectvoorstel aangepast				x	x	x	x	
15		Figuur 2 aangepast			x					
16		Bijlage beschrijving dierproeven 2 aangepast				x		x		
17		Bijlage beschrijving dierproeven 3 aangepast				x		x		
18		Bijlage beschrijving dierproeven 4 aangepast				x		x		
19		Adviesnota CCD		x						x
20		Beschikking en vergunning				x		x		
21		Verzoek herziening beschikking				x		x		
22		Herziene beschikking				x		x		

1047

Centrale Commissie Dierproeven

1.

18 MEI 2017

Aanvraag
Projectvergunning Dierproeven
Administratieve gegevens

- U bent van plan om één of meerdere dierproeven uit te voeren.
- Met dit formulier vraagt u een vergunning aan voor het project dat u wilt uitvoeren. Of u geeft aan wat u in het vergunde project wilt wijzigen.
- Meer informatie over de voorwaarden vindt u op de website www.centralecommissiedierproeven.nl. of in de toelichting op de website.
- Of bel met 0900-2800028 (10 ct/min).

1 Gegevens aanvrager

1.1 Heeft u een deelnemernummer van de NVWA?
Neem voor meer informatie over het verkrijgen van een deelnemernummer contact op met de NVWA.

☒ Ja > *Vul uw deelnemernummer in* 80100 KNAW
plaats van uitvoering: [redacted]

☐ Nee > *U kunt geen aanvraag doen*

1.2 Vul de gegevens in van de instellingsvergunninghouder die de projectvergunning aanvraagt.

Naam instelling of organisatie	[redacted]
Naam van de portefeuillehouder of diens gemachtigde	KNAW [redacted]
KvK-nummer	54667089
Straat en huisnummer	
Postbus	19121
Postcode en plaats	1000GC Amsterdam
IBAN	
Tenaamstelling van het rekeningnummer	

1.3 Vul de gegevens van het postadres in.
Alle correspondentie van de CCD gaat naar de portefeuillehouder of diens gemachtigde en de verantwoordelijke onderzoeker.

1.4 Vul de gegevens in van de verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	[redacted]
Functie	Groepsleider
Afdeling	[redacted]
Telefoonnummer	[redacted]
E-mailadres	[redacted]

1.5 *(Optioneel)* Vul hier de gegevens in van de plaatsvervangende verantwoordelijke onderzoeker.

(Titel) Naam en voorletters	☐ Dhr. ☐ Mw.
Functie	
Afdeling	
Telefoonnummer	
E-mailadres	

1.6	*(Optioneel)* Vul hier de gegevens in van de persoon die er verantwoordelijk voor is dat de uitvoering van het project in overeenstemming is met de projectvergunning.	(Titel) Naam en voorletters	☐ Dhr. ☐ Mw.
		Functie	
		Afdeling	
		Telefoonnummer	
		E-mailadres	
1.7	Is er voor deze projectaanvraag een gemachtigde?	☐ Ja > *Stuur dan het ingevulde formulier Melding Machtiging mee met deze aanvraag*	
		☒ Nee	

2 Over uw aanvraag

2.1	Wat voor aanvraag doet u?	☒ Nieuwe aanvraag > Ga verder met vraag 3
		☐ Wijziging op (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn Vul uw vergunde projectnummer in en ga verder met vraag 2.2
		☐ Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn Vul uw vergunde projectnummer in en ga verder met vraag 2.3
2.2	Is dit een *wijziging* voor een project of dierproef waar al een vergunning voor verleend is?	☐ Ja > Beantwoord dan in het projectplan en de niet-technische samenvatting alleen de vragen waarop de wijziging betrekking heeft en onderteken het aanvraagformulier
		☒ Nee > Ga verder met vraag 3
2.3	Is dit een *melding* voor een project of dierproef waar al een vergunning voor is verleend?	☒ Nee > Ga verder met vraag 3
		☐ Ja > Geef hier onder een toelichting en ga verder met vraag 6

3 Over uw project

3.1	Wat is de geplande start- en einddatum van het project?	Startdatum	1 - 6 - 2017
		Einddatum	1 - 6 - 2022
3.2	Wat is de titel van het project?	The cellular and molecular basis of the hematopoietic production	
3.3	Wat is de titel van de niet-technische samenvatting?	De cellulaire en moleculaire basis van de vorming van bloedvormende stamcellen	
3.4	Wat is de naam van de Dierexperimentencommissie (DEC) aan wie de instellingsvergunninghouder doorgaans haar projecten ter toetsing voorlegt?	Naam DEC	DEC-KNAW
		Postadres	Meibergdreef 47, 1105 BA Amsterdam
		E-mailadres	decsecr@knaw.nl

4 Betaalgegevens

4.1 Om welk type aanvraag gaat het?

☒ Nieuwe aanvraag Projectvergunning € 1684 Lege

☐ Wijziging € Lege

4.2 Op welke wijze wilt u dit bedrag aan de CCD voldoen.
Bij een eenmalige incasso geeft u toestemming aan de CCD om eenmalig het bij 4.1 genoemde bedrag af te schrijven van het bij 1.2 opgegeven rekeningnummer.

☐ Via een eenmalige incasso

☒ Na ontvangst van de factuur

5 Checklist bijlagen

5.1 Welke bijlagen stuurt u mee?

Verplicht

☒ Projectvoorstel

☒ Niet-technische samenvatting

Overige bijlagen, indien van toepassing

☐ Melding Machtiging

☐

6 Ondertekening

6.1 Print het formulier uit, onderteken het en stuur het inclusief bijlagen via de beveiligde e-mailverbinding naar de CCD of per post naar:

Centrale Commissie
Dierproeven
Postbus 20401
2500 EK Den Haag

Ondertekening door de instellingsvergunninghouder of gemachtigde (zie 1.7). De ondergetekende verklaart:

- dat het projectvoorstel is afgestemd met de Instantie voor Dierenwelzijn.
- dat de personen die verantwoordelijk zijn voor de opzet van het project en de dierproef, de personen die de dieren verzorgen en/of doden en de personen die de dierproeven verrichten voldoen aan de wettelijke eisen gesteld aan deskundigheid en bekwaamheid.
- dat de dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van richtlijn 2010/63/EU, behalve in het voorkomende geval de in onderdeel F van de bijlage bij het bij de aanvraag gevoegde projectvoorstel gemotiveerde uitzonderingen.
- dat door het ondertekenen van dit formulier de verplichting wordt aangegaan de leges te betalen voor de behandeling van de aanvraag.
- dat het formulier volledig en naar waarheid is ingevuld.

Naam	[redacted]
Functie	[redacted] - KNAW
Plaats	Amsterdam
Datum	15 - 5 - 2017
Handtekening	[redacted]

Format
Niet-technische samenvatting

- Dit format gebruikt u om uw niet-technische samenvatting te schrijven
- Meer informatie over de niet-technische samenvatting vindt u op de website www.centralecommissiedierproeven.nl.
- Of neem telefonisch contact op. (0900-2800028).

1 Algemene gegevens

1.1	Titel van het project	De cellulaire en moleculaire basis van de vorming van bloedvormende stamcellen
1.2	Looptijd van het project	5 jaar
1.3	Trefwoorden (maximaal 5)	bloedvorming , stamcellen, genregulatie, muis, zebravis

2 Categorie van het project

2.1 In welke categorie valt het project.

U kunt meerdere mogelijkheden kiezen.

- ☒ Fundamenteel onderzoek
- ☐ Translationeel of toegepast onderzoek
- ☐ Wettelijk vereist onderzoek of routinematige productie
- ☐ Onderzoek ter bescherming van het milieu in het belang van de gezondheid
- ☐ Onderzoek gericht op het behoud van de diersoort
- ☐ Hoger onderwijs of opleiding
- ☐ Forensisch onderzoek
- ☐ Instandhouding van kolonies van genetisch gemodificeerde dieren, niet gebruikt in andere dierproeven

3 Projectbeschrijving

3.1 Beschrijf de doelstellingen van het project (bv de wetenschappelijke vraagstelling of het wetenschappelijk en/of maatschappelijke belang)

Elke dag produceren bloedvormende stamcellen (*hematopoietic stem cells* ofwel HSC) miljarden nieuwe bloedcellen. Defecten in HSC leiden tot verscheidene bloedgerelateerde aandoeningen (zoals bloedarmoede) en soorten kanker (zoals leukemie).

De transplantatie van gezonde HSC is een belangrijk deel van de behandeling van bloedziektes. Soms is het voor de patiënten de enige mogelijke behandeling. De vraag naar stamceltransplantatie groeit, maar het is echter heel moeilijk de juiste donors te vinden. Daardoor is er een tekort aan HSC, wat een groeiend probleem is. Ondanks enorme inspanningen lukt het tot dusverre maar matig om nieuwe bronnen van HSC *in vitro* te genereren. Door de beperkte kennis van het natuurlijke productieproces (in vivo) is het moeizaam om HSC in een laboratoriumsituatie (in vitro) te

		kweken. Het doel van dit project is meer inzicht te krijgen in de *in vivo* HSC-productie, noodzakelijk om in de toekomst HSC *in vitro* te kunnen kweken.
3.2	Welke opbrengsten worden van dit project verwacht en hoe dragen deze bij aan het wetenschappelijke en/of maatschappelijke belang?	Wetenschappelijk belang: inzicht krijgen in de processen die betrokken zijn bij de productie en de regulatie van bloedvormende stamcellen. Maatschappelijk belang: Meer kennis over HSC-productie *in vivo* is van belang om *in vitro* kweken van HSC in de toekomst mogelijk te maken. De HSC zijn nodig in de kliniek voor het behandelen van patiënten met bloedgerelateerde ziekten.
3.3	Welke diersoorten en geschatte aantallen zullen worden gebruikt?	Muis: 12015 Zebravis: 4992
3.4	Wat zijn bij dit project de verwachte negatieve gevolgen voor het welzijn van de proefdieren?	Sommige dieren zullen worden gebruikt om donorcellen / weefsel te leveren die moeten worden getest. De overige dieren worden gebruikt als ontvangers van donorcellen en –weefsel. Om de donorcellen te testen, moeten we transplantaties uitvoeren bij dieren (ontvangers) die op lange termijn worden geanalyseerd (het in kaart brengen van de resultaten van onze experimenten). Kortdurend licht ongerief wordt verwacht als gevolg van het toedienen van stoffen, bijvoorbeeld door het geven van injecties (geen overlast door de gevolgen ervan). Licht ongerief wordt in een aantal gevallen ook verwacht door een kleine ingreep. In sommige gevallen zal een operatie worden uitgevoerd op een zwangere muis, wat een matig ongerief zal veroorzaken (bijvoorbeeld implantatie van genetisch gemodificeerde embryo's bij zwangere vrouwtjes). De meeste dieren in dit project zullen maximaal licht ongerief ondervinden; een aantal matig ongerief ten gevolge van de operaties.
3.5	Hoe worden de dierproeven in het project ingedeeld naar de verwachte ernst?	Muis: 3240 (matig ongerief, 27%), 8775 (licht ongerief, 73%) Zebravis: 4,992 (100%, licht ongerief)
3.6	Wat is de bestemming van de dieren na afloop?	Zowel de muizen als zebravissen worden geëuthanaseerd, waarna het weefsel uitgebreid wordt geanalyseerd.

4 Drie V's

4.1	**Vervanging** Geef aan waarom het gebruik van dieren nodig is voor de beschreven doelstelling en waarom proefdiervrije alternatieven niet gebruikt kunnen worden.	Om het aantal dierproeven tot een minimum te beperken, hebben we weefsels en cellen van dieren *in vitro* geanalyseerd. Zo kan gericht worden bepaald welke dierproeven nodig zijn. De complexe processen die betrokken zijn bij de productie van bloedvormende stamcellen worden bestudeerd in een levend organisme zodat de conclusies relevant zijn voor de mens. Deze complexe processen kunnen nog niet *in vitro* worden nagebootst.

4.2	**Vermindering** Leg uit hoe kan worden verzekerd dat een zo gering mogelijk aantal dieren wordt gebruikt.	Op basis van onze eerdere data, een goede statistische basis en een goede uitvoering van experimenten, in combinatie met jarenlange ervaring, kunnen wij strikt wetenschappelijke studies met een minimumaantal dieren uitvoeren. Er zullen zoveel mogelijk *ex vivo* analyses gecombineerd op de verkregen weefsels van een enkel dier om zo het aantal dieren te beperken tot het benodigde minimum. Zebravissen en muizen worden internationaal veelvuldig gebruikt voor onderzoek, waardoor een eenvoudige vergelijking te maken is met de gegevens uit andere studies. Hierdoor wordt herhaling voorkomen.
4.3	**Verfijning** Verklaar de keuze voor de diersoort(en). Verklaar waarom de gekozen diermodel(len) de meest verfijnde zijn, gelet op de doelstellingen van het project.	De dierexperimenten zullen worden uitgevoerd met muizen en zebravissen. Voor deze proefdieren geldt dat de kennis en expertise om het onderzoek uit te voeren groot zijn. Beide soorten zijn uitermate geschikt voor de studie van biologische processen. Beide diersoorten hebben elk hun eigen specifieke voordelen, zodat de studieopzet optimaal kan worden verfijnd. We gebruiken verschillende diersoorten om factoren/mechanismen te vinden die de productie van bloedstamcellen regelen en bovenal belangrijk zijn in alle diersoorten. Dergelijke factoren / mechanismen zullen zeer waarschijnlijk ook in de mens een rol spelen.
	Vermeld welke algemene maatregelen genomen worden om de negatieve (schadelijke) gevolgen voor het welzijn van de proefdieren zo beperkt mogelijk te houden.	De dieren krijgen adequate anesthesie en pijnbestrijding. De muizen en zebravissen worden dagelijks gecontroleerd op welzijn. De experimenten worden uitgevoerd door gekwalificeerd en bevoegd personeel. De omstandigheden waaronder het lijden van een proefdier actief moet worden beëindigd door euthanasie zijn nauwkeurig vastgelegd.

5 In te vullen door de CCD

Publicatie datum	
Beoordeling achteraf	
Andere opmerkingen	

Form
Project proposal

- This form should be used to write the project proposal for animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed.
- For more information on the project proposal, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80100 - KNAW
1.2	Provide the name of the licenced establishment.	[redacted]
1.3	Provide the title of the project.	The cellular and molecular basis of the hematopoietic production

2 Categories

2.1 Please tick each of the following boxes that applies to your project.

- ☒ Basic research
- ☐ Translational or applied research
- ☐ Regulatory use or routine production
- ☐ Research into environmental protection in the interest of human or
- ☐ Research aimed at preserving the species subjected to procedures
- ☐ Higher education or training
- ☐ Forensic enquiries
- ☐ Maintenance of colonies of genetically altered animals not used in other animal procedures

3 General description of the project

3.1 Background

Describe the project (motivation, background and context) with respect to the categories selected in 2.

- For legally required animal procedures, indicate which statutory or regulatory requirements apply (with respect to the intended use and market authorisation).
- For routine production, describe what will be produced and for which uses.
- For higher education or training, explain why this project is part of the educational program and describe the learning targets.

Every day, blood stem cells (also named hematopoietic stem cells or HSCs) produce billions of new blood cells, which are needed for an organism to survive. This massive cell production is possible due to two important properties inherent in HSCs, multipotency and self-renewal. The multipotency property allows single HSCs to produce all the different blood cell types (e.g. erythrocytes that carry oxygen in tissues,

platelets that provide coagulation in case of bleeding, lymphocytes that protect the organism against infections). The self-renewal property allows HSCs to produce all blood cells without exhaustion of the HSC pool, which remains constant during life. These inherent properties confer to HSCs the capacity to maintain blood homeostasis under physiological condition. HSCs play also a crucial role in the clinic. Indeed, defects in HSCs lead to blood-related disorders and various cancers (e.g. anemia, leukemia). The transplantation of healthy donor HSCs to replace the patient defective ones is an important part of the treatment and sometimes the only cure. However, less than 30% of the patients have matched donors in their family. Therefore, successful transplantation in most patients relies on finding unrelated volunteer donors with the highest compatibility (the chance of an optimal match being very low)[1]. Since the number of transplantations increases every year, the limitation in compatible HSCs available for transplantation procedures has become a major hurdle. More over, HSCs are very rare cells present in bone marrow, cord blood and mobilized peripheral blood (all tissues being used as source of HSCs in clinic). To circumvent the HSC shortage, research efforts have been made to expand donor HSCs *ex vivo* or to generate new sources of HSCs *in vitro* (e.g. from pluripotent stem cells or somatic cells)[2]. Despite some progresses, success has been limited and it remains impossible to date to produce large quantities of tailor-made HSCs. Because HSC production, as it occurs *in vivo*, is not fully understood yet, it is very difficult to mimic this process *in vitro*. To circumvent this issue, it is crucial (1) to better characterise the HSCs and their precursors, and (2) to identify the intrinsic factors (e.g. transcription factors) and extrinsic factors (e.g. growth factors provided by the surrounding microenvironment) that promote and regulate HSC fate determination, generation and expansion *in vivo*.

Adult HSCs are initially produced during embryonic development. They are first generated in the main arteries (aorta, vitelline and umbilical arteries) of the embryo[3-5] (**Fig. 1A**). They derive from hemogenic endothelial cells that are embedded in the arteries'wall[6,7] (**Fig. 1B**). The dynamic transition of an endothelial cell into a HSC has been observed in the aorta of chicken, mouse and zebrafish embryos[8,9,10,11]. Such finding demonstrated the conservation of the HSC production process in between species and the importance of the aorta microenvironment in supporting/regulating this important process. In arteries, HSCs are part of cell clusters[12] that will then colonize the fetal liver and placenta where the pool of HSCs expands before colonizing the bone marrow before birth (**Fig. 1A**,**B**). The important role of the endothelium in HSC production was an important fundamental discovery that paved the way to improve HSC production *in vitro*. For example, *in vitro* vascular induction was recently used to successfully reprogram human endothelial cells in HSC-like cells[13] and to induce mouse embryo aorta endothelial cells into HSC-like cells[14]. The produced cells are named HSC-like cells because it remains difficult to present to generate large quantities of fully functional HSCs that are able to engraft and to provide a long-term multilineage hematopoietic reconstitution when transplanted in adult recipients (the assay to experimentally identify HSCs). Therefore, research must continue to understand how a cell becomes a HSC, how it is regulated and how HSCs can expand without losing their stemness (**Fig. 1C**).

The **main research goal** in my lab is to better understand the production of HSCs, as it occurs *in vivo* during embryonic life. Our **sub-goal 1** is to determine the anatomical origin (intra- or extra-embryonic) of hemogenic endothelial cells (the cells producing HSCs). So far, it is uncertain because the blood is already circulating at the time of HSC detection in the embryo. Therefore, it cannot be excluded that hemogenic endothelial cells (or their precursors) emerge in one site and reach another anatomical site via the circulation or throughout tissues before to actually produce HSCs. It is an important sub-goal since the microenvironment of their site of origin determines the fate of these cells. Our **sub-goal 2** is to understand the function of specific genes in HSC production during embryonic life. HSC emergence and expansion are highly regulated processes both in time (as it occurs at specific time points of development) and space (in restricted regions of the vessels and organs) (**Fig. 1A**). However, the complex network of extrinsic and intrinsic regulatory factors involved *in vivo* in HSC production is still poorly understood. Our **sub-goal 3** is to find new markers for HSCs and cells from the supportive microenvironment to be able to precisely localise and follow the fate and behaviour of these cells during development.

The state-of-affairs on the production of HSCs *in vitro* is a worldwide concern since it remains impossible to achieve so far, despite extensive research. Understanding the process as it occurs *in vivo* is a very important research topic that several international labs are trying to achieve. However, only few labs study the embryonic development when the first HSCs are generated. Moreover, we are most likely the only lab able to perform a multi-species comparative study since we have the unique expertise, state-of-the-art technology and full access to the three most relevant animal models in the lab (i.e. chicken, mouse and zebrafish).

The use of the different animal models is needed because:
(i) They are complementary models. Indeed, each animal model allows different *ex vivo* analyses due to different technical advantages. For example, live imaging and cell tracing can only be performed in zebrafish embryos. Also, tissue grafting can only be performed with chicken embryos while HSC transplantation is used in the mouse and zebrafish models.
(ii) The use and comparison of different animal models allow finding conserved processes and mechanisms in between species. It reinforces the possibility that such processes and mechanisms also occur in human. In this project, chicken, mouse and zebrafish embryos will be used as reliable alternatives to human embryos to analyse in details the different aspects of HSC production during embryonic life.
(iii) The study of developmental hematopoiesis cannot be performed in human embryos due to the difficulty to access human embryo samples. When available, such samples are rare and often damaged because of the embryo collection procedure (i.e. from abortion). The use of human embryo is not a possible/realistic alternative for our project. The only alternative is to use animal models instead.
(iv) There is an increased request from the scientific community to provide and compare data in different animal models (e.g. for publication).

Because there is no need of licence to work on chicken embryos (no adult chicken will be used for the project), the requested licence only concerns the mouse and zebrafish models. Our three sub-goals are of equivalent importance. Therefore, our strategy is to conduct experiments in all three animal models in parallel to answer all sub-goals (and not sequentially since all models will provide, via different assays and *ex vivo* analysis, pieces of answers to the same question).

[redacted]

[redacted]

Overall, a better understanding of HSC generation/expansion in the successive physiological niches as it occurs *in vivo* during the embryonic development of various animal species is needed to understand the core mechanisms of HSC production that will most likely be conserved in human. It will help to engineer appropriate *in vitro* culture conditions to produce in the future autologous hematopoietic grafts needed to treat patients who suffer from hematopoietic disorders, the long-term target group of our proposal[17]. Deciphering the physiological development of HSCs will also prove useful for understanding the deregulation of HSCs.

References

1 [redacted]

3.2 Purpose

Describe the project's main objective and explain why this objective is achievable.

- If the project is focussed on one or more research objectives, which research questions should be addressed during this project?
- If the main objective is not a research objective, which specific need(s) does this project respond to?

The work described here aims to increase our understanding of the processes leading to HSC production as it occurs *in vivo* during embryonic development. It will identify which genes and signalling pathways

are functionally important during the successive steps of HSC production (such as endothelial cell specification into hemogenic endothelial cells, endothelial to hematopoietic transition, pre-HSC maturation, HSC survival and expansion). The goal of using and comparing different animal models (chicken, mouse, zebrafish) is to find conserved HSC regulators that will therefore most likely be involved also in human. Ultimately this knowledge should lead in the future to the production of tailor-made HSCs that are needed to treat patients who suffer from hematopoietic disorders and diseases.

Our main goal is to identify the molecular and cellular mechanisms involved in HSC production and regulation during the embryonic development of zebrafish, mouse and chicken (the three main and most reliable animal models used to study developmental hematopoiesis).

Our specific research sub-goals are:
1- To determine the anatomical origin of hemogenic endothelial cells (or precursors).
We wish to identify the anatomical site (yolk sac, allantois or embryo proper) at the origin of hemogenic endothelial cells (or precursors). We expect to identify within the 5 years of the project the microenvironment that determines the fate of hemogenic endothelial cells, which produce later on HSCs.

2- To understand the function of specific genes in HSC production during embryonic life under basal conditions by looking at the effect on cell specialization, emergence, viability, maturation, proliferation and differentiation.
We wish to identify the genes involved in the regulation of HSC production and we expect to prove their functionality within the 5 years of the project.

3- To find new markers for HSCs and cells from the supportive microenvironment.
We wish to identify new markers to better identify, isolate and locate HSCs and the cells in the surrounding microenvironment that constitute a supportive niche. We expect to identify new markers within the 5 years of the project.

This research will be performed in a lab, [redacted] The institute houses large zebrafish and mouse facilities, provides core facilities for various high-end techniques (e.g. sequencing, histology, confocal microscopy, flow cytometry), and expertise on animal models with dedicated and experienced animal caretakers. As principle investigator for this project, I have more that 20 years of experience with the use of the mouse model and 2 years with the zebrafish model, in biomedical research. In the lab, we have one dedicated *in vivo* technician and very experienced scientists that oversee the breeding of all animal lines, experiments and procedures, and new people are trained when required. The research lab has experience with the different experiments and techniques that are proposed for the project (e.g. intra-uterus injection in embryos, intravenous injection in adult, embryos and tissues collection, whole embryo multicolour staining, immunostaining on cryosections, confocal imaging of embryo/tissue slices after immunostaining).

There are several reasons why we think that we can achieve our objectives within 5 years:
- Part of the research described here was included in research proposals that were reviewed by independent experts in the field and were granted for funding ([redacted] and were previously approved as DEC protocols. All animal procedures are currently being performed and are part of the ongoing projects. It is important to note, that there is overlap between the mouse studies described in this project and those in earlier DEC-approved protocols. After a license for this project has been obtained, all experiments will formally be executed under this new license.
- Work from my group on [redacted] has resulted in seminal papers that described the required techniques.
- Our research and experiments are continuously being evaluated by various other researchers within and outside our institute. The scientists working in my group are selected based on their excellence and their commitment to our goals.

Our previous achievements make it very likely that we will be able to finish the project proposal in 5 years.

3.3 Relevance

What is the scientific and/or social relevance of the objectives described above?

The proposed work will result in the identification of molecular and cellular processes that drive

hematopoietic stem cell (HSC) production in vivo. The **scientific relevance** of the objectives is to improve our fundamental scientific knowledge of the processes and mechanisms driving and regulating HSC and hematopoietic production in zebrafish, and mouse and chicken throughout development and in adult. The **social relevance** of our objectives is that we can use this knowledge in the future to improve HSC production and expansion in vitro and therefore to supply in tailor-made HSCs that are in such need to cure patients with blood related disorders and diseases. Allogeneic and autologous HSC transplantations are used widely in the treatment of hematologic and genetic conditions. Induced pluripotent stem (IPS)-derived HSCs could be an ideal source for resolving current limitations related to HSC transplantation procedures. Current efforts to derive HSCs from pluripotent stem cells have largely focused on recapitulating normal developmental progression in an ex vivo setting. Lessons can certainly be learned from the study of HSC production during embryonic development. In addition, an ability to generate bona fide HSCs from iPS lines derived from patients with genetic diseases could be invaluable for disease modelling and drug screening. It would certainly tremendously reduce research on animals and the need to collect cells from donors or embryos. Although generating human HSCs from pluripotent stem cells still remains elusive, a better understanding of the network of regulatory factors governing HSC identity and production as it occurs during embryonic development (our proposal goal) should help to overcome the current hurdles in generating functional (i.e. transplantable) HSCs.

3.4 Research strategy

3.4.1 Provide an overview of the overall design of the project (strategy).

A flowchart that summarizes the workflow of experiments is provided in attachment.
Our research strategy is to study and compare the hematopoietic development in chicken, mouse and zebrafish embryos to find conserved mechanisms and regulatory genes. Parallel studies will therefore be performed in mouse, zebrafish and chicken species. For chicken embryo experiments, no AP is required. The goal is to find mechanisms and genes that will be conserved in the three species and therefore will be most likely also conserved in human embryos, which are difficult to study directly (due to the difficulties to obtain human embryo samples). Our research strategy provides a reliable alternative to learn more about human developmental hematopoiesis.
[redacted] Based on these data generated in previous in vivo and in vitro studies, available literature and interactions with other scientists, we therefore identified potent interesting intrinsic and extrinsic regulators of HSC production [redacted] In this project, we now wish to test their functional role on chicken, mouse and zebrafish hematopoiesis (licence required only for the two last animal models). We will test their precise mechanism of action in vivo in wild type, genetically modified (GM) animals or animals in which a specific gene or type of (stem) cell has been modified with a compound. Animals will be injected with compounds (e.g. Tamoxifen) to increase or inhibit a gene. In case a line with the required genetic modification is not available we will generate new animal lines via standard oocyte injection, blastocyst injection (mouse), or via the CRISPR/Cas9 system (see Appendix 3.4.4.1 (mouse)). We will only use well-established reagents and protocols to induce expression or deletion of the candidate gene(s). Our considerations and findings that support the use of mice and zebrafish will be part of our application that we submit to the IvD.
We will set up pilot studies with the minimum amount of animals possible to test the validity of our hypothesis (HSC regulator or not) in vivo. For that, we will collect tissues at the time when the gene candidate(s) or cell type should have the greatest effect. Each experiment is very carefully designed with clear go-no-go decisions to reduce the amount of cumulative discomfort and/or the number of animals. For every each experiment, the best trade-off will be made.

We will perform the following experiments for:
Sub-goal 1 (see Appendix 3.4.4.3). To identify the anatomical site at the origin of hemogenic endothelial cells (or precursors), we will perform in utero transplantation of donor cells isolated from yolk sac, allantois and embryo proper (potent sites of origin). The donor cells will carry a marker (e.g.

fluorescent, as GFP-positive cells transplanted in wild type recipients) that will allow the traceability of these cells and their progeny after transplantation. The fluorescent donor cells will also be easily distinguishable from the wild type recipients. Donor cells will be injected in the heart of mouse recipient embryos developing in the mother uterus. We will analyse ex vivo the tissues and cells collected from the growing recipients (cellular, molecular and histological analysis) to determine the long-term multi-lineage hematopoietic contribution of the donor cells. Only the tissues that will contain hemogenic endothelial cells or precursors will be able to produce HSCs in the recipient embryo and to provide long-term multi-lineage hematopoietic reconstitution in the growing recipients to adult age (see below the overall summary of the procedure). These experiments will determine which tissue/microenvironment (yolk sac, allantois or embryo proper) is at the origin of hemogenic endothelial cells, which produce later on HSCs.

Sub-goal 2. To identify the genes involved in the regulation of HSC production and to prove their functionality, we will modify candidate genes to test their function in HSC production in mice and zebrafish. During gene modification, a fluorescent marker (e.g. GFP) will be introduced to mark the genetically modified cells. After gene modification (see Appendix 3.4.4.1), tissues and cells will be isolated and analyzed *ex vivo* (See Appendix 3.4.4.2). Donor cells will also be used to perform *in vivo* transplantation assay (transplantation of WT or modified cells in adult or neonate recipients) (see Appendix 3.4.4.3 and 3.4.4.4). HSCs are identified in an *in vivo* assay where cells are transplanted intra-venously in adult recipient mice. Pre-HSCs are too immature to engraft adult recipients but they are identified in an *in vivo* assay where cells are transplanted in the liver of neonates, which constitutes a more permissive environment than the adult bone marrow. Recipients (adults and neonates) are irradiated prior transplantation to clear out the bone marrow and liver from all proliferating cells and therefore to make space. It allows the engraftment of the injected donor cells. The hematopoietic tissues and cells of the transplanted recipients will then be analyzed *ex vivo* for donor (fluorescent) contribution (see Appendix 3.4.4.3 and 3.4.4.4). The presence of donor cells in all hematopoietic lineages and all hematopoietic tissues of the recipient at long-term will prove the presence of pre-HSCs or HSCs (see below the overall summaries of the procedures).

We will also generate genetically modified zebrafish but since no hampered phenotypes are expected, this activity does not require a CCD license. After gene modification, the zebrafish will be analyzed *in vivo* by live confocal imaging of the embryo (e.g. by imaging cell emergence, maturation, survival, proliferation and/or differentiation) and by *ex vivo* analysis of the hematopoietic embryonic/adult tissues. The observation of HSC or hematopoietic defect in the hematopoietic organs/cells of the animals where

gene modifications have been performed will indicate that these specific genes are important HSC regulators.

Sub-goal 3. The sub-goal 3 is to identify new markers to better identify, isolate and locate HSC precursors (pre-HSCs), HSCs and the cells in the surrounding microenvironment that constitute the supportive niche. For this purpose, cells will be sorted based on the expression of the potent new surface markers and transplantated in neonate (assay to identify pre-HSCs; see Appendix 3.4.4.3) and adult mice (assay to identify HSCs; see Appendix 3.4.4.3), and adult zebrafish (assay to identify HSCs; see Appendix 3.4.4.4). Hematopoietic tissues will also be collected during embryonic development and adult to locate and trace the cells based on the expression of the new markers. Pre-HSCs and HSCs must localise in the clusters in the aorta, and should be thereafter be present in the successive hematopoietic organs (placenta, yolk sac, liver and bone marrow). Cells from the supportive niche should be located close by to the pre-HSCs and HSCs (e.g. in the mesenchyme underneath the clusters in the aorta of the embryo). The correct localisation and function of the cells based on the expression of the new markers will indicate that these markers are reliable markers of pre-HSCs, HSCs or supporting cells from the microenvironment.

3.4.2 Provide a basic outline of the different components of the project and the type(s) of animal procedures that will be performed.

The specific protocols that we apply to achieve our research goals are outlined below, in the flowchart and Appendix. All these animal procedures and their components are currently already on going in our lab.

Generation, welfare assessment and breeding of wild type and genetically modified mice (Appendix 3.4.4.1)
To study the function or behavior of a gene or a cell type relevant for HSC production, we will use appropriate mouse lines that are either already available or that need to be generated. New mouse line(s) will be generated via standard oocyte injection, blastocyst injection, or via the CRISPR/Cas9 system. The CRISPR/Cas9 system will especially be used as highly efficient tool for simultaneously multi-gene editing. This prevents the generation and breeding of multiple homozygotes from individually targeted ES cells (Reduction of the 3Rs).
In contrast to conventional gene-targeting strategy, the use of the Cre/LoxP recombination system in conjunction with gene targeting allows us to study the consequence of gene manipulation in a cell type specific manner. By incorporating Cre recombinase recognition sites (LoxP) into the genome, Cre expression from a specific promoter can drive gene disruption, activation or tracing in a cell type specific manner. New transgenic lines and/or KO lines generated via classical methods and/or novel combinations and used for breeding of these aforementioned lines will be monitored for 2 generations to determine the absence or presence of constitutional discomfort.
Cre recombinase expression can also be activated in an inducible manner by the addition of tamoxifen. To this end the Cre is flanked by 2 mutated estrogen receptors (Creert2, or merCremer) and will only allow for Cre activation when tamoxifen is administered.

Collection of tissues and cells from wild type and genetically modified animals for *ex vivo* analysis and collection of donor cells for transplantations (Appendix 3.4.4.2)
To study aspects of hemogenic endothelial cells or precursors origin, pre-HSC and HSC emergence and production as well as HSC progeny, we will isolate whole embryos, and hematopoietic tissues during embryonic development and in adults (from wild type or genetically modified mice and zebrafish). *Ex vivo* analysis will be performed on isolated tissues and cells. Donor cells will also be isolated to perform transplantations (see Appendix 3.4.4.3)

Transplantation in recipients (pregnant females (embryos), newborns and adult mice) and *ex vivo* analysis (Appendix 3.4.4.3).
To determine the anatomical origin of hemogenic endothelium, cells isolated from the embryo proper, allantois and yolk sac will be tested by performing *in utero* transplantations in embryos in pregnant mice. To test pre-HSCs and HSCs (wild-type and mutant), transplantation will be performed in neonates and adults, respectively.
Transplanted embryos and neonates will be analysed when they reach the adult stage. Adult mice will be analysed up to 4 months post-transplantation. Tissues and cells will be collected for *ex vivo* analysis (e.g. long-term multi-lineage hematopoietic reconstitution of donor origin).

Transplantation in zebrafish, live imaging and *ex vivo* analysis (Appendix 3.4.4.4).
Genetically modified and wild type animals will be analysed for HSC production. For this purpose, the zebrafish will be subjected to transplantation, *in vivo* analysis (live time-lapse confocal imaging) and *ex vivo* analysis (histology, DNA/RNA analysis…).

Generation, welfare assessment and breeding of wild type and genetically modified zebrafish
Overall, we will generate genetically modified zebrafish but since no hampered phenotypes are expected, this activity does not require a CCD license.
(see https://www.centralecommissiedierproeven.nl/actueel/nieuws/16/10/13/handreiking-genetisch-gewijzigde-dieren ; genereren, fokken, genotypen, monitoren en huisvesten van genetisch gewijzigde dieren).

3.4.3 Describe the coherence between the different components and the different steps of the project. If applicable, describe the milestones and selection points.

See also Flow Chart in attachment.

All experiments are based on the preliminary identification of candidate genes or cell types. The identified genes or cell types will initially be carefully tested in tissues from embryo samples and in *in vitro* experiments. If the identified genes or cell types show an interesting expression pattern or phenotype, we will consider the extensive and careful analysis of (compound) GMO for our *in vivo* experiments. If the desired genotype is not available, we will create them ourselves. We will only use well-established reagents and protocols to induce expression or deletion of the candidate gene/s in mice (Appendix 3.4.4.1) and zebrafish.

Our three sub-goals are of equivalent importance. Therefore, our strategy is to conduct experiments to try to answer all sub-goals in parallel (and not sequentially).

To address the role of candidate genes or cell types on hemogenic endothelium, pre-HSC and HSC production in the mouse model, *in vivo* transplantation will be performed in embryos, newborns and adult, respectively (Appendix 3.4.4.3). *Ex vivo* analysis on collected cells and tissues will be done to analyse the role of candidate genes or hematopoietic cells on the hematopoietic contribution.

In some cases we will use our animal lines without any intervention for the collection of tissues or cells for *ex vivo* analysis.

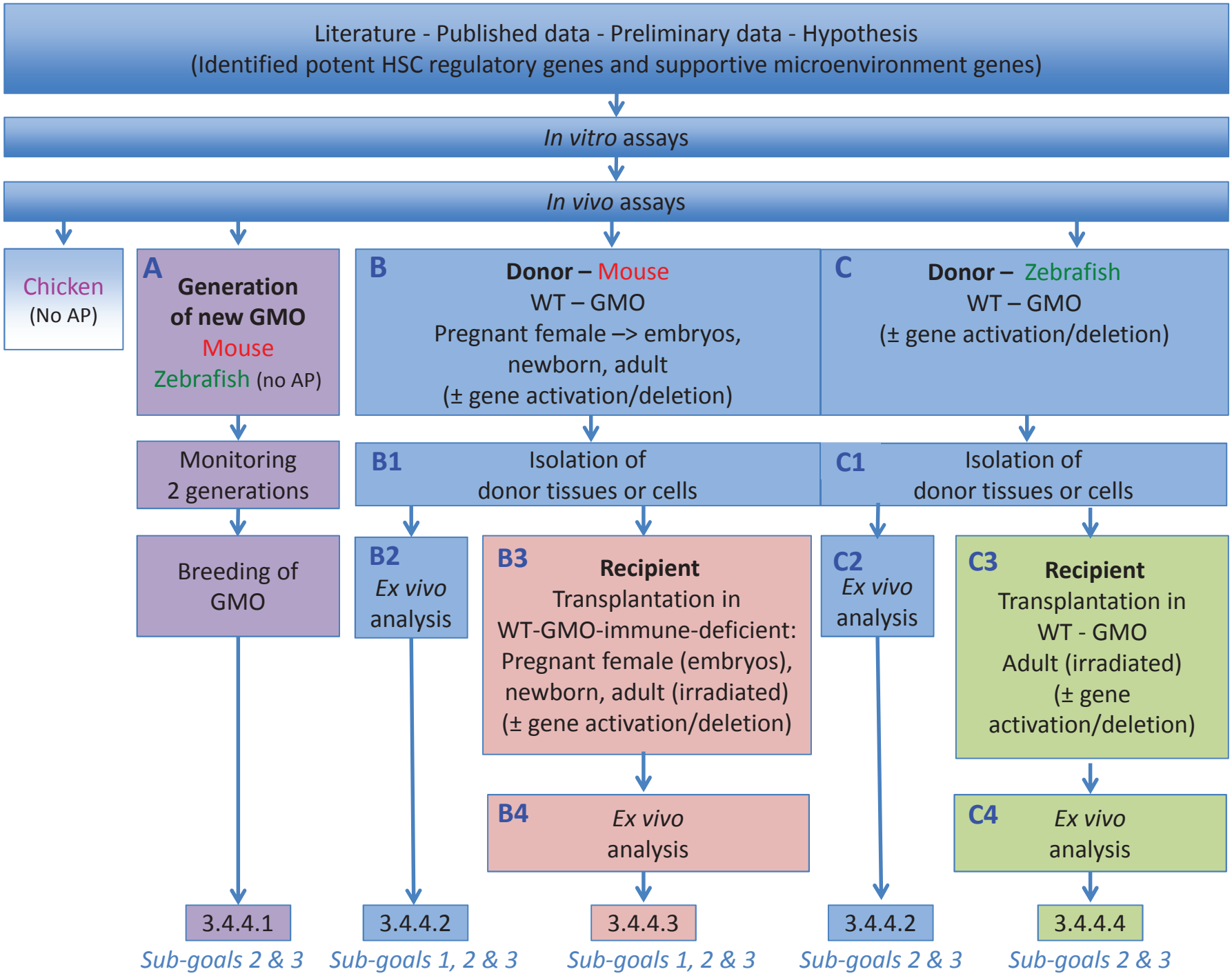
To address the role of candidate genes or cell types on HSC production in the zebrafish model, *in vivo* transplantation will be performed in adult (Appendix 3.4.4.4). Time-lapse live confocal imaging and *ex vivo* analysis on whole zebrafish and collected cells/tissues will be done to analyse the role of candidate genes or hematopoietic cells on the hematopoietic contribution, respectively.

Whenever possible, for all the *in vivo* experiments, we will perform pilot studies with the minimum amount of animals possible. It means that we will perform one experiment and will wait for the read-out result before to perform a complete set of experiments (e.g. we will wait for the result of the pilot experiment before to do the n=3 experiments requested to validate an analysis). We designed the experiments very carefully, to reduce the amount of cumulative discomfort and/or the number of animals. For each experiment, the best trade-off will be made.

3.4.4 List the different types of animal procedures. Use a different appendix 'description animal procedures' for each type of animal procedure.

Serial number	Type of animal procedure
1	Generation of new GMO (mice)
2	Donor – *Ex vivo* analysis (mice, zebrafish)
3	Recipient – *Ex vivo* analysis (Transplantation in pregnant females, newborns and adult mice)
4	Transplantation in zebrafish, live imaging and *ex vivo* analysis (embryo till adult)
5	
6	

7	
8	
9	
10	


Literature - Published data - Preliminary data - Hypothesis
(Identified potent HSC regulatory genes and supportive microenvironment genes)
In vitro assays
In vivo assays
Chicken
(No AP)
A
Generation of new GMO
Mouse
Zebrafish (no AP)
B
Donor – Mouse
WT – GMO
Pregnant female –> embryos, newborn, adult
(± gene activation/deletion)
C
Donor – Zebrafish
WT – GMO
(± gene activation/deletion)
Monitoring 2 generations
B1
Isolation of donor tissues or cells
C1
Isolation of donor tissues or cells
Breeding of GMO
B2
Ex vivo analysis
B3
Recipient
Transplantation in WT-GMO-immune-deficient:
Pregnant female (embryos), newborn, adult (irradiated)
(± gene activation/deletion)
C2
Ex vivo analysis
C3
Recipient
Transplantation in WT - GMO
Adult (irradiated)
(± gene activation/deletion)
B4
Ex vivo analysis
C4
Ex vivo analysis
3.4.4.1
Sub-goals 2 & 3
3.4.4.2
Sub-goals 1, 2 & 3
3.4.4.3
Sub-goals 1, 2 & 3
3.4.4.2
Sub-goals 2 & 3
3.4.4.4
Sub-goals 2 & 3

The cellular and molecular basis of the hematopoietic production

Overview number of animals and Animal Procedures

Procedure	Title	Species	Stage	Mild	Moderate
3.4.4.1	Generation of new GMO (mice)	Mouse	Adult		3000
3.4.4.2	*Donor – Ex vivo analysis (mice and zebrafish)*				
3.4.4.2a	Pregnant females for embryos	Mouse	Adult	2205	
3.4.4.2b	Newborns	Mouse	Newborn	720	
3.4.4.2c	Adults	Mouse	Adult	126	
				3051	
3.4.4.3	*Recipient – Ex vivo analysis*				
3.4.4.3a	Pregnant females for embryo transplants	Mouse	Adult		240
3.4.4.3b	Resulting embryos	Mouse	Adult	1440	
3.4.4.3c	Transplants in newborns	Mouse	Newborn	2268	
3.4.4.3d	Transplants in adults	Mouse	Adult	2016	
				5724	
3.4.4.4	Transplantation in zebrafish, live imaging and ex vivo analysis	Zebrafish	Adult	4992	

Total:

12.015 mice of which 3240 will have maximally moderate discomfort (27%) and 8775 with mild discomfort (73%)

4992 zebrafish with maximally mild discomfort = 100%

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80100
1.2	Provide the name of the licenced establishment.	[redacted]
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.1 — Type of animal procedure: Generation of new GMO (mice)

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The general design of the experiments in this appendix is shown in the attached flowchart (A).

Generation of new mouse lines in general by injecting DNA/RNA into oocyte, injection of genetically modified ES cells into blastocysts and/or via the CRISPR/Cas9 system. The CRISPR/Cas9 system will especially be used as a highly efficient tool for simultaneously multi-gene editing. We will do zygote injections with Cas9 and sgRNA to create a genetic deletion or zygote injection with Cas9, sgRNA and DNA template to create knock in mice.

Welfare assessment according to the Consensus documents on genetically altered animals. New compound mouse models and new created transgenic lines and/or KO lines generated via classical methods and/or novel combinations of these aforementioned lines will be monitored for 2 generations to determine the absence or presence of a phenotype with constitutional discomfort. We will daily check the mice on several parameters like overall appearance, size, growth, coat conditions, behaviour and clinical signs.
For some transplantation experiments, immune-deficient or wild type mice are required as recipients. We breed our own wild type, immune-deficient, transgenic and knock out mouse lines. In some mouse lines we make use of the Cre/Loxp recombination system. For that we had to cross transgenic or knock out mouse lines, who are caring LoxP site in their genome, with a Cre or tamoxifen inducible Cre mouse line. We have 4-8 breeding pairs per mouse line that will be retain for a maximum of 6 months. The offspring will be used for experiments. Since our immune-deficient mice are housed under proper barrier conditions, they do not have a hampered phenotype.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Generation of new lines

1. Superovulation: Administration of gonadotropin's (2 times) in female mice by subcutaneous or intra-peritoneal injections followed by mating. Females will be killed for the isolation of early embryos.
2. Embryo recipients: Recipients for embryo transfer will be rendered pseudo-pregnant by mating with a sterile (vasectomized) male. Genetically modified embryos will be implanted surgically or non-surgically into the reproductive tract. Embryo recipients, not as part of an experiment, will be killed after weaning of the pups at three weeks of age.
3. Weaned pups at 3 weeks of age: Tissue sampling for genotyping and/or identification via tail or ear cut, respectively, under anesthesia (in general with isoflurane).

Welfare assessment
We will daily check the mice on several parameters (overall appearance, size, growth, coat conditions, behaviour, clinical signs, relative size and numbers) as has been described in the Directive 2010/63/EU: corrigendum of 24 Jan. 2013.

Breeding wild type, immune-deficient, transgenic and knock out mouse lines
Mice will be housed under normal conditions with free access to food and water. Start breeding with a minimum of 8 weeks old mice. Breeding will be retaining for a maximum of 6 months. Depending on the amount of experimental animals, 4-8 breeding pairs per genotype will be needed.

Killing animals
In case of discomfort or surplus, animals will be euthanized by O2/CO2 method or by isoflurane and confirmed by cervical dislocation.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

Statistical analysis does not play a role for these types of experiments. We will use state of art techniques. All techniques are proven to be effective in generating genetic modified mice with a minimum number of mice possible.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Animal
Mus musculus; Wild type, GMO
Genetic background: C57Bl6
Age: embryo, newborn, adult
Origin: All vasectomized males, which will be obtained from a registered commercial company, all other mice are obtained from our own Institute, an establishment licensed breeder by the NVWA, or from a registered commercial company.

Generation of genetically modified mice
We expect to generate a maximum of 20 new mouse lines over the next 5 years (corresponding to the 20 candidate HSC regulatory genes identified, see Proposal). For the creation of new mouse line, we will use on average max 150 mice (according to the 'besluit biotechnologie'). Therefore, a total of max:
→ 150 (mice) x 20 (mouse lines) = **3000 mice** is requested.

Welfare assessment: a maximum number of 5120 mice will be used for welfare assessment (not part of the license project).

We do not expect hampered phenotype in our new GM lines. All new lines will be monitored for a hampered phenotype and the outcome of the monitoring will be reported to the IvD. We intend no breeding with mice showing a hampered phenotype. If we observe mice with a hampered phenotype during the monitoring phase, we will sacrifice these animals immediately.
The offspring of these breeding pairs will be used in appendix 3.4.4.2 and 3.4.4.3.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☒ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Before considering the generation of a new (compound) GM mouse line, we will first extensively analyse data from previous studies [redacted] to determine whether our research hypothesis is valid. Only if the embryo/tissue analysis and/or in vitro experiments are insufficient to completely address the research question/hypothesis, we will consider the generation of a novel GM mouse line.
We make extensive use of in vitro experiments where possible, which extensively reduces the animal numbers. The use of in vitro cultures/assays allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the mice numbers to be used to a minimum. Indeed, our in vitro data, consisting of flow cytometry analysis and in situ hybridization experiments, have permitted to tremendously reduce our long list of potent HSC regulators to 20 (see Preliminary data in part 3.1)).
Based on these data, available literature and interactions with other scientists, we will now study the functional role of these genes during endothelial specialization, endothelial into hematopoietic transition, pre-HSC maturation, HSC survival and/or expansion or involved in the supportive surrounding microenvironment. The functionality of these gene scan only be performed in vivo (in transplantation assays).
Animal studies are unavoidable if we seek comprehensive knowledge and understanding of molecular and cellular mechanisms of HSC production.
The CRISPR/Cas9 system allows us, if required, to genetically modify up to 5 different genes at the same time. This strongly reduce the number of mice used for the generation and/or breeding of these compound mice.
Since we are dealing with complex systems where all different cell types contribute to the outcome, it is not possible to appropriately study HSC biology without using animals.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects; all mice will be housed under strict D1 conditions.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

If required, adequate anesthesia and analgesia will be used.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

We do not expect to find other adverse effect. This is the direct result of how we create our constructs for the generation of GM mice

Explain why these effects may emerge.

n.a.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Daily monitoring of the mice combined with an experimental design aiming at reducing the discomfort of the animals.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, and signs of general sickness and/or discomfort.

Indicate the likely incidence.

We do not expect a hampered phenotype.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

3,000 mice, Moderate 100%; caused by the induction of superovulation and mating with a relative large male.

The true discomfort will be assessed for each individual experiment.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

Animals are killed when no longer needed anymore (surplus or fosters) or for analysis (to collect cells or tissues for *ex vivo* analysis, the animals will then be used under Animal Procedure 3.4.4.2.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
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- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80100 - KNAW
1.2	Provide the name of the licenced establishment.	[redacted]
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.2 Type of animal procedure: Donor – Ex vivo analysis (mice, zebrafish)

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The general design of the experiments in this appendix is shown in the attached flowchart (B).

To identify, localize and test hemogenic endothelial cells or precursors, HSC precursors (pre-HSCs) and HSCs, these cells will be isolated from WT or GMO. Cells and tissues will be collected from embryos, neonates and adults and tested in ex vivo assays and in transplantations (Flowchart B [mouse] and C [zebrafish]).

*** Animal models (mouse, zebrafish)**
There are several considerations to choose for a specific animal type:
- WT. They will be used to provide WT tissues or cells for ex vivo analysis (control) and to provide donor cells for transplantation experiments (see Appendix 3.4.4.3 [mouse] and 3.4.4.4 [zebrafish]).

- GMO will be used to provide donor tissues or cells for ex vivo analysis and to provide donor cells for transplantation experiments (see Appendix 3.4.4.3 [mouse] and 3.4.4.4 [zebrafish]).
GMO will be used to study the HSC function of a specific gene or type of (stem) cell. Several transgenic or mutant strains will be used. They are needed for several reasons: (1) the presence of a genetic marker is needed to trace the cells and their progenitors after transplantation; (2) to study a specific marker; (3) to study the influence of a gene (known to be important for hematopoiesis) upon the biology of (stem) cells.

In some cases, transgene inducing or deleting agents (e.g. tamoxifen) are administered to induce the expression of the fluorescent marker in a specific cell type/lineage or to change the expression of the functional gene (transgene knockout). The hematopoietic tissues and cells of the GMO will be collected (see Flowchart B1 and C1) and analysed ex vivo (see Flowchart B2 and C2). We will also use endogenous

and exogenous promotors that are tissue or cell specific to drive expression of genes. The presence of e.g. a fluorescent marker in a (putative) stem cell allows us to localize these cells by confocal imaging. The introduction of e.g. a toxin receptor (e.g., Diphtheria toxin receptor) in the cells allows us to specifically kill these cells upon the administration of the toxin (e.g. Diphtheria toxin). It allows us to determine the consequence of the loss of the toxin receptor expressing cells during development and for tissue homeostasis.

If a required GMO is not available, we will obtain this model by generating, importing or by crossing existing models (see Appendix 3.4.4.1).

The choice of animals will in all cases be based on the combination of the following considerations:
- Aim/ specific question (e.g. Identification of pre-HSCs, of HSCs, of hemogenic endothelial cells or precursors, of a cell type specific expression of a fluorescent marker for an immune-histochemical study)
- Aim/readout parameters (e.g. ex vivo assay)

*** Interventions:**
- Donor cells and tissues will be collected from embryos, neonates and adults (see Flowchart B and C).
- With the administration of small molecule compounds/drugs/chemicals/toxins, we might be able to rescue or mimic the phenotypes of the in vivo genetic deletion(s) and/or activation(s) and therefore further identify the function of these cells. The animals might be injected with DNA labelling agents shortly before euthanasia to measure the proliferation capacity of the stem cells and their derivatives ex vivo.

*** Readout parameters /endpoint:**
- To study the molecular and/or cellular mechanisms of HSC production, we need to analyse tissues and cells ex vivo. Cells and hematopoietic tissues will be collected from embryos, newborns and adults (WT and GMO).

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

- Animals are ordered from a commercial NVMA licensed breeder or from a registered commercial company, or are generated by us under appendix 3.4.4.1.
- Animals will be used as donor of tissues/cells for ex vivo studies or as donor for subsequent transplantations

*** Mouse model:**
- Donor pregnant females used to collect donor tissues/cells from embryos (see Flowchart B and B1). Pregnant female will be used to generate the donor embryos. The pregnant females will be euthanized by CO2/O2 followed by CO2 inhalation. After disinfection of the fur with 70% ethanol, a two-sided incision will be made in the fur and skin in the abdominal area. Using sterile forceps, the uterus will be pulled out and place in a culture dish-containing medium. The embryos and extra-embryonic tissues (allantois and yolk sac) will be isolated. Embryos from different ages (E8, E9, E10, E11, E12, E14 and E18) will be used to test the presence of pre-HSCs and HSCs in intra- and extra-embryonic tissues.

- Newborns and adult mice used as donors (see Flowchart B and B1)
Newborns and adult mice will be used to collect hematopoietic cells and tissues to test the presence of pre-HSCs and HSCs.

In some cases, we will perform the administration:
- transgene inducing or deleting agents or control substances, alone or in combination, continuously or intermittently by one or more of the following routes:
  a) in diet or drinking water (maximally 1 time, < 2 wks)
  b) subcutaneous (in general 2 times but maximally 3 times)
  c) intraperitoneal (in general 4 times but maximally 7 times)
  d) implantation of a slow release pellet subcutaneously under adequate anesthesia and analgesia (maximally 1 time)
  e) implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia (maximally 1 time)
  f) oral (in general 5 times but maximally 10 times)

- small molecule compounds, drugs, toxin, chemicals or control substances, alone or in combination, continuously or intermittently by one or more of the following routes:
  a) in diet or drinking water (maximally 1 time, < 2 wks)
  b) subcutaneous (in general 5 times but maximally 10 times)
  c) intraperitoneal (in general 5 times but maximally 10 times)
  d) implantation of a slow release pellet subcutaneously under adequate anaesthesia and analgesia (maximally 1 time)
  e) implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia (maximally 1 time)
  f) oral (in general 5 times but maximally 10 times)

- labeling agent (e.g. BrdU) via one of the following routes:
  a) intraperitoneal (maximally 1 time but maximally 3 times)
  b) implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia (maximally 1 time)
  c) intravenous (maximally 1 time)

None of the presented administration procedures will result in higher than cumulative mild discomfort.

Readout parameters /endpoint (see Flowchart B2)
The pregnant females will be kept under observation until delivery of the newborns. After birth and short weaning of the newborns (< 5 days), the mother will be euthanized by CO2/O2 followed by CO2 inhalation. Embryos and neonates (< 5 days) will be put on melting ice water for 10 min (but not in contact with) after which they will be decapitated and the head immediately frozen. The adult mice are sacrificed by CO2/O2 followed by CO2 inhalation or via cervical dislocation. Embryos and embryonic tissues (yolk sac, allantois), and neonate and adult tissues (e.g. blood, bone marrow, spleen, thymus, lymph nodes) will be collected for ex vivo analysis (e.g. in situ hybridization, flow cytometry analysis, molecular biological analysis (PCR), histological analysis to look at aspects of cell size, survival, proliferation and differentiation).

* Zebrafish model:
All donor tissues and cells will be collected before 5 dpf and, therefore, these zebrafish are not part of the license.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

The animal numbers are mainly based on our preliminary experience. We know that the analysis of the requested number of animal per experiment guarantees us a proper analysis of these animals. We have tried to reduce the number of animals per experiments to a minimum. Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$.
Qualitative analysis (most of the animal procedures described in this appendix): the number of animals (group size) is based in literature and/or years of experience with similar type of experiments. Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of animals per group that will be informative. The very low number of cells available from early embryonic tissues (embryo proper, allantois or yolk sac) implicates the use of a higher number of embryos and therefore of pregnant female mice. All mice will be euthanized to collect the embryonic tissues and therefore will only be used once. The number of pregnant female mice (carrying donor embryos) needed has also been calculated according to the average number of embryos per litter (6 embryos/litter), to the number of embryos needed to test the different tissues at different embryonic stages, and the number of cells that will be available for analyses.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

All animals will be used to provide tissues/cells for ex vivo analysis and to provide donor cells for transplantation experiments (see Appendix 3.4.4.3 [mouse] and 3.4.4.4 [zebrafish]).
To note, the number of experiments needed is based on your experience over the last years and my expectation that my workgroup has a rather stable number of experienced researchers involved.

1- Mus musculus, (WT, GMO)
Genetic background: C57Bl6
Age; embryos, newborns, adults
Origin: Mice are obtained from our own Institute or from external licensed breeders.
Number of animals (Total in 3.4.4.2): max. 3051

For the majority of the proposed studies, the mouse (beside the zebrafish) is one of the most appropriate animal model to use because: (1) like humans, mice are mammals; (2) physiology is more extensively characterized; (3) mice are amenable to transgenic modification; (4) a large number of relevant transgenic and knock out lines are already available.

- Donor pregnant females used to *collect donor tissues/cells from embryos and neonates*
Donor pregnant females are needed:
(i) to generate embryo, yolk sac and allantois.
Based on our previous experience, there is an average of 6 embryos/litter.
Seven embryonic stages (E8, E9, E10, E11, E12, E14 and E18) will be tested because they correspond to a specific time points of hematopoietic (stem) cell specification, emergence, maturation, colonization and expansion. Pluggings will be performed with wild type and GMO. The experiments will be performed three times for each assay to be scientifically reliable.
The cell populations of putative precursors in the embryo proper, allantois and yolk sac are very small at early time points of development. Thus, many embryos will be needed to be able to perform *ex vivo* analysis. Moreover, only up to 50% of the pups in the litters will carry a donor marker (e.g. fluorescent marker) and thus an average of 3 pups will be used per litter, with 5 litters needed per experiment (for a total of 3 experiments). The mothers will be killed for each experiment.
Therefore we need:
→ 7 (embryonic stages) x 5 (females/experiments) x 21 (1 WT and 20 GMO) x 3 (experiments) = **2205 mice**.

- Newborns used to *collect donor tissues/cells*
To determine the presence of Pre-HSCs in any given sample (Boisset *et al*. Blood, 2015), an average of 12 newborns will be used per experiments.
Therefore we need:
→ 120 (pregnant females – see above) x 6 (neonates) = **720 newborns**.

- Adults used to *collect donor tissues/cells*
Different hematopoietic tissues will be collected from WT and GMO for ex vivo analysis. An average of two mice will be needed per experiment to collect enough cells and tissues. The experiments will be performed three times for each assay to be scientifically reliable.
Therefore we need:
→ 2 (mice) x 21 (1 WT and 20 GMO) x 3 (experiments) = **126 mice**.

2- Zebrafish (WT, GMO):
All donor tissues and cells will be collected before 5dpf and therefore these zebrafish are not part of the license.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☒ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Animal studies are unavoidable if we seek for the fundamental insight into the molecular and cellular mechanisms that drive HSC production and regulation.
We make extensive use of *in vitro* experiments where possible, which extensively reduces the animal numbers. The use of *in vitro* cultures/assays allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the mice numbers to be used to a minimum. Indeed, our *in vitro* data, consisting of flow cytometry analysis and *in situ* hybridization experiments, have permitted to tremendously reduce our long list of potent HSC regulators to 20 (see Preliminary data in part 3.1)).
Based on these data, available literature and interactions with other scientists, we will now study the functional role of these genes during endothelial specialization, endothelial into hematopoietic transition, pre-HSC maturation, HSC survival and/or expansion or involved in the supportive surrounding microenvironment. The functionality of these gene scan only be performed in vivo (in transplantation assays).
Whenever possible, we will perform pilot studies with the minimum number of animals possible.
Where possible, mice with inducible alleles will be used, so mice should not display a phenotype before the induction of the alleles.
Experiments will be done sequential, where on basis of the results, decisions will be made on the next steps.
If homozygous mice do not show any discomfort, we will keep the mice on a homozygous background, thereby reducing the number of mice.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

- Anaesthesia will be administered (by inhalation) to mice in case of blood collection (in general: Isofluraan (4%)).

- Analgesia will be administered to mice in case of pain (in general: Rimadyl)

- Parameters for health monitoring: Mice will be kept as groups. Food and drinking water will be provided ad libitum. Animals will be monitored for health status (welzijnsdagboek) by the animal caretakers. Animals with a decreased health status (such as weight loss, bristly fur, bent back) will be checked by the researcher as well. In case of 15% loss of body weight within 2 days, the mice will be euthanized. In case of 20% loss of body weight compared to the littermates, the mice will be euthanized.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

Other than in terminally anaesthetized animals dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that will result in no more than transient, mild discomfort. In less than 3% of all experiments, pumps/pellets will be implanted which leads to a moderate discomfort.
Due to administration of inducing agents or other substances, animals will be experiencing no follow up effects.
The scientific endpoints of all studies are much earlier than the humane endpoints.
It is not expected that the activation or inactivation of genes will lead to discomfort. However, it is impossible to predict the nature or severity of any potential genetic defect. So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day and analysed.

Explain why these effects may emerge.

Mice might have sickness and/or disorientation signs after waking up from anesthesia (after blood collection) and have some pain due to the surgery. Analgesia will be administered accordingly.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Monitoring of the animal combined with an experimental design aiming at reducing the discomfort of the animals. If we unexpectedly observed weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort animals will be taken out of experiment and sacrificed.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behaviour or body posture, and signs of general sickness and/or discomfort.

Indicate the likely incidence.

Expected <5%, mild <1day.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Pregnant females; n = 2205; Mild
Newborns; n = 720; Mild
Adult mice; n = 126; Mild
Total = 2331 adult mice and 720 newborns

- Waking up from anaesthesia (after blood collection): Mice might have sickness and/or disorientation signs. Mild, < 1 day, expected 100%.

- Sacrifice: Mild, < 10 minutes, 100% animals

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

The animals need to be killed to collect embryos, cells or tissues for *ex vivo* analysis and to provide donor cells for transplantation experiments.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80100 - KNAW
1.2	Provide the name of the licenced establishment.	[redacted]
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.3 Type of animal procedure: Recipient – *Ex vivo* analysis (Transplantation in pregnant females, newborns and adult mice)

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The general design of the experiments in this appendix is shown in the attached flowchart (B3 and B4).

To identify and test hemogenic endothelial cells or precursors, HSC precursors (pre-HSCs) and HSCs isolated from WT or GMO (see Appendix 3.4.4.2), we need to perform in vivo transplantation assays in embryos in pregnant females (1), newborns (2) and adults (3), respectively (Flowchart B3). The different ages of the recipients provide different permissive microenvironment and allow the engraftment of various cell types (from the embryo to adult age). Hematopoietic tissues and cells of all transplanted recipients (embryos, neonates, adults) will be analysed ex vivo at 4 months post-transplantation (Flowchart B4).

(1) To determine the anatomical origin of hemogenic endothelial cells or precursors, cells (isolated from donor embryos, see Appendix 3.4.4.2) will be tested by performing transplantations in embryo recipients directly in pregnant recipient females (E7-E10). The recipient embryos will be analysed when they reach the adult stage for long-term donor multi-lineage hematopoietic reconstitution.
(2) To identify pre-HSCs, isolated candidate donor cells (see Appendix 3.4.4.2) are transplanted in newborns or immuno-deficient recipient mice (a more permissive environment for immature cells).
(3) To identify HSCs, isolated candidate donor cells (see Appendix 3.4.4.2) are transplanted in adult recipient mice.
In all cases, the primary recipients are analysed up to 4 months post-transplantation (to prove the multi-lineage property of the donor cells). Secondary transplantations are performed to prove the self-renewal property of donor cells. These secondary recipients are also analysed 4 months post-transplantation.

*** Mouse model**

There are several considerations to choose for a specific mouse model. WT and GMO mice will be used as recipients.

- WT and GMO:

GMO will be used to study the HSC function of a specific gene or type of (stem) cell. Several transgenic or mutant mouse strains will be used. They are needed for several reasons: (1) the presence of a genetic marker is needed to trace the cells and their progenitors after transplantation; (2) to study a specific marker; (3) to study the influence of a gene (known to be important for hematopoiesis) upon the biology of (stem) cells.

In some cases, transgene inducing or deleting agents (e.g. tamoxifen) are administered to induce the expression of the fluorescent marker in a specific cell type/lineage or to change the expression of the functional gene (transgene knockout). We will also use endogenous and exogenous promotors that are tissue or cell specific to drive expression of genes. The presence of e.g. a fluorescent marker in a (putative) stem cell allows us to localize these cells by confocal imaging. The introduction of e.g. a toxin receptor (e.g., Diphtheria toxin receptor) in the cells allows us to specifically kill these cells upon the administration of the toxin (e.g. Diphtheria toxin). It allows us to determine the consequence of the loss of the toxin receptor expressing cells during development and for tissue homeostasis.

To identify/validate for example which molecular and cellular processes are important for HSC production and regulation, we will analyse GMO in which cells/tissues are transplanted and the gene(s) will be activated, inactivated, overexpressed and/or misexpressed. These mice will be used as recipients (see Flowchart B3). We will use inducible systems including e.g. the well-known Cre-LoxP system in which a floxed gene can be deleted, overexpressed or misexpressed upon the induction of the Cre enzyme via the administration of tamoxifen. In particular, when Cre is expressed in mice harbouring a LoxP-containing target gene, the desired gene modification can be restricted to certain developmental stage, organ, cell type (e.g. stem cell) of the mouse depending on the specificity and timing of recombinase expression. In this way the desired gene modification can be restricted to certain developmental stage, organ, cell type (e.g. stem cell). Successful deletion/activation via (e.g.) Cre enzyme induction might be monitored via expression of a reporter gene (e.g. LacZ or fluorescent protein(s)).

If a required genetically modified mouse is not available, we will obtain this model by generating, importing or by crossing existing models (see Appendix 3.4.4.1). WT recipients serve as control.

- Immune-deficient or newborn recipients. Immune-deficient mice are required in some cases as recipients since they are more permissive to the engraftment of very immature cells.

The choice will in all cases be based on the combination of the following considerations:

- Aim/ specific question (e.g. Identification of pre-HSCs, of HSCs, of hemogenic endothelial cells or precursors, of a cell type specific expression of a fluorescent marker for an immune-histochemical study)
- Aim/readout parameters (e.g. to test long-term hematopoietic repopulation)

**** Interventions:***

- Cells will be transplanted intra-cardiac in embryo recipients, intra-liver in newborn recipients and intravenously in adult recipients (see Flowchart B3). Newborns and adult recipients will be irradiated. The irradiation is performed to clear out the bone marrow and liver from all proliferating cells and therefore to make space. It allows the engraftment of the injected donor cells.
- To identify/validate for example which molecular and cellular processes are important for HSC production and regulation, we will analyze wild type mice or GMO (in which functional gene(s) will be activated, inactivated, overexpressed and/or misexpressed) after transplantation. The presence of e.g. a fluorescent marker in transplanted cells allows us to visualize these cells and their progeny.
- With the administration of small molecule compounds/drugs/chemicals/toxins, we might be able to rescue or mimic the phenotypes of the *in vivo* genetic deletion(s) and/or activation(s) and therefore further identify the function of these cells *in vivo*. The mice might be injected with DNA labeling agents shortly before euthanasia to measure the proliferation capacity of the stem cells and their derivatives.

**** Readout parameters /endpoint:***

To study the molecular and/or cellular mechanisms of HSC production, we need to analyze tissues and cells *ex vivo*. Cells and hematopoietic tissues will be collected from up to 4 months after transplantation (see Flowchart B4).

In some cases, we will need to collect cells with a certain genetic constitution to transplant back into wild type or GMO (secondary transplantation).

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Mice are ordered from a commercial NVMA licensed breeder or from a registered commercial company, or are generated by us under appendix 3.4.4.1.

- Recipient pregnant females used for the in-uterus transplantation in E7-E10 embryos (see Flowchart B3)

Pregnant females will be used to generate recipient embryos (immune-deficient or wild type). Between E7 to E10 post-coitus, the pregnant females will be anaesthetized in a knock down box and then maintained by facemask with 2-3% Isoflurane. Hairs will be removed on the abdomen using a depilatory cream and then the abdomen will be rinsed with water. The female will be positioned on a platform ventral side up. Eye lubricant will be placed on each eye to prevent drying. A small amount of ECG gel will be placed on the copper leads on the platforms and the paws taped on them. This will provide the ECG and respiratory physiology of the mouse. A rectal probe will be inserted to monitor the temperature of the animal during the imaging session and will be maintained at 35-38ºC using a heating pad and a lamp. The stage upon which the mouse is placed is tilted head down (between 35–45°) to displace the bowel. Maternal well-being will be carefully monitored to maintain an appropriate body temperature, heart rate, respiratory vigour, and anaesthetic depth throughout the procedure. After sterilizing the skin with 70% ethanol, the abdomen will be opened with a 2 cm vertical midline incision along the linea alba (midline avascular region). A short segment of the uterine horns containing 1-3 embryos will be gently exteriorized from the abdominal cavity by using sterile forceps. With an injection needle, a small hole will be made in the uterus. Through this hole, the cells will be injected into the heart cavity of the embryo by using a glass transfer needle in combination with a micro-injector (guided by a high resolution ultrasound apparatus). This will allow the injection of very small volumes of cells (50 nL) while causing little damage to the tissue. The embryo is positioned in such a way that the heart is visible with the high-resolution ultrasound detector. Special care will be made for the tissues through which the needle will pass through before hitting the target of interest (the heart), especially by avoiding passing through the placenta (that might compromised the embryo development or generate bleeding). All embryos from the litter will be injected. After injection, the uterus will be guided back into the abdomen, muscle layer will be stitched and the skin will be closed with staples by using a wound clamp. The whole procedure will take about 20-30 minutes. To prevent cooling down of the recovery mouse, the cage will be put on a heating plate.

- Newborns and adult mice used as recipients (see Flowchart B3)

Transplantation of cells will be performed under adequate anaesthesia and analgesia.

→ Transplantation in **newborns**

* Wild-type or immuno-deficient newborns (1 to 5 days old) will be used. WT newborns will be treated to compromise their hematopoietic/immune system (by irradiation of the newborns [using a gamma-source] or by busulfan treatment of the pregnant females [to compromise the hematopoietic/immune system of the litter while still inside the uterus of the mother]). For irradiation, neonates will receive a single dose of 5 Gy. During irradiation, mice will be kept as a group in a cage without anaesthesia.

* Donor cells are injected in the livers of the newborns (max. 0.05 ml).

* Mice will receive antibiotics (such as neomycin) in the drinking water for the first 3 months post transplantation. The animal being irradiated, they are more subject to bacterial infections (until their immune system is restored. The antibiotics will prevent such possible infection.

→ Transplantation in **adults** (primary and secondary transplantation)

* Adult wild-type mice (>8 weeks) will be treated to eliminate their hematopoietic/immune system by irradiation using a gamma-source or 5-FU treatment. For irradiation, adults will receive a sub-lethal dose as a split dose (4 and 5 Gy with 3 hours in between). During irradiation, mice will be kept as a group in a cage without anaesthesia.

* Donor cells are injected intravenously (max. 0.2 ml) in the tail vein.

* Mice will receive antibiotics (such as neomycin) in the drinking water for the first 3 months

post transplantation to prevent bacterial infections.
Cells of reconstituted recipients will serve as donor cells for secondary transplantations (to test the self-renewal of the cells, second specific feature of a HSC). The adult secondary recipients will receive a sub-lethal dose of irradiation as a split dose (4 and 5 Gy with 3 hours in between), as described above.

All transplanted animals will be kept under observation and analysed at adult stage when they are older than 8 weeks old (see Flowchart B4).

Summary of the transplantation procedures:

Assay	**To test/To identify**	**Donor cells** *(see Appendix 3.4.4.2)*	**Recipient**
In uterus transplantation	Hemogenic endothelial cells (or precursors)	E8 embryo, yolk sac, allantois	Embryo (E7-E10)
Newborn transplantation	Pre-HSCs	Embryonic tissues (E8-E10)	Newborn (P1-P5)
Adult transplantation	HSCs	Embryonic tissues (E10-E12, E14 and E18)	Adult mice (> 8 weeks)

In some cases, we will perform the administration of:
- transgene inducing or deleting agents or control substances, alone or in combination, continuously or intermittently by one or more of the following routes:
  a) in diet or drinking water (maximally 1 time, < 2 wks)
  b) subcutaneous (in general 2 times but maximally 3 times)
  c) intraperitoneal(in general 4 times but maximally 7 times)
  d) implantation of a slow release pellet subcutaneously under adequate anesthesia and analgesia (maximally 1 time)
  e) implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia (maximally 1 time)
  f) oral (5 times but maximally 10 times)

- small molecule compounds, drugs, toxin, chemicals or control substances, alone or in combination, continuously or intermittently by one or more of the following routes:
  a) in diet or drinking water (maximally 1 time,, <2 wks)
  b) subcutaneous (in general 5 times but maximally 10 time)
  c) intraperitoneal (in general 5 times but maximally 10 times)
  d) implantation of a slow release pellet subcutaneously under adequate anaesthesia and analgesia (maximally 1 time)
  e) implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia (maximally 1 time)
  f) oral (in general 5 times but maximally 10 times)

- labeling agent (e.g. BrdU) via one of the following routes:
  a) intraperitoneal (in general 1 time maximally 1 times)
  b) implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia (maximally 1 time)
  c) intravenous (maximally 1 time)

None of the presented administration procedures will result in higher than cumulative mild discomfort.

Readout parameters /endpoint (see Flowchart B4)
After transplantation, the recipient animals will be kept under observation. In the case of transplantation in embryos, the pregnant females will be kept under observation until delivery of the pups. The pups will be checked at birth for viability. Pups will be kept (males and females separated) and analyzed at adult stage.
Blood (maximum volume at the different time points: 300 µl) will be collected at 1 or 2 time points: 1-2

months and/or 4-6 months post transplantation to determine the donor cell contribution in the peripheral blood. At the end of the experiment (4-6 months post transplantation), all mice are sacrificed by CO2/O2 followed by CO2 inhalation or via cervical dislocation, and tissues/cells are collected for ex vivo analysis. The long-term multi-lineage hematopoietic reconstitution will be tested by flow cytometry analysis or by molecular biological analysis (PCR on GFP). For this, cells or DNA will be isolated from the different adult mouse hematopoietic tissues (blood, bone marrow, spleen, thymus, lymph nodes). Tissue will be collected for histological and molecular analysis to look at aspects of cell size, survival, proliferation and differentiation. In some cases, cells will be collected to perform secondary transplantations.
Embryos and newborns (< 5 days) will be put on melting ice water for 10 min. (but not in contact with) after which they will be decapitated and the head immediately frozen.
After birth and weaning of the pups (that are used as embryo recipients), the mother will be euthanized by CO2/O2 followed by CO2 inhalation. For post-surgery pain relief, when anaesthetised, a s.c. dose of Rimadyl will be given in general. One injection will be made once pre-operatively and repeated once 24 hours after surgery. We have expertise in all the surgical procedure steps needed for the experiments.

The cumulative discomfort in the genetic models will maximally consist of discomfort due to:
- the genetic modification (< 5% of lines)
- transplantation of cells under adequate anaesthesia and analgesia
- sacrifice

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

The mice numbers are mainly based on our preliminary experience. We know that the analysis of the requested number of mice per experiment guarantees us a proper analysis of these mice. We have tried to reduce the number of animals per experiments to a minimum. Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$.
Qualitative analysis (most of the animal procedures described in this appendix): the number of animals (group size) is based in literature and/or years of experience with similar type of experiments.
Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be informative. The very low number of cells available from E8 tissues (embryo proper, allantois and yolk sac) implicates the use of a higher number of embryos and therefore of pregnant females. All mice will be euthanized to collect the embryonic tissues and therefore will only be used once. The number of pregnant females (carrying recipient embryos) needed has also been calculated according to the average number of embryos per litter (6 embryos/litter), to the number of embryos needed to test the different tissues at E8, and the number of cells that will be available for analyses (very low at E8).

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mus musculus, (WT, genetically modified, mutants, immuno-deficient mice)
Genetic background: C57Bl6
Age; embryos, newborns, adults
Origin: Mice are obtained from our own Institute or from external licensed breeders.

Number of animals (Total): max. 5964

Up to today, there are no alternative methods than in vivo transplantation to fully study HSC production and regulation in the context of the whole organism. For the majority of the proposed studies, the mouse is one of the most appropriate animal model because: (1) like humans, mice are mammals; (2) physiology is more extensively characterized; (3) mice are amenable to transgenic modification; (4) a large number of relevant transgenic and knock out lines are already available.
To note, the number of experiments needed is based on your experience over the last years and my

expectation that my workgroup has a rather stable number of experienced researchers involved.

A) In utero transplantation in embryos in pregnant females

- Pregnant females used to generate E7-E10 recipient embryos

The recipient females give an average litter size of 6. We need a minimum of 12 pups (2 litters) injected for a valid and reliable conclusion. Pups will be injected with 3 different cell populations from E8 allantois, yolk sac and embryo proper (= 3 kind of tissues). All embryos from the litter will be injected by the same cell type (since embryos cannot be marked at that stage to be discriminated at birth). The pregnant mice will be injected at E7, E8, E9 and E10) because the stage that will provide the best engraftment is unknown yet. Because the intra-cardiac injection in embryos is difficult, we know by experience that an average of 10 experiments are needed per tissue tested to achieve a reliable (and therefore publishable) data.
Therefore we need:
→ 4 (embryonic stages) x 2 (females/experiments) x 3 (kind of tissues) x 10[#] (experiments) = **240 transplanted pregnant mice**.
[#] Intra-cardiac injection in developing embryos is very difficult to achieve. Sometimes (i) the needle misses the heart and cells are not injected properly, (ii) the embryo does not survive to the procedure or (iii) there is a blockage in the needle and all cells cannot be injected. For these reasons, we estimated that 10 experiments are needed for a reliable conclusion (in comparison to all other experiments [that are technically easier and 100% successful in our hands] where 3 experiments are needed for statistical analysis).

- Growing transplanted embryo recipients (that will be analysed at adult age)

The amount of transplanted embryo recipients to analyse for long-term multi-lineage reconstitution at adult age is:
→ 240 (transplanted pregnant females – see above) x 6 (embryos/litter) = **1440 adults transplanted when at embryonal stage**.

B) Transplantation in newborns (pre-HSC assay)

- Newborn recipients for transplantation:

To determine the presence of Pre-HSCs in any given sample, we need to do transplantations in newborn recipients (Boisset *et al*. Blood, 2015).
We need a minimum of 12 newborns injected for a valid and reliable conclusion. Newborns will be injected with cells isolated at 3 different time points (E8, E9 and E10; 3 time points when pre-HSCs are detectable; isolated under AP 3.4.4.2). The experiments will be performed three times for each assay to be scientifically reliable with WT and GMO lines.
Therefore we need:
→ 3 (embryonic stages) x 12 (newborns) x 21 (1 WT and 20 GMO) x 3 (experiments) = **2268 newborn recipients**.

C) Transplantation in adults (HSC assay)

- Adult recipients for transplantation:

To determine the presence of HSCs in any given sample, we need to do transplantations in adult recipients (Harrison et al. Experimental Hematology, 1993; Szilvassy et al. PNAS, 1990). Adults will be injected with donor cells isolated at 5 different time points (E10, E11, E12, E14 and E18; 5 time points when HSCs are detectable in different tissues; isolated under AP 3.4.4.2). Based on literature and our own experience we estimate the variation per group at 15% (= anticipated coefficient of variation). The difference that we need to be able to detect is 15% (= difference considered meaningful). To get a power of 0.8 with the aforementioned percentages, we will need a group size of 18 animals in total (Van Zutphen et al., Principles of laboratory animal science). For secondary transplantations (a requirement for the self-renewal feature of a HSC), we use 6 recipients in total (standard in the field).
Therefore we need:
→ 5 (embryonic stages) x 18 (6 mice x 3 experiments) x 21 (1 WT and 21 GMO) = **1890 adult**

recipients (1st Tx)
→ 6 (2 mice x 3 experiments) x 21 (1 WT and 20 GMO) = **126 adult recipients** (2nd Tx)

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☒ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Animal studies are unavoidable if we seek for the fundamental insight into the molecular and cellular mechanisms that drive HSC production and regulation.
We make extensive use of *in vitro* experiments where possible, which extensively reduces the animal numbers. The use of *in vitro* cultures/assays allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the mice numbers to be used to a minimum. Indeed, our *in vitro* data, consisting of flow cytometry analysis and *in situ* hybridization experiments, have permitted to tremendously reduce our long list of potent HSC regulators to 20 (see Preliminary data in part 3.1)).
Based on these data, available literature and interactions with other scientists, we will now study the functional role of these genes during endothelial specialization, endothelial into hematopoietic transition, pre-HSC maturation, HSC survival and/or expansion or involved in the supportive surrounding microenvironment. The functionality of these gene scan only be performed in vivo (in transplantation assays).
Whenever possible, we will perform pilot studies with the minimum number of animals possible.
Where possible, mice with inducible alleles will be used, so mice should not display a phenotype before the induction of the alleles.
Experiments will be done sequential, where on basis of the results, decisions will be made on the next steps.
If homozygous mice do not show any discomfort, we will keep the mice on a homozygous background, thereby reducing the number of mice.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

- Anaesthesia will be administered (by inhalation) in case of:
* blood collection (at 1 and/or 4 months post transplantation) – Isofluraan (4%), in general.
* to the pregnant females during all the intra-uterus transplantation procedure – Isofluraan (2-3%) , in general.

- Analgesia will be administered in case of pain (such as Rimadyl) to:
* the transplanted mice
* the pregnant females. One injection will be made once pre-operatively and repeated once 24 hours after surgery. The pregnant females will be checked regularly (until delivery) after in utero cell transplantation and also on the delivery day (and the following week). Pups should develop normally. If abnormality or discomfort is visible, the pups will be sacrificed by CO2/O2 followed by CO2 inhalation. Animals will be sacrificed (and tissues collected) if unexpected illness occurs (by CO2/O2 followed by CO2 inhalation).
- Parameters for health monitoring: Mice will be kept as groups. Food and antibiotic drinking water (such as neomycin) will be provided ad libitum. Animals will be monitored for health status (welzijnsdagboek) by the animal caretakers. Animals with a decreased health status (such as weight loss, bristly fur, bent back) will be checked by the researcher as well. In case of 15% loss of body weight within 2 days, the mice will be euthanized. In case of 20% loss of body weight compared to the littermates, the mice will be euthanized.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

Other than in terminally anaesthetized animals dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that will result in no more than transient, light mild discomfort. In less than 3% of all experiments, pumps/pellets will be implanted which leads to a mild discomfort.
Due to administration of inducing agents or other substances, animals will be experiencing no follow up effects.

The scientific endpoints of all studies are much earlier than the humane endpoints.

It is not expected that the activation or inactivation of genes will lead to discomfort. However, it is impossible to predict the nature or severity of any potential genetic defect. So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day and analysed.

Explain why these effects may emerge.

Mice might have sickness and/or disorientation signs after waking up from anesthesia and have some pain due to the surgery. Analgesia will be administrated accordingly.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Monitoring of the mice combined with an experimental design aiming at reducing the discomfort of the animals. If we unexpectedly observed weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort animals will be taken out of experiment and sacrificed.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behaviour or body posture, and signs of general sickness and/or discomfort.

Indicate the likely incidence.

Expected <5%, moderate <1day.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Recipient pregnant females; n = 240 mice; Moderate
Growing transplanted embryo recipients; n = 1440 mice; Mild
Newborn recipients; n = 2268 mice; Mild
Adult primary recipients; n = 1890 mice; Mild
Adult secondary recipients; n = 126 mice; Mild
Total = 5964 mice

- After irradiation: The animals might develop a decreased health status during the first month (loss of weight). Mild, 7-30 days, expected <10%.
- After transplantation in adult: Mice might be more sensitive to infections. Mild, 7-30 days, expected <10% (because of antibiotics in the drinking water).
- After in utero transplantation (because of the surgery): Moderate, 1 day, 100% animals
- Waking up from anaesthesia (after in utero transplantation, blood collection): Mice might have sickness and/or disorientation signs. Mild, < 1 day, expected 100%.
- Sacrifice: Mild, < 1 minute, 100% animals

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

The animals need to be killed to collect embryos, cells or tissues and for ex vivo analysis.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.
☒ Yes

Centrale Commissie Dierproeven

9.

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80100 - KNAW
1.2	Provide the name of the licenced establishment.	[redacted]
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.4 Type of animal procedure: Transplantation in Zebrafish, live imaging and ex vivo analysis.

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

Wild type or genetically modified zebrafish will be used to study HSC production and regulation during embryonic development and in adult.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

The zebrafish (as the mouse) is now firmly established as a reliable developmental and genetic model to study vertebrate hematopoiesis. To enable the study of HSC production and regulation in zebrafish, we will perform HSC manipulation into embryos (e.g. tamoxifen injection) and/or transplantation in adult recipient animals (hematopoietic mutant or wild type conditioned by gamma-irradiation (as described in Traver *et al.* Blood. 2004; Henninger et al. Nat. Cell Biol. 2016) (see Flowchart C3). HSC transplantation will allow to study cell autonomy of mutant gene function and to rescue multi-lineage hematopoiesis in embryonic lethal mutants. Transplant recipients are anesthetized in tricaine (in general) and injected intra-cardiac by using glass capillary needles.

The transplanted adult animals (>3 months old) are visualized weekly under an inverted fluorescent microscope to monitor donor fluorescent cells over the first 30 days after transplantation.
In some cases (analyze the function of specific genes), fish treated between 48-72 hours post fertilization with a tamoxifen injection will be grown to 5 months. They will be then subjected to split dose 30Gy irradiation and transplanted. This high dose of irradiation is provided to eliminate the hematopoietic cells of the fish recipient, which is necessary to allow the engraftment of the donor cells. The irradiation dose has been chosen based on the literature published by various laboratories

performing transplantation experiments in zebrafish.

At a chosen end point (depending of the research question, between 6 to 20 weeks post irradiation/transplantation), the animals will be anesthetized (in general) with 0.02% tricaine prior to collect the hematopoietic tissues for *ex vivo* analysis (e.g. histology, *in situ* hybridization, RNA/DNA isolation) (see Flowchart C4). Different *ex vivo* analysis techniques require different handling of the extracted tissue (e.g. cryo-sectioning or paraffin embedding). If possible the *ex vivo* techniques will be combined.

Some zebrafish embryo will also be used for time-lapse live confocal imaging. In our experience the imaging method described in (Renaud et *al.* (2011), Nature Protocols) is the most reliable method. Direct visualization of fluorescent donor cells in embryonic recipients allows engraftment and homing events to be imaged in real time. These results provide a cellular context in which to study the genetics of hematopoiesis. During imaging, embryos are anaesthetized (in general) with 0.02% tricaine in embryo buffer. The imaging will be done once for each zebrafish embryo for up to 24h (age of embryos: below 5 days post fertilization (dpf) and, therefore, are no part of the licence project).

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

- It is our experience (in our institute and in the literature) that in general 16 (transplanted) adult animals are needed to collect tissues/cells (Flow chart C4) and 30 embryos for live confocal imaging per condition. These numbers are based on common scientific practice in our research field to allow publication.
- Different ex vivo analysis techniques require different handling of the extracted tissue (e.g. cryo-sectioning or paraffin embedding). To reduce the number of animals that are required we will combine ex vivo assays for different parameters as much as possible.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Maximum of zebrafish: 4,992
To note, the number of experiments needed is based on your experience over the last years and my expectation that my workgroup has a rather stable number of experienced researchers involved.
- Zebrafish (Danio rerio) is one of the most well characterized fish. Zebrafish are well maintained and bred in a laboratory condition. Furthermore, the zebrafish model comes with numerous tools, which can be used to fully exploit its potential, including a fully annotated genome and efficient genome editing strategies.
- The zebrafish used here are bred in [redacted]
- To test HSC engraftment or to test the effect of a treatment or genetic condition on HSC production a maximum of 16 animals (8 controls and 8 treated) are required for each type of analysis. To test HSC engraftment, the animals will be analysed at 1 time point (adult). To test the effect of a treatment or genetic condition, it will be required to analyse up to 3 time points (time point of HSC emergence in the embryo aorta, time point of HSC colonization/expansion in the embryo CHT, time point of colonization of adult hematopoietic organs). In all cases, we will measure up to 4 different *ex vivo* parameters (e.g. gene expression, lineage tracing, cell proliferation, cell apoptosis,) requiring different fixation and embedding techniques (See Flowchart C4).
Experiments will be performed on embryos and adults. However, experiments on zebrafish < 5 dpf is not considered as an animal experiment. Therefore, only the experiments on adult animals are part of the license and only these animals are counted. Therefore, we will use:
16 animals (8 controls and 8 treated) x 1 time point (adult) x 4 (*ex vivo* parameters) x 3 (experiments) = **192 fish**

It is expected that during the duration of this project we will test 25 genetically modified or knockout lines. Tissues and cells will be collected on all animals. Therefore we will use:
16 animals (8 controls and 8 treated) x 1 time point (adult) x 4 (*ex vivo* parameters) x 25 (genetically modified or knockout lines) x 3 (experiments) = **4800 fish**

- Live confocal imaging will be performed on embryos (<5dpf and therefore no part of license project). It is our experience that 30 embryos per condition are needed to analyse EHT and HSC production. This embryo number is common scientific practice in the research field to achieve reliable and publishable results.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☒ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

- Replacement: HSC production needs to be studied *in vivo*. There are no *in vitro* models available to study HSC production. Zebrafish are easy to maintain in an animal facility and are a well-characterized model to study HSC production.
- Reduction: *Ex vivo* assays will be combined as much as possible to reduce the number of animals that are required. The experiments will be executed in a phased order so that follow up experiments are only executed when pilot experiments show a positive outcome.
- Refinement: Animals will be anaesthetized in general in Tricaine solution to reduce the discomfort of the treatment.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

In order to minimize the animal suffering, all procedures to reduce pain, fear or suffering will be used. There are no negative environmental effects. Procedures will only be performed by competent personnel.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The proposed procedures are for fundamental research. It does not consist of legally required research.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

The animals will be anaesthetized during transplantation with proper anaesthesia (in general with MS222 - Tricaine methanesulfonate, 0.02%). We do not expect stress and discomfort in animals during transplantation.
Anesthesia will also be performed (in general with 0.02% tricaine) before blood (obtained by cardiac punction), kidney and spleen collection.
Transplanted recipients will be anesthetized in general in tricaine and immobilised in individual conical wells made in 2% agarose. Cells will be injected into the sinus venosus of the embryo at 48-72hpf or in adults. Recipients will be maintained in medium containing penicillin and streptomycin during and for several hours after transplantation to prevent infection.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

No other adverse effects on the animals' welfare are expected.

Explain why these effects may emerge.

n.a.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

The animals will be checked daily for signs of general sickness and discomfort (e.g. laying on the bottom of the tank, rapid gill movements).

Indicate the likely incidence.

Expected in <1%; mild discomfort no longer than 1 day (due to the irradiation and transplantation).

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

n = 4,992 zebrafish; Mild
Animals reaching the humane end-point will be euthanized.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

Transplanted zebrafish will be killed to collect hematopoietic tissues/cells for ex vivo analysis.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☒ No > Describe the method of killing that will be used and provide justifications for this choice.

Zebrafish will be killed by submersion in ice-cold water for at least 10 minutes. This procedure is currently common practice and is part of the guidelines by the Animal Research Advisory Committee (ARAC) of the NIH.

☐ Yes

10.

Format DEC-advies

Maak bij de toepassing van dit format gebruik van de Praktische Handreiking: Ethisch Toetsingskader voor proefdiergebruik. Voor voorbeelden, zie bijlage I.

Herhaling van antwoorden is niet nodig. Indien van toepassing kan verwezen worden naar een bij een eerdere vraag verstrekt antwoord.

A. Algemene gegevens over de procedure

1. Aanvraagnummer: AVD80100 2017 1047

2. Titel van het project: The cellular and molecular basis of the hematopoietic production

3. Titel van de NTS: De cellulaire en moleculaire basis van de vorming van bloedvormende stamcellen

4. Type aanvraag:
   - ■ nieuwe aanvraag projectvergunning
   - □ wijziging van vergunning met nummer

5. Contactgegevens DEC:
   - naam DEC: KNAW
   - telefoonnummer contactpersoon: 020 5664427
   - e-mailadres contactpersoon: DECsecr@knaw.nl

6. Adviestraject (data dd-mm-jjjj):
   - ■ ontvangen door DEC: 12-04-2017
   - ■ aanvraag compleet: 28-04-2017
   - ■ in vergadering besproken: 20-04-2017
   - ■ anderszins behandeld: niet van toepassing
   - ■ termijnonderbreking(en) van 21-04-2017 tot 28-04-2017
   - ■ besluit van CCD tot verlenging van de totale adviestermijn met maximaal 15 werkdagen: niet van toepassing
   - ■ aanpassing aanvraag: finale herziene versie ontvangen op 28-04-2017
   - ■ advies aan CCD: 17-05-2017

7. Geef aan of de aanvraag is afgestemd met de IvD en deze de instemming heeft van de IvD.
   De IvD geeft aan dat de aanvrager de aanvraag met de IvD heeft afgestemd en dat de aanvraag de instemming heeft van de IvD.

Bij de punten 8 t/m 10 kan worden volstaan met 'n.v.t.' wanneer de betreffende acties niet aan de orde zijn geweest. Bij vragen die gericht zijn op het compleet maken van de aanvraag (aanvullingen achtergrond informatie etc) kan bij punten 8 en 9 worden volstaan met de vermelding van het type vragen en de vermelding dat de aanvraag op de desbetreffende onderdelen is aangepast of dat de antwoorden in de aanvraag zijn verwerkt. Bij vragen die gericht zijn op het verkrijgen van verklaringen voor keuzes die door de aanvrager gemaakt worden, kan niet worden volstaan met het weergeven van de strekking van de antwoorden tenzij de antwoorden volledig in de aanvraag zijn opgenomen. Als dat het geval is, moet dat in het DEC advies worden benoemd en in de aanvraag inzichtelijk worden gemaakt.

8. Eventueel horen van aanvrager:
   - Datum: 20-04-2017
   - Plaats: [redacted]
   - Aantal aanwezige DEC-leden: 7
   - Aanwezige (namens) aanvrager: de hoofdaanvrager
   - Gestelde vraag / vragen: de mondeling gestelde vragen zijn later tevens schriftelijk aan de aanvrager gestuurd en hebben geleid tot aanpassing van de aanvraag
   - Verstrekt(e) antwoord(en): zie onder vraag 9
     Het horen van de aanvrager heeft wel/niet geleid tot aanpassing van de aanvraag: wel; zie onder vraag 9

9. Correspondentie met de aanvrager
   - Datum: 21-04-2017; n.a.v. de bespreking van de eerste versie van de aanvraag
   - Gestelde vraag/vragen: De vragen zijn gericht op het verkrijgen van aanvullende informatie ter completering van de aanvraag.
     Proposal
     The section on preliminary data is somewhat difficult to understand; perhaps some of the more technical terms could be deleted or replaced by a somewhat more simplified description?
   - *In the background section a summary on the state-of-affairs within the international field on HSC research is lacking. What are other people working on and why is your approach unique?*
   - *There are 3 sub goals; the DEC advises to make clear in 3.4.3 of the Proposal what the strategy will be. Will you first address subgoal 1, followed by subgoal 2 etc. Or will you follow a more parallel strategy?*
   - *Concerning 3.3, the Relevance of the project. It seems to the DEC that the long-term perspective is somewhat wider than stated. Would the outcome of the project not also allow the use of induced pluripotent stem cells; this would avoid the procedure of harvesting stem cells from donors or from embryo's. The DEC challenges you to sketch the wider long-term potential relevance of the project but keeping in mind that it should be a realistic perspective.*
   - *The DEC understands that a list of interesting candidates has been generated (proposal page 4). The DEC would like to understand the reasoning to reach the decision to actually start studies on a particular target. Assuming the list includes several hundreds of genes; which one to select? Could you make the entry criteria more clear?*
   - *As discussed after the presentation; the figure in your presentation donor > transplantation > read out seems to be quite helpful to understand your strategy (Proposal 3.4.1). We suggest including this scheme/figure in the application.*
   - *The arguments to use three different species is that you want to identify conserved mechanisms since these are more likely represent the mechanisms also relevant for the human situation. This seems a valid reason but in your presentation you also mentioned that for publication most journals also require the use of multiple species. In addition, you also pointed out that the three species are used in a complementary manner; certain techniques are most suited for a particular species. As explained by you: chicken for tissue grafting, mice for TG studies and the zebrafish for longitudinal studies. The DEC wants you to incorporate these arguments in the application. Also address whether or not you will start out to address a specific question in all three species at the same time or whether you will select one of the species and later on include the other species for validation.*
   - *The DEC advises to discuss the use of human embryos as a possible alternative in the 3R's. You made clear that this material is difficult to get, that only a few of your studies could be performed and that there are ethical issues involved. The conclusion would be that this is not a reasonable alternative for the proposed animal experiments.*
   - *In the Proposal 3.4.1. it is stated that "for every experiment, we design the experiment with a clear go-no-go decisions …." However, this is not substantiated in the application. This could be included in 3.4.1 or under the description of the strategy coherence (Section 3.4.3).*
   - *A similar issue is noted with the statement in Proposal 3.4.1. "we will set up pilot studies…" . Again this is not substantiated and section 3.4.3 is most suited to explain this.*
   - *Related to the above; under the three R's (section D) it is stated that "the use of in vitro cultures allows this project to ….." However, in the application this is not explained. The DEC assumes that the outcome of preliminary in vitro experiments may be one of the factors on which it is decided to start in vivo experiments or to start generating one of the 20 new TG*

mouse lines. Section 3.4.1 seems the best place to explain the involvement/role of the in vitro experiments.

Animal Procedures:

- *In your presentation the motivation to use irradiation was "to clear out the bone marrow". Please make this clear in the application. The impression was raised that the irradiation was to generate an animal deprived of an immune system to prevent host versus graft responses.*
- *It is state that no hampered phenotypes (mice or fish) are expected. However, given the role of the HSCs, it seems more logical that a dysfunctional HSC system would result in a hampered phenotype. On which facts or consideration do you think that it is justified to make such a statement and the decision to not include breeding animals with hampered phenotype?*

- Gezien de aard van de vragen en de mondeling gegeven toelichting op vragen van de DEC tijdens de bespreking is de DEC van mening dat de aanvrager naar alle waarschijnlijkheid een afdoend antwoord zal kunnen geven en dan een discussie van de herziene versie van het project in een plenaire DEC bijeenkomst niet nodig is.

- Datum antwoord: 28-04-2017; de aanvrager heeft de aanvraag herzien en separaat gereageerd op de vragen en opmerkingen van de DEC. De herziene finale versie is via een schriftelijke ronde door de DEC leden positief beoordeeld.
- Verstrekt(e) antwoord(en): De aanvrager heeft de aanvraag gecomplementeerd en de gevraagde aanpassingen zijn doorgevoerd in de finale versie.

- De antwoorden hebben wel/niet geleid tot aanpassing van de aanvraag: wel

10. Eventuele adviezen door experts (niet lid van de DEC): niet van toepassing
    - Aard expertise
    - Deskundigheid expert
    - Datum verzoek
    - Strekking van het verzoek
    - Datum expert advies
    - Advies expert

B. Beoordeling (adviesvraag en behandeling)

1. Is het project vergunningplichtig (dierproeven in de zin der wet)? Indien van toepassing, licht toe waarom het project niet vergunningplichtig is en of daar discussie over geweest is.
   Indien niet vergunningplichtig, ga verder met onderdeel E. Advies.
   Het project is vergunningplichtig.

2. De aanvraag betreft een nieuwe aanvraag / een wijziging op een bestaande vergunning.
   Nieuwe aanvraag – Zie A4

3. Is de DEC competent om hierover te adviseren?
   Ja

4. Geef aan of DEC-leden, met het oog op onafhankelijkheid en onpartijdigheid, zijn uitgesloten van de behandeling van de aanvraag en het opstellen van het advies. Indien van toepassing, licht toe waarom.
   Er zijn geen DEC-leden uitgesloten van de behandeling en het opstellen van het advies.

C. Beoordeling (inhoud)

1. Beoordeel of de aanvraag toetsbaar is en voldoende samenhang heeft *(Zie handreiking 'Invulling definitie project'; zie bijlage I voor toelichting en voorbeeld).*
   Deze aanvraag heeft een concrete en duidelijke hoofddoelstelling, te weten: het identificeren van de moleculaire en cellulaire mechanismen betrokken bij ontstaan van bloedvormende stamcellen en de regulatie van deze mechanismen tijdens de embryonale ontwikkeling van zebravis, muis en kip. Deze hoofddoelstelling zal worden bereikt door het verrichten van studies naar de anatomische plaats van het ontstaan van de allereerste stamcellen (subdoel 1), wat de functionele rol is van een aantal genen waarvan, op basis van vooronderzoek, vermoed wordt dat die een rol spelen in het ontstaan van de stamcellen (subdoel 2) en het identificeren van nieuwe markers om de stamcellen en cellen betrokken bij de stamcelproductie beter te kunnen identificeren, isoleren en lokaliseren (subdoel 3).

   De strategie om de drie subdoelen is duidelijk uitgewerkt. In de geplande uitvoering van het project zullen de experimenten gericht op het behalen van de subdoelen geen directe onderlinge relatie of tijdsafhankelijkheid hebben, de doelstelling zal binnen de looptijd van 5 jaar kunnen worden gehaald, de subdoelen zijn uitgewerkt en duidelijk, elk subdoel levert een bijdrage aan het hoofddoel. De DEC komt tot de conclusie dat de aanvraag overeen komt met voorbeeld 4B van de handreiking 'Invulling definitie project'. De aanvraag heeft naar de mening van de DEC voldoende samenhang en is te typeren als een project.

   Het is helder welke handelingen individuele dieren zullen ondergaan. Hierdoor is ook duidelijk welk ongerief individuele dieren zullen ondergaan. De DEC is er daardoor van overtuigd dat de aanvrager gedurende het project op zorgvuldige wijze besluiten zal nemen over de voortgang van het project en dat er niet onnodig dieren gebruikt zullen worden.

   Gezien het bovenstaande komt de DEC tot de conclusie dat de aanvraag voldoende samenhang heeft en daarmee toetsbaar is.

2. Signaleer of er mogelijk tegenstrijdige wetgeving is die het uitvoeren van de proef in de weg zou kunnen staan. Het gaat hier om wetgeving die gericht is op de gezondheid en welzijn van het dier of het voortbestaan van de soort (bijvoorbeeld Wet dieren en Wet Natuurbescherming).
   Dit valt buiten de taakstelling van de DEC als beschreven in artikel 18a.2.b van de Wod. Naar deze specifieke informatie wordt in het aanvraagformulier en de bijbehorende toelichting niet gevraagd en de aanvrager heeft deze informatie dan ook niet verstrekt. Het is voor de DEC daarom niet mogelijk om op dit punt een onderbouwde uitspraak te doen. De DEC wil erop wijzen dat mocht dit in sommige omstandigheden wel het geval zijn dat de CCD in een procedure voorziet waarin de aanvrager inzage krijgt en verweer kan voeren.

3. Beoordeel of de in de projectaanvraag aangekruiste doelcategorie(ën) aansluit(en) bij de hoofddoelstelling. Nevendoelstellingen van beperkt belang hoeven niet te worden aangekruist in het projectvoorstel.
   De doelcategorie sluit aan bij de hoofddoelstelling.

Belangen en waarden

4. Benoem zowel het directe doel als het uiteindelijke doel en geef aan of er een directe en reële relatie is tussen beide doelstellingen. Beoordeel of het directe doel gerechtvaardigd is binnen de context van het onderzoeksveld *(Zie Praktische handreiking ETK: Stap*

1.C4; zie bijlage I voor voorbeeld).
Het *directe doel* van het project is het identificeren van de moleculaire en cellulaire mechanismen betrokken bij ontstaan van bloedvormende stamcellen en de regulatie van deze mechanismen tijdens de embryonale ontwikkeling van zebravis, muis en kip. Het uiteindelijke doel is om op basis van de mechanismen die in alle onderzochte diersoorten gevonden worden ("conservering") mechanismen de vorming van bloedvormende stamcellen in de mens beter te begrijpen en om met de nieuw verworven kennis de productie van patiëntgebonden bloedvormende stamcellen te kunnen opzetten. De verwachting is dat met de nieuwe kennis het genereren van bloedvormende stamcellen uit lichaamseigen geïnduceerde pluripotente stamcellen een stap dichterbij zal komen. Dit zal bijdragen aan therapieën om patiënten met afwijkingen in de bloedvorming te kunnen genezen via transplantatie van de gegenereerde cellen.

De DEC is ervan overtuigd dat het doel gerechtvaardigd is binnen de context van het onderzoeksveld. De aanvrager heeft duidelijk gemaakt wat de status van het onderzoeksveld is, wat de bijdrage van het al verrichte werk van de onderzoeksgroep is geweest, en wat de bijdrage van dit project aan het onderzoeksveld naar verwachting zal zijn. Uit de aanvraag blijkt dat de fundamenteel wetenschappelijke kennis over de productie van bloedvormende stamcellen nog beperkt is en dat het project zal resulteren in hete vergroten van de wetenschappelijke inzichten op dit terrein.

5. Benoem de belanghebbenden in het project en beschrijf voor elk van de belanghebbenden welke morele waarden in het geding zijn of bevorderd worden *(Zie Praktische handreiking ETK: Stap 2.B en tabel 1; zie bijlage I voor voorbeeld)*
   De belangrijkste belanghebbenden in dit onderzoeksproject zijn: (i) *De proefdieren.* De integriteit van de dieren zal in geringe mate worden aangetast. Het merendeel van de muizen (72%) zal mild ongerief ondervinden door de proeven en een kleiner deel (28%) maximaal matig ongerief. De zebravissen zullen mild ongerief ondervinden. In het kader van de proeven zullen de dieren worden gedood. (ii) *De bij de uitvoering van het project betrokken onderzoekers.* De onderzoekers zullen een substantiële toename in kennis en vaardigheden verkrijgen. De carrièremogelijkheden van de onderzoekers zullen verbeteren door publicaties. Ook de kans op het behouden en verkrijgen van nieuwe onderzoeksmogelijkheden, veelal deels gebaseerd op een goede wetenschappelijke reputatie, zal toenemen. Deze waarden zijn naar opvatting van de DEC echter van gering gewicht in de ethische afweging. (iii) *Onderzoekers in veld van de ontwikkelingsbiologie.* Dit onderzoek is in de eerste plaats fundamenteel van aard. Het zal resulteren in een toename van de ontwikkelingsbiologische inzichten hoe bloedvormende stamcellen worden gevormd in het lichaam en welke factoren van belang zijn in de regulatie van dit proces. Indien het mogelijk wordt om stamcellen in vitro te kunnen vormen en bestuderen zal dit resulteren in een vermindering van het gebruik van proefdieren en proefdierembryo's. (iv) *De doelgroepen in de maatschappij.* De nieuwe kennis draagt bij aan inzichten hoe lichaamseigen bloedvormende stamcellen buiten het lichaam kunnen worden geproduceerd voor transplantatiedoeleinden bij patiënten met afwijkingen in bloedvormende processen (o.a. anemie- en leukemiepatiënten). Op dit moment zijn deze patiënten geheel afhankelijk van de beschikbaarheid van geschikte donoren wat maar in 30% van de gevallen mogelijk is. Op basis van de nieuw verworven kennis kan een transplantatietherapie beschikbaar worden voor een grotere groep patiënten en is het risico op complicaties kleiner.

   Is er aanleiding voor de DEC om de in de aanvraag beschreven effecten op het milieu in twijfel te trekken?

   Nee

Proefopzet en haalbaarheid

6. Beoordeel of de kennis en kunde van de onderzoeksgroep en andere betrokkenen bij de dierproeven voldoende gewaarborgd zijn. Licht uw beoordeling toe. *(Zie Praktische handreiking ETK: Stap 1.C5).*
   De DEC is ervan overtuigd dat de aanvrager over voldoende expertise en de geschikte voorzieningen beschikt om de projectdoelstelling met de gekozen strategie/aanpak binnen de gevraagde termijn te realiseren. De DEC is er van overtuigd dat de aanvrager voldoende expertise heeft om gedurende het project te kunnen blijven voldoen aan de 3V's.

7. Beoordeel of het project goed is opgezet, de voorgestelde experimentele opzet en uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstellingen en of de gekozen strategie en experimentele aanpak kan leiden tot het behalen van de doelstelling binnen het kader van het project. Licht uw beoordeling toe. *Zie Praktische handreiking ETK: Stap 1.C6)*.
   De DEC is van mening dat de voorgestelde experimentele opzet en uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstellingen van het project en bij recente wetenschappelijke inzichten. De DEC acht het reëel om te veronderstellen dat op basis van de resultaten van de voorgenomen reeks experimenten zoals beschreven in het project, nieuwe en/of aanvullende fundamenteel wetenschappelijke kennis zal worden verkregen en dat de gekozen strategie en experimentele aanpak zal leiden tot het behalen van de doelstelling van het project. De gevraagde looptijd van 5 jaar acht de DEC reëel gezien de opbouw, de grootte van de onderzoeksgroep en de beschikbare financiële ondersteuning door [redacted]. Het project sluit goed aan bij de resultaten van het vooronderzoek dat de groep heeft verricht.
   Tijdens de uitvoering van het project zullen de in de aanvraag beschreven kaders, inclusief de kaders van ongerief, nauwgezet door de IvD bewaakt worden. De inzet van de vier verschillende Type Dierproeven is duidelijk en is goed onderbouwd.
   De keuze voor het parallel gebruik van de muis en zebravis als proefdier, naast het gebruik van kipembryo's) is gemotiveerd: het zijn complementaire modellen met elk specifieke technische voordelen, de mogelijkheid tot het vinden van geconserveerde mechanismen.
   De gegevens uit het vooronderzoek zijn verkregen in de drie soorten en biedt de continuering van het onderzoek aan dezelfde proefdiersoorten een grotere kans op het behalen van de doelstellingen en de acceptatie/publicatie van de resultaten binnen het wetenschappelijke veld.

Welzijn dieren

8. Geef aan of er sprake is van één of meerdere bijzondere categorieën van dieren, omstandigheden of behandeling van de dieren. Beoordeel of de keuze hiervoor voldoende wetenschappelijk is onderbouwd en of de aanvrager voldoet aan de in de Wet op de Dierproeven (Wod). voor de desbetreffende categorie genoemde beperkende voorwaarden. Licht uw beoordeling toe *(Zie Praktische handreiking ETK: Stap 1.C1; zie bijlage I voor toelichting en voorbeelden).*

   ☐ Bedreigde diersoort(en) (10e, lid 4)
   ☐ Niet-menselijke primaten (10e)
   ☐ Dieren in/uit het wild (10f)
   ☐ Niet gefokt voor dierproeven (11, bijlage I richtlijn)
   ☐ Zwerfdieren (10h)
   ☐ Hergebruik (1e, lid 2)
   ☐ Locatie: buiten instelling vergunninghouder (10g)
   ☐ Geen toepassing verdoving/pijnbestrijding (13)
   ☐ Dodingsmethode niet volgens bijlage IV richtlijn (13c, lid 3)

Er is geen sprake van bijzondere categorieën van dieren, omstandigheden of behandeling van de dieren.

9. Geef aan of de dieren gehuisvest en verzorgd worden op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van richtlijn 2010/63/EU. Indien niet aan deze minimale eisen kan worden voldaan, omdat het, om redenen van dierenwelzijn of diergezondheid of om wetenschappelijke redenen, noodzakelijk is hiervan af te wijken, beoordeel of dit in voldoende mate is onderbouwd. Licht uw beoordeling toe.
   De dieren worden gehuisvest en verzorgd op een wijze die voldoet aan de eisen die zijn opgenomen in bijlage III van de richtlijn.

10. Beoordeel of het cumulatieve ongerief als gevolg van de dierproeven voor elk dier realistisch is ingeschat en geclassificeerd. Licht uw beoordeling toe *(Zie Praktische handreiking ETK: Stap 1.C2)*.
    De DEC heeft zich ervan verzekerd dat de aanvrager al het mogelijke zal doen om het eventuele ongerief voor de proefdieren te identificeren, te verminderen en waar mogelijk te voorkomen.
    Het ongerief is door de onderzoekers ingeschat als mild ongerief voor het merendeel (72%) van de in totaal 11.700 muizen en matig ongerief voor 28% van de dieren. De muizen ondervinden maximaal matig ongerief voornamelijk door de gevolgen van een chirurgische ingreep, de gevolgen van hormooninjecties of dekking door een relatief groot mannetje. De dieren worden in het kader van of aan het einde van de proef gedood en het weefsel wordt ex vivo onderzocht. De zebravissen (4992) zullen maximaal mild ongerief ondervinden.

    Gegeven de zorgvuldige beschrijving van de procedures in de verschillende bijlagen Type Dierproeven is de DEC van mening dat het beschreven ongerief en de classificatie van het cumulatieve ongerief een realistische inschatting is.

11. Het uitvoeren van dierproeven zal naast het ongerief vaak gepaard gaan met aantasting van de integriteit van het dier. Beschrijf op welke wijze er sprake is van aantasting van integriteit. *(Zie Praktische handreiking ETK: Stap 1.C2). (zie bijlage I voor voorbeeld).*
    De integriteit van de dieren zal in geringe mate en tijdelijk worden aangetast tijdens de uitvoering van de proeven waarbij het herstel van een chirurgische ingreep, de gevolgen van een hormooninjectie op jonge leeftijd en de gedwongen paring met een relatief grote man de belangrijkste factoren zijn.

12. Beoordeel of de criteria voor humane eindpunten goed zijn gedefinieerd en of goed is ingeschat welk percentage dieren naar verwachting een humaan eindpunt zal bereiken. Licht uw beoordeling toe *(Zie Praktische handreiking ETK: Stap 1.C3)*.
    De humane eindpunten zijn duidelijk gedefinieerd. De DEC is het met de aanvrager eens dat de kans klein is dat de dieren ten gevolge van de procedures een humaan eindpunt zullen bereiken. De verwachting is dat er geen sprake is van een aangetast fenotype t.g.v. de genetische modificaties. Indien de genetische modificaties leiden tot een aantasting van de vorming van bloedvormende stamcellen zal dit resulteren in de dood van het dier in de embryonale fase. Indien het aantal bloedvormende stamcellen minder is dan normaal dan zullen de dieren anemisch zijn maar dit zal niet resulteren in een aantasting van hun gezondheid door de afwezigheid van fysieke uitdagingen en door de huisvesting in een schone omgeving.

3V's

13. Beoordeel of de aanvrager voldoende aannemelijk heeft gemaakt dat er geen geschikte vervangingsalternatieven zijn. Licht uw beoordeling toe *(Zie Praktische handreiking ETK:*

Stap 1.C3).
De DEC is van mening dat de aanvrager voldoende aannemelijk heeft gemaakt dat er geen vervangingsalternatieven zijn. Om de fundamenteel wetenschappelijke kennis te vergroten over het identificeren van de moleculaire en cellulaire mechanismen betrokken bij ontstaan van bloedvormende stamcellen en de regulatie van deze mechanismen tijdens de embryonale ontwikkeling van zebravis, muis en kip, is het noodzakelijk om proefdieren te gebruiken voor transplantatie-experimenten. De reden is dat voor de beschreven doelen de interactie tussen stamcellen en micro-omgeving een rol speelt en dat verschillende ontwikkelingsstadia zich in verschillende organen afspelen. Hiervoor bestaan geen alternatieven op basis van (stam)cellijnen of computermodellen. Waar mogelijk maken de onderzoekers gebruik van in vitro experimenten.

14. Beoordeel of het aantal te gebruiken dieren realistisch is ingeschat en of er een heldere strategie is om ervoor te zorgen dat tijdens het project met zo min mogelijk dieren wordt gewerkt waarmee een betrouwbaar resultaat kan worden verkregen. Licht uw beoordeling toe *(Zie Praktische handreiking ETK: Stap 1.C3)*.
De DEC is van mening dat het maximale aantal van 11.700 muizen en 4992 zebravissen te gebruiken dieren realistisch is geraamd en proportioneel is ten opzichte van de gekozen strategie en de looptijd. Er is sprake van een sequentiële gefaseerde aanpak om de subdoelen te behalen waarbij iedere volgende stap wordt afgewogen op basis van de verkregen resultaten. Waar mogelijk worden pilot experimenten gedaan voorafgaand aan een volledig experiment met drie herhalingen.

15. Beoordeel of het project in overeenstemming is met de vereiste van verfijning van dierproeven en het project zodanig is opgezet dat de dierproeven zo humaan mogelijk kunnen worden uitgevoerd. Licht uw beoordeling toe *(Zie Praktische handreiking ETK: Stap 1.C3)*.
De DEC heeft zich ervan verzekerd dat de aanvrager al het mogelijke heeft gedaan om het eventuele ongerief voor de proefdieren te identificeren, te verminderen en waar mogelijk te voorkomen. De verwachting is dat humane eindpunten zelden zullen worden bereikt.

16. Beoordeel, indien het wettelijk vereist onderzoek betreft, of voldoende aannemelijk is gemaakt dat er geen duplicatie plaats zal vinden en of de aanvrager beschikt over voldoende expertise en informatie om tijdens de uitvoering van het project te voorkomen dat onnodige duplicatie plaatsvindt. Licht uw beoordeling toe.

Dieren in voorraad gedood en bestemming dieren na afloop proef
Er is geen sprake van wettelijk vereist onderzoek. Alle dieren in proef worden na afloop gedood en het weefsel wordt voor ex-vivo studies gebruikt.

17. Geef aan of dieren van beide geslachten in gelijke mate ingezet zullen worden. Indien alleen dieren van één geslacht gebruikt worden, beoordeel of de aanvrager dat in voldoende mate wetenschappelijk heeft onderbouwd. *(Zie Praktische handreiking ETK: Stap 1.C3; zie bijlage I voor voorbeeld).*
De aanvrager gebruikt waar mogelijk mannelijke en vrouwelijke dieren behalve als er zwangere dieren nodig zijn.

18. Geef aan of dieren gedood worden in kader van het project (tijdens of na afloop van de dierproef). Indien dieren gedood worden, geef aan of en waarom dit noodzakelijk is voor het behalen van de doelstellingen van het project. Indien dieren gedood worden, geef aan of er een voor de diersoort passende dodingsmethode gebruikt wordt die vermeld staat in bijlage IV van richtlijn 2010/63/EU. Zo niet, beoordeel of dit in voldoende mate is onderbouwd. Licht uw beoordeling toe. Indien van toepassing, geeft ook aan of er door de aanvrager ontheffing is aangevraagd *(Zie Praktische handreiking ETK: Stap 1.C3).*
De aanvrager geeft aan dat het noodzakelijk is om dieren te doden in het kader van het

project. Het weefsel wordt voor ex-vivo studies gebruikt en de verkregen gegevens dragen bij aan het behalen van het doel. De aanvrager gebruikt voor het doden een methode die beschreven is in bijlage IV van de richtlijn en waarvoor geen aanvullende voorwaarden gelden.

Indien niet-humane primaten, honden, katten of landbouwhuisdieren worden gedood om niet-wetenschappelijke redenen, is herplaatsing of hergebruik overwogen? Licht toe waarom dit wel/niet mogelijk is.
Niet van toepassing.

NTS

19. Is de niet-technische samenvatting een evenwichtige weergave van het project en begrijpelijk geformuleerd?
De niet-technische samenvatting is een evenwichtige weergave van het project en begrijpelijk geformuleerd.

D. Ethische afweging

1. Benoem de centrale morele vraag *(Zie Praktische handreiking ETK: Stap 3.A).*
Rechtvaardigt het identificeren van de moleculaire en cellulaire mechanismen betrokken bij ontstaan van bloedvormende stamcellen en de regulatie van deze mechanismen tijdens de embryonale ontwikkeling van zebravis, muis en kip het merendeels (72% voor muizen; 100% voor de zebravissen) milde ongerief en matige ongerief (28% muizen) dat dieren wordt aangedaan in het voorliggende project?

2. Weeg voor de verschillende belanghebbenden, zoals beschreven onder C5, de sociale en morele waarden waaraan tegemoet gekomen wordt of die juist in het geding zijn, ten opzichte van elkaar af. Om dit proces te vergemakkelijken, kunt u de belangrijkste belanghebbenden en de belangrijkste waarden die in het geding zijn waarderen. U kunt dit verwoorden in termen van gering, matig of veel/ernstig voordeel of nadeel. Geef aan waarom de DEC bevordering van waarden (baten) voor de ene belanghebbende prevaleert boven de aantasting van waarden (kosten) voor de andere belanghebbende *(Zie Praktische handreiking ETK: Stap 3.B; zie bijlage I voor voorbeelden).*
De volgende waarden/belangen zijn in het geding (zie onderdeel C5): Waarden/belangen met betrekking tot de proefdieren: de dieren ondervinden *maximaal matig ongerief* dit wordt *door de DEC beschouwd als veel nadeel*. De belangen voor de uitvoerende onderzoekers: *veel voordeel*. De belangen met betrekking tot de doelgroepen binnen het veld van de neurobiologie: de kennisvergroting wordt door de DEC gezien als *zwaarwegend en veel voordeel*. De belangen met betrekking tot de maatschappij (patiëntengroepen): op korte termijn *gering voordeel maar op lange termijn mogelijk veel voordeel.*

3. Beantwoord de centrale morele vraag. Maak voor het beantwoorden van deze vraag gebruik van bovenstaande afweging van morele waarden. Maak daarnaast gebruik van de volgende moreel relevante feiten: belang onderzoek (C4), kennis en kunde van betrokkenen (C7), haalbaarheid doelstellingen (C8), categorieën en herkomst dieren (C9), 3V's (C14-C18), ongerief (C10-13 en C19) en relevante wet en regelgeving (C2). Onderbouw hoe al deze elementen zijn meegewogen bij de beantwoording van de centrale morele vraag, zodanig dat het navolgbaar is zonder gedetailleerde kennis te hebben van het projectvoorstel *(Zie Praktische handreiking ETK: Stap 3.C; zie bijlage I voor voorbeeld)*.
De DEC is van mening dat de benoemde belangen van de wetenschap en samenleving in dit project zwaarder wegen dan de belangen/waarden van de proefdieren. De volgende

overwegingen hebben bijgedragen tot deze conclusie:

- Indien de doelstellingen bereikt worden, zal dit resulteren in een aanmerkelijke toename van de ontwikkelingsbiologische inzichten hoe bloedvormende stamcellen worden gevormd en welke regulatiesystemen daarbij betrokken zijn. Deze kennis is van belang voor het opzetten van het methoden om deze stamcellen in vitro te kunnen opgroeien voor transplantatiedoeleinden. Dit zal een einde kunnen maken aan het huidige tekort aan het aantal geschikte transplantatiedonoren in het kader van behandelingen van ondermeer anemie en leukemie. De kennis hiertoe ontbreekt nog en de verwachting is dat het project een grote bijdrage zal leveren om die leemte te vullen.

- De DEC is van mening dat de voorgestelde experimentele opzet en uitkomstparameters logisch en helder aansluiten bij de aangegeven doelstellingen en recente wetenschappelijke inzichten. De gekozen strategie en experimentele aanpak kan leiden tot het behalen van de doelstelling binnen het kader van het project.

- Het is naar de mening van de DEC aannemelijk dat de doelstellingen behaald zullen worden. Om dit doel te bereiken is het nodig proefdieren te gebruiken. De onderzoekers doen er echter alles aan om het lijden van de dieren te beperken waardoor het uiteindelijk ongerief van elk individueel dier, naar verwachting, voor een deel van de dieren beperkt blijft tot maximaal matig ongerief en mild ongerief voor de rest.

- De DEC is overtuigd van het belang van de wetenschappelijke doelstelling en het belang van de nieuwe kennis.

- De DEC is er van overtuigd dat de aanvrager voldoende kennis en kunde heeft om de doelstellingen te behalen en, tijdens de uitvoering van het project, te kunnen voldoen aan de 3V-beginselen.

- De DEC is van mening dat de aanvrager bij de uitvoering van het project alle mogelijke maatregelen treft om het ongerief van de dieren te beperken en het aantal dieren tot een minimum te beperken.

Gezien bovenstaande overwegingen is de DEC van opvatting dat het bereiken van de doelstelling, op de wijze zoals beschreven in deze projectaanvraag, het gebruik van proefdieren rechtvaardigt.

E. Advies

1. Advies aan de CCD
   - ■ De DEC adviseert de vergunning te verlenen.
   - ☐ De DEC adviseert de vergunning te verlenen onder de volgende voorwaarden
     - ☐ Op grond van het wettelijk vereiste dient de projectleider bij beëindiging van het project een beoordeling achteraf aan te leveren die is afgestemd met de IvD.
     - ☐ Voor de uitvoering van dit project is tevens ministeriële ontheffing vereist
     - ☐ Overige door de DEC aan de uitvoering verbonden voorwaarden, te weten: geen.
   - ☐ De DEC adviseert de vergunning niet te verlenen vanwege:
     - ☐ De vaststelling dat het project niet vergunningplichtig is om de volgende redenen:…
     - ☐ De volgende doorslaggevende ethische bezwaren:…

☐ De volgende tekortkomingen in de aanvraag:…

2. Het uitgebrachte advies kan unaniem tot stand zijn gekomen dan wel gebaseerd zijn op een meerderheidsstandpunt in de DEC. Indien gebaseerd op een meerderheidsstandpunt, specificeer het minderheidsstandpunt op het niveau van verschillende belanghebbenden en de waarden die in het geding zijn *(Zie Praktische handreiking ETK: Stap 4.A; zie bijlage I voor voorbeeld).*
   Het advies is unaniem

3. Omschrijf de knelpunten/dilemma's die naar voren zijn gekomen tijdens het beoordelen van de aanvraag en het opstellen van het advies zowel binnen als buiten de context van het project *(Zie Praktische handreiking ETK: Stap 4.B)*.
   De DEC heeft geen dilemma's gesignaleerd die binnen of buiten de context van het project vallen.

Centrale Commissie Dierproeven

11.

> Retouradres Postbus 20401 2500 EK Den Haag

Kon. Ned. Academie van Wetenschappen (KNAW)
[redacted]
Postbus 19121
1000 GC AMSTERDAM

Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag
centralecommissiedierproeven.nl
0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD8010020171047

Bijlagen
2

Datum 17 mei 2017
Betreft Ontvangstbevestiging aanvraag projectvergunning Dierproeven

Geachte [redacted],

Wij hebben uw aanvraag voor een projectvergunning dierproeven ontvangen op 17 mei 2017. Het gaat om uw project "The cellular and molecular basis of the hematopoietic production". Het aanvraagnummer dat wij aan deze aanvraag hebben toegekend is AVD8010020171047. Gebruik dit nummer wanneer u contact met de CCD opneemt.

Wacht met de uitvoering van uw project
Als wij nog informatie van u nodig hebben dan ontvangt u daarover bericht. Uw aanvraag is in ieder geval niet compleet als de leges niet zijn bijgeschreven op de rekening van de CCD. U ontvangt binnen veertig werkdagen een beslissing op uw aanvraag. Als wij nog informatie van u nodig hebben, wordt deze termijn opgeschort. In geval van een complexe aanvraag kan deze termijn met maximaal vijftien werkdagen verlengd worden. U krijgt bericht als de beslisperiode van uw aanvraag vanwege complexiteit wordt verlengd. Als u goedkeuring krijgt op uw aanvraag, kunt u daarna beginnen met het project.

Factuur
Bijgaand treft u de factuur aan voor de betaling van de leges. Wij verzoeken u de leges zo spoedig mogelijk te voldoen, zodat we uw aanvraag in behandeling kunnen nemen. Is uw betaling niet binnen dertig dagen ontvangen, dan kan uw aanvraag buiten behandeling worden gesteld. Dit betekent dat uw aanvraag niet beoordeeld wordt en u uw project niet mag starten.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Datum:
17 mei 2017
Aanvraagnummer:
AVD8010020171047

Met vriendelijke groet,

Centrale Commissie Dierproeven

Deze brief is automatisch aangemaakt en daarom niet ondertekend.

Bijlagen:
- Gegevens aanvraagformulier
- Factuur

Datum:
17 mei 2017
Aanvraagnummer:
AVD8010020171047

Gegevens aanvrager

Uw gegevens	
Deelnemersnummer NVWA:	80100
Naam instelling of organisatie:	Kon. Ned. Academie van Wetenschappen (KNAW)
Naam portefeuillehouder of diens gemachtigde:	[redacted]
KvK-nummer:	54667089
Postbus:	19121
Postcode en plaats:	1000 GC AMSTERDAM
IBAN:	NL47DEUT0436465302
Tenaamstelling van het rekeningnummer:	KNAW

Gegevens verantwoordelijke onderzoeker	
Naam:	[redacted]
Functie:	Groepsleider
Afdeling:	[redacted]
Telefoonnummer:	[redacted]
E-mailadres:	[redacted]

Datum:
17 mei 2017
Aanvraagnummer:
AVD2010020171047

Over uw aanvraag

Wat voor aanvraag doet u?	[x] Nieuwe aanvraag [ ] Wijziging op een (verleende) vergunning die negatieve gevolgen kan hebben voor het dierenwelzijn [ ] Melding op (verleende) vergunning die geen negatieve gevolgen kan hebben voor het dierenwelzijn

Over uw project

Geplande startdatum:	1 juni 2017
Geplande einddatum:	1 juni 2022
Titel project:	The cellular and molecular basis of the hematopoietic production
Titel niet-technische samenvatting:	De cellulaire en moleculaire basis van de vorming van bloedvormende stamcellen
Naam DEC:	DEC-KNAW
Postadres DEC:	Meibergdreef 47 1105 BA Amsterdam
E-mailadres DEC:	decsecr@knaw.nl

Betaalgegevens

De leges bedragen:	€ 1.684,-
De leges voldoet u:	na ontvangst van de factuur

Checklist bijlagen

Verplichte bijlagen:	[x] Projectvoorstel [x] Beschrijving Dierproeven [x] Niet-technische samenvatting
Overige bijlagen:	[x] DEC-advies

Ondertekening

Naam:	[redacted]
Functie:	[redacted]
Plaats:	Amsterdam
Datum:	15 mei 2017

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Kon. Ned. Academie van Wetenschappen (KNAW)
[redacted]
Postbus 19121
1000 GC AMSTERDAM

Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag
centralecommissiedierproeven.nl
0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD8010020171047

Bijlagen
2

Datum 17 mei 2017
Betreft Factuur aanvraag projectvergunning Dierproeven

Factuur
Factuurdatum: 17 mei 2017
Vervaldatum: 16 juni 2017
Factuurnummer: 171047

Omschrijving	**Bedrag**
Betaling leges projectvergunning dierproeven Betreft aanvraag AVD8010020171047	€ 1.684,00

Wij verzoeken u het totaalbedrag vóór de gestelde vervaldatum over te maken op rekening NL29INGB 070.500.1512 onder vermelding van het factuurnummer en aanvraagnummer, ten name van Centrale Commissie Dierproeven, Postbus 93144, 2509 AC te 's Gravenhage.

Response to: Vraag bij de behandeling van AVD8010020171047

U heeft bij de CCD een aanvraag tot projectvergunning gedaan. Bij de behandeling hiervan hebben wij nog een paar vragen. Het betreft uw project: 'The cellular and molecular basis of the hematopoietic production' met aanvraagnummer AVD8010020171047.

→ All comments and questions have been answered and all changes appear in red in the revised version of the documents.

In bijlage 3.4.4.2 moet het aantal foeten van E18 in aantal worden meegeteld als dierproef. Bij zoogdieren worden dieren vanaf het derde trimester van de dracht gezien als proefdier. U maakt de foeten dood ten behoeve van het verzamelen van organen, dit is een dierproef met licht ongerief. Wanneer het dieraantal veranderd zal de Niet Technische Samenvatting ook aangepast moeten worden.

→ The following paragraph has been added in Appendix 3.4.4.2:
"- E18 embryos used to collect donor tissues/cells
Different hematopoietic tissues will be collected from WT and GMO E18 embryos for ex vivo analysis. An average of 6 embryos will be needed per experiment to collect enough cells and tissues. The experiments will be performed three times for each assay to be scientifically reliable.
Therefore we need:
→ 6 (E18 embryos) x 21 (1 WT and 20 GMO) x 3 (experiments) = **378 embryos**."

The number of mice has been adjusted in the appendix 3.4.4.2 and in the "Niet Technische Samenvatting" document and in the "Overview number of animals" table.

Worden in de bijlagen 3.4.4.3 en 3.4.4.4 beide geslachten zebra vissen en muizen ingezet?

→ Both genders for fish and mice will be used. The following sentence has been added in section 2B of the appendix 3.4.4.2, 3.4.4.3 and 3.4.4.4:
"Genders: both males and females will be used."

U beschrijft dat er in het project go/no go beslismomenten zijn. De criteria hiervoor beschrijft u niet, kunt u deze beschrijven?

→ The following paragraph is now included in the revised version of the project proposal (section 3.4 Research strategy):
"Before generating a new GMO line, the gene of interest will be studied based on the literature. The expression pattern of the gene in embryonic tissues will be carefully examined by in vitro analysis (e.g. in situ hybridization, flow cytometry analysis). When the GMO lines will be synthesized with the most promising regulator genes, their effect will be tested on HSCs by in vivo transplantation assays. One transplantation experiment will be performed and analyzed to decide if the gene is a potent interesting HSC regulator (e.g. observation of less mice reconstituted 4 months post-transplantation of GMO cells [where the gene is inactivated] compared to WT cells). Only then, more mice will be transplanted to reach n=3 independent experiments."

Form
Project proposal

- This form should be used to write the project proposal for animal procedures.
- The appendix 'description animal procedures' is an appendix to this form. For each type of animal procedure, a separate appendix 'description animal procedures' should be enclosed.
- For more information on the project proposal, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80100 - KNAW
1.2	Provide the name of the licenced establishment.	[redacted]
1.3	Provide the title of the project.	The cellular and molecular basis of the hematopoietic production

2 Categories

2.1 Please tick each of the following boxes that applies to your project.

- ☒ Basic research
- ☐ Translational or applied research
- ☐ Regulatory use or routine production
- ☐ Research into environmental protection in the interest of human or
- ☐ Research aimed at preserving the species subjected to procedures
- ☐ Higher education or training
- ☐ Forensic enquiries
- ☐ Maintenance of colonies of genetically altered animals not used in other animal procedures

3 General description of the project

3.1 Background

Describe the project (motivation, background and context) with respect to the categories selected in 2.

- For legally required animal procedures, indicate which statutory or regulatory requirements apply (with respect to the intended use and market authorisation).
- For routine production, describe what will be produced and for which uses.
- For higher education or training, explain why this project is part of the educational program and describe the learning targets.

Every day, blood stem cells (also named hematopoietic stem cells or HSCs) produce billions of new blood cells, which are needed for an organism to survive. This massive cell production is possible due to two important properties inherent in HSCs, multipotency and self-renewal. The multipotency property allows single HSCs to produce all the different blood cell types (e.g. erythrocytes that carry oxygen in tissues,

platelets that provide coagulation in case of bleeding, lymphocytes that protect the organism against infections). The self-renewal property allows HSCs to produce all blood cells without exhaustion of the HSC pool, which remains constant during life. These inherent properties confer to HSCs the capacity to maintain blood homeostasis under physiological condition. HSCs play also a crucial role in the clinic. Indeed, defects in HSCs lead to blood-related disorders and various cancers (e.g. anemia, leukemia). The transplantation of healthy donor HSCs to replace the patient defective ones is an important part of the treatment and sometimes the only cure. However, less than 30% of the patients have matched donors in their family. Therefore, successful transplantation in most patients relies on finding unrelated volunteer donors with the highest compatibility (the chance of an optimal match being very low)[1]. Since the number of transplantations increases every year, the limitation in compatible HSCs available for transplantation procedures has become a major hurdle. More over, HSCs are very rare cells present in bone marrow, cord blood and mobilized peripheral blood (all tissues being used as source of HSCs in clinic). To circumvent the HSC shortage, research efforts have been made to expand donor HSCs *ex vivo* or to generate new sources of HSCs *in vitro* (e.g. from pluripotent stem cells or somatic cells)[2]. Despite some progresses, success has been limited and it remains impossible to date to produce large quantities of tailor-made HSCs. Because HSC production, as it occurs *in vivo*, is not fully understood yet, it is very difficult to mimic this process *in vitro*. To circumvent this issue, it is crucial (1) to better characterise the HSCs and their precursors, and (2) to identify the intrinsic factors (e.g. transcription factors) and extrinsic factors (e.g. growth factors provided by the surrounding microenvironment) that promote and regulate HSC fate determination, generation and expansion *in vivo*.

Adult HSCs are initially produced during embryonic development. They are first generated in the main arteries (aorta, vitelline and umbilical arteries) of the embryo[3-5] (**Fig. 1A**). They derive from hemogenic endothelial cells that are embedded in the arteries'wall[6,7] (**Fig. 1B**). The dynamic transition of an endothelial cell into a HSC has been observed in the aorta of chicken, mouse and zebrafish embryos[8,9,10,11]. Such finding demonstrated the conservation of the HSC production process in between species and the importance of the aorta microenvironment in supporting/regulating this important process. In arteries, HSCs are part of cell clusters[12] that will then colonize the fetal liver and placenta where the pool of HSCs expands before colonizing the bone marrow before birth (**Fig. 1A**,**B**). The important role of the endothelium in HSC production was an important fundamental discovery that paved the way to improve HSC production *in vitro*. For example, *in vitro* vascular induction was recently used to successfully reprogram human endothelial cells in HSC-like cells[13] and to induce mouse embryo aorta endothelial cells into HSC-like cells[14]. The produced cells are named HSC-like cells because it remains difficult to present to generate large quantities of fully functional HSCs that are able to engraft and to provide a long-term multilineage hematopoietic reconstitution when transplanted in adult recipients (the assay to experimentally identify HSCs). Therefore, research must continue to understand how a cell becomes a HSC, how it is regulated and how HSCs can expand without losing their stemness (**Fig. 1C**).

The **main research goal** in my lab is to better understand the production of HSCs, as it occurs *in vivo* during embryonic life. Our **sub-goal 1** is to determine the anatomical origin (intra- or extra-embryonic) of hemogenic endothelial cells (the cells producing HSCs). So far, it is uncertain because the blood is already circulating at the time of HSC detection in the embryo. Therefore, it cannot be excluded that hemogenic endothelial cells (or their precursors) emerge in one site and reach another anatomical site via the circulation or throughout tissues before to actually produce HSCs. It is an important sub-goal since the microenvironment of their site of origin determines the fate of these cells. Our **sub-goal 2** is to understand the function of specific genes in HSC production during embryonic life. HSC emergence and expansion are highly regulated processes both in time (as it occurs at specific time points of development) and space (in restricted regions of the vessels and organs) (**Fig. 1A**). However, the complex network of extrinsic and intrinsic regulatory factors involved *in vivo* in HSC production is still poorly understood. Our **sub-goal 3** is to find new markers for HSCs and cells from the supportive microenvironment to be able to precisely localise and follow the fate and behaviour of these cells during development.

The state-of-affairs on the production of HSCs *in vitro* is a worldwide concern since it remains impossible to achieve so far, despite extensive research. Understanding the process as it occurs *in vivo* is a very important research topic that several international labs are trying to achieve. However, only few labs study the embryonic development when the first HSCs are generated. Moreover, we are most likely the only lab able to perform a multi-species comparative study since we have the unique expertise, state-of-the-art technology and full access to the three most relevant animal models in the lab (i.e. chicken, mouse and zebrafish).

The use of the different animal models is needed because:
(i) They are complementary models. Indeed, each animal model allows different *ex vivo* analyses due to different technical advantages. For example, live imaging and cell tracing can only be performed in zebrafish embryos. Also, tissue grafting can only be performed with chicken embryos while HSC transplantation is used in the mouse and zebrafish models.
(ii) The use and comparison of different animal models allow finding conserved processes and mechanisms in between species. It reinforces the possibility that such processes and mechanisms also occur in human. In this project, chicken, mouse and zebrafish embryos will be used as reliable alternatives to human embryos to analyse in details the different aspects of HSC production during embryonic life.
(iii) The study of developmental hematopoiesis cannot be performed in human embryos due to the difficulty to access human embryo samples. When available, such samples are rare and often damaged because of the embryo collection procedure (i.e. from abortion). The use of human embryo is not a possible/realistic alternative for our project. The only alternative is to use animal models instead.
(iv) There is an increased request from the scientific community to provide and compare data in different animal models (e.g. for publication).

Because there is no need of licence to work on chicken embryos (no adult chicken will be used for the project), the requested licence only concerns the mouse and zebrafish models. Our three sub-goals are of equivalent importance. Therefore, our strategy is to conduct experiments in all three animal models in parallel to answer all sub-goals (and not sequentially since all models will provide, via different assays and *ex vivo* analysis, pieces of answers to the same question).

[redacted]

[redacted]

Overall, a better understanding of HSC generation/expansion in the successive physiological niches as it occurs *in vivo* during the embryonic development of various animal species is needed to understand the core mechanisms of HSC production that will most likely be conserved in human. It will help to engineer appropriate *in vitro* culture conditions to produce in the future autologous hematopoietic grafts needed to treat patients who suffer from hematopoietic disorders, the long-term target group of our proposal[17]. Deciphering the physiological development of HSCs will also prove useful for understanding the deregulation of HSCs.

References

1 [redacted]

3.2 Purpose

Describe the project's main objective and explain why this objective is achievable.

- If the project is focussed on one or more research objectives, which research questions should be addressed during this project?
- If the main objective is not a research objective, which specific need(s) does this project respond to?

The work described here aims to increase our understanding of the processes leading to HSC production as it occurs *in vivo* during embryonic development. It will identify which genes and signalling pathways

are functionally important during the successive steps of HSC production (such as endothelial cell specification into hemogenic endothelial cells, endothelial to hematopoietic transition, pre-HSC maturation, HSC survival and expansion). The goal of using and comparing different animal models (chicken, mouse, zebrafish) is to find conserved HSC regulators that will therefore most likely be involved also in human. Ultimately this knowledge should lead in the future to the production of tailor-made HSCs that are needed to treat patients who suffer from hematopoietic disorders and diseases.

Our main goal is to identify the molecular and cellular mechanisms involved in HSC production and regulation during the embryonic development of zebrafish, mouse and chicken (the three main and most reliable animal models used to study developmental hematopoiesis).

Our specific research sub-goals are:
1- To determine the anatomical origin of hemogenic endothelial cells (or precursors).
We wish to identify the anatomical site (yolk sac, allantois or embryo proper) at the origin of hemogenic endothelial cells (or precursors). We expect to identify within the 5 years of the project the microenvironment that determines the fate of hemogenic endothelial cells, which produce later on HSCs.

2- To understand the function of specific genes in HSC production during embryonic life under basal conditions by looking at the effect on cell specialization, emergence, viability, maturation, proliferation and differentiation.
We wish to identify the genes involved in the regulation of HSC production and we expect to prove their functionality within the 5 years of the project.

3- To find new markers for HSCs and cells from the supportive microenvironment.
We wish to identify new markers to better identify, isolate and locate HSCs and the cells in the surrounding microenvironment that constitute a supportive niche. We expect to identify new markers within the 5 years of the project.

This research will be performed in a lab, [redacted] The institute houses large zebrafish and mouse facilities, provides core facilities for various high-end techniques (e.g. sequencing, histology, confocal microscopy, flow cytometry), and expertise on animal models with dedicated and experienced animal caretakers. As principle investigator for this project, I have more that 20 years of experience with the use of the mouse model and 2 years with the zebrafish model, in biomedical research. In the lab, we have one dedicated *in vivo* technician and very experienced scientists that oversee the breeding of all animal lines, experiments and procedures, and new people are trained when required. The research lab has experience with the different experiments and techniques that are proposed for the project (e.g. intra-uterus injection in embryos, intravenous injection in adult, embryos and tissues collection, whole embryo multicolour staining, immunostaining on cryosections, confocal imaging of embryo/tissue slices after immunostaining).

There are several reasons why we think that we can achieve our objectives within 5 years:
- Part of the research described here was included in research proposals that were reviewed by independent experts in the field and were granted for funding [redacted]) and were previously approved as DEC protocols. All animal procedures are currently being performed and are part of the ongoing projects. It is important to note, that there is overlap between the mouse studies described in this project and those in earlier DEC-approved protocols. After a license for this project has been obtained, all experiments will formally be executed under this new license.
- Work from my group [redacted] has resulted in seminal papers that described the required techniques.
- Our research and experiments are continuously being evaluated by various other researchers within and outside our institute. The scientists working in my group are selected based on their excellence and their commitment to our goals.

Our previous achievements make it very likely that we will be able to finish the project proposal in 5 years.

3.3 Relevance

What is the scientific and/or social relevance of the objectives described above?

The proposed work will result in the identification of molecular and cellular processes that drive

hematopoietic stem cell (HSC) production in vivo. The **scientific relevance** of the objectives is to improve our fundamental scientific knowledge of the processes and mechanisms driving and regulating HSC and hematopoietic production in zebrafish, and mouse and chicken throughout development and in adult. The **social relevance** of our objectives is that we can use this knowledge in the future to improve HSC production and expansion in vitro and therefore to supply in tailor-made HSCs that are in such need to cure patients with blood related disorders and diseases. Allogeneic and autologous HSC transplantations are used widely in the treatment of hematologic and genetic conditions. Induced pluripotent stem (IPS)-derived HSCs could be an ideal source for resolving current limitations related to HSC transplantation procedures. Current efforts to derive HSCs from pluripotent stem cells have largely focused on recapitulating normal developmental progression in an ex vivo setting. Lessons can certainly be learned from the study of HSC production during embryonic development. In addition, an ability to generate bona fide HSCs from iPS lines derived from patients with genetic diseases could be invaluable for disease modelling and drug screening. It would certainly tremendously reduce research on animals and the need to collect cells from donors or embryos. Although generating human HSCs from pluripotent stem cells still remains elusive, a better understanding of the network of regulatory factors governing HSC identity and production as it occurs during embryonic development (our proposal goal) should help to overcome the current hurdles in generating functional (i.e. transplantable) HSCs.

3.4 Research strategy

3.4.1 Provide an overview of the overall design of the project (strategy).

A flowchart that summarizes the workflow of experiments is provided in attachment.
Our research strategy is to study and compare the hematopoietic development in chicken, mouse and zebrafish embryos to find conserved mechanisms and regulatory genes. Parallel studies will therefore be performed in mouse, zebrafish and chicken species. For chicken embryo experiments, no AP is required. The goal is to find mechanisms and genes that will be conserved in the three species and therefore will be most likely also conserved in human embryos, which are difficult to study directly (due to the difficulties to obtain human embryo samples). Our research strategy provides a reliable alternative to learn more about human developmental hematopoiesis.
[redacted] Based on these data generated in previous in vivo and in vitro studies, available literature and interactions with other scientists, we therefore identified potent interesting intrinsic and extrinsic regulators of HSC production [redacted]. In this project, we now wish to test their functional role on chicken, mouse and zebrafish hematopoiesis (licence required only for the two last animal models). We will test their precise mechanism of action in vivo in wild type, genetically modified (GM) animals or animals in which a specific gene or type of (stem) cell has been modified with a compound. Animals will be injected with compounds (e.g. Tamoxifen) to increase or inhibit a gene. In case a line with the required genetic modification is not available we will generate new animal lines via standard oocyte injection, blastocyst injection (mouse), or via the CRISPR/Cas9 system (see Appendix 3.4.4.1 (mouse)). We will only use well-established reagents and protocols to induce expression or deletion of the candidate gene(s). Our considerations and findings that support the use of mice and zebrafish will be part of our application that we submit to the IvD.
We will set up pilot studies with the minimum amount of animals possible to test the validity of our hypothesis (HSC regulator or not) in vivo. For that, we will collect tissues at the time when the gene candidate(s) or cell type should have the greatest effect. Each experiment is very carefully designed with clear go-no-go decisions to reduce the amount of cumulative discomfort and/or the number of animals. For every each experiment, the best trade-off will be made.

We will perform the following experiments for:
Sub-goal 1 (see Appendix 3.4.4.3). To identify the anatomical site at the origin of hemogenic endothelial cells (or precursors), we will perform in utero transplantation of donor cells isolated from yolk sac, allantois and embryo proper (potent sites of origin). The donor cells will carry a marker (e.g.

fluorescent, as GFP-positive cells transplanted in wild type recipients) that will allow the traceability of these cells and their progeny after transplantation. The fluorescent donor cells will also be easily distinguishable from the wild type recipients. Donor cells will be injected in the heart of mouse recipient embryos developing in the mother uterus. We will analyse ex vivo the tissues and cells collected from the growing recipients (cellular, molecular and histological analysis) to determine the long-term multi-lineage hematopoietic contribution of the donor cells. Only the tissues that will contain hemogenic endothelial cells or precursors will be able to produce HSCs in the recipient embryo and to provide long-term multi-lineage hematopoietic reconstitution in the growing recipients to adult age (see below the overall summary of the procedure). These experiments will determine which tissue/microenvironment (yolk sac, allantois or embryo proper) is at the origin of hemogenic endothelial cells, which produce later on HSCs.

Sub-goal 2. To identify the genes involved in the regulation of HSC production and to prove their functionality, we will modify candidate genes to test their function in HSC production in mice and zebrafish. During gene modification, a fluorescent marker (e.g. GFP) will be introduced to mark the genetically modified cells. After gene modification (see Appendix 3.4.4.1), tissues and cells will be isolated and analyzed *ex vivo* (See Appendix 3.4.4.2). Donor cells will also be used to perform *in vivo* transplantation assay (transplantation of WT or modified cells in adult or neonate recipients) (see Appendix 3.4.4.3 and 3.4.4.4). HSCs are identified in an *in vivo* assay where cells are transplanted intra-venously in adult recipient mice. Pre-HSCs are too immature to engraft adult recipients but they are identified in an *in vivo* assay where cells are transplanted in the liver of neonates, which constitutes a more permissive environment than the adult bone marrow. Recipients (adults and neonates) are irradiated prior transplantation to clear out the bone marrow and liver from all proliferating cells and therefore to make space. It allows the engraftment of the injected donor cells. The hematopoietic tissues and cells of the transplanted recipients will then be analyzed *ex vivo* for donor (fluorescent) contribution (see Appendix 3.4.4.3 and 3.4.4.4). The presence of donor cells in all hematopoietic lineages and all hematopoietic tissues of the recipient at long-term will prove the presence of pre-HSCs or HSCs (see below the overall summaries of the procedures).

We will also generate genetically modified zebrafish but since no hampered phenotypes are expected, this activity does not require a CCD license. After gene modification, the zebrafish will be analyzed *in vivo* by live confocal imaging of the embryo (e.g. by imaging cell emergence, maturation, survival, proliferation and/or differentiation) and by *ex vivo* analysis of the hematopoietic embryonic/adult tissues. The observation of HSC or hematopoietic defect in the hematopoietic organs/cells of the animals where

gene modifications have been performed will indicate that these specific genes are important HSC regulators.

Sub-goal 3. The sub-goal 3 is to identify new markers to better identify, isolate and locate HSC precursors (pre-HSCs), HSCs and the cells in the surrounding microenvironment that constitute the supportive niche. For this purpose, cells will be sorted based on the expression of the potent new surface markers and transplantated in neonate (assay to identify pre-HSCs; see Appendix 3.4.4.3) and adult mice (assay to identify HSCs; see Appendix 3.4.4.3), and adult zebrafish (assay to identify HSCs; see Appendix 3.4.4.4). Hematopoietic tissues will also be collected during embryonic development and adult to locate and trace the cells based on the expression of the new markers. Pre-HSCs and HSCs must localise in the clusters in the aorta, and should be thereafter be present in the successive hematopoietic organs (placenta, yolk sac, liver and bone marrow). Cells from the supportive niche should be located close by to the pre-HSCs and HSCs (e.g. in the mesenchyme underneath the clusters in the aorta of the embryo). The correct localisation and function of the cells based on the expression of the new markers will indicate that these markers are reliable markers of pre-HSCs, HSCs or supporting cells from the microenvironment.

3.4.2 Provide a basic outline of the different components of the project and the type(s) of animal procedures that will be performed.

The specific protocols that we apply to achieve our research goals are outlined below, in the flowchart and Appendix. All these animal procedures and their components are currently already on going in our lab.

Generation, welfare assessment and breeding of wild type and genetically modified mice (Appendix 3.4.4.1)
To study the function or behavior of a gene or a cell type relevant for HSC production, we will use appropriate mouse lines that are either already available or that need to be generated. New mouse line(s) will be generated via standard oocyte injection, blastocyst injection, or via the CRISPR/Cas9 system. The CRISPR/Cas9 system will especially be used as highly efficient tool for simultaneously multi-gene editing. This prevents the generation and breeding of multiple homozygotes from individually targeted ES cells (Reduction of the 3Rs).
In contrast to conventional gene-targeting strategy, the use of the Cre/LoxP recombination system in conjunction with gene targeting allows us to study the consequence of gene manipulation in a cell type specific manner. By incorporating Cre recombinase recognition sites (LoxP) into the genome, Cre expression from a specific promoter can drive gene disruption, activation or tracing in a cell type specific manner. New transgenic lines and/or KO lines generated via classical methods and/or novel combinations and used for breeding of these aforementioned lines will be monitored for 2 generations to determine the absence or presence of constitutional discomfort.
Cre recombinase expression can also be activated in an inducible manner by the addition of tamoxifen. To this end the Cre is flanked by 2 mutated estrogen receptors (Creert2, or merCremer) and will only allow for Cre activation when tamoxifen is administered.

Collection of tissues and cells from wild type and genetically modified animals for *ex vivo* analysis and collection of donor cells for transplantations (Appendix 3.4.4.2)
To study aspects of hemogenic endothelial cells or precursors origin, pre-HSC and HSC emergence and production as well as HSC progeny, we will isolate whole embryos, and hematopoietic tissues during embryonic development and in adults (from wild type or genetically modified mice and zebrafish). *Ex vivo* analysis will be performed on isolated tissues and cells. Donor cells will also be isolated to perform transplantations (see Appendix 3.4.4.3)

Transplantation in recipients (pregnant females (embryos), newborns and adult mice) and *ex vivo* analysis (Appendix 3.4.4.3).
To determine the anatomical origin of hemogenic endothelium, cells isolated from the embryo proper, allantois and yolk sac will be tested by performing *in utero* transplantations in embryos in pregnant mice. To test pre-HSCs and HSCs (wild-type and mutant), transplantation will be performed in neonates and adults, respectively.
Transplanted embryos and neonates will be analysed when they reach the adult stage. Adult mice will be analysed up to 4 months post-transplantation. Tissues and cells will be collected for *ex vivo* analysis (e.g. long-term multi-lineage hematopoietic reconstitution of donor origin).

Transplantation in zebrafish, live imaging and *ex vivo* analysis (Appendix 3.4.4.4).
Genetically modified and wild type animals will be analysed for HSC production. For this purpose, the zebrafish will be subjected to transplantation, *in vivo* analysis (live time-lapse confocal imaging) and *ex vivo* analysis (histology, DNA/RNA analysis…).

Generation, welfare assessment and breeding of wild type and genetically modified zebrafish
Overall, we will generate genetically modified zebrafish but since no hampered phenotypes are expected, this activity does not require a CCD license.
(see https://www.centralecommissiedierproeven.nl/actueel/nieuws/16/10/13/handreiking-genetisch-gewijzigde-dieren ; genereren, fokken, genotypen, monitoren en huisvesten van genetisch gewijzigde dieren).

3.4.3 Describe the coherence between the different components and the different steps of the project. If applicable, describe the milestones and selection points.

See also Flow Chart in attachment.

All experiments are based on the preliminary identification of candidate genes or cell types. The identified genes or cell types will initially be carefully tested in tissues from embryo samples and in *in vitro* experiments. If the identified genes or cell types show an interesting expression pattern or phenotype, we will consider the extensive and careful analysis of (compound) GMO for our *in vivo* experiments. If the desired genotype is not available, we will create them ourselves. Before generating a new GMO line, the gene of interest will be studied based on the literature. The expression pattern of the gene in embryonic tissues will be carefully examined by in vitro analysis (e.g. in situ hybridization, flow cytometry analysis); go/nogo for the generation of a new GMO

We will only use well-established reagents and protocols to induce expression or deletion of the candidate gene/s in mice (Appendix 3.4.4.1) and zebrafish.

New GMO lines will only be generated for the most promising regulator genes. Their role will be tested on HSCs by in vivo transplantation assays. One transplantation experiment will be performed and analyzed to decide if the gene is indeed a potent interesting HSC regulator (e.g. observation of less mice reconstituted 4 months post-transplantation of GMO cells [where the gene is inactivated] compared to WT cells). Only then, more mice will be transplanted to reach n=3 independent experiments."

Our three sub-goals are of equivalent importance. Therefore, our strategy is to conduct experiments to try to answer all sub-goals in parallel (and not sequentially).

To address the role of candidate genes or cell types on hemogenic endothelium, pre-HSC and HSC production in the mouse model, *in vivo* transplantation will be performed in embryos, newborns and adult, respectively (Appendix 3.4.4.3). *Ex vivo* analysis on collected cells and tissues will be done to analyse the role of candidate genes or hematopoietic cells on the hematopoietic contribution.

In some cases we will use our animal lines without any intervention for the collection of tissues or cells for *ex vivo* analysis.

To address the role of candidate genes or cell types on HSC production in the zebrafish model, *in vivo* transplantation will be performed in adult (Appendix 3.4.4.4). Time-lapse live confocal imaging and *ex vivo* analysis on whole zebrafish and collected cells/tissues will be done to analyse the role of candidate genes or hematopoietic cells on the hematopoietic contribution, respectively.

Whenever possible, for all the *in vivo* experiments, we will perform pilot studies with the minimum amount of animals possible. It means that we will perform one experiment and will wait for the read-out result before to perform a complete set of experiments (e.g. we will wait for the result of the pilot experiment before to do the n=3 experiments requested to validate an analysis). We designed the experiments very carefully, to reduce the amount of cumulative discomfort and/or the number of animals. For each experiment, the best trade-off will be made.

3.4.4 List the different types of animal procedures. Use a different appendix 'description animal

procedures' for each type of animal procedure.

Serial number	Type of animal procedure
1	Generation of new GMO (mice)
2	Donor – *Ex vivo* analysis (mice, zebrafish)
3	Recipient – *Ex vivo* analysis (Transplantation in pregnant females, newborns and adult mice)
4	Transplantation in zebrafish, live imaging and *ex vivo* analysis (embryo till adult)
5	
6	
7	
8	
9	
10	

14.

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The cellular and molecular basis of the hematopoietic production

Overview number of animals and Animal Procedures

Procedure	Title	Species	Stage	Mild	Moderate
3.4.4.1	Generation of new GMO (mice)	Mouse	Adult		3000
3.4.4.2	*Donor – Ex vivo analysis (mice and zebrafish)*				
3.4.4.2a	Pregnant females for embryos	Mouse	Adult	2205	
3.4.4.2b	E18 embryos	Mouse	Embryos	378	
3.4.4.2c	Newborns	Mouse	Newborn	720	
3.4.4.2d	Adults	Mouse	Adult	126	
				3429	
3.4.4.3	*Recipient – Ex vivo analysis*				
3.4.4.3a	Pregnant females for embryo transplants	Mouse	Adult		240
3.4.4.3b	Resulting embryos	Mouse	Adult	1440	
3.4.4.3c	Transplants in newborns	Mouse	Newborn	2268	
3.4.4.3d	Transplants in adults	Mouse	Adult	2016	
				5724	
3.4.4.4	Transplantation in zebrafish, live imaging and ex vivo analysis	Zebrafish	Adult	4992	

Total:

12.393 mice of which 3240 will have maximally moderate discomfort (27%) and 9153 with mild discomfort (73%)

4992 zebrafish with maximally mild discomfort = 100%

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80100 - KNAW
1.2	Provide the name of the licenced establishment.	[redacted]
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.2 — Type of animal procedure: Donor – Ex vivo analysis (mice, zebrafish)

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The general design of the experiments in this appendix is shown in the attached flowchart (B).

To identify, localize and test hemogenic endothelial cells or precursors, HSC precursors (pre-HSCs) and HSCs, these cells will be isolated from WT or GMO. Cells and tissues will be collected from embryos, neonates and adults and tested in ex vivo assays and in transplantations (Flowchart B [mouse] and C [zebrafish]).

*** Animal models (mouse, zebrafish)**
There are several considerations to choose for a specific animal type:
- WT. They will be used to provide WT tissues or cells for ex vivo analysis (control) and to provide donor cells for transplantation experiments (see Appendix 3.4.4.3 [mouse] and 3.4.4.4 [zebrafish]).

- GMO will be used to provide donor tissues or cells for ex vivo analysis and to provide donor cells for transplantation experiments (see Appendix 3.4.4.3 [mouse] and 3.4.4.4 [zebrafish]).
GMO will be used to study the HSC function of a specific gene or type of (stem) cell. Several transgenic or mutant strains will be used. They are needed for several reasons: (1) the presence of a genetic marker is needed to trace the cells and their progenitors after transplantation; (2) to study a specific marker; (3) to study the influence of a gene (known to be important for hematopoiesis) upon the biology of (stem) cells.

In some cases, transgene inducing or deleting agents (e.g. tamoxifen) are administered to induce the expression of the fluorescent marker in a specific cell type/lineage or to change the expression of the functional gene (transgene knockout). The hematopoietic tissues and cells of the GMO will be collected (see Flowchart B1 and C1) and analysed ex vivo (see Flowchart B2 and C2). We will also use endogenous

and exogenous promotors that are tissue or cell specific to drive expression of genes. The presence of e.g. a fluorescent marker in a (putative) stem cell allows us to localize these cells by confocal imaging. The introduction of e.g. a toxin receptor (e.g., Diphtheria toxin receptor) in the cells allows us to specifically kill these cells upon the administration of the toxin (e.g. Diphtheria toxin). It allows us to determine the consequence of the loss of the toxin receptor expressing cells during development and for tissue homeostasis.

If a required GMO is not available, we will obtain this model by generating, importing or by crossing existing models (see Appendix 3.4.4.1).

The choice of animals will in all cases be based on the combination of the following considerations:
- Aim/ specific question (e.g. Identification of pre-HSCs, of HSCs, of hemogenic endothelial cells or precursors, of a cell type specific expression of a fluorescent marker for an immune-histochemical study)
- Aim/readout parameters (e.g. ex vivo assay)

*** Interventions:**
- Donor cells and tissues will be collected from embryos, neonates and adults (see Flowchart B and C).
- With the administration of small molecule compounds/drugs/chemicals/toxins, we might be able to rescue or mimic the phenotypes of the in vivo genetic deletion(s) and/or activation(s) and therefore further identify the function of these cells. The animals might be injected with DNA labelling agents shortly before euthanasia to measure the proliferation capacity of the stem cells and their derivatives ex vivo.

*** Readout parameters /endpoint:**
- To study the molecular and/or cellular mechanisms of HSC production, we need to analyse tissues and cells ex vivo. Cells and hematopoietic tissues will be collected from embryos, newborns and adults (WT and GMO).

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

- Animals are ordered from a commercial NVMA licensed breeder or from a registered commercial company, or are generated by us under appendix 3.4.4.1.
- Animals will be used as donor of tissues/cells for ex vivo studies or as donor for subsequent transplantations

*** Mouse model:**
- Donor pregnant females used to collect donor tissues/cells from embryos (see Flowchart B and B1). Pregnant female will be used to generate the donor embryos. The pregnant females will be euthanized by CO2/O2 followed by CO2 inhalation. After disinfection of the fur with 70% ethanol, a two-sided incision will be made in the fur and skin in the abdominal area. Using sterile forceps, the uterus will be pulled out and place in a culture dish-containing medium. The embryos and extra-embryonic tissues (allantois and yolk sac) will be isolated. Embryos from different ages (E8, E9, E10, E11, E12, E14 and E18) will be used to test the presence of pre-HSCs and HSCs in intra- and extra-embryonic tissues.

- Newborns and adult mice used as donors (see Flowchart B and B1)
Newborns and adult mice will be used to collect hematopoietic cells and tissues to test the presence of pre-HSCs and HSCs.

In some cases, we will perform the administration:
- transgene inducing or deleting agents or control substances, alone or in combination, continuously or intermittently by one or more of the following routes:
  a) in diet or drinking water (maximally 1 time, < 2 wks)
  b) subcutaneous (in general 2 times but maximally 3 times)
  c) intraperitoneal (in general 4 times but maximally 7 times)
  d) implantation of a slow release pellet subcutaneously under adequate anesthesia and analgesia (maximally 1 time)
  e) implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia (maximally 1 time)
  f) oral (in general 5 times but maximally 10 times)

- small molecule compounds, drugs, toxin, chemicals or control substances, alone or in combination, continuously or intermittently by one or more of the following routes:
  a) in diet or drinking water (maximally 1 time, < 2 wks)
  b) subcutaneous (in general 5 times but maximally 10 times)
  c) intraperitoneal (in general 5 times but maximally 10 times)
  d) implantation of a slow release pellet subcutaneously under adequate anaesthesia and analgesia (maximally 1 time)
  e) implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia (maximally 1 time)
  f) oral (in general 5 times but maximally 10 times)

- labeling agent (e.g. BrdU) via one of the following routes:
  a) intraperitoneal (maximally 1 time but maximally 3 times)
  b) implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia (maximally 1 time)
  c) intravenous (maximally 1 time)

None of the presented administration procedures will result in higher than cumulative mild discomfort.

Readout parameters /endpoint (see Flowchart B2)
The pregnant females will be kept under observation until delivery of the newborns. After birth and short weaning of the newborns (< 5 days), the mother will be euthanized by CO2/O2 followed by CO2 inhalation. Embryos and neonates (< 5 days) will be put on melting ice water for 10 min (but not in contact with) after which they will be decapitated and the head immediately frozen. The adult mice are sacrificed by CO2/O2 followed by CO2 inhalation or via cervical dislocation. Embryos and embryonic tissues (yolk sac, allantois), and neonate and adult tissues (e.g. blood, bone marrow, spleen, thymus, lymph nodes) will be collected for ex vivo analysis (e.g. in situ hybridization, flow cytometry analysis, molecular biological analysis (PCR), histological analysis to look at aspects of cell size, survival, proliferation and differentiation).

* Zebrafish model:
All donor tissues and cells will be collected before 5 dpf and, therefore, these zebrafish are not part of the license.

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

The animal numbers are mainly based on our preliminary experience. We know that the analysis of the requested number of animal per experiment guarantees us a proper analysis of these animals. We have tried to reduce the number of animals per experiments to a minimum. Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$.
Qualitative analysis (most of the animal procedures described in this appendix): the number of animals (group size) is based in literature and/or years of experience with similar type of experiments. Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of animals per group that will be informative. The very low number of cells available from early embryonic tissues (embryo proper, allantois or yolk sac) implicates the use of a higher number of embryos and therefore of pregnant female mice. All mice will be euthanized to collect the embryonic tissues and therefore will only be used once. The number of pregnant female mice (carrying donor embryos) needed has also been calculated according to the average number of embryos per litter (6 embryos/litter), to the number of embryos needed to test the different tissues at different embryonic stages, and the number of cells that will be available for analyses.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

All animals will be used to provide tissues/cells for ex vivo analysis and to provide donor cells for transplantation experiments (see Appendix 3.4.4.3 [mouse] and 3.4.4.4 [zebrafish]).
To note, the number of experiments needed is based on your experience over the last years and my expectation that my workgroup has a rather stable number of experienced researchers involved.

1- Mus musculus, (WT, GMO)
Genetic background: C57Bl6
Age; embryos, newborns, adults
Genders: both males and females will be used.
Origin: Mice are obtained from our own Institute or from external licensed breeders.
Number of animals (Total in 3.4.4.2): max. 3429

For the majority of the proposed studies, the mouse (beside the zebrafish) is one of the most appropriate animal model to use because: (1) like humans, mice are mammals; (2) physiology is more extensively characterized; (3) mice are amenable to transgenic modification; (4) a large number of relevant transgenic and knock out lines are already available.

- Donor pregnant females used to *collect donor tissues/cells from embryos and neonates*
Donor pregnant females are needed:
(i) to generate embryo, yolk sac and allantois.
Based on our previous experience, there is an average of 6 embryos/litter.
Seven embryonic stages (E8, E9, E10, E11, E12, E14 and **E18; see below**) will be tested because they correspond to a specific time points of hematopoietic (stem) cell specification, emergence, maturation, colonization and expansion. Pluggings will be performed with wild type and GMO. The experiments will be performed three times for each assay to be scientifically reliable.
The cell populations of putative precursors in the embryo proper, allantois and yolk sac are very small at early time points of development. Thus, many embryos will be needed to be able to perform *ex vivo* analysis. Moreover, only up to 50% of the pups in the litters will carry a donor marker (e.g. fluorescent marker) and thus an average of 3 pups will be used per litter, with 5 litters needed per experiment (for a total of 3 experiments). The mothers will be killed for each experiment.
Therefore we need:
→ 7 (embryonic stages) x 5 (females/experiments) x 21 (1 WT and 20 GMO) x 3 (experiments) = **2205 mice**.

- E18 embryos used to *collect donor tissues/cells*
Different hematopoietic tissues will be collected from WT and GMO E18 embryos for ex vivo analysis. An average of 6 embryos will be needed per experiment to collect enough cells and tissues. The experiments will be performed three times for each assay to be scientifically reliable.
Therefore we need:
→ 6 (E18 embryos) x 21 (1 WT and 20 GMO) x 3 (experiments) = **378 embryos**.

- Newborns used to *collect donor tissues/cells*
To determine the presence of Pre-HSCs in any given sample (Boisset *et al*. Blood, 2015), an average of 12 newborns will be used per experiments.
Therefore we need:
→ 120 (pregnant females – see above) x 6 (neonates) = **720 newborns**.

- Adults used to *collect donor tissues/cells*
Different hematopoietic tissues will be collected from WT and GMO for ex vivo analysis. An average of two mice will be needed per experiment to collect enough cells and tissues. The experiments will be performed three times for each assay to be scientifically reliable.
Therefore we need:
→ 2 (mice) x 21 (1 WT and 20 GMO) x 3 (experiments) = **126 mice**.

2- Zebrafish (WT, GMO):
All donor tissues and cells will be collected before 5dpf and therefore these zebrafish are not part of the

license.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☒ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Animal studies are unavoidable if we seek for the fundamental insight into the molecular and cellular mechanisms that drive HSC production and regulation.
We make extensive use of *in vitro* experiments where possible, which extensively reduces the animal numbers. The use of *in vitro* cultures/assays allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the mice numbers to be used to a minimum. Indeed, our *in vitro* data, consisting of flow cytometry analysis and *in situ* hybridization experiments, have permitted to tremendously reduce our long list of potent HSC regulators to 20 (see Preliminary data in part 3.1)).
Based on these data, available literature and interactions with other scientists, we will now study the functional role of these genes during endothelial specialization, endothelial into hematopoietic transition, pre-HSC maturation, HSC survival and/or expansion or involved in the supportive surrounding microenvironment. The functionality of these gene scan only be performed in vivo (in transplantation assays).
Whenever possible, we will perform pilot studies with the minimum number of animals possible.
Where possible, mice with inducible alleles will be used, so mice should not display a phenotype before the induction of the alleles.
Experiments will be done sequential, where on basis of the results, decisions will be made on the next steps.
If homozygous mice do not show any discomfort, we will keep the mice on a homozygous background, thereby reducing the number of mice.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

- Anaesthesia will be administered (by inhalation) to mice in case of blood collection (in general: Isofluraan (4%)).

- Analgesia will be administered to mice in case of pain (in general: Rimadyl)

- Parameters for health monitoring: Mice will be kept as groups. Food and drinking water will be provided ad libitum. Animals will be monitored for health status (welzijnsdagboek) by the animal caretakers. Animals with a decreased health status (such as weight loss, bristly fur, bent back) will be checked by the researcher as well. In case of 15% loss of body weight within 2 days, the mice will be euthanized. In case of 20% loss of body weight compared to the littermates, the mice will be euthanized.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

Other than in terminally anaesthetized animals dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that will result in no more than transient, mild discomfort. In less than 3% of all experiments, pumps/pellets will be implanted which leads to a moderate discomfort.
Due to administration of inducing agents or other substances, animals will be experiencing no follow up effects.
The scientific endpoints of all studies are much earlier than the humane endpoints.
It is not expected that the activation or inactivation of genes will lead to discomfort. However, it is impossible to predict the nature or severity of any potential genetic defect. So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day and analysed.

Explain why these effects may emerge.

Mice might have sickness and/or disorientation signs after waking up from anesthesia (after blood

collection) and have some pain due to the surgery. Analgesia will be administered accordingly.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Monitoring of the animal combined with an experimental design aiming at reducing the discomfort of the animals. If we unexpectedly observed weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort animals will be taken out of experiment and sacrificed.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behaviour or body posture, and signs of general sickness and/or discomfort.

Indicate the likely incidence.

Expected <5%, mild <1day.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Pregnant females; n = 2205; Mild
E18 embryos; n=378; Mild
Newborns; n = 720; Mild
Adult mice; n = 126; Mild
Total = 2331 adult mice and 720 newborns

- Waking up from anaesthesia (after blood collection): Mice might have sickness and/or disorientation signs. Mild, < 1 day, expected 100%.

- Sacrifice: Mild, < 10 minutes, 100% animals

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

The animals need to be killed to collect embryos, cells or tissues for *ex vivo* analysis and to provide donor cells for transplantation experiments.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☐ No > Describe the method of killing that will be used and provide justifications for this choice.

☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80100 - KNAW
1.2	Provide the name of the licenced establishment.	[redacted]
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.3 Type of animal procedure: Recipient – *Ex vivo* analysis (Transplantation in pregnant females, newborns and adult mice)

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

The general design of the experiments in this appendix is shown in the attached flowchart (B3 and B4).

To identify and test hemogenic endothelial cells or precursors, HSC precursors (pre-HSCs) and HSCs isolated from WT or GMO (see Appendix 3.4.4.2), we need to perform in vivo transplantation assays in embryos in pregnant females (1), newborns (2) and adults (3), respectively (Flowchart B3). The different ages of the recipients provide different permissive microenvironment and allow the engraftment of various cell types (from the embryo to adult age). Hematopoietic tissues and cells of all transplanted recipients (embryos, neonates, adults) will be analysed ex vivo at 4 months post-transplantation (Flowchart B4).

(1) To determine the anatomical origin of hemogenic endothelial cells or precursors, cells (isolated from donor embryos, see Appendix 3.4.4.2) will be tested by performing transplantations in embryo recipients directly in pregnant recipient females (E7-E10). The recipient embryos will be analysed when they reach the adult stage for long-term donor multi-lineage hematopoietic reconstitution.
(2) To identify pre-HSCs, isolated candidate donor cells (see Appendix 3.4.4.2) are transplanted in newborns or immuno-deficient recipient mice (a more permissive environment for immature cells).
(3) To identify HSCs, isolated candidate donor cells (see Appendix 3.4.4.2) are transplanted in adult recipient mice.
In all cases, the primary recipients are analysed up to 4 months post-transplantation (to prove the multi-lineage property of the donor cells). Secondary transplantations are performed to prove the self-renewal property of donor cells. These secondary recipients are also analysed 4 months post-transplantation.

*** Mouse model**

There are several considerations to choose for a specific mouse model. WT and GMO mice will be used as recipients.

- WT and GMO:

GMO will be used to study the HSC function of a specific gene or type of (stem) cell. Several transgenic or mutant mouse strains will be used. They are needed for several reasons: (1) the presence of a genetic marker is needed to trace the cells and their progenitors after transplantation; (2) to study a specific marker; (3) to study the influence of a gene (known to be important for hematopoiesis) upon the biology of (stem) cells.

In some cases, transgene inducing or deleting agents (e.g. tamoxifen) are administered to induce the expression of the fluorescent marker in a specific cell type/lineage or to change the expression of the functional gene (transgene knockout). We will also use endogenous and exogenous promotors that are tissue or cell specific to drive expression of genes. The presence of e.g. a fluorescent marker in a (putative) stem cell allows us to localize these cells by confocal imaging. The introduction of e.g. a toxin receptor (e.g., Diphtheria toxin receptor) in the cells allows us to specifically kill these cells upon the administration of the toxin (e.g. Diphtheria toxin). It allows us to determine the consequence of the loss of the toxin receptor expressing cells during development and for tissue homeostasis.

To identify/validate for example which molecular and cellular processes are important for HSC production and regulation, we will analyse GMO in which cells/tissues are transplanted and the gene(s) will be activated, inactivated, overexpressed and/or misexpressed. These mice will be used as recipients (see Flowchart B3). We will use inducible systems including e.g. the well-known Cre-LoxP system in which a floxed gene can be deleted, overexpressed or misexpressed upon the induction of the Cre enzyme via the administration of tamoxifen. In particular, when Cre is expressed in mice harbouring a LoxP-containing target gene, the desired gene modification can be restricted to certain developmental stage, organ, cell type (e.g. stem cell) of the mouse depending on the specificity and timing of recombinase expression. In this way the desired gene modification can be restricted to certain developmental stage, organ, cell type (e.g. stem cell). Successful deletion/activation via (e.g.) Cre enzyme induction might be monitored via expression of a reporter gene (e.g. LacZ or fluorescent protein(s)).

If a required genetically modified mouse is not available, we will obtain this model by generating, importing or by crossing existing models (see Appendix 3.4.4.1). WT recipients serve as control.

- Immune-deficient or newborn recipients. Immune-deficient mice are required in some cases as recipients since they are more permissive to the engraftment of very immature cells.

The choice will in all cases be based on the combination of the following considerations:

- Aim/ specific question (e.g. Identification of pre-HSCs, of HSCs, of hemogenic endothelial cells or precursors, of a cell type specific expression of a fluorescent marker for an immune-histochemical study)
- Aim/readout parameters (e.g. to test long-term hematopoietic repopulation)

**** Interventions:***

- Cells will be transplanted intra-cardiac in embryo recipients, intra-liver in newborn recipients and intravenously in adult recipients (see Flowchart B3). Newborns and adult recipients will be irradiated. The irradiation is performed to clear out the bone marrow and liver from all proliferating cells and therefore to make space. It allows the engraftment of the injected donor cells.
- To identify/validate for example which molecular and cellular processes are important for HSC production and regulation, we will analyze wild type mice or GMO (in which functional gene(s) will be activated, inactivated, overexpressed and/or misexpressed) after transplantation. The presence of e.g. a fluorescent marker in transplanted cells allows us to visualize these cells and their progeny.
- With the administration of small molecule compounds/drugs/chemicals/toxins, we might be able to rescue or mimic the phenotypes of the *in vivo* genetic deletion(s) and/or activation(s) and therefore further identify the function of these cells *in vivo*. The mice might be injected with DNA labeling agents shortly before euthanasia to measure the proliferation capacity of the stem cells and their derivatives.

**** Readout parameters /endpoint:***

To study the molecular and/or cellular mechanisms of HSC production, we need to analyze tissues and cells *ex vivo*. Cells and hematopoietic tissues will be collected from up to 4 months after transplantation (see Flowchart B4).

In some cases, we will need to collect cells with a certain genetic constitution to transplant back into wild type or GMO (secondary transplantation).

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

Mice are ordered from a commercial NVMA licensed breeder or from a registered commercial company, or are generated by us under appendix 3.4.4.1.

- Recipient pregnant females used for the in-uterus transplantation in E7-E10 embryos (see Flowchart B3)

Pregnant females will be used to generate recipient embryos (immune-deficient or wild type). Between E7 to E10 post-coitus, the pregnant females will be anaesthetized in a knock down box and then maintained by facemask with 2-3% Isoflurane. Hairs will be removed on the abdomen using a depilatory cream and then the abdomen will be rinsed with water. The female will be positioned on a platform ventral side up. Eye lubricant will be placed on each eye to prevent drying. A small amount of ECG gel will be placed on the copper leads on the platforms and the paws taped on them. This will provide the ECG and respiratory physiology of the mouse. A rectal probe will be inserted to monitor the temperature of the animal during the imaging session and will be maintained at 35-38ºC using a heating pad and a lamp. The stage upon which the mouse is placed is tilted head down (between 35–45°) to displace the bowel. Maternal well-being will be carefully monitored to maintain an appropriate body temperature, heart rate, respiratory vigour, and anaesthetic depth throughout the procedure. After sterilizing the skin with 70% ethanol, the abdomen will be opened with a 2 cm vertical midline incision along the linea alba (midline avascular region). A short segment of the uterine horns containing 1-3 embryos will be gently exteriorized from the abdominal cavity by using sterile forceps. With an injection needle, a small hole will be made in the uterus. Through this hole, the cells will be injected into the heart cavity of the embryo by using a glass transfer needle in combination with a micro-injector (guided by a high resolution ultrasound apparatus). This will allow the injection of very small volumes of cells (50 nL) while causing little damage to the tissue. The embryo is positioned in such a way that the heart is visible with the high-resolution ultrasound detector. Special care will be made for the tissues through which the needle will pass through before hitting the target of interest (the heart), especially by avoiding passing through the placenta (that might compromised the embryo development or generate bleeding). All embryos from the litter will be injected. After injection, the uterus will be guided back into the abdomen, muscle layer will be stitched and the skin will be closed with staples by using a wound clamp. The whole procedure will take about 20-30 minutes. To prevent cooling down of the recovery mouse, the cage will be put on a heating plate.

- Newborns and adult mice used as recipients (see Flowchart B3)

Transplantation of cells will be performed under adequate anaesthesia and analgesia.

→ Transplantation in **newborns**

* Wild-type or immuno-deficient newborns (1 to 5 days old) will be used. WT newborns will be treated to compromise their hematopoietic/immune system (by irradiation of the newborns [using a gamma-source] or by busulfan treatment of the pregnant females [to compromise the hematopoietic/immune system of the litter while still inside the uterus of the mother]). For irradiation, neonates will receive a single dose of 5 Gy. During irradiation, mice will be kept as a group in a cage without anaesthesia.

* Donor cells are injected in the livers of the newborns (max. 0.05 ml).

* Mice will receive antibiotics (such as neomycin) in the drinking water for the first 3 months post transplantation. The animal being irradiated, they are more subject to bacterial infections (until their immune system is restored. The antibiotics will prevent such possible infection.

→ Transplantation in **adults** (primary and secondary transplantation)

* Adult wild-type mice (>8 weeks) will be treated to eliminate their hematopoietic/immune system by irradiation using a gamma-source or 5-FU treatment. For irradiation, adults will receive a sub-lethal dose as a split dose (4 and 5 Gy with 3 hours in between). During irradiation, mice will be kept as a group in a cage without anaesthesia.

* Donor cells are injected intravenously (max. 0.2 ml) in the tail vein.

* Mice will receive antibiotics (such as neomycin) in the drinking water for the first 3 months

post transplantation to prevent bacterial infections.
Cells of reconstituted recipients will serve as donor cells for secondary transplantations (to test the self-renewal of the cells, second specific feature of a HSC). The adult secondary recipients will receive a sub-lethal dose of irradiation as a split dose (4 and 5 Gy with 3 hours in between), as described above.

All transplanted animals will be kept under observation and analysed at adult stage when they are older than 8 weeks old (see Flowchart B4).

Summary of the transplantation procedures:

Assay	**To test/To identify**	**Donor cells** *(see Appendix 3.4.4.2)*	**Recipient**
In uterus transplantation	Hemogenic endothelial cells (or precursors)	E8 embryo, yolk sac, allantois	Embryo (E7-E10)
Newborn transplantation	Pre-HSCs	Embryonic tissues (E8-E10)	Newborn (P1-P5)
Adult transplantation	HSCs	Embryonic tissues (E10-E12, E14 and E18)	Adult mice (> 8 weeks)

In some cases, we will perform the administration of:
- transgene inducing or deleting agents or control substances, alone or in combination, continuously or intermittently by one or more of the following routes:
  a) in diet or drinking water (maximally 1 time, < 2 wks)
  b) subcutaneous (in general 2 times but maximally 3 times)
  c) intraperitoneal(in general 4 times but maximally 7 times)
  d) implantation of a slow release pellet subcutaneously under adequate anesthesia and analgesia (maximally 1 time)
  e) implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia (maximally 1 time)
  f) oral (5 times but maximally 10 times)

- small molecule compounds, drugs, toxin, chemicals or control substances, alone or in combination, continuously or intermittently by one or more of the following routes:
  a) in diet or drinking water (maximally 1 time,, <2 wks)
  b) subcutaneous (in general 5 times but maximally 10 time)
  c) intraperitoneal (in general 5 times but maximally 10 times)
  d) implantation of a slow release pellet subcutaneously under adequate anaesthesia and analgesia (maximally 1 time)
  e) implantation of a slow release pump subcutaneously under adequate anesthesia and analgesia (maximally 1 time)
  f) oral (in general 5 times but maximally 10 times)

- labeling agent (e.g. BrdU) via one of the following routes:
  a) intraperitoneal (in general 1 time maximally 1 times)
  b) implantation of a slow release pump subcutaneously under adequate anaesthesia and analgesia (maximally 1 time)
  c) intravenous (maximally 1 time)

None of the presented administration procedures will result in higher than cumulative mild discomfort.

Readout parameters /endpoint (see Flowchart B4)
After transplantation, the recipient animals will be kept under observation. In the case of transplantation in embryos, the pregnant females will be kept under observation until delivery of the pups. The pups will be checked at birth for viability. Pups will be kept (males and females separated) and analyzed at adult stage.
Blood (maximum volume at the different time points: 300 µl) will be collected at 1 or 2 time points: 1-2

months and/or 4-6 months post transplantation to determine the donor cell contribution in the peripheral blood. At the end of the experiment (4-6 months post transplantation), all mice are sacrificed by CO2/O2 followed by CO2 inhalation or via cervical dislocation, and tissues/cells are collected for ex vivo analysis. The long-term multi-lineage hematopoietic reconstitution will be tested by flow cytometry analysis or by molecular biological analysis (PCR on GFP). For this, cells or DNA will be isolated from the different adult mouse hematopoietic tissues (blood, bone marrow, spleen, thymus, lymph nodes). Tissue will be collected for histological and molecular analysis to look at aspects of cell size, survival, proliferation and differentiation. In some cases, cells will be collected to perform secondary transplantations.
Embryos and newborns (< 5 days) will be put on melting ice water for 10 min. (but not in contact with) after which they will be decapitated and the head immediately frozen.
After birth and weaning of the pups (that are used as embryo recipients), the mother will be euthanized by CO2/O2 followed by CO2 inhalation. For post-surgery pain relief, when anaesthetised, a s.c. dose of Rimadyl will be given in general. One injection will be made once pre-operatively and repeated once 24 hours after surgery. We have expertise in all the surgical procedure steps needed for the experiments.

The cumulative discomfort in the genetic models will maximally consist of discomfort due to:
- the genetic modification (< 5% of lines)
- transplantation of cells under adequate anaesthesia and analgesia
- sacrifice

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

The mice numbers are mainly based on our preliminary experience. We know that the analysis of the requested number of mice per experiment guarantees us a proper analysis of these mice. We have tried to reduce the number of animals per experiments to a minimum. Whenever possible we strive for a situation to express the outcome of our experiments in quantitative terms. To estimate the number of animals to be used in an experiment, we use the effect size (if known, e.g., from data in the literature, from our own historical data or if unknown from small scale pilots) to estimate the sample size needed to achieve a certain power (usually around 0.8) with appropriate statistical test like the t test with a $p<0.05$.
Qualitative analysis (most of the animal procedures described in this appendix): the number of animals (group size) is based in literature and/or years of experience with similar type of experiments.
Moreover, these types of experiments will be performed sequentially via which we ensure that we will use the minimum number of mice per group that will be informative. The very low number of cells available from E8 tissues (embryo proper, allantois and yolk sac) implicates the use of a higher number of embryos and therefore of pregnant females. All mice will be euthanized to collect the embryonic tissues and therefore will only be used once. The number of pregnant females (carrying recipient embryos) needed has also been calculated according to the average number of embryos per litter (6 embryos/litter), to the number of embryos needed to test the different tissues at E8, and the number of cells that will be available for analyses (very low at E8).

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Mus musculus, (WT, genetically modified, mutants, immuno-deficient mice)
Genetic background: C57Bl6
Age; embryos, newborns, adults
Origin: Mice are obtained from our own Institute or from external licensed breeders.
Genders: both males and females will be used.

Number of animals (Total): max. 5964

Up to today, there are no alternative methods than in vivo transplantation to fully study HSC production and regulation in the context of the whole organism. For the majority of the proposed studies, the mouse is one of the most appropriate animal model because: (1) like humans, mice are mammals; (2) physiology is more extensively characterized; (3) mice are amenable to transgenic modification; (4) a large number of relevant transgenic and knock out lines are already available.

To note, the number of experiments needed is based on your experience over the last years and my

expectation that my workgroup has a rather stable number of experienced researchers involved.

A) In utero transplantation in embryos in pregnant females

- Pregnant females used to generate E7-E10 recipient embryos

The recipient females give an average litter size of 6. We need a minimum of 12 pups (2 litters) injected for a valid and reliable conclusion. Pups will be injected with 3 different cell populations from E8 allantois, yolk sac and embryo proper (= 3 kind of tissues). All embryos from the litter will be injected by the same cell type (since embryos cannot be marked at that stage to be discriminated at birth). The pregnant mice will be injected at E7, E8, E9 and E10) because the stage that will provide the best engraftment is unknown yet. Because the intra-cardiac injection in embryos is difficult, we know by experience that an average of 10 experiments are needed per tissue tested to achieve a reliable (and therefore publishable) data.
Therefore we need:
→ 4 (embryonic stages) x 2 (females/experiments) x 3 (kind of tissues) x 10[#] (experiments) = **240 transplanted pregnant mice**.
[#] Intra-cardiac injection in developing embryos is very difficult to achieve. Sometimes (i) the needle misses the heart and cells are not injected properly, (ii) the embryo does not survive to the procedure or (iii) there is a blockage in the needle and all cells cannot be injected. For these reasons, we estimated that 10 experiments are needed for a reliable conclusion (in comparison to all other experiments [that are technically easier and 100% successful in our hands] where 3 experiments are needed for statistical analysis).

- Growing transplanted embryo recipients (that will be analysed at adult age)

The amount of transplanted embryo recipients to analyse for long-term multi-lineage reconstitution at adult age is:
→ 240 (transplanted pregnant females – see above) x 6 (embryos/litter) = **1440 adults transplanted when at embryonal stage**.

B) Transplantation in newborns (pre-HSC assay)

- Newborn recipients for transplantation:

To determine the presence of Pre-HSCs in any given sample, we need to do transplantations in newborn recipients (Boisset *et al*. Blood, 2015).
We need a minimum of 12 newborns injected for a valid and reliable conclusion. Newborns will be injected with cells isolated at 3 different time points (E8, E9 and E10; 3 time points when pre-HSCs are detectable; isolated under AP 3.4.4.2). The experiments will be performed three times for each assay to be scientifically reliable with WT and GMO lines.
Therefore we need:
→ 3 (embryonic stages) x 12 (newborns) x 21 (1 WT and 20 GMO) x 3 (experiments) = **2268 newborn recipients**.

C) Transplantation in adults (HSC assay)

- Adult recipients for transplantation:

To determine the presence of HSCs in any given sample, we need to do transplantations in adult recipients (Harrison et al. Experimental Hematology, 1993; Szilvassy et al. PNAS, 1990). Adults will be injected with donor cells isolated at 5 different time points (E10, E11, E12, E14 and E18; 5 time points when HSCs are detectable in different tissues; isolated under AP 3.4.4.2). Based on literature and our own experience we estimate the variation per group at 15% (= anticipated coefficient of variation). The difference that we need to be able to detect is 15% (= difference considered meaningful). To get a power of 0.8 with the aforementioned percentages, we will need a group size of 18 animals in total (Van Zutphen et al., Principles of laboratory animal science). For secondary transplantations (a requirement for the self-renewal feature of a HSC), we use 6 recipients in total (standard in the field).
Therefore we need:

→ 5 (embryonic stages) x 18 (6 mice x 3 experiments) x 21 (1 WT and 21 GMO) = **1890 adult recipients** (1st Tx)
→ 6 (2 mice x 3 experiments) x 21 (1 WT and 20 GMO) = **126 adult recipients** (2nd Tx)

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☒ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

Animal studies are unavoidable if we seek for the fundamental insight into the molecular and cellular mechanisms that drive HSC production and regulation.
We make extensive use of *in vitro* experiments where possible, which extensively reduces the animal numbers. The use of *in vitro* cultures/assays allows this project to comply with the 3Rs (replacement, reduction and refinement) by keeping the mice numbers to be used to a minimum. Indeed, our *in vitro* data, consisting of flow cytometry analysis and *in situ* hybridization experiments, have permitted to tremendously reduce our long list of potent HSC regulators to 20 (see Preliminary data in part 3.1)).
Based on these data, available literature and interactions with other scientists, we will now study the functional role of these genes during endothelial specialization, endothelial into hematopoietic transition, pre-HSC maturation, HSC survival and/or expansion or involved in the supportive surrounding microenvironment. The functionality of these gene scan only be performed in vivo (in transplantation assays).
Whenever possible, we will perform pilot studies with the minimum number of animals possible.
Where possible, mice with inducible alleles will be used, so mice should not display a phenotype before the induction of the alleles.
Experiments will be done sequential, where on basis of the results, decisions will be made on the next steps.
If homozygous mice do not show any discomfort, we will keep the mice on a homozygous background, thereby reducing the number of mice.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

All possibilities to reduce pain, fear or suffering will be used. There are no negative environmental effects.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The experiments in this project are not carried out due to legal requirements.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III

of the Directive 2010/63/EU?

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

- Anaesthesia will be administered (by inhalation) in case of:
* blood collection (at 1 and/or 4 months post transplantation) – Isofluraan (4%), in general.
* to the pregnant females during all the intra-uterus transplantation procedure – Isofluraan (2-3%) , in general.

- Analgesia will be administered in case of pain (such as Rimadyl) to:
* the transplanted mice
* the pregnant females. One injection will be made once pre-operatively and repeated once 24 hours after surgery. The pregnant females will be checked regularly (until delivery) after in utero cell transplantation and also on the delivery day (and the following week). Pups should develop normally. If abnormality or discomfort is visible, the pups will be sacrificed by CO2/O2 followed by CO2 inhalation. Animals will be sacrificed (and tissues collected) if unexpected illness occurs (by CO2/O2 followed by CO2 inhalation).
- Parameters for health monitoring: Mice will be kept as groups. Food and antibiotic drinking water (such as neomycin) will be provided ad libitum. Animals will be monitored for health status (welzijnsdagboek) by the animal caretakers. Animals with a decreased health status (such as weight loss, bristly fur, bent back) will be checked by the researcher as well. In case of 15% loss of body weight within 2 days, the mice will be euthanized. In case of 20% loss of body weight compared to the littermates, the mice will be euthanized.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

Other than in terminally anaesthetized animals dosing and sampling procedures will be undertaken using a combination of volumes, routes and frequencies that will result in no more than transient, light mild discomfort. In less than 3% of all experiments, pumps/pellets will be implanted which leads to a mild discomfort.

Due to administration of inducing agents or other substances, animals will be experiencing no follow up effects.
The scientific endpoints of all studies are much earlier than the humane endpoints.

It is not expected that the activation or inactivation of genes will lead to discomfort. However, it is impossible to predict the nature or severity of any potential genetic defect. So, in all cases, animals will be careful monitored for possible side effects. Animals exhibiting any unexpected harmful phenotypes will be killed within a day and analysed.

Explain why these effects may emerge.

Mice might have sickness and/or disorientation signs after waking up from anesthesia and have some pain due to the surgery. Analgesia will be administrated accordingly.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

Monitoring of the mice combined with an experimental design aiming at reducing the discomfort of the animals. If we unexpectedly observed weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behavior or body posture, signs of general sickness and/or discomfort animals will be taken out of experiment and sacrificed.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

Weight loss (>20% in comparison with control or 15% weight loss in two days), changes in behaviour or body posture, and signs of general sickness and/or discomfort.

Indicate the likely incidence.

Expected <5%, moderate <1day.

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

Recipient pregnant females; n = 240 mice; Moderate
Growing transplanted embryo recipients; n = 1440 mice; Mild
Newborn recipients; n = 2268 mice; Mild
Adult primary recipients; n = 1890 mice; Mild
Adult secondary recipients; n = 126 mice; Mild
Total = 5964 mice

- After irradiation: The animals might develop a decreased health status during the first month (loss of weight). Mild, 7-30 days, expected <10%.
- After transplantation in adult: Mice might be more sensitive to infections. Mild, 7-30 days, expected <10% (because of antibiotics in the drinking water).
- After in utero transplantation (because of the surgery): Moderate, 1 day, 100% animals
- Waking up from anaesthesia (after in utero transplantation, blood collection): Mice might have sickness and/or disorientation signs. Mild, < 1 day, expected 100%.
- Sacrifice: Mild, < 1 minute, 100% animals

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

The animals need to be killed to collect embryos, cells or tissues and for ex vivo analysis.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?
☐ No > Describe the method of killing that will be used and provide justifications for this choice.
☒ Yes

Appendix
Description animal procedures

- This appendix should be enclosed with the project proposal for animal procedures.
- A different appendix 'description animal procedures' should be enclosed for each type of animal procedure.
- For more information, see our website (www.centralecommissiedierproeven.nl).
- Or contact us by phone (0900-2800028).

1 General information

1.1	Provide the approval number of the 'Netherlands Food and Consumer Product Safety Authority'.	80100 - KNAW
1.2	Provide the name of the licenced establishment.	[redacted]
1.3	List the serial number and type of animal procedure. *Use the serial numbers provided in Section 3.4.4 of the Project Proposal form.*	Serial number: 3.4.4.4 Type of animal procedure: Transplantation in Zebrafish, live imaging and ex vivo analysis.

2 Description of animal procedures

A. Experimental approach and primary outcome parameters

Describe the general design of the animal procedures in relation to the primary outcome parameters. Justify the choice of these parameters.

Wild type or genetically modified zebrafish will be used to study HSC production and regulation during embryonic development and in adult.

Describe the proposed animal procedures, including the nature, frequency and duration of the treatment. Provide justifications for the selected approach.

The zebrafish (as the mouse) is now firmly established as a reliable developmental and genetic model to study vertebrate hematopoiesis. To enable the study of HSC production and regulation in zebrafish, we will perform HSC manipulation into embryos (e.g. tamoxifen injection) and/or transplantation in adult recipient animals (hematopoietic mutant or wild type conditioned by gamma-irradiation ([redacted] (see Flowchart C3). HSC transplantation will allow to study cell autonomy of mutant gene function and to rescue multi-lineage hematopoiesis in embryonic lethal mutants. Transplant recipients are anesthetized in tricaine (in general) and injected intra-cardiac by using glass capillary needles.

The transplanted adult animals (>3 months old) are visualized weekly under an inverted fluorescent microscope to monitor donor fluorescent cells over the first 30 days after transplantation.
In some cases (analyze the function of specific genes), fish treated between 48-72 hours post fertilization with a tamoxifen injection will be grown to 5 months. They will be then subjected to split dose 30Gy irradiation and transplanted. This high dose of irradiation is provided to eliminate the hematopoietic cells of the fish recipient, which is necessary to allow the engraftment of the donor cells. The irradiation dose has been chosen based on the literature published by various laboratories

performing transplantation experiments in zebrafish.

At a chosen end point (depending of the research question, between 6 to 20 weeks post irradiation/transplantation), the animals will be anesthetized (in general) with 0.02% tricaine prior to collect the hematopoietic tissues for *ex vivo* analysis (e.g. histology, *in situ* hybridization, RNA/DNA isolation) (see Flowchart C4). Different *ex vivo* analysis techniques require different handling of the extracted tissue (e.g. cryo-sectioning or paraffin embedding). If possible the *ex vivo* techniques will be combined.

Some zebrafish embryo will also be used for time-lapse live confocal imaging. In our experience the imaging method [redacted] is the most reliable method. Direct visualization of fluorescent donor cells in embryonic recipients allows engraftment and homing events to be imaged in real time. These results provide a cellular context in which to study the genetics of hematopoiesis. During imaging, embryos are anaesthetized (in general) with 0.02% tricaine in embryo buffer. The imaging will be done once for each zebrafish embryo for up to 24h (age of embryos: below 5 days post fertilization (dpf) and, therefore, are no part of the licence project).

Describe which statistical methods have been used and which other considerations have been taken into account to minimise the number of animals.

- It is our experience (in our institute and in the literature) that in general 16 (transplanted) adult animals are needed to collect tissues/cells (Flow chart C4) and 30 embryos for live confocal imaging per condition. These numbers are based on common scientific practice in our research field to allow publication.
- Different ex vivo analysis techniques require different handling of the extracted tissue (e.g. cryo-sectioning or paraffin embedding). To reduce the number of animals that are required we will combine ex vivo assays for different parameters as much as possible.

B. The animals

Specify the species, origin, estimated numbers, and life stages. Provide justifications for these choices.

Maximum of zebrafish: 4,992
Genders: both males and females will be used.

To note, the number of experiments needed is based on your experience over the last years and my expectation that my workgroup has a rather stable number of experienced researchers involved.
- Zebrafish (Danio rerio) is one of the most well characterized fish. Zebrafish are well maintained and bred in a laboratory condition. Furthermore, the zebrafish model comes with numerous tools, which can be used to fully exploit its potential, including a fully annotated genome and efficient genome editing strategies.
- The zebrafish used here are bred [redacted].
- To test HSC engraftment or to test the effect of a treatment or genetic condition on HSC production a maximum of 16 animals (8 controls and 8 treated) are required for each type of analysis. To test HSC engraftment, the animals will be analysed at 1 time point (adult). To test the effect of a treatment or genetic condition, it will be required to analyse up to 3 time points (time point of HSC emergence in the embryo aorta, time point of HSC colonization/expansion in the embryo CHT, time point of colonization of adult hematopoietic organs). In all cases, we will measure up to 4 different *ex vivo* parameters (e.g. gene expression, lineage tracing, cell proliferation, cell apoptosis,) requiring different fixation and embedding techniques (See Flowchart C4).
Experiments will be performed on embryos and adults. However, experiments on zebrafish < 5 dpf is not considered as an animal experiment. Therefore, only the experiments on adult animals are part of the license and only these animals are counted. Therefore, we will use:
16 animals (8 controls and 8 treated) x 1 time point (adult) x 4 (*ex vivo* parameters) x 3 (experiments) = **192 fish**

It is expected that during the duration of this project we will test 25 genetically modified or knockout lines. Tissues and cells will be collected on all animals. Therefore we will use:
16 animals (8 controls and 8 treated) x 1 time point (adult) x 4 (*ex vivo* parameters) x 25 (genetically

modified or knockout lines) x 3 (experiments) = **4800 fish**

- Live confocal imaging will be performed on embryos (<5dpf and therefore no part of license project). It is our experience that 30 embryos per condition are needed to analyse EHT and HSC production. This embryo number is common scientific practice in the research field to achieve reliable and publishable results.

C. Re-use

Will the animals be re-used?

☒ No, continue with question D.

☐ Yes > Explain why re-use is considered acceptable for this animal procedure.

Are the previous or proposed animal procedures classified as 'severe'?

☒ No

☐ Yes> Provide specific justifications for the re-use of these animals during the procedures.

D. Replacement, reduction, refinement

Describe how the principles of replacement, reduction and refinement were included in the research strategy, e.g. the selection of the animals, the design of the procedures and the number of animals.

- Replacement: HSC production needs to be studied *in vivo*. There are no *in vitro* models available to study HSC production. Zebrafish are easy to maintain in an animal facility and are a well-characterized model to study HSC production.
- Reduction: *Ex vivo* assays will be combined as much as possible to reduce the number of animals that are required. The experiments will be executed in a phased order so that follow up experiments are only executed when pilot experiments show a positive outcome.
- Refinement: Animals will be anaesthetized in general in Tricaine solution to reduce the discomfort of the treatment.

Explain what measures will be taken to minimise 1) animal suffering, pain or fear and 2) adverse effects on the environment.

In order to minimize the animal suffering, all procedures to reduce pain, fear or suffering will be used. There are no negative environmental effects. Procedures will only be performed by competent personnel.

Repetition and duplication

E. Repetition

Explain what measures have been taken to ensure that the proposed procedures have not already been performed. If applicable, explain why repetition is required.

The proposed procedures are for fundamental research. It does not consist of legally required research.

Accommodation and care

F. Accommodation and care

Is the housing and care of the animals used in experimental procedures not in accordance with Annex III of the Directive 2010/63/EU?

☒ No

☐ Yes > If this may adversely affect animal welfare, describe how the animals will be housed and provide specific justifications for these choices.

G. Location where the animals procedures are performed

Will the animal procedures be carried out in an establishment that is not licenced by the NVWA?

☒ No > Continue with question H.

☐ Yes > Describe this establishment.

Provide justifications for the choice of this establishment. Explain how adequate housing, care and treatment of the animals will be ensured.

Classification of discomfort/humane endpoints

H. Pain and pain relief

Will the animals experience pain during or after the procedures?

☐ No > Continue with question I.

☒ Yes > Will anaesthesia, analgesia or other pain relieving methods be used?

☐ No > Justify why pain relieving methods will not be used.

☒ Yes > Indicate what relieving methods will be used and specify what measures will be taken to ensure that optimal procedures are used.

The animals will be anaesthetized during transplantation with proper anaesthesia (in general with MS222 - Tricaine methanesulfonate, 0.02%). We do not expect stress and discomfort in animals during transplantation.
Anesthesia will also be performed (in general with 0.02% tricaine) before blood (obtained by cardiac punction), kidney and spleen collection.
Transplanted recipients will be anesthetized in general in tricaine and immobilised in individual conical wells made in 2% agarose. Cells will be injected into the sinus venosus of the embryo at 48-72hpf or in adults. Recipients will be maintained in medium containing penicillin and streptomycin during and for several hours after transplantation to prevent infection.

I. Other aspects compromising the welfare of the animals

Describe which other adverse effects on the animals' welfare may be expected?

No other adverse effects on the animals' welfare are expected.

Explain why these effects may emerge.

n.a.

Indicate which measures will be adopted to prevent occurrence or minimise severity.

J. Humane endpoints

May circumstances arise during the animal procedures which would require the implementation of humane endpoints to prevent further distress?

☐ No > Continue with question K.

☒ Yes > Describe the criteria that will be used to identify the humane endpoints.

The animals will be checked daily for signs of general sickness and discomfort (e.g. laying on the bottom of the tank, rapid gill movements).

Indicate the likely incidence.

Expected in <1%; mild discomfort no longer than 1 day (due to the irradiation and transplantation).

K. Classification of severity of procedures

Provide information on the expected levels of discomfort and indicate to which category the procedures are assigned ('non-recovery', 'mild', 'moderate', 'severe').

n = 4,992 zebrafish; Mild
Animals reaching the humane end-point will be euthanized.

End of experiment

L. Method of killing

Will the animals be killed during or after the procedures?

☐ No

☒ Yes > Explain why it is necessary to kill the animals during or after the procedures.

Transplanted zebrafish will be killed to collect hematopoietic tissues/cells for ex vivo analysis.

Is the proposed method of killing listed in Annex IV of Directive 2010/63/EU?

☒ No > Describe the method of killing that will be used and provide justifications for this choice.

Zebrafish will be killed by submersion in ice-cold water for at least 10 minutes. This procedure is currently common practice and is part of the guidelines by the Animal Research Advisory Committee (ARAC) of the NIH.

☐ Yes

18.

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Kon. Ned. Academie van Wetenschappen (KNAW)
[redacted]
Postbus 19121
1000 GC AMSTERDAM

Centrale Commissie Dierproeven
Postbus 20401
2500 EK Den Haag
centralecommissiedierproeven.nl
0900 28 000 28 (10 ct/min)
info@zbo-ccd.nl

Onze referentie
Aanvraagnummer
AVD8010020171047
Bijlagen
1

Datum 6 juni 2017
Betreft Beslissing aanvraag projectvergunning Dierproeven

Geachte [redacted],

Op 17 mei 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project "The cellular and molecular basis of the hematopoietic production" met aanvraagnummer AVD8010020171047. Wij hebben uw aanvraag beoordeeld.

Op 29 mei 2017 heeft u uw aanvraag aangevuld. Wij hebben u gevraagd de dieren vanaf E18 op te nemen als proefdier, of beide geslachten dieren worden ingezet en of u de go/no go criteria meer kunt beschrijven. U heeft de documenten van uw projectaanvraag aangepast op basis van de antwoorden. Alleen bijlage dierproeven 3.4.4.1 is niet aangepast.

Beslissing
Wij keuren uw aanvraag goed op grond van artikel 10a van de Wet op de Dierproeven (hierna: de wet). Hierbij gelden de voorwaarden zoals genoemd in de vergunning.
Met het oog op artikel 10a, lid 1, zijn er algemene voorwaarden gesteld.

U kunt met uw project "The cellular and molecular basis of the hematopoietic production" starten. De vergunning wordt afgegeven van 6 juni 2017 tot en met 1 juni 2022.

Overige wettelijke bepalingen blijven van kracht.

Procedure
Bij uw aanvraag heeft u een advies van de Dierexperimentencommissie DEC-KNAW gevoegd. Dit advies is opgesteld op 17 mei 2017. Bij de beoordeling van uw aanvraag is dit advies betrokken overeenkomstig artikel

10a, lid 3 van de wet.
Wij kunnen ons vinden in de inhoud van het advies van de Dierexperimentencommissie. Dit advies van de commissie nemen wij over, inclusief de daaraan ten grondslag liggende motivering. Er worden aanvullende algemene voorwaarde(n) gesteld.
Het DEC-advies en de in de bijlage opgenomen beschrijving van de artikelen van de wet- en regelgeving zijn de grondslag van dit besluit.

Bezwaar
Als u het niet eens bent met deze beslissing, kunt u binnen zes weken na verzending van deze brief schriftelijk een bezwaarschrift indienen.
Een bezwaarschrift kunt u sturen naar Centrale Commissie Dierproeven, afdeling Juridische Zaken, postbus 20401, 2500 EK Den Haag.

Bij het indienen van een bezwaarschrift vragen we u in ieder geval de datum van de beslissing waartegen u bezwaar maakt en het aanvraagnummer te vermelden. U vindt deze nummers in de rechter kantlijn in deze brief.

Bezwaar schorst niet de werking van het besluit waar u het niet mee eens bent. Dat betekent dat dat besluit wel in werking treedt en geldig is. U kunt tijdens deze procedure een voorlopige voorziening vragen bij de Voorzieningenrechter van de rechtbank in de woonplaats van de aanvrager. U moet dan wel kunnen aantonen dat er sprake is van een spoedeisend belang.

Voor de behandeling van een voorlopige voorziening is griffierecht verschuldigd. Op http://www.rechtspraak.nl/Organisatie/Rechtbanken/Pages/default.aspx kunt u zien onder welke rechtbank de vestigingsplaats van de aanvrager valt.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

Datum:
6 juni 2017
Aanvraagnummer:
AVD8010020171047

Centrale Commissie Dierproeven
namens deze:

[redacted]

ir. G. de Peute[redacted]
Algemeen Secretaris

Bijlagen:
- Vergunning
  Hiervan deel uitmakend:
  - DEC-advies
  - Weergave wet- en regelgeving

Centrale Commissie Dierproeven

Projectvergunning

gelet op artikel 10a van de Wet op de Dierproeven

Verleent de Centrale Commissie Dierproeven aan

Naam:	Kon. Ned. Academie van Wetenschappen (KNAW)
Adres:	Postbus 19121
Postcode en plaats:	1000 GC AMSTERDAM
Deelnemersnummer:	80100

deze projectvergunning voor het tijdvak 6 juni 2017 tot en met 1 juni 2022, voor het project "The cellular and molecular basis of the hematopoietic production" met aanvraagnummer AVD8010020171047, volgens advies van Dierexperimentencommissie DEC-KNAW. Er worden aanvullende algemene voorwaarde(n) gesteld.
De functie van de verantwoordelijk onderzoeker is Groepsleider.
De aanvraag omvat de volgende bescheiden:

1 een aanvraagformulier projectvergunning dierproeven, ontvangen op 17 mei 2017
2 de bij het aanvraagformulier behorende bijlagen:
a Projectvoorstel, zoals ontvangen per digitale indiening op 29 mei 2017;
b Niet-technische Samenvatting van het project, zoals ontvangen per digitale indiening op 29 mei 2017;
c Advies van dierexperimentencommissie d.d. 17 mei 2017, ontvangen op 17 mei 2017.
d De aanvullingen op uw aanvraag, ontvangen op 29 mei 2017

Aanvraagnummer:
AVD8010020171047

Naam proef	Diersoort/ Stam	Aantal dieren	Ernst	Opmerkingen
3.4.4.1 Generation of new GMO (mice)				
	Muizen (Mus musculus) /	3.000	100% Matig	
3.4.4.2 Donor – Ex vivo analysis (mice, zebrafish)				drachtige vrouwen n=2205, jonge dieren n=720, volwassen muizen n=126
	Muizen (Mus musculus) /	3.051	100% Licht	
3.4.4.3 Recipient – Ex vivo analysis (Transplantation in pregnant females, newborns and adult mice)				
	Muizen (Mus musculus) /	5.964	4% Matig 96% Licht	
3.4.4.4 Transplantation in Zebrafish, live imaging and ex vivo analysis				
	Zebravissen (Danio rerio) /	4.992	100% Licht	

Voorwaarden

Op grond van artikel 10a1 lid 2 van de Wet op de dierproeven zijn aan een projectvergunning voorwaarden te stellen

De vergunning wordt verleend onder de voorwaarde dat go/no go momenten worden afgestemd met de IvD.

Aanvraagnummer:
AVD8010020171047

In artikel 10, lid 1 sub a van de wet, wordt bepaald dat het verboden is een dierproef te verrichten voor een doel dat, naar de algemeen kenbare, onder deskundigen heersende opvatting, ook kan worden bereikt anders dan door middel van een dierproef, of door middel van een dierproef waarbij minder dieren kunnen worden gebruikt of minder ongerief wordt berokkend dan bij de in het geding zijnde proef het geval is. Nieuwe onderzoeken naar alternatieven kunnen tot gevolg hebben dat inzichten en/of omstandigheden van het aangevraagde project in de vergunningsperiode wijzigen, gedurende de looptijd van deze vergunning. Indien bovenstaande zich voordoet dient aanvrager dit in afstemming met de IvD te melden bij de CCD. De CCD kan in een dergelijke situatie aan de vergunning nieuwe voorwaarden verbinden en gestelde voorwaarde wijzigen of intrekken.

Centrale Commissie Dierproeven

Aanvraagnummer:
AVD8010020171047

Weergave wet- en regelgeving

Dit project en wijzigingen
Volgens artikel 10c van de Wet op de Dierproeven (hierna de wet) is het verboden om andere dierproeven uit te voeren dan waar de vergunning voor is verleend. De dierproeven mogen slechts worden verricht in het kader van een project, volgens artikel 10g. Uit artikel 10b volgt dat de dierproeven zijn ingedeeld in de categorieën terminaal, licht, matig of ernstig. Als er wijzigingen in een dierproef plaatsvinden, moeten deze gemeld worden aan de Centrale Commissie Dierproeven. Hebben de wijzigingen negatieve gevolgen voor het dierenwelzijn, dan moet volgens artikel 10a5 de wijziging eerst voorgelegd worden en mag deze pas doorgevoerd worden na goedkeuren door de Centrale Commissie Dierproeven.
Artikel 10b schrijft voor dat het verboden is een dierproef te verrichten die leidt tot ernstige mate van pijn, lijden, angst of blijvende schade die waarschijnlijk langdurig zal zijn en niet kan worden verzacht, tenzij hiervoor door de Minister een ontheffing is verleend.

Verzorging
De fokker, leverancier en gebruiker moeten volgens artikel 13f van de wet over voldoende personeel beschikken en ervoor zorgen dat de dieren behoorlijk worden verzorgd, behandeld en gehuisvest. Er moeten ook personen zijn die toezicht houden op het welzijn en de verzorging van de dieren in de inrichting, personeel dat met de dieren omgaat moet toegang hebben tot informatie over de in de inrichting gehuisveste soorten en personeel moet voldoende geschoold en bekwaam zijn. Ook moeten er personen zijn die een eind kunnen maken aan onnodige pijn, lijden, angst of blijvende schade die tijdens een dierproef bij een dier wordt veroorzaakt. Daarnaast zijn er personen die zorgen dat een project volgens deze vergunning wordt uitgevoerd en als dat niet mogelijk is zorgen dat er passende maatregelen worden getroffen.
In artikel 9 staat dat de persoon die het project en de dierproef opzet deskundig en bekwaam moet zijn. In artikel 8 van het Dierproevenbesluit 2014 staat dat personen die dierproeven verrichten, de dieren verzorgen of de dieren doden, hiervoor een opleiding moeten hebben afgerond.
Voordat een dierproef die onderdeel uitmaakt van dit project start, moet volgens artikel 10a3 van de wet de uitvoering afgestemd worden met de instantie voor dierenwelzijn.

Pijnbestrijding en verdoving
In artikel 13 van de wet staat dat een dierproef onder algehele of plaatselijke verdoving wordt uitgevoerd tenzij dat niet mogelijk is, dan wel bij het verrichten van een dierproef worden pijnstillers toegediend of andere goede methoden gebruikt die de pijn, het lijden, de angst of de blijvende schade bij het dier tot een minimum beperken. Een dierproef die bij het dier gepaard gaat met zwaar letsel dat hevige pijn kan veroorzaken, wordt niet zonder verdoving uitgevoerd. Hierbij wordt afgewogen of het toedienen van verdoving voor het dier traumatischer is dan de dierproef zelf en het toedienen van verdoving onverenigbaar is met het doel van de dierproef. Bij een dier wordt geen stof toegediend waardoor het dier niet meer of slechts in verminderde mate in staat is pijn te tonen, wanneer het dier niet tegelijkertijd voldoende verdoving of pijnstilling krijgt toegediend, tenzij wetenschappelijk gemotiveerd. Dieren die pijn

kunnen lijden als de verdoving eenmaal is uitgewerkt, moeten preventief en postoperatief behandeld worden met pijnstillers of andere geschikte pijnbestrijdingsmethoden, mits die verenigbaar zijn met het doel van de dierproef. Zodra het doel van de dierproef is bereikt, moeten passende maatregelen worden genomen om het lijden van het dier tot een minimum te beperken.

Einde van een dierproef
Artikel 13a van de wet bepaalt dat een dierproef is afgelopen wanneer voor die dierproef geen verdere waarnemingen hoeven te worden verricht of, voor wat betreft nieuwe genetisch gemodificeerde dierenlijnen, wanneer bij de nakomelingen niet evenveel of meer, pijn, lijden, angst, of blijvende schade wordt waargenomen of verwacht dan bij het inbrengen van een naald. Er wordt dan door een dierenarts of een andere ter zake deskundige beslist of het dier in leven zal worden gehouden. Een dier wordt gedood als aannemelijk is dat het een matige of ernstige vorm van pijn, lijden, angst of blijven schade zal blijven ondervinden. Als een dier in leven wordt gehouden, krijgt het de verzorging en huisvesting die past bij zijn gezondheidstoestand.
Volgens artikel 13b moet de dood als eindpunt van een dierproef zoveel mogelijk worden vermeden en vervangen door in een vroege fase vaststelbare, humane eindpunten. Als de dood als eindpunt onvermijdelijk is, moeten er zo weinig mogelijk dieren sterven en het lijden zo veel mogelijk beperkt blijven.
Uit artikel 13d volgt dat het doden van dieren door een deskundig persoon moet worden gedaan, wat zo min mogelijk pijn, lijden en angst met zich meebrengt. De methode om te doden is vastgesteld in de Europese richtlijn artikel 6.
In artikel 13c is vastgesteld dat proefdieren geadopteerd kunnen worden, teruggeplaatst in hun habitat of in een geschikt dierhouderijsysteem, als de gezondheidstoestand van het dier het toelaat, er geen gevaar is voor volksgezondheid, diergezondheid of milieu en er passende maatregelen zijn genomen om het welzijn van het dier te waarborgen.
De Minister heeft vrijstelling verleend volgens artikel 13c, die de afwijkende methode van doden op basis van wetenschappelijke motivering ten minste even humaan acht als de in de richtlijn opgenomen passende methoden.

19.

[redacted]

Van: Info-zbo
Verzonden: donderdag 8 juni 2017 9:16
Aan: 'secretariaat DEC'
CC: [redacted]
Onderwerp: RE: Beschikking AVD80100 2017 1047 > correctie aantallen gevraagd

Beste [redacted]

Dank voor uw bericht. Er is inderdaad sprake van een vergissing, onze excuses hiervoor. Een correctie van de beschikking wordt u later vandaag toegezonden,

Vriendelijke groet, [redacted]

Namens Centrale Commissie Dierproeven
www.centralecommissiedierproeven.nl
.....
Postbus 20401 | 2500 EK | Den Haag
.....
T: 0900 2800028
E: info@zbo-ccd.nl (let op: nieuw emailadres!) -----Oorspronkelijk bericht-----
Van: secretariaat DEC [mailto:DECsecr@knaw.nl]
Verzonden: woensdag 7 juni 2017 15:18
Aan: 'info@zbo-ccd.nl'
CC: '[redacted]'
Onderwerp: FW: Beschikking AVD80100 2017 1047 > correctie aantallen gevraagd

Geachte CCD,

We willen u graag bedanken voor de vlotte afhandeling van onze aanvraag en het sturen van de positieve beschikking over AVD80100 2017 1047.

Bij controle hebben we echter bemerkt dat er een fout in de tabel van de beschikking staat. Op verzoek van de CCD is een extra groep onder 3.4.4.2 toegevoegd van 378 E18 embryo's. Deze zijn echter niet in de beschikking opgenomen; bijgaande de naar u op 29 mei 2017 gestuurde tabel met in rood de aanpassingen. Wij gaan ervan uit dat het om een vergissing gaat.

Namens de onderzoeker en de IvD-HI wil ik vragen om de beschikking aan te passen en de 378 dieren toe te voegen.

Wij horen graag van u.

Met vriendelijke groeten,
[redacted]

[redacted]
Onderwerp: Beschikking 1047

Geachte [redacted],

Deze beschikking is ook per post verzonden.

Met vriendelijke groet,

Namens,

Centrale Commissie Dierproeven www.centralecommissiedierproeven.nl

Nationaal Comité advies dierproevenbeleid www.ncadierproevenbeleid.nl
.....
Bezuidenhoutseweg 73 | 2594 AC | Den Haag Postbus 20401 | 2500 EK | Den Haag
.....
T: 0900 2800028
E: info@zbo-ccd.nl

20.

Centrale Commissie Dierproeven

> Retouradres Postbus 20401 2500 EK Den Haag

Kon. Ned. Academie van Wetenschappen (KNAW)
[redacted]
Postbus 19121
1000GC Amsterdam

Centrale Commissie Dierproeven

Postbus 20401
2500 EK Den Haag
www.centralecommissiedierproeven.nl

T 0900-28 000 28 (10 ct /min)

info@zbo-ccd.nl

Onze referentie
AVD8010020171047

Uw referentie

Bijlagen
geen

Datum 8 juni 2017
Betreft Correctie beslissing Aanvraag projectvergunning dierproeven

Geachte [redacted],

Op 17 mei 2017 hebben wij uw aanvraag voor een projectvergunning dierproeven ontvangen. Het gaat om uw project 'The cellular and molecular basis of the hematopoietic production' met aanvraagnummer AVD8010020171047

Beslissing
Op 6 juni 2017 hebben wij u de beschikking en vergunning van uw aanvraag toegezonden. Op 7 juni 2017 hebben de DEC en de verantwoordelijk onderzoeker contact met ons opgenomen, omdat het dieraantal voor bijlage 3.4.4.2 niet correct is.
In de vergunning staan n=3051 genoemd, terwijl in de aanvraag n=3429 staat beschreven.

Zoals in de beschikkingsbrief van 6 juni 2017 genoemd, hebben wij uw aanvraag beoordeeld. Bij de beoordeling van uw aanvraag hebben wij het aantal dieren zoals genoemd in uw aanvraag meegenomen. De aan u verstuurde vergunning bevat dus een kennelijke verschrijving en kan gecorrigeerd worden naar n=3429 proefdieren voor bijlage 3.4.4.2 'donor- Ex vivo analysis (mice / zebrafish'.
Voor het overige blijft het besluit van 6 juni 2017 ongewijzigd.
Deze brief dient u bij uw vergunning te voegen.

Bezwaar
Als u het niet eens bent met deze beslissing, kunt u binnen zes weken na verzending van deze brief schriftelijk een bezwaarschrift indienen.
Een bezwaarschrift kunt u sturen naar Centrale Commissie Dierproeven, afdeling Juridische Zaken, postbus 20401, 2500 EK Den Haag.

Bij het indienen van een bezwaarschrift vragen we u in ieder geval de datum van de beslissing waartegen u bezwaar maakt en het aanvraagnummer te vermelden. U vindt deze gegevens in het colofon.

Bezwaar schorst niet de werking van het besluit waar u het niet mee eens bent. Dat betekent dat dat besluit wel in werking treedt en geldig is. U kunt tijdens deze procedure een voorlopige

voorziening vragen bij de Voorzieningenrechter van de rechtbank in de woonplaats van de aanvrager. U moet dan wel kunnen aantonen dat er sprake is van een spoedeisend belang.

Voor de behandeling van een voorlopige voorziening is griffierecht verschuldigd. Op http://www.rechtspraak.nl/Organisatie/Rechtbanken/Pages/default.aspx kunt u zien onder welke rechtbank de vestigingsplaats van de aanvrager valt.

Meer informatie
Heeft u vragen, kijk dan op www.centralecommissiedierproeven.nl. Of neem telefonisch contact met ons op: 0900 28 000 28 (10 ct/minuut).

De Centrale Commissie Dierproeven
namens deze:

[redacted]

Algemeen Secretaris

Dit besluit is genomen met inachtneming van het Besluit mandaat, volmacht en machtiging van de Centrale Commissie Dierproeven CCD 2014 zoals de Centrale Commissie Dierproeven heeft vastgesteld op 19 december 2014, ref 2014-04 en is gepubliceerd in de Staatscourant van 2 januari 2015, Nr. 163