From Mesenchymal Stem Cells To Insulin producing cells

Mesenchymal Stem Cells

Advantages

- Availability
- Proliferative ability
- Multipotent / Pluripotent ?
- Autologous application
- Possible allogenic application

Disadvantages:

- Difficulties in their differentiation

Four main steps

1 . Proving of a principle .
2 . Application in vivo
3 . An answer for a dilemma .
4 . Experiment in larger animals

Proving of a principle

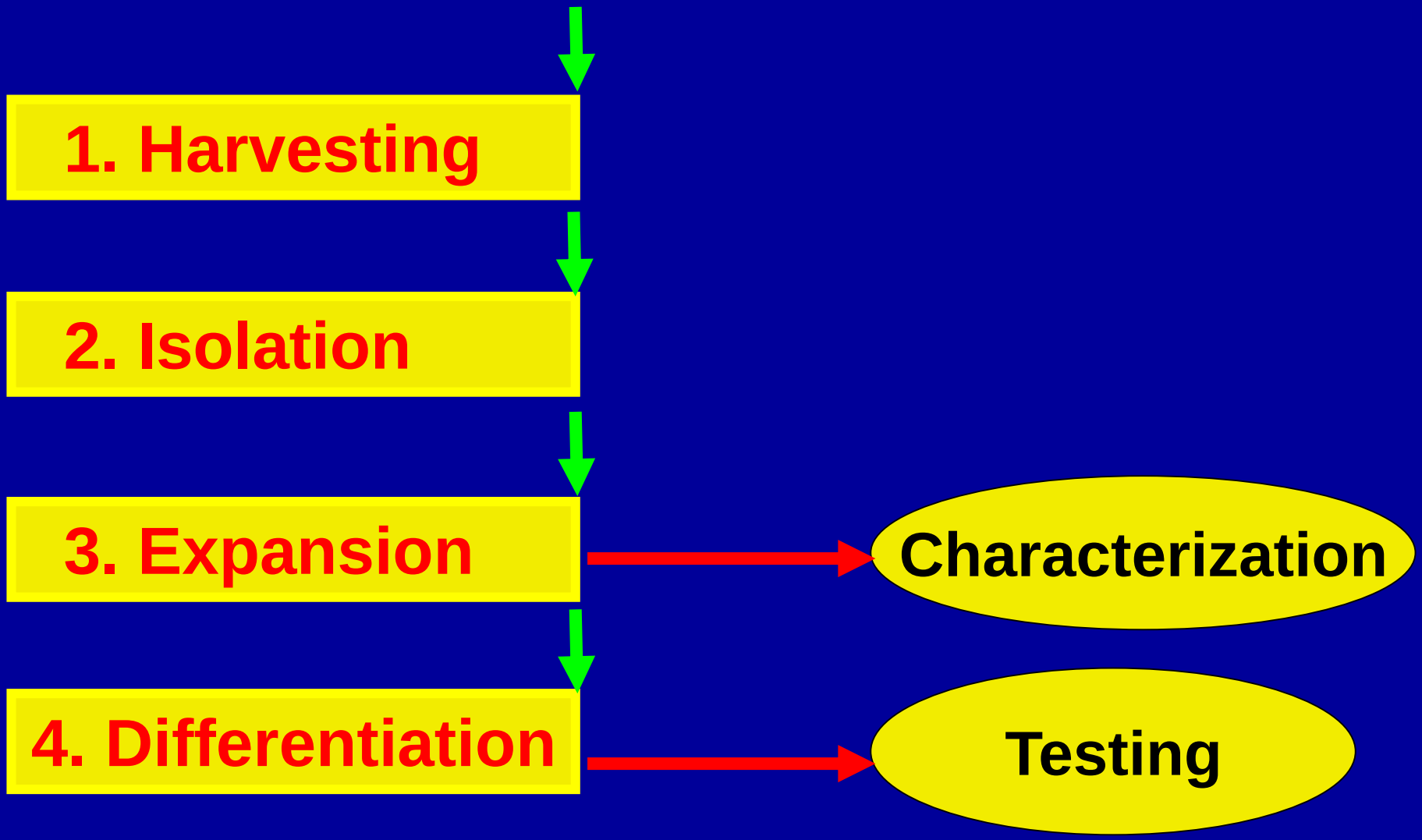
From MSCs to IPCS

INSULIN-PRODUCING, ISLET-LIKE CELLS DERIVED FROM ADULT HUMAN BONE MARROW MSCs

a. In-vitro production.

b. In-vivo application .

IN-VITRO PRODUCTION



1. HARVESTING

- **Bone marrow aspirates were obtained from healthy subjects during open orthopedic surgery for correction of closed fractures.**
- **The aspirating syringe was loaded with heparin.**
- **The aspirate was diluted 1:1 with low-glucose (5 mmol/L) DMEM.**

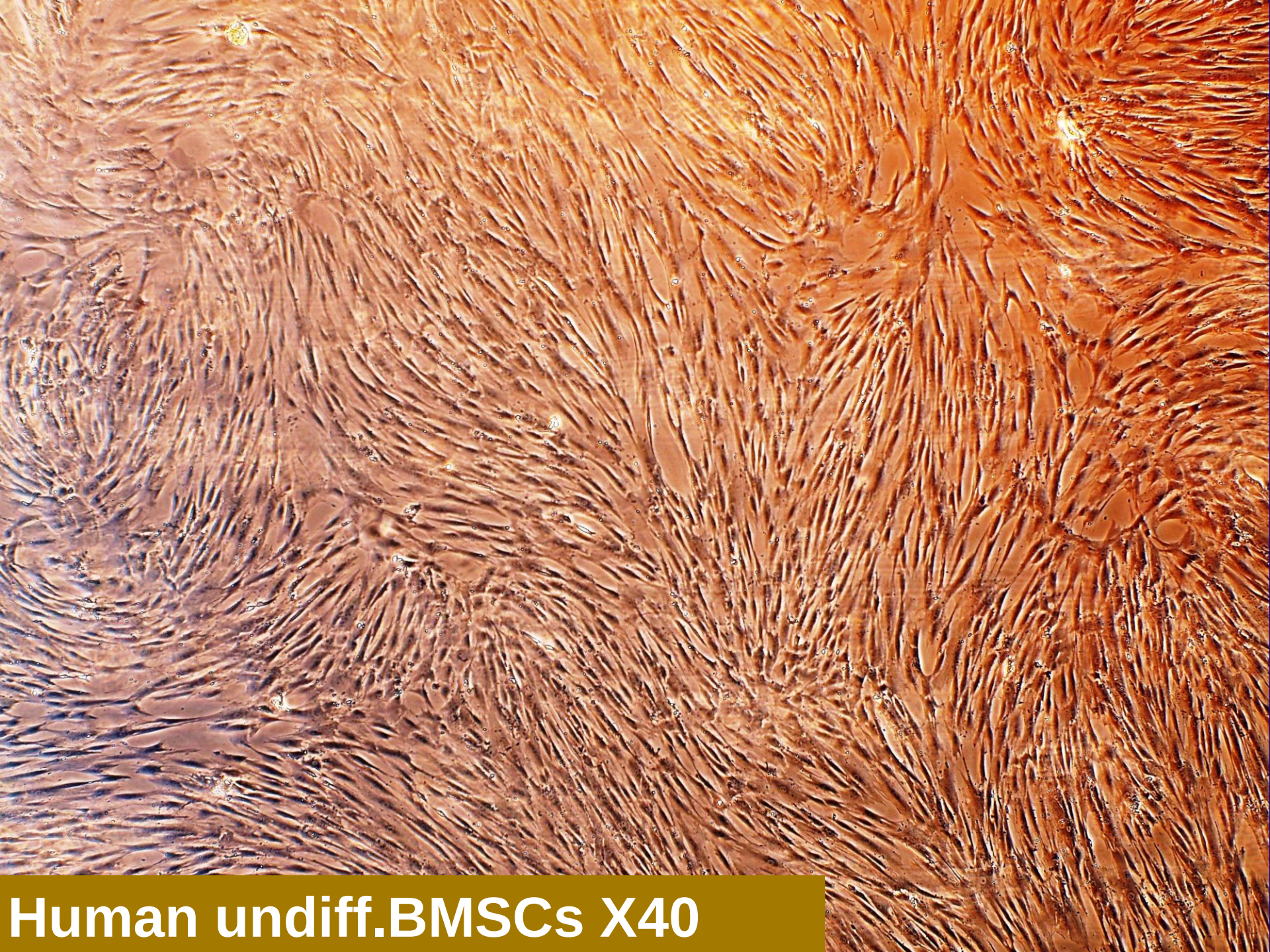
2. ISOLATION OF MSCs

- The cell suspension is layered on top of a density gradient solution.
- The cells are collected from the plasma/Ficoll interface. One ml of the bone marrow aspirate yields ~ $1.5x10^6$ nucleated cells.
- The collected cells are then cultured in complete DMEM at a density of $5x10^5$ cells /ml (10 ml in 25 cm^2 culture flasks) and incubated at 37° in a 5% Co^2 incubator.
- After 3 days, the non-adherent cells are discarded. The adherent ones represent the MSCs.

3. EXPANSION

- The adherent cells (MSCs-rich) are fed twice weekly with complete DMEM.

- When 80% confluence is reached , the cells are detached and re-suspended in complete DMEM for 2 passages; approximately for one week each.


Human undiff.BMSCs X40

Testing of the expanded MSCs

1) **Phenotyping**

- Flow cytometry.

2) **Trilineage differentiation**

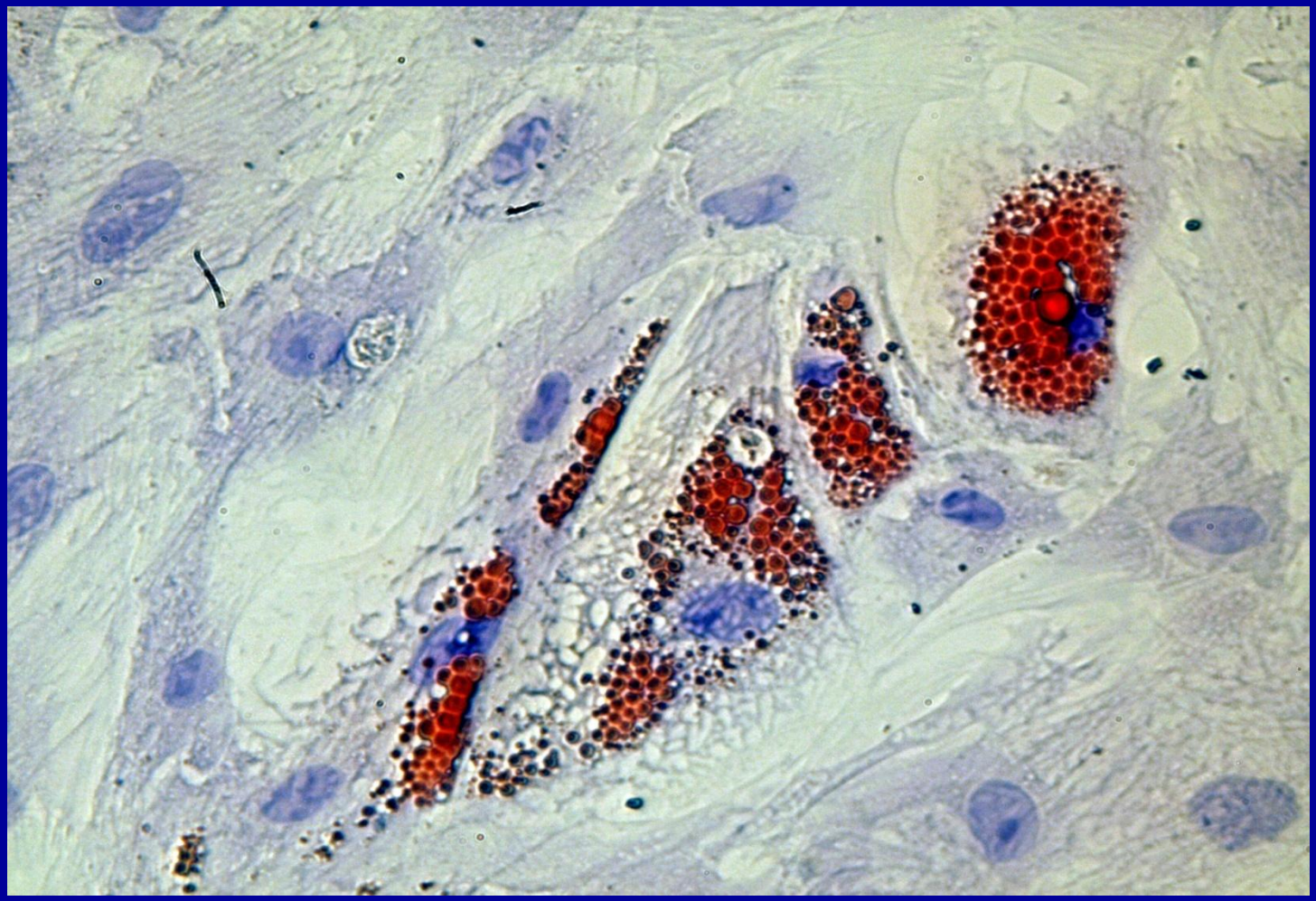
- adipogenic differ.
- chondrogenic differ.
- osteogenic differ.

Testing for purity : Flow cytometric analysis

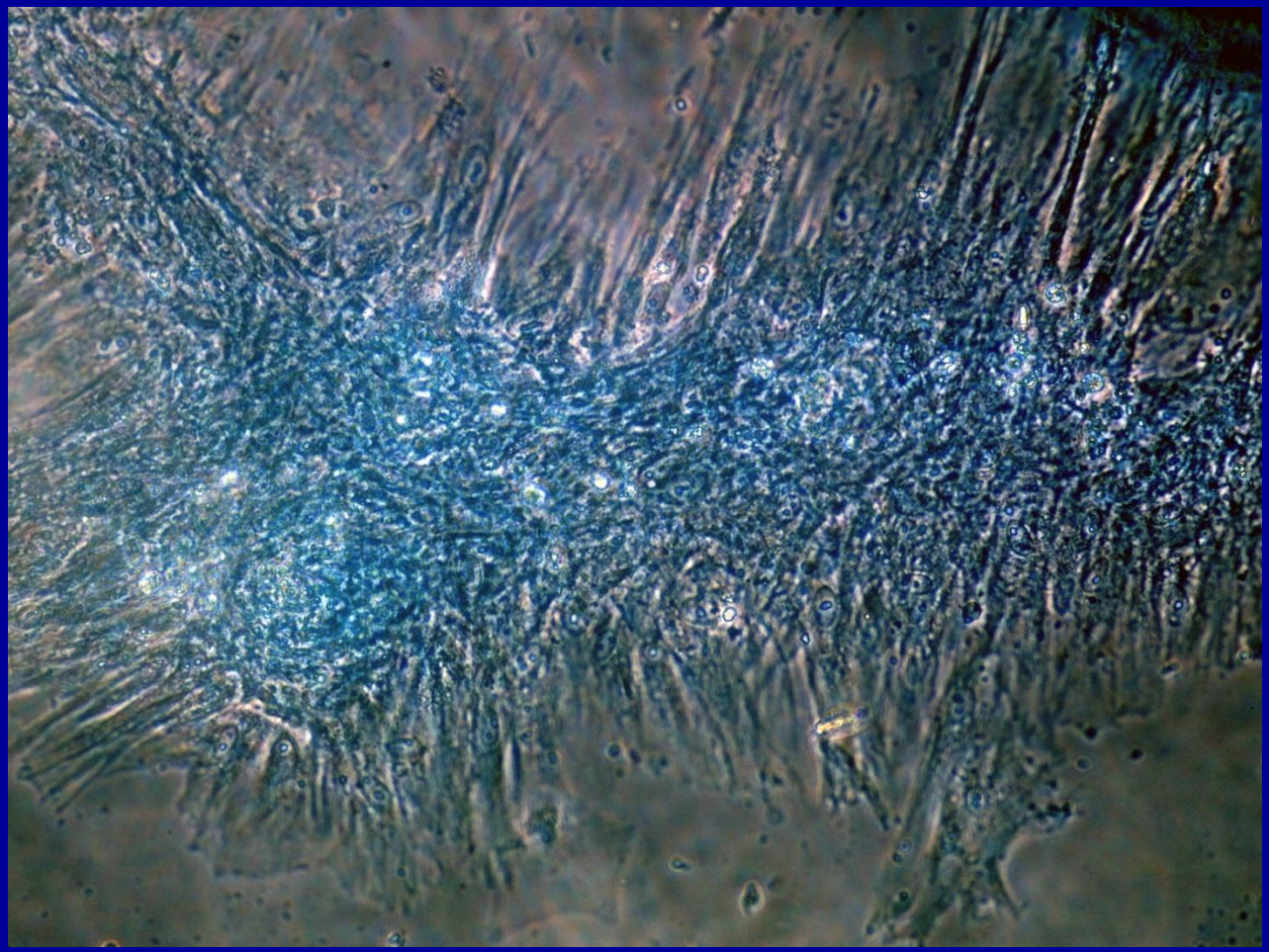
Cell surface markers

Negative		Positive	
CD 14	(2.6%)	CD 73	(99.5%)
CD 34	(0.2%)	CD 90	(99.4%)
CD 45	(0.1%)	CD 105	(96.4%)

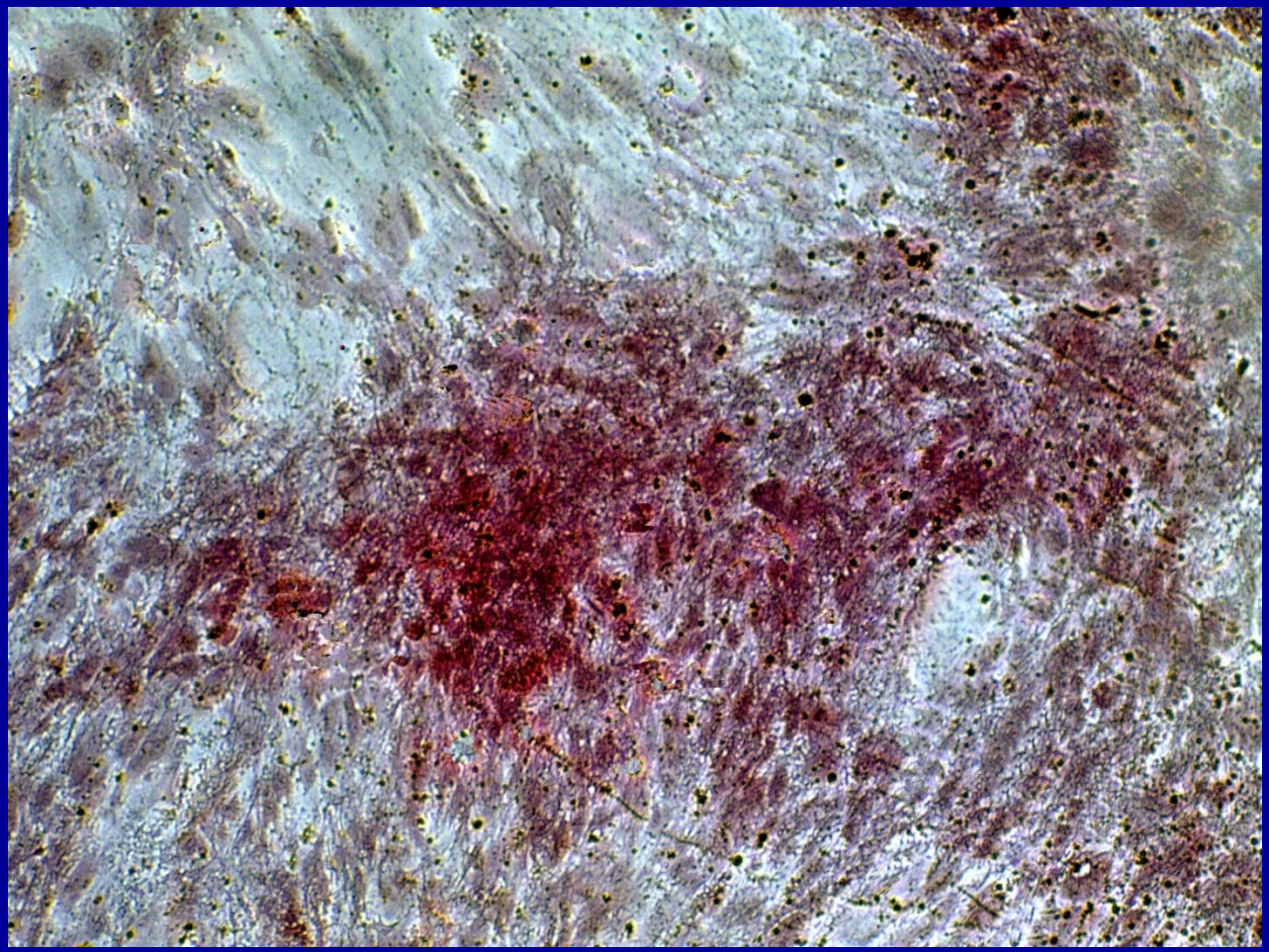
Adipogenic Differentiation
Oil red O stain (x 400)



Chondrogenic differentiation in MSCs (Alcian blue stain)(X600)



Osteogenic differentiation in MSCs (Alizarin red stain)(X400)



4.1 DIFFERENTIATION

At passage 3, MSCs are cultured in 30 ml of serum free, glucose-rich (25 mmol/L) DMEM containing 0.5 mmol/L ß - mercaptoethanol. These cells are cultured at a density of $1x10^5$ cells/ml ($3x10^6$ cells in the 75 cm^2 flask) and incubated for 2 days.

4.2 DIFFERENTIATION

The cells are then cultured for 8 days in a serum-free, glucose-rich DMEM, supplemented with :

- **Non essential amino acids.**
- **Fibroblast growth factor.**
- **Epidermal growth factor.**
- **B 27 supplement.**
- **L-glutamine.**

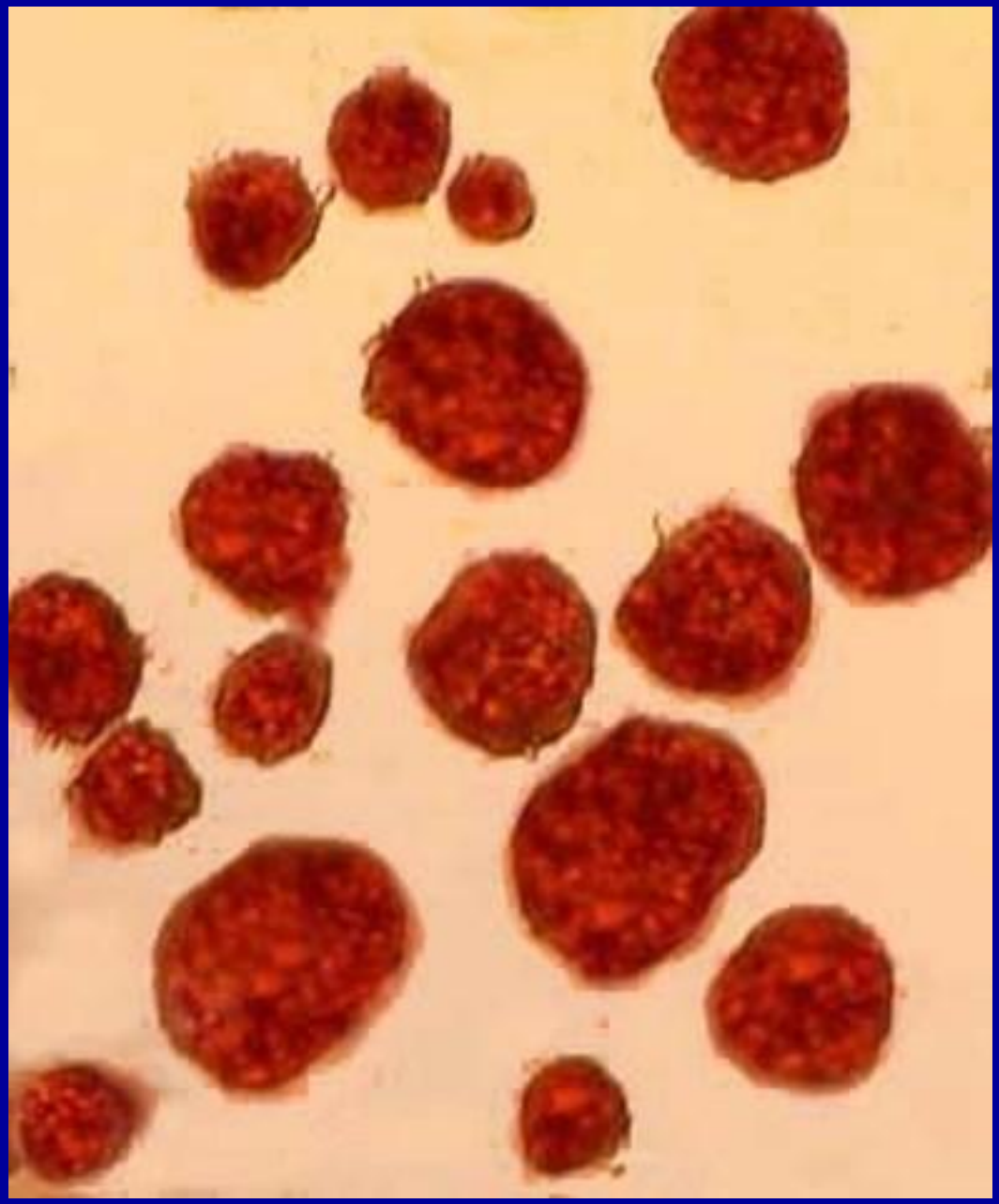
4.3 DIFFERENTIATION

Finally, the cells are cultured for an additional 8 days in a fresh serum-free glucose-rich DMEM supplemented with:

- ß-Cellulin.
- Activin A.
- B27 supplement.
- Nicotinamide.

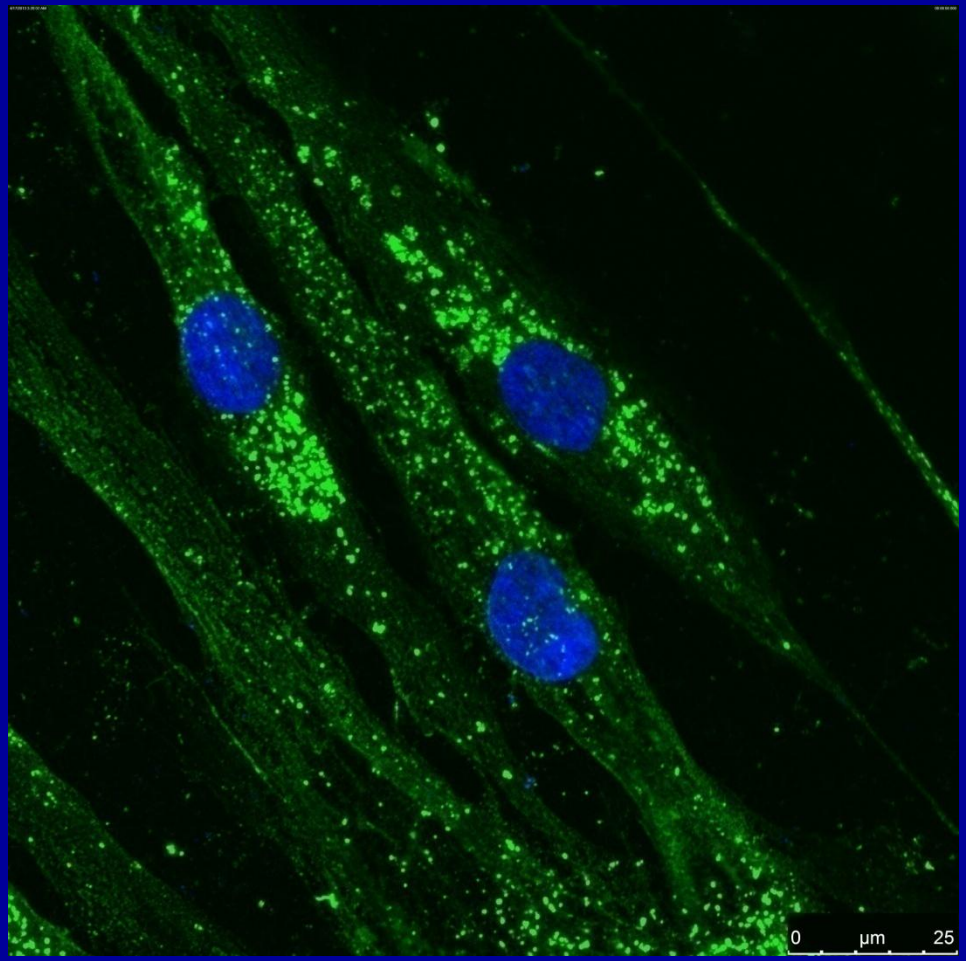
Testing the cells at end of differentiation

- DTZ staining .
- Immuno-fluorescent staining.
- Reverse transcription-PCR.
- Electron microscopy(*conventional & functional*)
- In-vitro insulin release in response to increasing glucose concentrations.

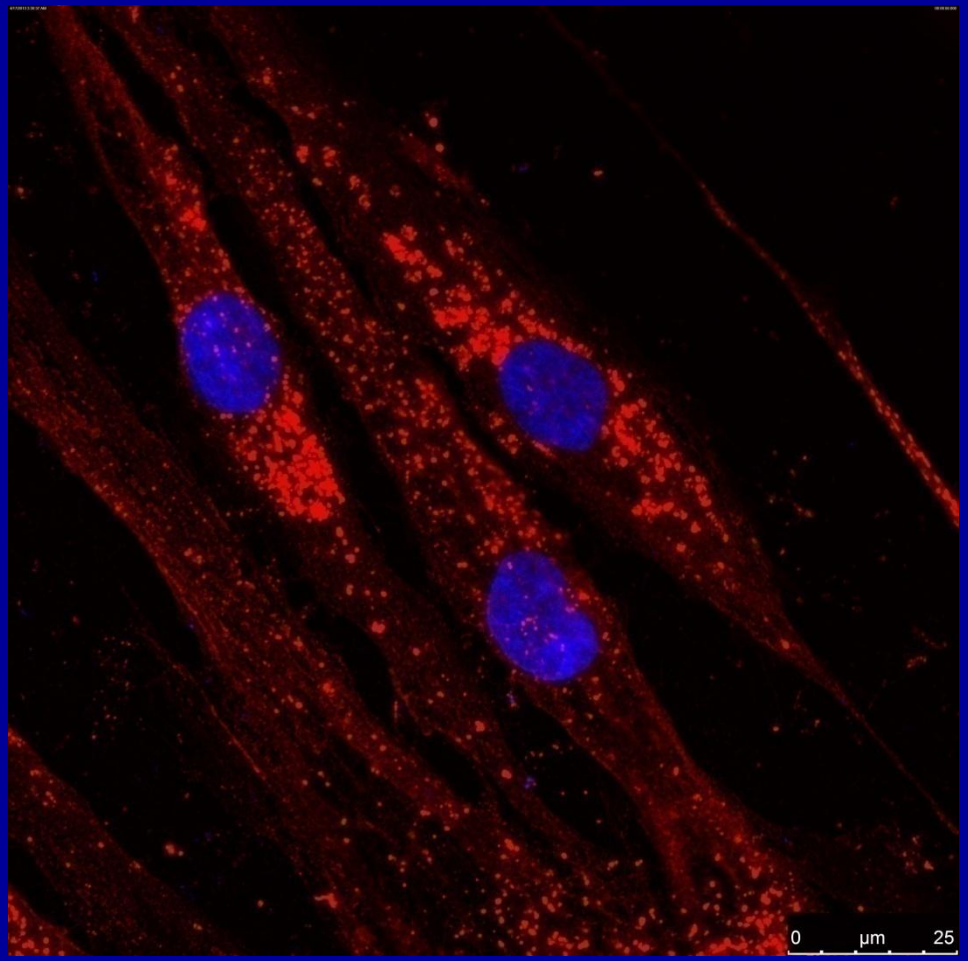


Islet-like clusters ,DTZ positive x 100

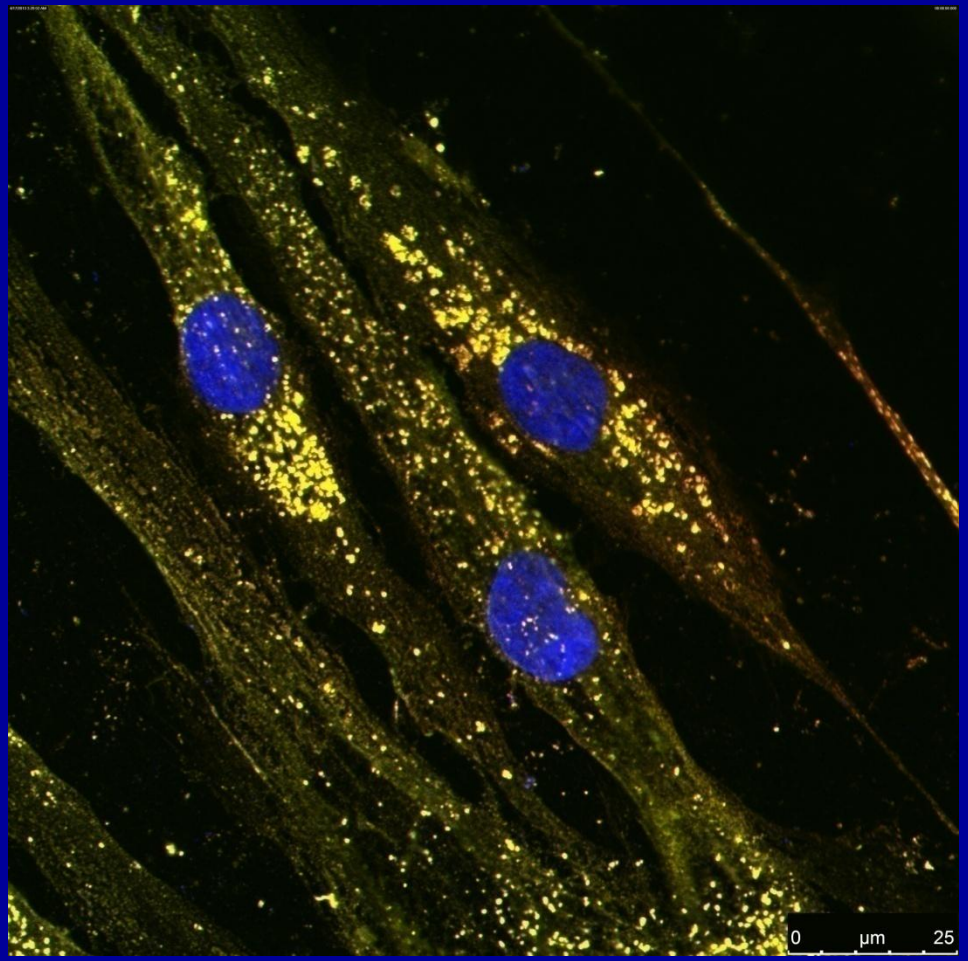
Differentiatedn MSCs Positive for insulin (green)



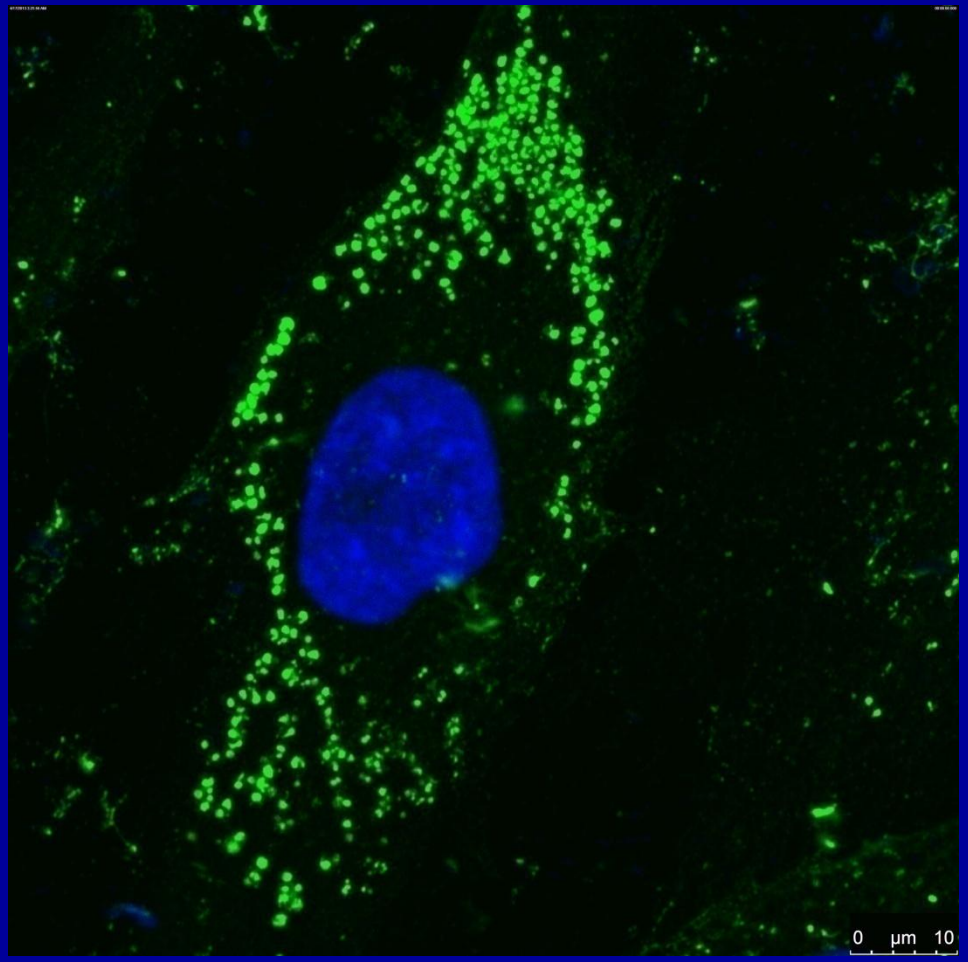
Differentiated MSCs
Positive for C-peptide (red)



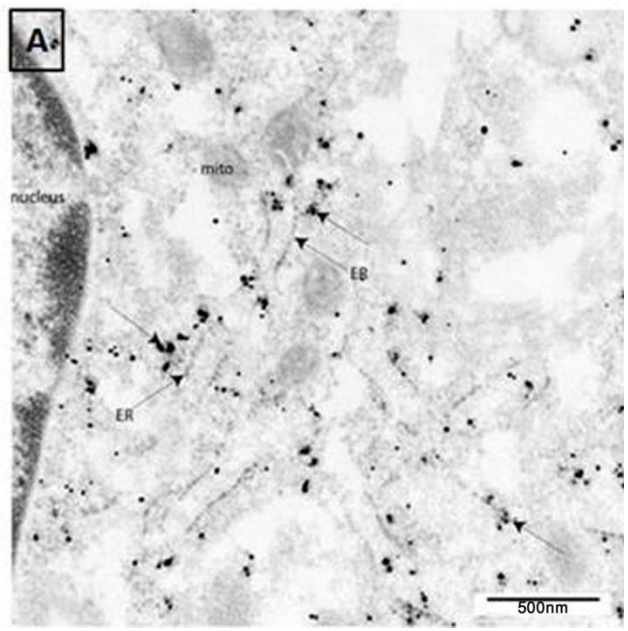
Differentiated MSCs
Insulin / C-peptide merge (yellow)

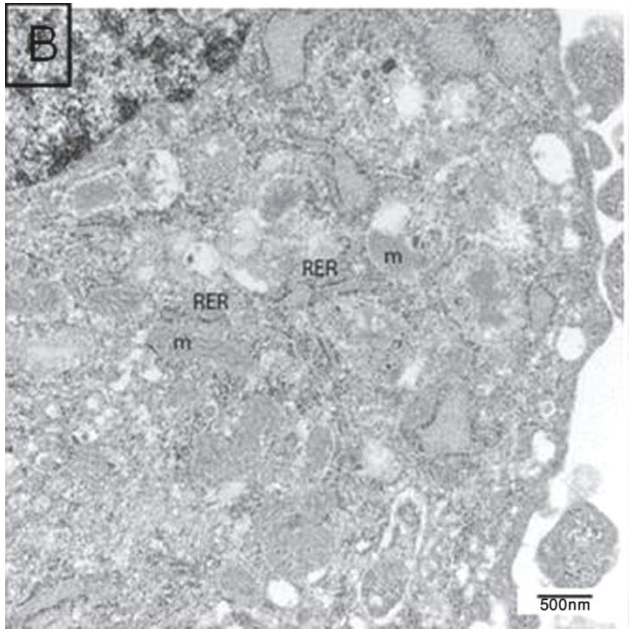


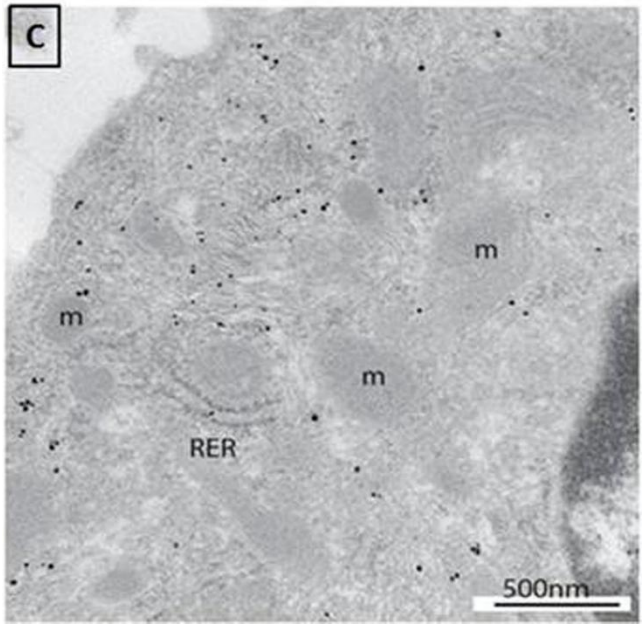
Hassan MSCs differentiation by confocal microscope (insulin positive cells)



Immune- nanogold staining for c-peptide (EM)







Insulin producing cells

(Positive)

Undifferentiated MSCs

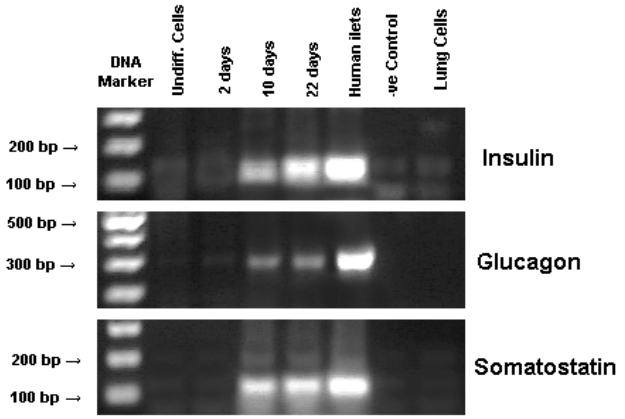
(Negative control)

Human islets

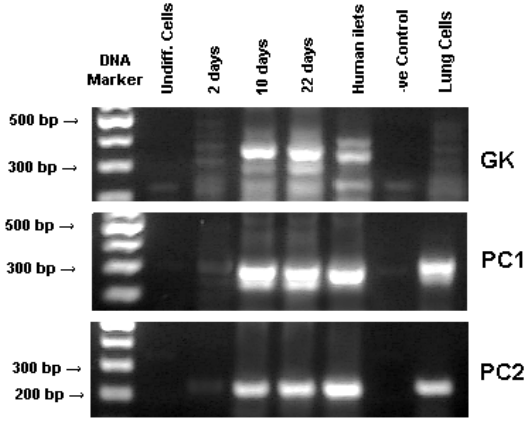
(Positive control)

Gene Expression of the Differentiated IPCs (Donor 2)

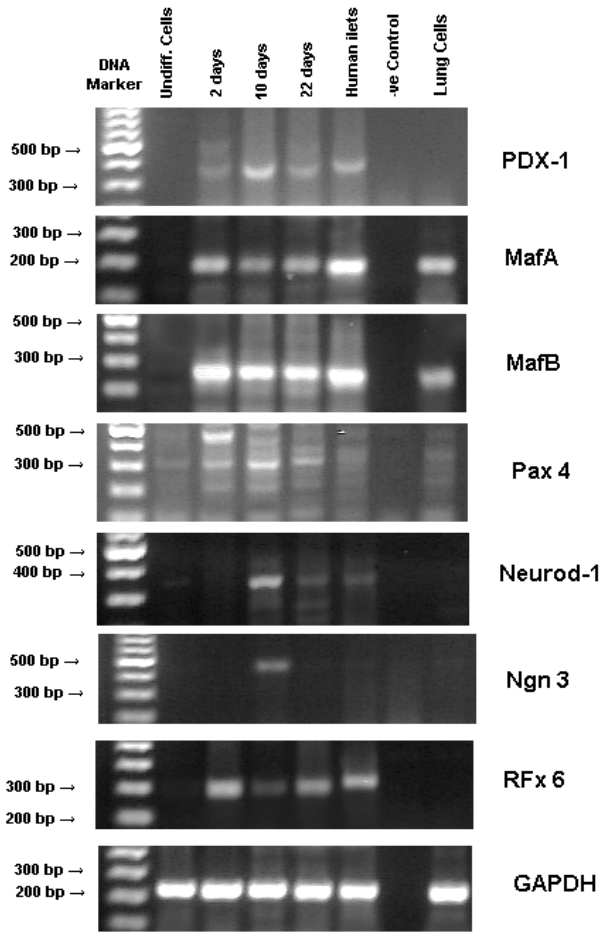
Endocrine Hormones



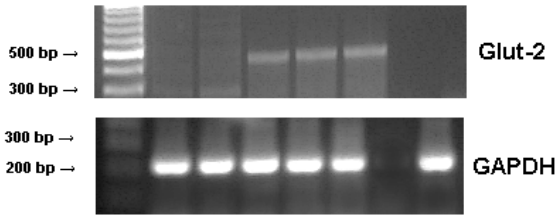
Pancreatic Enzymes



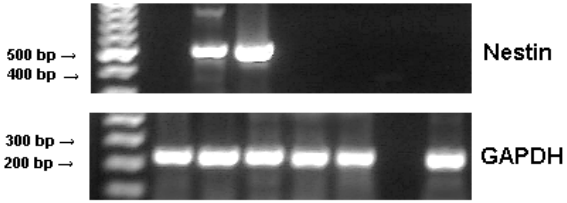
Transcription Factors



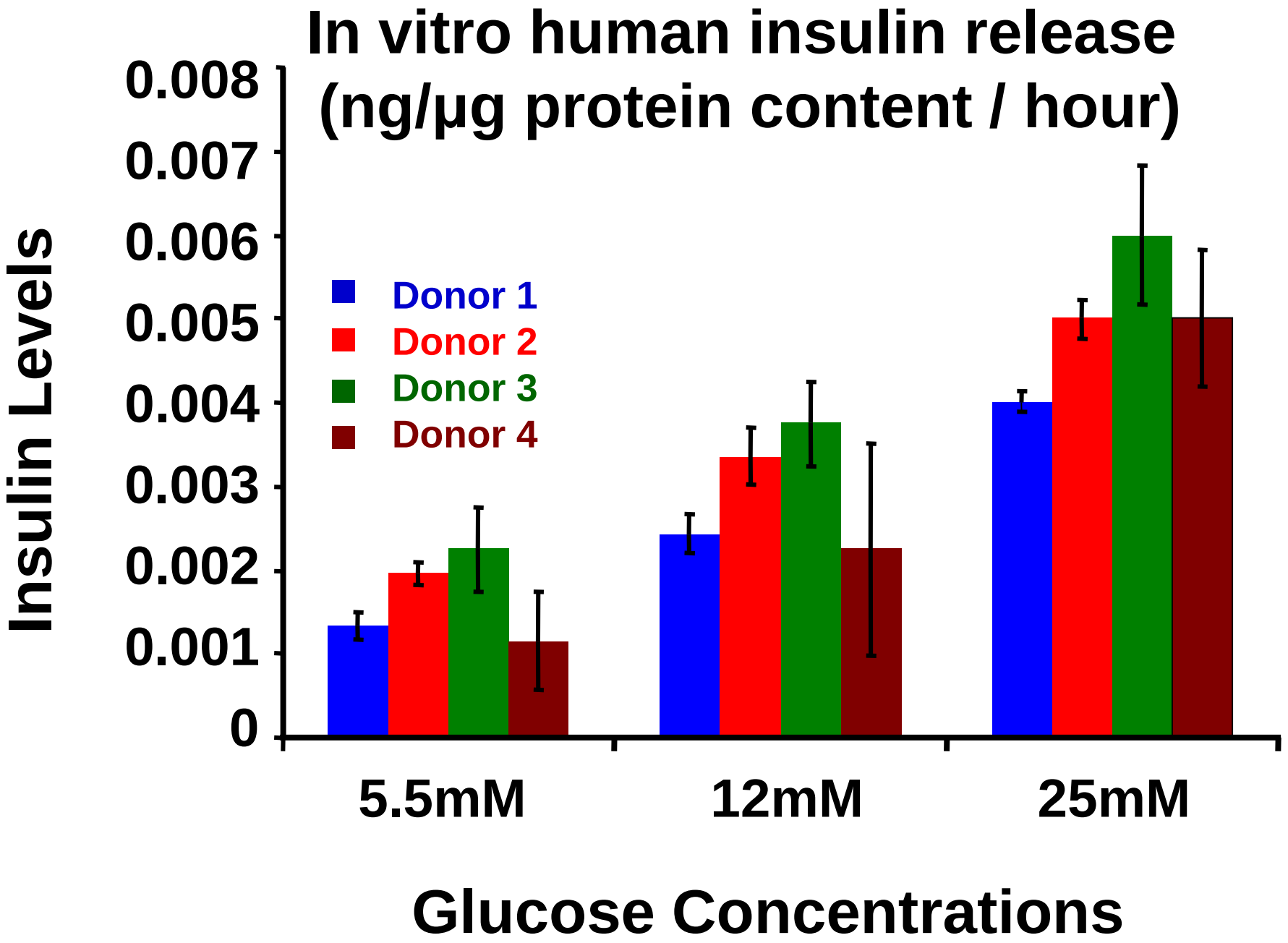
Glucose Transporter



Endocrine Precursor Marker



- Negative Controls : No Template
  Lung Cells (cell line; ATTC CCL75)
- Positive Control : Human Ilets
- Endogenous Control : Reference gene GAPDH


In vitro human insulin release
(ng/µg protein content / hour)
Insulin Levels
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0
Donor 1
Donor 2
Donor 3
Donor 4
5.5mM
12mM
25mM
Glucose Concentrations

Conclusion

MSCs can be differentiated to form IPCs

However, the proportion of the formed IPCs with the used protocol was modest (2-3 %).

Trials of other differentiation protocols

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Hindawi

Research Article

Generation of Insulin-Producing Cells from Human Bone Marrow-Derived Mesenchymal Stem Cells: Comparison of Three Differentiation Protocols

Mahmoud M. Gabr,[1] Mahmoud M. Zakaria,[1] Ayman F. Refaie,[2] Sherry M. Khater,[3] Sylvia A. Ashamallah,[3] Amani M. Ismail,[4] Nagwa El-Badri,[5] and Mohamed A. Ghoneim[6]

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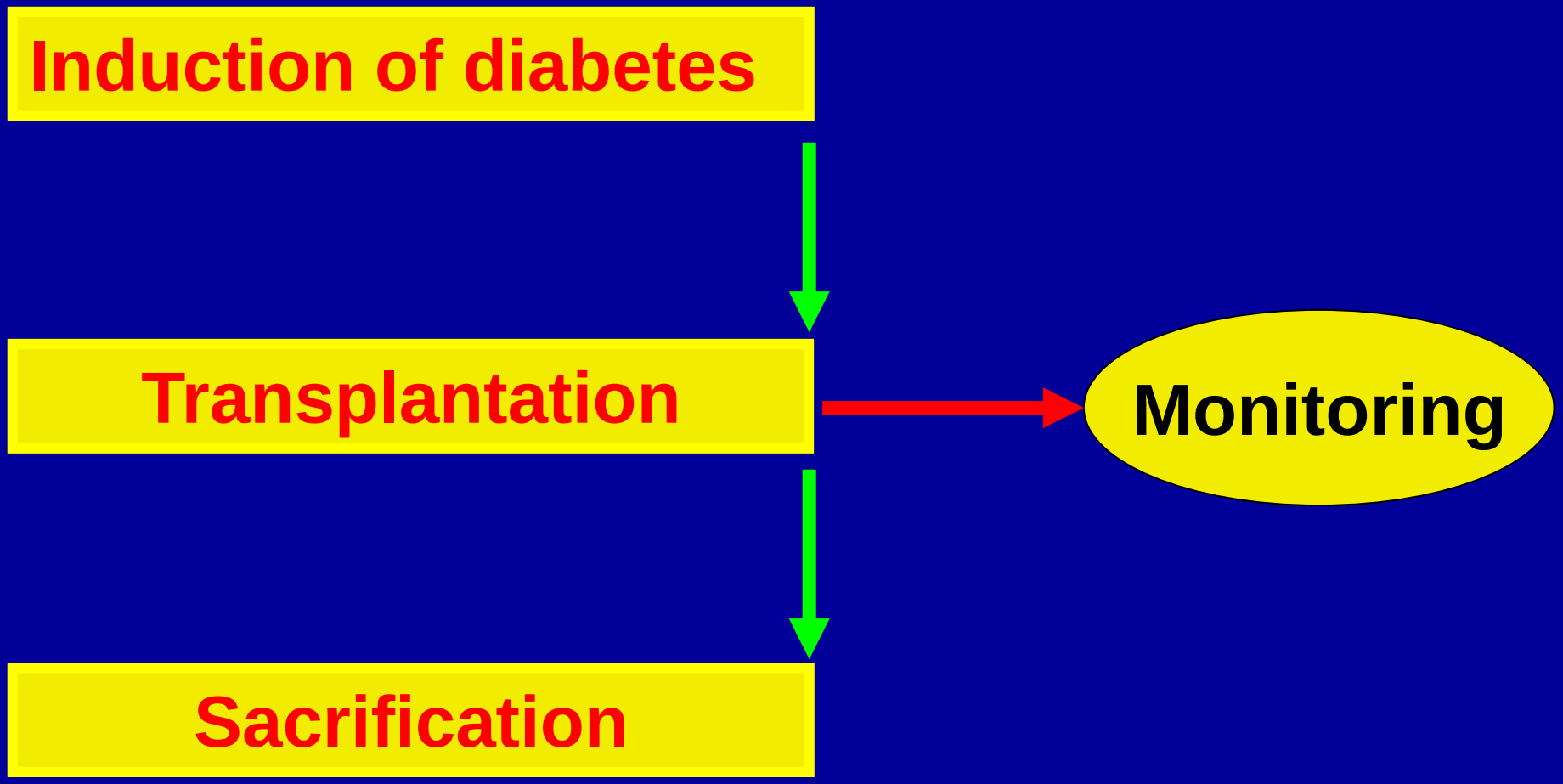
All the tested protocols were comparable

IN - VIVO Transplantation

IN-VIVO TRANSPLANTATION AND TESTING



INDUCTION OF DIABETES

- Nude mice.
- Intra-peritoneal STZ (220 mg/Kg).
- Evidence:

  Blood sugar > 350 mg/dL (two consecutive readings).

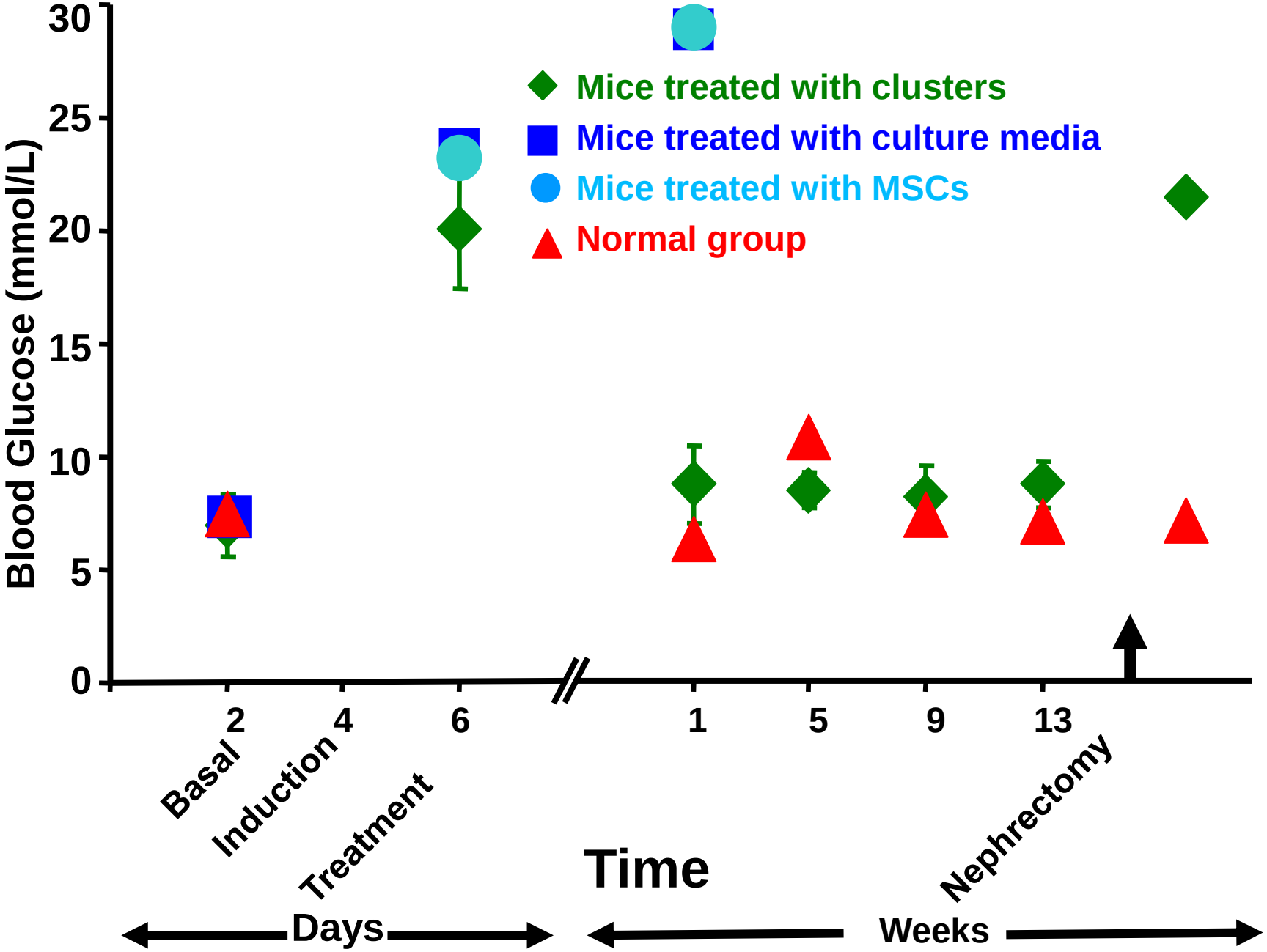
Transplantation in the renal subcapsular space :The experimental groups

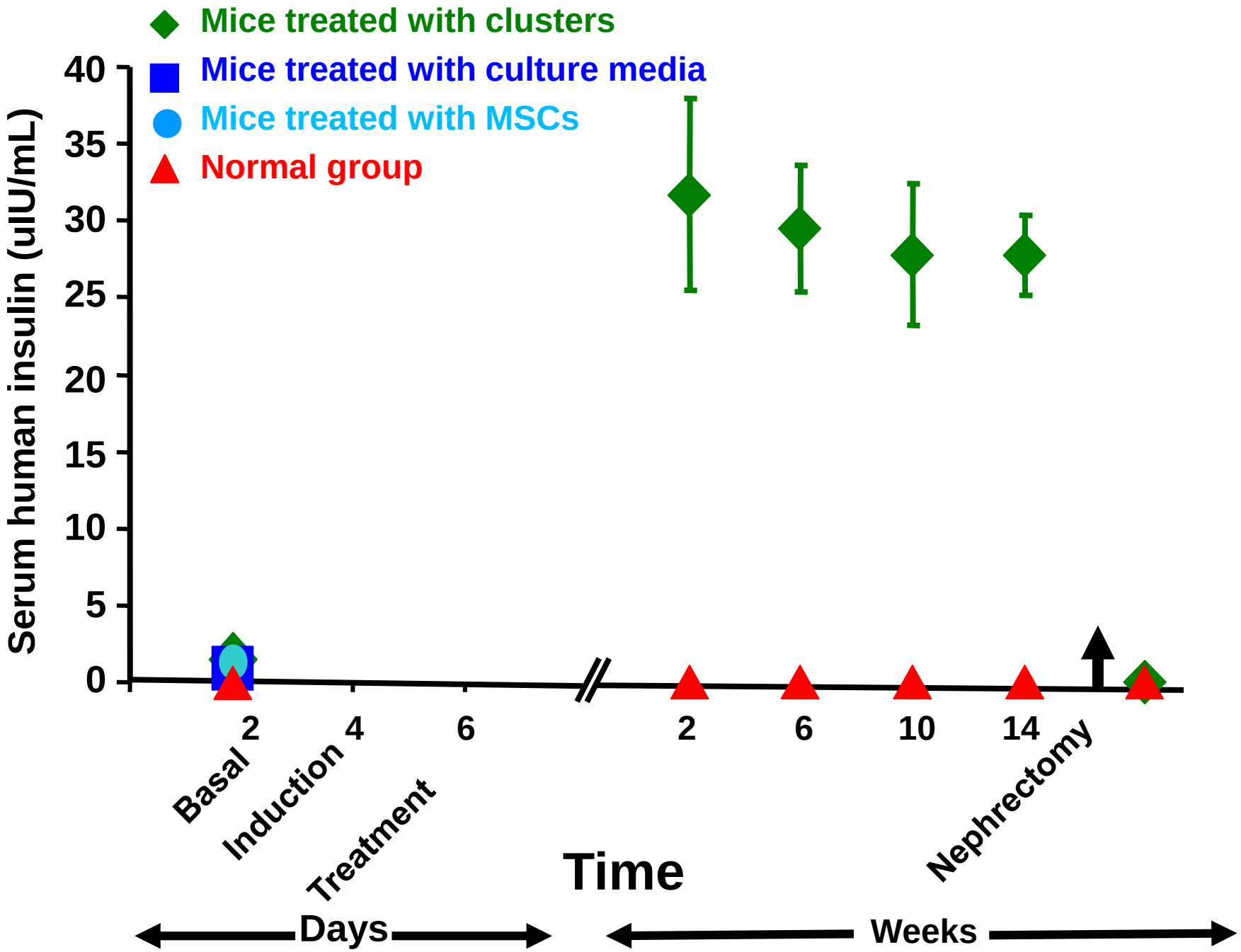
1) Diabetic animals treated with the culture media only(10 mice).

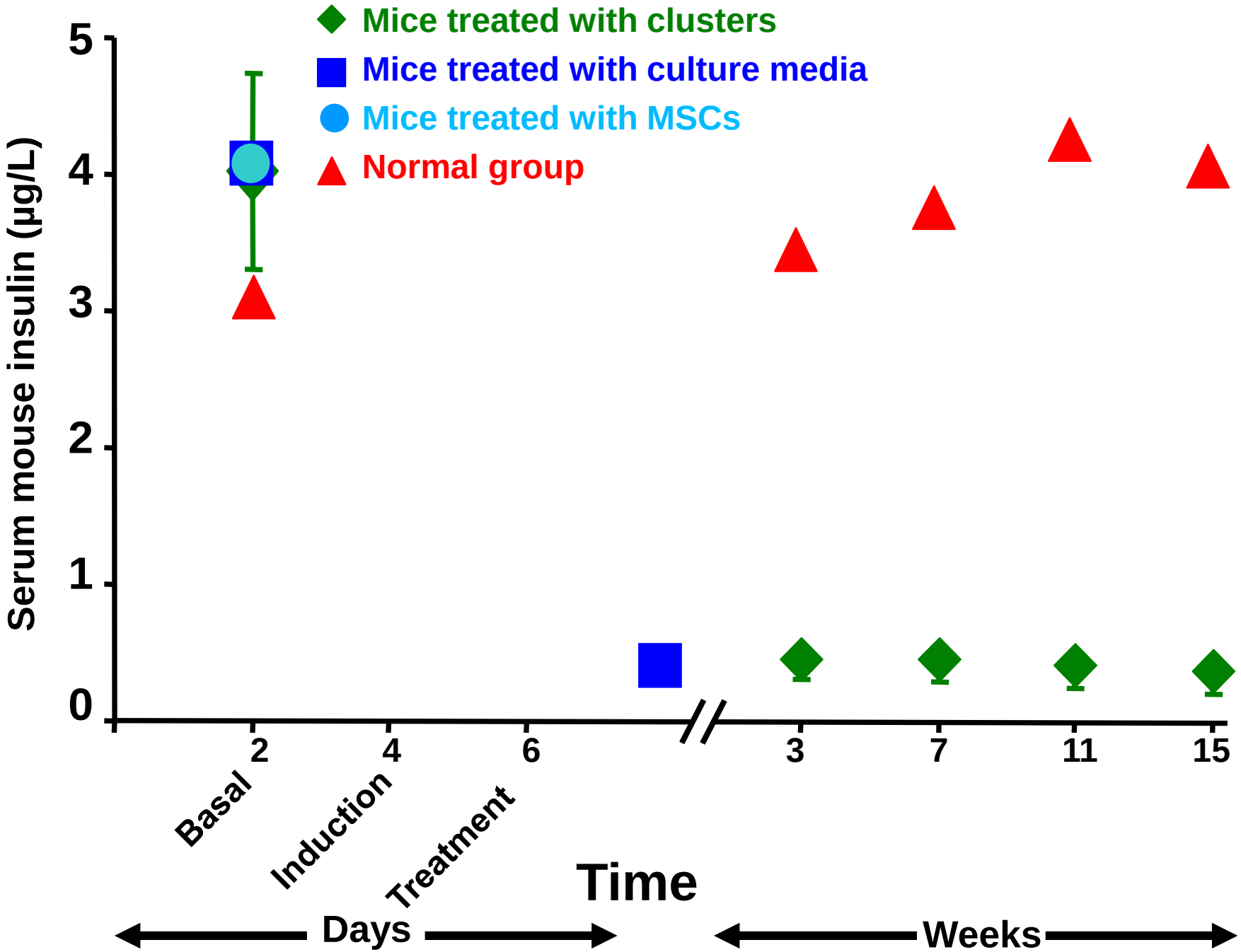
2)Diabetic animals treated with unmodified MSCs :$1x10^6$ cells (10 mice).

3)Diabetic animals treated with IPCs : $1x10^6$ under the renal capsule. (20 mice)

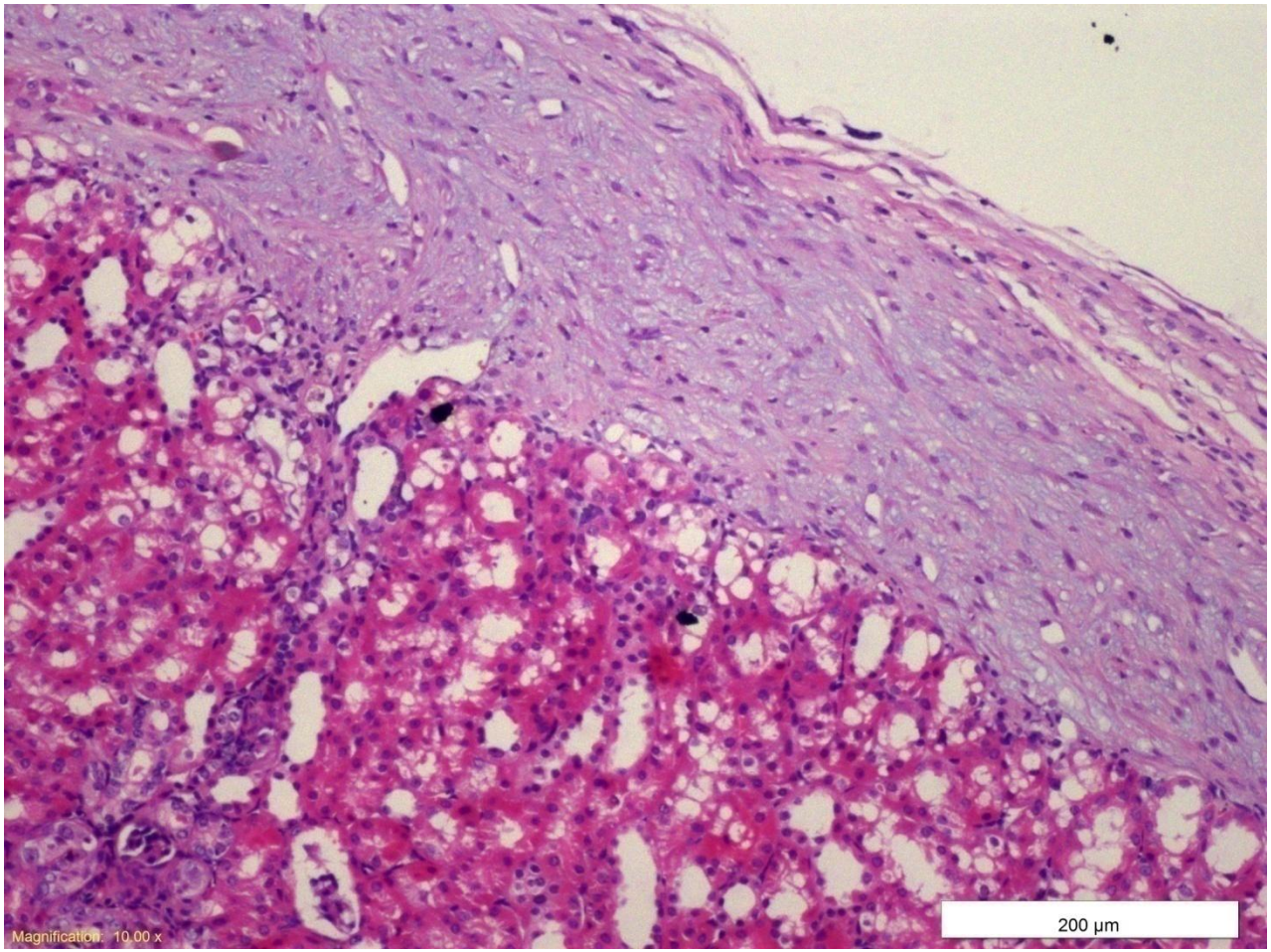
4)Non diabetic untreated animals :normal controls (10 mice)


Blood Glucose (mmol/L)
30
25
20
15
10
5
0
Mice treated with clusters
Mice treated with culture media
Mice treated with MSCs
Normal group
2
4
6
1
5
9
13
Basal
Induction
Treatment
Nephrectomy
Time
Days
Weeks

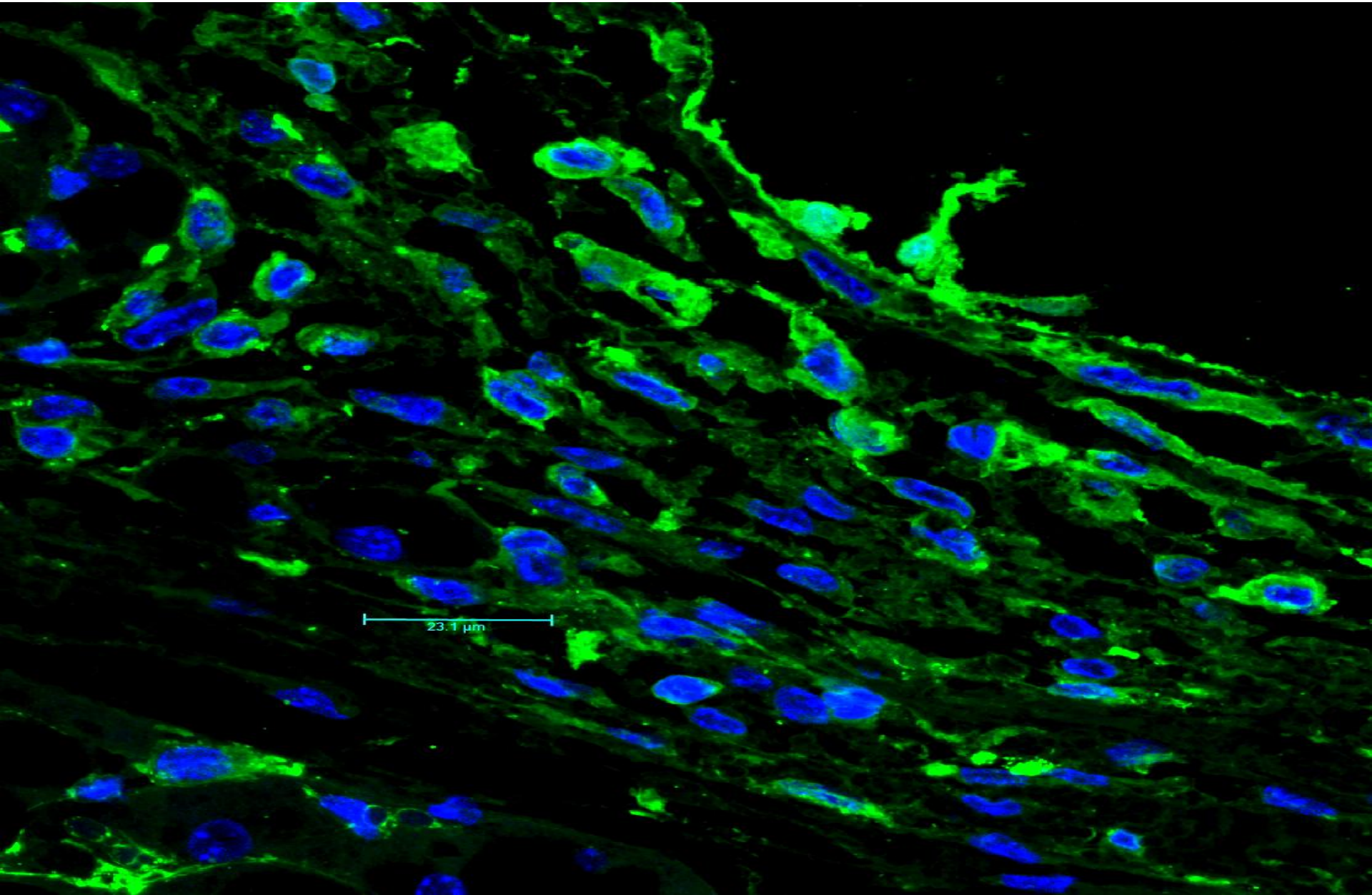

Mice treated with clusters
Mice treated with culture media
Mice treated with MSCs
Normal group
Serum human insulin (uIU/mL)
40
35
30
25
20
15
10
5
0
2
4
6
2
6
10
14
Basal
Induction
Treatment
Nephrectomy
Time
Days
Weeks


Mice treated with clusters
Mice treated with culture media
Mice treated with MSCs
Normal group
Serum mouse insulin (µg/L)
5
4
3
2
1
0
2
4
6
3
7
11
15
Basal
Induction
Treatment
Time
Days
Weeks

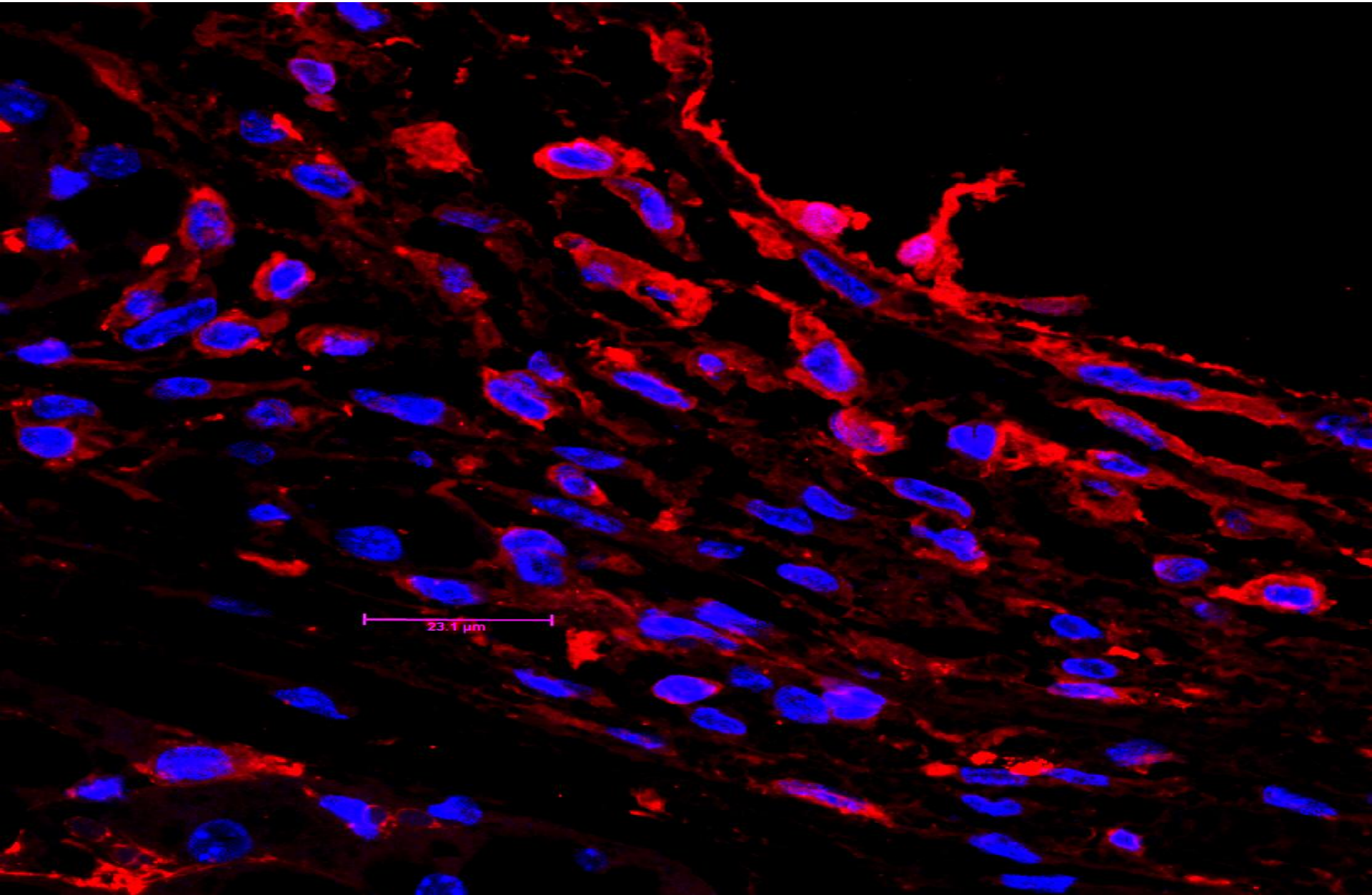
MSCs under renal capsule of Kidney (Hx & E)



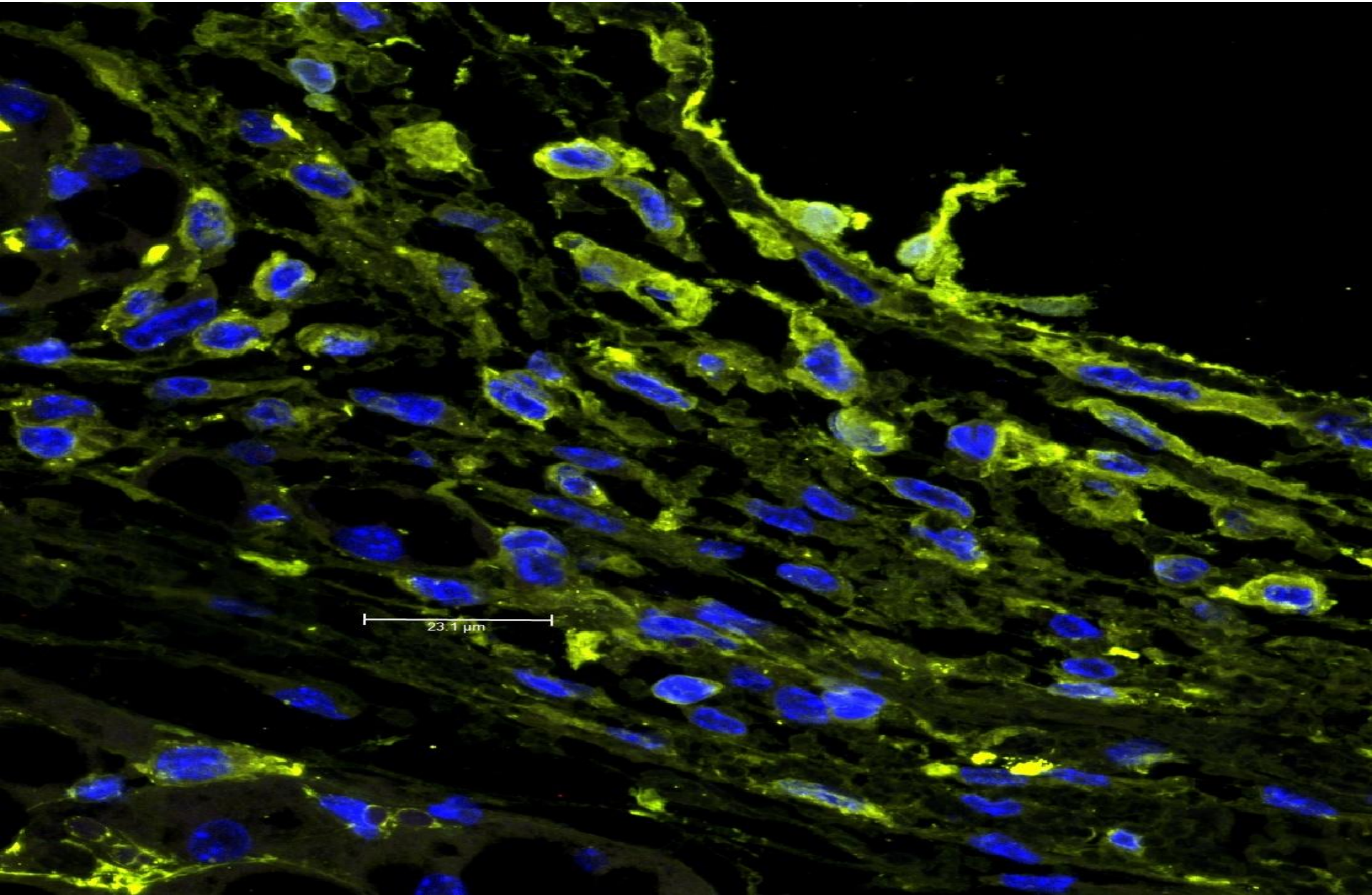
Eissa Trichostatin protocol 10 days (with laminin) 3 month post-transplant (positive intracytoplasmic insulin - green)

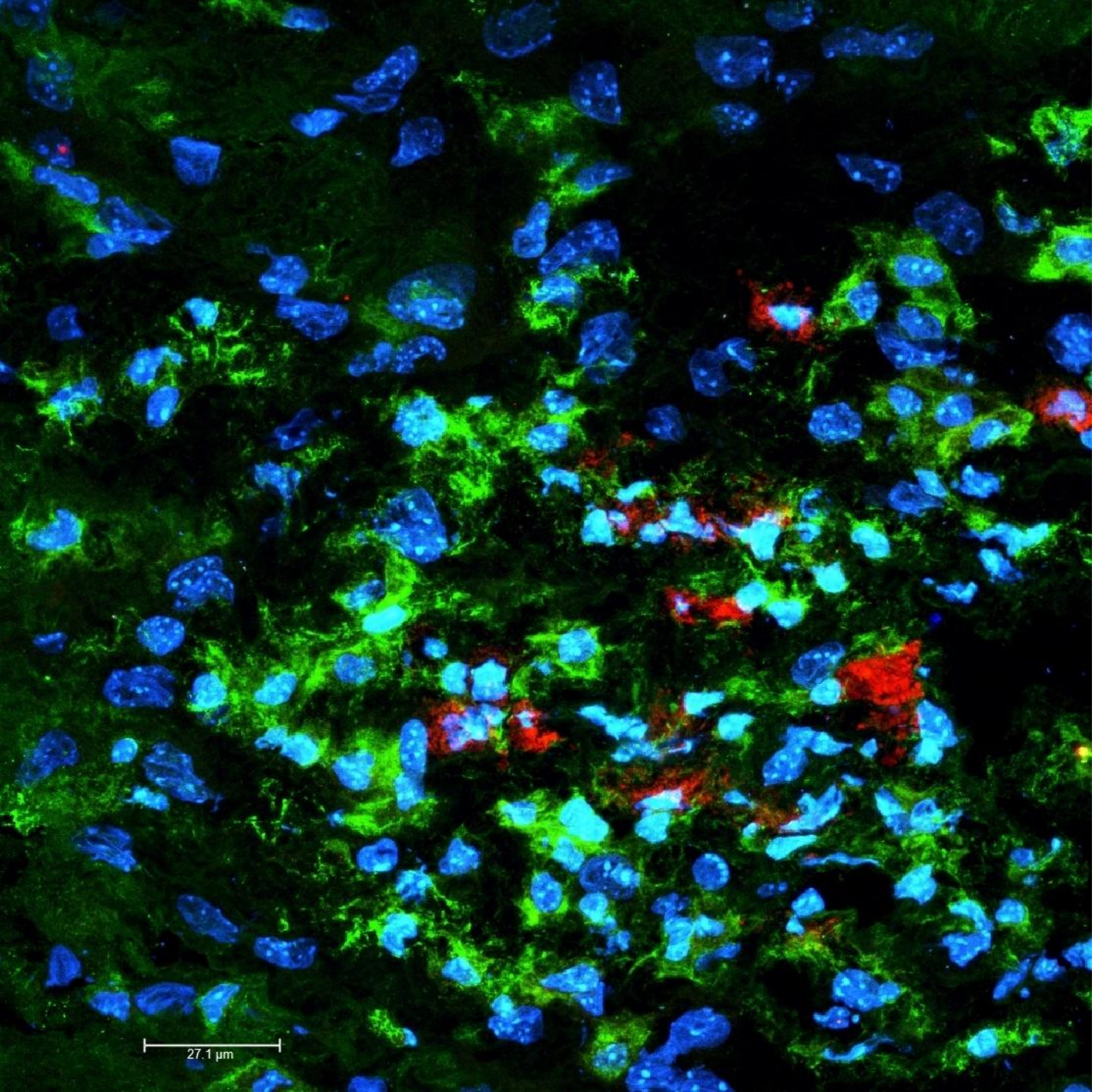


Eissa Trichostatin protocol 10 days (with laminin) 3 month post-transplant (positive intracytoplasmic c-peptide - red)

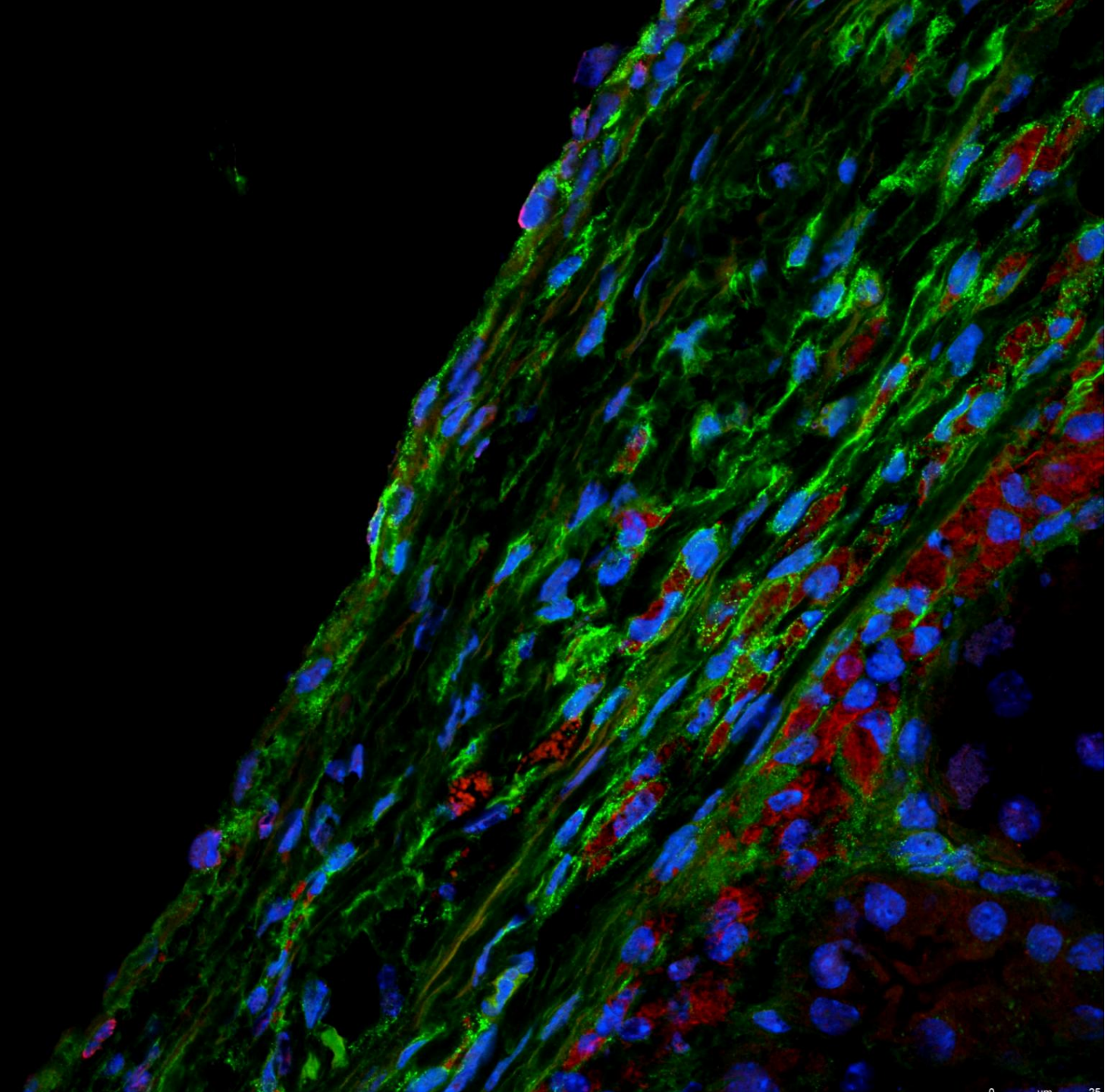


Eissa Trichostatin protocol 10 days (with laminin) 3 month post-transplant (intracytoplasmic insulin & c-peptide colocalization - yellow)

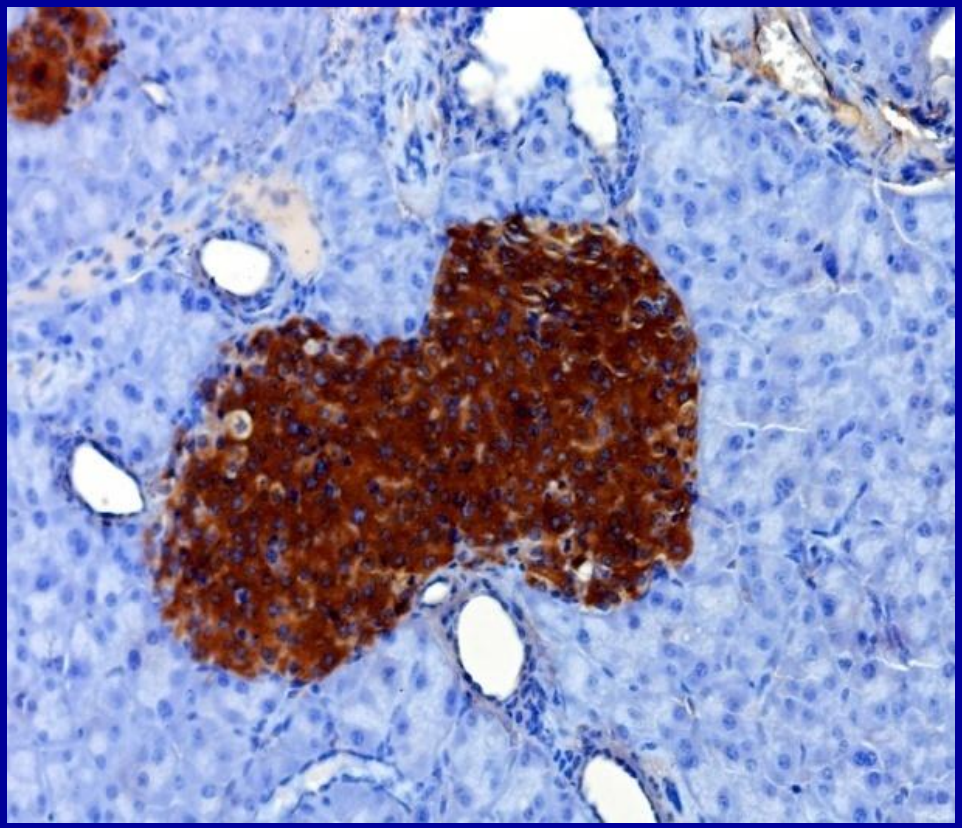


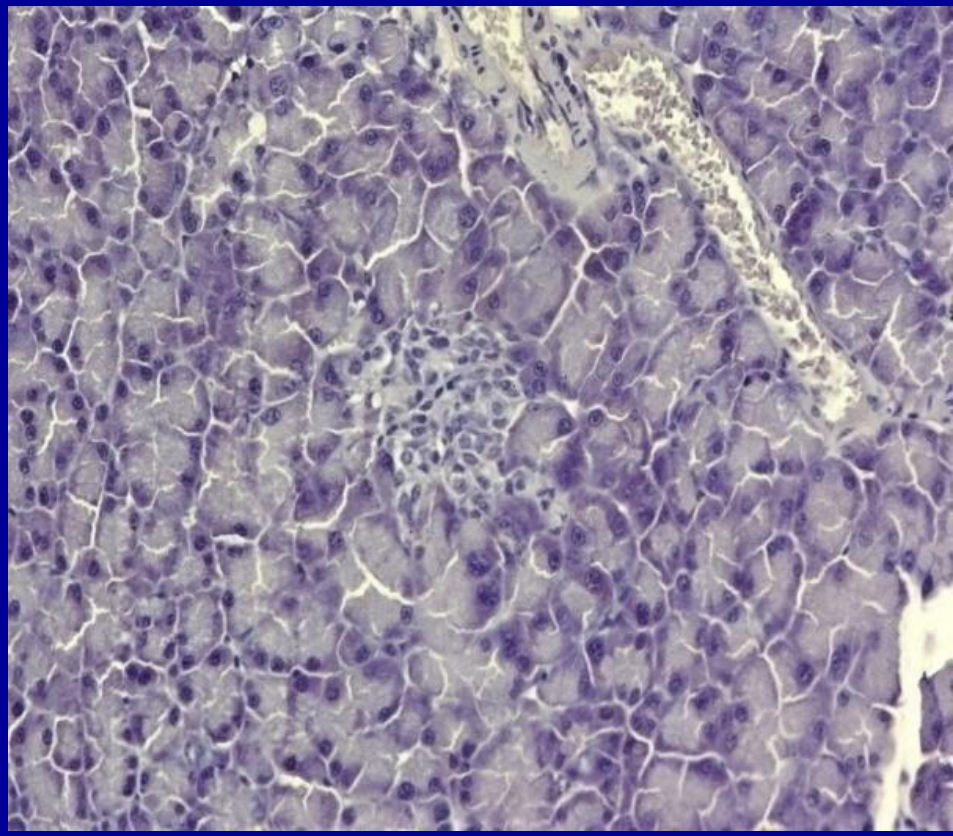


Insulin +ve (GREEN) & glucagon +ve (RED) differentiated MSCs transplanted under renal capsule (63X/1.40 OIL)



Insulin +ve (Green) & Somatostatin +ve (RED) differentiated MSCs transplanted under renal capsule (63X/1.40 OIL)





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Insulin-Producing Cells From Adult Human Bone Marrow Mesenchymal Stem Cells Control Streptozotocin-Induced Diabetes in Nude Mice

Mahmoud M. Gabr,* Mahmoud M. Zakaria,* Ayman F. Refaie,* Amani M. Ismail,* Mona A. Abou-El-Mahasen,* Sylvia A. Ashamallah,* Sherry M. Khater,* Sawsan M. El-Halawani,* Rana Y. Ibrahim,* Gan Shu Uin,† Malgorzata Kloc,‡ Roy Y. Calne,†§ and Mohamed A. Ghoneim*

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†Department of Surgery, Yong Loo Lin School of Medicine, National University of Singapore, Singapore
‡The Methodist Hospital, Department of Surgery and The Methodist Hospital Research Institute, Houston, TX, USA
§Department of Surgery, Cambridge University, Cambridge, UK

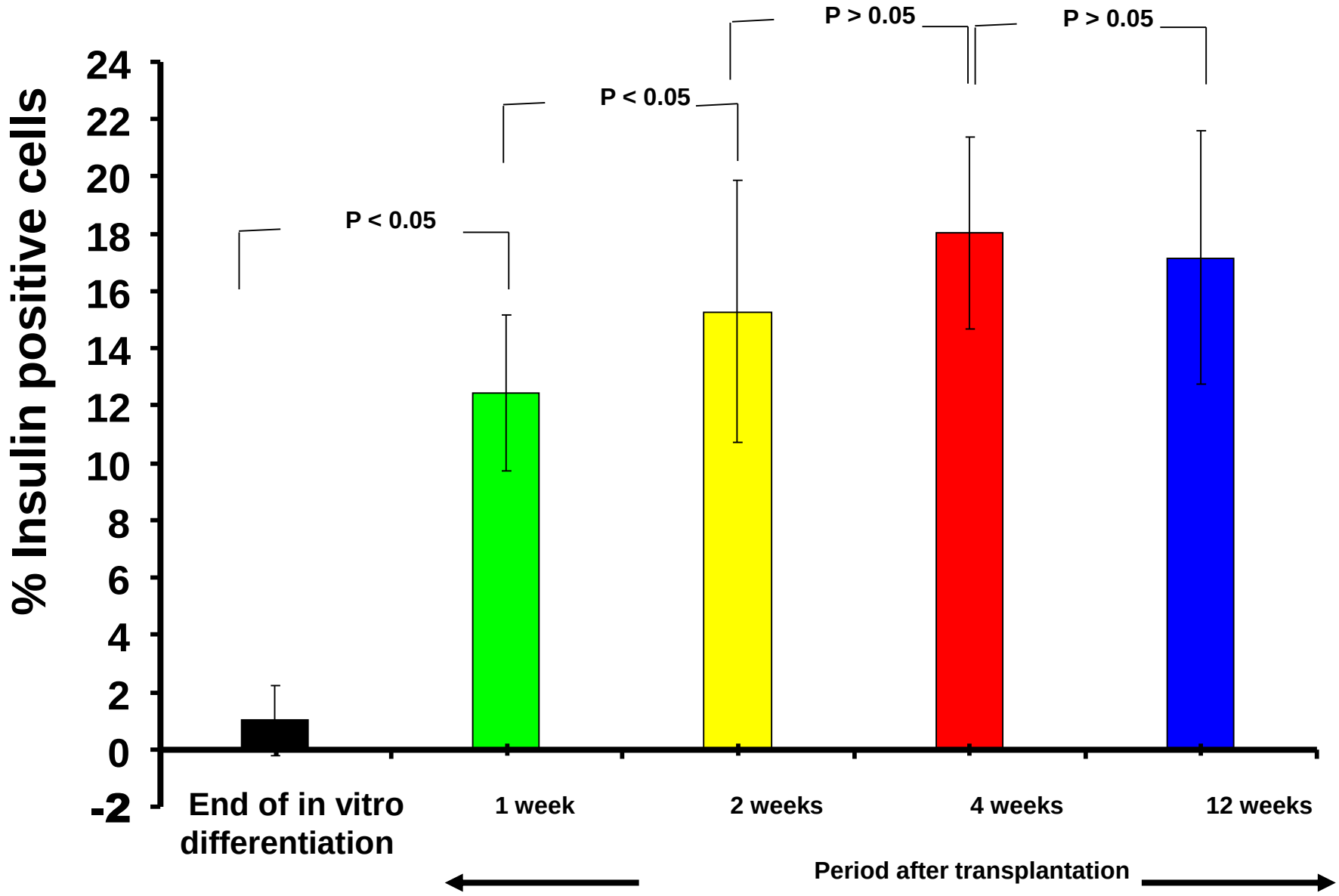
A clue for a dilemma

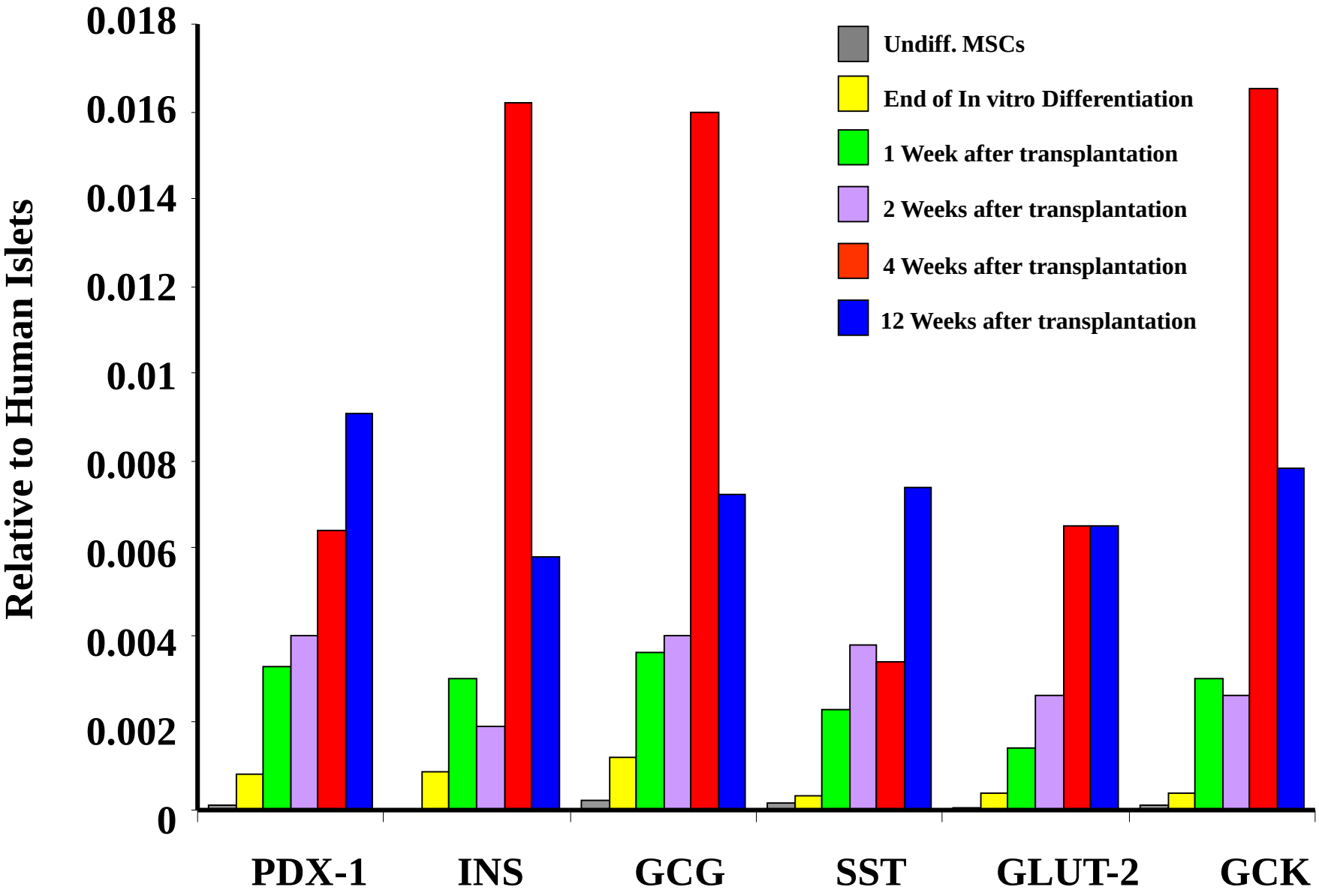
The Dilemma

- The yield of IPCs after directed differentiation of HB-MSCs was modest
- Nevertheless , transplantation of these cells cured diabetic nude mice

Material and Method

1) HBM-MSCs were differentiated to form IPCs.
2)Induction of diabetes in nude mice
3)Transplantation of differentiated MSC under the renal capsule of diabetic mice.
4) Harvesting of the transplanted kidneys : one,2,4 and12 weeks after transplantation.
5) examination of the harvested kidneys :
cell count of IPCS and relative gene expression of relevant pancreatic genes


% Insulin positive cells
24
22
20
18
16
14
12
10
8
6
4
2
0
-2
P < 0.05
P < 0.05
P > 0.05
P > 0.05
End of in vitro differentiation
1 week
2 weeks
4 weeks
12 weeks
Period after transplantation


Relative to Human Islets
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
Undiff. MSCs
End of In vitro Differentiation
1 Week after transplantation
2 Weeks after transplantation
4 Weeks after transplantation
12 Weeks after transplantation
PDX-1
INS
GCG
SST
GLUT-2
GCK

The Harvest

- The proportion of IPCs increased to reach a maximum at 4 weeks (20%).There was no changes thereafter.
- There was a parallel increase in the relative expression of the relevant pancreatic endocrine genes.
- Further maturation is the result of favorable factors in the in vivo microenvironment .

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Research Article

Differentiation of Human Bone Marrow-Derived Mesenchymal Stem Cells into Insulin-Producing Cells: Evidence for Further Maturation In Vivo

Mahmoud M. Gabr,[1] Mahmoud M. Zakaria,[1] Ayman F. Refaie,[2] Sherry M. Khater,[3] Sylvia A. Ashamallah,[3] Amani M. Ismail,[4] Sawsan M. El-Halawani,[1] and Mohamed A. Ghoneim[5]

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[4] *Department of Immunology, Urology and Nephrology Center, Mansoura 35516, Egypt*
[5] *Department of Urology, Urology and Nephrology Center, Mansoura 35516, Egypt*

Experiments with dogs

Justifications :

- A large animal .
- Calculation of the optimal number of cells / kg .
- Testing of encapsulation
- Long term follow up.

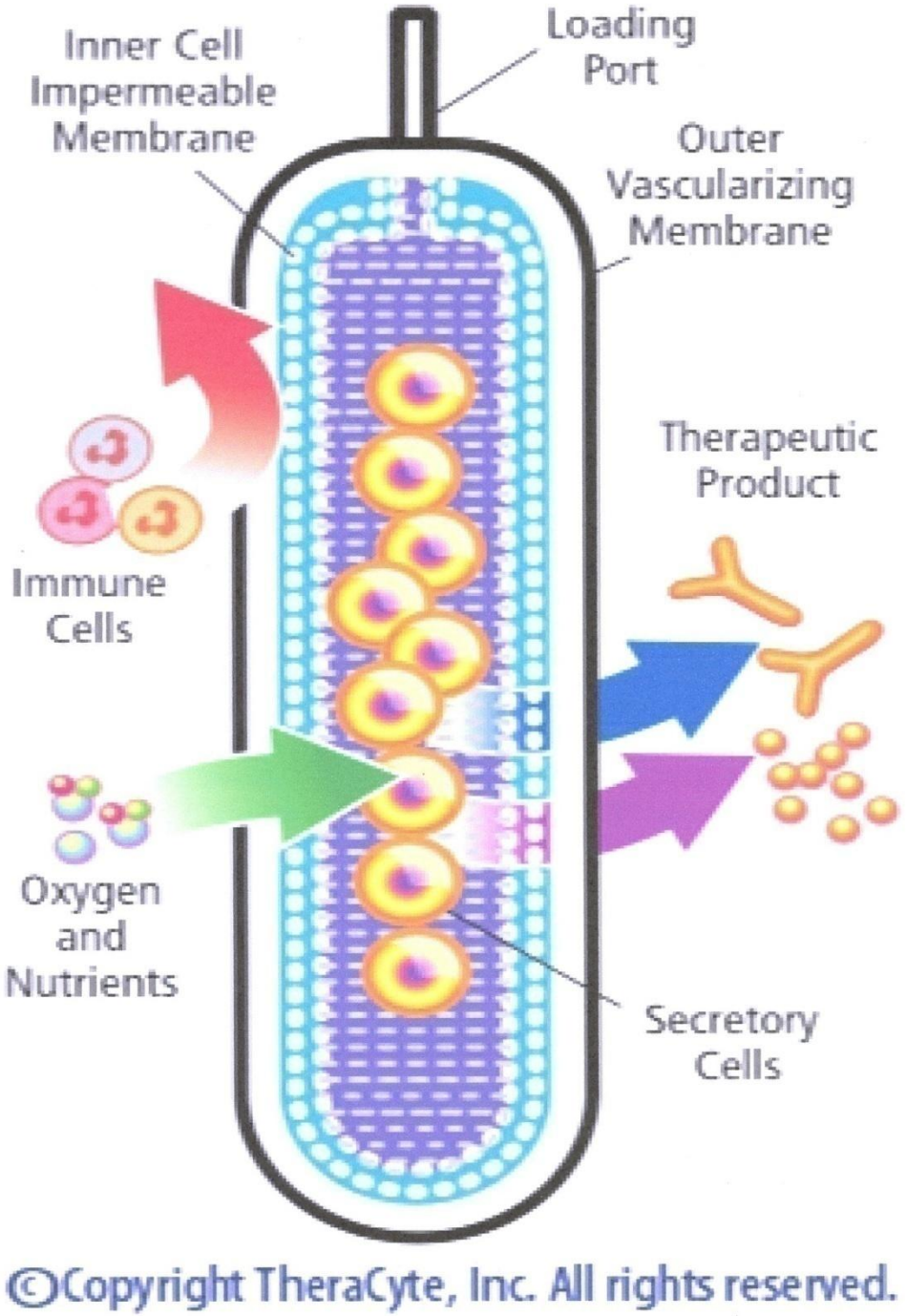
Induction of diabetes

- A mixture of Alloxan (40mg /kg) and STZ (35mg / kg).
- The mixture is freshly prepared and dissolved in citrate buffer and given I.V.
- Animals were considered diabetic when their fasting blood sugar is more than 350 mg / ml .

The Procedure

- The procedure was carried under general anesthesia with intubation.
- Five million differentiated cells were loaded in 2 Theracyte capsules .
- The capsules were transplanted in a pocket created under the anterior rectus sheath.

5 million cells /kg were loaded into:
a Theracyte capsules.



Follow -up

-Azathiopurine (1 mg/kg) / day was given orally.

-Monthly :

- Fasting blood sugar
- Serum human and canine insulin
- Serum human and canine c-peptide
- Body weight

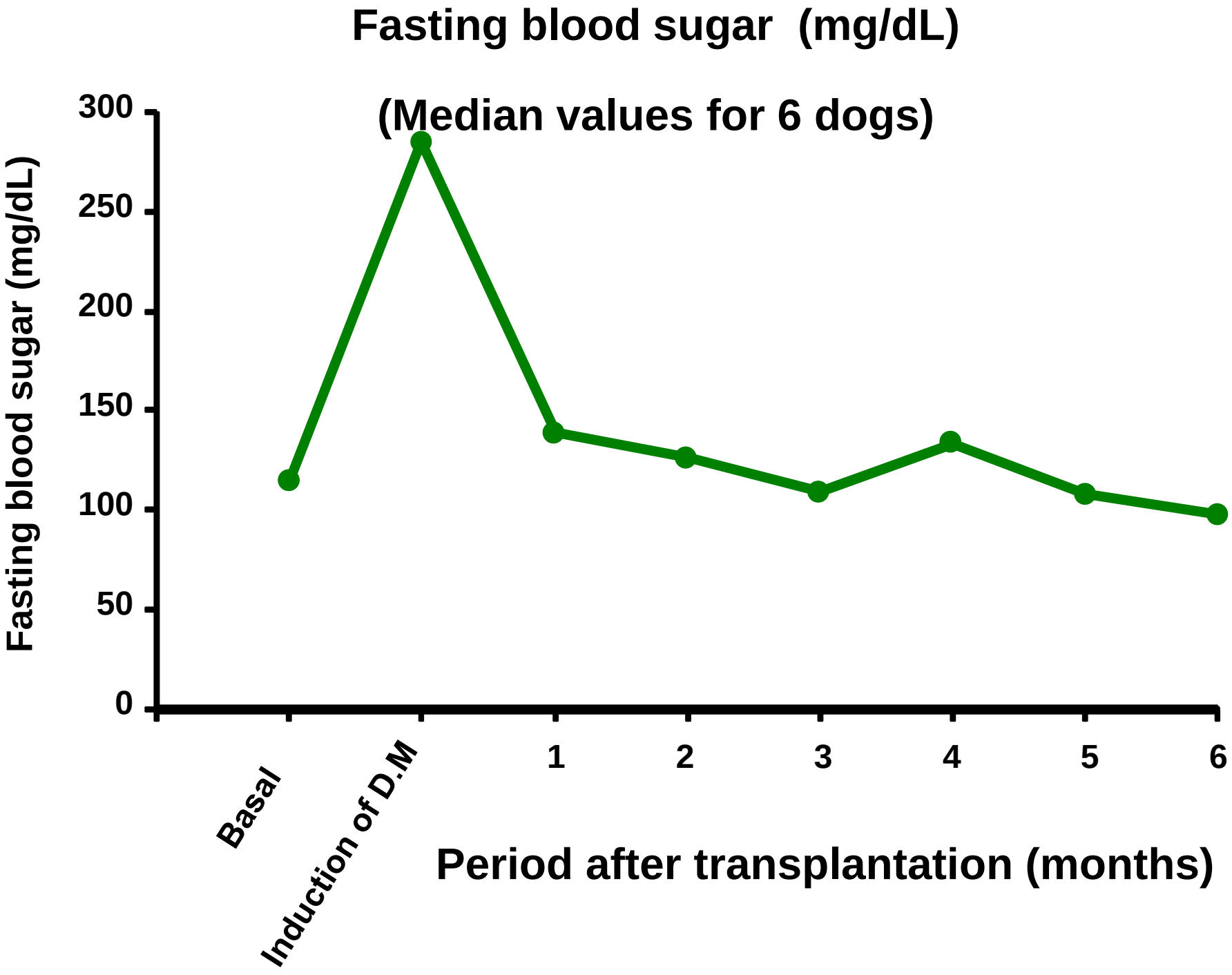
- Three & 6 monthly

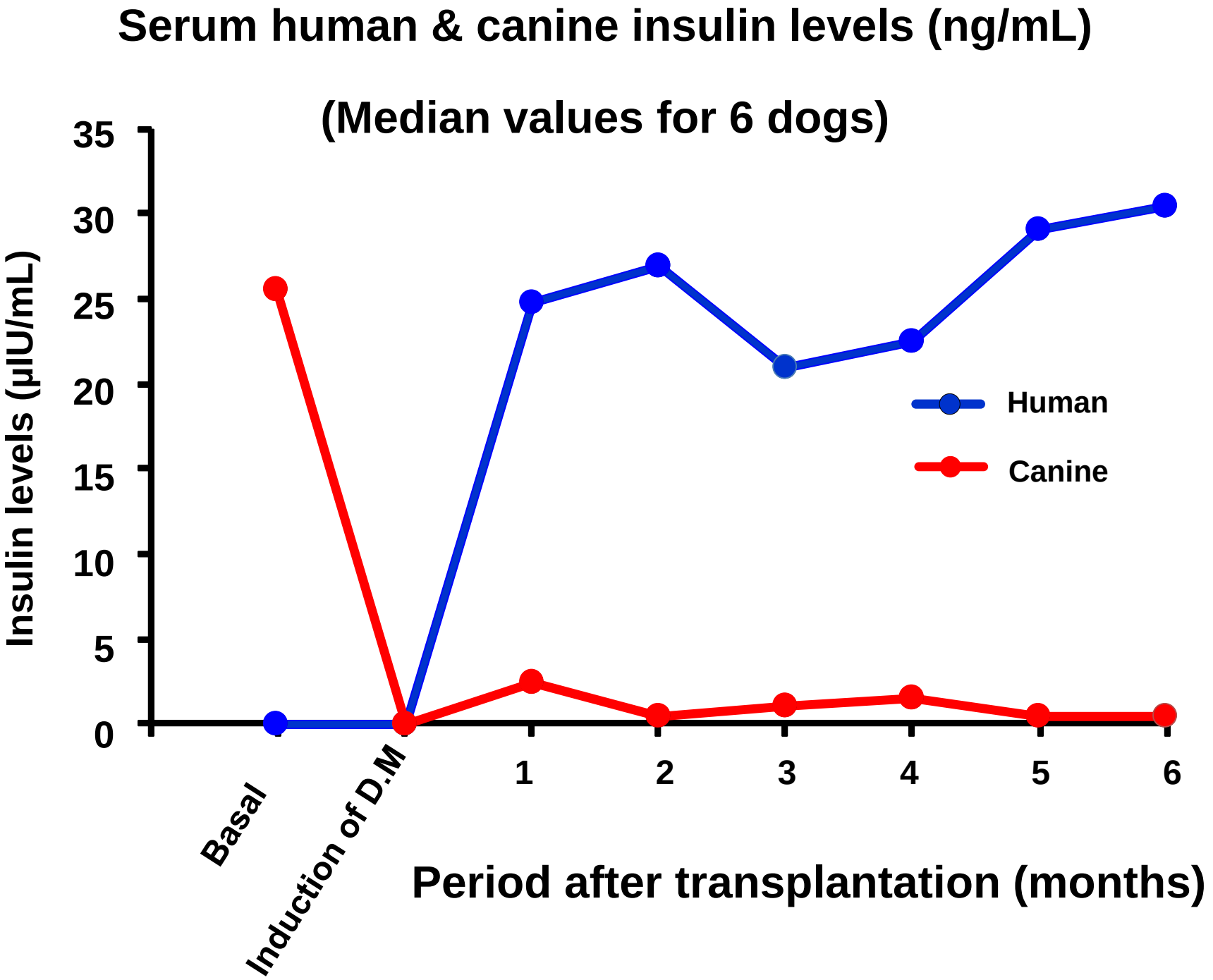
glucose tolerance curve

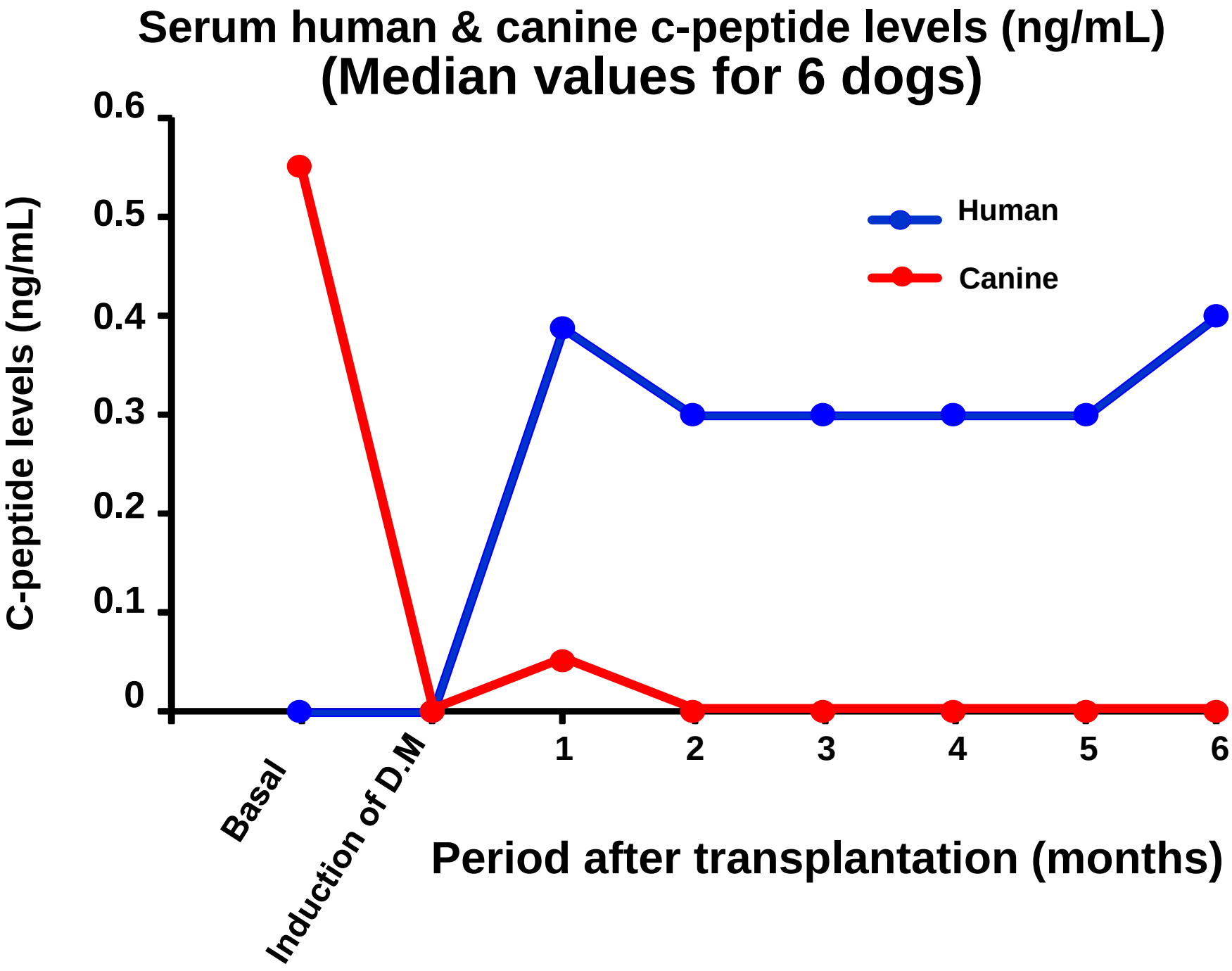
A brief summary of results

The experiment was carried so far for 6 dogs . Results are for a follow up of a minimum of 6 months .

- Four dogs are euglycemic with a normal glucose tolerance curves.
- Two dogs are partially controlled .

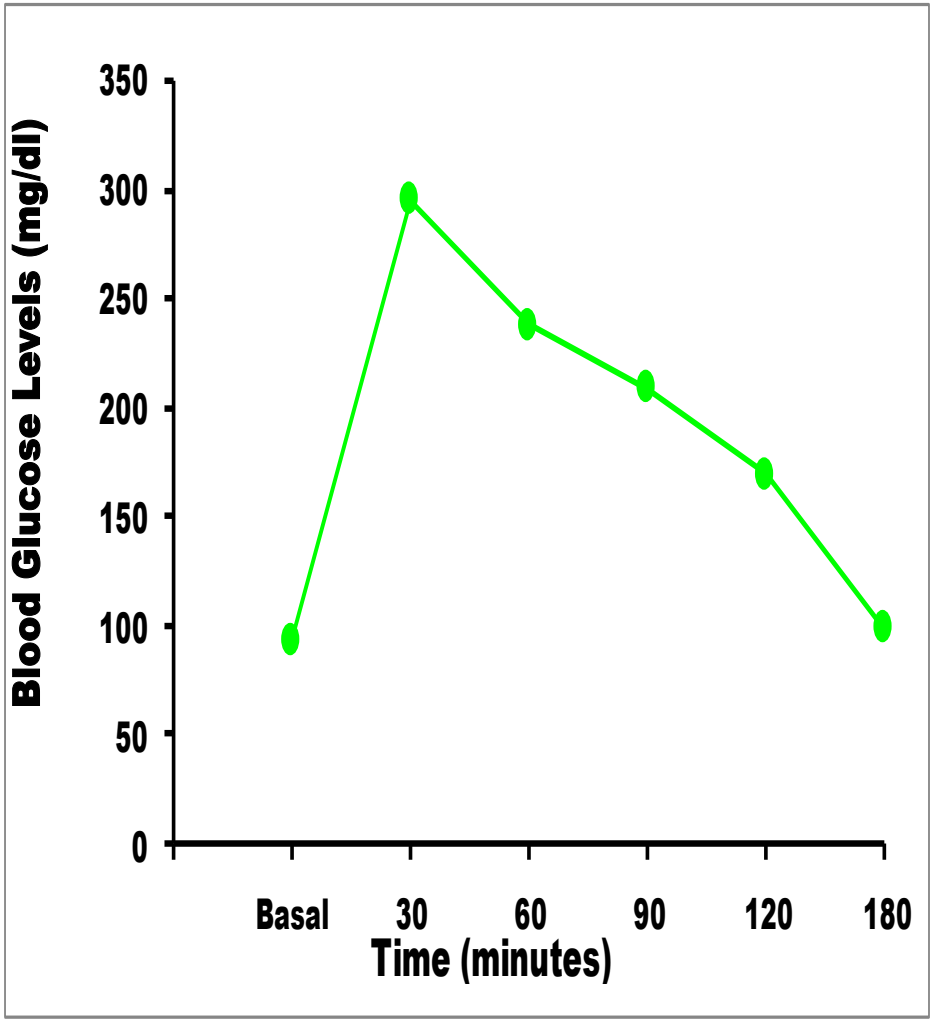

Fasting blood sugar (mg/dL)
(Median values for 6 dogs)
Fasting blood sugar (mg/dL)
300
250
200
150
100
50
0
Basal
Induction of D.M
1
2
3
4
5
6
Period after transplantation (months)

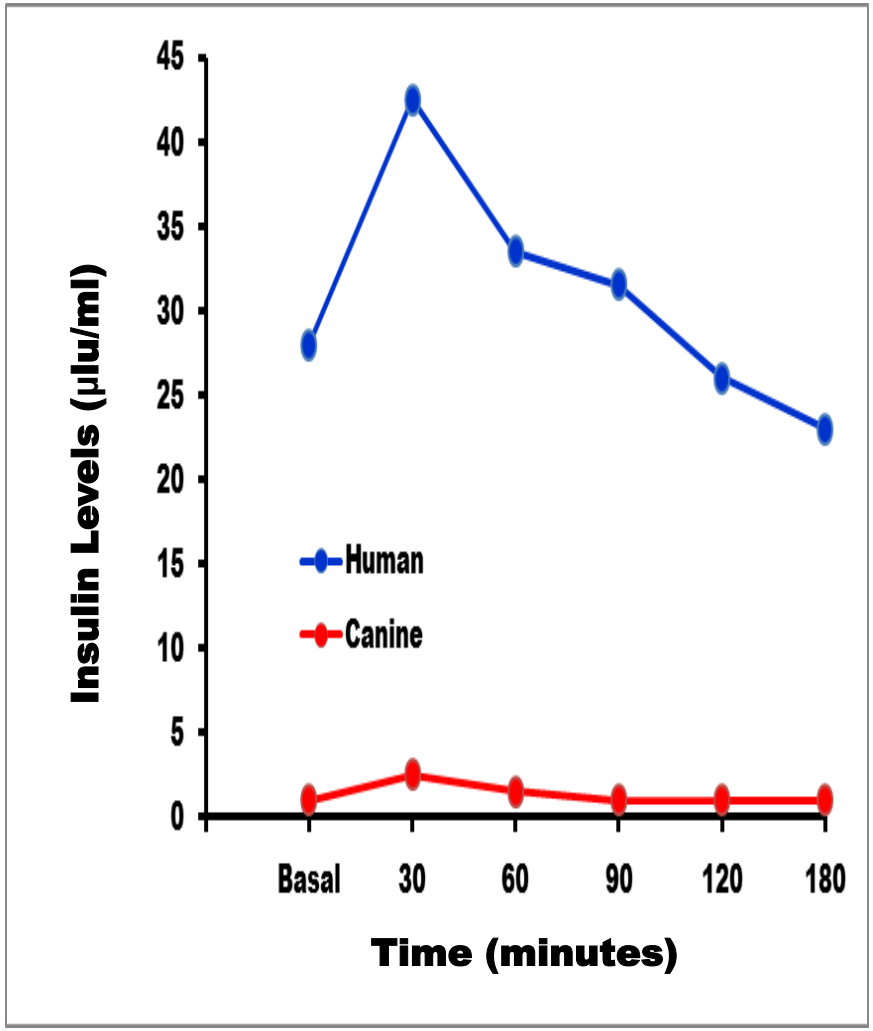

Serum human & canine insulin levels (ng/mL)
(Median values for 6 dogs)
Insulin levels (μIU/mL)
35
30
25
20
15
10
5
0
Human
Canine
Basal
Induction of D.M
1
2
3
4
5
6
Period after transplantation (months)


Serum human & canine c-peptide levels (ng/mL)
(Median values for 6 dogs)
C-peptide levels (ng/mL)
0.6
0.5
0.4
0.3
0.2
0.1
0
Human
Canine
Basal
Induction of D.M
1
2
3
4
5
6
Period after transplantation (months)

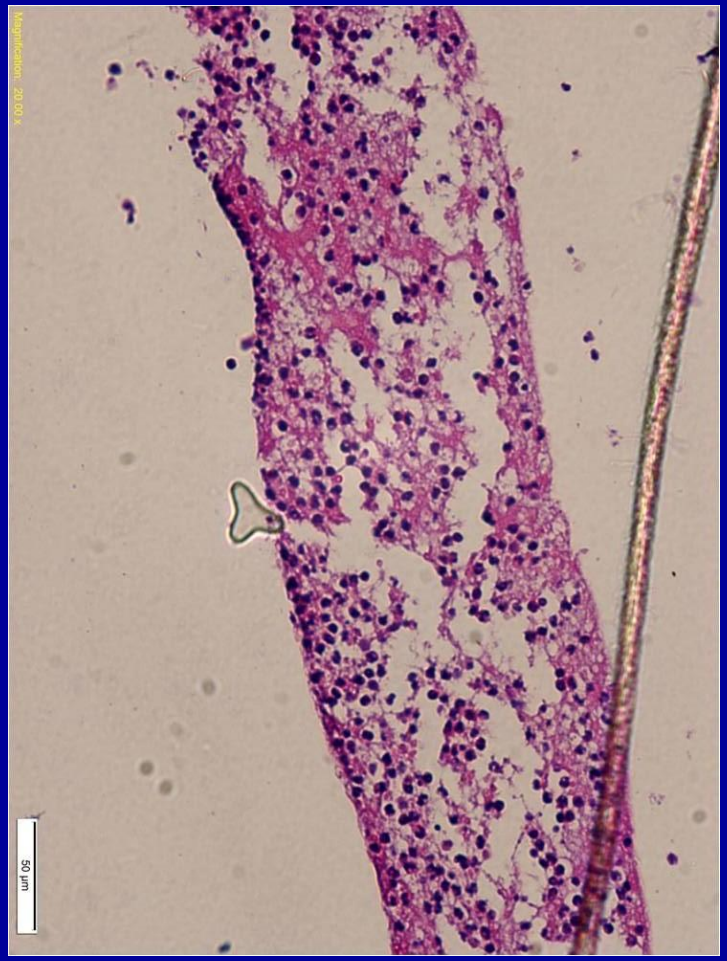
Intravenous glucose tolerance test of the 4 cured dogs

(Median values)

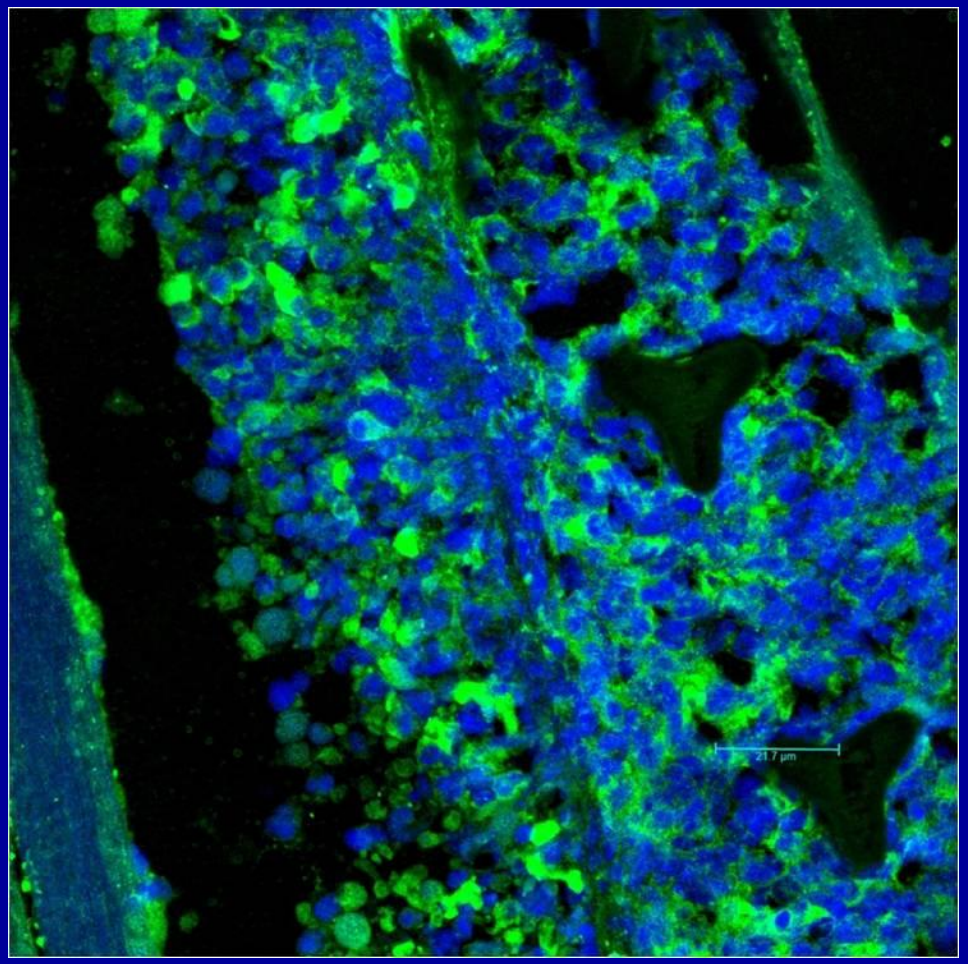




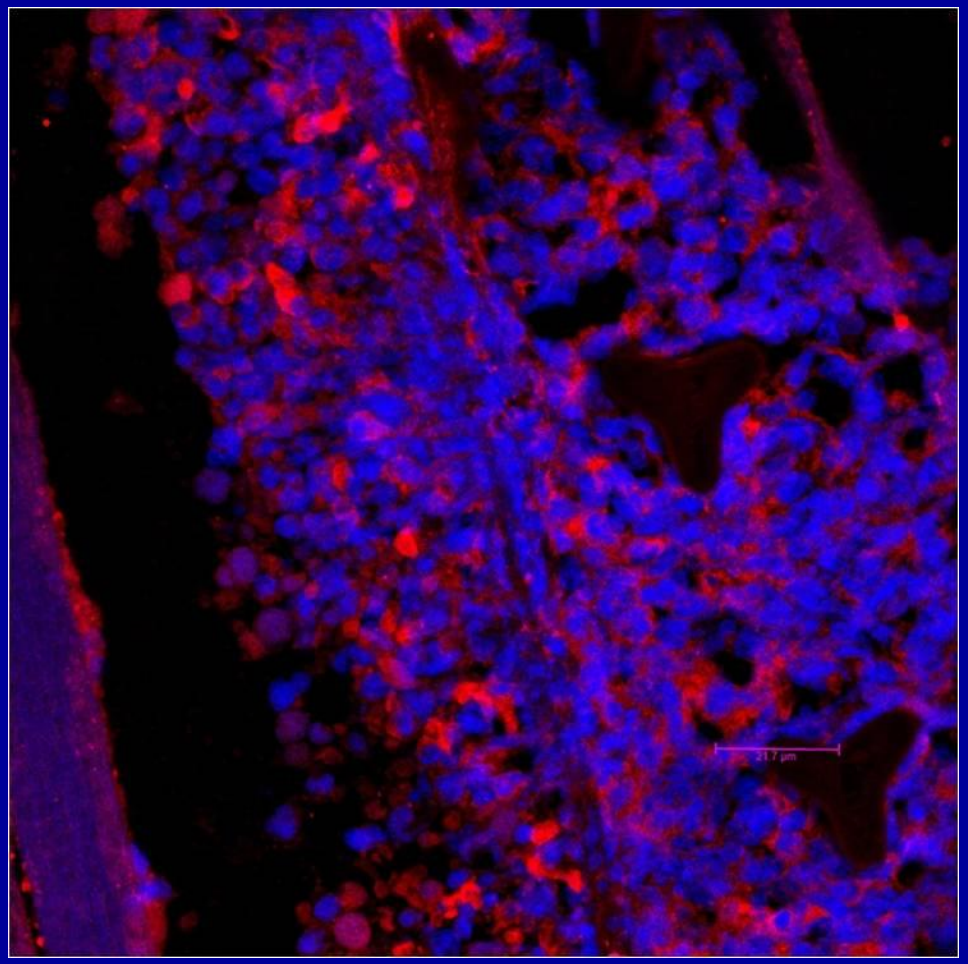
Explanted theracyte capsule 6 months after transplantation (Hx&Es.):



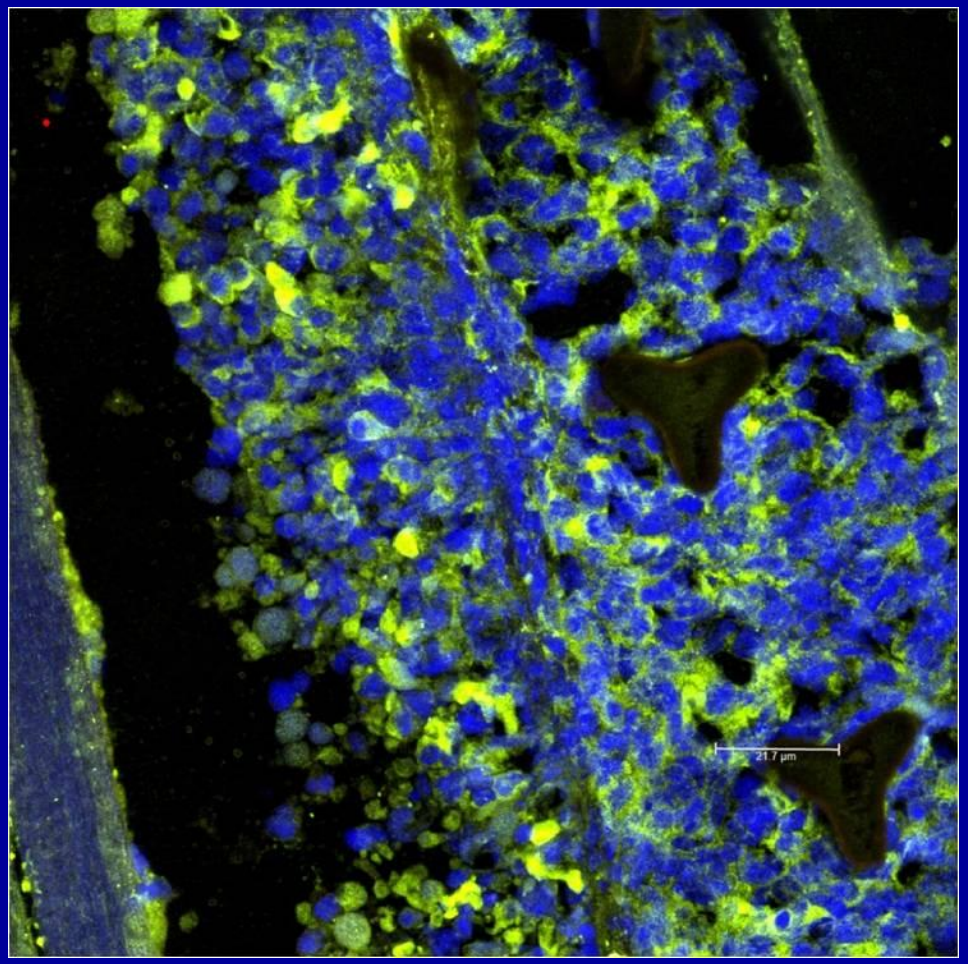
Theracyte capsule :
Intracytoplasic insulin positive granules (green)



Theracyte capsule:
Intracytoplasmic positive c-peptide granules (red)

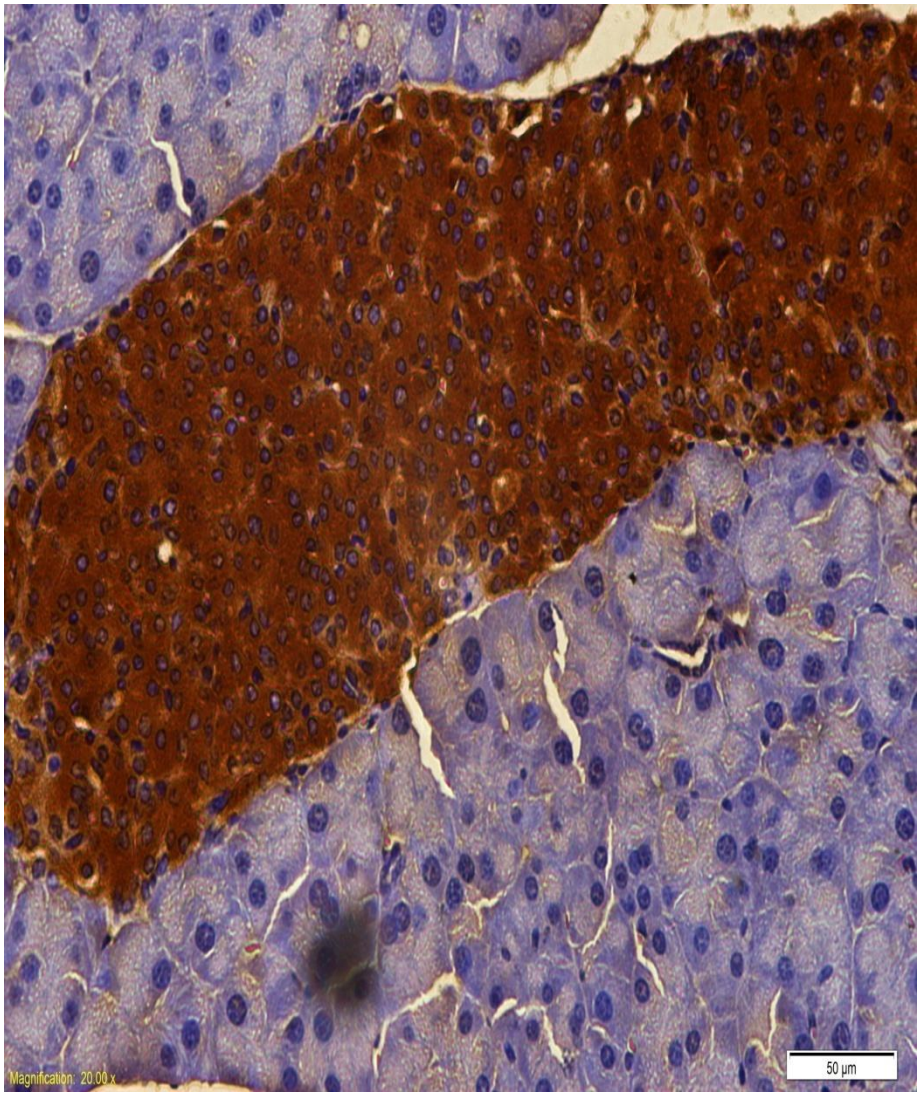


Theracyte capsule :
Co-localization of insulin & c-peptide (yellow)

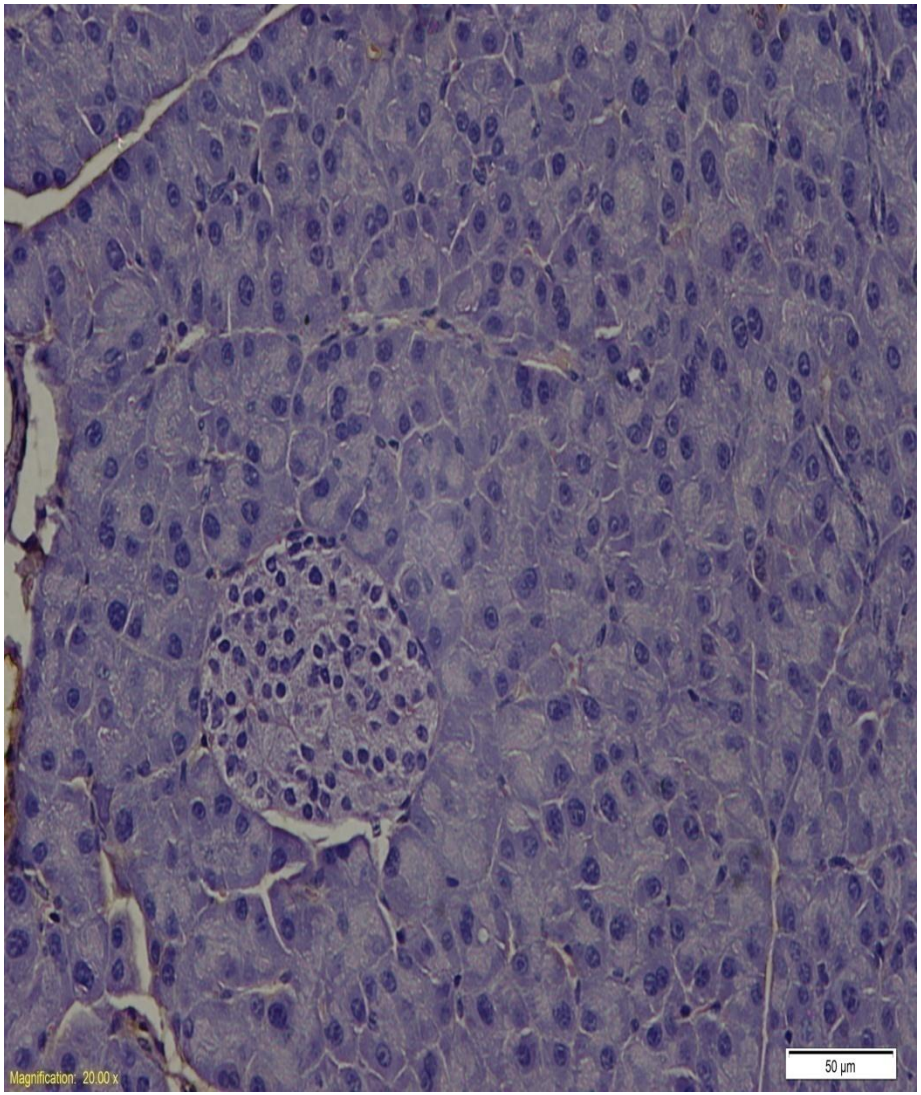


Immuno-cytochemistry

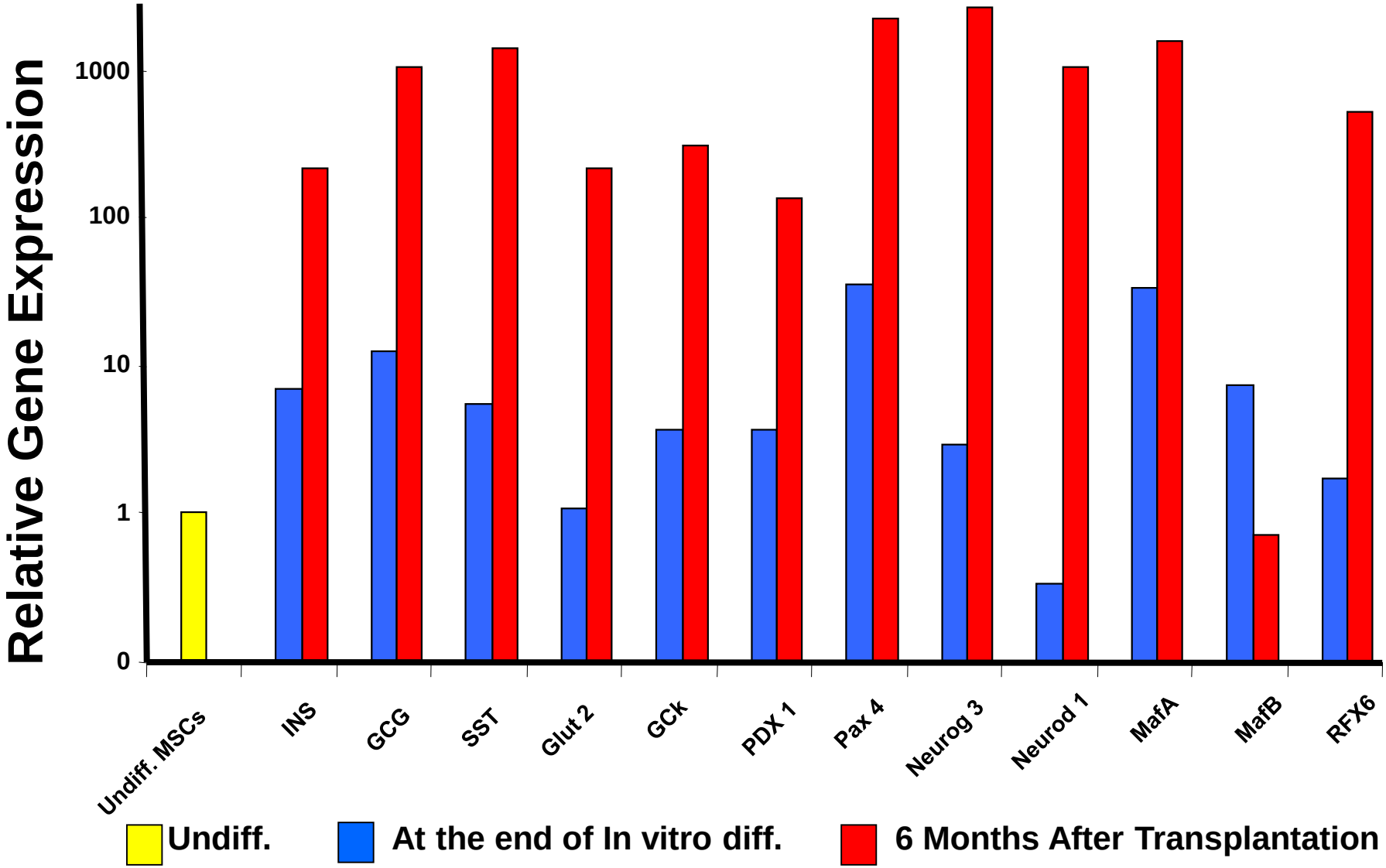
Pancreas of a normal dog



Pancreas of a dog 6 months after induction of diabetes



Gene Expression:
Mean values from 6 dogs



Other Important Trials

TRIALS WITH EMBRYONIC STEM CELLS

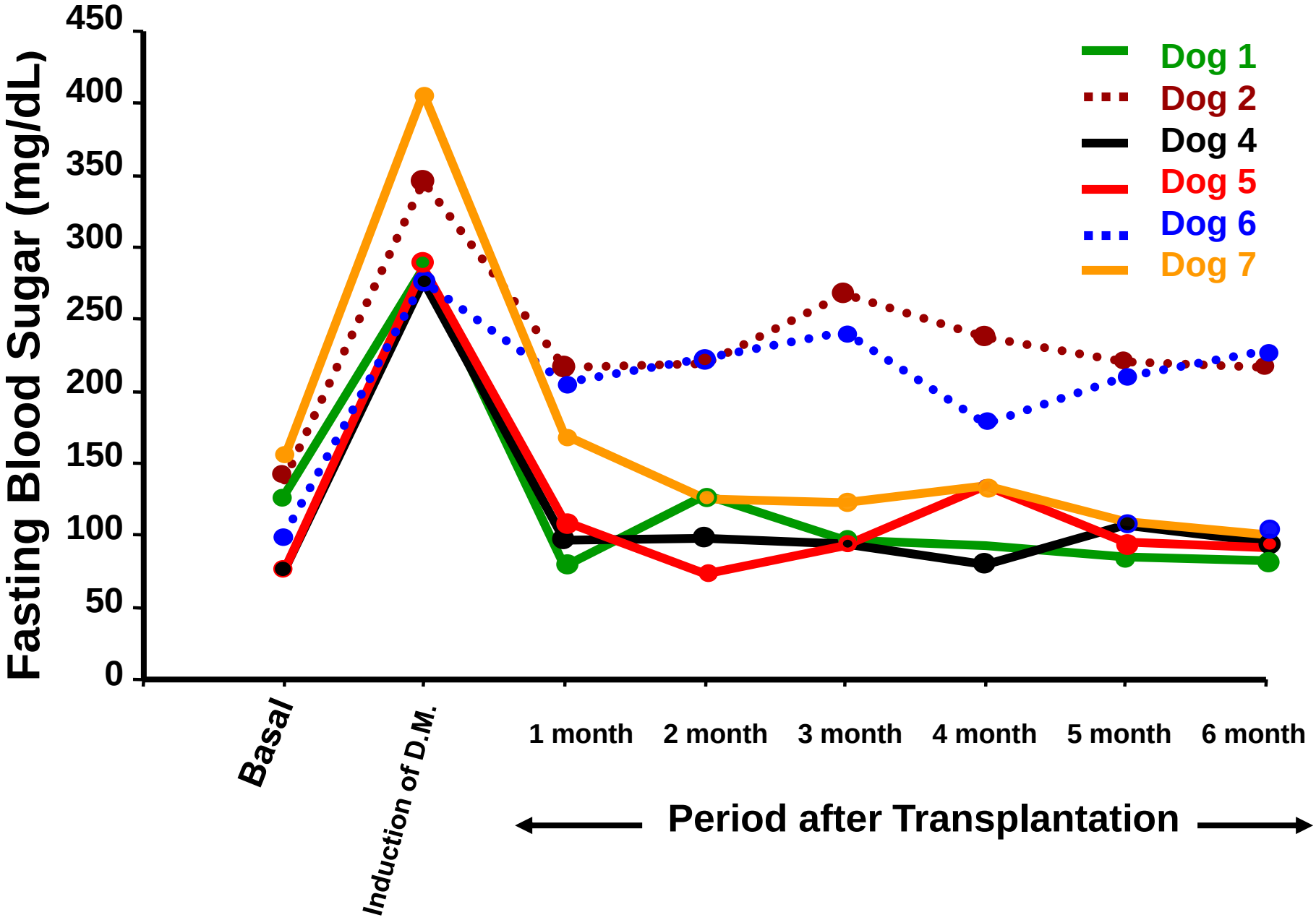
	Kieffer (Betarlogics)	Melton (Semma)	Schulz (Viacyte)
Type of cells	embryonic	iPCs	embryonic
Differentiation	7 steps	6 steps	4 steps
Duration	27 – 42 days	27 – 42 days	8 week
Time for correction of diabetes in mice :			
	50 days (kc)	75 days (kc)	50 days(sc)
Clinical trial	----	----	Yes

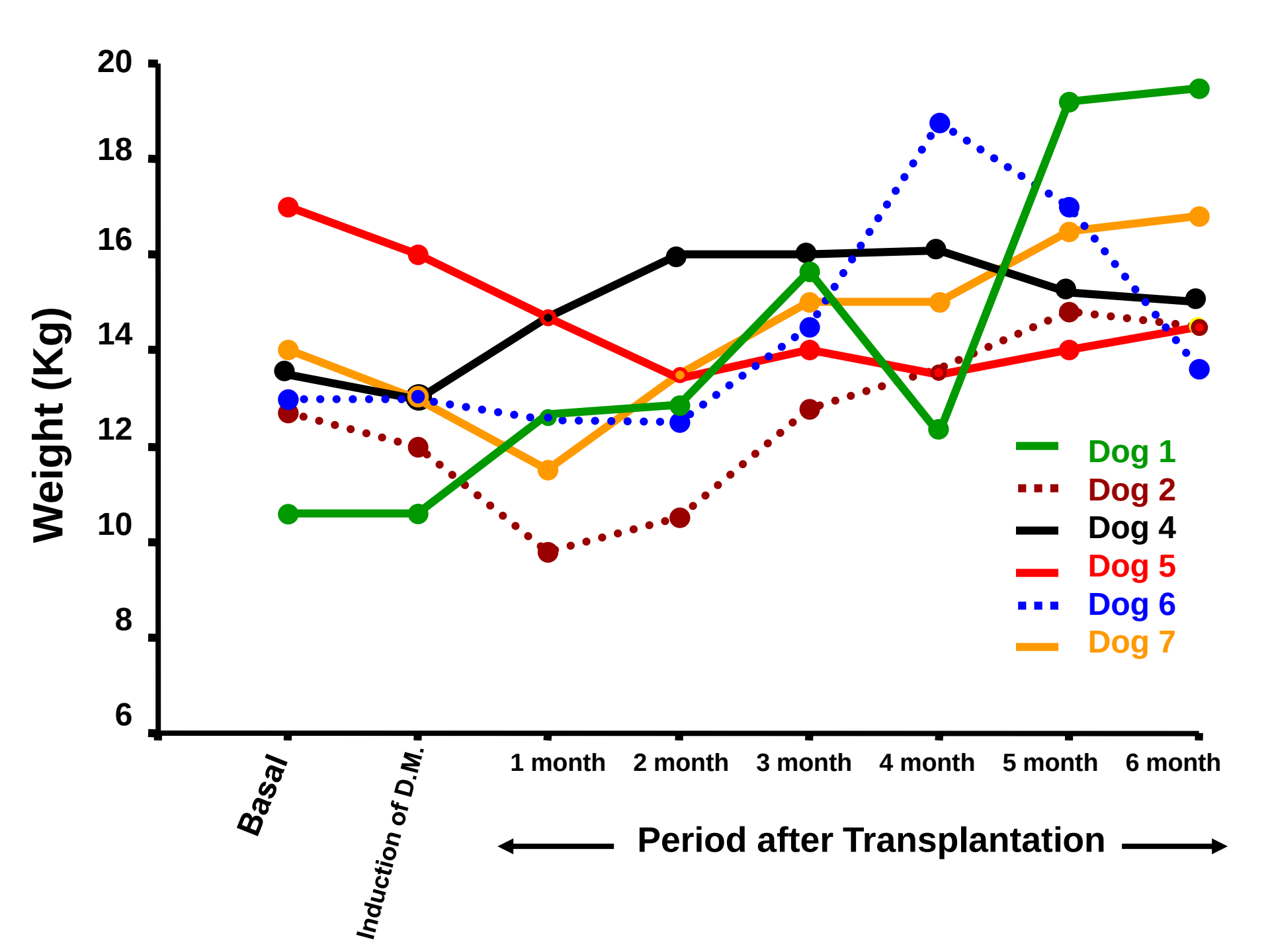
A long way (but not too long) before clinical application

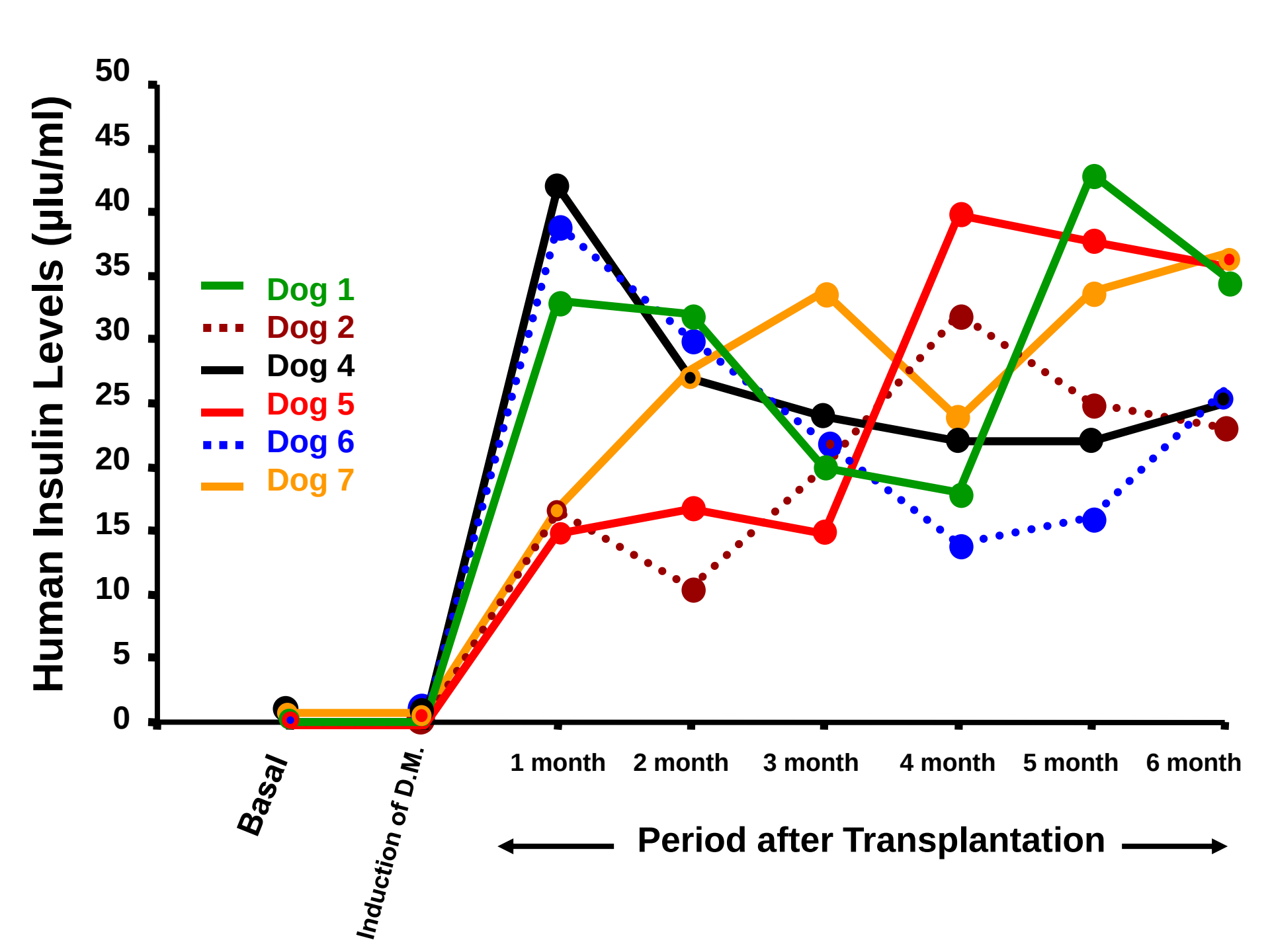
- How long these cells can maintain their function ?
- How many cells are needed per kg of body weight?
- What is the optimal site for their transplantation ?

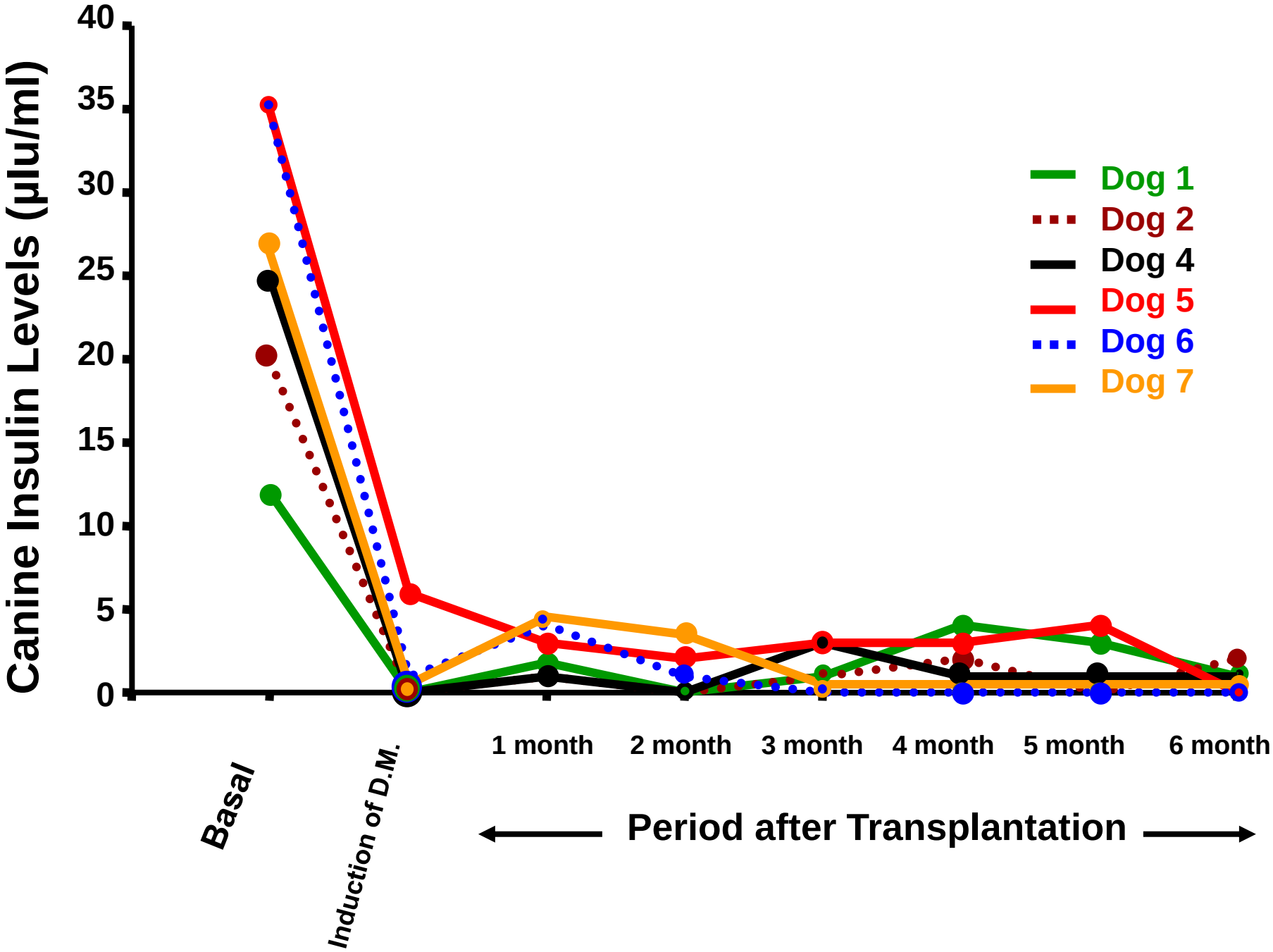
Acknowledgment

The project was generously supported by a grant from **Misr El-Kheir Foundation.**


Fasting Blood Sugar (mg/dL)
450
400
350
300
250
200
150
100
50
0
Dog 1
Dog 2
Dog 4
Dog 5
Dog 6
Dog 7
Basal
Induction of D.M.
1 month
2 month
3 month
4 month
5 month
6 month
Period after Transplantation


Weight (Kg)
20
18
16
14
12
10
8
6
Basal
Induction of D.M.
1 month
2 month
3 month
4 month
5 month
6 month
Period after Transplantation
Dog 1
Dog 2
Dog 4
Dog 5
Dog 6
Dog 7


Human Insulin Levels (μlu/ml)
50
45
40
35
30
25
20
15
10
5
0
Dog 1
Dog 2
Dog 4
Dog 5
Dog 6
Dog 7
Basal
Induction of D.M.
1 month
2 month
3 month
4 month
5 month
6 month
Period after Transplantation


Canine Insulin Levels (μlu/ml)
40
35
30
25
20
15
10
5
0
Dog 1
Dog 2
Dog 4
Dog 5
Dog 6
Dog 7
Basal
Induction of D.M.
1 month
2 month
3 month
4 month
5 month
6 month
Period after Transplantation