Treatment of perianal fistulas with human embryonic stem cell-derived MSCs: a canine model of human fistulizing Crohn’s disease

Aim: To evaluate the safety and efficacy of intralesional injection of human embryonic stem cell (hESC)-derived mesenchymal stem/stromal cells (MSCs) in canine anal furunculosis dogs. Materials & methods: Dogs naturally develop an immune-mediated disease called canine anal furunculosis, which shares many features with human fistulizing Crohn’s disease. Results: The hESC-MSCs were well tolerated and 1-month postinjection, accompanied by reduced serum levels of IL-2 and IL-6, two inflammatory cytokines associated with Crohn’s disease. All six dogs were found to be completely free of fistulas at 3 months postinjection. However, at 6 months, two dogs had some fistula relapse. Conclusion: Results of this study provide the first evidence of the safety and therapeutic potential of hESC-MSCs in a large animal model.

Keywords: canine perianal furunculosis • Crohn’s disease • human embryonic stem cells • mesenchymal stem cells • perianal fistulas • pluripotent stem cells

Crohn’s disease (CD) is a chronic inflammatory bowel disorder that affects an estimated 1 to 1.3 million Americans [1]. A combination of environmental and genetic factors is thought to lead to an autoimmune attack against cells of the GI tract or associated microbial antigens [2]. Affected regions of the GI tract vary from patient to patient and ulceration with cutaneous or rectocutaneous fistulae within the perianal tissues (perianal fistulas) is present in up to a third of CD patients [3]. The most widely used treatments for CD-associated perianal fistulas include antibiotics and immunosuppressants such as azathioprine and cyclosporine, yet long-term use comes with serious adverse effects or potential reemergence of fistulas if the dose of such drugs is lowered [3]. Anti-TNFα therapies have also been used to heal perianal fistulas yet many patients are either nonresponsive or experience relapse [4], highlighting the need for alternative therapies to help manage this difficult disease feature. Having an animal model in which the clinical symptoms and underlying pathogenesis parallel those of the human disease would be very helpful to explore and screen new treatment approaches for this severe condition.

Canine anal furunculosis (CAF) is a chronic, progressive inflammatory disease of dogs characterized by the development of perianal fistulas [5]. It shares several features with fistulizing CD in humans in terms of the clinical signs, histopathology [6], cytokine profile of the lesions [7–9] as well as the clinical response to cyclosporine therapy [10,11]. Therefore, it has been considered a clinically relevant model for fistulizing CD [12,13], although it is unknown whether lesions are patent enterocutaneous fistulas in dogs as they may be in humans. CAF is diagnosed most commonly in middle-aged German shepherd dogs, with this breed accounting for over 80% of diagnosed cases [14]. Similar to humans, a genetic background predisposing to the development of the disease has been identified [13,15], but the etiopathogenesis is only partially understood. A specific causative pathogen has not been identified for CAF and it has been proposed that the inflammation is due to inappropriate immune responses to commensal organisms...
CD, some of them with encouraging results. The professional injection of MSCs as a treatment for fistulizing CD, specifically anal fistulas as a model for fistulizing CD, specifically to evaluate the usefulness of spontaneous canine peri-anal fistulas. Here, we performed an exploratory study to test the MSCs more closely resembles that tested in larger animals whose physiology (and sophistication) more closely resembles that of humans. Having a realistic animal model for the evaluation of new treatments for fistulizing CD would help to assess the safety and efficacy of potential stem cell therapies and also to explore many other aspects of this approach including mechanism of action, most effective dose, frequency of injection and potential side-effects.

Small numbers of MSCs can be isolated from primary tissue such as bone marrow, adipose tissue and umbilical cord and expanded to achieve clinically useful quantities. However, the therapeutic potency of MSCs can vary from donor to donor and be negatively impacted during the in vitro expansion process. Recently, we described the derivation of a mesenchymal stem cell population from hESC-derived hembangioblasts (HBs) [30]. These cells have similar immunomodulatory properties to adult tissue-derived MSCs, but unlike adult MSCs, hESC-MSCs can be generated in large-scale quantities without loss of therapeutic potency, making them an attractive cell-based therapy for commercial development. hESCs are a self-renewing source of starting material, capable of generating a virtually unlimited supply of minimally expanded hESC-MSCs, offering a distinct advantage to reduce culture-associated senescence. hESC-derived MSCs have well-documented immunomodulatory and therapeutic properties in rodent models of autoimmune disease [30,31]. However, to date, they have not been tested in larger animals whose physiology (and sophisticated immune system) more closely resembles that of humans. Here, we performed an exploratory study to evaluate the usefulness of spontaneous canine peri-anal fistulas as a model for fistulizing CD, specifically for the evaluation of therapeutic potential and safety of an intralesional injection of stem cells of different sources and characteristics. In this study, we hypothesized that hESC-MSCs would significantly reduce the frequency of fistulas and the dosage of cyclosporine A required to sustain remission (healing and closure) in affected dogs.

**Materials & methods**

**Study design & dogs**

This was an open-label study in the treatment of CAF with intraleisional injections of hESC-MSCs into each fistula at one time point. Six canine patients from the Cummings Veterinary Medical Center at Tufts University were enrolled in the study. The inclusion criteria were adult dogs (either gender, any breed) with a clinical diagnosis of anal fistulas and presence of perianal sinus and fistulous tracts and/or ulcers. Inclusion also required that fistulas failed to completely close or relapsed periodically while the dogs were administered standard dosages of cyclosporine. Eligible patients also displayed clinical signs of tenesmus and dyschezia. Dogs younger than 1 year or older than 10 years, dogs with other diseases or treatments apart from anal furunculosis and dogs that have had surgery (cryosurgery, anal sac resection, tail amputation) to treat the anal fistulas were excluded.

The signalment of the six dogs can be seen in Table 1. All dogs were partially or completely refractory to standard of care which included oral administration of cyclosporine A (doses ranging from 4.8 to 15.38 mg/kg/q 24 h) for at least 6 months prior enrolment. Before entering the study, all dogs had a full physical examination and the number and size of perianal fistulas and ulcers were recorded. The dogs were maintained on the pretreatment dose of cyclosporine for at least 30 days after hESC-MSC cell injections. The end points of the study included the number and depth of the fistulas at 6 months after treatment, and the average daily dose of cyclosporine necessary to maintain the dogs without lesions. The study was approved by Clinical Sciences Research Committee of the Cummings School of Veterinary Medicine and all owners received detailed information about the treatment and signed a written consent form.

**Generation of hESC-MSCs**

hESC-MSCs were generated from MA09 hESCs, a US FDA-approved hESC line established using single blastomere technology [32]. hESC-MSCs were generated through the differentiation of hESCs first into embryoid bodies, then into HBs and subsequently into hESC-MSCs, as previously described [30]. A sample of HBs from a cryopreserved bank were thawed and
subjected to mycoplasma, sterility and human virus testing. Upon successfully passing these tests, a single bank of HB intermediates was used to manufacture bulk hESC-MSCs in an ISO 7 (class 10,000) controlled Cell Processing Cleanroom. Full gowning, raw material specifications and batch record documentation procedures were followed from the HB intermediate to the final, formulated hESC-MSC product in the spirit of GMP. For each patient, bulk hESC-MSC product was thawed and plated at 4000 cells/cm² for 4 days prior to harvest, washing and final formulation in a glucose-saline solution supplemented with 1% autologous serum. Formulated hESC-MSC product, termed HMC™ (Ocata, MA, USA) for hemangio-derived mesenchymal cells was preloaded into syringes, delivered in a temperature-monitored 4°C cooler to the Cummings Veterinary Medical Center, and administered within 4 h of formulation to ensure minimal loss in viability.

hESC-MSC quality control & characterization
As part of hESC-MSC quality control, bulk hESC-MSC product was tested and passed GMP sterility and mycoplasma testing (WuXiApptech), as well as karyotype with FISH analysis (Molecular Diagnostic Services, CA, USA). For qRT-PCR, mRNA was harvested by using a RNAeasy kit and cDNA synthesis was performed with Superscript III First-strand Synthesis SuperMix (both from Life Technologies). Taqman-based qRT-PCR was performed in triplicate with SsoAdvanced Mastermix on a Biorad CFX96 using a 40 cycle reaction. GAPDH was used as an internal control, and normalized mRNA expression was calculated based on the ΔΔCt method with CFX Manager 3.0 software (Biorad). For samples in which there was no detectable gene expression (i.e., 1–3 hESC-MSC replicates for Nanog, Oct4, Sox2), a Ct value of 39 was used to allow graphical representation. Gene expression in hESCs was set at 1 and expression in hESC-MSCs as relative to that. Flow cytometry staining was performed by using standard methods and samples were run on an Accuri C6 cytometer with CFlow software. Shown in Figure 1, antibodies for Tra-1–60 and Tra-1–81 were from Biolegend and BDBiosciences, respectively. In addition, flow cytometry was used to confirm that bulk hESC-MSCs contained >95% expression of CD73, CD90, CD105, CD13, CD29, CD54, CD44 and CD166 and <5% non-MSC markers CD31, CD34 and CD45 (data not shown). Additional release assays include specifications for population doubling time and cell density at harvest [30; and data not shown].

As part of establishing the safety of hESC-MSC treatment, hESC-MSCs generated in the GMP facility were evaluated for tumorigenicity potential. A single dose of two million hESC-MSCs per mouse was IV. injected into 8-week-old NOD/SCID mice and a subset was euthanized at either 3 months (n = 12) or 6 months (n = 12) postinjection for histopathologic evaluation (BioMedical Research Models, MA, USA). No evidence of masses or teratomas were found at either time point.

Mixed lymphocyte reaction assay: canine PBMCs & human hESC-MSCs
Peripheral blood was drawn from three experimental dogs at the Cummings Veterinary Medical Center. PBMCs were isolated using Accuspin tubes containing Histopaque 1077 according to manufacturer’s instructions. Nonplastic adherent PBMCs were labeled with carboxyfluorescein succinimidyl ester (CFSE) according to manufacturer’s instructions. hESC-MSCs, plated at varying concentrations in 24 well plates, were...
Figure 1: Human embryonic stem cell-derived mesenchymal stem/stromal cell quality control and activity toward canine immune cells. Normal karyotype of bulk hESC-MSC product (A). qRT-PCR analysis of a panel of genes associated with either pluripotency or mesenchymal cells. Normalized expression values were set to 1 for hESCs and the level in hESC-MSCs graphed as relative to hESC. Bars represent triplicate reactions, error bars are standard error (B). Flow cytometry for surface markers associated with pluripotency. Black lined samples are unstained, red lined samples are stained for Tra-1–60 or Tra-1–81, as indicated. Gated regions (blue line) show the presence of positively staining cells in hESC samples and their absence in hESC-MSC samples (C). Dose-dependent inhibition of canine T-cell proliferation by human hESC-MSCs. Mitotically inactivated hESC-MSCs were cocultured with carboxyfluorescein succinimidyl ester-labeled canine PBMC at the indicated ratios and stimulated with 5 μg/ml PHA on day 3. On day 6, cultures were subjected to flow cytometry staining for canine CD4, CD8 and analyzed for a concomitant reduction in carboxyfluorescein succinimidyl ester signal, indicative of cellular proliferation. Data points represent the average of three biological replicates, error bars indicate standard deviation (D).

hESC-MSC: Human embryonic stem cell-derived mesenchymal stem/stromal cell; PBMC: Peripheral blood mononuclear cell.
Treatment of perianal fistulas with human embryonic stem cell-derived MSCs

Intrallesional hESC-MSC injections
Under sedation with dexmedetomidine (5 μg/kg/IV), all six patients received a single injection of 2 × 10^7 human embryonic stem cells in 10 ml volume of sterile glucose-saline solution distributed equally between lesions. The cells were injected using a 21-g needle into the lumen, and within the dermis and subcutaneous tissue around the perianal fistulas. After the injection, all fistula openings were coated with a fibrin glue (Evicel®, Ethicon), according to manufacture guidelines to prevent spillage of treatments. Sedation was reversed with atipamezole and the dogs remained in the hospital on observation for 4–6 h, to detect eventual immediate side-effects before discharging to owners to take home.

Follow-up of patients
At days 0 (injection), 7, 30, 60, 90 and 180, the dogs were thoroughly examined and the number, extension and depth of the fistulas recorded. Complete blood count and serum chemistry analysis were performed at each time point. The dose of cyclosporine was adjusted at days 30, 60 and 90. In dogs showing improvement in fistulas numbers or depth, the dose of cyclosporine was reduced, to reach the lowest dose that was effective to maintain the disease under remission.

Serum cytokine analysis
Preinjection (day 0) and day 30 post-hESC-MSC injection serum was collected for cytokine analysis from dogs 1, 3, 5 and 6. Serum samples and standard curve cocktails were incubated on a quantibody array according to manufacturer’s instructions. Fluorescence laser scanning and data extraction service were provided by RayBiotech. Background was subtracted from densitometry values and plotted against a standard curve to determine the actual concentration of each analyte for each dog using GraphPad Prism software. To account for variability in day 0 (preinjection) levels among the 4 dogs, day 0 levels are set at 100% and each dog’s corresponding day 30 level is expressed as a percentage of its day 0 level.

Statistical analysis
The comparison of the doses of cyclosporine before and 3 and 6 months after the stem cell injection was done using the paired t-test. Analysis of the percent change in serum cytokine concentrations was done using an unpaired Student’s t-test.

Results
hESC-MSC quality control & immunomodulatory capacity of hESC-MSCs in a large animal, discordant xenograft system
As part of quality control and the establishment of release criteria, hESC-MSCs were subjected to and passed mycoplasma and sterility testing and were confirmed to have a normal karyotype (Figure 1A). qRT-PCR analysis showed that there was a profound down-regulation of pluripotency-associated genes (hTERT, Nanog, Oct4, Sox2) with a concomitant upregulation of MSC-associated genes (CD73, CD105, Angl, HGF, VEGF) in bulk hESC-MSC (Figure 1B). While not tested here, further upregulation of MSC-expressing genes can be found upon environmental stimulation, as previously described [30]. In addition, flow cytometry was used to confirm the absence of pluripotency cell surface markers, Tra-1–60 and Tra-1–81 (Figure 1C), as well as >95% expression for a panel of eight different MSC markers and <5% expression of hematopoietic/endothelial cell markers (data not shown).

Our previous data have demonstrated that human hESC-MSCs can specifically modulate the function of mouse immune cells and in particular, inhibit the proliferation of activated mouse T cells [31]. Given that canines are a higher level organism with a more sophisticated immune system, we wanted to determine if our human hESC-MSCs can similarly inhibit canine T-cell proliferation. To do so, we performed a mixed leukocyte reaction assay with CFSE-labeled canine PBMCs and used phytohemagglutinin (PHA) to stimulate their proliferation in the presence of hESC-MSCs. hESC-MSCs were found to inhibit the PHA-induced proliferation of canine CD4+ and CD8+ T cells in a dose-dependent manner (Figure 1D). This confirmed that despite being a discordant xenograft system, human hESC-MSCs retain their ability to modulate canine T-cell function.
Clinical presentation of dogs & hESC-MSC intralesional injection
At the time of injection, all six dogs presented active perianal ulcers or fistulas (Table 1). Three dogs (2, 4 and 6) presented with a single ulcer-fistula and one dog presented with two (dog 5), five (dog 1) and six ulcers or fistulas (dog 3) each (Figures 2 & 3). All six dogs were on treatment with cyclosporine (doses ranging from 4.8 to 15.38 mg/kg/q24 h; X = 8.22 mg/kg/q24 h). The dogs were sedated and injected uneventfully. No side-effects were observed after injection, except in one animal (dog 4), which developed a mild perianal erythema that resolved in 24 h. At 1 week after injection, the lesions looked very similar and no side-effects of the injections were detected. In one dog (case 6) at 1 week, the fistula had improved markedly and was almost closed (Figure 3).

Alterations in serum cytokine levels 1-month post-hESC-MSC injection
Inflammatory cytokines are thought to be involved in the disease process underlying development of perianal fistulas [33]. To determine if hESC-MSC injection affects circulating cytokine levels, we performed a quantitative cytokine antibody array using available serum from dogs 1, 3, 5 and 6 at day 0 (preinjection) and day 30 of treatment. This allowed us to examine cytokine changes during a time period when the level of cyclosporine did not change. Ten different cytokines were analyzed: IL-2, -6, -8, -10, GM-CSF, MCP-1, RAGE, SCF, TNFα, and VEGF. The day 0 serum levels of IL-2 varied among the four dogs, with levels being >1000, 39, 39 and 102 pg/ml for dogs 1, 3, 5 and 6, respectively. Similar to IL-2, there was a significant decrease in the percentage of IL-6 by day 30 post-hESC-MSC injection. IL-6 was present at only 1, 22, 23 and 54% of day 0 levels for dogs 1, 3, 5 and 6, respectively (Figure 4, second set of bars). There was no consistent or significant pattern of change for any of the eight other cytokines analyzed (data not shown).

hESC-MSC-injected dogs show signs of clinical improvement during 6 month follow-up
One month after hESC-MSC injection, all dogs demonstrated improvement and the fistulas completely resolved in three dogs. A tapering of the cyclosporine dose after 30 days was started on all dogs, except dog number 2, which suffered from atopic dermatitis requiring that cyclosporine continue for controlling the severe clinical signs of the allergy. At 3 months after injection, none of the dogs showed open ulcers or fistulas (Table 2). The mean dose of cyclosporine, that had been adjusted 1 and 2 months after treatment, was 3.59 mg/kg/q24 h at 3 months postinjection, significantly lower than the dose at day 0 (8.22 mg/kg/q24 h) (p = 0.036) (Table 2). At 6 months postinjection, two of the dog had developed small fistulas (dogs 1 and 4) (Table 1). Three dogs were still on the same cyclosporine dose (dogs 2, 5 and 6), two were on a lower dose (dogs 3 and 4) and one was on a higher dose, as prescribed by its family veterinarian to control the development of small fistulas that redeveloped 5 months after the stem cell injection (dog 1). Nevertheless, the mean dose of cyclosporine 6 months after the stem cell injection (4.22 mg/kg/q24 h) was lower (p = 0.0512) than the initial dose. None of the dogs showed adverse changes in hematological or serum chemistry end points.

Discussion
This is the first report of treatment of fistula activity using a pluripotent stem cell-derived therapy in a large animal model. Although this was designed as an open-label trial, and therefore no comparison was made with a placebo controlled group to evaluate efficacy, the results are striking and imply that treatment with hESC-MSCs has therapeutic potential. As an off-the-shelf xenogeneic treatment, the hESC-MSCs were also found to be safe as a single injection. No serious local

![Figure 2. Dog 3. This patient presented six small ulcers-fistulas, most surrounded by severe scarring and hyperpigmentation (A). One month after the stem, the ulcers have disappeared and the scarring markedly reduced (B).](image-url)
or systemic safety issues related to the transplanted cells were observed. The only 'adverse event' was noted in dog #4 which showed mild, transient perianal erythema after the injection but this could also be a consequence of the trauma promoted by the shaving and scrubbing performed in preparation for the hESC-MSC injections. Furthermore, no changes in the CBC and serum chemistry were identified in any of the dogs.

Spontaneous healing in CAF is an extremely uncommon event and lifelong therapy is usually required [33]. In the long term, CAF presents as a chronic progressive inflammatory condition increasing in severity and showing periodic flares [33]. The observation that fistulas completely healed with hESC-MSC injection in dogs which were previously refractory to standard of care, including cyclosporine, is a strong indication that the hESC-MSCs initiated the healing process either through immune modulation or tissue repair or both. Three months after the injection, all dogs were free of lesions and the dose of cyclosporine was more than halved (from 8.22 mg/kg/q24 h pretreatment to 3.59 mg/kg/q24 h at 3 months). The observation that at 6 months postinjection, some dogs started to relapse further supports the interpretation that the improvement was evoked by hESC-MSC injection and implies that multiple or periodic injections of MSCs may be required for long-term control of this disease manifestation in some animals. Since hESC-MSCs were transplanted xenogeneically, repeated injections were deferred to avoid the risk of sensitization, which could impose safety risks on the patients and confound interpretation of the results. In reports of treatment of fistulizing CD with autologous, bone marrow-derived MSCs, it is evident that some patients require a second or a third MSC injection to resolve the lesions [19].

The physiopathology of the fistula formation in the dog is not clearly understood, but likely has some differences with the process for fistulizing CD in humans. Based on histologic evaluation, early CAF lesions show an inflammatory reaction associated with epidermal appendages without concomitant epidermal ulceration. As the inflammatory reaction intensifies, folliculitis/furunculosis and nonarborizing sinus tracts develop in the perianal dermis. Perifollicular, superficial epidermal ulceration and arborizing dissecting cellulitis soon follow throughout the perianal tissue. The sinus tracts are typically lined by squamous epithelium and are infiltrated with a mixture of lymphocytes, plasma cells, macrophages, neutrophils and eosinophils. As perianal lesions progress, peripheral lymphoid nodules develop, along with extensive granulating fibrosis [34]. In the physical examination, dogs present ulcers of different sizes, sinus tracts and true fistulous tracts [33]. In our trial, hESC-MSCs were injected into the sinus and fistulous tracts, as deep as possible and also in the tissue around the lesions. We believe that most of the effects were local, on the perianal tissue. It is also possible that local injection of hESC-MSCs can be used in combination with systemic treatment to improve healing in fistulizing CD.

Figure 3. Dog 6. This patient presented an open fistula right and ventral to the anus (A). One week after the stem cell injection, the fistula was almost completely closed (B).

Figure 4: Serum levels of IL-2 and IL-6 are reduced following human embryonic stem cell-derived mesenchymal stem/stromal cell injection. d0 and d30 serum samples were available from dogs 1, 3, 5 and 6 and processed on a ten-analyte canine cytokine antibody array. A standard cytokine cocktail was run on the same array and used to generate standard curves for each analyte. The concentration (pg/ml) of each analyte was determined based on its respective standard curve. To account for variability in preinjection cytokine levels among the four dogs, the d0 level for each dog is set at 100% (dotted line on graph) and the corresponding d30 level for each dog is expressed as a percentage of its own d0 level. For dog 1, the level of IL-2 was below the limit of detection (5 pg/ml) at both d0 and at d30, therefore it was excluded from IL-2 analysis. Unpaired Student’s t-test was used to determine significance for d30 vs d0 levels for IL-2 and IL-6, p-values are indicated. Other analytes did not show a significant trend or change among the four dogs (data not shown).

d0: Day 0; d30: Day 30.
that the observed improvement was the consequence of the natural time course and biology of the disease or of the application of the fibrin glue at the skin surface. The effect of fibrin at the surface is not likely to have evoked the substantial improvements observed in this study. However, a randomized, double-blind, controlled study is required to study these effects.

Although the etiology of canine perianal fistulas is poorly understood, there is evidence for an underlying immune dysfunction. This is illustrated by the presence of a T helper type 1 cytokine mRNA profile in CAF lesions and the clinical response to cyclosporine therapy [10] demonstrated increased levels of expression of IL-2 and IFNγ mRNA in CAF lesions compared with control tissue. Expression of these cytokines in the diseased tissue indicates the presence of activated T cells and suggests that the pathogenesis of CAF is associated with T-cell-mediated inflammation. The trigger of this T-cell response remains to be identified but some investigators have suggested that components of the skin or fecal flora are responsible for driving the T-cell response in affected tissues [17,33]. In this study, we confirmed that human hESC-MSCs can inhibit the proliferation of canine CD4+ and CD8+ T cells at least in vitro. Moreover, 1 month after injection, serum levels of the inflammatory cytokines, IL-2 and IL-6 were significantly reduced. IL-2 is a key cytokine driving T cell activation/proliferation, therefore a reduction in its circulating levels may be beneficial for reducing the proliferation of autoreactive T cells. This is thought to be a major mechanism for the therapeutic activity of cyclosporine A in CAF, which can lower IL-2 through inhibition of its transcription [33]. IL-6 is a pleiotrophic cytokine whose serum levels have been found to correlate with CD-associated inflammation as well as the presence of perianal fistulas in humans [35,36]. Treatment of CD patients with anti-IL-6 receptor antibodies showed that blocking IL-6 signaling led to clinical improvement [37]. Given these observations, the reduction in IL-6 levels observed here after hESC-MSC injection is intriguing. Moreover, it is consistent with our data showing reductions in IL-6 levels in rodent models for two other autoimmune disorders: multiple sclerosis and lupus [31].

These results can help to develop more effective strategies to treat perianal fistulas in humans and dogs. At this point, it will be important to run double blind controlled clinical trials, but in dogs, our data imply that a therapeutic approach combining standard of care (cyclosporine) and intralesional injections of hESC-MSC could be more effective, and reduce costs and side-effects. The combination of systemic chemotherapy with local injection of stem cell-based therapies has also been advocated as one of the most effective treatments for fistulizing CD in humans [38].

The treatment of CAF could be a useful model for advancing the understanding of cell-based therapies in human diseases. Although several studies have demonstrated the efficacy and safety of various primary tissue-derived stem cells for treating fistulizing CD,

<table>
<thead>
<tr>
<th>Dog</th>
<th>Dose of cyclosporine before SC injection</th>
<th>Dose of cyclosporine 3 months after SC injection</th>
<th>Dose of cyclosporine 6 months after SC injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>One Australian terrier, FS, 14 years</td>
<td>15.38</td>
<td>7.69</td>
<td>15.38</td>
</tr>
<tr>
<td>Two GSDs, MN, 9 years</td>
<td>4.8</td>
<td>4.8</td>
<td>4.8</td>
</tr>
<tr>
<td>Three GSDs, M, 3 years</td>
<td>10.25</td>
<td>2.93</td>
<td>0</td>
</tr>
<tr>
<td>Four GSDs, MN, 10 years</td>
<td>5.88</td>
<td>2.94</td>
<td>1.96</td>
</tr>
<tr>
<td>Five Australian shepherds, MN, 9 years</td>
<td>7.89</td>
<td>2.25</td>
<td>2.25</td>
</tr>
<tr>
<td>Six GSDs, MN, 11 years</td>
<td>5.17</td>
<td>0.98</td>
<td>0.98</td>
</tr>
<tr>
<td>X</td>
<td>X = 8.22</td>
<td>X = 3.59</td>
<td>X = 4.22</td>
</tr>
</tbody>
</table>

FS: Female spayed; GSD: German shepherd dog; M: Male; MN: Male neutered; SC: Stem cell; X: Average cyclosporine doses before the SC injection, and then 3 and 6 months afterwards.
the local injection of adipose or hematopoietic stem cells has been criticized because their mechanisms of action are incompletely understood [38] and the donor cells can vary significantly in quality, especially from autologous sources. This canine model can help to investigate the cellular and molecular mechanisms of stem cell-derived therapies and also evaluate the route of administration, dose, frequency and safety of administration. At the same time, the use of hESC-derived MSCs can avoid issues with variable quality and represents a consistent, replenishable and scalable alternative to primary tissue-derived MSCs.

Conclusion
Here, we show that hESC-MSCs have therapeutic value for treating cyclosporine-resistant canine perianal fistulas. Fistulas are a chronic manifestation of canine anal furunculosis, which shares similarities with fistulizing CD in humans. We found that a single intralesional injection of hESC-MSCs was safe and well tolerated and by 1 month postinjection, accompanied by a reduction in serum levels of the proinflammatory cytokines IL-2 and IL-6. Cyclosporine doses were successfully dropped in several of the dogs at 1 month postinjection without adverse effects. All six dogs in the trial had complete fistula closing by 3 months post-treatment. Two of the six dogs experienced some recurrence of fistulas by 6 months, suggesting that more than one injection may be needed in some cases. Nonetheless, the data is encouraging and the first to demonstrate the safety and potential efficacy of hESC-MSCs in a large animal autoimmune-like disease setting.

Future perspective
The future of cell therapy will continue to depend on animal models for evaluation of biologic activity and safety. This study demonstrates that a naturally occurring canine disease in veterinary medicine can serve as an important preclinical model of human disease and provides a way to evaluate the effects of a novel stem cell-based therapy. With respect to fistulizing CD, dogs are the only species that naturally develop similar symptoms, pathology and a subset (similar to humans) as studied herein are also refractory to immunosuppressive therapies. Many other human disease conditions occur naturally in dogs, suggesting that veterinary species will serve as important preclinical models for a variety of novel applications. These data also show that single injection xenotransplantation is an effective strategy to investigate the safety and efficacy of hESC-MSCs in canine patients. Finally, the results of this study add to the encouraging series of results from MSC-based trials for fistulizing CD, suggesting that MSCs have important paracrine signals that modify this disease consistent with their in vitro immunomodulatory effects. In the future, it will be important to better

Executive summary
- Dogs naturally develop an immune-mediated disease called canine anal furunculosis (CAF), which shares many features with fistulizing Crohn’s disease (CD) of humans, including recurrent fistula activity.
- We evaluated the safety and efficacy of intralesional injection of human embryonic stem cell (hESC)-derived mesenchymal stem/stromal cells (MSCs) in CAF dogs refractory to standard treatment, including cyclosporine therapy.
- We also wanted to explore the usefulness of the anal furunculosis in dogs as a model to test new treatments for human with CD.
- Six dogs with a sound diagnosis of perianal fistulas and that were refractory to standard of care were injected intralesionally with $2 \times 10^7$ hESC-MSCs.
- hESC-MSCs were generated through the differentiation of hESCs first into embryoid bodies, then into hemangioblasts and subsequently into hESC-MSCs, as previously described.
- Previous studies have demonstrated that human hESC-MSCs can specifically modulate the function of mouse immune cells and in particular, inhibit the proliferation of activated mouse T cells.
- In a mixed leukocyte reaction assay with canine peripheral blood mononuclear cells, the hESC-MSCs were found also to inhibit the phytohemagglutinin-induced proliferation of canine CD4$^+$ and CD8$^+$ T cells in a dose-dependent manner. This confirmed that despite being a discordant xenograft system, human hESC-MSCs retain their ability to modulate canine T-cell function.
- The hESC-MSCs were well tolerated and by 1 month postinjection, were accompanied by a reduction in serum levels of IL-2 and IL-6, two inflammatory cytokines associated with CD in humans. All six dogs were found to be completely free of fistulas at 3 months postinjection.
- However, at 6 months, two of the dogs had some fistula relapse, indicating that repeated injections may be necessary to sustain the effects of treatment in some patients.
- The results of this study provide the first evidence of the safety and therapeutic potential of hESC-MSCs in a large animal model.
understand the mechanisms by which hESC-MSCs and other MSCs exert effects on perianal fistulas. Are intact cells or paracrine signals (soluble mediators, extracellular vesicles) responsible for this effect? Are interactions with T cells, dendritic cells or other immune effectors key to their biological activity? Do MSCs alter the response to the microbiome? The canine model will be particular useful to explore these possible mechanisms.

Acknowledgements
The authors thank Diane Welsh, Dawn Meola, Deborah Peak, Gregory Yavanian and Nicholas Kouris for technical assistance.

Financial & competing interests disclosure
EA Kimbrel and R Lanza are employees of Ocata Therapeutics (formerly Advanced Cell Technology), a biotechnology company engaged in stem cell and regenerative medicine research.

References
Papers of special note have been highlighted as:
• of interest; •• of considerable interest
1 Inflammatory bowel disease: epidemiology of the IBD. http://www.cdc.gov/ibd/ibd-epidemiology.htm

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research
The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Open access
This work is licensed under the Attribution-NonCommercial-NoDerivatives 4.0 Unported License. To view a copy of this license, visit http://creativecommons.org/licenses/by-nc-nd/4.0/


• Demonstration of long-term efficacy of intralesional stem cell injections in human Crohn’s disease.


• Recent trial demonstrating the efficacy of bone marrow-derived mesenchymal stromal cells in refractory perianal fistulas in patients with Crohn’s disease.


27 Rombouts Wj, Ploemacher RE. Primary murine MSC show highly efficient homing to the bone marrow but lose homing ability following culture. Leukemia 17(1), 160–170 (2003).


30 Kimbrel E, Kouris NA, Yavanian G et al. Mesenchymal stem cell population derived from human pluripotent stem cells displays potent immunomodulatory and therapeutic properties. Stem Cells Dev. 23(14), 1611–1624 (2014).

• Development and characterization of the mesenchymal stem cells used in the present clinical trial.


• Very informative and comprehensive review of the canine entity.


