Programming the Software Functions of Adipose-Derived Stem Cells to Direct Hardware Repair

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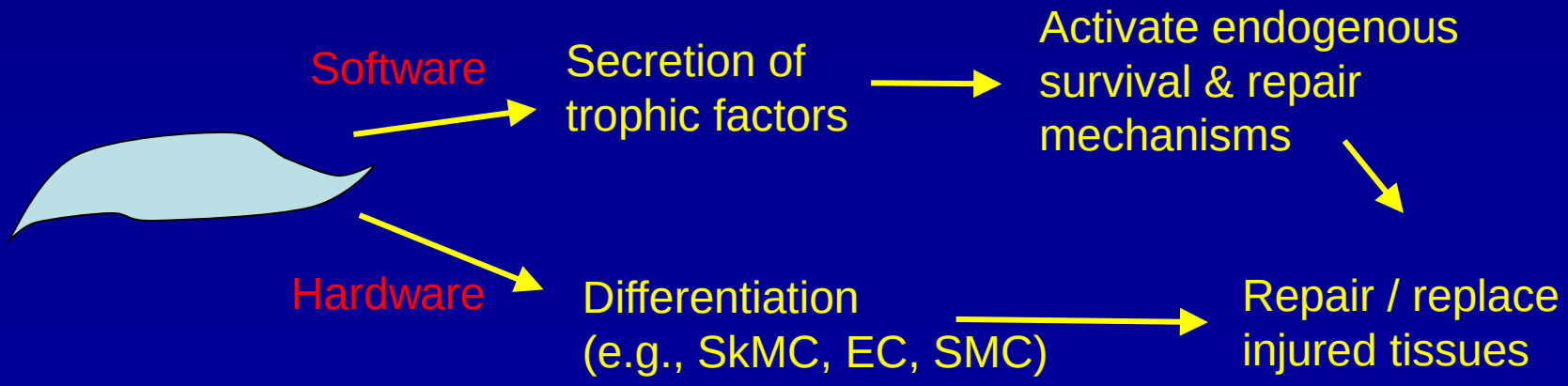
Emerging Hypothesis of Adult Stem Cell Function

Paracrine (software) stimulation of endogenous repair, rather than direct tissue regeneration (hardware), is increasingly accepted as a major mode of therapeutic stem and progenitor cell action



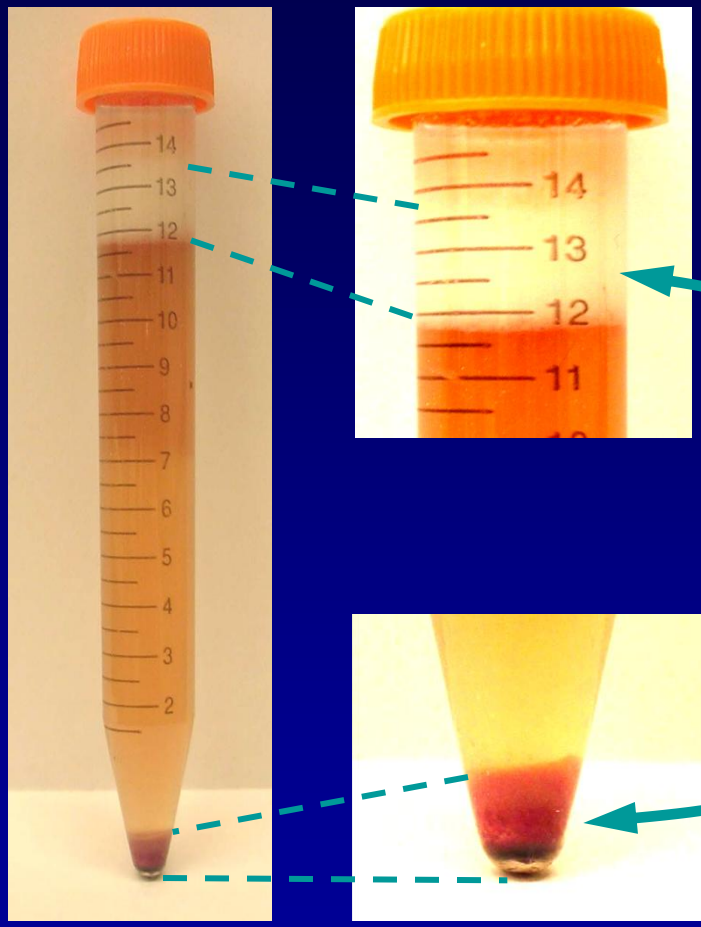
Overview of Talk

- Introduction to adipose-derived stem (stromal) cells (ASCs)
- Evidence supporting paracrine mechanisms of action for ASCs… (with a twist)
- Preclinical studies for application to:
  - peripheral vascular diseases
  - neurological disorders
  - wound healing

Adipose-Derived Stem (Stromal) Cells

- **Subcutaneous Adipose Tissue contains pluripotent cells that can differentiate into multiple cell lineages *in vitro*, including neurons, skeletal myocytes, osteoblasts, chondroblasts, adipocytes, vascular wall cells, and possibly cardiomyocytes**
- **These cells are found in the stromal (non-adipocyte) fraction of the adipose tissue**
- **They may be readily expanded *in vitro***
- **They can be harvested from a patient in a simple outpatient procedure in large numbers (100-300 million in a single procedure from ~100-500 g fat)**

Isolation of ASCs



adipocytes

stromal-vascular fraction (SVF)

abdominoplasty

lipoaspirate

finely mince

filter through 0.2 mm mesh

collagenase at 37° with shaking

low speed centrifugation

remove top layer

collect pellet and wash 3x

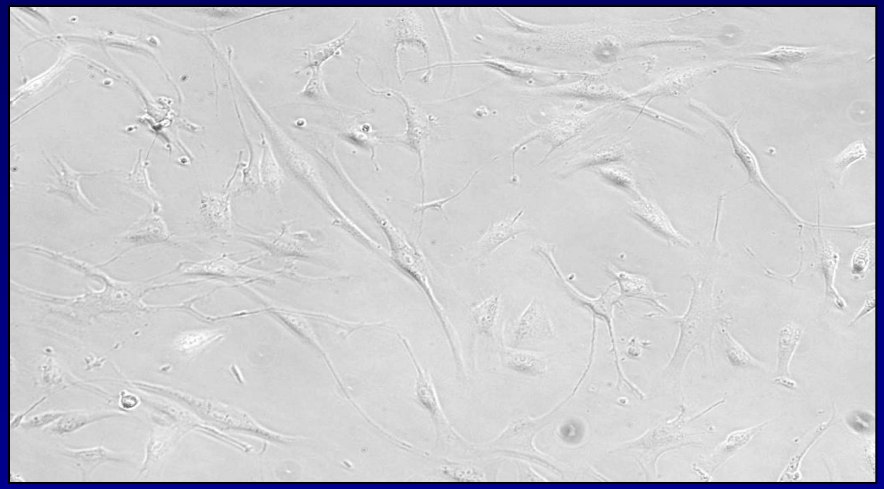
lyse residual RBC

culture 1 hr to overnight on plastic

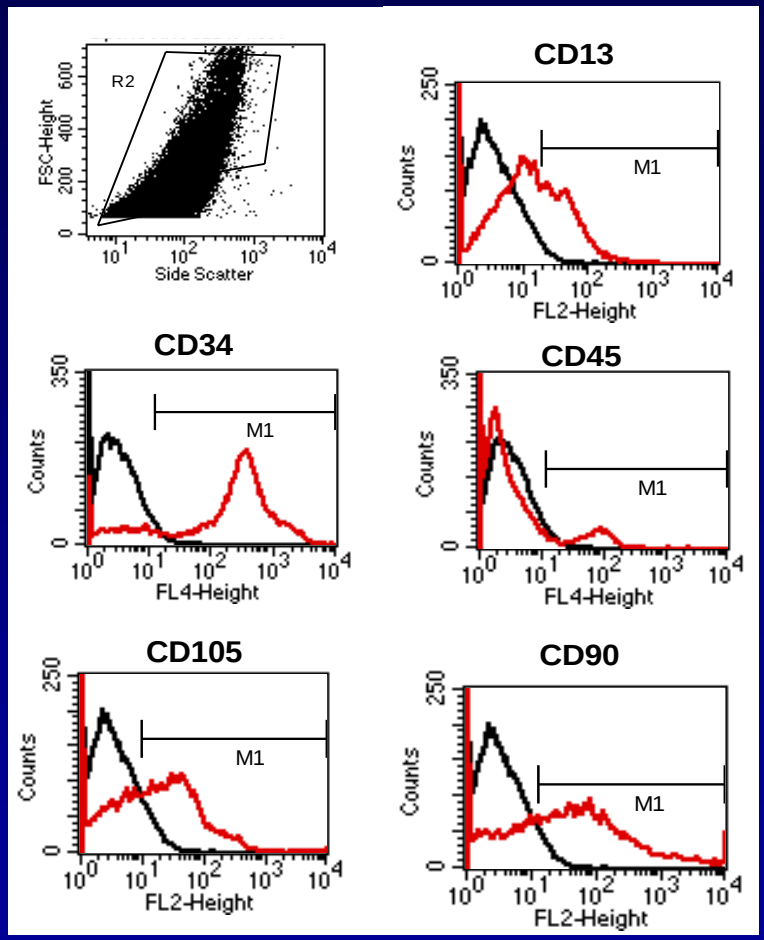
wash away non-adherent cells

ASCs

Human ASC Phenotype



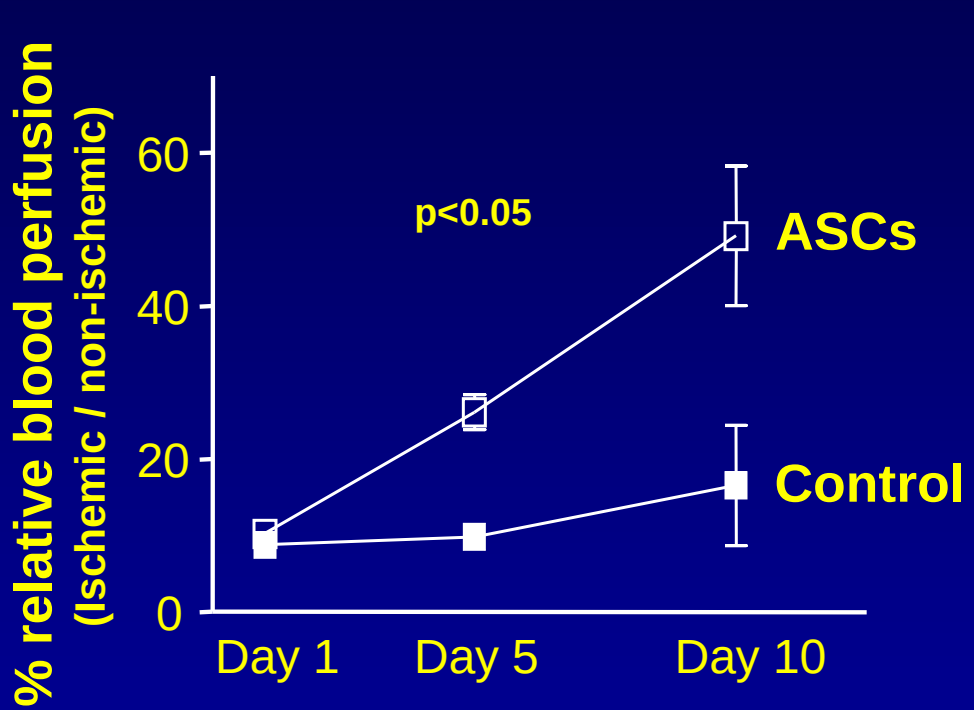
Cultured in EGM2-MV medium

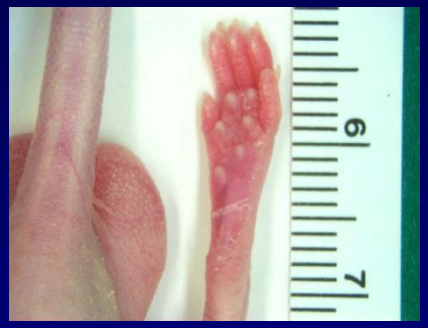


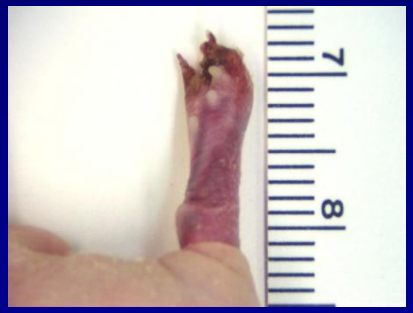
Evidence Supporting Paracrine Effects of Stem Cells

- Rehman et al., 2003. Circ. 107:1164
  - Blood-derived “EPC” are actually non-replicating monocytes/macrophages
  - Rich source of growth factors and cytokines
- Rehman et al, 2004. Circ. 109:1292
- Kinnaird et al. 2004. Circ Res. 94:678
  - Conditioned medium from cultured MSCs effectively promotes ischemia reperfusion
- Victor Dzau and colleagues
  - MSCs genetically modified to overexpress Akt demonstrate enhanced efficacy primarily through elevated secretion of trophic factors
- Cai et al. 2007. Stem Cells. 25:3234

Kinetics of Repair Suggest a Mechanism Distinct From Regeneration



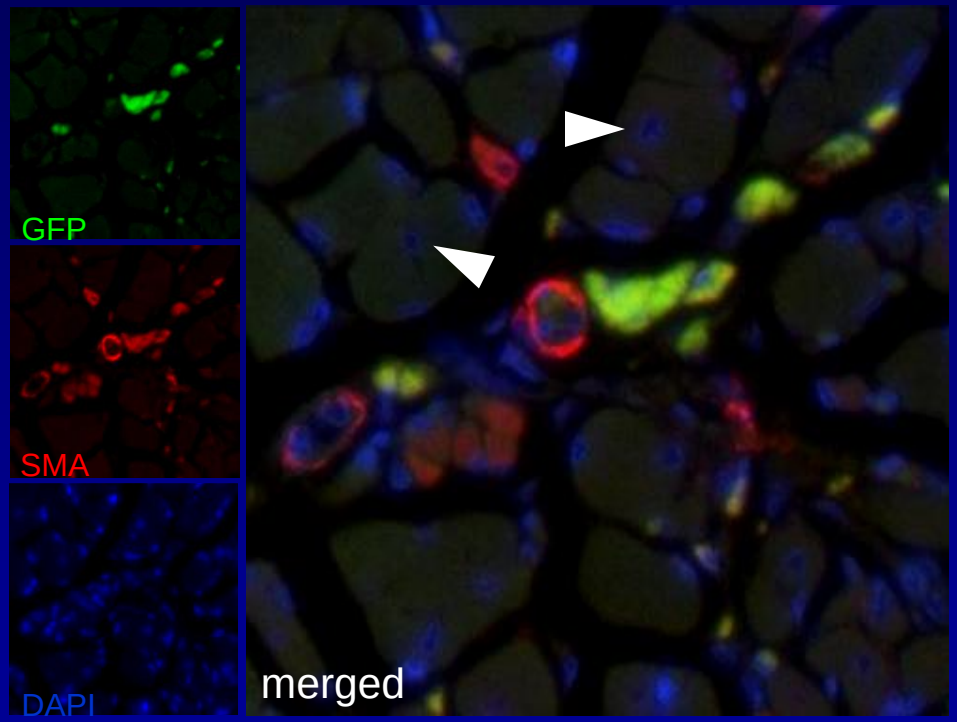


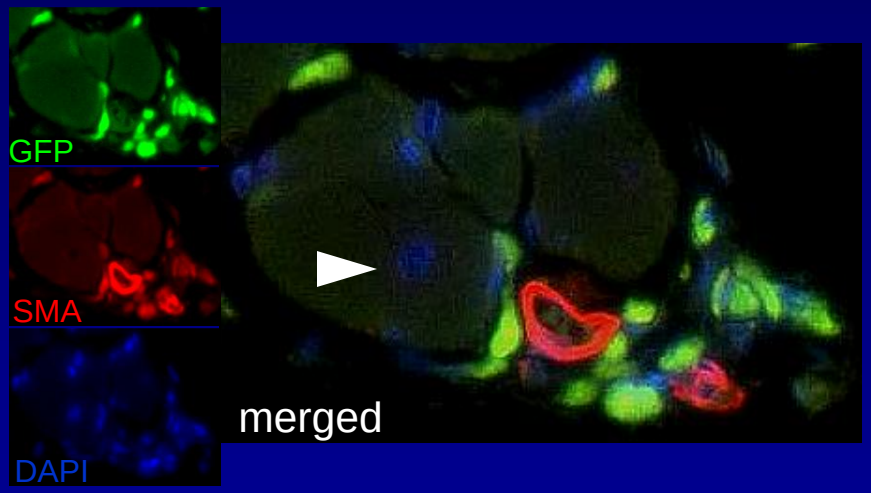


Rehman J, et al. Circulation. 2004. 109:1292.

Rapidity of the effect suggests mechanisms in addition to contributing directly to regeneration of damaged tissues

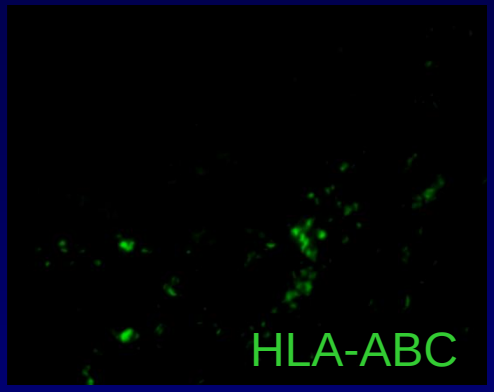
ASCs Are Frequently Detected at Borders of Regenerating Tissues

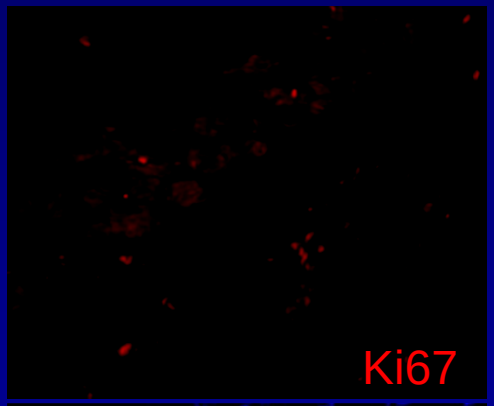


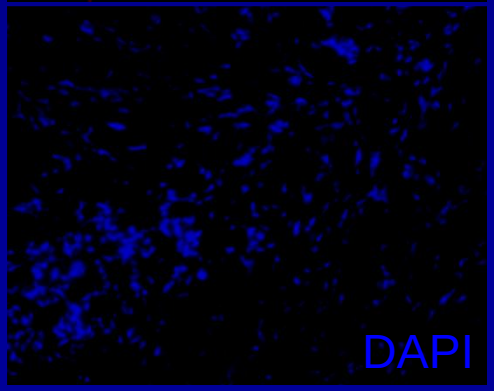


Ischemic Skeletal Muscles

ASCs Injected into Ischemic Rat Myocardium Persist in Border Zones

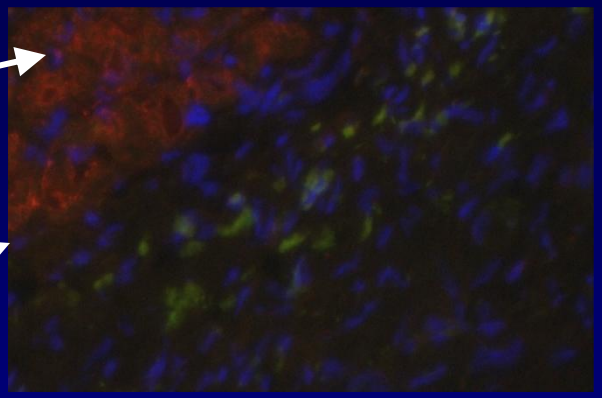


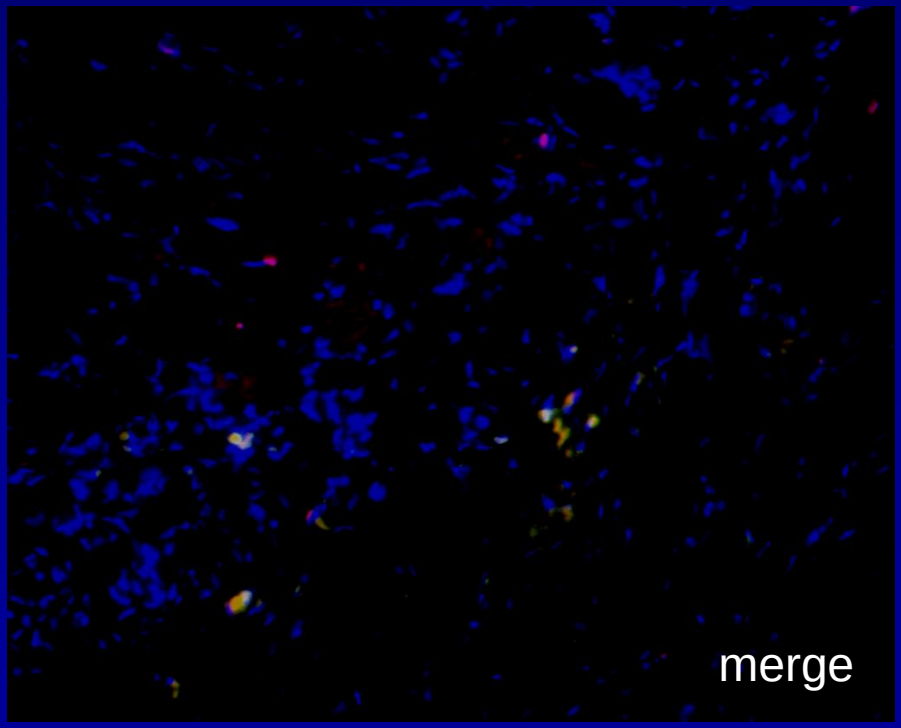




α-sarcomeric actin (viable cardiomyocytes)

Border zone



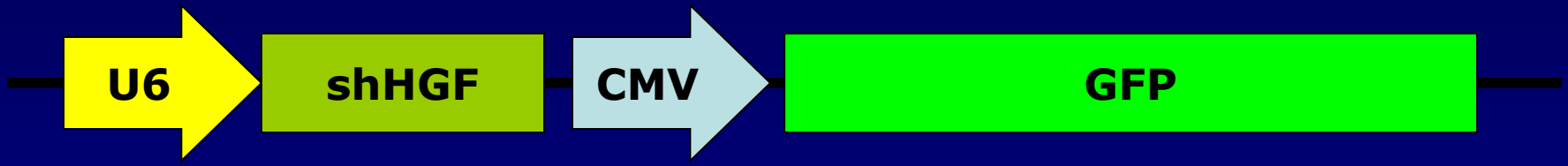


First Direct Demonstration *In Vivo* of the Paracrine Principle

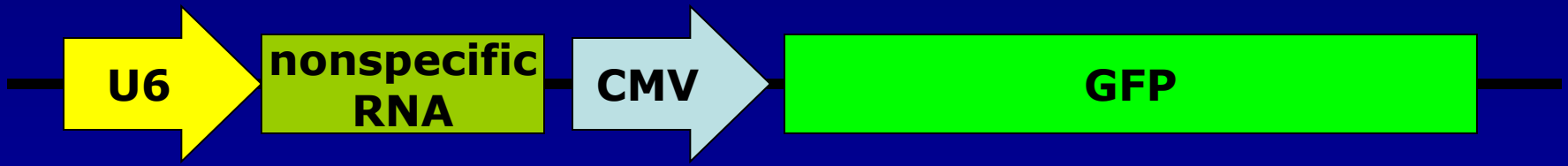
- Hepatocyte Growth Factor (HGF) is produced at relatively high levels by ASCs
  - HGF is a potent angiogenic factor that also possess pro-survival and anti-apoptotic actions on various cell types.
- RNA interference was used to specifically silence HGF expression to determine its direct contribution to ASC potency *in vivo*.

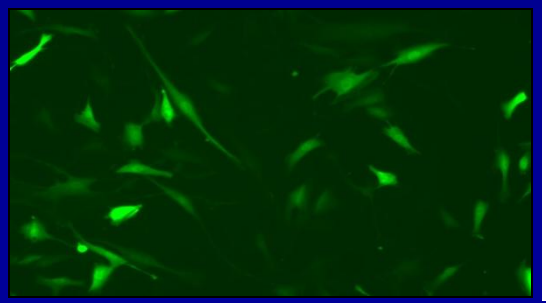
Lentivirus Vectors Used to stably Express shHGF and shCtrl RNAs

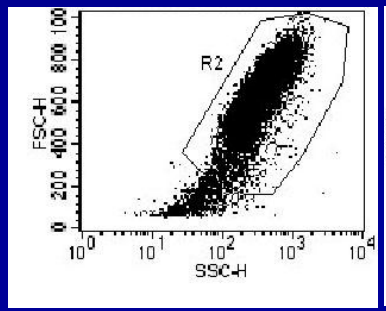
shHGF

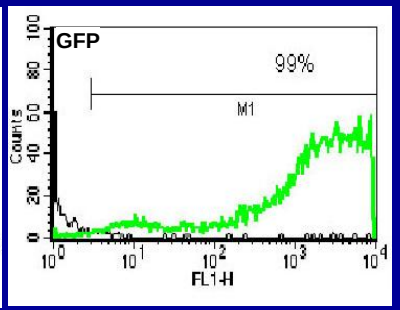


shCtrl





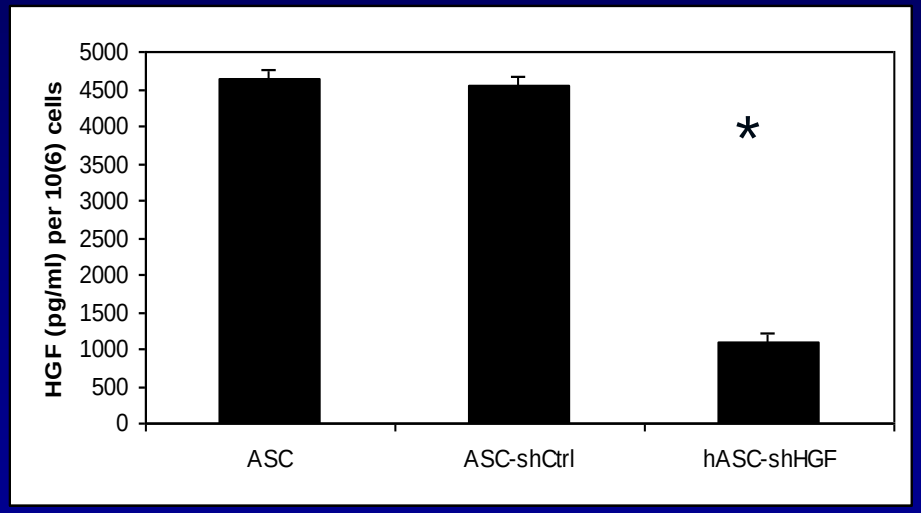




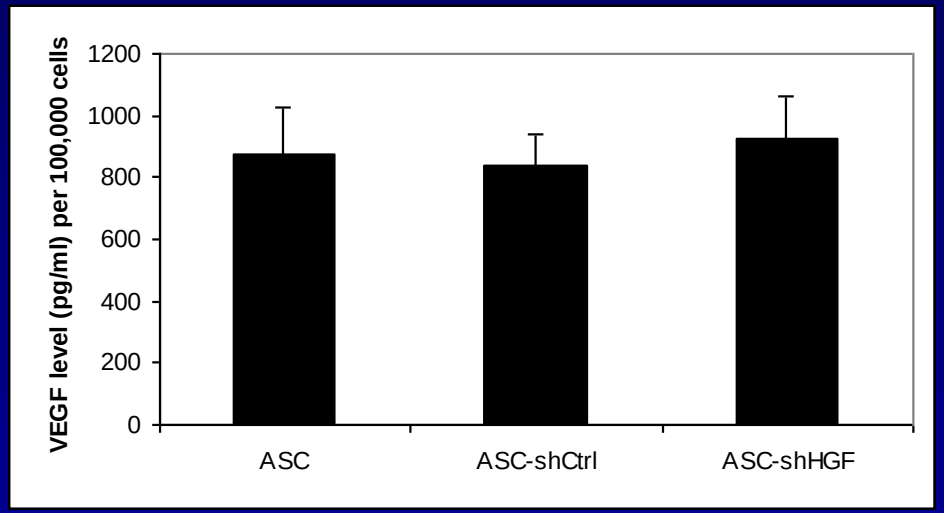
Cai et al. 2007. Stem Cells

shHGF Specifically Knocks-Down HGF Expression

HGF



VEGF

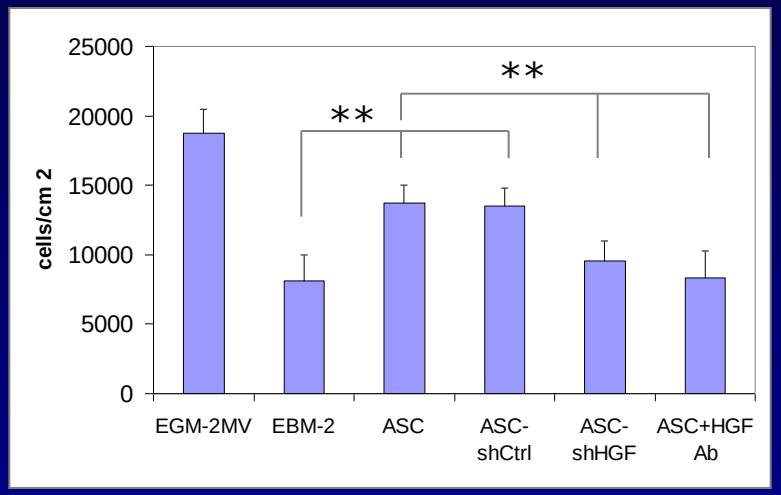


Transduction with shRNA for HGF selectively reduced HGF levels by 5-fold (*p<0.01) without affecting ASC expression of VEGF.

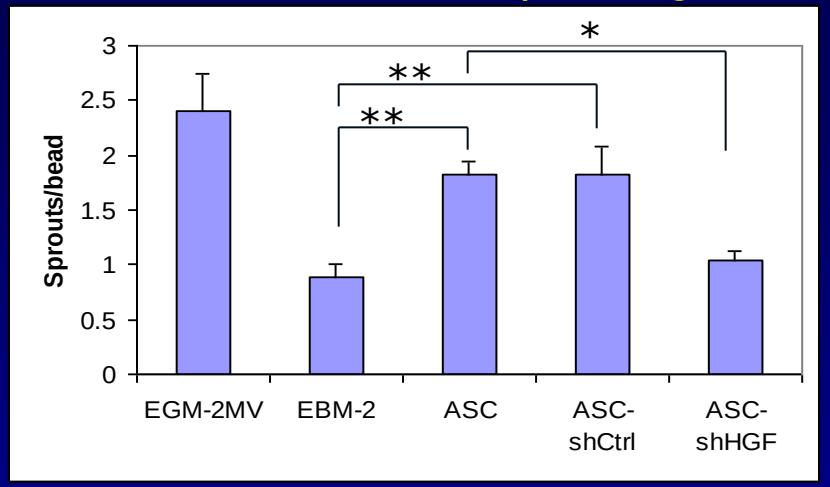
Cai et al. 2007. Stem Cells

Conditioned Medium from ASC-shHGF Has a Reduced Angiogenic Activity

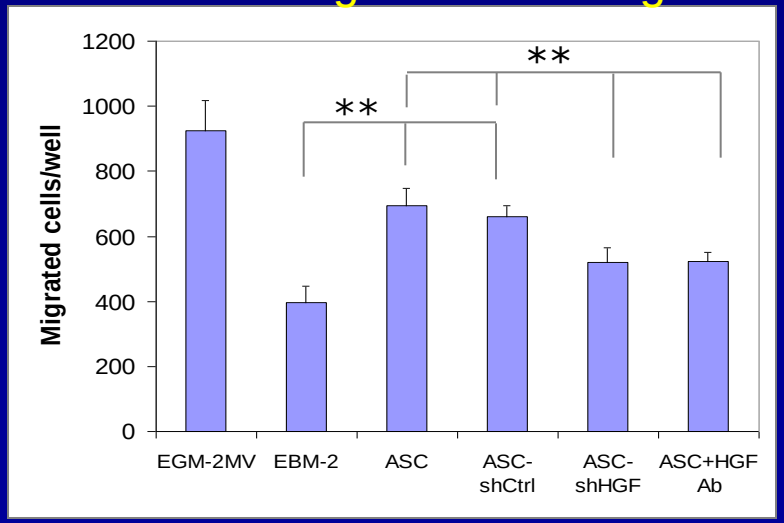
Endothelial Cell Proliferation



Endothelial Cell Sprouting

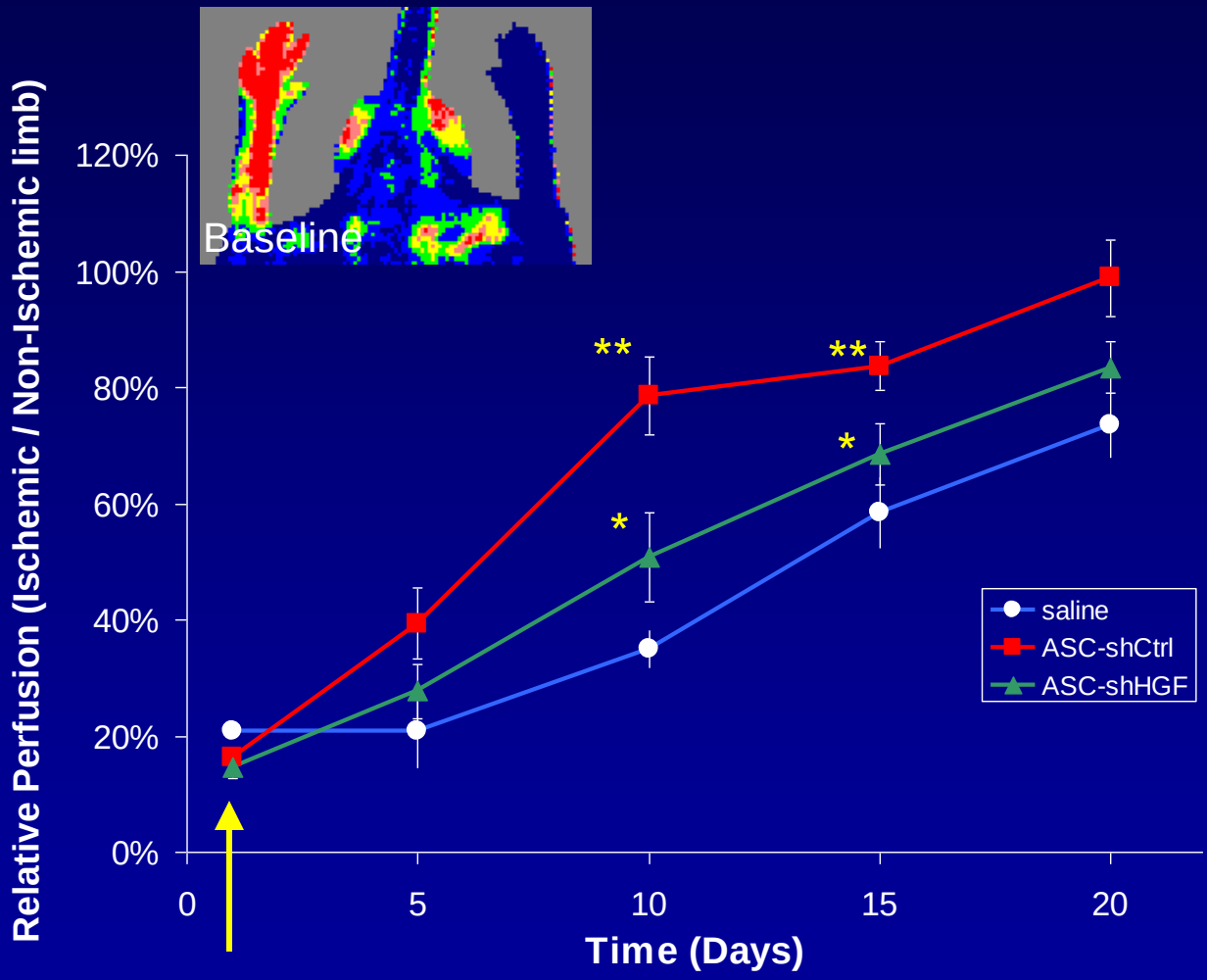


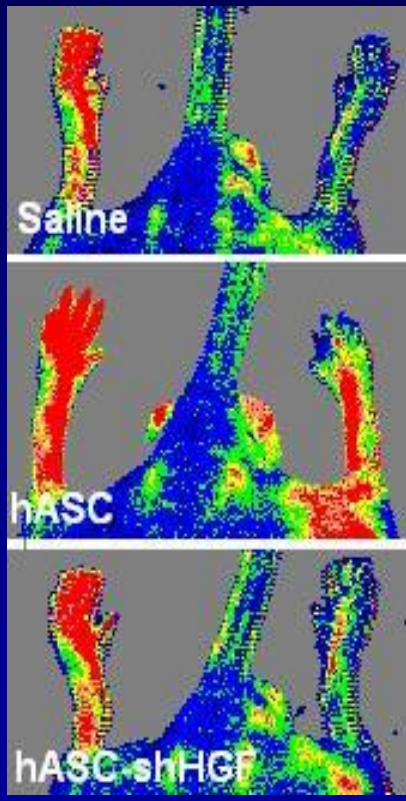
Endothelial Progenitor Cell Migration



Cai et al. 2007. Stem Cells

HGF Knock-Down Reduces ASC Ability to Promote Reperfusion





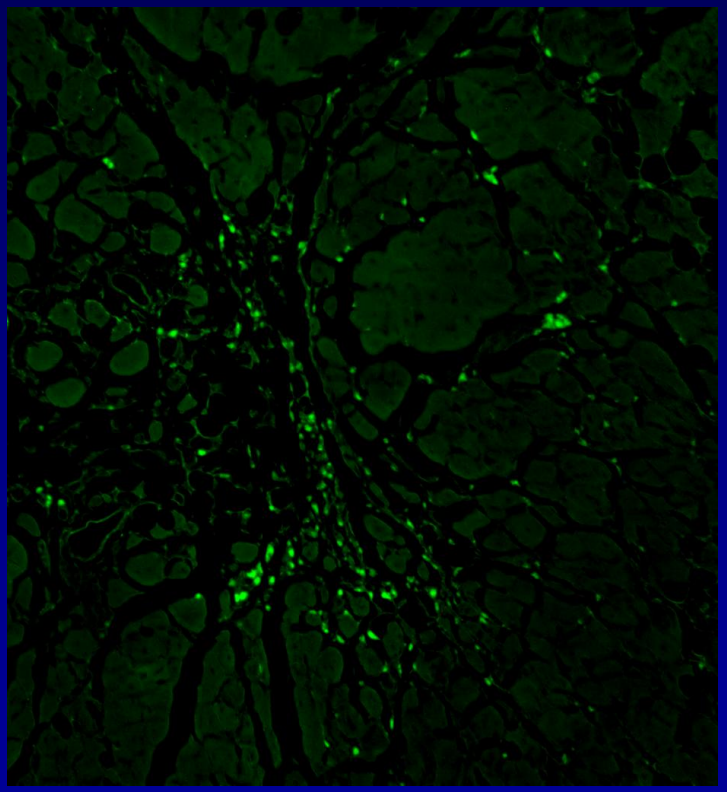
Systemic infusion of ASCs

$p<0.05$ (*) and $p<0.1$ (**) compared to saline

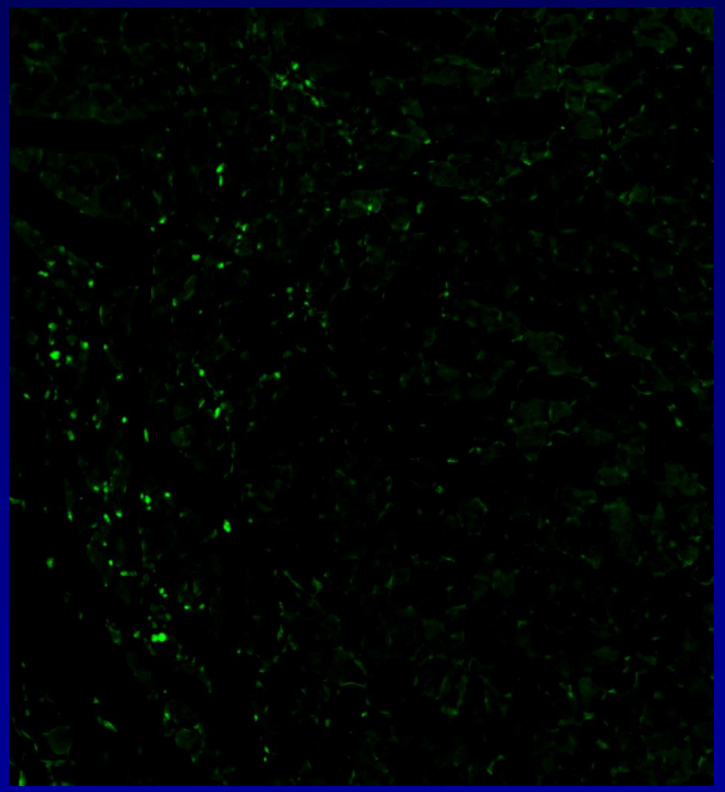
Cai et al. 2007. Stem Cells

Tracking the Fate of GFP-Expressing ASCs in Ischemic Tissues

ASC-shCtrl

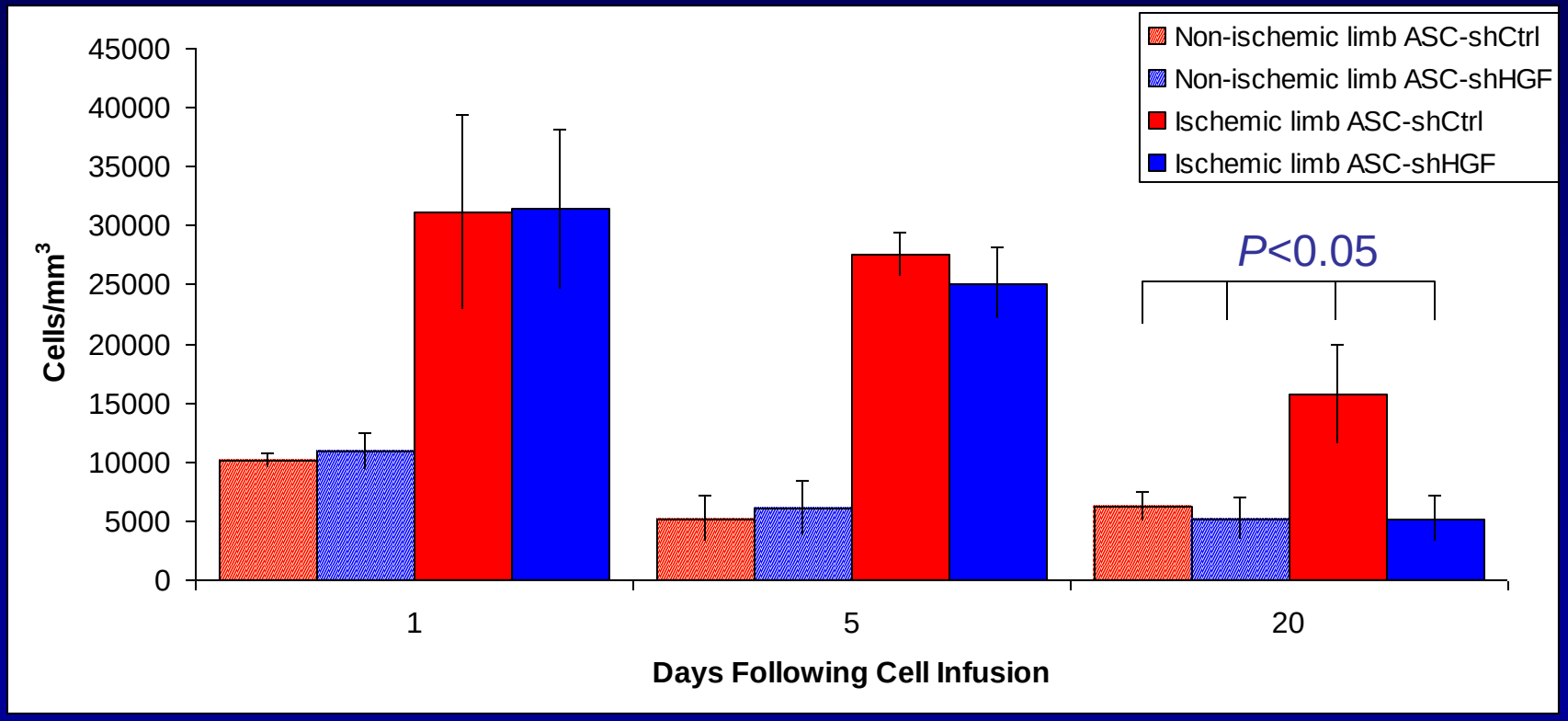


ASC-shHGF

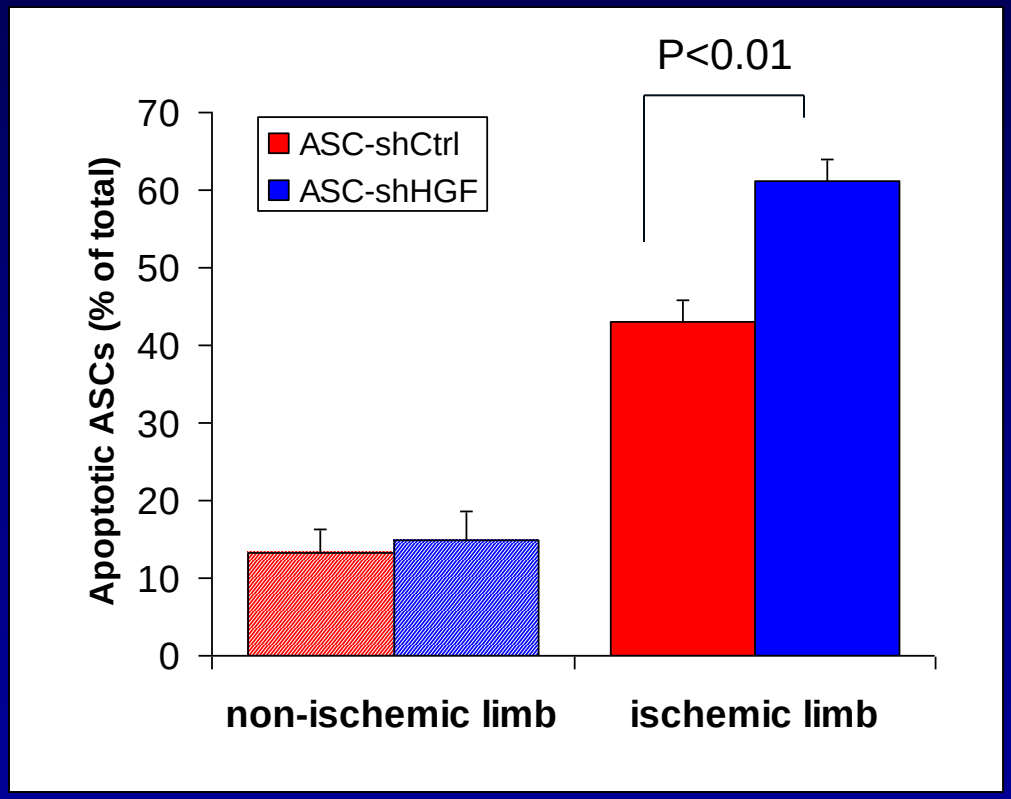


Gastrocnemius muscles harvested at 3 weeks following systemic infusion

ASCs Expressing Normal Levels of HGF (ASC-Ctrl) Have a Clear Survival Advantage *In Vivo* Over ASC-HGF



The Reduced Survival of ASC-shHGF Is Related to a Higher Frequency of Apoptosis Compared to ASC-Ctrl



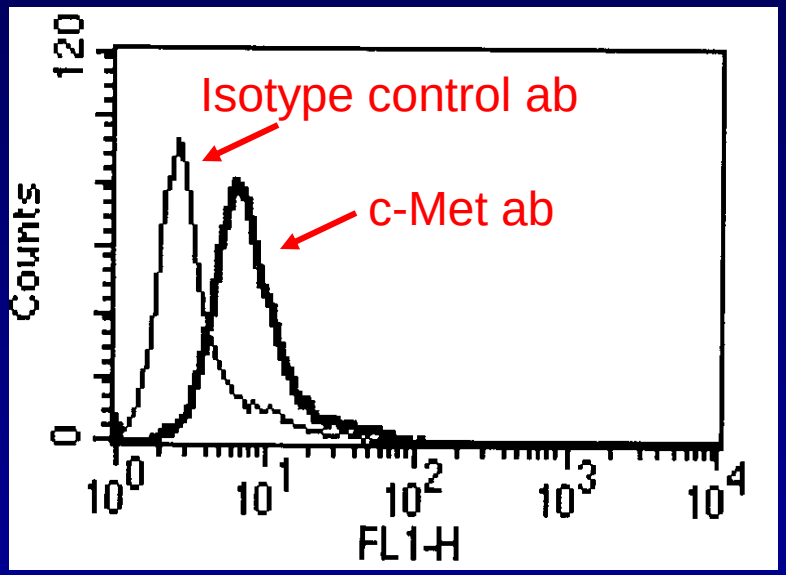
TUNEL^{+}/GFP^{+} cells were quantitated in ischemic and non-ischemic tissues at 5 d after systemic infusion.

Cai et al. 2007. Stem Cells

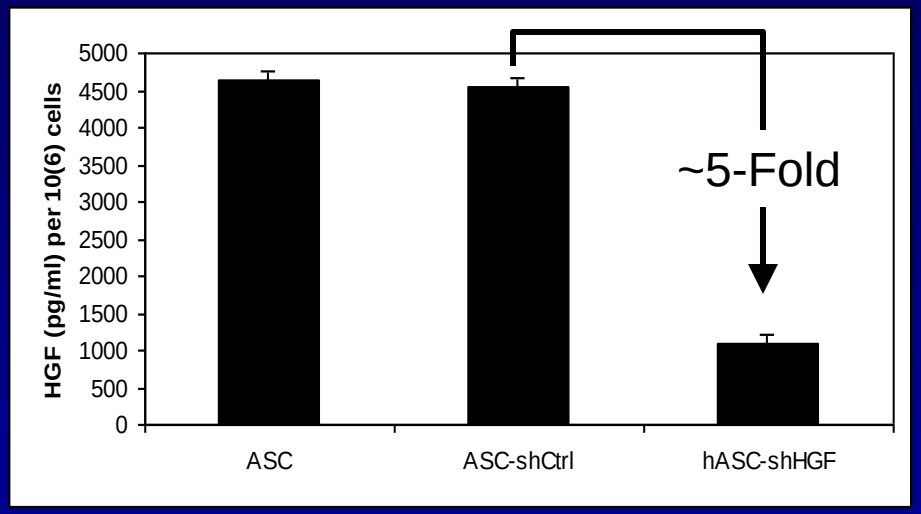
Possible Explanations

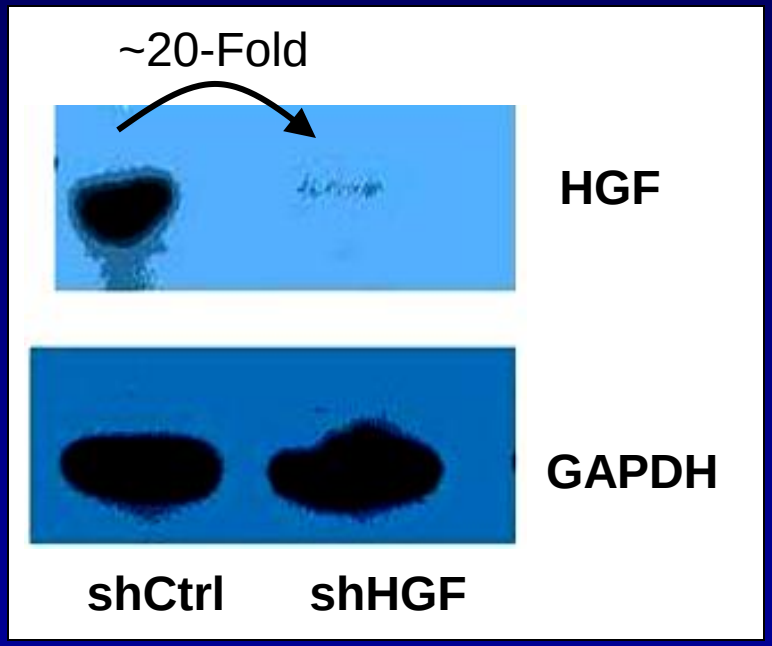
- Disruption of a paracrine action on surrounding tissues
  - Secretion of HGF by ASCs provides local support and recruits circulating progenitor cells
  - This induces more rapid reperfusion, which in turn enhances survival of engrafted ASCs
- Disruption of an autocrine loop in ASCs
  - ASCs express HGF receptor (c-Met)
  - Knock-down of HGF could reduce prosurvival signaling by c-Met and reduce ASC survival

C-Met is Expressed on the Surface of ASCs

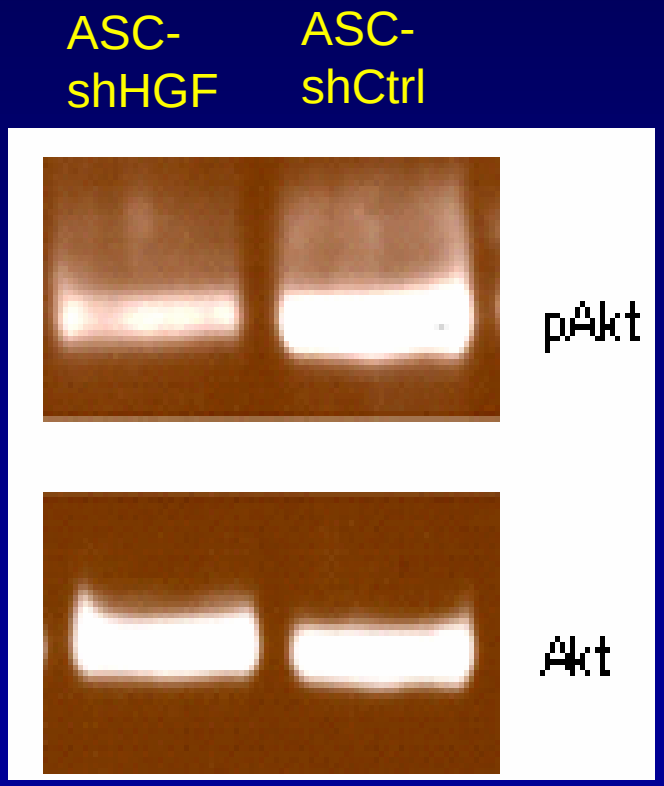


Levels of Cell-Associated HGF Are Suppressed by a Much Greater Degree Than 5-Fold Seen in Secreted HGF

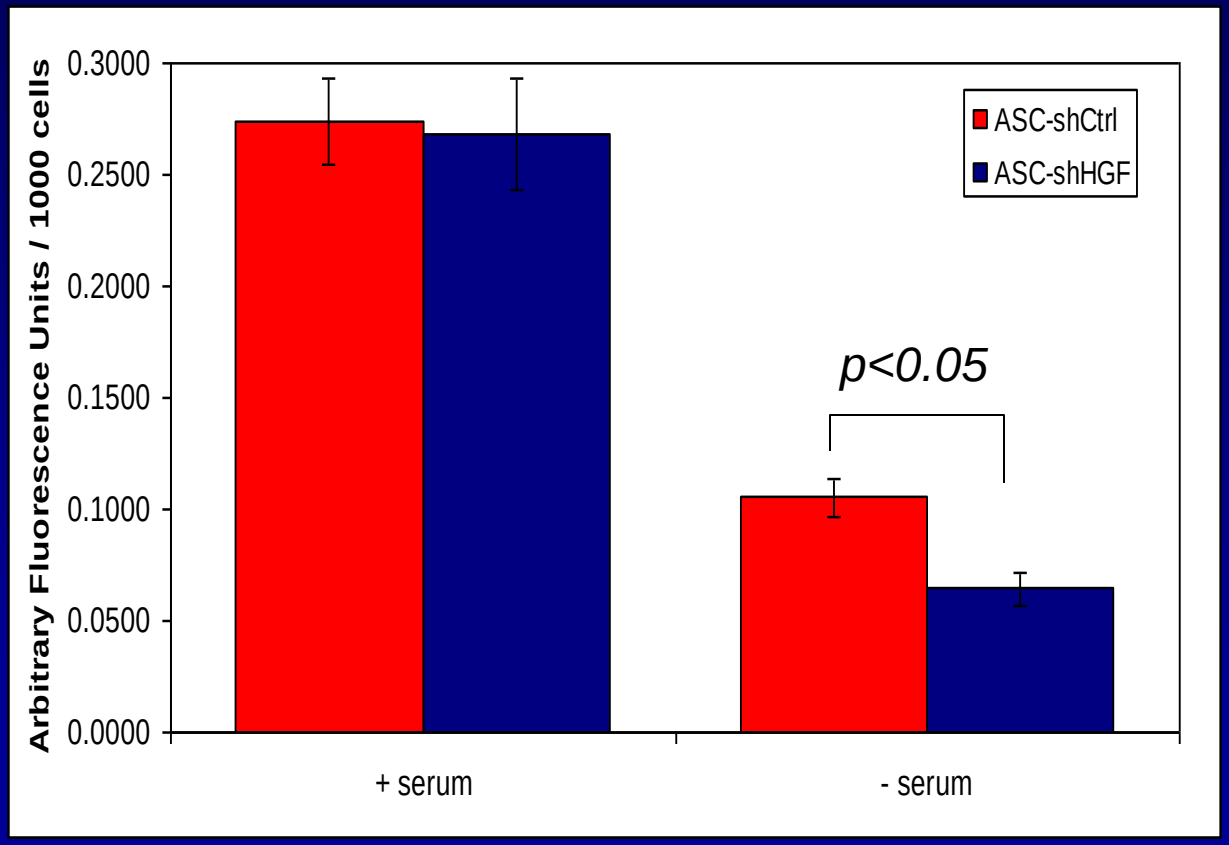




HGF Knock-Down Suppresses Activation of the PI3K/Akt Survival Pathway

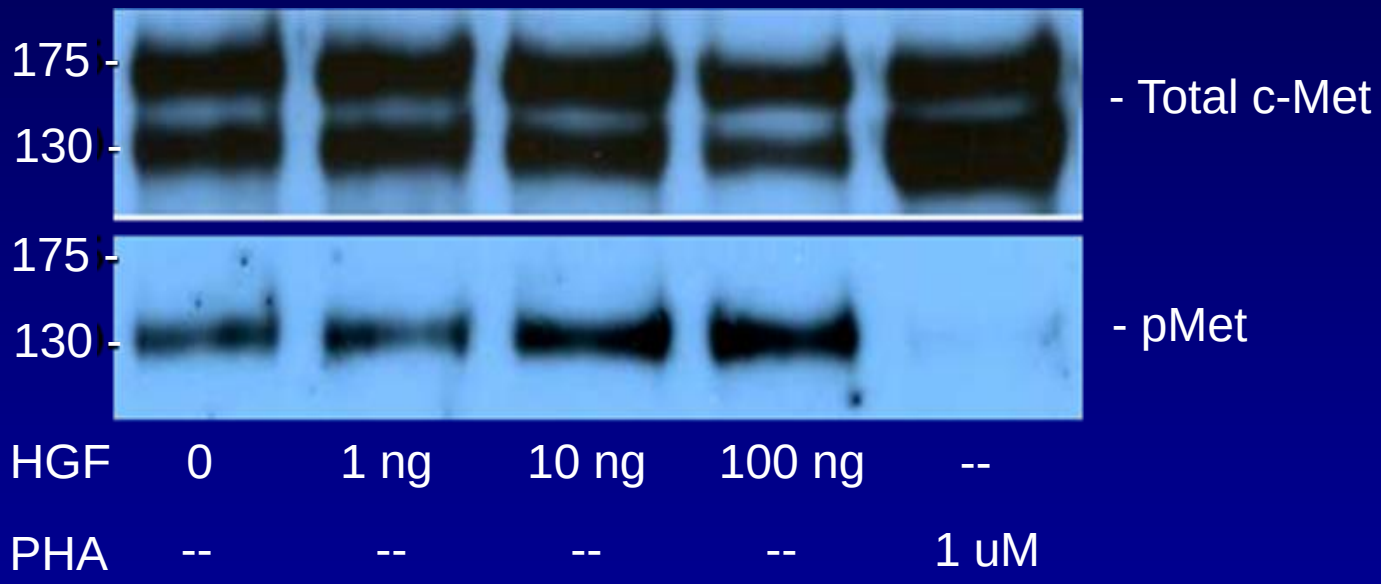


HGF Knock-Down Reduces the Metabolic Activity of ASCs Subjected to Serum Withdrawal



MTS assay for metabolic activity

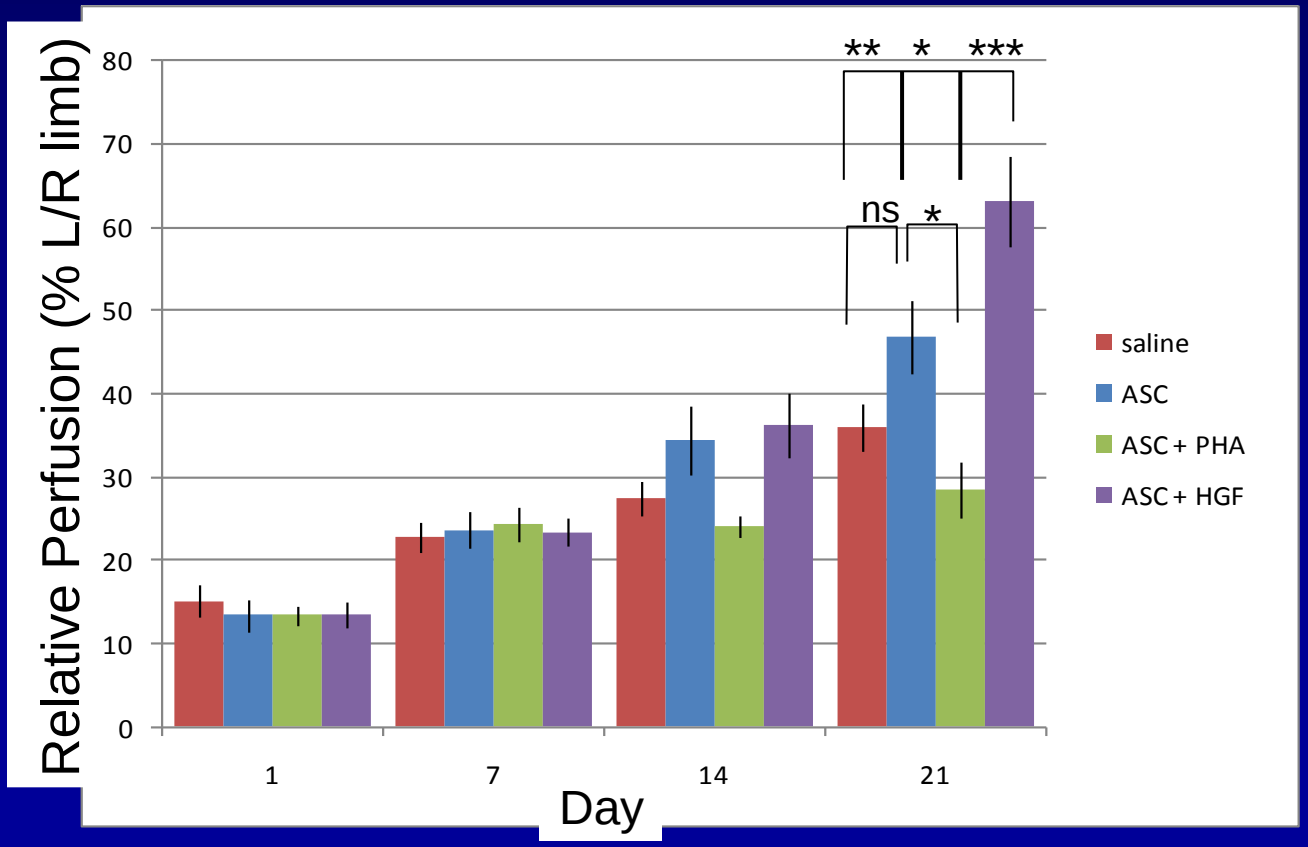
Pharmacological Inactivation of C-Met



PHA-665752 (Pfizer)

Inhibition of C-Met Abolishes the Potency of ASC In-Vivo

- Unilateral hindlimb ischemia model
- Sub-efficacious dose of ASC infused iv



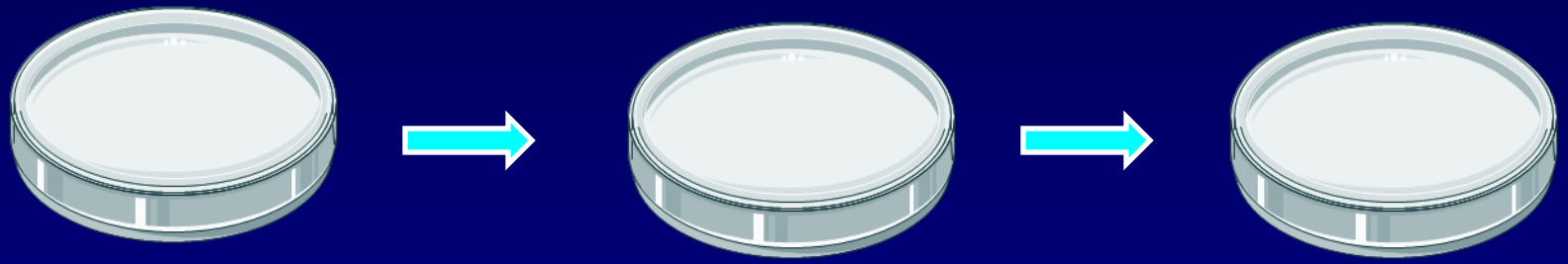
Conclusions I

- HGF plays multiple roles in promoting repair of ischemic tissues by ASCs
  - promotes survival of damaged host tissues
  - promotes revascularization of ischemic tissues
  - is a key autocrine survival factor that promotes ASC survival under adverse conditions such as ischemia

Development of Neurological Applications

- Brain presents unique issues for stem cell therapies due to the impenetrability of the blood brain barrier (BBB)
- Therefore, we chose to initially investigate the potential of conditioned medium derived from cultured ASCs

Preparation of Concentrated ASC-CM



ASCs cultured to confluence

Wash with PBS

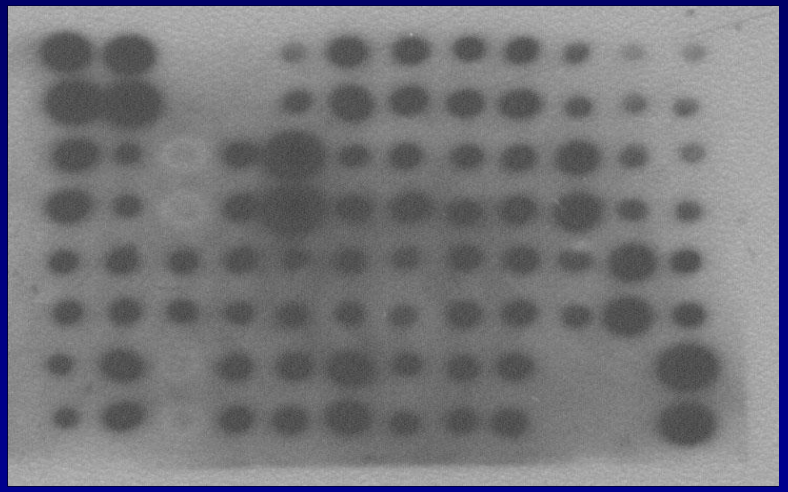
Add Fresh Basal Medium

24hrs

Conditioned medium was collected and concentrated 250 times

ASCs Secrete Many Neutrophic Factors During Culture

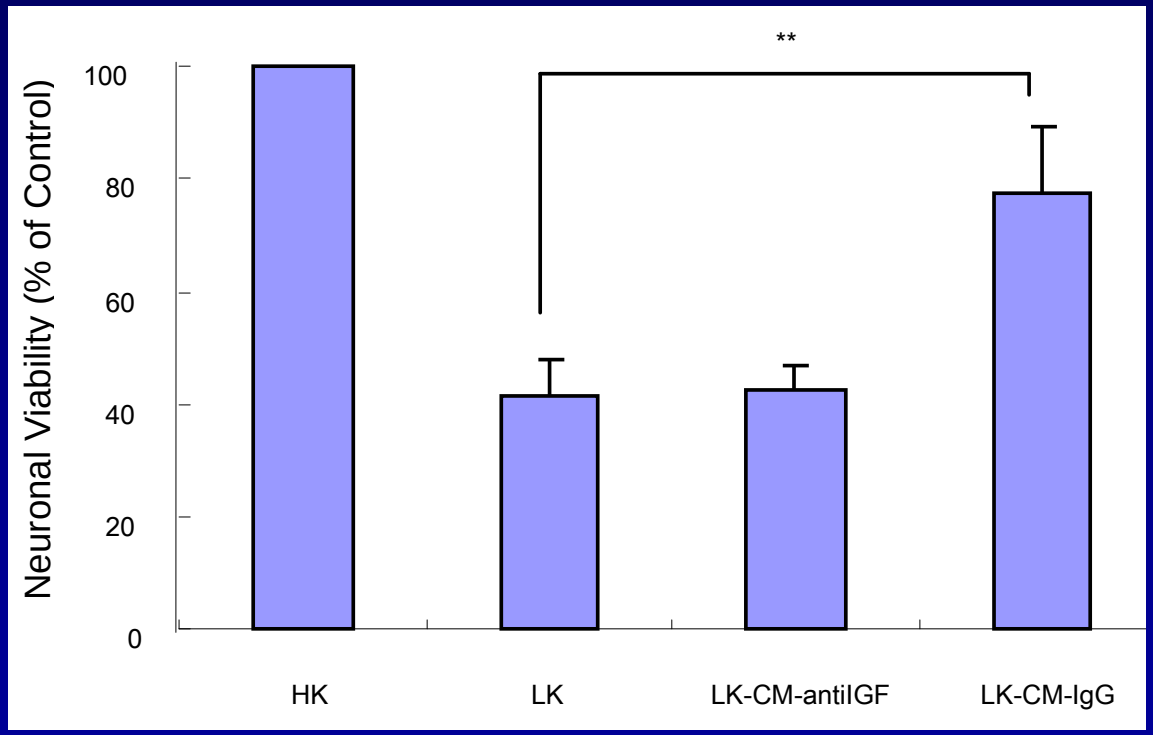
IGF-1, BDNF and VEGF have been reported to protect against ischemic injury



Multiple growth factors were identified by RayBiotech antibody array

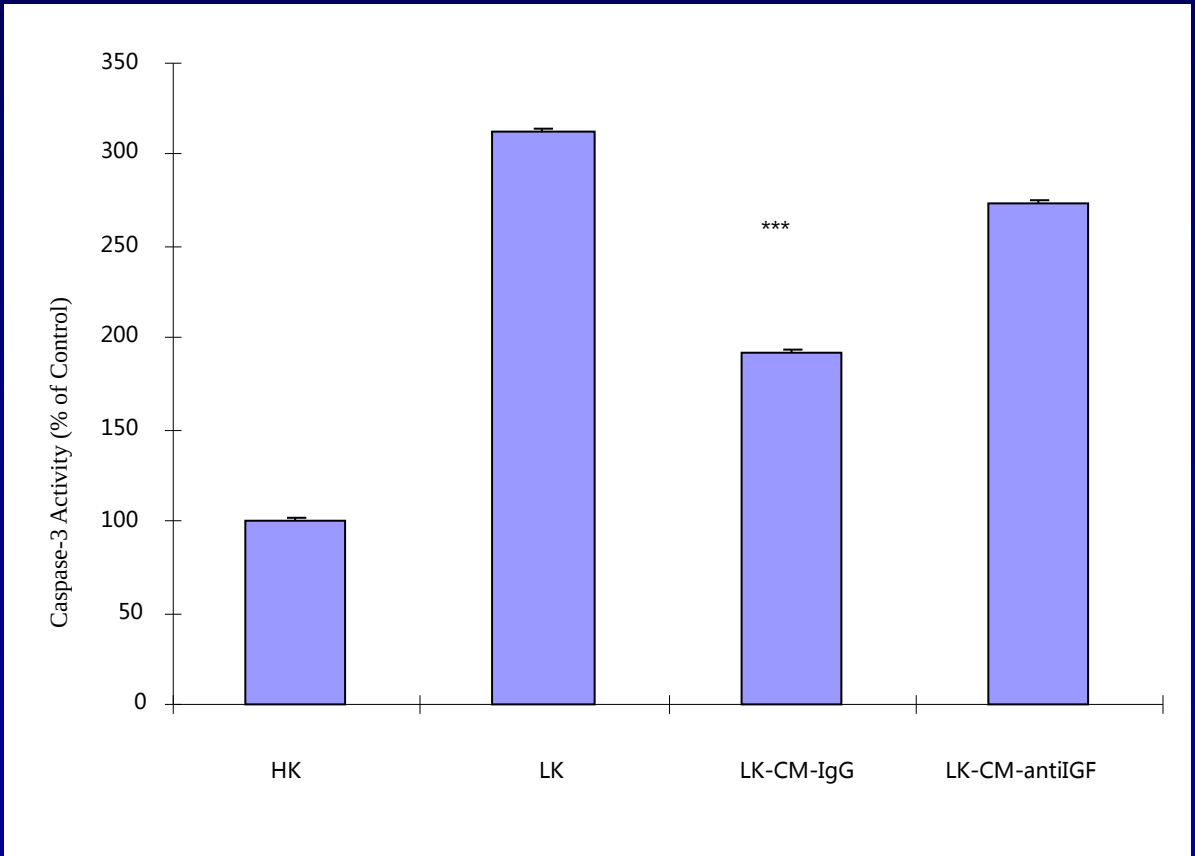
ASC Conditioned Medium (ASC-CM) Potently Protects Neurons From Apoptosis

Low K apoptosis model using primary neonatal rat cerebellar granule neurons (CGN)

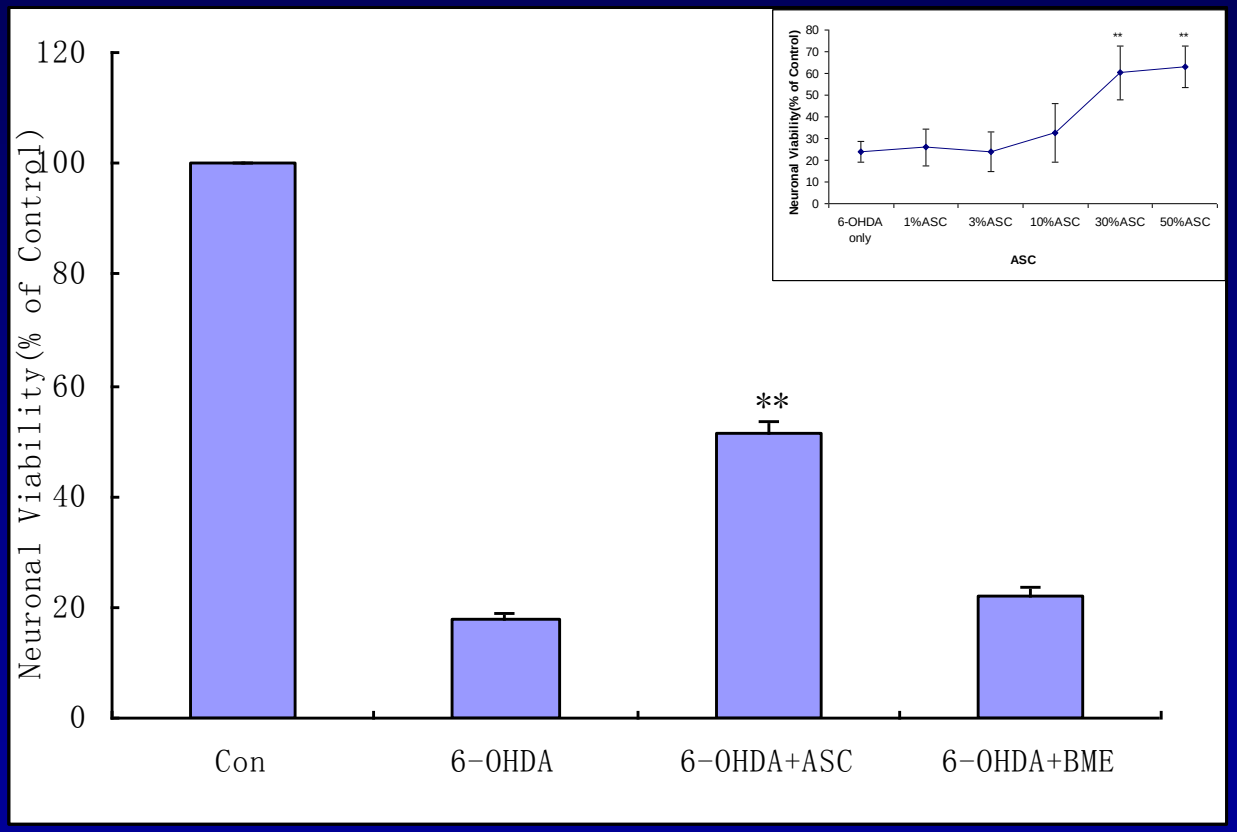


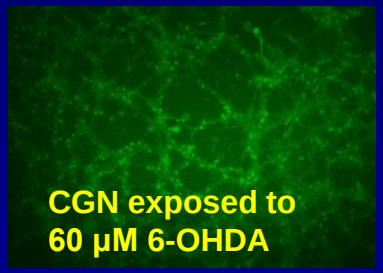
IGF-1 activity in ASC-CM essential for protecting primary neurons from apoptosis

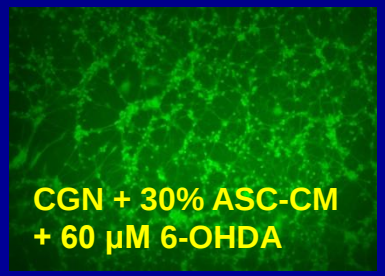
IGF-1 Protects Through Akt/PI3K-Mediated Inhibition of Caspase-3



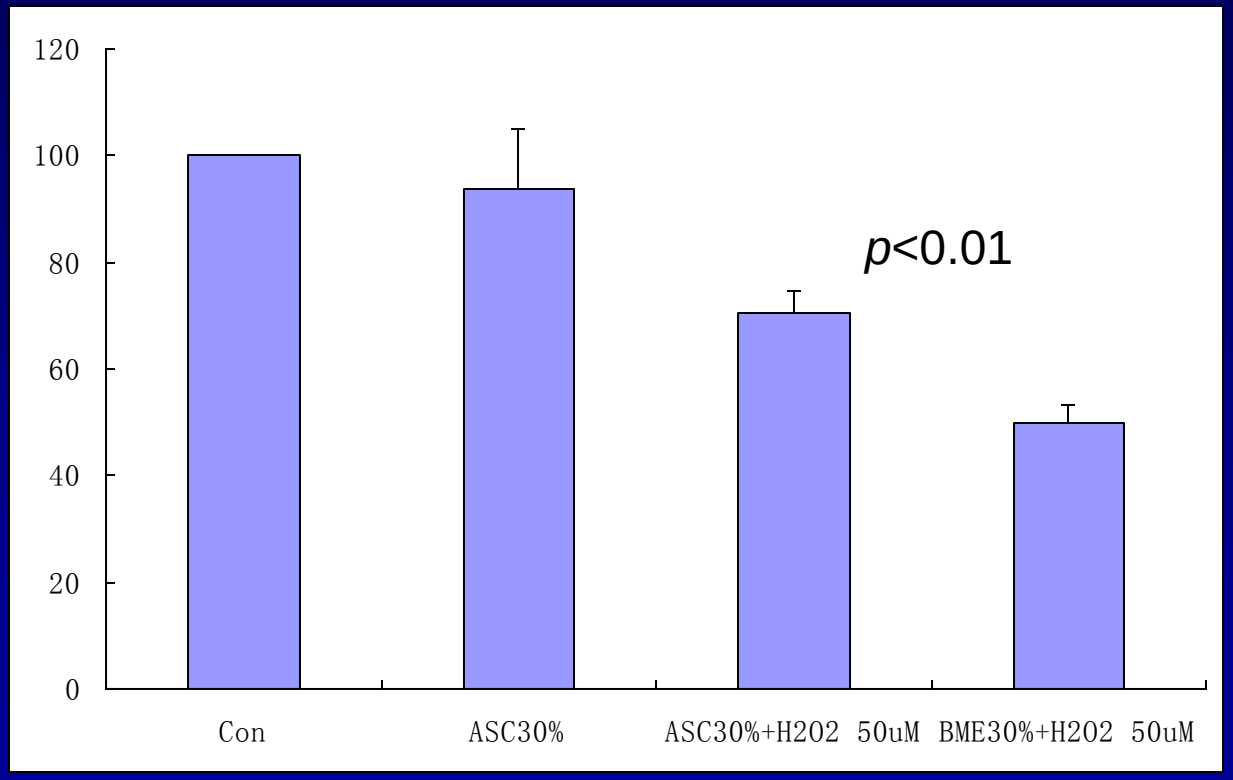
ASC-CM Blocks 6-OHDA-Induced Oxidative Damage in Primary Neurons







ASC-CM Blocks Other Oxidative Agents (e.g., H_2O_2)



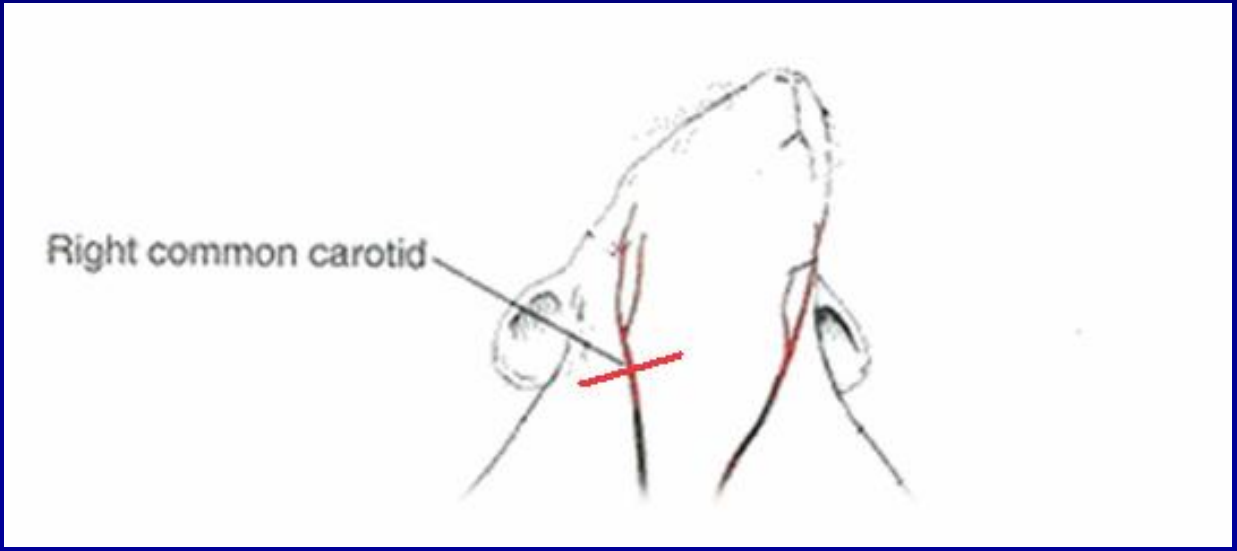
Perinatal Hypoxic-Ischemic Encephalopathy (HIE)

- Major cause of acute mortality and chronic neurological morbidity in infants and children.
- Mortality: 20-50%
- Life-long neurological deficit (~ 25% of survivors)
  - Cerebral palsy,
  - Epilepsy
  - Mental retardation
- No effective clinical treatment available

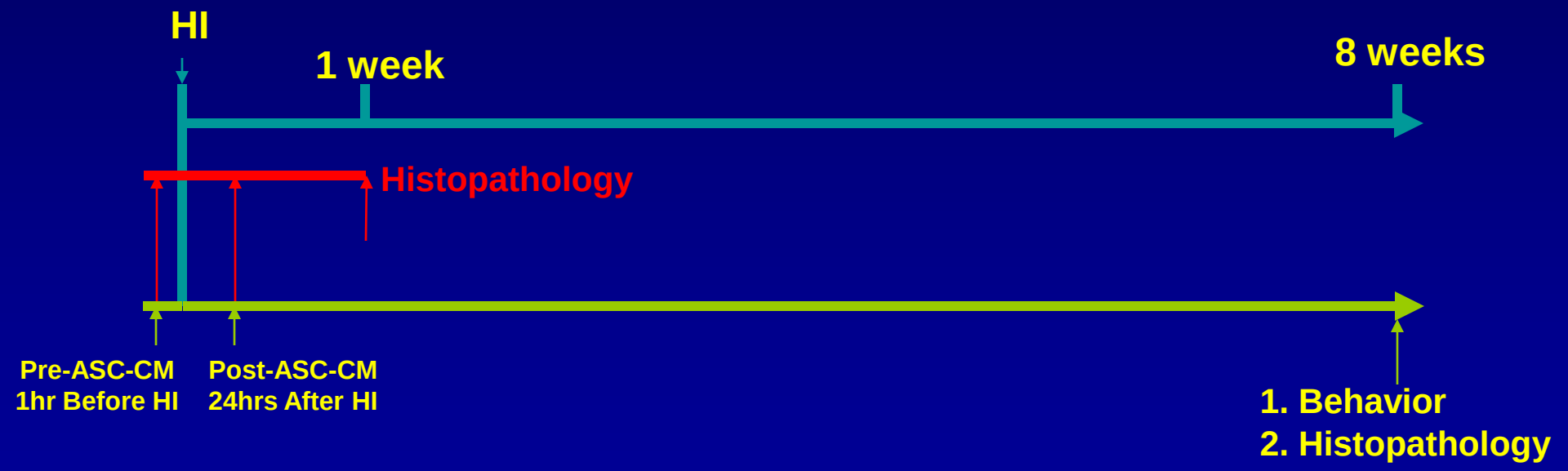
Hypoxia-Ischemia (HI) Rat Model

7 day-old rats (P7)

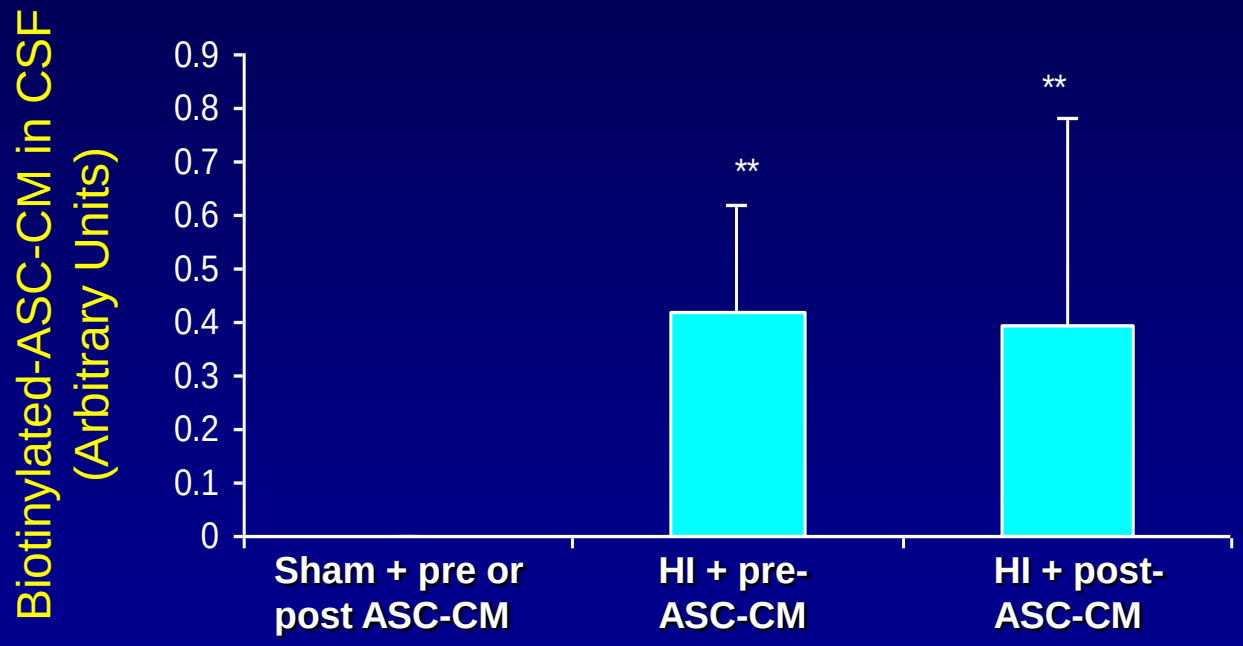
1. Unilateral common carotid artery ligation
2. Hypoxia exposure: 8% O_2, 92% N_2
   37 ^oC
   2 hr

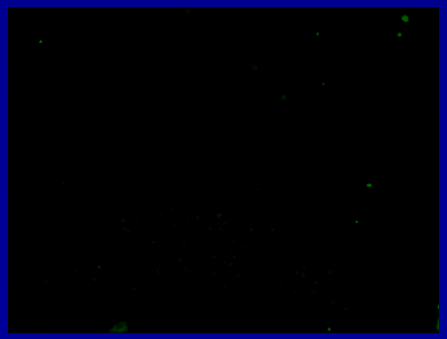


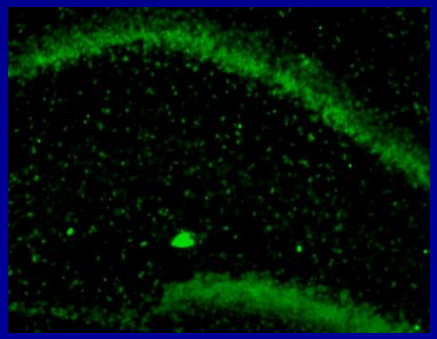
Experimental Protocol



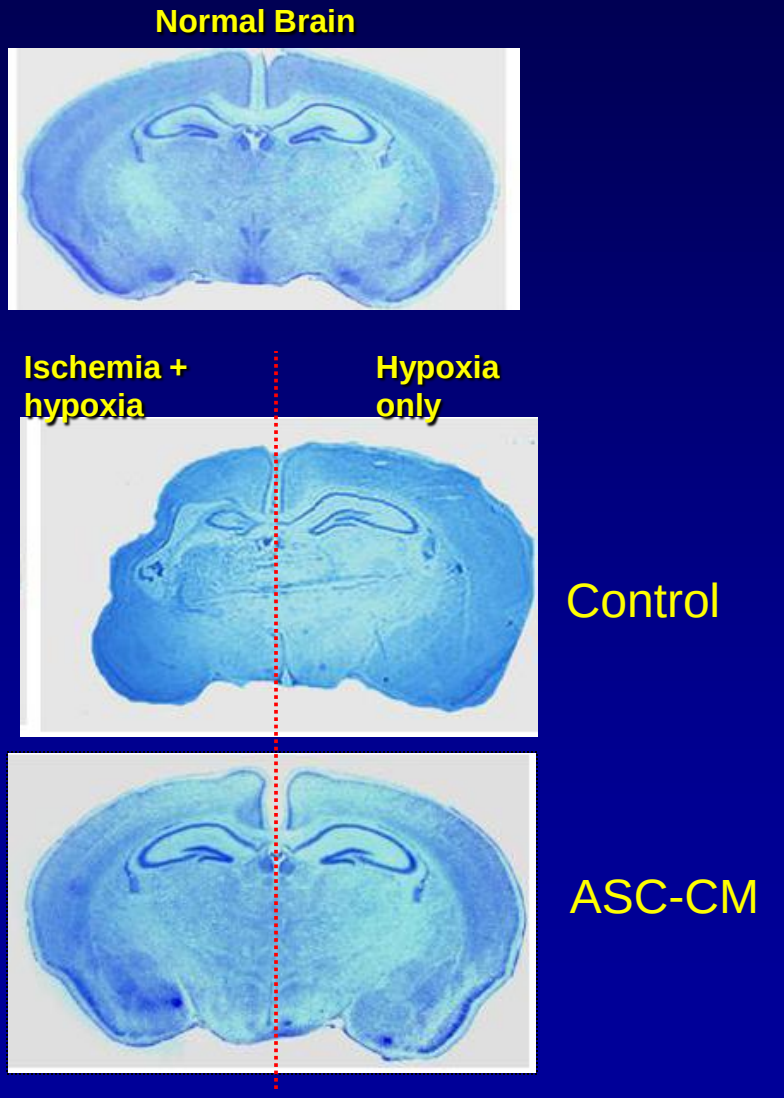
Protein Factors in ASC-CM Do Cross the BBB

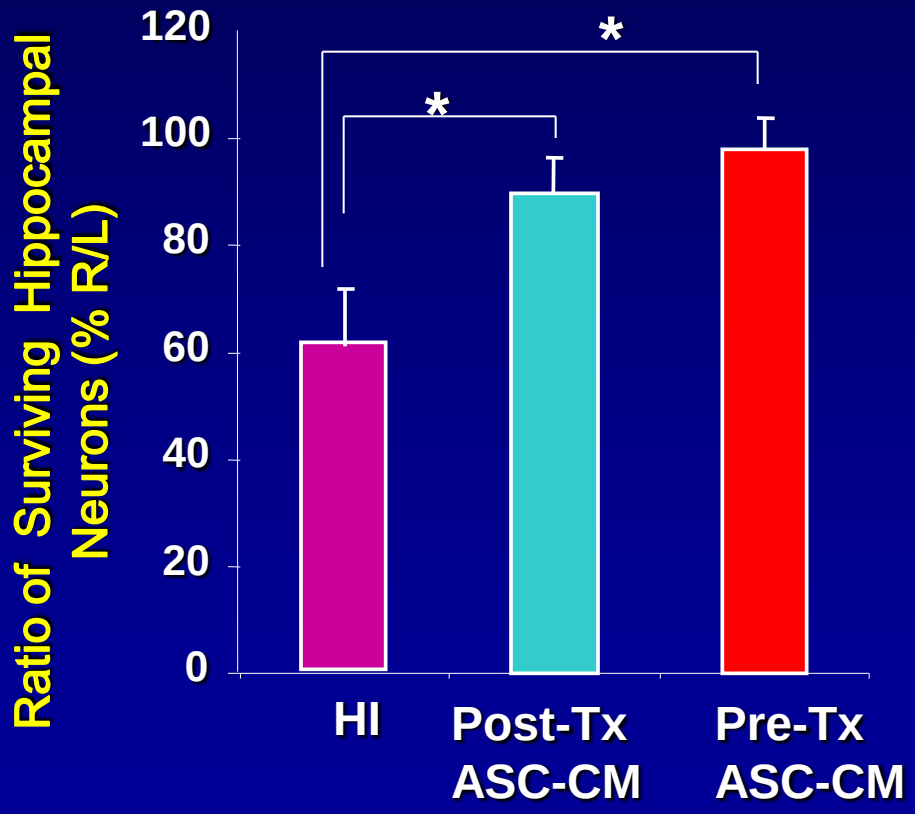




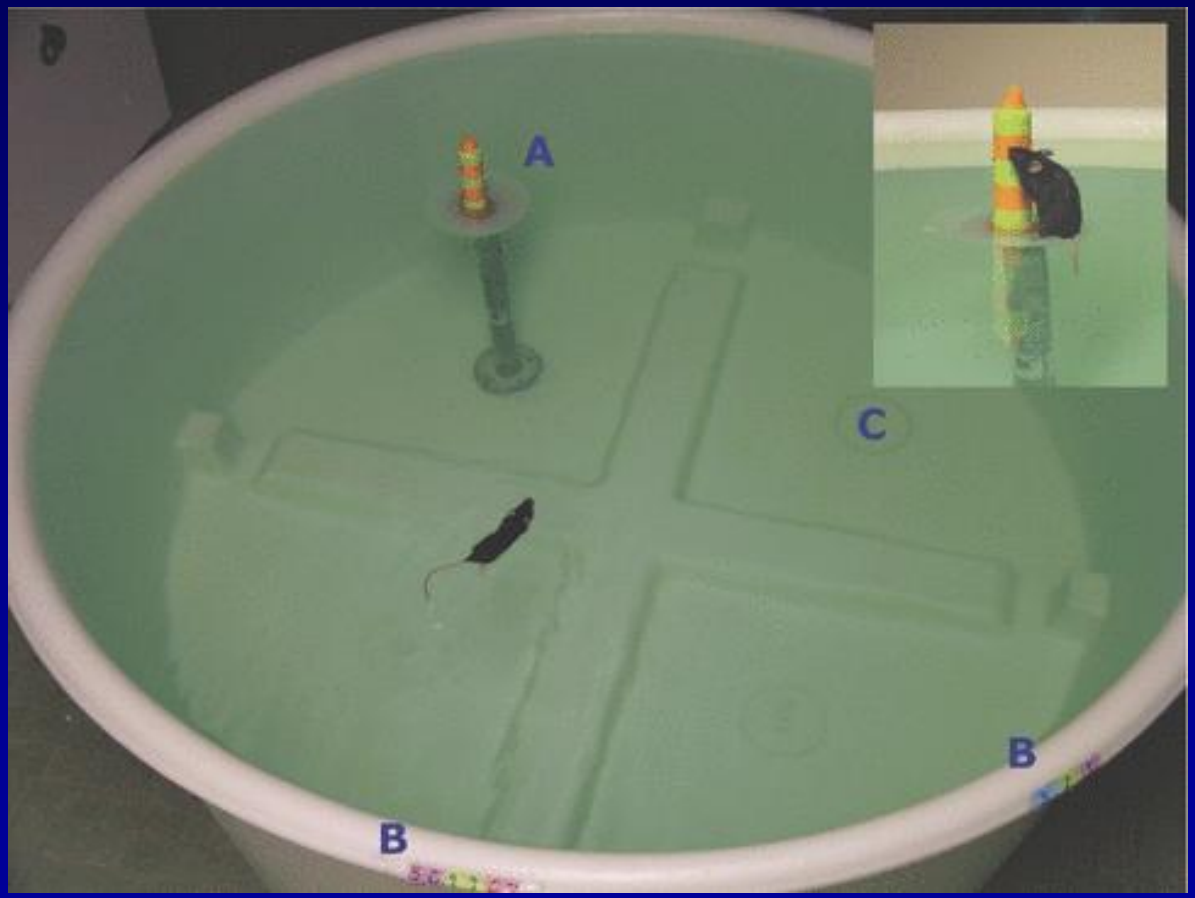


ASC-CM prevents neuronal loss following hypoxic-ischemic injury

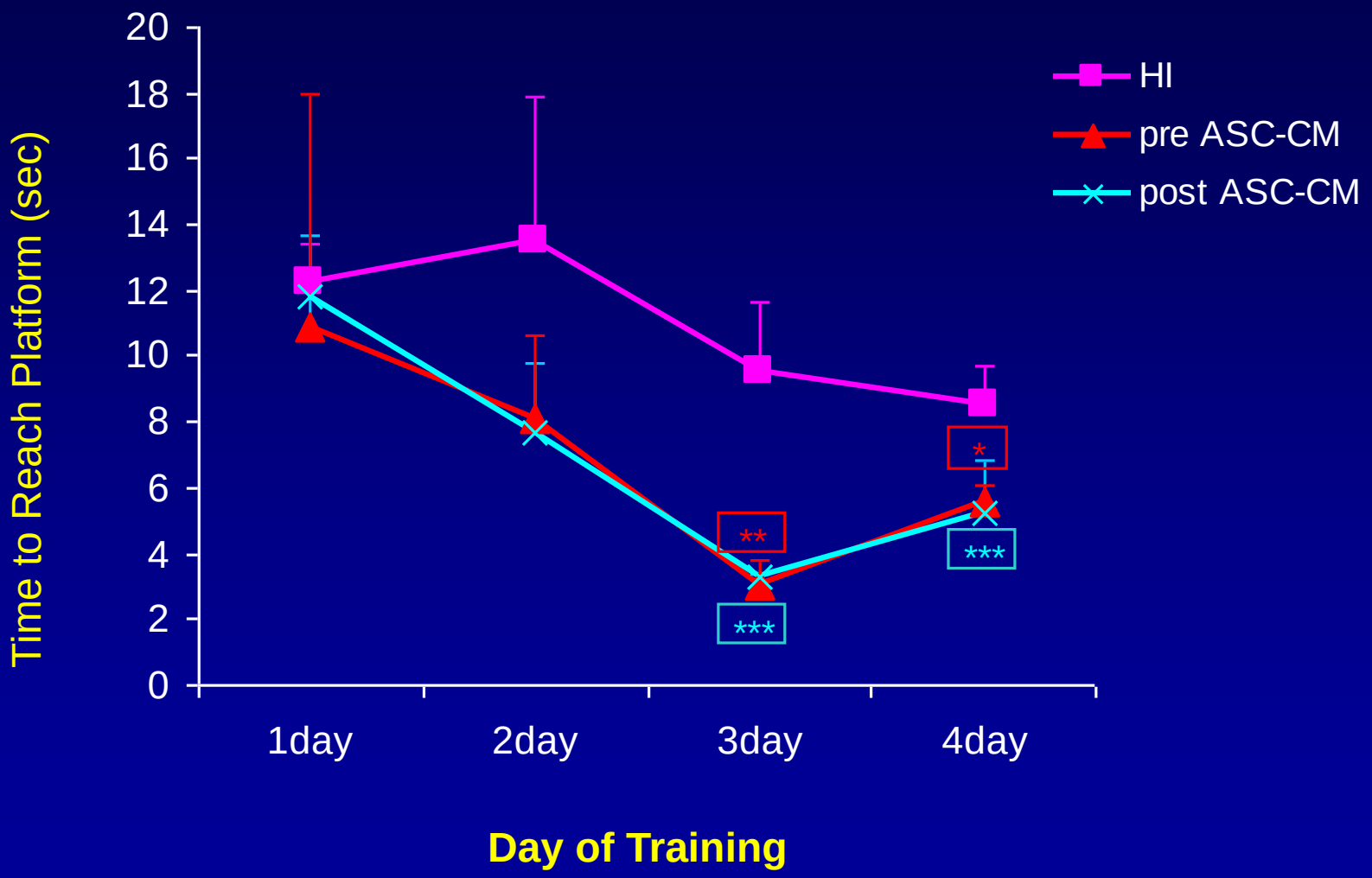




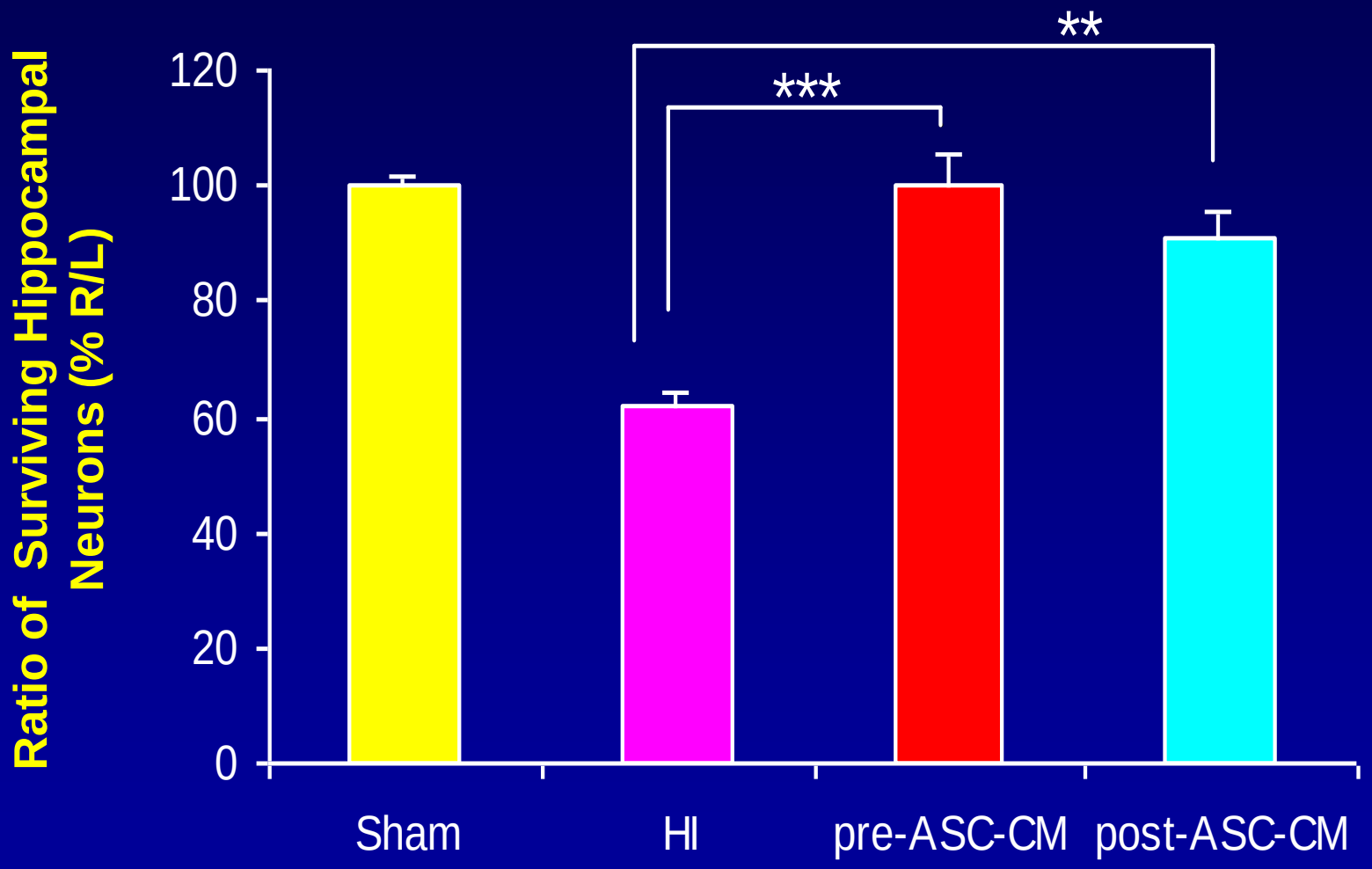
Morris Water Maze:
Spatial Learning and Memory



Escape Latency: Invisible Platform



ASC-CM Improved Hippocampal Neuronal Survival at 8 Weeks



Conclusions II

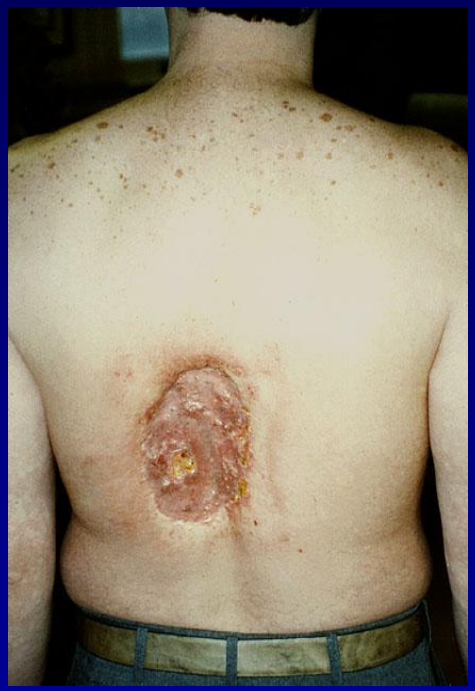
Following HI exposure of neonatal rat brain, neuroprotective substances in ASC-CM:

- **Penetrate BBB and bind to hippocampal neurons**
- **Promote survival of hippocampal neurons**
- **Improve learning and memory**

Development of a Combination ASC and Autologous Matrix for Wound Healing Applications

The Need for New Therapies to Promote Wound Healing

- Delayed wound healing often observed following radiation therapy in combination with:
  - Breast reconstruction post-mastectomy & RT
  - Neck dissection & reconstruction post-RT
- There are 1.4 million new cancer cases each year in the U.S.
- 70% of patients require irradiation
- Wound complication rates can be as high as 67% due to fibrosis and loss of the microvascular network ing the skin



Source: Cancer Facts and Figures, ACS, 2007

ASCs & Autologous Platelet Rich Fibrin-Rich Matrix as a Combination Therapy

- Adipose Stromal Cells
  - Secrete many growth factors and cytokines beneficial for healing (e.g., VEGF, HGF, FGF-2)
- Platelet Rich Plasma
  - Rich source of PDGF (which ASCs do not produce)
  - Has been shown to increase proliferation of fibroblasts and Type I collagen production
  - Combined with thrombin, PRP creates a fibrin gel scaffold; thus, providing physical support for “trapping” ASCs in wound

Eppley,et al. 2004

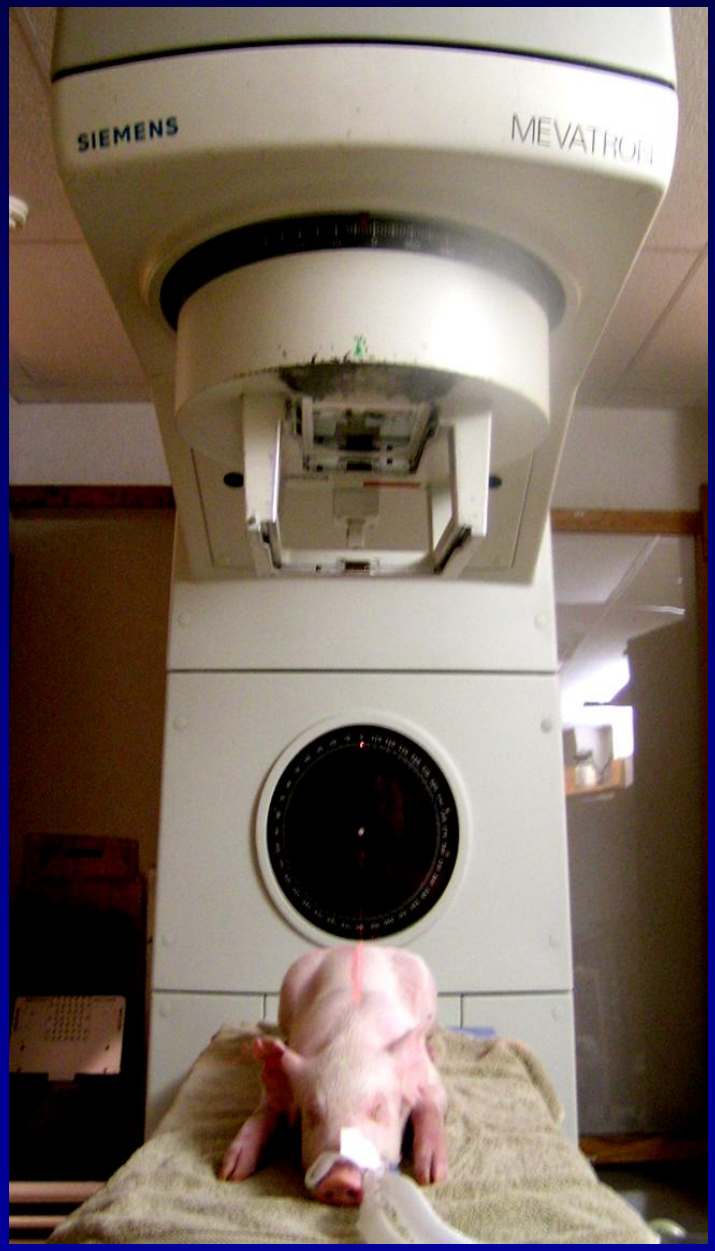
Complementarity of Factors in Plasma and ASC-CM

Growth Factor (pg/ml)	Wound Treatments				
	ASC+PRP	ASC+PPP	ASC	PRP	PPP
VEGF	1819 ± 70	974 ± 12	1103 ± 190	136 ± 7	0
TGF-β1	15982 ± 575	1630 ± 56	1844 ± 188	12003 ± 941	287 ± 31
PDGF	1168 ± 102	1 ± 0.33	0	856 ± 105	2 ± 0.34

Porcine Delayed Wound Healing Study

- First task was to establish a irradiation-induced delayed wound healing model using clinically relevant radiation dosages and delivery methods
- Models had used out-dated modalities
- Pig skin possesses similarities to human skin– at least much more so than rodent.

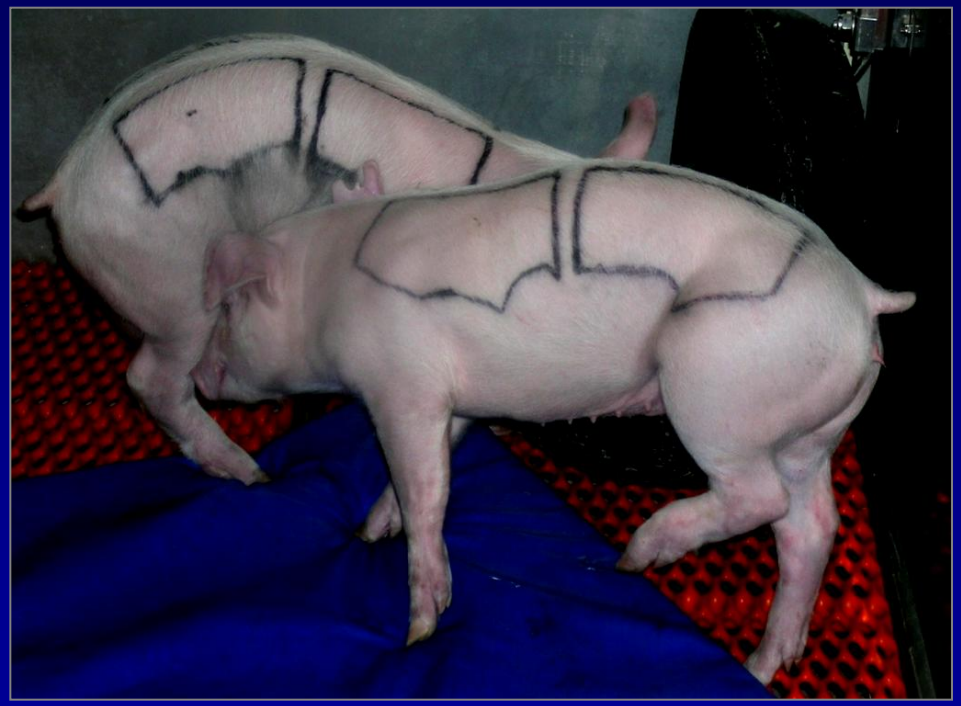
Radiation Injury Pilot Study



Goals:

- Create a reliable, clinically-relevant radiation injury model *in vivo*
  - Replicate skin treated to 60 – 70 Gy with conventional fractionation
- Observe and quantify acute & chronic radiation effects
- Quantify irradiation effects on porcine skin microvasculature
- Determine appropriate timing for subsequent wounding

Radiation Injury Pilot Study



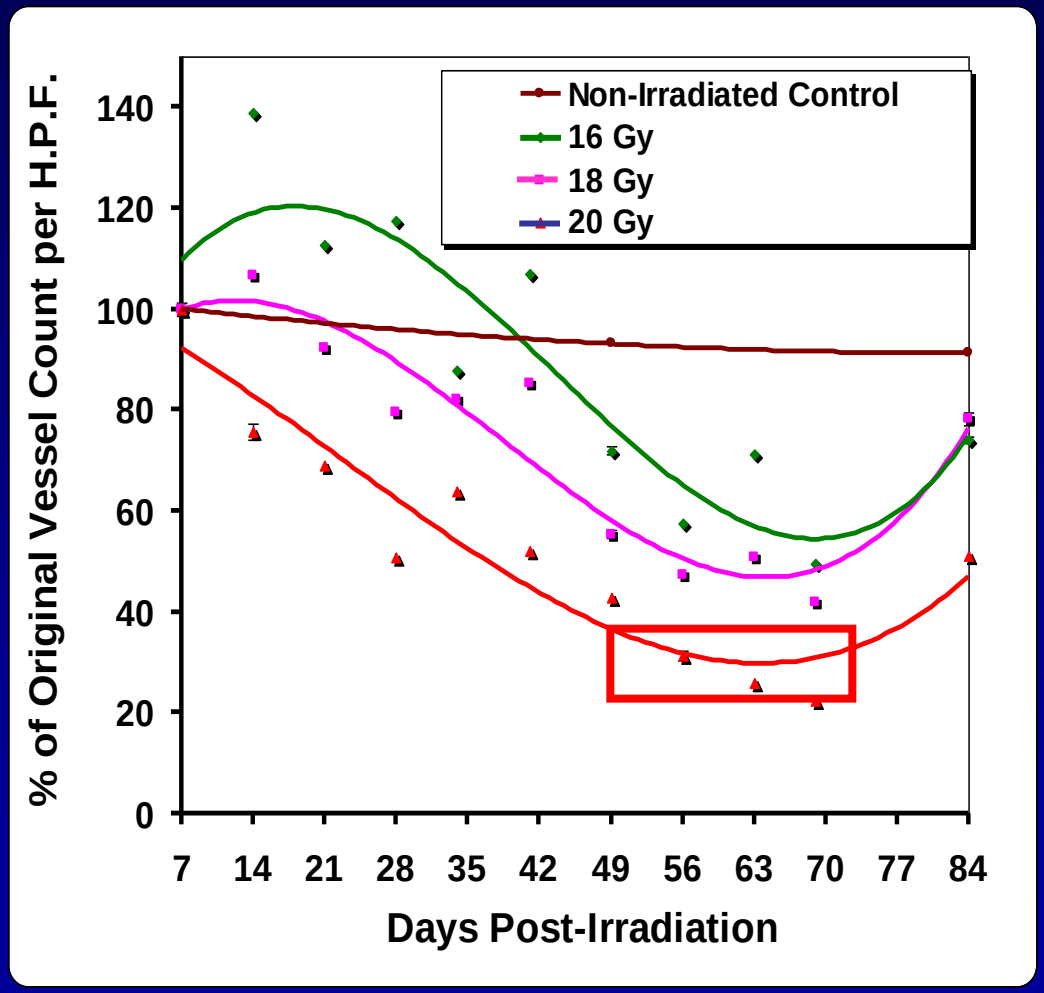
Design:

- 3 pigs receiving a single fraction of 16, 18 or 20 Gy
- Weekly biopsies of radiated and normal skin
- Immunohistochemistry for alpha-smooth muscle actin to quantify the number of arterioles per high powered field.
- Follow the wounds from biopsy sites for signs of delayed healing

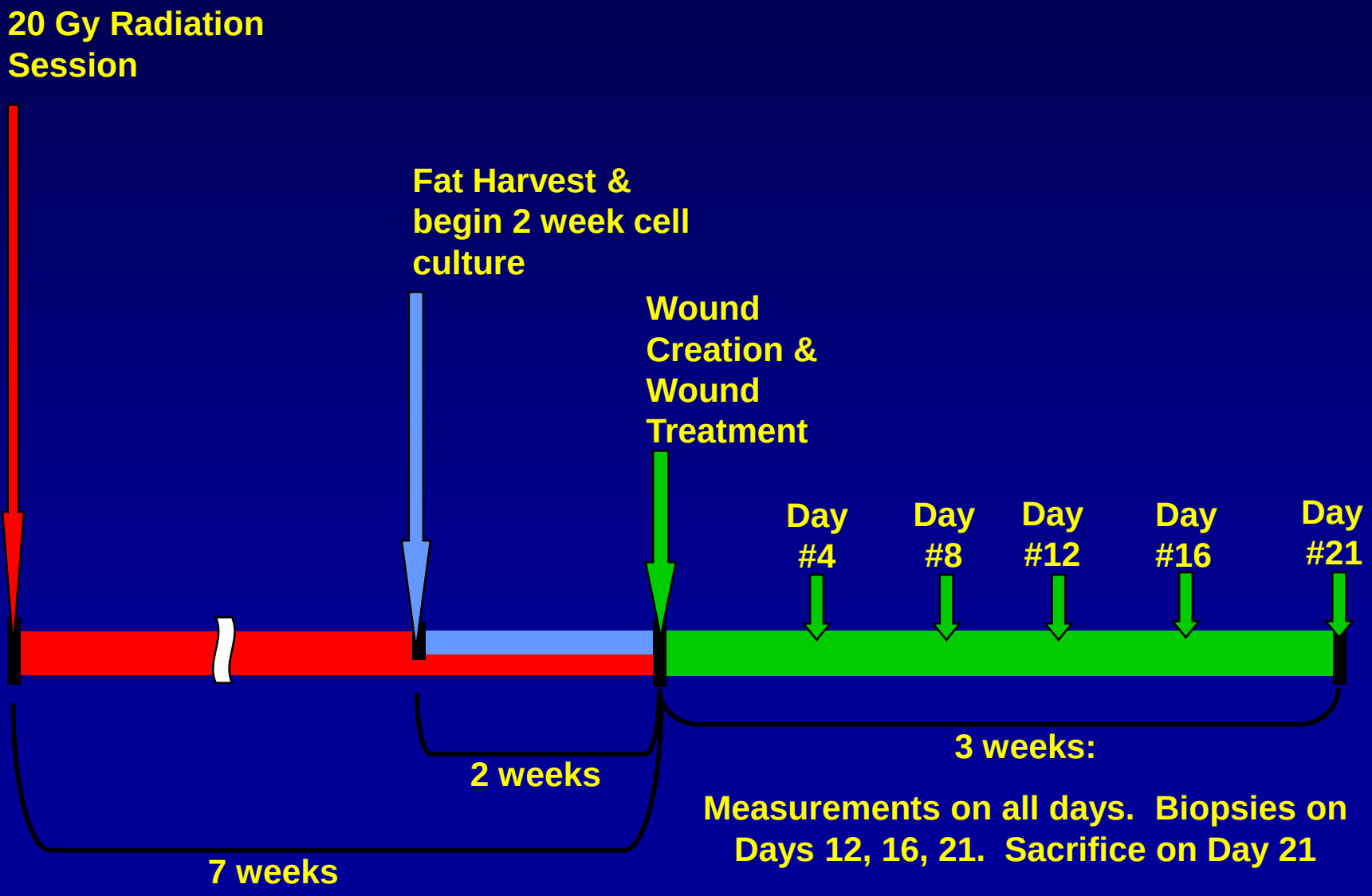
Observed radiation effects

- Acute radiation effects (e.g. erythema) were mild and well tolerated
- Chronic effects seen were an increase in skin dryness, and a decrease in hair density
- The 20 Gy subject had visibly decreased healing of the 8 mm biopsy wounds.

Arteriole Histology: α-smooth muscle actin

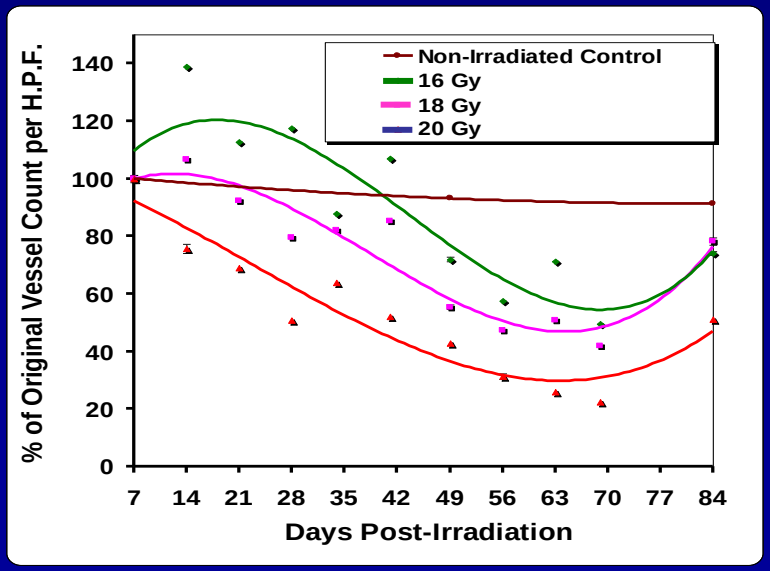


Full Wounding Study: Project Timeline

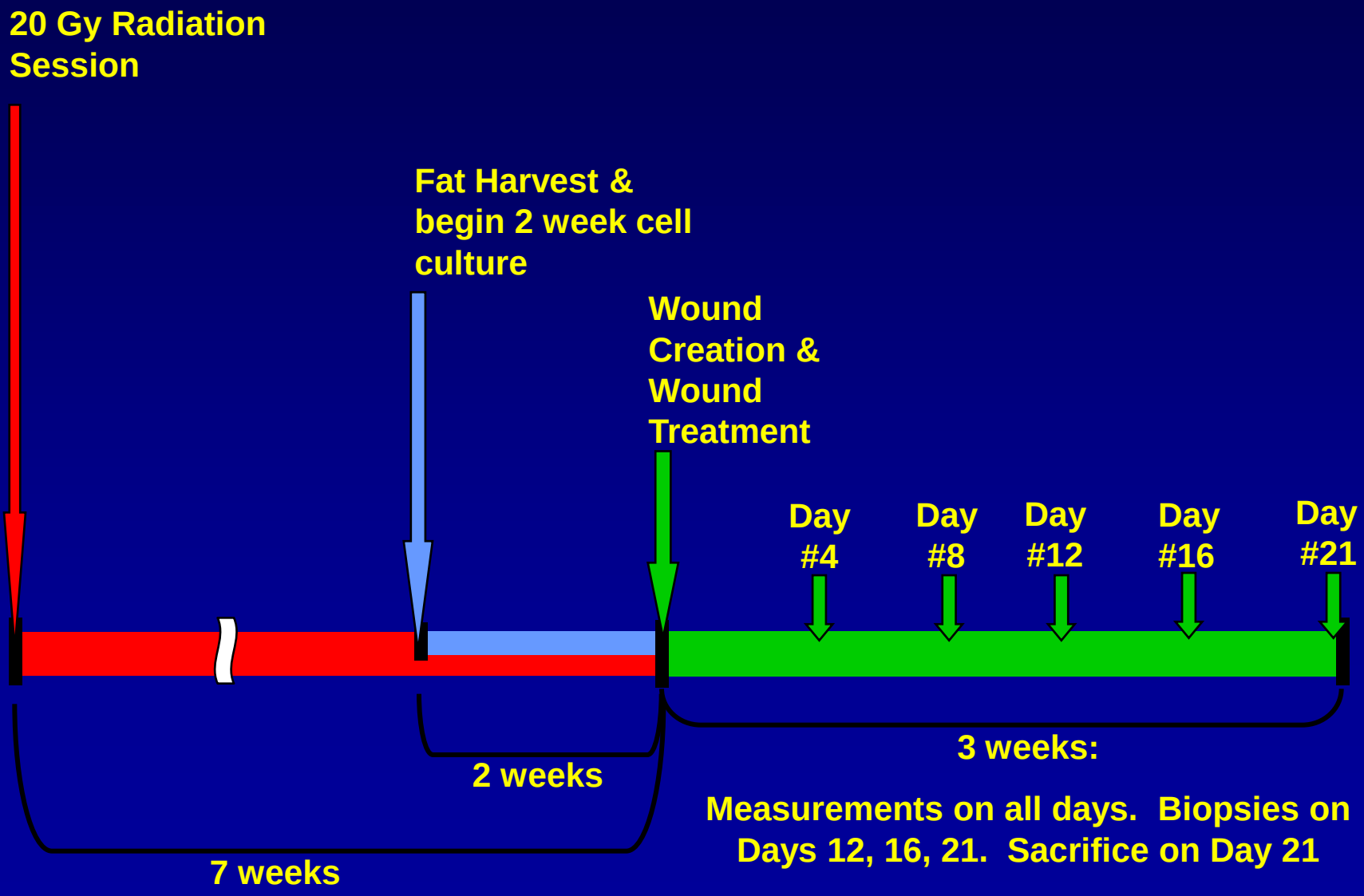


20 Gy Radiation Injury

- 7 weeks prior to wounding

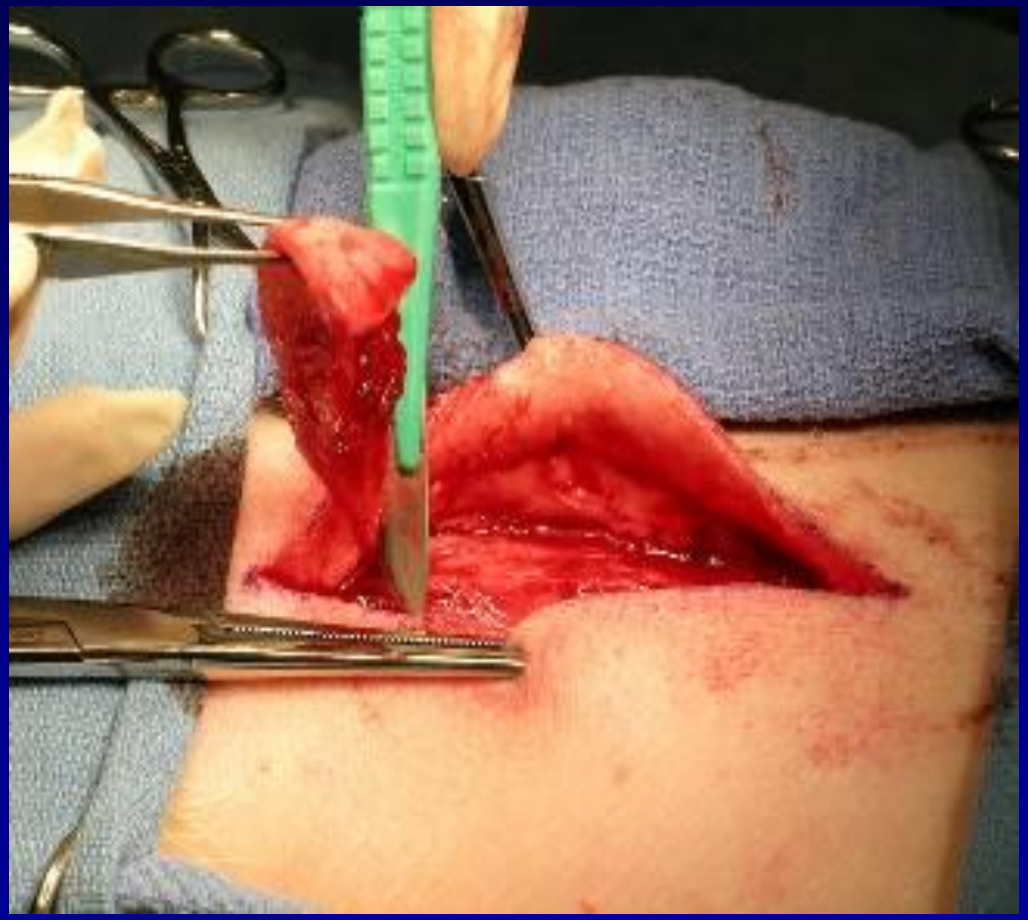


Full Wounding Study: Project Timeline



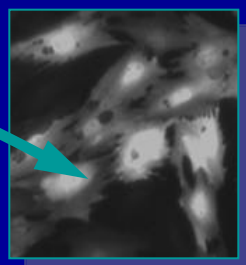
Fat Harvest

- 2 weeks prior to wounding



ASC Isolation

Stromal-Vascular Fraction (SVF)



Dorsal hump fat

⬇

Collagenase / Hyaluronidase Digestion at 37º with shaking (50 min)

⬇

Plate on plastic dishes

⬇

Adipose Stromal Cells

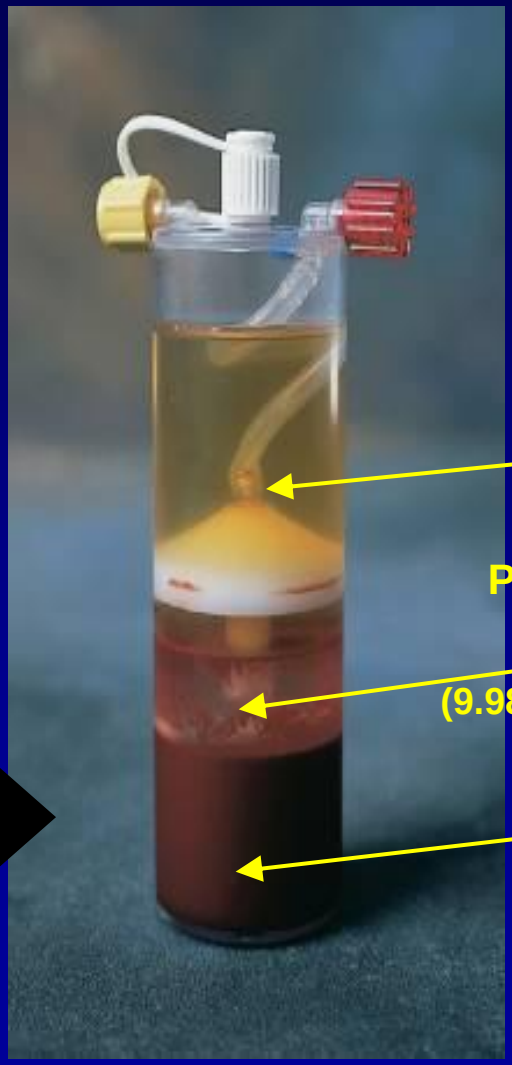
⬇

Freeze at -80 C

⬇

Thaw & expand ASCs for 5 days in culture

Platelet Rich Plasma Treatment Preparation



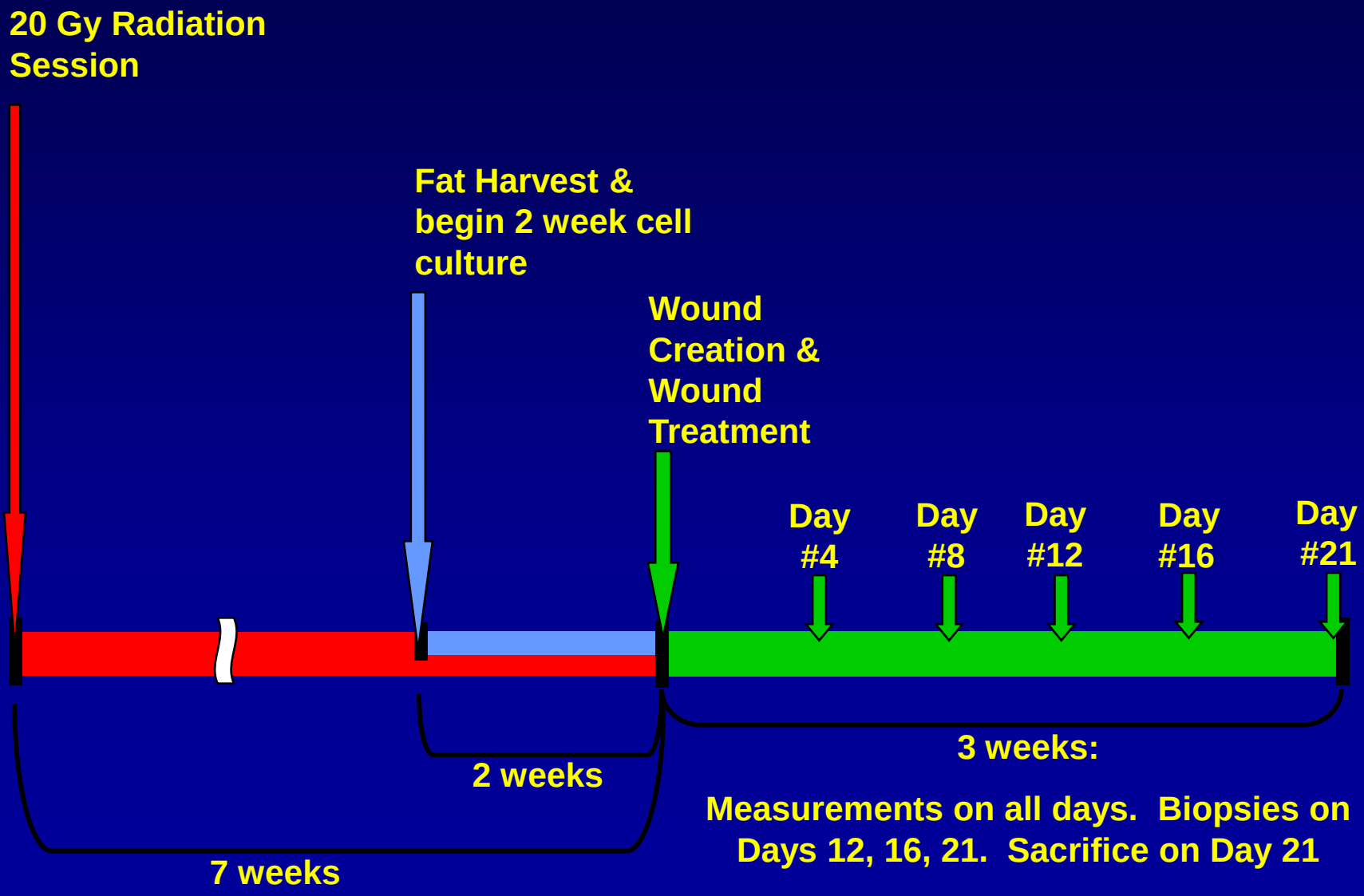
Platelet Poor Plasma (PPP)

Platelet Rich Plasma (PRP)

($9.98 \pm 0.40 \times 10^5$ platelets /µL)

Packed Red Blood Cells

Full Wounding Study: Project Timeline

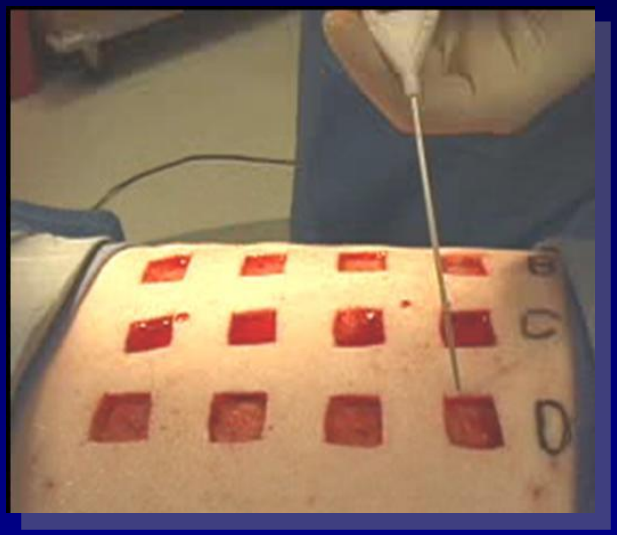


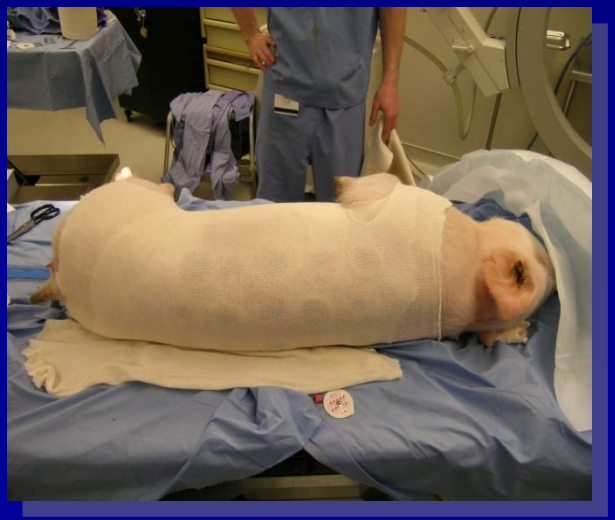
Treatment Groups

1. Saline (also applied to non-irradiated skin)
2. ASCs in Saline
3. Platelet Rich Plasma (PRP)
4. ASCs in PRP

Wounding & Treatment

- 4 pigs
- 30-33 irradiated wounds per pig
- 4 non-irradiated wounds per pig (treated with saline)
- Approx. 4 million ASCs in each wound
- Wounds covered with a Tegaderm dressing and a protective vest
- Treatments are applied only on the day of wounding
- Pain treated with a narcotic patch



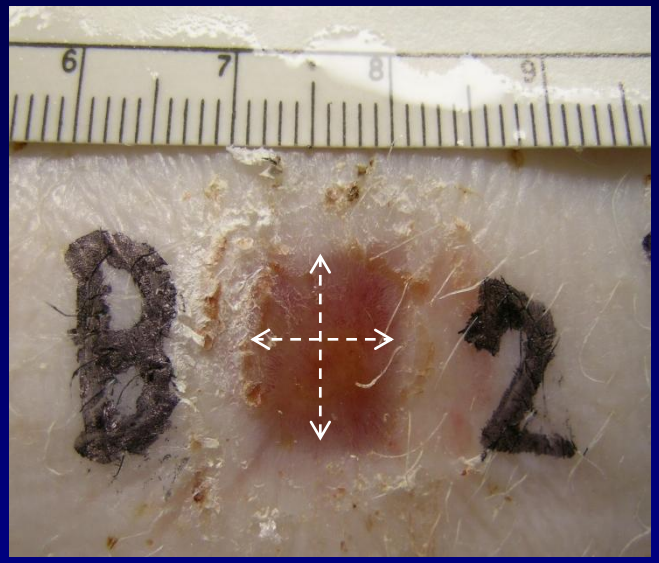


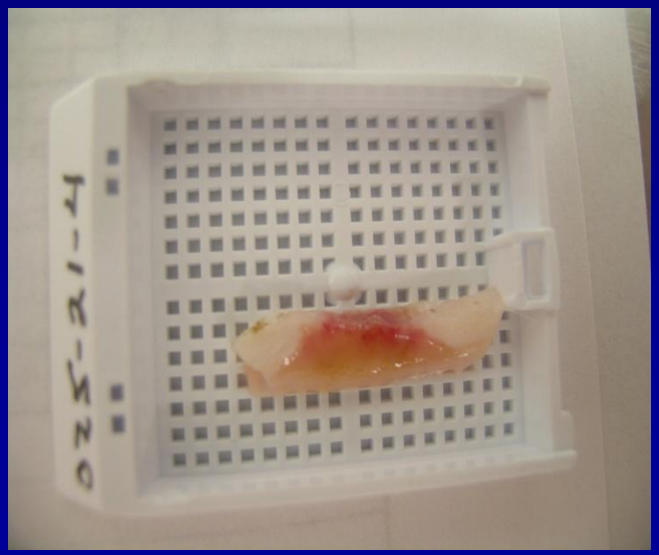
Full-Thickness Wounding

each wound 1.5 x 1.5 cm

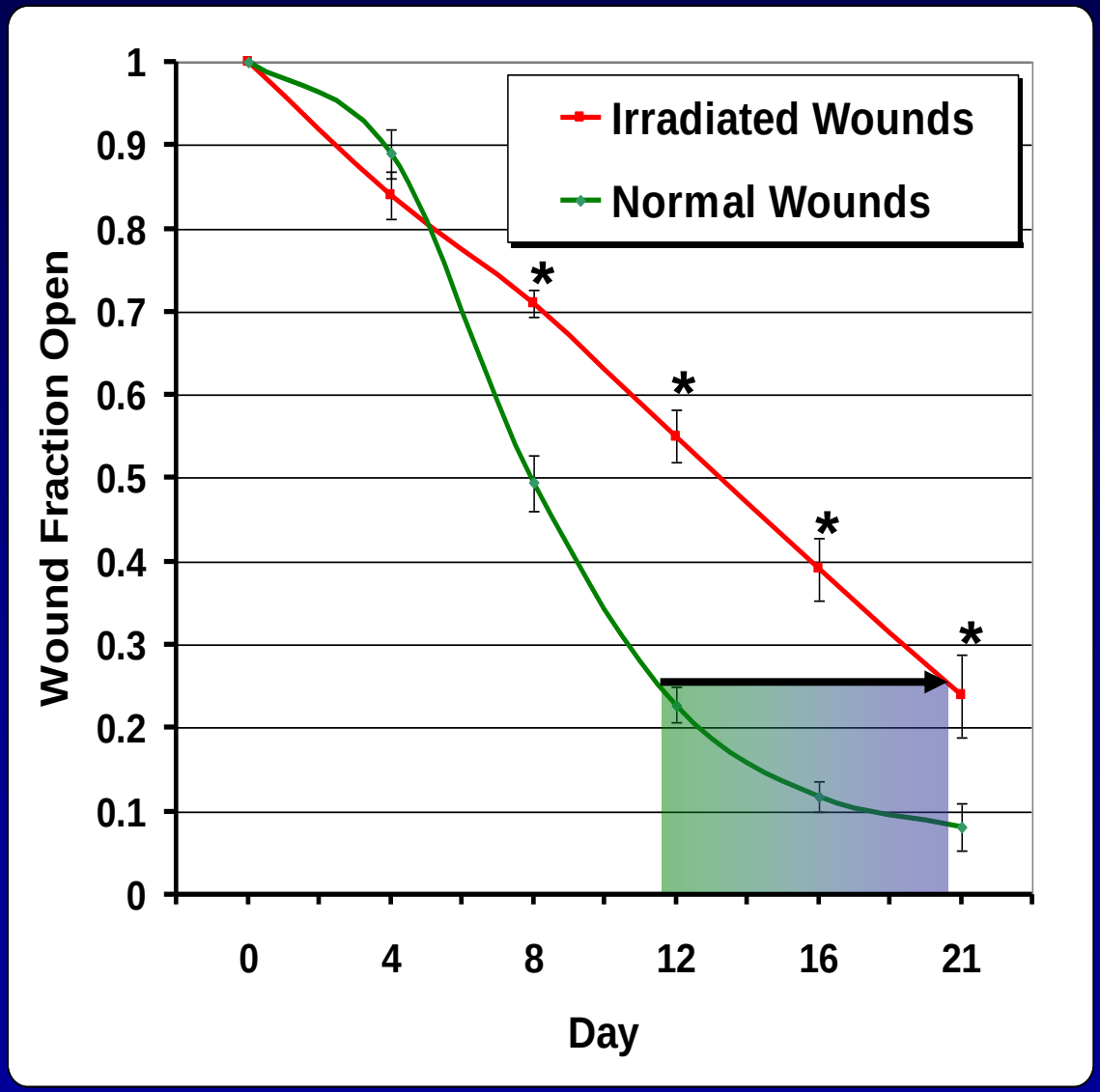
Wound Assessments

- All dressings removed
- "No Touch Technique" employed
- Dimensions measured to determine surface area of each wound on days 4, 8, 12, 16, and 21
- On days 12, 16, and 21, full thickness longitudinal biopsies taken for histologic analysis
- Re-bandaging of pig





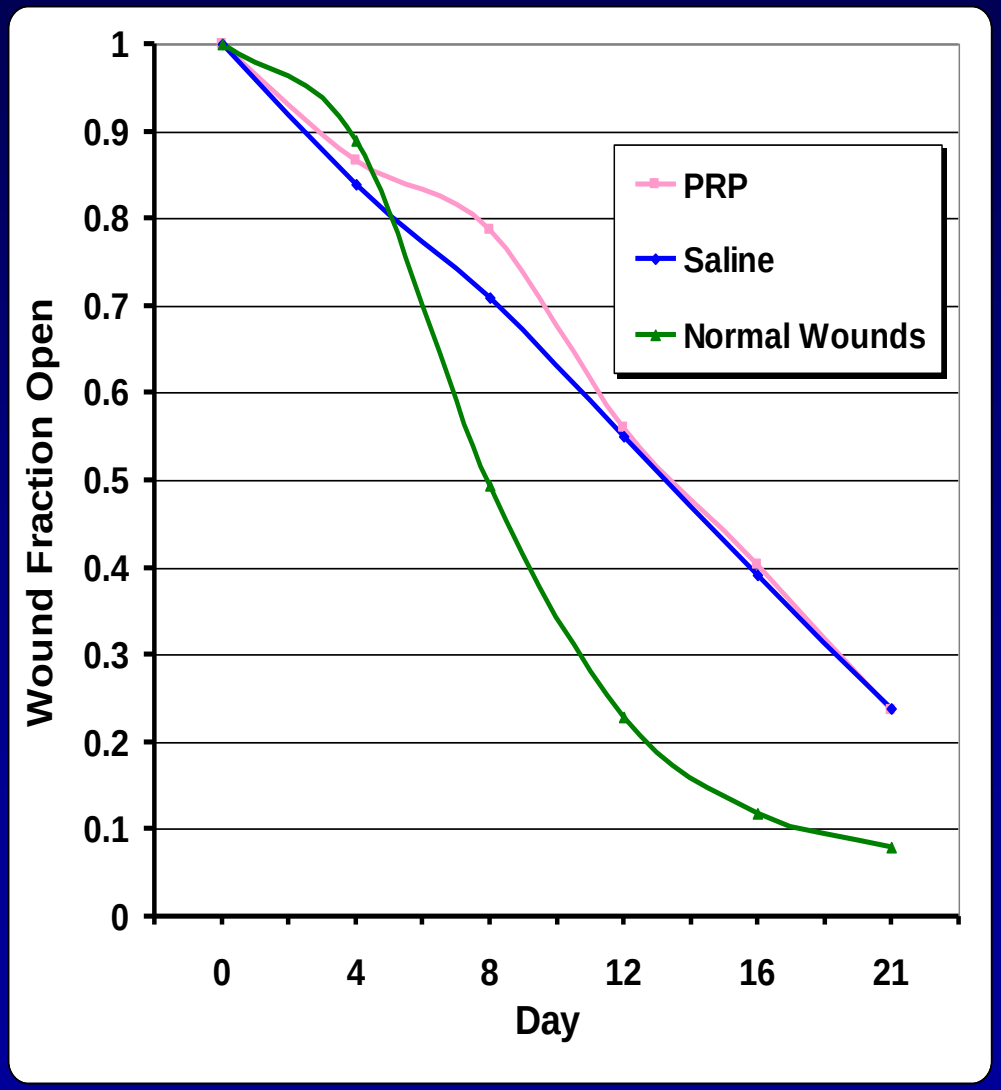
20 Gy of a Single Electron Radiation Fraction Creates Significant Healing Delays



- Saline treated normal skin wounds vs. saline treated irradiated skin wounds ($p<0.05$)

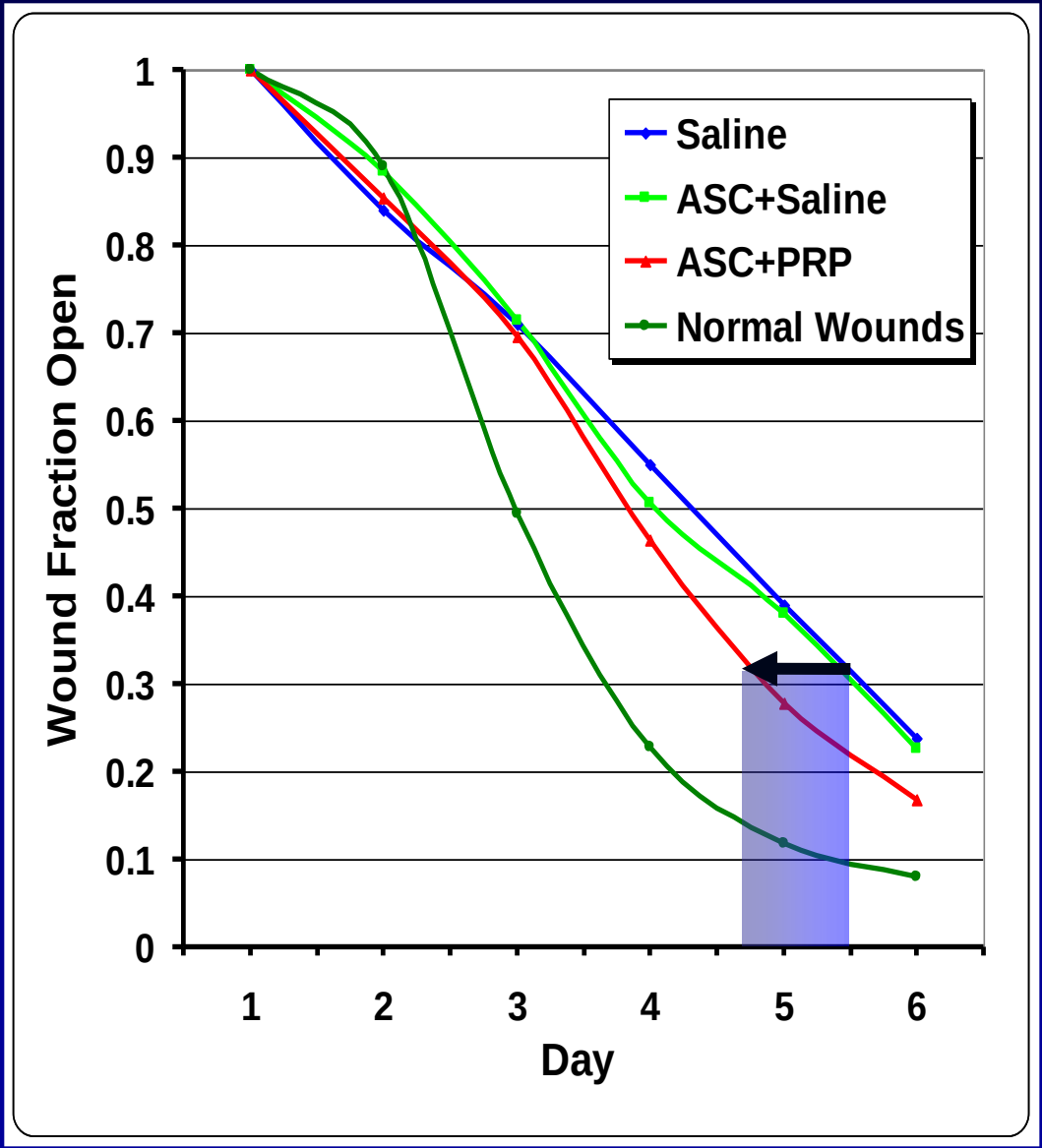
- **The model creates a 10 day delay in wound healing**

PRP fibrin gel alone does not induce improved healing



- No improvement in the rate of healing was observed with PRP alone

PRP Matrix Was Required For ASC-Induced Healing



- No significant difference was found between Saline and ASC+Saline
- A significant improvement in healing was found between Saline and ASC+PRP ($p < 0.05$)
- ASC+PRP treatment accelerated healing by 2.5 days

Conclusions for ASC & PRP Based Wound Therapy

- ASC-based therapy improves the healing rate of irradiated, vascularly-depleted tissues by 25%
- ASCs appear to require a fibrin gel vehicle (PRP) to persist in tissues and improve healing
- PRP alone does not appear to improve healing

Overall Summary

- A significant portion of the therapeutic potential of ASCs can be attributed to secreted factors
- Further studies need to be conducted to establish whether differentiation and incorporation occurs to a significant degree
- We intend to seek or are currently seeking FDA-approval for human trials for each of these applications

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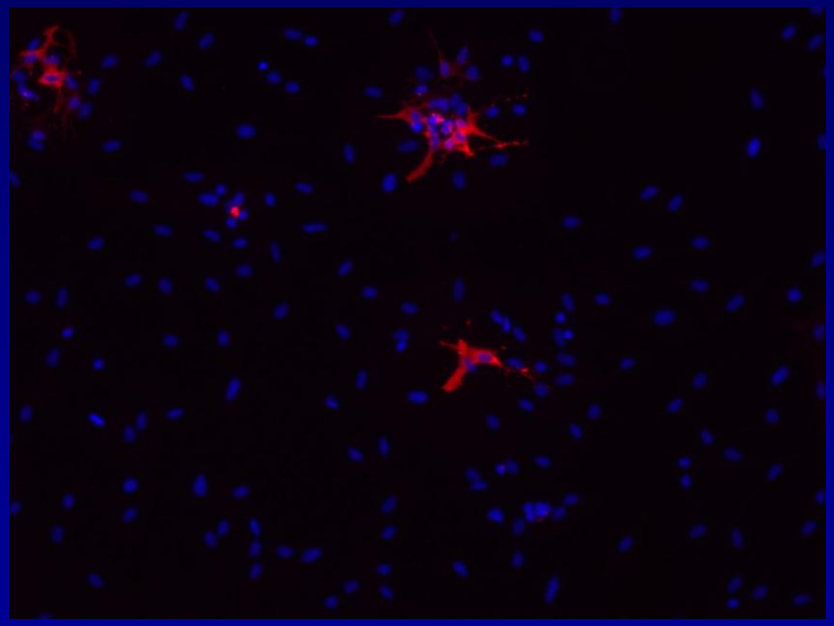
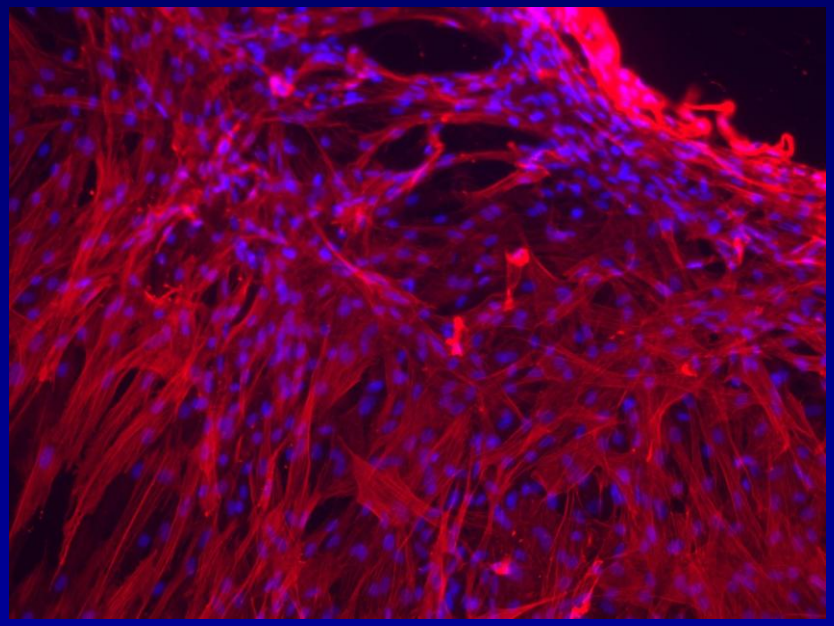
ASCs Can Be Induced to Adopt a Smooth Muscle Cell Phenotype



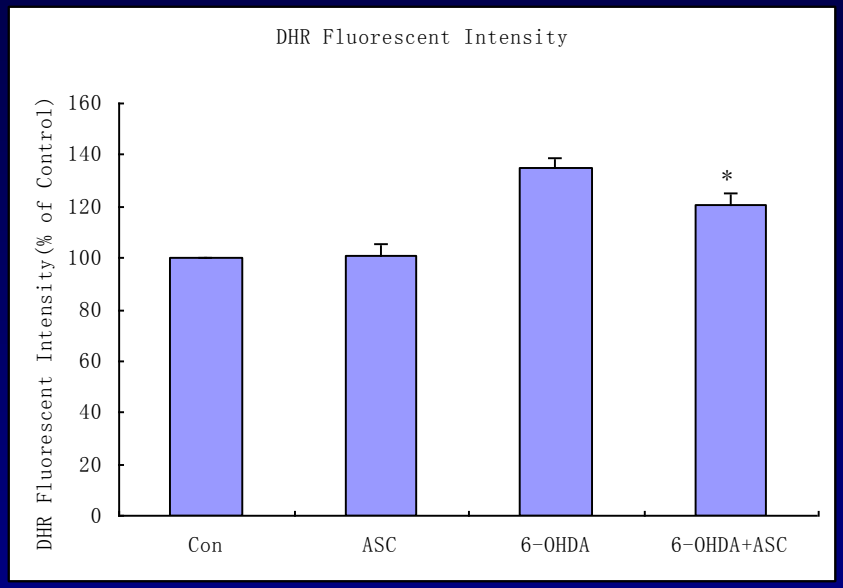
Low serum



High serum

3. *ASC blocks 6-OHDA-induced free radicalformation in CGN*

3A



3B

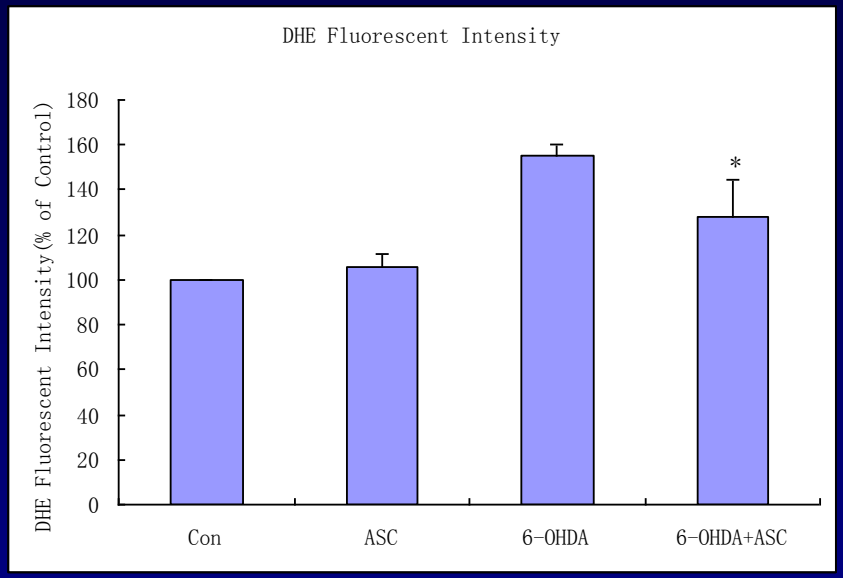
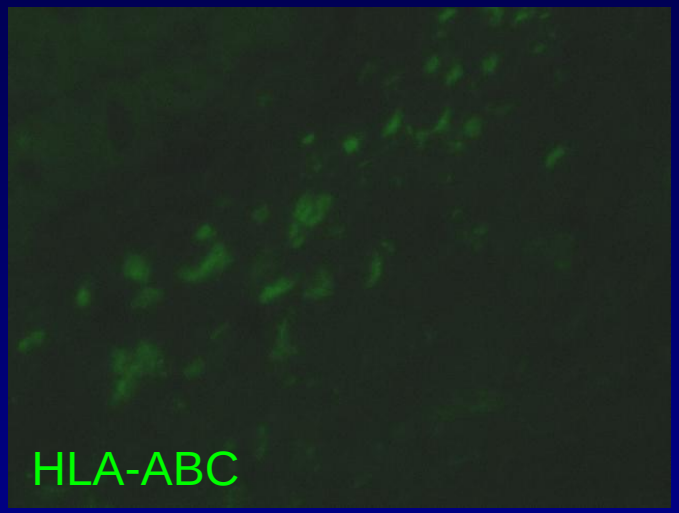
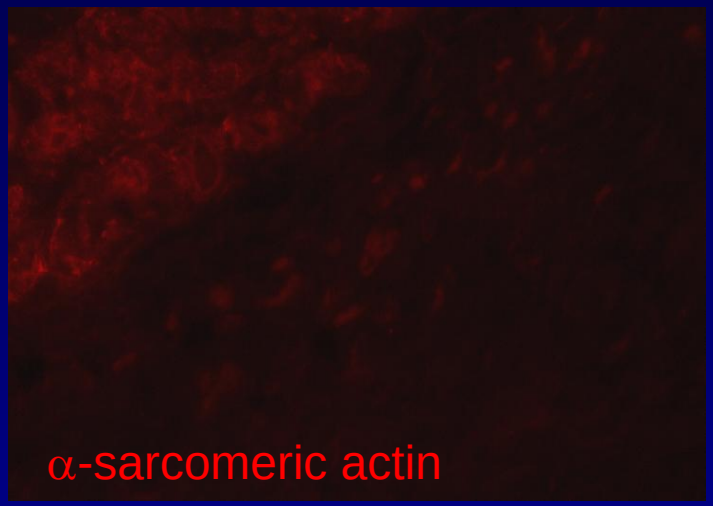
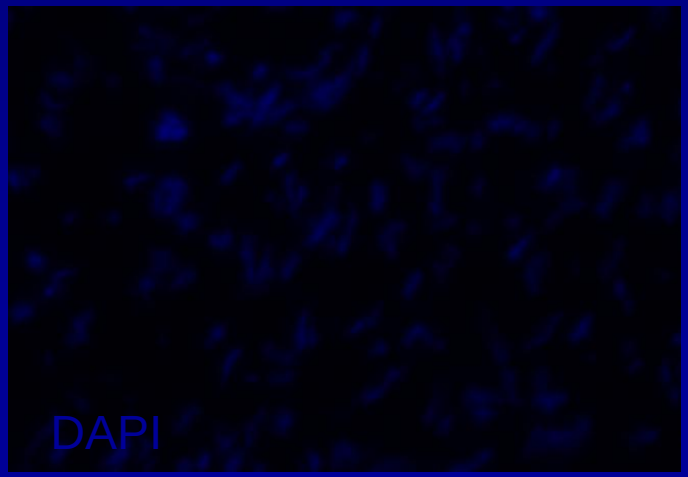
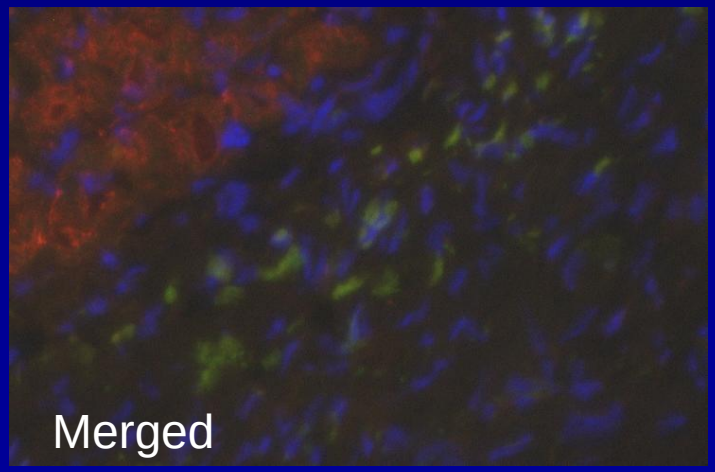


Fig. 3.ASC-CM blocks 6-OHDA-induced free radical generation in CGN.
Treatment with 6-OHDA (60 μM) for 6h significantly increased free radical generation (determined by dihydrorhodamine 123 staining(3A) and dihydroethidium(3B)) in CGN compared to non-treated controls. 30% ASC-CM replacement significantly decreased 6-OHDA-induced free radical production in CGN. Bars represent the mean±S.E.M. (*$p<0.05$, compared to 6-OHDA treatment) .


HLA-ABC


α-sarcomeric actin


DAPI


Merged