1 Reconstructive Urology

2 Differentiation of smooth muscle cells from

3 human amniotic mesenchymal cells implanted in

4 the freeze-injured mouse urinary bladder

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Abstract

Objectives: To determine if cells derived from human amniotic mesenchymal cells (HAMCs) support the structural and functional reconstruction of freeze-injured mouse bladders.

Design, Setting and Participants: HAMCs were harvested from an amnion membrane and cells cultured for seven days prior to injection into the freeze-injured bladder walls of nude mice.

Intervention: Three days prior to implantation, the posterior bladder walls of the mice were freeze-injured for 30 seconds. The cultured HAMC-derived cells $(0.5 \times 10^5 \text{cells}/50 \mu l)$ were implanted into the injured regions. Control bladders received a cell-free injection. At 1, 2, 4 and 6 weeks after the cell-implantation, the experimental bladders were extirpated.

Measurements: The bladder tissues were examined by immunohistochemistry for alpha-smooth muscle actin (SMA). The HAMC-derived cells were detected by anti-human nuclei antibody (HuNu). Separately, bladder muscle strips were examined for contractile responses to potassium.

Results and Limitation: At 1 week after implantation, the HAMC-derived cells, which were detected by HuNu, differentiated into muscular layers composed of SMA-positive cells. From 2 – 6 weeks after implantation, abundant layers of SMA-positive and HuNu-positive cells developed. In control bladders, few SMA-positive cells remained at the injured regions at 1 week, but by 6 weeks, more were present. At 1 week, the

64 contractile responses to potassium of the cell-implanted bladders were significantly

65 higher than those of the control-injected ones. Control-injected bladders also recovered

66 by six weeks, but the rate of recovery was slower.

67 **Conclusion:** Freeze-injured mouse bladders implanted with HAMC-derived cells

68 recovered morphology and function more rapidly than did control-injected bladders.

69

70 # 1. Introduction

71   In urology, regenerative medicine has been vigorously pursued to provide effective

72 treatments for severe and/or irreversibly injured bladders due to radiation, diabetes

73 mellitus, or perforation. The establishment of reliable and effective cell sources for

74 regenerative medicine is extremely important. There are many stem or progenitor cells,

75 such as embryonic stem cells [1-5]. However, clinical use of these cells can be

76 problematic for several reasons including securing consent of tissue donors and

77 recipients, immunological antigenicity, and ethical concerns. Human amniotic

78 mesenchymal cells (HAMCs) might be a source that can overcome these problems. The

79 HAMCs are multipotent and can differentiate into various cell types, including

80 adipocytes, osteocytes, neural cells [6], and chondrocytes [7], and they have low

81 immunological antigenicity [8, 9]. Furthermore, the HAMCs have few ethical concerns

82 because the cells are derived from amnions after parturition.

83   Our ultimate aim is to develop clinical applications using HAMC-derived cells for

84 the treatment of partially irreversibly damaged bladders. As the first step toward that

85 aim, we implanted HAMC-derived cells into mouse freeze-injured bladders and

86 assessed the effect on structural and functional recovery.

87

88

2. Materials and methods

2.1. Materials

This study and the use of human amniotic tissue were approved by the Ethics

Committee of Shinshu University School of Medicine. The amnion was provided with

informed consent by a woman who underwent an uncomplicated cesarean section.

BALB/C nu/nu female nude mice (Japan SLC INC., Shizuoka, Japan) at postnatal

week 5 were used for the experiments. The mice were treated in accordance with

National Institutes of Health Animal Care Guidelines and the guidelines approved by

the Animal Ethics Committee of Shinshu University School of Medicine.

2.2. Isolation and culture of HAMCs

The HAMCs were isolated from the amnion, which was mechanically peeled from

the placenta, described in a previous report [8]. Briefly, the amnion was cut into pieces,

and to separate the epithelial cells from the amnion, the resulting minced membrane was

digested with 0.2% trypsin (Sigma-Aldrich Co.) in Dulbecco's Modified Eagle's

Medium (DMEM, Sigma-Aldrich Co.) and incubated for 30 min at 37°C. This

trypsinization was repeated several times.

For cultivation, the HAMCs were resuspended in DMEM supplemented with 10%

regular fetal bovine serum (Biowest, Nuaille, France). They were then seeded onto

collagen-coated 10-cm culture dishes at a density of 3.0×10^4 cells/cm^2. The HAMCs

were cultured at 37°C in humid air with 5% CO_2 for 7 days.

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2.3. Freeze-injury of urinary bladders and implantation of HAMC-derived cells

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Seventy-five mice were used in this study. To obtain baseline values, eight of them

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were killed without any interventions, and the bladders were removed for histological

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analysis (n=3) and muscle strip investigation (n=5).

114

Three days prior to cell implantation, 67 mice were anesthetized with a

115

pentobarbital sodium solution (0.05 mg/g body weight), and the bladders were exposed

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through abdominal midline incisions. After all urine in the bladders was evacuated

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through the anterior side using 29G-microsyringe, 0.1 mL of saline at 38°C was injected

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in the same manner to create a uniform bladder volume. The posterior walls of the

119

bladders were freeze-injured by application for 30 seconds of the 10×3 mm end of an

120

iron bar chilled by dry ice. The bladders were returned to the pelvic cavity and the

121

abdomens were closed.

122

At 3 days after the freeze injury, 3 of the 67 mice were killed and the bladders were

123

removed for histological analysis. Sixty-four mice were randomly divided into the

124

cell-implantation group and the control-injection group (n=32 each). After anesthetizing

125

and making a midline abdominal incision as above, the HAMC-derived cells

126

$(0.5\mathrm{x}10^5$cells/50 µl of DMEM) were implanted into the freeze-injured regions of the

127

bladders using a 30G-microsyringe. In the control-injection group, 50 µl of cell-free

128

solution was injected in the same manner.

129

At 1, 2, 4, and 6 weeks after the cell implantation and the control injection, 8

130

131   experimental mice of each group were killed with a pentobarbital sodium solution, and

132   the bladders were removed. The bladders were subdivided into groups for

133   immunohistochemistry (n=3) and muscle strip investigation (n=5).

134

135   **2.4. Immunohistochemistry**

136   They were fixed in 4% paraformaldehyde and 4% sucrose in 0.1 M phosphate

137   buffer, pH 7.4, for 12 hours at 4°C, and then embedded in paraffin. The sections were

138   deparaffinized, rehydrated, rinsed three times with PBS. For antigen retrieval, they were

139   then microwaved at 100°C for 5 min. The specimens were coated with 1.5% normal

140   donkey serum (Chemicon Internatinal Inc., Temecula, CA, USA) and then incubated

141   with the primary antibody, mouse anti-human nuclei monoclonal antibody (HuNu,

142   1:100; Chemicon). The specimens were incubated with PBS at 4°C for 1 hour, and then

143   secondary antibody consisting of donkey anti-mouse IgG conjugated with Alexa fluor

144   594 (1:250, Molecular Probes, Eugene, OR, USA) was added for 1 hour at 4°C.

145   Subsequently, the specimens were incubated for 12 hours at 4°C with the primary

146   antibodies to detect alpha-smooth muscle actin (SMA, 1:100, mouse monoclonal,

147   Progen Biotechnik GmbH, Heidelberg, Germany), a marker of smooth muscle cell

148   differentiation. These sections were then incubated with secondary antibody donkey

149   anti-mouse IgG conjugated with Alexa fluor 488 (1:250, Molecular Probes) for 1 hour

150   at 4°C. Other sections from each sample (n=3 each), in addition to normal bladders

151   (n=3) and 3-day injured bladders (n=3), were stained by hematoxylin and eosin (H&E).

152   The cultured HAMCs on the dishes were also stained.

153

154   **2.5. Muscle strip investigation**

155   Five mice from each group were killed at 1, 2, 4, and 6 weeks, and the bladders

156   were removed for muscle strip investigation. The bladders were equally separated into

157   anterior and posterior sides. The strips were transferred to 5 mL organ baths containing

158   Krebs solution (see below for composition) maintained at 37°C. The Krebs solution was

159   bubbled with a mixture of 95% O_2 and 5% CO_2, giving a pH of 7.4. The strips were

160   attached at one end to a tissue holder and at the other end to a force displacement

161   transducer (Type 7923; NEC San-Ei instruments Ltd., Tokyo, Japan). Data were

162   recorded and analyzed using WinDaq data analysis software (DATAQ Instruments,

163   Akron, OH, USA). Each strip was stretched until a stable tension of 1 g was obtained.

164   After the equilibration period, the experiment was started by exposing the strips to a 124

165   mM potassium-Krebs solution for 1 minute (see below for composition). After washing

166   out the K^+-Krebs solution by standard Krebs solution, contractions were evoked using

167   the muscarinic cholinergic agonist carbachol (10^{-8} – 10^{-3} M, Sigma-Aldrich Chemical

168   Co.). Based upon the concentration-response curve, pD2 values were calculated as the

169   negative logarithm of the molar concentration that produced a response that was 50% of

170   the maximal contraction.

171   The Krebs solution consisted of sodium chloride (NaCl) 119 mM, potassium

172   chloride (KCl) 4.6 mM, calcium chloride 1.5 mM, sodium bicarbonate 15 mM, sodium

173   dihydrogen phosphate 1.2 mM, magnesium chloride 1.2 mM, glucose 5.55 mM

174   (Sigma-Aldrich Co.). To make the 124 mM K^+-Krebs solution, NaCl was replaced with

175   an equimolar amount of KCl.

176

177   **2.6. Statistical analysis**

178   Statistical analyses were performed with Excel Statistical Program File

179   ystat2006.xls (Igakutosho Shuppan Ltd., Tokyo, Japan). Differences between groups

180   and periods were evaluated by analysis of variance (ANOVA) tests. When ANOVA

181   indicated a significant difference, groups were further compared using the unpaired

182   nonparametric Mann-Whitney test. Values of $p < 0.05$ were considered statistically

183   significant.

184

185 ## 3. Results

186 ### 3.1. Cultured cells

187   At 7 days after plating in collagen-coated dishes, the attached HAMC-derived cells

188 achieved confluence (Fig. 1A). Immunohistochemically, the nuclei of the cultured

189 HAMC-derived cells before implantation were positive for HuNu and negative for SMA

190 (Fig. 1B).

191

192 ### 3.2. Bladder wall 3 days after injury

193   Just prior to the cell implantation or the control injection, the freeze-injured regions

194 of each bladder were identified by the presence of a hematoma or scar tissue. Compared

195 to the normal bladders (Fig.1 C), 3-day injured bladders were edematous and inflamed

196 and had few typical smooth muscle layer structures (Fig. 1D).

197

198 ### 3.3. Immunohistochemistry

199   At 1 week, the presence of hematoma, edema, and inflammation in the injured

200 bladders receiving the HAMC-derived cells were slightly decreased (Fig. 2A). These

201 bladders also had thin, fragile layers of smooth muscle structures composed of some

202 SMA-positive cells in the injured regions (Fig. 3A). At 2 and 4 weeks, the injured

203 regions had little hematoma, edema, and inflammation (Fig. 2B, C), and SMA-positive

204 cells formed layered smooth muscle structures in the injured regions (Fig. 3B, C)

205 similar to normal bladders. At 6 weeks, the layered smooth muscle structures of the

206 cell-implanted bladders (Fig. 2D, Fig. 3D) were similar to normal ones. In contrast, 1

207 week after the control injection, the hematoma, edema, and inflammation remained

208 present without any notable decrease (Fig. 2E). These bladders had few distinct layered

209 structures (Fig. 3E) compared to cell-implanted ones. At 2 and 4 weeks, the control

210 regions partially showed spontaneous recovery (Fig. 2F, G, Fig. 3F, G). At 6 weeks after,

211 the control-injected bladders had layered smooth muscle structures composed of

212 SMA-positive smooth muscle cells (Fig. 2H, Fig. 3H) similar to normal ones. In

213 addition, at each period, cells positive for HuNu were present at the injured site in the

214 walls of all cell-injected bladders (Fig. 3A - D). On the other hand, no HuNu-positive

215 cells were observed in the walls of the control-injected bladders (Fig. 3E - H).

216

217 **3.4. Differentiation of HAMC-derived cells into smooth muscle cells**

218   At 1 and 2 weeks, in the regions receiving the HAMC-derived cells, some

219 SMA-positive cells were also positive for HuNu (Fig. 4A, B). These cells were round in

220 shape and interspersed among the other cells in the wounded region. At 4 and 6 weeks,

221 both SMA and HuNu-positive cells were spindle shaped (Fig. 4C, D), similar to typical

222 smooth muscle cells. These cells formed components of the layered smooth muscle

223 structures.

224

225 **3.5. Muscle-strip investigation**

226   The high K^+-Krebs solution evoked a sustained contraction in mouse bladder strips

227   and the contractions of the both posterior and anterior sides were diminished after the

228   freeze injury (Fig. 5A, B). At 1 week, the contractile responses to potassium of the

229   cell-implanted bladders were significantly higher than those of the control-injected ones.

230   Control-injected bladders also recovered by six weeks, but the rate of recovery was

231   slower. The muscarinic cholinergic agonist carbachol also elicited contractions. While

232   the maximum contraction in response to carbachol was diminished by the freeze injury,

233   the percentage of the maximum contraction compared to that induced by the high

234   K^+-Krebs solution was not altered at any time (Fig. 6A, B). In addition, the pD2 values-

235   of both the posterior and the anterior sides were not significantly different between the

236   cell-implanted and the control-injected groups (Fig. 7).

237

4. Discussion

238

239   The HAMCs have great potential as a source of cells for the development of

240 regenerative medicine. Human amnions have the advantages of few ethical questions,

241 multipotency, and low immunological antigenicity [8-15]. HAMCs have been

242 successfully used as cell therapies for injured nerve [16], infarcted heart [17], and stroke

243 [18]. However, to our knowledge, investigations of the HAMCs in the treatment of

244 injured urinary tracts have not been reported.

245   In this study, we showed that the HAMC-derived cells implanted into the walls of

246 freeze-injured mouse bladders survived and differentiated into smooth muscle cells. At

247 1 week, the hematoma, edema, and inflammation in the cell-implanted bladders were

248 significantly decreased compared to the 3-day injured bladders without treatment. In

249 contrast, edema and inflammation remained in the control-injected bladders and few

250 smooth muscle layers were observed.

251   The contractile responses to potassium by the cell-implanted bladders were

252 significantly higher than those of the control-injected bladders. However the maximum

253 contractile responses to carbachol were not different between the normal bladders and

254 the freeze-injured bladders. Additionally, the pD2 values for contractile responses to

255 carbachol did not differ between the cell-implantation and the control-injection groups.

256 These results suggest that freeze injury destroyed much of the structure of the smooth

257 muscle layers and decreased the contractile response to potassium, but did not affect the

258 proportion of the contraction activated by muscarinic receptors. Therefore, the

259   contractile dysfunction of the bladders induced by freeze injury was mainly due to a

260   decrease in the number of smooth muscle cells.

261   It is likely that the implanted HAMC-derived cells facilitated the recovery of the

262   layered smooth muscle structures of the freeze-injured bladders. However, even without

263   the HAMC-derived cells, the damage of the freeze injury was reversed as seen by the

264   spontaneous recovery of tissue morphology and contractions at 4-6 weeks after injury.

265   We previously reported details of the microenvironment within the freeze-injured

266   bladders that promote the differentiation of the bone marrow-derived cells into smooth

267   muscle cells [19]. At 3 days after freezing, the injured region contains numerous large

268   pores that are not present in the uninjured normal tissue. It is possible that those pores

269   promote the high rate of implanted HAMC-derived cell survival. Compared to the intact

270   bladders, the injured bladders significantly upregulate some growth factors supporting

271   differentiation of smooth muscle cells [19]. For these reasons, the 3-day freeze-injured

272   bladder is especially suitable for differentiation of the implanted HAMC-derived cells.

273

5. Conclusions

274

275   HAMC-derived cells implanted into freeze-injured mouse bladders differentiate

276   into smooth muscle cells and promote morphological and functional recovery. The

277   results of the present study suggest that HAMC-derived cells might be useful as a

278   source of cells in the regeneration of damaged or diseased urinary tracts.

279

Acknowledgments

287 **References**

288 [1] Frimberger D, Morales N, Shamblott M, Gearhart JD, Gearhart JP, Lakshmanan Y.

289   Human embryoid body-derived stem cells in bladder regeneration using rodent

290   model. Urology 2005; 65: 827-32.

291 [2] Cannon TW, Lee JY, Somogyi G, et al. Improved sphincter contractility after

292   allogenic muscle-derived progenitor cell injection into the denervated rat urethra.

293   Urology 2003; 62: 958-63.

294 [3] De Coppi P, Callegari A, Chiavegato A, et al. Amniotic fluid and bone marrow

295   derived mesenchymal stem cells can be converted to smooth muscle cells in the

296   cryo-injured rat bladder and prevent compensatory hypertrophy of surviving

297   smooth muscle cells. J Urol 2007; 177: 369-76.

298 [4] Imamura T, Kinebuchi Y, Ishizuka O, Seki S, Igawa Y, Nishizawa O. Implanted

299   mouse bone marrow-derived cells reconstruct layered smooth muscle structures

300   in injured urinary bladders. Cell Transplantation 2008; 17: 267-78.

301 [5] Jack GS, Almeida FG, Zhang R, Alfonso ZC, Zuk PA. Rodriguez LV. Processed

302   lipoaspirate cells for tissue engineering of the lower urinary tract: implications

303   for the treatment of stress urinary incontinence and bladder reconstruction. J

304   Urol 2005; 174: 2041-5.

305 [6] Tsia MS, Lee JL, Chang YJ, Hwang SM. Isolation of human multipotent

306   mesenchymal stem cells from second-trimester amniotic fluid using a novel

307   two-stage culture protocol. Hum Reprod 2004; 19: 1450-6.

308   [7] Wei JP, Nawata M, Wakitani S, et al. Human amniotic mesenchymal cells

309   differentiate into chondrocytes. Cloning Stem Cells 2009; 11: 19-26.

310   [8] Wei JP, ZhangTS, Kawa S et al. Human amnion-isolated cells normalize blood

311   glucose in streptozoticin-induced diabetic mice. Cell Transplant 2003; 12:

312   545-52.

313   [9] Akle CA, Adinolfi M, Welsh KI, Leibowitz S, McColl I. Immunogenicity of

314   human amniotic epithelial cells after transplantation into volunteers. Lancet

315   1981; 2: 1003-5.

316   [10]Prusa AR, Marton E, Rosner M, ~~Nernaschek~~ Bernaschek G, Hengstschlager M.

317   Oct-4-expressing cells in human amniotic fluid: a new source for stem cell

318   research? Hum. Reprod 2003; 18: 1489-93.

319   [11]Miki T, Strom SC. Amnion-derived pluripotent/multipotent stem cells. Stem Cell

320   Rev. 2006; 2: 133-42.

321   [12]Toda A, Okabe M, Yoshida T, Nikaido T. The potential of amniotic

322   membrane/amnion-derived cells for regeneration of various tissues. J Pharmacol

323   Sci. 2007; 105:215-28.

324   [13]Parolini O, Soncini M, Evangelista M, Schmidt D. Amniotic membrane and

325   amniotic fluid-derived cells: potential tools for regenerative medicine? Regen

326   Med 2009; 4: 275-91.

327   [14]Rooney IA, Morgan BP. Characterization of the membrane attack complex

328   inhibitory protein CD59 antigen on human amniotic cells and in amniotic fluid.

329   Immunology 1992; 76: 541-7.

330   [15]Rebmann V, Pfeiffer K, Passler M, et al. Detection of soluble HLA-G molecules

331   in plasma and amiotic fluid. Tissue Antigens 1999; 53: 14-22.

332   [16]Pan HC, Yang ~~DU~~ DY, Chiu YT, et al. Enhanced regeneration in injured sciatic

333   nerve by human amniotic mesenchymal stem cell. J Clin Neuroscience 2006; 13:

334   570-575.

335   [17]Fujimoto KL, Miki T, Liu LJ, et al. Naïve rat amnion-derived cell transplantation

336   improved left ventricular function and reduced myocardial scar of post infarcted

337   heart. Cell Transplant 2009. In press.

338   [18]Yu SJ, Soncini M, Kaneko Y, Hess DC, Parolini O, Borlongan CV. Amnion: a

339   potent graft source for cell therapy in stroke. Cell Transplant 2009; 18: 111-8.

340   [19]Imamura T, Yamamoto T, Ishizuka O, Gotoh M, Nishizawa O. The

341   microenvironment of freeze-injured mouse urinary bladders enables successful

342   tissue engineering. Tissue Engin 2009. In press.

343

Figure Legends

Figure 1. Cultured cells and freeze-injured bladders. (A) After 7 days of culture, the attached HAMCs, which were isolated from a human amnion, were spindle-shaped and achieved confluence. x100. (B) The cultured HAMCs were positive for HuNu (arrow); however, they were negative for SMA. x640. Comparison of longitudinal sections through (C) a normal bladder and (D) one 3 days after freeze injury, just prior to implantation, showed that the posterior wall (C, D arrowheads) of the injured bladder had a hematoma, with edema and inflammation spreading to the anterior side. H&E, x50.

Figure 2. The histopathological course of recovery for freeze-injured bladders. Longitudinal sections of the bladders at 1, 2, 4 and 6 weeks after the cell-implantation or the control-injection showed the injury site on the posterior sides (arrowheads). (A) At 1 week after the HAMC-derived cell implantation, the hematoma and inflammation were decreased, but a small degree of edema remained. At 2 (B), 4 (C) and 6 (D) weeks after the cell implantation, the hematoma, edema, and inflammation disappeared. (E) On the other hand, at 1 week after the control injection, hematoma, edema, and inflammation of the bladders remained. At 2, 4, and 6 weeks (F – G respectively) after the control injection, the bladders showed spontaneous recovery. H&E, x50.

Figure 3. The immunohistochemical course of recovery for freeze injured bladders.

365 Representative cell-implanted (A - D) or control-injected (E – H) bladders are shown in

366 merged images of HuNu (red) and SMA (green). (A) At 1 week after cell implantation,

367 a continuous layer composed of SMA-positive cells was formed. At 2 (B), 4 (C), and 6

368 (D) weeks after the cell-implantation, the formed layer of smooth muscle structures

369 were similar to those in normal bladders. In addition, cells positive for HuNu were

370 present in the injury sites of the cell-implanted bladders at each period. (E - G) On the

371 other hand, at 1, 2, and 4 weeks after the control injection, the muscular layer of the

372 posterior bladder was still disrupted and had few SMA-positive cells. (H) At 6 weeks

373 after the control injection, the bladders showed spontaneous recovery. No

374 HuNu-positive cells were in the walls of control-injected bladders. x640.

375

376 **Figure 4.** Differentiation of HAMC-derived cells into smooth muscle cells. (A) At 1

377 week after the HAMC-derived cell implantation, SMA-positive cells were located in the

378 muscular layer of the posterior side of the bladder, and many of these cells were positive

379 for HuNu (arrows). This suggested that the implanted HAMC-derived cells

380 differentiated into SMA-positive smooth muscle cells. (B) At 2 weeks, these cells still

381 had a rounded shape (arrows). (C, D) At 4 and 6 weeks after the cell-implantation, these

382 cells were spindle shaped and formed layered structures (arrows) similar to those in

383 normal bladders. x640.

384

385 **Figure 5.** Contractile responses of muscle strip preparations to high K^+-Krebs solution.

386   (A) At one week after cell-implantation into the injured posterior bladder wall,

387   contractions in response to the high K^+-Krebs solution were reduced significantly in

388   both the cell-implanted and the control-injected bladder strips compared to the

389   uninjured normal bladder strips. However, the contractile responses of the

390   cell-implanted bladder strips were significantly greater than those of the control injected

391   bladder strips.   The functional damages after the freeze-injury were spontaneously

392   recovered at 6 weeks. (B) At one week after cell-implantation into the injured posterior

393   bladder wall, contractions of the anterior bladder wall strips in response to the high

394   K^+-Krebs solution were not significantly different from the uninjured anterior bladder

395   strips. However contractions of the anterior strips from the control-injected bladders

396   were significantly reduced compared to both the anterior uninjured bladder strips and

397   the cell-implanted bladder strips. N/g = Newton/grams; *, $p < 0.05$.

398

399   Figure 6. Maximum contractile response to carbachol. The maximum contractile

400   response to the muscarinic cholinergic agonist carbachol was determined as a percent of

401   the maximum contraction to the response induced by the high-K solution. (A) There

402   were no significant changes in the response to carbachol for either the cell-implanted

403   bladder strips or the control-injected bladder strips during the 6-week study period.

404   Further, there were no differences between the two groups. (B) Similar results were

405   obtained for the contractile response of the anterior bladder strips.

406

407   **Figure 7.** Concentration-dependence of contractile responses to carbachol. (A) There

408   were no significant differences among the pD2 values for the posterior wounded sides

409   when compared with normal bladder muscle strips or between groups at each week. (B)

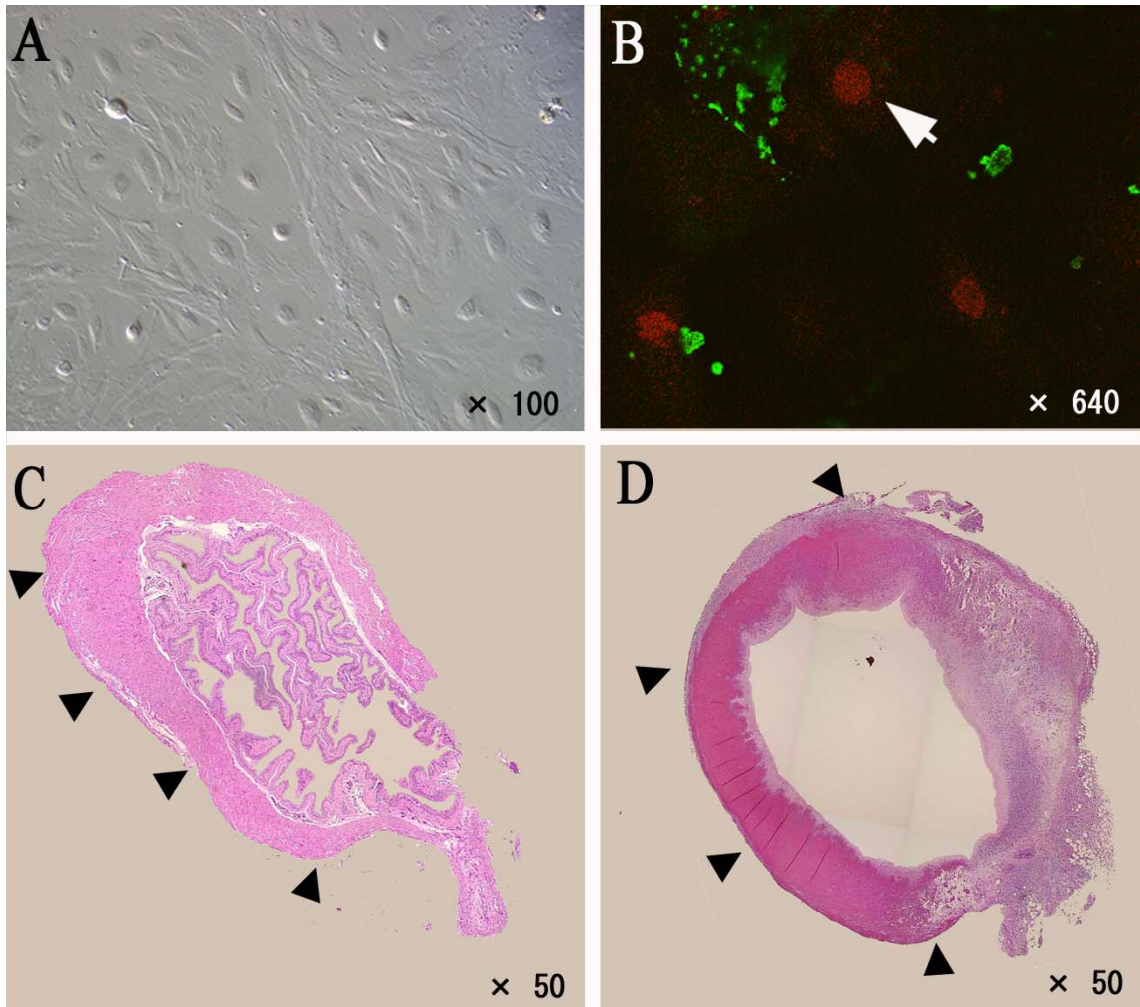410   Similarly, no differences in pD2 values were present for the anterior bladder muscle
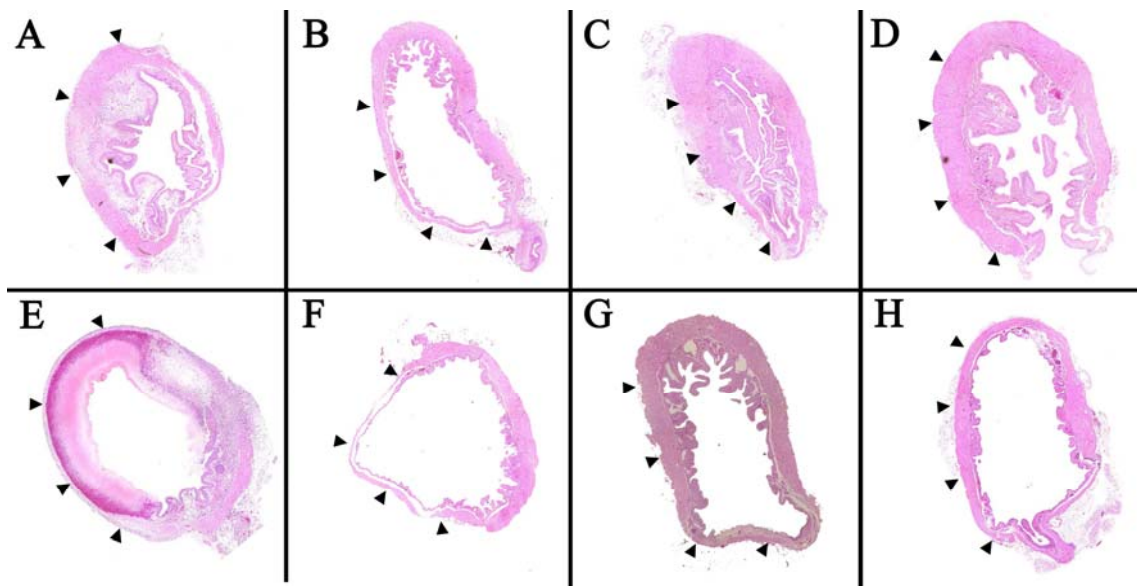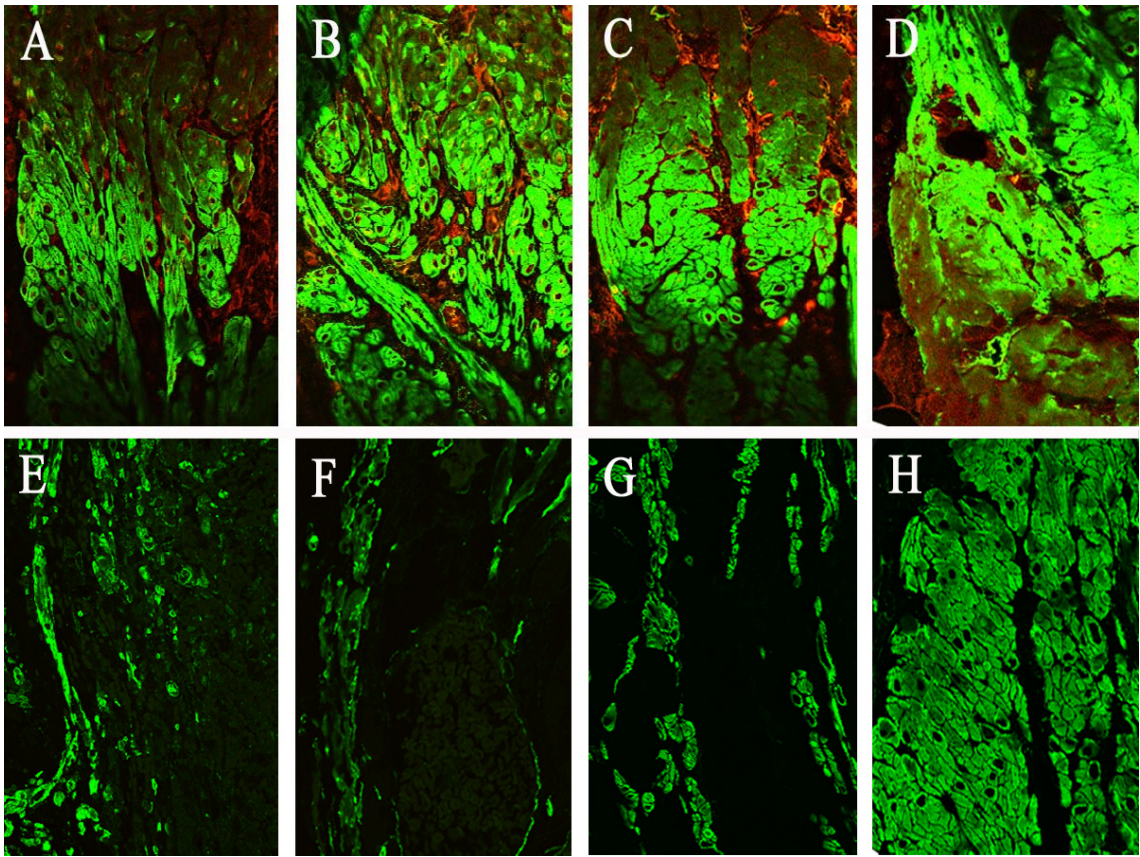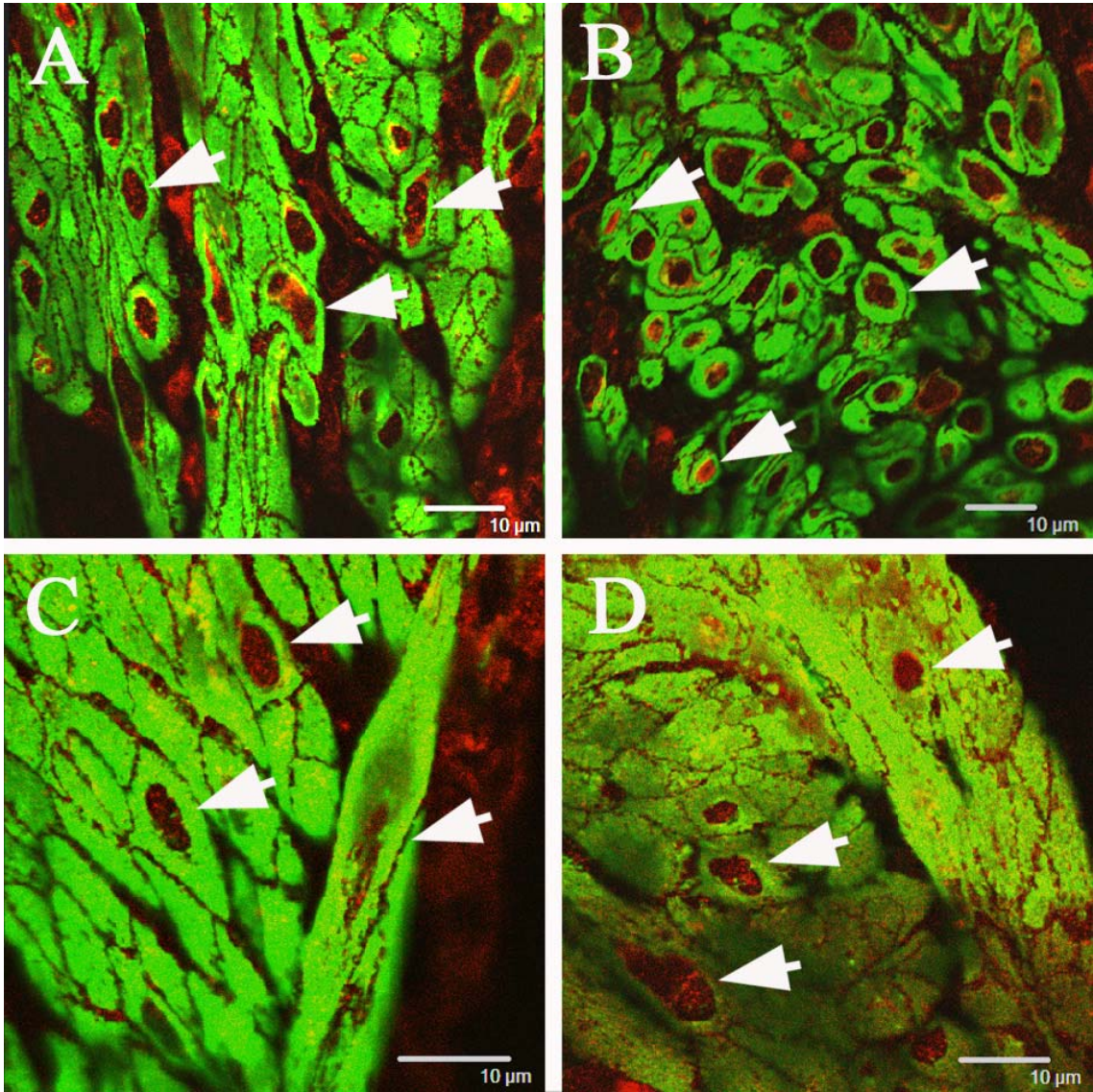
411   strips.

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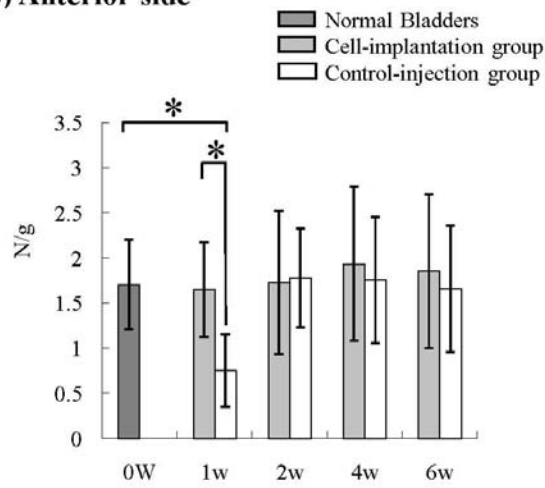
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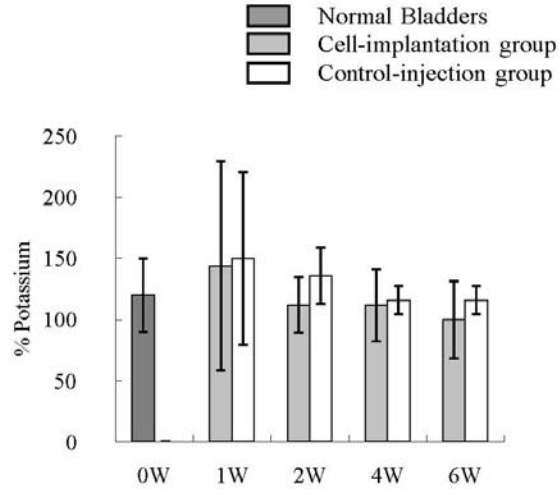
Figure 2

Figure 3

Figure 4
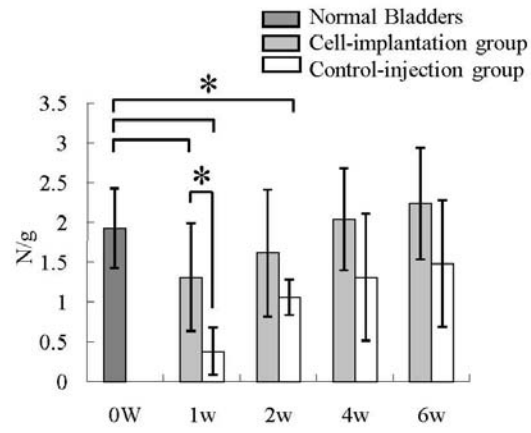
(A) Posterior side

(B) Anterior side
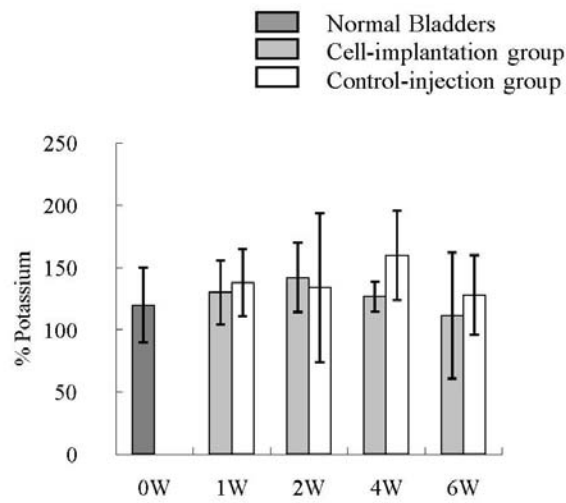
Figure 5

Figure 6

(A) Posterior side

Normal Bladders
Cell-implantation group
Control-injection group

(B) Anterior side

Normal Bladders
Cell-implantation group
Control-injection group

Figure 7