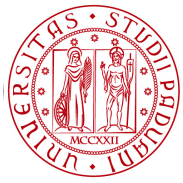

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Amniotic Fluid Stem Cells Improve Survival And Enhance Repair Of Damaged Intestine In Experimental Necrotizing Enterocolitis Via A Cox-2 Dependent Mechanism.

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SUMMARY

Background. Necrotizing enterocolitis (NEC) is a major cause of morbidity and death in neonates. No specific therapy is available and the treatment is only supportive. Amniotic Fluid Stem (AFS) cells represent a novel class of pluripotent stem cells with intermediate characteristics between embryonic and adult stem cells, as they are able to differentiate into lineages representative of all embryonic germ layers and do not form tumors after implantation *in vivo.* These characteristics, together with the absence of ethical issues concerning their obtainment, make AFS cells good candidates for cell therapy of human diseases.

Aim. The aim of this study was to explore the therapeutic potential of Amniotic Fluid Stem (AFS) cells in a rat model of NEC.

Methods. AFS cells were obtained from green fluorescent (GFP+) transgenic pregnant rats at 16 days p.c. by c-kit selection. NEC was induced in newborn rats by hyperosmolar milk formula, oral lipopolysaccharide and hypoxia. Rats were divided into 2 groups receiving at 24 and 48 hours of life an intraperitoneal injection of: (i) phosphate buffered saline (PBS; n=120) or (ii) $2x10^6$ AFS cells (n= 121). Additional groups of animals, either injected with bone marrow-derived mesenchymal stem cells (i.e. rat BM-MSCs) or committed cells (i.e. rat myoblasts), or non subjected to NEC induction (i.e. healthy breast fed newborn rats), were used as additional controls. All groups were blindly compared regarding survival, clinical status, radiological features (abdominal MRI), gut motility (carmine red transit time) and intestinal permeability (plasma lactulose/mannitol ratio). Intestines were blindly analyzed for macro- and microscopic appearance, transcriptional profile (microarray-based expression analysis), neutrophil infiltration (myeloperoxidase activity), enterocyte proliferation (EdU assay) and apoptosis (cleaved caspase 3 immunohistochemistry). AFS cell integration in the gut was evaluated by GFP amplification and immunostaining. Cyclooxygenase 2 (COX2+) cells in the lamina propria were evaluated by immunofluorescence. COX2 activity was inhibited in vivo using selective (celecoxib) and non-selective (ibuprofen) inhibitors; the effects of COX2 pharmacological inhibition on rat survival and clinical status were evaluated.

Results. Compared to animals injected with PBS, rats receiving AFS cells survived longer ($p<0.0001$), and showed: improved clinical conditions ($p<0.001$), better abdominal appearance at MRI, restored intestinal transit ($p<0.01$), decreased intestinal permeability ($p<0.05$), reduced macroscopical ($p<0.001$) and histological gut damage ($p<0.001$). These beneficial effects were specific to AFS cells as neither BM-MSCs nor myoblasts were able to improve animal morbidity/mortality in comparison to PBS (p=n.s.). AFS cells integrated in the intestine with various degrees of spreading in all the animals. cDNA arrays comparing the intestinal transcriptional profile of PBS vs. AFS cell rats showed differences in the expression of genes involved in inflammation, apoptosis and cell proliferation which were respectively down-regulated (inflammation and apoptosis) and up-regulated (proliferation) in the AFS cell group. At a protein level, AFS cell rats had lower gut neutrophil infiltration ($p<0.05$), reduced enterocyte apoptosis ($p<0.05$) and increased enterocyte proliferation ($p<0.0001$) compared to PBS rats. In rats treated with AFS cells vs. rats injected with PBS, COX2+ cells in the lamina propria were increased ($p<0.001$) and repositioned under crypts ($p<0.001$). Moreover, both the total number of COX-2+ cells per villus unit and the number of cryptal COX-2+ cells inversely correlated with the degree of intestinal damage ($p=0.014$). The pharmacological inhibition of COX2 activity did not exert any effect in PBS rats, whereas it completely abolished AFS cell beneficial effects on animal survival and clinical behavior.

Conclusions. In experimental NEC, AFS cell administration via the intraperitoneal route is associated with reduced animal morbidity/mortality and decreased incidence of NEC. AFS cell beneficial effects seems to be related to decreased intestinal neutrophil infiltration, enhanced enterocyte proliferation and reduced epithelial apoptosis. We hypothesize that is achieved through activation of COX2+ cells in the lamina propria. Stem cell therapy may represent a new therapeutic option for infants with NEC.

RIASSUNTO

Premesse. L'enterocolite necrotizzante (NEC) rappresenta la causa più frequente di insufficienza intestinale in età pediatrica. Non esistono tuttora terapie specifiche per la NEC ed il suo trattamento si basa unicamente sulla terapia medica di supporto e sulla rimozione chirurgica delle porzioni di intestino affetto. Le cellule staminali derivanti da liquido amniotico (AFSC) sono una popolazione di cellule staminali di origine fetale descritta per la prima volta nel 2007. Esse possiedono delle caratteristiche intermedie fra le cellule staminali embrionali (i.e. pluripotenza) e le cellule staminali adulte (i.e. mancata tumorigenicità dopo iniezione in vivo) che le rendono candidati ideali per la terapia cellulare.

Scopo dello studio. Valutare il potenziale terapeutico delle cellule staminali derivanti da liquido amniotico (AFSC) in un modello animale di NEC.

Materiali e metodi. AFSC sono state derivate da ratti Sprague-Dawley GFP+ (i.e. esprimenti in modo costitutivo la proteina reporter "Green Fluorescent Protein") al 16^ giorno p.c. tramite immunoselezione per il loro caratterstico marcatore di superficie (i.e. c-kit/CD117). Le cellule ottenute sono state caratterizzate per morfologia e immunofenotipo. La NEC e' stata indotta in ratti neonati tramite l'utilizzo di elementi simili ai fattori patogenetici implicati nell'insorgenza della NEC umana: alimentazione con latte formulato iperosmolare, eventi ipossici, somministrazione di lipopolisaccaride. I ratti, suddivisi in due gruppi principali, hanno ricevuto a 24 e 48 ore di vita, per via intraperitoneale: i. 50 ul di soluzione salina (PBS; n=120) o ii. $2x10^6$ AFSC (n= 121). Altri gruppi di animali, trattati con cellule staminali mesenchimali di ratto derivanti da midollo osseo o con mioblasti, oppure non sottoposti all'induzione di NEC (i.e. neonati sani allattati al seno), sono stati utilizzati come gruppi aggiuntivi di controllo. I diversi gruppi di animali sono stati valutati in cieco per i seguenti parametri: sopravvivenza, stato clinico, aspetto radiologico intestinale (RM ad alta risoluzione), motilita' intestinale (studio del tempo di transito con coloranti vitali), permeabilita' intestinale (rapporto lattulosio/mannitolo plasmatici). L'intestino e' stato valutato in cieco per: aspetto macroscopico ed istologico, profilo di espressione genica (tramite tecnologia cDNA-microarray), infiltrazione neutrofila (saggio di attivita' della mieloperossidasi), proliferazione (EdU) e apoptosi degli enterociti (immunoistochimica per caspasi 3 attivata). L'integrazione di AFSC nell'intestino e' stata analizzata sia tramite PCR (amplificazione del gene *gfp*) che tramite immunoistochimica (immunofluorescenza per GFP). Il numero e la localizzazione delle cellule stromali esprimenti COX2 nella mucosa sono stati valutati con immunofluorescenza. L'attivita' di COX2, in vivo, e' stata inibita farmacologicamente con inibitori selettivi (celecoxib) e non selettivi di COX2 (ibuprofene); gli effetti di tale inibizione sulla sopravvivenza e sulla morbidita' degli animali trattai con AFSC o PBS sono stati analizzati in cieco.

Risultati. La somministrazione di AFSC, per via intraperitoneale a ratti neonati affetti da NEC: migliora significativamente la sopravvivenza degli animali sia rispetto alla somministrazione di PBS ($p<0.0001$) che di linee cellulari di controllo (i.e. cellule staminali mesenchimali di ratto derivanti da midollo osseo [$p=0.024$] e mioblasti di ratto [$p<0.0001$]). Rispetto alla somministrazione di PBS, inoltre, il trattamento con AFSC: i. riduce la morbidita' degli animali migliorandone l'aspetto clinico ($p<0.001$); ii. riduce significativamente il danno intestinale sia alla valutazione dell'addome con RM ad alta risoluzione che all'esame macroscopico ($p<0.001$) ed istologico dell'intestino ($p<0.001$); iii. migliora significativamente la funzionalità dell'intestino sia per quanto concerne la motilità ($p<0.01$) che l'assorbimento di nutrienti ($p<0.05$). AFSC somministrate per via intraperitoneale migrano preferenzialmente verso l'intestino dove, seppur con un basso tasso di integrazione tissutale, sono in grado di localizzarsi in tutti gli strati della parete e talora di differenziarsi in cellule con fenotipo mesenchimale (i.e. cellule muscolari lisce). La somministrazione di AFSC in ratti neonati affetti da NEC è in grado di modificare il profilo di espressione genica dell'intestino incrementando l'espressione di geni coinvolti nella proliferazione e riducendo l'espressione di geni coinvolti in apoptosi e

infiammazione. Tali dati di espressione sono stati confermati a livello proteico dimostrando che nell'intestino dei ratti affetti da NEC trattati con AFSC v.s. PBS è maggiore la proliferazione delle cellule epiteliali ($p<0.0001$), minore l'apoptosi degli enterociti ($p<0.05$) e ridotta l'infiltrazione neutrofila tissutale ($p<0.05$). La somministrazione di AFSC, inoltre, determina l'attivazione di una popolazione di cellule stromali esprimenti la ciclossigenasi 2 (COX2) nella lamina propria della mucosa intestinale. Più in dettaglio la somministrazione di AFSC v.s. PBS causa un significativo aumento del numero delle cellule COX2+ nella lamina propria ($p<0.001$) e un loro spostamento dall'asse del villo alla *niche* delle cripte intestinali ($p<0.001$). Tale effetto costituisce il meccanismo d'azione di AFSC poiché la somministrazione in vivo di inibitori selettivi e non selettivi di COX2 (ma non di COX1) a ratti affetti da NEC abolisce gli effetti positivi di AFSC su morbidità e mortalità degli animali ma non ha alcun effetto sugli animali trattati con PBS.

Conclusioni. In un modello animale di NEC, AFSC sono in grado di migliorare in modo significativo la mortalita' e la morbidita' degli animali e il danno intestinale. AFSC non determinano direttamente tali effetti rigenerando di per sé l'intestino ma indirettamente attivando le cellule stromali esprimenti COX2 presenti nella lamina propria le quali a loro volta stimolano la proliferazione e riducono l'apoptosi delle cellule epiteliali intestinali residenti. Sebbene ulteriori studi siano necessari (e.g. per identificare i fattori/meccanismi molecolari responsabili dell'attivazione delle cellule COX2+), riteniamo che la terapia con cellule staminali derivanti da liquido amniotico possa rappresentare una nuova prospettiva terapeutica per i pazienti affetti da NEC.

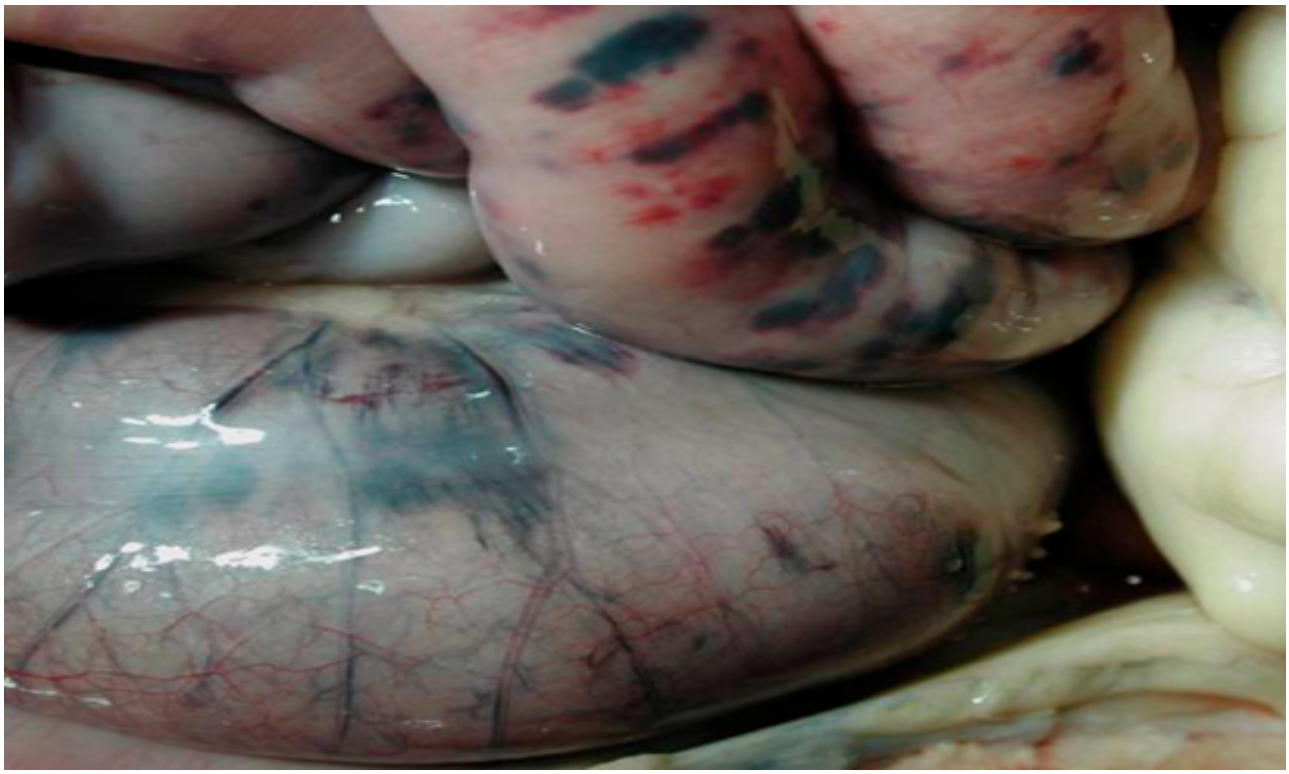
INTRODUCTION

A. Necrotizing Enterocolitis (NEC)

Necrotizing enterocolitis (NEC) is an acute ischemic-inflammatory disease of the neonatal intestine characterised by variable degrees of intestinal necrosis and inflammation (***Figure 1***). It is a devastating disease of newborn infants that mainly affects the ileum and the proximal colon but which can involve any segment of the gastrointestinal tract.

NEC is the most common acquired gastrointestinal emergency in infants and one of the most common surgical emergencies in neonatal intensive care units (NICUs). Despite more than three decades of intensive research efforts, the pathogenesis of NEC is still unproven, its treatment is difficult and often inadequate and NEC remains a major cause of morbidity and death in neonates. As advances in neonatology have improved survival of smaller, more premature infants, the incidence of NEC has increased (*Lin, 2008*).

Figure 1. *Intestinal segments affected by necrotizing enterocolitis (NEC). The classic findings of diffuse NEC are seen in this image. Patchy areas of necrosis are seen with questionable intestinal viability. Adapted from Hunter, 2008.*



Epidemiology

NEC mainly affects preterm babies (>90% of those affected were born prior to 36 weeks of gestation and weighed less than 2000gr), in which the risk of developing NEC is inversely related to birth weight and gestational age. NEC, however, can also occur in full term infants especially if predisposing risk factors, such as perinatal asphyxia, polycythaemia, respiratory distress and congenital cardiac diseases, are present (*Lin, 2006*).

Population-based studies have shown that NEC occurs in 1–3 babies per 1000 live births and in 3–7% of preterm and low-birth-weight infants with the highest rates occuring in very-low-birth-weight (VLBW) and black infants. Among full-term infants, the estimated incidence of NEC is 0.05 per 1000 live births. Reported NEC mortality rates range from 10% to 50%. Smaller infants, infants with more severe disease, and infants requiring surgery, especially if black and male, have higher case fatality rates (*Henry, 2009*).

Pathophysiology

The pathophysiology of NEC remains elusive but is likely multifactorial. Since premature infants are at higher risk for NEC, immaturity of the intestinal tract has been implicated as the crucial element for the development of the disease. Current theories suggest that NEC is initiated by perinatal insults (e.g. formula feeding, abnormal bacterial colonization of the immature gut, lack of appropriate gut colonization with commensal organisms, hypoxia and hypoperfusion) in response to which the immature intestine mounts an exaggerated and inappropriate inflammatory response leading to intestinal injury. Genetic factors have also been recently advocated as predisposing factors involved in the pathogenesis of NEC (***Figure 2***) (*Lin, 2006 and 2008; Petrosyan, 2009*).

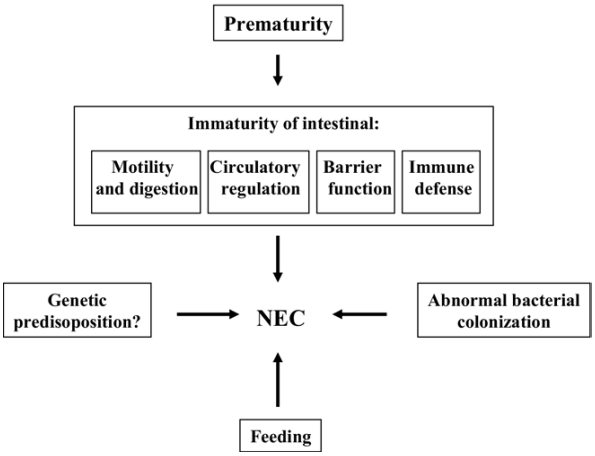


Figure 2. *Pathophysiology of NEC. Adapted from Lin, 2008.*

Gut immaturity involves different functions of the intestinal tract:

- **Motility.** GI motility development begins in the 2nd trimester but does not complete until the 3rd trimester. Indeed, migrating motor complexes are not present until about 34 weeks of gestation. External factors can also alter intestinal motility; perinatal asphyxia for example decreases intestinal transit. With a decreased propulsive action of the intestine, there is an increased likelihood of an accumulation of noxious substances in contact with the epithelium, which may lead to intestinal damage (*Guner, 2008; Lin, 2006 and 2008; Petrosyan, 2009; Schnabl, 2008; Thompson, 2008*).
- **Barrier function.** The intestinal barrier consists of structural and biochemical defenses, both of which are underdeveloped in preterms. Breach of this barrier activates inflammatory processes. The **structural barrier** is composed by epithelial tight junctions and by mucus production. Tight junctional complexes, which zip enterocytes together, are formed by 10 weeks of gestation, but completely mature in terms of nutrient absorption and fluid secretion from 26 weeks to term. Secretory ability is particularly important to host defense since enterocytes use Cl^- and water secretion to flush pathogens or toxins from the intestinal lumen. The integrity of the intestinal barrier is reinforced by a thick mucin layer secreted by goblet cells. In addition to mechanical protection, this layer provides other functions such as aggregating and facilitating removal of adherent bacteria, as well as trapping enzymes near the epithelial surface to aid in digestion. Mucin gene expression mimics adult expression by 23 to 27 weeks gestation, and mucin becomes more viscous and therefore more effective as it matures. An immature mucin layer leads to increased intestinal permeability and enhanced bacterial adherence. **Biochemical defenses** consist in the production by Paneth cells of lysozyme and antimicrobial peptides (defensins). Paneth cell number and defensin expression are lower in the intestine of premature neonates than in adults thus determining a lower defense capacity against bacteria (***Figure 3***) (*Guner, 2008; Lin, 2006 and 2008; Petrosyan, 2009; Schnabl, 2008; Thompson, 2008*).

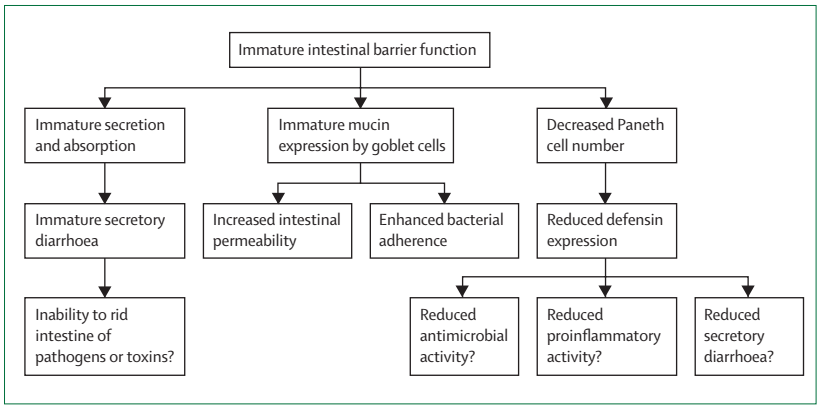


Figure 3. *Immature intestinal barrier function. Adapted from Lin, 2006.*

- **Circulatory regulation.** Although hypoxic-ischemic stress can cause intestinal injury, the hypothesis that this stress is the primary inciting factor in the pathogenesis of NEC has been seriously questioned. Despite convincing evidence that hypoxic–ischemic stress is involved in term NEC, evidences are lacking to support this hypothesis for preterm NEC. Whereas term NEC occurs within the first few days of life, in fact, preterm NEC occurs much later, making perinatal hypoxic–ischemic events less plausible in the pathogenesis of the disease. However, hypoxia–ischemia may still play a secondary role due to immature circulatory regulation. Recent studies, in fact, have demonstrated an imbalance between vasoconstriction and vasodilatation mediators (e.g. NO) in the immature intestine which may predispose the gut to ischemic injury in response to feeding or abnormal bacterial colonization (*Guner, 2008; Lin, 2006 and 2008; Petrosyan, 2009; Schnabl, 2008; Thompson, 2008*).
- **Innate immunity.** The immature intestine responds to injury on the one hand with excessive inflammation and on the other with inadequate inflammatory responses respectively leading to a massive gut damage and to enterocyte apoptosis and bacterial overgrowth (***Figure 4***). Inflammatory mediators implicated in the pathogenesis of necrotising enterocolitis include PAF, tumour necrosis factor (TNFα), and interleukins (IL-1, IL-6, IL-8, IL-10, IL-12, and IL-18) (*Guner, 2008; Lin, 2006 and 2008; Petrosyan, 2009; Schnabl, 2008; Thompson, 2008*).

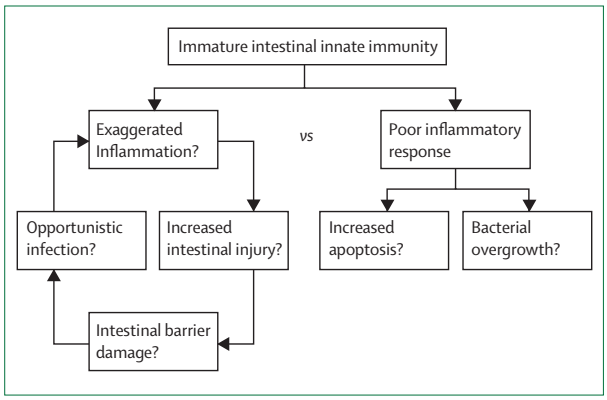


Figure 4. *Immature intestinal innate immunity. Adapted from Lin, 2006.*

Among environmental factors, abnormal bacterial colonization of the intestine and feeding with milk artificial formulas are widely recognized triggering factors involved in the pathogenesis of NEC.

- **Abnormal bacterial colonization.** NEC does not occur in utero, when the gut is sterile, implicating bacterial involvement in its pathogenesis (*Lin, 2008*). Since most cases of NEC are sporadic, with no clear seasonal distribution, a specific infectious

agent is not suspected. It is more likely that abnormal colonization of the gastrointestinal tract, or an unfavorable balance between commensal and pathogenic bacteria, is significant in the pathogenesis of NEC. VLBW infants may be particularly susceptible to dysbiosis (an inappropriate composition of gut microflora). In healthy term breastfed infants, the intestine, which was sterile in utero, becomes colonized with organisms acquired from maternal flora and the surrounding environment. Within two weeks, a predominance of Bifidobacteria and other facultative anaerobes is seen (*Fanaro, 2003*). Premature infants, however, are at greater risk of developing abnormal intestinal bacterial colonization, secondary to contact with nosocomial flora and frequent exposure to antibiotics in the NICU (*Stoll, 1996*). Indeed, studies have revealed that VLBW infants have abnormal duodenal colonization of Enterobacteriaceae (*Hoy, 2000*). Further, early abnormal stool colonization with Clostridium Perfringes has been associated with later development of NEC (*De la Cochetiere, 2004)*. When considering neonatal intestinal defenses, one must bear in mind that the gut is totally naive to MAMPs in utero and is instantly challenged at birth with the introduction of normal flora. Not only is the premature intestine predisposed to injury from abnormal bacterial colonization, it may not be ready to respond appropriately to normal colonization. Recent reports suggest an increasingly important role of the PRR, toll-like receptor 4 (TLR4), in the pathogenesis of NEC (*Fusunyan, 2001; Jilling, 2006; Lu, 2007*). Increased TLR4 expression in the immature intestine may predispose to inappropriately increased apoptotic (*Jilling, 2006)* and proinflammatory responses (*Fusunyan, 2001; Lu, 2007*), and modulation of TLR4 expression can reduce the incidence of experimental NEC (*Leaphart, 2007*) (***Figure 5***).

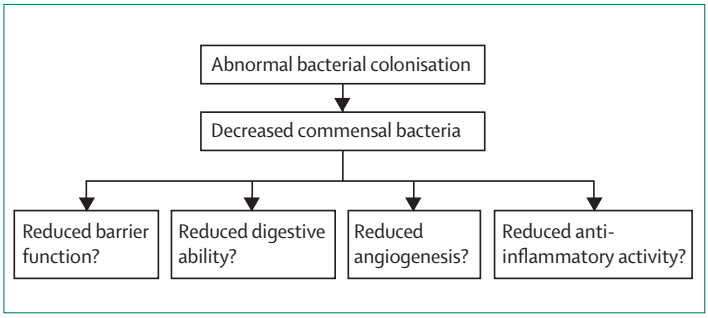


Figure 5. *Abnormal bacterial colonization. Adapted from Lin, 2006.*

- **Feeding with milk artificial formulas.** Enteral feeds have a firm association with NEC as 90-95% of NEC cases occur in infants with initiation/reinitiation of enteral feeds or recent volume advances (*Lee, 2003*). Infants receiving hyperosmolar formulas or rapid volume advances are at greatest risk (*Caplan, 2003*). Although the mechanism is not

well understood, enteral feeding has been reported to contribute to the development of NEC through disruption of mucosal integrity, blood flow and motility and through provision of a bacterial substrate (*Horton, 2005*). Raising milk intake increases metabolic demands, making it difficult for the infant to expand mesenteric blood flow to meet demands. As a result, intestinal hypoxiemia may occur. Human milk has been reported to reduce the incidence of NEC by up to 10 folds compared with infant formula whether using mother's own or donor milk (*Lucas, 1996; McGuire, 2003; Quingley, 2007; Schanler, 2005*). Breast milk also reduces the severity of NEC. The protective effect of breast milk has been correlated with its anti-inflammatory components (IL-10), growth factors (EGF), lysozyme, immunoglobulins as well as pre- and probiotics that modulate intestinal microflora composition to the advantage of the host (*Hanson, 1999*). Whether human milk reduces the incidence of NEC is not clear at present. Despite its advantages, human milk alone does not eliminate NEC as cases are reported in neonates who have been exclusively breast-fed with human milk (*Patole, 2005*).

Recently, the presence of genetic factors predisposing to the development of NEC have been hypothesized. In particular, the presence of genetic variations (polimorphisms) of genes involved in inflammatory signaling and circulatory regulation are under investigation. A recent study found that a carrier state for a mutant allele of vascular endothelial growth factor (VEGF), an important protein involved in angiogenesis and vasodilation, is an independent risk factor for NEC (*Banyasz, 2006*). Another study focused on single nucleotide polymorphisms in the Carbamoyl Phosphate Synthetase 1 (CPS 1) gene (*Moonen, 2007*). CPS 1 is the rate-limiting enzyme in the urea cycle production of the NO precursor L-arginine. Premature infants are commonly deficient in arginine, which may play a role in intestinal wound healing. Moonen and coworkers demonstrated that CPS1 T1405N polimorphism is associated with a higher risk of NEC in preterm babies. Since many immunoregulatory cytokines have been implicated in the pathogenesis of NEC, variations in many cytokine encoding genes have been studied. However, most cytokine genetic variations studied, including TNF-alpha, IL-1, IL-6, and IL-10, have not been associated with an increased risk of NEC (*Henderson, 2007; Treszl, 2001 and 2003*). A recent study suggests that infants with a mutant promoter allele causing lower levels of IL-12 may be at increased risk for NEC as well as pneumonia (*Bokodi, 2007*). However, the experiments conducted so far regarding the role of genetic factors in the development of NEC likely represent the tip of the iceberg and much remains still to investigate concerning this topic (*Lin, 2008*).

Clinical picture, diagnosis and management

Necrotising enterocolitis presents with gastrointestinal and systemic signs. Neonates most commonly present with feeding intolerance, delayed gastric emptying, abdominal distention or tenderness, occult or gross blood in the stool, lethargy, apnoea, respiratory distress, or poor perfusion. Infants might either have a benign disease with mainly gastrointestinal symptoms or a catastrophic illness characterized by a sudden fulminant onset with circulatory compromise, respiratory and metabolic acidosis, disseminated intravascular coagulopathy, grossly bloody stools, and multiorgan failure (***Figure 6a***). In severe cases of the disease, there is intestinal perforation, peritonitis, and profound shock (*Henry, 2009*).

Laboratory tests reveal nonspecific indicators of an inflammatory process, thus no one laboratory examination is diagnostic of NEC (*Lin, 2006*).

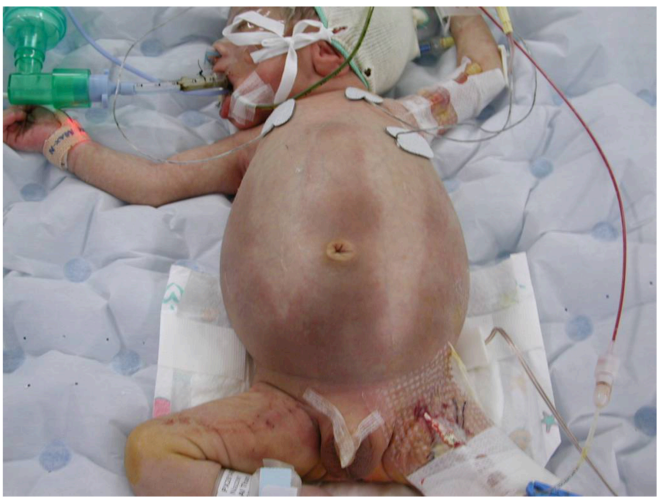
The diagnosis and staging of NEC rely on radiographic findings. Standard antero-posterior and dependent views (cross-table lateral or left lateral decubitus) of the abdomen are the cornerstones of NEC diagnosis. Early in the disease process, a plain abdominal radiograph may be normal or may show mild intestinal distension. As the disease progresses, intestinal dilatation worsens and pneumatosis intestinalis may be evident (***Figure 6b***). With further progression, evidence of portal venous gas may be found. Pneumoperitoneum indicates intestinal perforation (*Epelman, 2007*).

The combination of physical signs, physiologic abnormalities, and radiographic findings has been used to create the classic Bell staging system for NEC which is used to describe the severity of NEC (***Figure 6c***) (*Henry, 2009*).

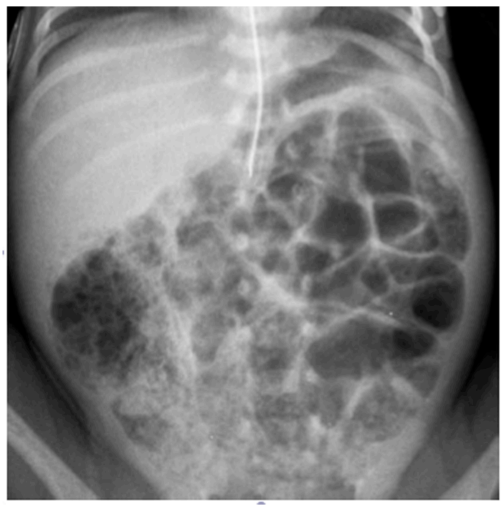
Most cases of NEC can be managed medically if care is initiated as soon as NEC is suspected. The first steps in medical management include bowel rest and decompression by placing an orogastric tube. Intravenous fluids should be started for resuscitation and broad-spectrum antibiotic coverage should begin after cultures have been obtained. Approximately one third of infants with NEC will require surgical intervention. Most of these cases occur after progression of disease during medical management, but many infants present acutely with severe disease requiring immediate operation. Laparotomy with resection of affected bowel and the creation of ostomies is the standard of care for the surgical treatment of NEC (*Lin, 2006 and 2008; Henry, 2009; Thompson, 2008*).

Figure 6. a. *Neonate affected by a severe form of NEC associated to multiorgan failure.* ***b.*** *Supine abdominal radiograph of a neonate with NEC showing dilatation of the bowel with gas and extensive intramural gas, which is seen as a bubbly pattern of radiolucency mainly in the right lower quadrant. Adapted from Epelman, 2007.* ***c.*** *Bell staging system for NEC. Adapted from Henry, 2009.*

a.



b.



c.

Stage	Systemic criteria	Abdominal criteria	Radiographic criteria
stage 1a = suspected NEC	temperature instability, apnea, bradycardia	elevated pregavage residuals, mild abdominal distension, occult blood in stool	normal or intestinal dilatation, mild ileus
stage 1b = suspected NEC	same as above	grossly bloody stool	same as above
stage 2a = definite NEC; mildly ill	same as above	same as stage 1 plus lack of bowel sounds, possible abdominal tenderness	ileus, pneumatosis intestinalis
stage 2b = Definite NEC; moderately ill	same as stage 1 plus mild metabolic acidosis, mild thrombocytopenia	same as above plus peritonitis, definite abdominal tenderness, possible abdominal cellulitis, right lower quadrant mass	same as above plus possible portal venous gas
stage 3a = advanced NEC; severely ill, intact bowel	same as stage 2b plus hypotension, bradycardia, severe apnea, combined respiratory and metabolic acidosis, DIC,[a] and neutropenia	same as above, with marked tenderness and abdominal distention	same as above plus ascites
stage 3b = advanced NEC; severely ill, perforated bowel	same as stage 3a	same as stage 3a	pneumoperitoneum

Long-term outcome

Despite advances in neonatal intensive care, mortality from NEC remains high, ranging from 10% to 50%. Moreover, 10-30% of surviving children experience significant morbidity including neurodevelopmental impairment, vision and hearing impairment, failure to thrive, feeding abnormalities, bowel obstruction and short bowel syndrome (*Schnabl, 2008*). In particular NEC is the main cause of short bowel syndrome in patients born with a normal gastrointestinal tract and one of the principal causes of intestinal failure in childhood.

Current demands and future perspectives

Despite the high clinical impact of NEC in the pediatric population, many questions remain to be addressed concerning its etiology, diagnosis and treatment. In everyday practice, there clearly is the need of:

- novel biological markers of early disease allowing more timely diagnoses and earlier interventions;
- new medical approaches able to prevent the progression of the disease and long-term complications;
- innovative surgical approaches minimizing the incidence of short bowel syndrome.

Current research efforts are mainly focused on the one hand on unraveling the pathogenesis of NEC and on the other on developing new preventive and therapeutic strategies. At present arginine supplementation, administration of probiotics and treatment with growth factors (i.e. EGF) are among the most attractive therapeutic alternatives being explored in the field of NEC.

Arginine. Endothelial nitric oxide (NO) is an anti-inflammatory agent and vasodilator involved in the maintenance of intestinal vascular permeability, mucosal integrity and barrier function. Plasmatic levels of arginine, a substrate for NOS, have been shown to be low in neonates with NEC (*Zamora, 1997*). Arginine supplementation has recently been shown to reduce the incidence of all stages of NEC in randomized, double blind, placebo controlled studies in preterm neonates (*Amin, 2002*). Wheter the beneficial effects of arginine supplementation in NEC are related to synthesis of glutamine or to its free radical scavenging action is currently unknown (*Neu, 2002*). Guidelines have not been yet established for the safety and efficacy of arginine at doses above standard dietary practices in NEC (*Shah, 2004*).

Probiotics. Probiotics (i.e. living microorganisms which upon administration in sufficient numbers colonize the gut and exert health benefits on the host) have been considered of potential help in NEC given the known role of bacterial inappropriate gut colonization in its pathogenesis. Several studies have used different strains of probiotics and different administration regimens in preterm infants. None of the trials reported adverse effects and no episodes of pathogenetic infection caused by a probiotic organism have been observed (*Dani, 2002; Lin, 2005*). Clinical trials showed that probiotic supplements (lactobacillus acidophilus, bifidobacterium bifidus, Streptococcus thermophilus) reduce the incidence and the severity of NEC (*Bin-Nun, 2005; Lin, 2005*). Larger trials are necessary to confirm

the safety and the efficacy of this intervention to better define the benefits and risks for premature infants before wider use can be recommended.

EGF. EGF has multiple effects upon gut epithelial cells including cytoprotection, stimulatory effects on cell proliferation and migration, induction of mucosal enzyme and trefoil peptide expression, and inhibitory effects on gastric acid secretion (*Wong, 1999*). Preterm neonates with NEC have diminished levels of salivary and serum EGF (*Shin, 2000*). The presence of immunoreactive EGF receptors in gut epithelial cells of preterm neonates raised the possibility of using EGF for the prevention and treatment of NEC (*Fagbemi, 2001*). In a neonatal rat model of NEC, EGF treatment maintained intestinal integrity by accelerating goblet cell maturation and normalizing expression of tight junctions (*Clark, 2006*). Further studies are needed to evaluate EGF efficacy and safety profile.

B. Regenerative Medicine

Regeneration after damage remains a major challenge. “Regenerative medicine is an interdisciplinary field of research and clinical applications focused on the repair, replacement or regeneration of cells, tissues or organs to restore impaired function resulting from any cause” (*Daar, 2007*). Main strategies include cell-based therapies, the use of biomaterials (scaffolds) alone and the use of scaffolds seeded with cells (*Hipp, 2008*). However, the employment of cells for transplantation and tissue engineering has been restrained by the limited proliferation and differentiation capabilities of somatic differentiated cells.

In the last decades, major advancements have been permitted by the discovery of cells, defined as “stem cells”, capable of widely expanding *ex vivo* and of differentiating to various cell lineages. According to The United States National Academies of Science report, “Stem Cells and the Future of Regenerative Medicine”, more than a hundred million of patients in the U.S. are affected by diseases that, in the future, may be potentially treated with stem cell-based therapies (*Commission on Life Sciences, 2002*). Stem cells have been isolated from embryonic, fetal and adult tissues and, more recently, also from extra-embryonic adnexa such as umbilical cord, placenta, fetal membranes and amniotic fluid. Among the different stem cell populations described, each one carries peculiar features, values and limitations.

Stem cells: a variety of diverse populations

The term “stem cell” identifies cells that share the dual ability to proliferate indefinitely (i.e. self-renewal) and to differentiate into one or more types of specialized cells (i.e. potency) (*Mimeault, 2006*). Considering their differentiation potential, stem cells are classified into pluripotent (i.e. able to differentiate into all derivatives of the three primary germ layers) and multipotent (i.e. able to lead to multiple cell types deriving from a single germ layer) (*Solter, 2006*). There is still no consensus whether cells with the capacity to regenerate and/or contribute to only a single lineage should be referred to as unipotent stem cells, unipotent progenitors, or simply cells that have retained a proliferative potential (*Blanpain, 2007*).

Stem cells, depending on their origin, are further divided in two main groups: embryonic and adult stem cells. Embryonic stem (ES) cells are pluripotent cells deriving from the inner cell mass of a blastocyst (*Bongso, 1994; Cole, 1965 and 1966; Evans, 1981; Martin, 1981; Thomson 1995 and 1998*). Because of their plasticity and potentially unlimited capacity for self-renewal (*Niwa, 2007; Ying, 2008*), ES cells have been proposed as a potential treatment for several disorders such as diabetes and Parkinson’s disease. However, to date, no approved medical treatment has been derived from embryonic stem cell research (*Edwards, 2008; Pucéat 2007*). ES cell clinical use has been mainly constrained so far by: 1. safety concerns regarding their observed tendency to form tumours when injected undifferentiated or only partially differentiated *in vivo* (*Hanson, 2005; Hentze, 2007; Kolossov, 2006; Lawrenz, 2004; Maitra, 2005; Shih, 2007; Teramoto 2005*); 2. possible host immune rejection of cellular allografts (*Grinnemo, 2008; Kofidis, 2005; Nussbaum, 2007; Saric’, 2008; Swijnenburg, 2005*); and, 3. ethical considerations concerning their obtainment from human embryos (*Daley, 2007; Edwards, 2007; Green, 2007*). On the other side of the spectrum, adult stem (AS) cells reside in specific locations, called niches, in adult tissues in which they are able to maintain their multipotency (*Jones, 2008*). Their physiological role consists of constantly renewing and, in case of damage, repopulating the tissues in which they reside (*Yamashita, 2007*). At present AS cells have been identified in almost all organs and tissues other than the gonads (*Barker, 2007; Bonner-Weir 2005; Griffiths, 2005; Guettier, 2005; Gupta, 2006; Kim CF, 2005; Lyngbaek, 2007; Scoville, 2008*). Although their differentiation potential and proliferative capacities are limited compared to that of ES cells along with the possibility that genetic alterations occurring with ageing may lead to a loss of their functions, AS cells represent a possible resource both for research and medical purposes as their derivation can be performed in

an autologous setting and does not involve the destruction of human embryos (*Mimeault, 2007*).

In order to overcome the limitations associated with both ES and AS cells, attempts have been made to identify alternative stem cell sources.

On the one hand, methods capable of generating patient specific pluripotent stem cells from adult cells have been developed. Among these, nuclear reprogramming has been recently reported (*Hanna, 2007; Meissner, 2007; Takahashi, 2006 and 2007*); it consists in the de-differentiation of somatic cells through cell retroviral transduction of defined transcription factors (Oct4, Sox2, Klf-4, c-Myc) (*Maherali, 2007; Yamanaka, 2008*). The obtained cells (i.e. induced pluripotent stem, iPS, cells) are molecularly and functionally indistinguishable from ES cells in many respects as they exhibit similar morphology and growth properties, express ES cell markers, are able to generate germline-competent chimeras and form tumors when injected into nude mice. Current studies are investigating the safety profile of these cells for therapeutic application (*Liu, 2008*).

On the other hand, different multipotent progenitors have been recently isolated from the fetus (i.e. fetal stem cells, FSC). These cells, although more lineage-committed than ES cells, show better proliferation and differentiation capacities in comparison to adult progenitors, do not form teratomas *in vivo* and, if obtained before the 12th week of gestation, are poorly associated with rejection when transplanted into immunocompetent mice (*Campagnoli, 2001; Guillot, 2006 and 2007; Mimeault, 2006*). At present hematopoietic, mesenchymal, neural, pancreatic and lung progenitors have been obtained from fetal tissues (*Andrade, 2007; Brands, 2008; Chan, 2007; Gao, 2006 and 2007; Guillot, 2008; Kenzaki, 2006; Rollini, 2004*). However, the collection of fetal tissue during gestation is associated with high morbidity and mortality both for the fetus and the mother (*Deprest, 2008; Kohl, 2004; Walsh, 2000*).

Given the aforementioned, attempts have been made to obtain stem cells from extra-embryonic tissues. Umbilical cord blood (UCB) was the first to be investigated and is now a well established source of transplantable hematopoietic stem cells (HSC) with greater proliferative capacity and lower immunologic reactivity in comparison to bone marrow derived HSC (*Broxmeyer, 1989; Brunstein, 2007; Hwang, 2007; Schoemans, 2006; Yu, 2001*). Moreover, it has been recently demonstrated that UCB also contains mesenchymal (*Secco, 2008; Weiss, 2006*) and multipotent stem cells (*van de Ven, 2007*), as well as cells with ES cell-like characteristics (*Zhao, 2006*). In the last ten years, placenta, fetal membranes (i.e. amnion and chorion) and amniotic fluid have also been extensively investigated as a potential non controversial source of stem cells. They are

usually discarded after delivery and are accessible during pregnancy through amniocentesis and chorionic villus sampling (*Marcus, 2008-A*). Several populations of cells with multilineage differentiation potential and immunomodulatory properties have been isolated from the human placenta and fetal membranes; they have been classified by an International Workshop (*Parolini, 2007*) as human amniotic epithelial cells (hAEC) (*Kim J., 2007-A; Marcus, 2008-B; Miki, 2005 and 2006; Tamagawa, 2004*), human amniotic mesenchymal stromal cells (hAMSC) (*Alviano, 2007; Soncini, 2007*), human chorionic mesenchymal stromal cells (hCMSC) (*Igura, 2004; In't Anker 2004*), and human chorionic trophoblastic cells (hCTC). In the amniotic fluid (AF), two main populations of stem cells have been isolated so far: 1. amniotic fluid mesenchymal stem cells (AFMSCs); 2. amniotic fluid stem (AFS) cells. Although only recently described, these cells may, given the easier accessibility of the AF in comparison to other extra-embryonic tissues, hold much promise in regenerative medicine.

In the next pages we will describe the state of the art concerning AFS cell distinctive features, isolation modalities, culture techniques and applications in preclinical studies.

Amniotic Fluid Stem (AFS) Cells

The first evidence that the AF could contain pluripotent stem cells was provided in 2003 when Prusa described the presence of a distinct sub-population of proliferating AF cells (0.1-0.5% of the cells present in the AF) expressing the pluripotency marker Oct4 at both transcriptional and proteic levels (*Prusa, 2003*). Oct4 (i.e. octamer binding transcription factor 4) is a nuclear transcription factor which plays a critical role in maintaining ES cell differentiation potential and capacity of self-renewal (*Nichols, 1998; Niwa, 2000; Scholer, 1989*). Other than by ES cells, Oct4 is specifically expressed by germ cells, where its inactivation results in apoptosis, and by embryonal carcinoma cells and tumors of germ cell origin, where it acts as an oncogenic fate determinant (*Donovan, 2001; Gidekel, 2003; Looijenga, 2003; Pesce, 2001*). While its role in stem cells of fetal origin has not been completely addressed, it has been recently demonstrated that Oct4 is neither expressed nor required by somatic stem cells or progenitors (*Berg, 2007; Lengner, 2007; Liedtke, 2007*).

After Prusa, different groups confirmed the expression of Oct4 and of its transcriptional targets (e.g. Rex-1) in the AF (*Bossolasco, 2006; Stefanidis, 2007*). Remarkably, Karlmark transfected human AF cells with the green fluorescent protein gene under either the Oct4 or the Rex-1 promoter and established that some AF cells were able to activate these promoters (*Karlmark, 2005*). Several authors subsequently reported the possibility of harvesting AF cells displaying features of pluripotent stem cells (*Kim J., 2007-B; Tsai, 2006*). Thereafter, the presence of a cell population able to generate clonal cell lines capable of differentiating into lineages representative of all 3 embryonic germ layers was definitively demonstrated (*De Coppi, 2007-B*). These cells, named AF stem (AFS) cells, are characterized by the expression of the surface antigen c-kit (CD117), which is the type III tyrosine kinase receptor of the stem cell factor (*Zsebo, 1990*).

Isolation and culture

The proportion of hematopoietic lineage negative c-kit+ cells in the amniotic fluid varies over the course of gestation roughly describing a Gaussian curve; they appear at very early time points in gestation (i.e. at 7 weeks of amenorrhea in humans and at E9.5 in mice) and present a peak at midgestation corresponding to 90 x 10^4 cells/fetus at 20 weeks of pregnancy in humans and to 10.000 cells/fetus at E12.5 in mice (*Ditadi, 2009;* ***Figure 7***). Human AFS cells can be derived either from small volumes (5 ml) of second trimester AF (14-22 weeks of gestation) or from confluent back-up amniocentesis cultures.

Murine AFS cells are obtainable from the AF collected during the second week of gestation (E11.5-14.5) (*De Coppi, 2007-B; Kim J., 2007-B; Siegel, 2009-B; Tsai, 2006*). AFS cell isolation is based on a two-step protocol consisting in the prior immunological selection of c-kit positive cells from the AF (approximately 1% of total AF cells) and in the subsequent expansion of these cells in culture (*Chen, 2009; De Coppi, 2007-B; Kolambar, 2007; Perin, 2007; Siegel, 2009-B; Valli, 2009*). Isolated AFS cells can be expanded in feeder layer-free, serum-rich conditions without evidence of spontaneous differentiation in vitro. Cells are cultured in basic medium containing 15% of fetal bovine serum and Chang supplement (*De Coppi, 2007-B; Valli, 2009*).

Characterization

Karyotype analysis of human AFS cells deriving from pregnancies in which the fetus was male revealed the fetal origin of these cells (*De Coppi, 2007-B*).

AFS cells proliferate well during ex vivo expansion. When cultivated, they display a spectrum of morphologies ranging from a fibroblast-like to an oval-round shape (***Figure 8a***). As demonstrated by different authors, AFS cells possess a great clonogenic potential (*De Coppi, 2007-B; Tsai, 2006*). Clonal AFS cell lines expand rapidly in culture (doubling time = 36 h) and, more interestingly, maintain a constant telomere length (20 kbp) between early and late passages (***Figure 8b***). Almost all clonal AFS cell lines express markers of a pluripotent undifferentiated state: Oct4, NANOG (*Chambers, 2007; Chen, 2009; De Coppi, 2007-B; Tsai, 2006; Valli, 2009*). However, they have been proved not to form tumors when injected in severe combined immunodeficient (SCID) mice (*De Coppi, 2007-B*).

The cell-surface antigenic profile of AFS cells has been determined through flow cytometry by different investigators (***Table 1***). Cultured human AFS cells are positive for ES cell (e.g. SSEA-4) and mesenchymal markers (e.g. CD73, CD90, CD105), for several adhesion molecules (e.g. CD29, CD44) and for antigens belonging to the major histocompatibility complex I (MHC-I). They are negative for hematopoietic and endothelial markers (e.g. CD14, CD34, CD45, CD133, CD31), and for antigens belonging to the major histocompatibility complex II (MHC-II).

As stability of cell lines is a fundamental prerequisite for basic and translational research, AFS cell capacity of maintaining their baseline characteristics over passages has been evaluated based on multiple parameters. Despite their high proliferation rate, AFS cells and derived clonal lines show a homogeneous, diploid DNA content without evidence of chromosomal rearrangement even after expansion to 250 population doublings (*Chen,*

2009; De Coppi, 2007-B) (***Figure 8c***). Moreover, AFS cells maintain constant morphology, doubling time, apoptosis rate, cell cycle distribution and marker expression (e.g. Oct4, CD117, CD29, CD44) up to 25 passages (*Chen, 2009; Valli, 2009*). During in vitro expansion, however, cell volume tends to increase and significant fluctuations of proteins involved in different networks (i.e. signalling, antioxidant, proteasomal, cytoskeleton, connective tissue and chaperone proteins) can be observed using a gel-based proteomic approach (*Chen, 2009*); the significance of these modifications warrants further investigations but needs to be taken in consideration when interpreting experiments run over several passages and comparing results from different groups.

AFS cells and, more importantly, derived clonal cell lines are able to differentiate towards tissues representative of all 3 embryonic germ layers both, spontaneously, when cultured in suspension to form embryoid bodies (EBs) and when grown in specific differentiation conditions.

EBs consist in 3-dimensional aggregates of ES cells, which recapitulate the first steps of early mammalian embyogenesis (*Itskovitz-Eldor, 2000; Koike, 2007; Ungrin, 2008*). As ES cells, when cultured in suspension and without anti-differentiation factors, AFS cells harbor the potential to form EBs with high efficiency: the incidence of EB formation (i.e. % of number of EB recovered from 15 hanging drops) is estimated to be around 28% for AFS cell lines and around 67% for AFS cell clonal lines. Similarly to ES cells, EB generation by AFS cells is regulated by the mTor (i.e. mammalian Target of Rapamycin) pathway and is accompanied by a decrease of Oct4 and Nodal expression and by an induction of endodermal (GATA4), mesodermal (Brachyury, HBE1) and ectodermal (Nestin, Pax6) markers (*Siegel, 2009-A; Valli, 2009*).

In specific mesenchymal differentiation conditions AFS cells express molecular markers of adipose, bone, muscle and endothelial differentiated cells (e.g. LPL, desmin, osteocalcin and V-CAM1). In the adipogenic, chondrogenic and osteogenic medium, AFS cells respectively develop intracellular lipid droplets, secrete glycosaminoglycans and produce mineralized calcium (*Kim J. ,2007-B; Tsai, 2006*). In conditions inducing cell differentiation towards the hepatic lineage, AFS cells express hepatocyte specific transcripts (e.g. albumin, alpha-fetoprotein, multidrug resistance membrane transporter 1 and acquire the liver specific function of urea secretion (***Figure 9a***) (*De Coppi, 2007-B*). In neuronal conditions, AFS cells are capable of entering the neuroectodermal lineage. After induction, they express neuronal markers (e.g. GIRK potassium channels), exhibit barium-sensitive potassium current and release glutamate after stimulation (***Figure 9b***). Ongoing studies

are investigating AFS cell capacity to yield mature, functional neurons (*Donaldson, 2009;Santos, 2008; Toselli, 2008*).

AFS cells can be easily manipulated in vitro. They can be transduced with viral vectors more efficiently than adult MSCs, and, after infection, maintain their antigenic profile and the ability to differentiate into different lineages (*Grisafi, 2008*). AFS cells labelled with superparamagnetic micrometer sized iron oxide particles (MPIOs) retain their potency and can be non-invasively tracked by MRI for at least 4 weeks after injection in vivo (*Delo, 2008*).

Preclinical studies

Despite the very recent identification of AFS cells, several reports have investigated their potential applications in different settings.

Bone. Critical-sized segmental bone defects are one of the most challenging problems faced by orthopedics surgeons. Autologous and heterologous bone grafting are limited respectively by the small amount of tissue available for transplantation and by high re-fracture rates (*Beardi, 2008; Muscolo, 2009; Salgado, 2006*). Tissue engineering strategies that combine biodegradable scaffolds with stem cells capable of osteogenesis have been indicated as a promising alternatives to bone grafting (*Bianco, 2001*); however, bone regeneration through cell-based therapies has been limited so far by the insufficient availability of osteogenic cells (*Peister, 2009*).

The potential of AFS cells to synthesize mineralized extracellular matrix within porous scaffolds has been investigated by different groups. After exposure to osteogenic conditions in static 2D cultures, AFS cells differentiate into functional osteoblasts (i.e. activate the expression of osteogenic genes such as Runx2, Osx, Bsp, Opn, Ocn and produce alkaline phosphatase) and form dense layers of mineralized matrix (*De Coppi, 2007-B; Peister, 2009; Sun 2010*). As demonstrated by clonogenic mineralization assays, 85% of AFS cells vs. 50% of MSCs are capable of forming osteogenic colonies (*Morito, 2008; Peister, 2009; Sakaguchi, 2004*). When seeded into 3D biodegradable scaffolds and stimulated by osteogenic supplements (i.e. rhBMP-7 or dexamethasone), AFS cells remain highly viable up to several months in culture and produce extensive mineralization throughout the entire volume of the scaffold (*De Coppi, 2007-B; Peister ,2009; Sun, 2010*). In vivo, when subcutaneously injected into nude rodents, pre-differentiated AFS cell-scaffold constructs are able to generate ectopic bone structures in 4 weeks time (*De Coppi,2007-B; Peister, 2009; Sun, 2010*) (***Figure 9c***). AFS cells embedded in scaffolds, however, are not able to mineralize in vivo at ectopic sites unless previously pre-

differentiated in vitro (*Peister, 2009*). These studies demonstrate the potential of AFS cells to produce 3D mineralized bioengineered constructs and suggest that AFS cells may be an effective cell source for functional repair of large bone defects. Further studies are needed to explore AFS cell osteogenic potential when injected into sites of bone injury.

Cartilage. Enhancing the regeneration potential of hyaline cartilage is one of the most significant challenges for treating damaged cartilage (*Deans, 2009; Koelling, 2009*).

The capacity of AFS cells to differentiate into functional chondrocytes has been tested in vitro. Human AFS cells treated with TGF-β1 have been proven to produce significant amounts of cartilaginous matrix (i.e. sulfated glycosaminoglycans and type II collagen) both in pellet and alginate hydrogel cultures (*Kolambar, 2007*).

Skeletal muscle. Stem cell therapy is an attractive method to treat muscular degenerative diseases because only a small number of cells, together with a stimulatory signal for expansion, are required to obtain a therapeutic effect (*Price, 2007*). The identification of a stem cell population providing efficient muscle regeneration is critical for the progression of cell therapy for muscle diseases (*Farini, 2009*).

AFS cell capacity of differentiating into the myogenic lineage has recently started to be explored. Under the influence of specific induction media containing 5-Aza-2'-deoxycytidine, AFS cells are able to express myogenic-associated markers such as Mrf4, Myo-D, and desmin both at a molecular and proteic level (*De Coppi, 2007-B; Gekas, 2010*). However, when transplanted undifferentiated into damaged skeletal muscles of SCID mice, despite displaying a good tissue engraftment AFS cells did not differentiate towards the myogenic lineage (*Gekas, 2010*). Further studies are needed to confirm the results of this single report.

Heart. Cardiovascular diseases are the first cause of mortality in developed countries despite advances in pharmacological, interventional, and surgical therapies (*Walther, 2009*). Cell transplantation is an attractive strategy to replace endogenous cardiomyocytes lost by myocardial infarction. Fetal and neonatal cardiomyocites are the ideal cells for cardiac regeneration as they have been shown to integrate structurally and functionally into the myocardium after transplantation (*Ott, 2008; Yao, 2003*). However, their application is limited by the ethical restrictions involved in the use of fetal and neonatal cardiac tissues (*Dai, 2007*).

Chiavegato et al. investigated human AFS cell plasticity towards the cardiac lineage. Undifferentiated AFS cells express cardiac transcription factors at a molecular level (i.e. Nkx2.5 and GATA-4 mRNA) but do not produce any myocardial differentiation marker. The

authors proved that, under in vitro cardiovascular inducing conditions, human AFS cells express cardiomyocyte (i.e. Nkx2.5, MLC-2v, GATA-4, β-MyHC), endothelial (i.e. angiopoietin, CD146) and smooth muscle (i.e. smoothelin) markers. However, when xenotransplanted in the hearts of immunodeficient rats 20 minutes after creating a myocardial infarction, AFS cell differentiation capabilities were impaired by cell immune rejection (*Chiavegato, 2007; Dai, 2007*).

These results have been recently confirmed by the same group. When cultured with neonatal rat cardiomyocytes, rat AFS cells activated a “myocardial differentiation” program as confirmed using immunofluorescence, RT-PCR and single cell electrophysiology tecniques. Moreover, 3 weeks after transplantation in the heart of animals with ischemia/ reperfusion injury not only these cells showed the capacity of engtrafting in the myocardial tissue and to differentiate into endothelial or smooth muscle, but also of improving heart ejection fraction as measured by magnetic resonance imaging (*Bollini, 2010*).

Hematopoietic system. Hematopoietic stem cells (HSC) lie at the top of hematopoietic ontogeny and, if engrafted in the right niche, can theoretically reconstitute the organism's entire blood supply. Thus, the generation of autologous HSCs from pluripotent, patient-specificic stem cells offers real promise for cell-therapy of both genetic and malignant blood disorders (*Kim, 2009*).

The hematopoietic potential of c-kit+ hematopoietic lineage negative cells present the amniotic fluid (AFKL cells) has been recently explored (*Ditadi, 2009*). In vitro, human and murine AFKL cells exhibit strong multilineage hematopoietic potential. Cultured in semisolid media, these cells are able to generate erythroid, myeloid and lymphoid colonies. Moreover, murine cells exhibit the same clonogenic potential (0.03%) as hematopoietic progenitors present in the liver at the same stage of development. In vivo, mouse AFKL cells (i.e. $2x10^4$ cells i.v. injected) are able to generate all 3 hematopoietic lineages after primary and secondary transplantation into immunocompromised hosts (i.e. sublethally irradiated $Rag^{-/-}$ mice), demonstrating their ability to self-renew. These results clearly show that AFS c-kit+ cells present in the amniotic fluid have true hematopoietic potential, both in vitro and in vivo and support the idea that they may be a new source of stem cells for therapeutic applications.

Kidney. The incidence and prevalence of end stage renal disease (ESRD) continues to increase worldwide. Although renal transplantation represents a good treatment option, the shortage of compatible organs remains a critical issue for patients affected by ESRD. Therefore, the possibility of developing stem cell-based therapies for both glomerular and tubular repair is receiving intensive investigation (*Bussolati, 2009*). Different stem cell

types have shown some potential in the generation of functional nephrons (*Bruce, 2007; Bruno, 2009; Bussolati, 2005; Gupta, 2002; Kramer, 2006; Morigi, 2008 and 2010*) but the most appropriate cell type for transplantation is still to be established (*Murray, 2007*).

The potential of AFS cells in contributing to kidney development has been recently explored. Using a mesenchymal/epithelial differentiation protocol previously applied to demonstrate the renal differentiation potential of kidney stem cells (*Bussolati, 2005*), Sigel demonstrated that AFS cells and clonal-derived cell lines can differentiate towards the renal lineage; AFS cells sequentially grown in a mesenchymal differentiation medium containing EGF and PDGF-BB and in an epithelial differentiation medium containing HGF and FGF4, reduce the expression of pluripotency markers (i.e. Oct4 and c-Kit) and switch on the expression of epithelial (i.e. CD51, ZO-1) and podocyte markers (i.e. CD2AP, NPHS2) (*Sigel, 2009*). AFS cells have also been shown to contribute to the development of primordial kidney structures during in vitro organogenesis; undifferentiated human AFS cells injected into a mouse embryonic kidney cultured ex vivo are able to integrate in the renal tissue, participate in all steps of nephrogenesis and express molecular markers of early kidney differentiation such as ZO-1, claudin and GDNF (*Giuliani, 2008; Perin, 2007*). Preliminary, still unpublished, in vivo experiments show that AFS cells directly injected into damaged kidneys are able to survive, integrate into tubular structures, express mature kidney markers and restore renal function (*Perin, 2008*). These studies demonstrate the nephrogenic potential of AFS cells and warrant further investigation of their potential use for cell-based kidney therapies.

Lung. Chronic lung diseases are common and debilitating; medical therapies have restricted efficacy and lung transplantation is often the only effective treatment (*Loebinger, 2008*). The use of stem cells for lung repair and regeneration after injury holds promise as a potential therapeutic approach for many lung diseases; however, current studies are still in their infancy (*Weiss, 2008*).

AFS cell ability to integrate into the lung and to differentiate into pulmonary lineages has been elegantly investigated in different experimental models of lung damage and development. In vitro, human AFS cells injected into mouse embryonic lung explants engraft into the epithelium and into the mesenchyme and express the early pulmonary differentiation marker TFF1 (*Carraro, 2008*). In vivo, in the absence of lung damage, systemically administered AFS cells show the capacity to home to the lung but not to differentiate into specialized cells; whilst, in the presence of lung injury, AFS cells not only exhibit a strong tissue engraftment but also express specific alveolar and bronchiolar epithelial markers (e.g. TFF1, SPC, CC10). Remarkably cell fusion fenomena were

elegantly excluded and long-term experiments confirmed the absence of tumour formation in the treated animals up to 7 months after AFS cell injection (*Carraro, 2008*).

Pros and cons of Amniotic Fluid Stem (AFS) cells

Many stem cell populations (e.g. embryonic, adult and fetal stem cells) as well as methods for generating pluripotent cells (e.g. nuclear reprogramming) have been described to date. All of them carry specific advantages and disadvantages and, at present, it has yet to be established which type of stem cell represents the best candidate for cell therapy. However, although it is likely that one cell type may be better than another depending on the clinical scenario, the recent discovery of easily accessible cells of fetal derivation, not burden by ethical concerns, in the AF has the potential of opening new horizons in regenerative medicine. Amniocentesis, in fact, is routinely performed for the antenatal diagnosis of genetic diseases and its safety has been established by several studies documenting an extremely low overall fetal loss rate (0.06-0.83%) related to this procedure (*Caughey, 2006; Eddleman, 2006*). Moreover, stem cells can be obtained from AF samples without interfering with diagnostic procedures.

AFS cells represent a novel class of pluripotent stem cells with intermediate characteristics between ES cells and AS cells (*Bayada, 2008; Siegel, 2008*). They express both embryonic and mesenchymal stem cell markers, are able to differentiate into lineages representative of all embryonic germ layers and do not form tumours after implantation in vivo. However, AFS cells have been just recently identified and many questions need to be answered concerning their origin, epigenetic state, immunological reactivity, regeneration and differentiation potential in vivo. AFS cells, in fact, may not differentiate as promptly as ES cells and their lack of tumorigenesis can be argued against their pluripotency.

Although further studies are needed to better understand their biological properties and to define their therapeutic potential, stem cells present in the AF appear to be promising candidates for cell therapy and tissue engineering. In particular, they represent an attractive source for the treatment of perinatal disorders such as congenital malformations and acquired neonatal diseases requiring tissue repair/regeneration. In a future clinical scenario, AF cells collected during a routinely performed amniocentesis could be banked and, in case of need, subsequently expanded in culture or engineered in acellular grafts (*Kunisaki, 2007; Siegel, 2007*). In this way affected children could benefit from having autologous expanded/engineered cells ready for implantation either before birth or in the neonatal period.

Figure 7. *The percentage of AFKL cells as a function of the gestation stage is indicated in the left panel. The middle panel shows the total number of mAFKL cells per embryo equivalent (EE). The right panel indicates the percentage of mAmKL cells among total live cells. Means are represented by bars. Adapted from Ditadi, 2009.*

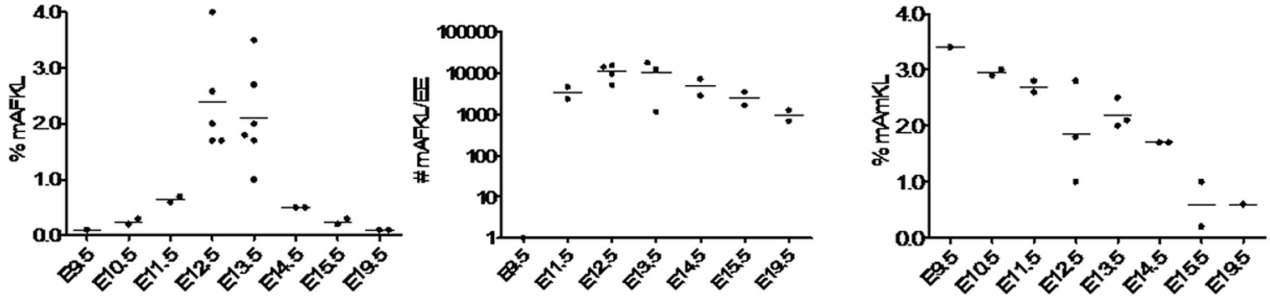


Figure 8. a. *Human AFS cells mainly display a spindle-shaped morphology during in-vitro cultivation under feeder layer-free, serum-rich conditions.* ***b***,***c****. Clonal human AFS cell lines retain long telomeres and a normal karyotype after more than 250 cell divisions.* ***b.*** *Conserved telomere length of AFS cells between early passage (20 population doublings, lane 3) and late passage (250 population doublings, lane 4). Short length (lane 1) and high length (lane 2) telomere standards provided in the assay kit.* ***c****. Giemsa band karyogram showing chromosomes of late passage (4250 population doublings) cells. Adapted from De Coppi, 2007b.*

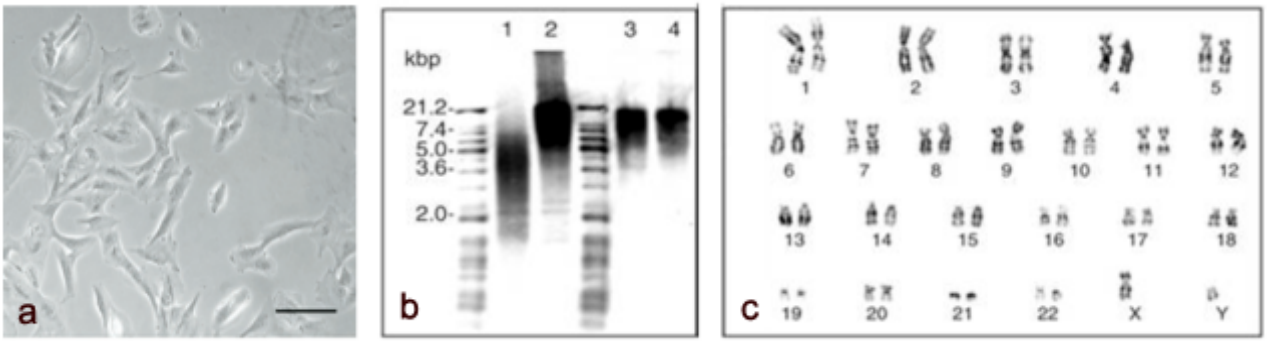


Figure 9. *AFS cells differentiation.* ***a.*** *Hepatogenic: urea secretion by human AFS cells before (rectangles) and after (diamonds) hepatogenic in-vitro differentiation.* ***b.*** *Neurogenic: secretion of neurotransmitter glutamic acid in response to potassium.* ***c.*** *Osteogenic: mouse micro-CT scan 18 weeks after implantation of printed constructs of engineered bone from human AFS cells; arrow head: region of implantation of control scaffold without AFS cells; rhombus: scaffolds seeded with AFS cells. Adapted from De Coppi, 2007b.*

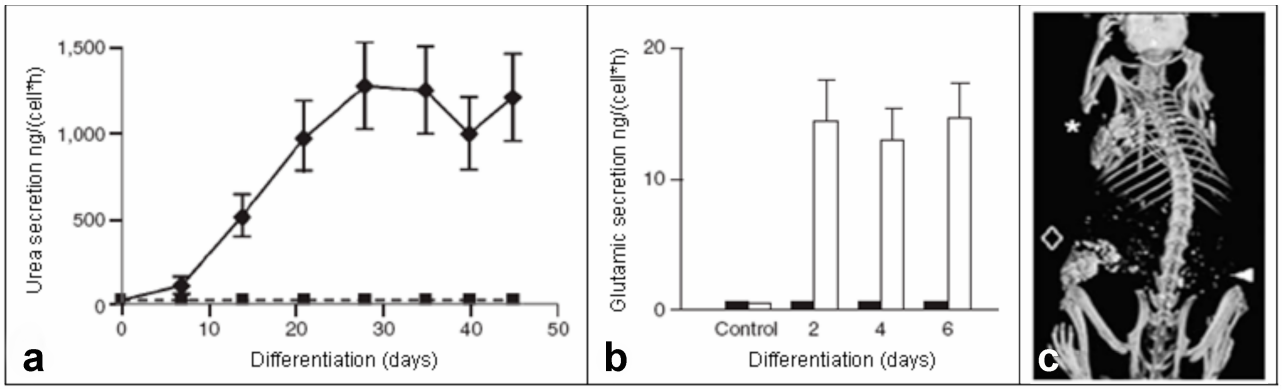


Table 1. *Surface markers expressed by human AFS cells: results by different groups.*

Markers	*Antigen*	*CD no.*	*De Coppi* et al. *(2007b)*	*Kim* et al. *(2007b)*	*Tsai* et al. *(2006)*
ESC	SSEA-3	none	–	+	nt
	SSEA-4	none	+	+	nt
	Tra-1–60	none	–	+	nt
	Tra-1–81	none	–	nt	nt
Mesenchymal	SH2, SH3, SH4	CD73	+	nt	+
	Thy1	CD90	+	nt	+
	Endoglin	CD105	+	nt	+
Endothelial and haematopoietic	LCA	CD14	nt	nt	–
	gp105–120	CD34	–	nt	–
	LPS-R	CD45	–	nt	nt
	Prominin-1	CD133	–	nt	nt
Integrins	β1-integrin	CD29	+	nt	+
Ig superfamily	PECAM-1	CD31	nt	+	nt
	ICAM-1	CD54	nt	+	nt
	VCAM-1	CD106	nt	+	nt
	HCAM-1	CD44	+	+	+
MHC	I (HLA-ABC)	none	+	+	+
	II (HLA-DR,DP,DQ)	none	–	–	–

nt = not tested.

C. Regenerative Medicine as a new strategy for the treatment of NEC

NEC is a devastating disease of newborns for which no specific treatment is currently available. Thus, there clearly is the need of new therapeutic strategies capable either to increase the functioning intestinal surface or to avoid its massive destruction. Among novel scientific frontiers in regenerative medicine, current research efforts are today focused on the evaluation of stem cell strategies to promote tissue repair/regeneration. Although still at initial stages and associated with numerous problematics, ever increasing experimental evidences support the intriguing hypothesis that stem cells on the one hand may modulate the inflammatory cascade that normally leads to irreversible damage in ischemic tissues (*Kale, 2003*; *Dawn, 2005, Janssens, 2006)* and on the other may be possible candidates to treat and/or prevent intestinal diseases *(Khalil, 2007; Srivastava, 2007; Kudo, 2007)*.

Stem cell-based approaches have already been advocated in treatment of inflammatory bowel diseases (IBD), such as Crohn's disease and ulcerative colitis. Following the first report in 1993, in which autologous stem cell transplantation used for haematopoietic malignancy caused regression of Crohn's disease (*Drakos, 1993*), stem cell therapy has become an option for refractory IBD (*Cassinotti, 2008; Garcia-Olmo, 2009*). The means by which bone marrow (BM) derived cells provide clinical benefit is still unclear, as stem cells could act either by immunoregulatory mechanisms, and/or by intestinal regeneration. BM derived cells have an anti-inflammatory effect in both IL-10 knockout mice (*Bamba, 2006*) and experimental colitis (*Tanaka, 2008*). They can also differentiate into epithelial cells of the gastro-intestinal (GI) tract, both in animals (*Krause, 2001*) and humans, in which repopulation of the GI tract epithelia by donor cells is related to the degree of epithelial damage (*Okamoto, 2002*). BM derived cells also integrate in the mucosa in experimental colitis (*Komori, 2005*) where they are involved in repair and formation of blood vessels, contributing to endothelial cells, vascular smooth muscle cells, and pericytes (*Brittan, 2005; Khalil, 2007*). These two mechanisms, namely anti-inflammatory and regenerative, could also operate together, as mesenchymal stem cells (MSCs) topically implanted in inflamed areas not only differentiate into colonic interstitial cells, but also provide VEGF and TGF-β1 to the injured area, which are responsible for fibroblast activation, angiogenesis and tissue repair (*Hayashi, 2008*). Given these promising results in other intestinal diseases, and the lack of clinical options other than medical support and surgery in human NEC, we investigated the potential use of stem cells in experimental NEC.

AIM

The **ultimate objective** of the project is to provide evidences as to whether stem cell strategies employing Amniotic Fluid Stem (AFS) cells may be of therapeutic relevance for the treatment of Necrotizing Enterocolitis (NEC).

Thus, the **main aim** of the study is to evaluate the therapeutic potential of rat AFS cells in a neonatal rat model of NEC.

To this end, the following project outline based on three main phases and consecutive intermediate objectives was pursued during the study period (2008-2010):

Phase	Objective
1	Establishment of rat AFS cell cultures and cell characterization
	Establishment of a neonatal rat model of NEC
2	Evaluation of AFS cell effect on animal morbidity and mortality
	Evaluation of AFS cell effect on gut morphology and function
3	Evaluation of AFS cell mechanism of action

MATERIALS AND METHODS

A. Cell cultures

A1. Amniotic Fluid Stem (AFS) cells

Samples of rat amniotic fluid were derived from time-mated GFP+ transgenic Sprague-Dawley rats at E16. AFS cells were isolated as previously described (*De Coppi, 2007; Ditadi, 2009*) to avoid problems of contamination with cells of different origines as stated in the recent paper by Dobreva and coworkers (*Dobreva, 2010*). Briefly, pregnant rats were sacrificed by CO_2 inhalation and the uterus was extracted by Caesarean section. The uterus was removed and the single fetuses with their membranes were dissected under a stereomicroscope (Leica Microsystems). After isolation of single amniotic sacs, the chorion was carefully removed and the placentas were gently pulled apart. Amnion rupture resulted in AF leakage, which was then harvested with a syringe fitted with a 19G needle (***Figure 10***). AFS cells were selected using c-kit (CD117; BD Biosciences) antibodies bound to sheep anti-rabbit antibodies conjugated with magnetic beads (Dynabeads, Invitrogen). After selection, AFS cells were seeded on non-tissue culture petri dishes (BD Biosciences) at a density of 2×10^3 cells/cm^2 and grown at 37°C with a 5% CO_2 atmosphere in α-MEM medium (Sigma-Aldrich) containing 15% fetal bovine serum (Gibco), 18% Chang medium B (Irvine Scientific), 2% Chang C supplement (Irvine Scientific), 1% streptomycin-penicillin solution (Gibco) and 1% glutamine (Gibco). After expansion, cells were routinely sub-cultured by trypsinization (0.25% trypsin-EDTA solution, Sigma-Aldrich) and not permitted to grow beyond 70% of confluence. Clonal AFS cell lines were generated by the limiting dilution method in non-tissue culture 96-well plates.

Cell morphology was studied using a phase contrast Zeiss Axiovert 135 microscope.

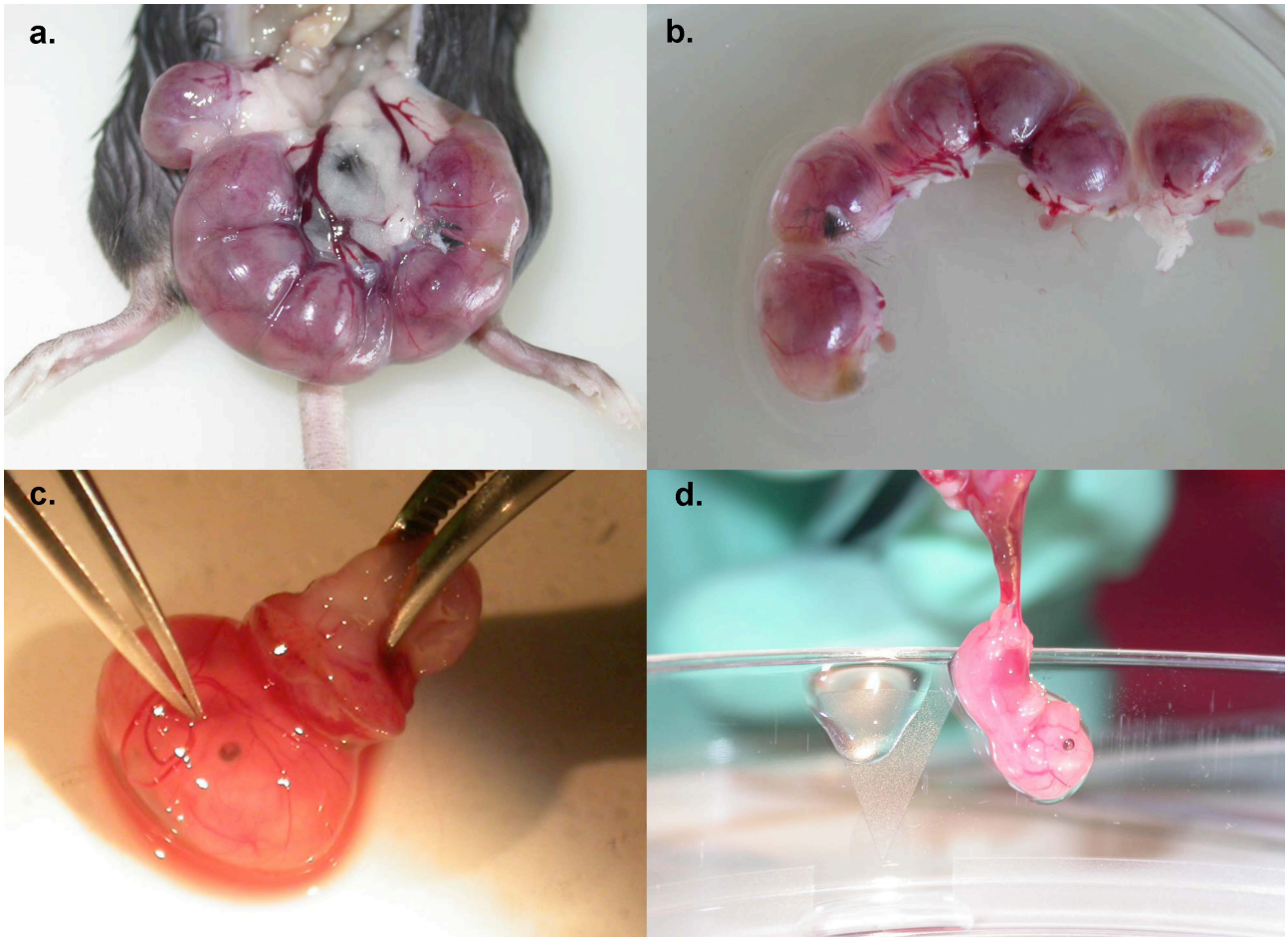
Immunophenotipic characterization was performed by collaborators as recently described (*Bollini, 2010*). AFS cell antigenic profile was determined by immunostaining of cytocentrifugates (cytoplasmic antigens) and flow cytometry (cell membrane antigens).
Cell cytospins were collected using a Shandon Cytospin 4 centrifuge (Thermo Fisher Scientific). Slides were fixed in 4% PFA (Sigma) at room temperature, permeabilized with a

0.1% Triton X-100 (Sigma) solution and then incubated with primary and secondary antibodies. Immunofluorescence was performed employing the following primary antibodies: anti-SSEA4 (mouse monoclonal IgG, Chemicon), anti-Oct 3/4 (rabbit polyclonal IgG, Santa Cruz Biotech), anti-c-kit (anti-CD117, rabbit polyclonal IgG, Santa Cruz Biotech), anti-CD34 (mouse monoclonal IgG, Sigma) anti-CD29 (mouse monoclonal IgG, Chemicon) anti-CD105 (mouse monoclonal IgG, Cymbus Bioscience), anti-CD90 (mouse monoclonal IgG Cymbus Bioscience), anti-Stro-1 (mouse monoclonal IgG Iowa Hybridoma Bank), anti-Flk-1 (mouse monoclonal IgG, Santa Cruz Biotech), anti-Smooth Muscle-Actin (SMA, mouse monoclonal IgG Sigma), anti-NGF receptor (mouse monoclonal IgG, Pharmigen BD Biosciences), anti-pan-cytokeratin (mouse monoclonal IgG, Sigma) and anti-vimentin (mouse monoclonal IgG Dako). Proper Alexa Fluorescence 594-coniugated IgG (Molecular Probes, Invitrogen, Italy) were used as secondary antibodies. Three distinct preparations of cytocenrifugates from AFS cells were examined by two independent operators. Immunofluorescence observations were carried out using a Zeiss Axioplan epifluorescence microscope (Zeiss, Oberkochen, Germany) and acquired by Leica IM 1000 software.

Flow cytometry characterization of AFS cells was performed in triplicates using cells re-suspended in PBS at concentration of 5×10^5 cells/100 using FITC-, PE- or Alexa Fluorescence 647-labeled monoclonal antibodies. The following antibodies were used: anti-CD45 (mouse monoclonal IgG, Immunotech), anti-CD73 (mouse monoclonal IgG, BD Pharmigen, BD Biosciences), anti-MHC I (mouse monoclonal IgG, AbD Serotec) and anti-MHC II (mouse monoclonal IgG, Immunotech). Analysis was performed by a COULTER Epics XL-MCL cytometer (Beckman Coulter) and data were elaborated by means of EXPOTM 32 ADC Software. Data are expressed as number of cells/10^6 cytometric events. Percentage of cells expressing the antigen was evaluated as follows: - 0%; +/- <10%; + 10-30%; ++ 31-60%; +++ 61-90%; ++++ >90%.

After several passages in culture (p15), despite absence of signs of cell transformation (conserved and uniform morphology, stable growth rate), rat AFS cells were subjected to a karyotype assessment following standard procedures (*De Coppi, 2007*)

Figure 10. *Phases of amniotic fluid collection (representative pictures).* ***a.*** *Uterus isolation and dissection.* ***b.*** *Uterus extraction and dissection of foetuses.* ***c.*** *Isolation of single amniotic sacs and chorion removal.* ***d.*** *Amnion rupture and amniotic fluid collection.*



A2. Bone marrow derived mesenchymal stem cells (BM-MSCs)

BM-MSCs were obtained from the femurs of adult Sprague-Dawley donor rats as previously reported (*Iop, 2008*). Briefly, adult rats were sacrificed by CO_2 inhalation and femurs were dissected in aseptic conditions under a stereomicroscope (Leica Microsystems). After removal of epiphysial ends and exposure of the medullary cavity, the bone marrow was flushed out employing a syringe fitted with a 19G needle and containing DMEM (Sigma) plus 1% penicillin/streptomycin (Gibco). Cells were seeded on non-tissue culture petri dishes (BD Biosciences) in DMEM (Sigma-Aldrich) containing 15% fetal bovine serum (Gibco), 2mM Glutamine (Gibco) and 1% penicillin/streptomycin (Gibco). After 3 days, non-adherent cells and debris were discarded and the adherent cells were cultivated until pre-confluency. Adherent cells were detached using a 0.25% trypsin-EDTA solution (Sigma-Aldrich) and passaged up to maximum 9 cell passages. Cell morphology

was studied using a phase contrast Zeiss Axiovert 135 microscope. Flow cytometric analysis was performed at the fifth passage. Cells were detached by adding citrate buffer (0.13M KCl + 0.014M $C_6H_9Na_3O_9/C_6H_5Na_3O_7x2H_2O$, Sigma-Aldrich) and resuspended in PBS at a concentration of $5x10^5$ cells/100 µl. Cells were stained directly with 10 µl of: FITC fluorochrome-labeled anti-rat CD29 or CD44 antibodies (BD Biosciences); PE-conjugated anti-rat CD31, CD73 or CD90 antibodies (BD Biosciences). Cytometric analysis was performed using a COULTER Epics XL-MCL cytometer (Beckman Coulter), and data were elaborated by means of EXPO^{TM} 32 ADC Software. Cell differentiation potential towards the adipogenic lineage was assessed at the fifth passage as previously described (*De Coppi, 2007*). Briefly, BM-MSCs were plated at a density of 3000 cells/cm^2 on tissue culture dishes (BD Biosciences) and cultured in DMEM (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Gibco), 1% penicillin/streptomycin (Gibco), 1% glutamine (Gibco), 1 mM dexamethasone, 1 mM 3-isobutyl-1-methylxanthine, 10 mg/ml insulin and 60 mM indomethacin (Sigma-Aldrich-Aldrich). Medium changes were carried out three times a week and adipogenesis was assessed at 7 and 14 days with Oil red O (Sigma-Aldrich) staining.

A3. Skeletal Myoblasts

Rat skeletal muscle myoblasts purchased from the Health Protection Agency (HPA, UK) . Cells were cultured on tissue-culture dishes (BD Biosciences) in DMEM (Sigma-Aldrich) containing 15% fetal bovine serum (Gibco), 2mM Glutamine (Gibco) and 1% penicillin/streptomycin (Gibco). Plates were incubated at 37°C in a 5% CO_2 atmosphere and repeatedly passaged using a 0.25% trypsin-EDTA solution (Sigma-Aldrich). Cell morphology was assessed using a phase contrast Zeiss Axiovert 135 microscope.

B. Neonatal rat model of NEC

B1. Animal study

The study was approved under the United Kingdom Home Office Regulations for Animals (Scientific Procedures) Act of 1986.

In the process of establishing a rat model of NEC, rats were divided in 2 groups (***Table 2***):

- **Treated group (NEC group)**, consisting of rats delivered by Caesarean section, forcedly fed by gavage with artificial milk and exposed to stress;
- **Control group (Breast fed-BF group),** consisting of rats born via vaginal delivery, breast fed and not subjected to any stress.

NEC group. Time-mated pregnant Sprague-Dawley rats were sacrificed at E21.5 by CO_2 inhalation and foetuses were immediately delivered by Caesarean section. To induce NEC, soon after birth and up to the 4th day of life newborn rats were subjected to:

- **Gavage feeding with hyperosmolar formula:** rats were gavage fed using a 2-Fr silicon catheter (Vygon UK Ltd, UK) 3 times a day, with a hyper-osmolar rat milk substitute prepared using 15 g of SMA Gold (SMA Nutrition, Berkshire, UK) in 75 ml Esbilac canine supplement (Pet-Ag Inc., Hampshire, IL), as described by Barlow (*Barlow, 1974;* ***Figure 11***). This milk formula provided 163 kcal/100 ml, close to mother rat milk which contains 152 kcal/100 ml.
- **Hypoxic stress:** before each feed, rats were subjected to 10 minutes' whole body hypoxic stress induced by inhalation of a gas mixture containing 5% O_2 and 95% N_2, verified by monitoring with an O_2 gas detector (BW O2 Gas Alert Clip Extreme, Rockall Confined & Safety, UK).
- **LPS administration:** on the 1st and the 2nd day of life, rats were gavaged with 4 mg/kg/day LPS (LPS from Escherichia Coli 0111:B4, Sigma-Aldrich) mixed within the formula feed.

NEC induction protocol is summarized in ***Table 3***. Rats surviving over the fourth day of life were gavage fed with hyperosmolar formula until death but no more subjected to hypoxia or LPS administration. Rats were followed up from birth until natural death occurred.
Throughout the experiment, pup rats were kept in a neonatal incubator (Neocare air-controlled incubator, Vickers Medical) to control temperature (30C) and humidity (40%).

Figure 11. *Gavage feeding of a newborn rat.*

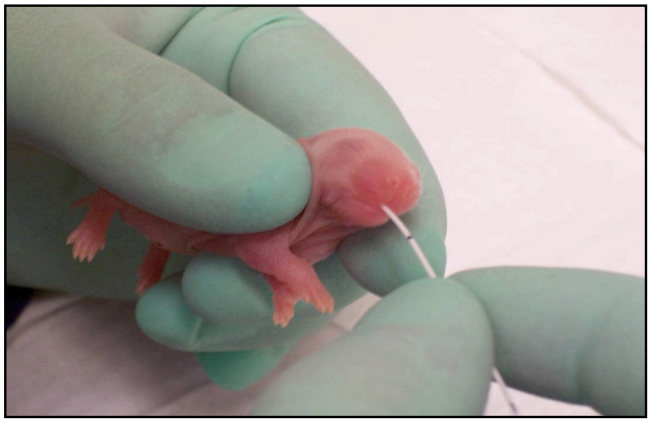


Table 2. *NEC induction protocol.*

	Day 1			Day 2			Day 3			Day 4		
	8 am	4 pm	0 am	8 am	4 pm	0 am	8 am	4 pm	0 am	8 am	4 pm	0 am
Gavage feeding	+	+	+	+	+	+	+	+	+	+	+	+
Hypoxic stress	-	+	+	+	+	+	+	+	+	+	+	+
LPS administration	-	+	-	-	+	-	-	-	-	-	-	-

BF group. Neonates were born at term (E21–22) by vaginal delivery from time-mated pregnant Sprague-Dawley rats. Throughout the experiment, rats were housed with their mothers and allowed breastfeeding ad libitum. Rats were followed up from birth until natural death occurred or up to maximum 3 weeks of life when they were killed by cervical dislocation.

Table 3. *Main differences among rats belonging to the NEC vs. the BF group.*

	NEC group	BF group
Gestational age	E21.5	E21-22
Delivery	Caesarean section	Vaginal delivery
Nutrition	Hyperosmolar milk formula	Mother rat milk
Hypoxic stress	+	-
LPS administration	+	-

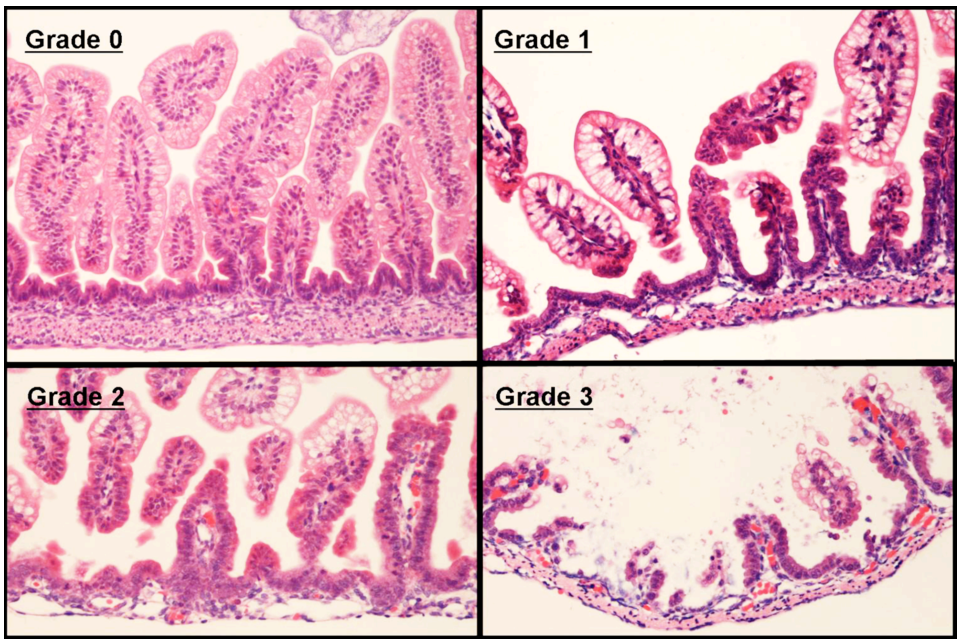
B2. Microscopic gut assessment

NEC incidence, employing the above described protocol, was assessed based on gut histology. NEC ratswere sacrificed via cervical dislocation at 4 different postnatal days (day 1, 2, 3 and 4) and compared with BF rats. Soon after sacrifice, the abdomen was opened and the gut dissected. The ileum, which is the area most affected by experimental NEC in rats (*Dvorak, 2002*), was excised, formalin-fixed, paraffin embedded, and stained for haematoxylin and eosin. Specimens were evaluated microscopically by 2 blinded independent scorers, including a consultant histopathologist, using a modified scoring system (*Nadler, 2000;* ***Table 4 and Figure 12***). NEC was defined as grade 2 or above (average of two independent scorers). Tukey's multiple comparison test was used to compare data from day 1 to day 4. Groups A and B were compared for the presence of NEC on day 4 using Fisher's exact test.

Table 4. *Scoring system for the assessment of gut microscopic appearance in neonatal rats.*

Grade	Description
0	normal intestine
1	disarrangement of villus enterocytes and mild villus core separation
2	disarrangement of villus enterocytes and severe villus core separation
3	epithelial sloughing of the villi
4	bowel necrosis/ perforation

Figure 12. *Histological appearance of the ileum of NEC and BF pup rats, showing representative sections for each grading score (H&E staining; original magnification 200X).*



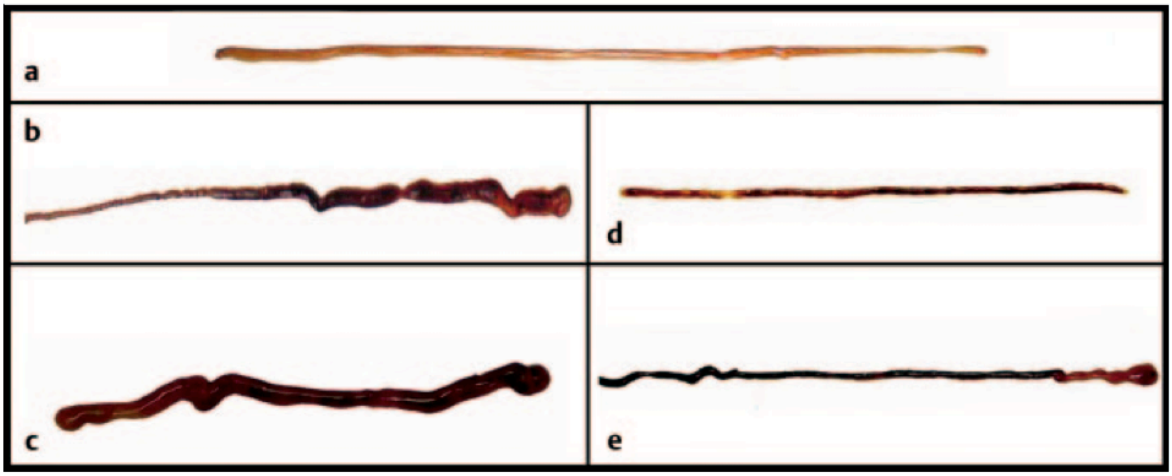
B3. Macroscopic gut assessment

NEC and BF rats from were sacrificed via cervical dislocation at 4 different postnatal days (day 1, 2, 3 and 4). Soon after sacrifice, the abdomen was opened and the gastrointestinal tract from the duodenum to the rectum was dissected free from the surrounding tissue, straightened and put on a PBS filled Petri dish. A macroscopic assessment of the resected gut was performed by 2 blinded and indipendent investigators using a scoring system based on gut colour, consistency and degree of dilation (*Zani, 2008-2;* ***Table 5***). "Gut colour" refers to the presence/absence of areas of discoloration which can be interpreted as less viable zones up to frank necrosis. "Gut consistency" is assessed by the friability encountered when unwrapping the bowel all way through its length. "Gut dilation" refers to the presence/absence of dilated gut segments, which could reflect abnormal gut motility up to paralytic ileus. Examples of macroscopic gut changes in colour and dilation are shown in ***Figure 13***. Tukey's multiple comparison test was used to compare data from day 1 to 4. Histology was used to validate the macroscopic gut assessment. The correlation between the macroscopic gut score and histology was evaluated by linear regression analysis.

Table 5. *Scoring system for the assessment of gut macroscopic appearance.*

	Colour	**Consistency**	**Dilatation**
0	Normal (white/pink)	Normal	No dilatation
1	Patchy discoloration	Moderately friable	Patchy dilatation
2	Extensive discoloration	Extremely friable	Extensive

Figure 13. *Macroscopic appearance of the gut, showing representative pictures of normal and damaged intestine:* ***a.*** *Normal intestine;* ***b.*** *Patchy dilatations;* ***c.*** *Extensive dilatation;* ***d.*** *Patchy discoloration;* ***e.*** *Extensive discoloration.*



B4. Animal clinical status assessment

At 96 hours of life, NEC and BF rats fwere assessed by two blinded and indipendent investigators using a clinical sickness score modified from that previously published by Wolfensohn and coworkers (*Wolfensohn, 2003*) and based on animal appearance, natural activity, response to touch and colour (***Table 6***). “Appearance” assesses rat general conditions. “Natural activity” gives information on rat behaviour in comparison with other rats in the cage: this allows a comparison between animals within a litter, and between litters. On the other hand, “Response to touch” assesses the activity of a single rat, investigating its righting reflex, as by the $3^{rd}/4^{th}$ day of life healthy rats are able to turn immediately. Finally, “Body colour” is a reliable marker of well-being. To assess body colour properly, the animal has to be rolled over and all body segments have to be thoroughly investigated looking for skin discoloration. We did not score body temperature and presence/absence of milk in the stomach, also recommended by Wolfensohn (*Wolfensohn, 2003)* for the following reasons. Body temperature was not considered as neonatal rats in this model are kept in a neonatal incubator at a preset temperature of 30C, and are subjected to temperature shifts when they are gavage fed or given hypoxia out of the incubator. Likewise, the presence/absence of milk in the stomach does not give information on animal well-being in this model as feed volumes and frequency are fixed by the operator. Results from each group were then compared using a Mann-Whitney test.

Animal (BF and NEC rats either treated with PBS or AFS cells) weight at was also evaluated at birth, 24 hours of life and at sacrifice (96 hours of life). Data expressed as (mean ± SD) were analyzed using ANOVA.

Table 6. *Clinical Sickness Score for newborn rats.*

	Appearance	**Natural activity**
0	The pup rat is tonic and well hydrated	The pup rat moves normally in the cage
1	The pup rat is slimmer, but still tonic and hydrated	The pup rat, if put supine, is able to wriggle
2	The pup rat is skinny, floppy and dehydrated	The pup rat, if put supine, is not able to wriggle
3	The pup rat is gasping and in agony	The pup rat does not move its limbs and lays still
	Response to touch	**Colour**
0	The pup rat is alert (without stimulation)	The pup rat skin colour is pink
1	The pup rat responds to mild stimulation	The pup rat skin colour is pale (extremities only)
2	The pup rat responds to vigorous stimulation	The pup rat skin colour is pale (whole body)
3	The pup rat is unresponsive notwithstanding a vigorous stimulation	The pup rat skin colour is grey

C. In vivo experiments

All the following experiments were approved under the United Kingdom Home Office regulations for Animals (Scientific Procedures) Act 1986.

C1. Survival studies employing Amniotic Fluid Stem (AFS) cells

In a first set of experiments (i.e. 2 indipendent experiments), the safety of AFS cells when injected intraperitoneally in newborn BF rats was evaluated. Rat neonates were born at term (E21–22) via vaginal delivery from time-mated pregnant Sprague-Dawley rats. Throughout the experiment, rats were housed with their mothers and allowed breastfeeding ad libitum. At 24 and 48 h of life, rats matched for body weight were randomised to receive an intraperitoneal (i.p.) injection of: (1) 50µl of phosphate buffer saline (PBS); or (2) $2x10^6$ AFS cells diluted in 50µl of PBS. The i.p. injection was performed in the right lower abdominal quadrant with rats in Trendelenburg position (head down and legs up) to decrease the risk of bowel perforation. Rats were followed up from birth until natural death occurred or up to maximum 3 weeks of life when they were killed by cervical dislocation.

In a second set of experiments (i.e. > than 10 indipendent experiments), the potential beneficial effect of AFS cell on animal morbidity ant mortality was evaluated comparing the survival time and the clinical sickness score of NEC rats injected with PBS vs. AFS cells. Further groups of rats were used as controls (i.e. BF rats and/or rats injected with other cell populations). NEC was induced in E21.5 Sprague-Dawley pups delivered by caesarean section, using the above described protocol (paragraph B1). After 24 hours of life, NEC rats were randomised to receive either: cells ($2x10^6$ AFS cells, BM-MSCs or myoblasts in 50µl of PBS) or 50µl of PBS alone at 24 and 48 hours of life via i.p. injection. A further control group consisted of BF animals. Rats were followed up from birth until natural death occurred, or rats were considered moribund, in which case they were sacrificed for ethical reasons. Survival curves were compared by logrank test. At 96 hours of life of the animals, rat clinical status was evaluated by two independent and blinded

investigators employing the validated clinical sickness score described in paragraph A4. Results from each group were compared using a Mann-Whitney test.

C2. Survival study employing medium conditioned by Amniotic Fluid Stem (AFS) Cells

In an indipendent set of experiments (i.e. 3 indipendent experiments), the effect on animal survival of medium conditioned by AFS cells was evaluated comparing mortality of NEC rats injected with AFS cells vs. AFS cell-conditioned medium.

The conditioned medium was prepared as follows:

1) Chang medium was removed from sub-confluent plates of AFS cells and replaced with an equal volume of serum-free medium (α-MEM, Sigma-Aldrich);
2) after 48 hours in culture, α-MEM was collected and filtered with 0.22 μm filters to avoid carry over of cells;
3) the obtained filtered conditioned medium was freshly administered to NEC rats.

NEC was induced in E21.5 Sprague-Dawley pups delivered by caesarean section, using the above described protocol (paragraph B1). After 24 hours of life, NEC rats were randomised to receive either: i. $2x10^6$ AFS cells in 50μl of PBS, ii. 50μl of serum-free medium conditioned by AFS cells (i.e. conditioned α-MEM), iii. 50μl of serum-free medium (i.e. α-MEM) not conditioned by AFS cells and iv. 50μl of PBS alone at 24 and 48 hours of life via intraperitoneal injection. Rats were followed up from birth until natural death occurred, or rats were considered moribund, in which case they were sacrificed for ethical reasons. Survival curves were compared by logrank test.

C3. Survival studies employing Cyclooxygenase (COX) inhibitors

In a first set of experiments (i.e. 2 indipendent experiments), the safety of the administration of COX inhibitors to newborn rats was evaluated. 32 pup rats were born at term (E21–22) via vaginal delivery from time-mated pregnant Sprague-Dawley rats. Throughout the experiment, rats were housed with their mothers and allowed breastfeeding ad libitum. At 24 hours of life, rats were randomly divided in 4 sub-groups receiving by gavage: (i) vehicle (1% DMSO, tid) (n=8); (ii) cox-1 inhibitor (sc-560, 20 mg/

kg, bid) (n=8); (iii) cox-1+2 inhibitor (ibuprofen, 120 mg/kg, tid) (n=8); (iv) cox-2 inhibitor (celecoxib, 60 mg/kg, bid) (n=8). Drug doses were chosen according to data from previous studies (*Menozzi, 2006*), while frequency of administration was decided based on the pharmacokinetic profile of individual molecules. Rats were followed up from birth until natural death occurred or up to maximum 3 weeks of life when they were killed by cervical dislocation.

In a second set of experiments (i.e. 3 indipendent experiments), the effects of selective vs. non selective COX inhibitors on NEC rats receiving PBS or AFS cells were evaluated. Thus, the following survival study was designed:

1) NEC was induced in E21.5 Sprague-Dawley pups delivered by caesarean section, using the above described protocol (paragraph B1);
2) at 24 hours of life, NEC rats were randomised to receive either: $2x10^6$ AFS cells (n=78) or 50µl of PBS alone (n=77) at 24 and 48 hours of life via intraperitoneal injection;
3) at 24 hours of life NEC rats receiving AFS cells and PBS were further equally and randomly divided in 4 sub-groups receiving by gavage: (i) vehicle (1% DMSO, tid); (ii) cox-1 inhibitor (sc-560, 20 mg/kg, bid); (iii) cox-1+2 inhibitor (ibuprofen, 120 mg/kg, tid); (iv) cox-2 inhibitor (celecoxib, 60 mg/kg, bid).

Animal Clinical Sickness Score (assessed at 96 hours and expressed as mean ± SEM) and survival were compared between groups respectively by one-way ANOVA (Kruskal-Wallis test) with Dunns' post-test and by log-rank test.

D. Assessment of intestinal morphology, function and inflammation

During the course of the study, AFS cell eventual beneficial effects on the intestine were evaluated based on three main parameters:

1. Intestinal morphology

2. Intestinal function

3. Gut inflammation

D1. Intestinal morphology

Intestinal morphology was evaluated in situ employing high resolution magnetic resonance imaging techniques (**a**) and, ex-vivo, assessing gut macroscopic (**b**) and microscopic appearance (**c**).

D1a. Magnetic resonance imaging (MRI) of the intestine

MRI studies were performed using a Varian 9.4 T VNMRS 20 cm horizontal-bore system (Varian Inc. Palo Alto, CA, USA), using 100 G cm^{-1} imaging gradients (***Figure 14***). A 26mm quadrature birdcage coil (RAPID Biomedical GmbH, Wurzburg, Germany) was used for both volume transmit and receive. Tuning and matching of the coil was performed manually. Images were acquired at two stages. In stage one, pups were imaged immediately after sacrifice. Multi-slice spin-echo axial images centred on the bowel were acquired at echo times of 15, 20, 30 and 45ms in order to generate T2 maps for clear visualisation of abdominal fluid. Other acquisition parameters were: no of slices =40; in plane matrix size =128 × 128; field of view = 19.2mm × 19.2mm; slice thickness =0.5mm; slice separation =0mm; repetition time =3.5s. T2 maps were generated by fitting the images acquired at increasing echo time to a mono-exponential decay model. In stage 2, 1 pup from each group was imaged using μMRI methods (Cleary, *2009*). Directly after stage 1 imaging, the animals were immersed in 4% formaldehyde solution, doped with 8mM gadolinium-diethylene triamine pentaacetic acid (Gd-DTPA, [Magnevist], Bayer–Schering) and placed on a rotator for 3 weeks. Pups (1 from each group) were scanned using a 3D gradient echo sequence. Acquisition parameters were: matrix size 512×512×512; imaging volume =27 × 27 × 27mm; echo time =6ms; repetition time = 20ms; flip angle =60^{o}; number of averages = 8; 10 dummy scans. From the high resolution

images, bowel wall thicknesses was measured in 5 axial slices in the same plane as the following anatomical landmarks: the top, bottom and midpoint of the right kidney, and the midpoint and bottom of the left kidney. Within these slices, only small bowel sections running perpendicular to the axial slice were considered to minimise partial volume effects. Large bowel sections were excluded from analysis. The thicknesses of bowel walls were evaluated by measuring the shortest distance between the inner diameter to the outer diameter at 4 points. The 4 points per bowel section were: the most dorsal, ventral, lateral (sinister and dexter). The extent of fluid accumulation outside the bowels was estimated by counting the number of voxels with a T2 greater than 160ms in the same 5 slices described above for each animal. Voxels with a T2>160ms within the bowel, bladder and spinal cord and kidney were excluded by manual segmentation based on visual inspection of the images. The threshold of 160ms was chosen based on the lower limit of the 95% confidence interval of the estimated T2 values within a region of fluid in the abdomen. A students T-test was used to test for possible differences between the wall thickness measurements as well as between the T2 voxel counts.

Figure 14. *Experimental MRI scanner (Varian 9.4T) employed in the study. It utilises a much higher magnetic field compared to a typical clinical scanner and acquires images with less than 40 micron resolution.*

D1b. Macroscopic gut assessment

NEC rats treated with PBS and AFS cells were sacrificed at 96 hours of life. Soon after sacrifice, the abdomen was opened and the gastrointestinal tract from the duodenum to the rectum was dissected free from the surrounding tissue, straightened and put on a PBS filled Petri dish. A macroscopic assessment of the resected gut was performed by 2 blinded and indipendent investigators using the scoring system reported in paragraph B3 (*Zani, 2008-2;* ***Table 5*** *and* ***Figure 13***). Data (mean ± SEM) from NEC vs. PBS rats were compared using ANOVA with Tukey post-test.

D1c. Microscopic gut assessment

NEC rats treated with PBS and AFS cells were sacrificed at 96 hours of life. Soon after sacrifice, the abdomen was opened and the gut dissected. The ileum, which is the area most affected by experimental NEC in rats (*Dvorak, 2002*), was excised, formalin-fixed, paraffin embedded, and stained for haematoxylin and eosin. Specimens were evaluated microscopically by 2 blinded independent scorers, including a consultant histopathologist, using the modified scoring system reported in paragraph B2 (*Nadler, 2000; Zani, 2008-2;* ***Table 4*** *and* ***Figure 12***). Data (mean ± SEM) were compared using ANOVA with Tukey post-test. NEC incidence was compared between NEC rats receiving PBS or AFS cells using Fisher's Exact Test.

Gut measures (length, weight, weight/length, mass) were also evaluated (BF rats and NEC rats treated with PBS or AFS cells); data (mean ± SEM) were compared using ANOVA with Tukey post-test.

D2. Intestinal function

During the course of the study, two intestinal functions were evaluated: transit (**a**) and absorption (**b**).

D2a. Gut transit

Rats (i.e. BF and NEC rats injected with either PBS or AFS cells) were gavaged with 0.1ml carmine red (10mg/ml) at 92 hours of life. Four hours later, the whole gut (stomach- rectum) was dissected and the percentage of red stained gut was assessed by

two independent and blinded investigators. Gut transit was considered as complete when at least 90% of intestine was stained red.

D2b. Gut absorption

At 92 h of life, rats from BF and NEC groups were gavaged with a 0.1 ml solution containing mannitol (20 mg/ml), and lactulose (30 mg/ml) in water. Four hours later, all rats were killed, blood was collected, placed in heparinised tubes, and centrifuged at 13,000 rpm at 4°C for 10 min to separate plasma. 10 nmol turanose and 10 nmol xylose were added to 25 ll plasma as internal standards, and proteins precipitated by adding 200 ll acetone. The samples were centrifuged (13,000 rpm 9 10 min) and the supernatants evaporated under N2 at 60°C. To avoid multiple chromatographic peaks for the sugar derivatives, we converted the sugars to oximes before silylation to their trimethylsilyl derivatives. Dried samples were then derivatized, first with 40 ll of 25 mg/ml hydroxylamine hydrochloride in pyridine at 75°C for 30 min, then with 40 ll N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 75°C for a further 30 min. Samples were then analysed by gas chromatography–mass spectrometry as follows: 2 ll derivatized sample was injected at 255°C at a 1:10 split ratio. The initial column temperature was 110°C and this was raised to 320°C over 12 min. The column was a ZB50 column (10 m 9 0.25 mm 9 0.25 lm, Phenomenex, Cheshire, UK) and the GC–MS was a Thermo-Scientific DSQ2 with a Trace GC. Xylose, mannitol, lactulose and turanose were detected at m/z 307, 319, 771 and 800, respectively. The monosaccharide mannitol was quantified using recovery of the monosaccharide xylose and the disaccharide lactulose quantified using the disaccharide turanose, and the lactulose:mannitol ratio calculated as an index of intestinal permeability. Data were not normally distributed, and so were transformed to $\log_{10}$, and compared by one-way ANOVA with Tukey post-test.

D3. Gut inflammation

At sacrifice, the intestine (from the jejunum to cecum) was removed from NEC rats receiving either PBS or AFS cells, and homogenised. Malondialdehyde (MDA; lipid peroxidation marker) and myeloperoxidase (MPO; measure of neutrophil infiltration) were measured as described previously (*Stefanutti, 2005 and 2008; Vinardi, 2003)*. Both MDA and MPO were normalised to protein (*Peterson, 1977*) and compared between groups by

one-way ANOVA with Tukey's post hoc test. MDA quantification and MPO activity assays are briefly reported hereafter.

Quantification of malondialdehyde (MDA). Levels of MDA (a marker of lipid peroxidation) in gut tissue were measured by high-performance liquid chromatography (HPLC). Intestines were homogenized using an Ultra-Turrax homogenizer in 2 mL of 50 mM potassium phosphate buffer (pH 6.0) containing 0.5% (w/v) hexadecyl-trimethylammonium bromide. Protein concentration of the homogenate was measured by the method of Peterson (*Peterson, 1977*). Twenty-five microliters of tissue homogenate was incubated with 2 mL of 0.2% (w/v) butylated hydroxytoluene in ethanol and 375 mL of 1% (v/v) phosphoric acid, and was then derivatized with 345 mL of 15 mM 2-thiobarbituric acid at 100°C for 60 min. Two hundred microliters of the derivatized solution was collected and mixed with 200 mL of methanol. After addition of 15 mL of 1 M KH2PO4 and 4 mL of 2 M KOH/2.4 M KHCO3, samples were centrifuged (13,000 rpm for 10 min at 4°C). HPLC was performed on a Hypersil 5 mODS column at a flow rate of 1 mL/min, isocratically with an eluant of 65% 50 mM KH2PO4 (pH 7.0)/35% methanol. Fluorescence was monitored by a FP-1520 detector (Jasco); excitation wavelength of 515 nm and emission wavelength of 553 nm), and values of molar concentration were calculated by comparison with reference solutions of MDA- tetrabutylammonium salt (Fluka) derivatized and analyzed in parallel. MDA was expressed as micromoles per milligram of protein for tissues and as micromoles per liter for plasma.

MPO activity assay. This biochemical assay is based on the spectrophotometrical measure of MPO reaction products. MPO acts as a catalyst in the production of hypochlorous acid (HOCl), a powerful oxidant produced from chloride ion (Cl^-) and hydrogen peroxide (H_2O_2). The samples were homogenised using an Ultra-Turrax homogeniser in 10 mL 50 mmol/L potassium phosphate buffer, pH 6.0, containing 0.5% (wt/vol) hexadecyltrimethylammonium bromide, 100 uL removed for protein estimation by the method of Peterson (*Peterson, 1977*), and the remainder centrifuged at 40,790*g* for 30 minutes. A total of 100 uL of supernatant then was added to 2.9 mL buffer containing 0.53 mmol/L *O*-dianisidine hydrochloride and 0.0005% hydrogen peroxide and MPO activity followed spectrophotometrically at 25°C at a wavelength of 460 nm. Myeloperoxidase activity was expressed as units (U) per mg protein.

E. IMMUNOHISTOCHEMISTRY

E1. Amniotic Fluid Stem (AFS) cell localisation and differentiation

AFS cell eventual engraftment and localisation in the intestine was evaluated by immunofluorescence employing the following protocol. At 96 hours of life, NEC rats (both treated with PBS and AFS cells) were sacriced and the intestine (jejunum to cecum) was isolated and fixed in formalin overnight at 4°C. After two rinses in PBS, specimens were dehydrated in ethanol and paraffin-embedded for microtome sectioning. After tissue rehydration and heat-induced antigen retrival with citrate buffer (10mM, pH6), slides containing 3 μm thick tissue sections were placed into blocking solution (1%BSA, 0.15% glycine, 0.1% Triton X-100 in PBS) for 30 minutes at room temperature, immunostained with anti-GFP (either raised in mouse or rabbit) and lineage-specific (anti-Cytokeratin, anti-SMA, anti-TujI) antibodies overnight at +4°C (***Table 9***). Anti-GFP antibodies were contourstained using appropriate green (488) fluorescent secondary antibodies while lineage markers were contourstained employing appropriate red (568) fluorescent secondary antibodies (***Table 9***); secondary antibodies, diluted in blocking solution, were separately incubated for 2 hours at room temperature. Slides were finally mounted in Vectashield with DAPI (Vector Laboratories). Negative and positive control slides were used. Sections were viewed with a Zeiss Axiophot microscope attached to a Leica DC500 digital colour camera employing the Leica Firecam software. Images were compiled using Adobe Photoshop CS4.

E2. Villus and crypt cell apoptosis

At 96 hours of life, NEC rats (both treated with PBS and AFS cells) were sacriced and the ileum was isolated and fixed in formalin overnight at 4°C. Immunohistochemistry was performed on 3 μm thick tissue sections. Incubation in primary antibody (i.e. anti-activated caspase-3; ***Table 9***) prepared in 5% heat-inactivated sheep serum, 0.15% glycine, 2% BSA in TBSTX was carried out overnight at 4°C. Following three washes in TBSTX, sections were incubated with a biotinylated secondary antibody (***Table 9***) diluted in 5% normal goat serum, 0.15% glycine, 2% BSA in TBSTX at room temperature for 1 hour. Slides were washed three times in TBSTX, then incubated with the avidin-biotin complex following manufacturer’s protocol (ABC Kit, Vectastain, Vectorlab). After three

washes in PBS, staining was revealed with DAB colorimetric reaction. Negative and positive control slides were used. Sections were viewed with a Zeiss Axiophot microscope attached to a Leica DC500 digital colour camera employing the Leica Firecam software. Images were compiled using Adobe Photoshop CS4. Groups were compared by two blinded investigators using a modified apoptotic index (*Feng 2006; Jilling, 2004*), reported in ***Table 8.*** Data were expressed as mean ± SEM and were compared by unpaired t test.

Table 8. *Scoring system for the assessment of epithelial cell apoptosis.*

Grade	Description
0	no apoptosis
1	few apoptotic cells at the villus tip
2	many apoptotic cells at the villus tip
3	few apoptotic cells in the villus axis
4	many apoptotic cells in the villus axis
5	apoptotic cells in the crypts

E3. Enterocyte migration/proliferation

PBS and AFS cell-injected NEC rats received an intraperitoneal injection of 5-ethynyl-2'-deoxyuridine (EdU) at 72 hours of life (Click-iT® EdU Cell Proliferation Assays, Invitrogen; 100 µg in 40 µl of PBS). Animals were sacrificed 24 hours later (i.e. at 96 hours of life)and the intestine harvested, formalin fixed and processed for EdU detection as described in the manufacturer's instructions (*Salic, 2008*). Negative and positive control slides were used. Sections were viewed with a Zeiss Axiophot microscope attached to a Leica DC500 digital colour camera employing the Leica Firecam software. Images were compiled using Adobe Photoshop CS4. Cell migration was calculated as described by Feng et al. (*Feng, 2007*) by measuring the distance from the base of the villus crypt to the foremost labelled enterocyte (FLE) and expressing this distance as a percentage of the mucosal thickness (FLE/total mucosal thickness X 100). Data were expressed as mean ± SEM and were compared by unpaired t test.

E4. COX-2 immunohistochemistry

At 96 hours of life, BF and NEC rats (both treated with PBS and AFS cells) were sacriced and the ileum was isolated and fixed in formalin overnight at 4°C.

Immunohistochemistry was performed on 3 μm thick tissue sections as previously described (see above paragraph E1) employing an anti-COX antibody and an appropriate red fluorescent secondary antibody (***Table 9***). Negative and positive control slides were used. Sections were viewed with a Zeiss Axiophot microscope attached to a Leica DC500 digital colour camera employing the Leica Firecam software. Images were compiled using Adobe Photoshop CS4. COX-2 positive cells in the lamina propria were counted under 63x magnification in at least 20 randomly chosen villi per rat by two independent and blinded investigators (n=8 in the BF group, n=8 in the PBS group, n=7 in the AFS cell group). Data were expressed as mean ± SEM and were compared by one-way Anova with Tukey's post-test. The average number of COX-2 positive cells per crypt in PBS vs. AFS cell NEC rats was correlated with the degree of intestinal histological damage by linear regression. Villus height was calculated from the villus tip to the crypt bottom under 63x magnification (n=58 in the BF group, n=56 in the PBS group, n=50 in the AFS cell group); data were compared by one-way Anova with Dunns' post-test and were expressed as mean ± SEM.

Table 9. *Details of primary and secondary antibodies employed in immunohistochemistry experiments.*

Antibody	Species	Brand	Dilution
Primaries			
anti-smooth muscle actin (SMA)	mouse	Dako	1:200
anti-cleaved caspase 3	rabbit	Cell Signalling	1:1000
anti-cyclooxygenase 2 (COX2)	mouse	BD Biosciences	1:50
anti-cytokeratine wide spectrum (CTK)	rabbit	Dako	1:100
anti-green fluorescent protein (GFP)	mouse	Invitrogen	1:200
anti-green fluorescent protein (GFP)	rabbit	Invitrogen	1:200
anti-neuron specific class III β-tubulin (TuJ1)	mouse	Covance	1:500
Secondaries			
anti-mouse 568 Alexa Fluor	goat	Molecular Probes	1:250
anti-rabbit 568 Alexa Fluor	goat	Molecular Probes	1:250
anti-mouse 488 Alexa Fluor	goat	Molecular Probes	1:250
anti-rabbit 488 Alexa Fluor	goat	Molecular Probes	1:250
anti-rabbit biotinylated	goat	Cell signalling	1:250

F. Molecular biology

F1. Genomic DNA extraction and *gfp* amplification

At sacrifice of BF and NEC rats injected with AFS cells (i.e. 96 hours of life), brain, lungs, heart, liver, spleen, kidneys, intestine and femur were removed and homogenizated. Genomic DNA was extracted employing the DNeasy cell and tissue kit (Qiagen) and quantified with a ND-1000 spectrophotometer (Nanodrop). DNA extracted from GFP+ rat AFS cells and organs of pup rats injected with PBS served respectively as positive and negative controls. *Gadph* was amplified in all samples prior to *gfp* to confirm the quality of the extracted DNA. Primer sequences were selected using PrimerExpress software (ABI PRISM): **gfp-fw** tga acc gca tcg agc tga agg g (Tm 65°C), **gfp-rv** tcc agc agg acc atg tga tcg c (Tm 65°C), **gadph-fw** ttg tgc agt gcc agc ctc g (Tm 66°C), **gadph-rv** tgc cgt tga act tgc cgt g (Tm 66°C). PCR reactions were carried out in duplicates in a reaction volume of 50 µl containing 20mMTris–HCl (pH 8.4), 50mM KCl, 1.25mM MgCl2, 0.2mM dNTPs, 25pmol of each primer and 2.5 U of recombinant Taq polymerase (Invitrogen). As template, 100 ng of genomic DNA were used. PCR was carried out as follows: initial denaturation at 94°C for 2 min; 40 amplification cycles of 30 s at 94°C, 30 s at 65°C, 45 s at 72°C; final extension at 72°C for 10 min. PCR products were separated on 2% agarose gels and visualized with ethidium bromide staining.

F2. Microarray-based gene expression analysis

A microarray-based gene expression analysis was performed as previously reported (D'Arrigo, 2005 and 2010; Marchet, 2007) to compare the intestinal transcriptional profile of NEC rats treated with PBS vs. AFS cells.

RNA extraction. After sacrifice of the animals at 96 hours, the entire intestine (jejunum-cecum) was isolated from NEC rats belonging to the PBS and the AFS cell group. A third group of BF rats was used as reference. Samples were snap-frozen in liquid nitrogen immediately after collection using RNase-free vials without other protective solutions. After tissue homogenization with a rotor-stator homogenizer with disposable probe tips (Ultra-Turrax, Ika), total RNA was extracted with TRIzol Reagent (Invitrogen) and quantified with a ND-1000 spectrophotometer (Nanodrop). RNA quality was assessed on the basis of the A260/280 ratio and through separation of 28S and 18S ribosomal bands on agarose gels.

Prior to gene expression analysis, contaminant genomic DNA was digested with DNase I (Sigma), according to manufacturer's instructions.

Array construction. Duplicated cDNA arrays comprising 3734 rodent genes (selected on the basis of their relevance to processes such as inflammation, apoptosis and cell cycle regulation) were assembled onto mirrored aminosilane-coated Amplislide (GeneWave) using a Lucidea Spotter (GE Healthcare). The cDNAs were purchased as purified, sequence-verified PCR products from RZPD (Berlin, Germany) and were diluted in 50% DMSO (list of genes available in table x). After deposition, slides were exposed to a brief pulse of UVC light to stabilize DNA attachment to the slide surface.

Probe preparation, hybridization and laser scanning. A dendrimer-based labeling system (3DNA Array50 version 2, Genisphere) was used for the preparation of fluorescent cDNA from intestinal samples and for slides hybridization. Briefly, 20 µg of total RNA were reverse transcribed into cDNA using 5'-tagged oligo$(dT)_{17}$ primers. Cy3 and Cy5 dyes were respectively used for test (PBS and AFS cell NEC guts) and reference sample labeling. A pool of total RNAs, deriving from the intestine of BF rats, was employed as a reference sample. The tagged cDNAs were hybridized with the arrays overnight, at 50°C, in a humidified chamber. The slides were then sequentially washed as follows: 2X SSC+0.2% SDS at 50°C, 2X SSC at room temperature and 0.2X SSC at room temperature (10 min per washing step). Each slide was rapidly dried by centrifugation and then laser-scanned with a Gen III Laser Scanner (Amersham-Pharmacia/Molecular Dynamics). Images were visualized and signals quantified by Array Vision (Imaging Research).

Data analysis. GeneSpring GX software (Agilent Technologies) was used for filtration and normalization of raw data and for statistical analysis. Data were expressed as sample-to-reference $\log_2$ ratios and LOWESS normalized. Normalized and filtered data were analysed by t-test and variations with a $p<0.05$ were ranked based on their average fold variation between the PBS and the AFS cell group. Heat maps were generated by centroid-linkage hierarchical clustering employing the Pearson correlation for distance measure.

RESULTS

A. Establishment of cell cultures

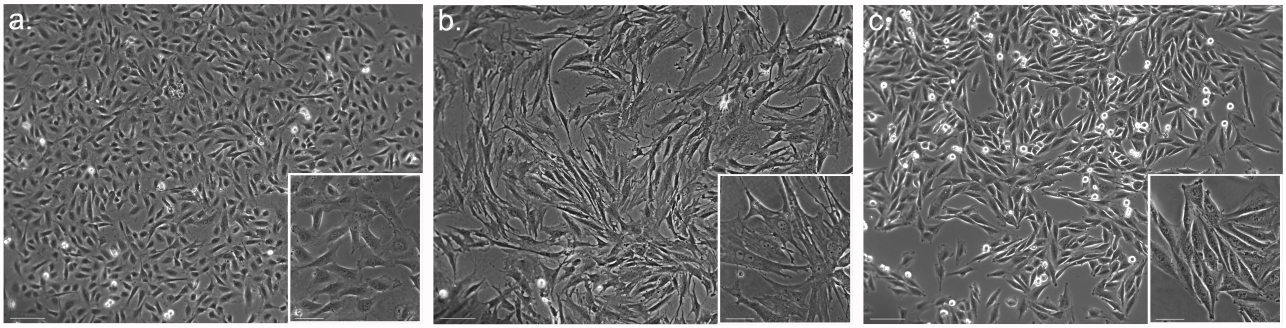
Three cell types were employed in the study (***Figure 15***):

1. Amniotic Fluid Stem (AFS) cells

2. Bone marrow-derived mesenchymal stem cells (BM-MSCs)

3. Skeletal Myoblasts

Figure 15. *Cell morphology as assessed using a phase contrast microscope:* ***a.*** *AFS cells.* ***b.*** *BM-MSCs.* ***c.*** *Skeletal myoblasts. 100µm scale bar in the 10x magnification photo; 50µm scale bar in the 32x magnification photo.*



A1. Amniotic Fluid Stem (AFS) cells

AFS cells were derived from time-mated GFP+ transgenic Sprague-Dawley rats at E16. Cells and clones could be readily expanded in culture as stable lines showing no evidence of spontaneous differentiation in vitro. Cells were characterized by morphology and immunophenotype. AFS cells displayed a homogeneous and constant morphology in culture (***Figure 16a***). Antigen profile was determined by immunofluorescence staining of cytocentrifugates and by flow cytometry analysis. According to other authors (*De Coppi, 2007; Ditadi, 2009; Kim, 2007; Tsai, 2006*), AFS cells: i. consistently expressed embryonic stem cell markers (SSEA4 and Oct 3/4) and mesenchymal stem cell markers (CD29, CD90, CD73, CD105, CD166, vimentin); ii. did not express endothelial and haematopoietic markers (CD34 and CD45); iii. expressed MHC I and MHC II at low levels (***Figure 16b***). After several passages in culture (p15), despite absence of signs of cell transformation (conserved and uniform morphology, stable growth rate), rat AFS cells were subjected to a

karyotype assessment which did not show obvious chromosomal rearrangements as judged by Giemsa banding (***data not shown***).

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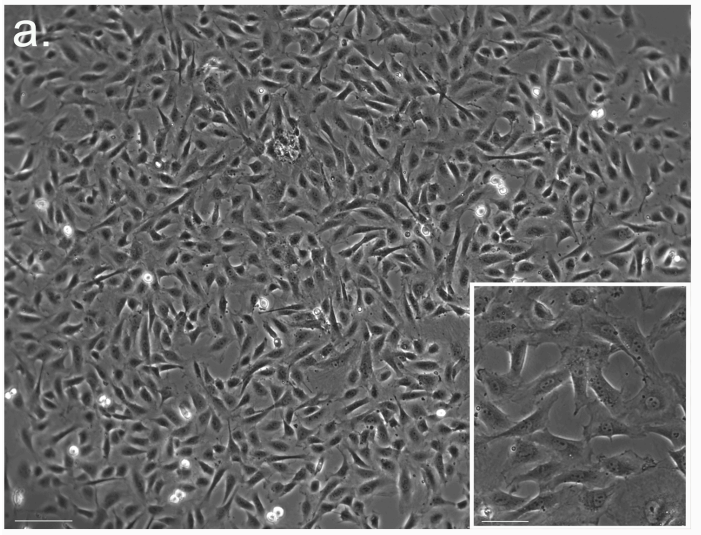
Figure 16. Characterization of AFS cells. a. *Morphology. Cells homogeneously displayed a characteristic AFS cell appearance (De Coppi, 2007). Cells were assessed at each passage using a phase contrast microscope (100μm scale bar in the 10x magnification photo; 50μm scale bar in the 32x magnification photo).* ***b.*** *Antigen profile was determined by immunofluorescence staining of cytocentrifugates and by flow cytometry analysis. Percentage of cells expressing the antigen was evaluated as follows: - 0%; +/- <10%; + 10-30%; ++ 31-60%; +++ 61-90%; ++++ >90% (adapted from Bollini, 2010).*



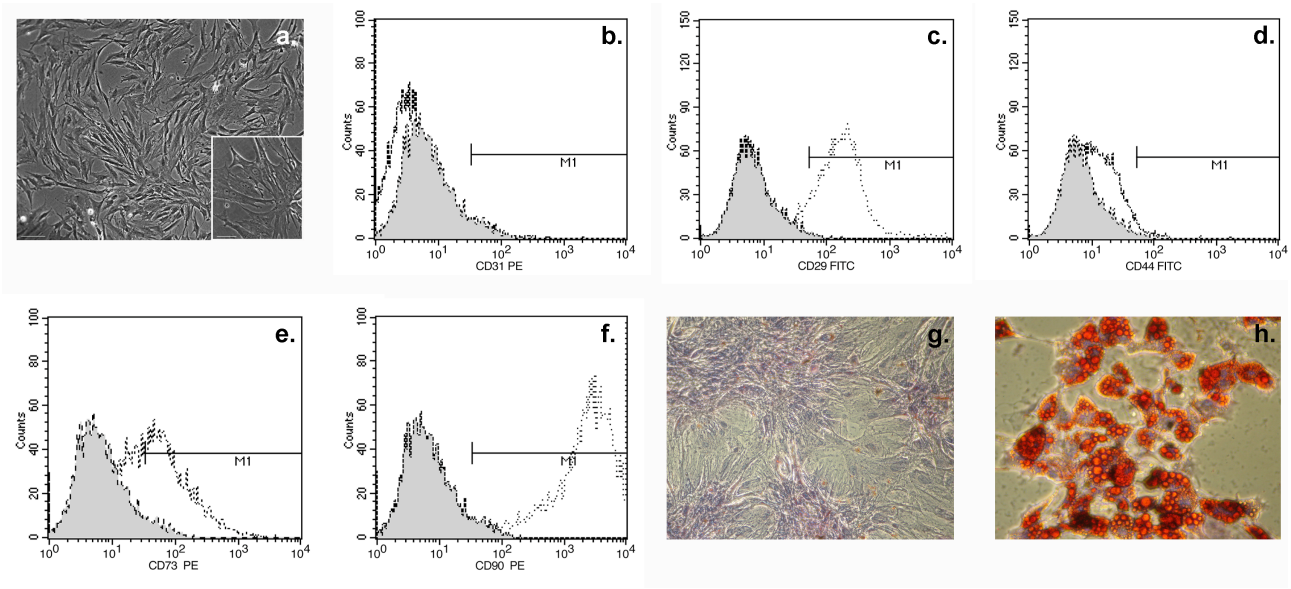
b.

Antigen	GFP-rAFS cells
c-kit	–
OCT ¾	+
SSEA4	++++
CD34	–
CD45	–
CD29	++++
CD105	++++
CD90	+/–
CD73	+/–
Stro-1	–
NGFr	+++
Flk-1	++++
αSMA	+++
Vimentin	++++
Pan-cytokeratin	–
MHC I	+/–
MHC II	+/–

A2. Bone Marrow-derived Mesenchymal Stem Cells

BM-MSCs were derived from femurs of adult Sprague-Dawley donor rats. Cells, cultured up to maximum 9 passages, were characterized by morphology, immunophenotype and differentiation potential. The obtained cell populations homogeneously displayed a characteristic MSC morphology (i.e. large, round nucleus with a prominent nucleolus and a small cell body with long processes) (***Figure 17a***). By flow-cytometric analysis, cells resulted negative for endothelial markers (CD31) and positive for mesenchymal stem cell markers (CD29, CD44, CD73, CD90) (***Figure 17b-f***). Cell multipotency was assessed demonstrating cell capacity to differentiate towards mesenchymal lineages (i.e. adipogenic lineage) under appropriate conditions (***Figure 17g-h***).

Figure 17. Characterization of BM-MSCs. a. *Morphology. Cells homogeneously displayed a characteristic MSC morphology. Cells were assessed at each passage using a phase contrast microscope (100µm scale bar in the 10x magnification photo; 50µm scale bar in the 32x magnification photo).* ***b-f.*** *Immunophenotype. BM-MSCs resulted negative for endothelial markers (CD31;* ***b****) and positive for mesenchymal stem cell markers (CD29, CD44, CD73, CD90;* ***c,d,e,f****). The control isotype is indicated in light gray.* ***g-h.*** *Mesenchymal differentiation potential. BM-MSCs resulted able to differentiate towards mature adipocytes as assessed with Oil red O staining on untreated (****g.****) and treated (****h.****) cells (20x).*



A3. Skeletal Myoblasts

A line of committed cells, i.e. rat skeletal muscle myoblasts, was used as a supplementary negative control vs. AFS cells during in vivo experiments (***Figure 15c***). These cells, purchased from the Health Protection Agency (HPA, UK) and expanded in Culture following the Manufacturer instructions, constantly maintained their original morphology and behavior.

B. Establishment of a consistent and reproducible rat model of NEC

Among the various experimental models of NEC described in the past 40 years, the one proposed by Barlow in 1974 remains the most reliable, as it reproduces the main contributory factors to the development of human NEC: 1) intestinal immaturity, 2) hyperosmolar feeding, 3) stress, and 4) bacteria or bacterial products. This model mainly consists in the administration of bacteria (i.e. Klebsiella spp.), hyperosmolar formula and hypoxic stress to newborn rats during their first 4 days of life (*Barlow, 1974*) and leads to a NEC-like gut damage. However, NEC reproducibility using this model varies among different studies with a prevalence ranging from 35% to 71% (*Grishin, 2006; Zamora, 2005*). In our experience, the incidence of NEC was even lower using this protocol and it appears that its success or failure in inducing NEC depends on the microbiological cleanliness of animal facilities. To overcome this source of variability and increase the severity of bowel damage in experimental NEC, we decided to administer oral bacterial lipopolysaccharide (LPS) mimicking the bacterial load that occurs in human babies affected by NEC. This approach as been used by other authors in similar NEC models (*Feng, 2006*).

Thus, we established a LPS-modified gavage model of NEC, closely resembling the one described by Barlow, in newborn rats based on:

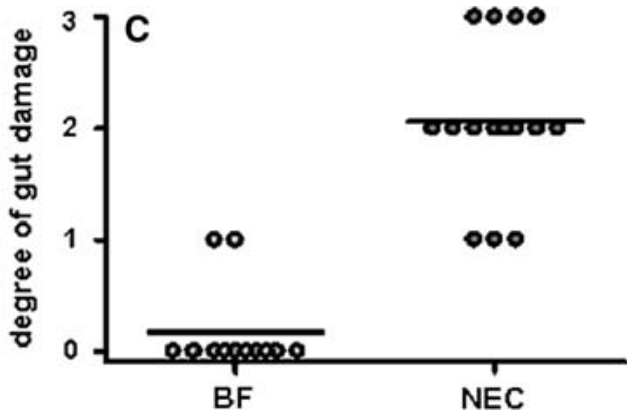
- term delivery by caesarean section (term rats born with an immature intestine similar to that of human preterm babies)
- gavage feeding with hyperosmolar formula
- hypoxic stress
- LPS administration

The consistency and reproducibility of our model, as well as NEC incidence, were assessed comparing control animals (**Breast fed-BF group**) vs. animals with induced NEC (**NEC group**) based on three main parameters: 1) gut microscopic appearance, 2) gut macroscopic appearance, 3) animal clinical status. Of these, gut histology is recognised as the “gold standard” to assess NEC and the severity of intestinal damage, whilst gut macroscopic appearance and animal clinical status are novel tools that we established and validated in order to facilitate the experimental design (i.e. to predict NEC presence/severity without the need of always assessing histology) (*Zani, 2008-2*).

B1. Gut microscopic appearance

The histological incidence of NEC was assessed by 2 blinded independent scorers, including a consultant histopathologist, using a modified scoring system (*see Materials and Methods, paragraph B2*). At 96 hours of life of the animals (i.e. end of NEC induction), the severity of bowel damage of NEC rats (n= 15) was significantly higher than in BF rats (n=12) (mean 2.1 ± 0.2 vs. 0.2 ± 0.1; $p < 0.0001$). Moreover, NEC was not present in any of BF rat, whereas in the NEC group the disease incidence was 81%. Finally, none of the NEC group rats had a histologically normal bowel (grade 0), which was present in 83% of BF rats. Two BF rats had very minor gut histological changes, with no pathological significance (**Figure 18**). Moreover, in a different experiment considering the progression of intestinal damage over time, we observed that NEC was not present in any BF rat at any of the 4 time points (n=3 for each time point), while, conversely, NEC (grade 2 or above) was seen in 50% of NEC rats from day 3 onwards, and in 75% of rats at sacrifice on day 4 ($p < 0.05$) (n=9 on day 1, n= 9 on day 2, n=8 on day 3, n=5 on day 4). We admit that only few NEC rats showed a grade 3 of intestinal damage (i.e. bowel perforation/ necrosis) that is a severity degree most similar to severe human NEC. However, our results overlap what experienced in the literature by other authors who hardly achieved severe patterns of intestinal injury. Moreover, in view of translating laboratory findings on experimental NEC to the bedside, a grade 3 intestinal damage (bowel necrosis/ perforation) benefit from surgery more than any pharmacological agent or cellular therapy. Therefore, we believe that a grade 2-3 intestinal damage is sufficient to experiment alternative strategies in patients with moderate NEC, which corresponds to Bell stage 1-2 human of NEC.

Figure 18. *Degree of histological gut damage scored for BF and NEC rats. NEC was defined as an average grade of ≥2. The severity of bowel damage in the NEC group was significantly higher than in BF rats (mean 2.1 ± 0.2 vs. 0.2 ± 0.1; p <0.0001). Picture adapted from Zani-Cananzi, 2010.*

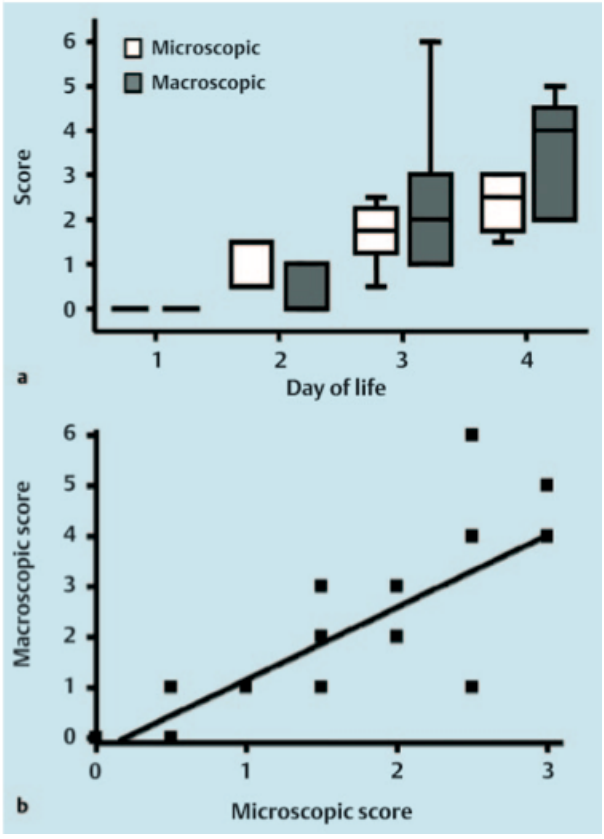


B2. Gut macroscopic appearance

A macroscopic assessment of the resected gut was performed by 2 blinded and independent investigators using a scoring system based on gut color, consistency and degree of dilatation (*see Materials and Methods, paragraph B3*). The macroscopic appearance of the gut of BF rats (n=3 for each time point) did not show any signs of bowel damage at any of the four time points. Conversely, NEC rats (n=9 on day 1, n= 9 on day 2, n=8 on day 3, n=5 on day 4) showed a significant increase in macroscopic bowel injuries from day 2 onwards ($p<0.0001$; ***Figure 19a***). Gut macroscopic and microscopic scores proved to be strongly correlated ($r^2=0.74$, $p<0.0001$; ***Figure 19b***).

Thus, the only difference between gut macroscopic and microscopic appearance was that gut damage was evident macroscopically from day 2, and, as expected, it was microscopically evident at an earlier stage (from day 1).

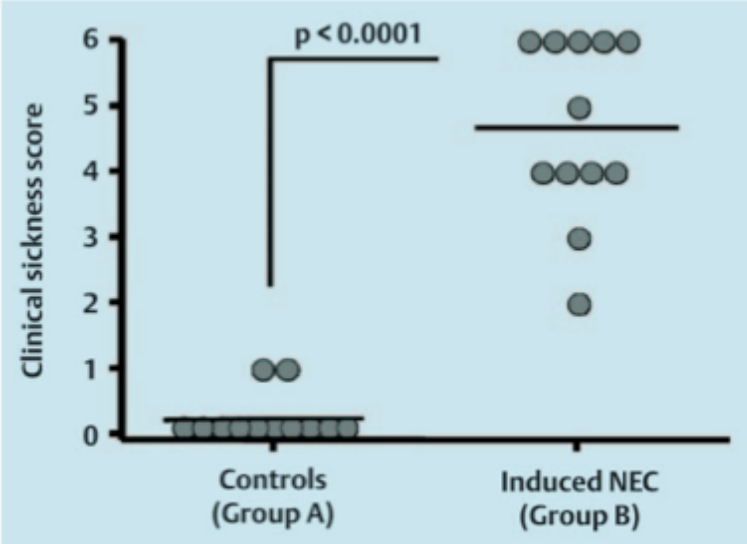
Figure 19. a. *Increase of intestinal changes detected by macroscopic assessment ($p<0.0001$ from day 2) and by microscopic assessment at histology ($p<0.0001$ from day 1) (0 = best, 6 = worst).* ***b.*** *When analyzed on the same specimens, the macroscopic and histologic scores showed a significant correlation as assessed by linear regression ($r^2 = 0.74$, $p < 0.0001$). Picture adapted from Zani-Cananzi, 2008-2.*



B3. Animal clinical status

At 96 hours of life, rats from the BF (n=12) and the NEC (n=12) groups were assessed by two blinded and indipendent investigators using a modified clinical sickness score based on animal appearance, natural activity, response to touch and colour (*see Materials and Methods, paragraph B4*). The clinical sickness score of NEC rats (median=4.5; range = 2 – 6) was higher than that of BF rats (median = 0; range=0–1; p<0.0001) (***Figure 20***).

Figure 20. *Clinical status of BF and NEC animals assessed by two blinded investigators using a clinical sickness score (0 = best, 12 = worst). NEC rats showed a worse clinical status in comparison to BF rats. Picture adapted from Zani-Cananzi, 2008-2.*



In conclusion, we established a consistent and reproducible rat model of NEC with a 70% incidence of gut disease at the end of the treatment. Moreover, we validated two novel parameters (i.e. gut macroscopic appearance and clinical status), other than histology, to judge rat well-being and occurrence of NEC with no need to process gut specimens for histology, thus allowing a higher throughput for testing new therapies.

C. The intraperitoneal administration of AFS cells to healthy newborn rats is a safe procedure and allows stem cell migration, homing and integration into various organs including the gut.

To assess the safety of AFS cell injection in vivo, 24 healthy newborn rats born at term (E21–22) via vaginal delivery were randomised to receive, at 24 and 48 hours of life ,an intraperitoneal (i.p.) injection of: (1) 50µl of phosphate buffer saline (PBS group, n=12); or (2) $2x10^6$ AFS cells diluted in 50µl of PBS (AFS group, n=12). Both groups of rats were daily monitored from birth up to 3 weeks of life. During the 21 days of observation, none of the animals injected with PBS or AFS cells died or showed adverse effects related to the procedure (i.e. i.p. injection) or to cell administration.

We then investigated if AFS cells were able to diffuse systemically after 48–72 h from the i.p. injection. This was assessed through amplification of the *gfp* gene, specific to AFS cells, in the genomic DNA extracted from organs of rat injected with AFS cells and sacrificed at 96 h of life (n=12). *Gadph* was amplified in every sample, indicating that genomic DNA had been properly extracted from every specimen (data not shown). G*fp* signal was detected in at least one organ in all pup rats except one (11 out of 12; ***Figure 21a and b***). None of the PBS injected rats (controls) resulted positive for *gfp*. In the majority of cases, *gfp* signal was detected in organs of the abdominal compartment. The intestine was the organ with the major occurrence of positivity (8 pup rats out of 12) followed by kidneys (4 rats out of 12), liver and spleen (3 and 2 animals, respectively out of 12). In a minority of cases, also organs of the thoracic compartment resulted positive for *gfp*: both heart and lungs in two rats out of 12. Only in one animal out of 12 the *gfp* gene was amplified in the DNA extracted from the femur. *Gfp* was never detected in DNA deriving from brain homogenates (***Figure 21 b and c***).

Finally, we investigated if, after their initial systemic spreading, AFS cells were able to integrate in tissues. This was assessed through immunohistochemistry for GFP performed on organs collected from rats injected with AFS cells and killed at 21 days of life. Confirming PCR results, GFP+ AFS cells were detected in different thoracic and abdominal organs (e.g. lung, liver; ***Figure 21d***), whereas cell integration was never observed in brain sections.

In conclusion, we demonstrated that, in the short term, the i.p. administration of AFS cells is a safe procedure and allow their migration, homing and integration into different

organs. In particular, AFS cells preferentially localized in the intestine, which resulted positive for *gfp* in more than 60% of the animals.

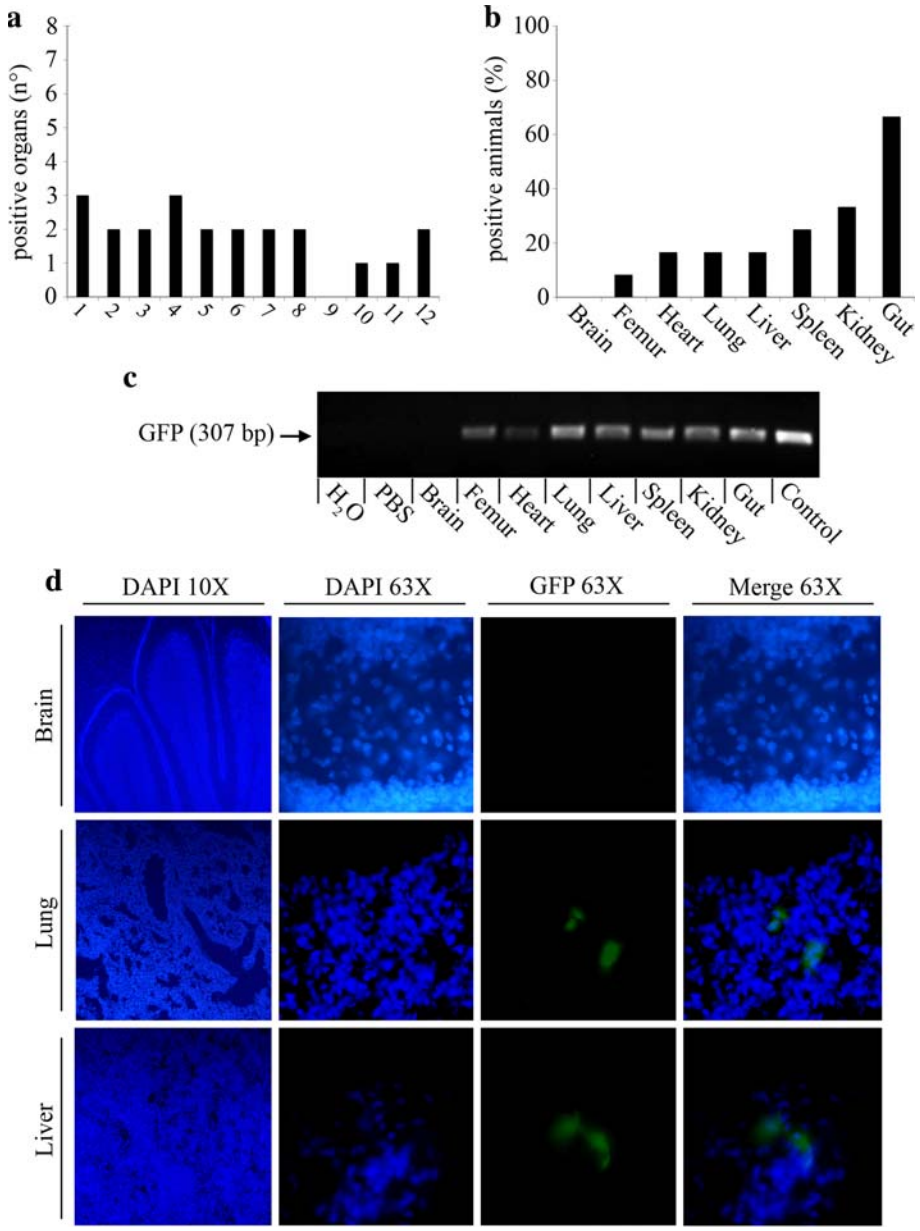


Figure 21. *Results of PCR analysis and immunohistochemistry for GFP on organs collected respectively at 96 h and at 3 weeks, of life of the animals.* ***a.*** *PCR analysis. The chart shows, on the x-axis individual treated animals and, on the y-axis, the number of gfp positive organs. Gfp signal was detected in at least one organ in all pup rats except one.* ***b.*** *PCR analysis. Gfp was never detected in the DNA deriving from brain homogenates. Organs of the thoracic compartment resulted positive for gfp in a minority of cases. Gfp was mainly amplified in the DNA deriving from organs of the abdominal compartment.* ***c.*** *PCR analysis. Representative agarose gel showing amplification of the gfp gene (307 bp) in different organs of rats injected with AFS cells. Control=gfp amplification in the genomic DNA extracted from GFP⁺AFS cells (positive control). PBS= absent gfp detection in the DNA extracted from organs of rats injected with PBS (negative control).* ***d.*** *Immunohistochemistry. Immunofluorescence for GFP on cryosections. AFS cell integration was never observed in brain. GFP⁺AFS cells were detected in different thoracic (e.g. lungs) and abdominal organs (e.g. liver). Picture adapted from Ghionzoli-Cananzi, 2010.*

D. AFS cells improve mortality and morbidity in rats with NEC by preserving gut function.

NEC rats injected intraperitoneally (i.p.) with BM-MSCs (n=26) at 24 and 48 hours of life did not show improved survival compared with animals injected with phosphate buffered saline (PBS; n=30) (***Figure 22a***). Age matched breastfed rats (BF= 12) not subjected to the NEC induction protocol and used as controls, had 94% survival ($p< 0.0001$) (***Figure 22a***). Considering this lack of effect, we therefore tested AFS cells (n=40), comparing them with BM-MSCs (n=17), BF (n=24) and PBS (n=43) (***Figure 22b***). A further group of animals, injected with a line of committed cells (myoblasts; n=21) was also used as a negative control **(*Figure 22b*)**. NEC rats injected with AFS cells survived significantly longer than rats injected with PBS ($p<0.0001$), myoblasts ($p<0.0001$), or BM-MSCs ($p=0.024$) (***Figure 22b***). Moreover, there were no differences between PBS, myoblast, and BM-MSC groups (p=n.s.; ***Figure 22b***). Finally, BF rats survived significantly longer than all other groups ($p<0.0001$), confirming that NEC induction is a source of mortality. This beneficial effect of AFS cells was proven to be robust and reproducible, when tested on a large number of animals (AFS cells, n=121 vs. PBS n=120 $p<0.0001$, ***Figure 22c***). When animals were evaluated at 96 hours of life by 2 independent and blinded investigators using the validated scoring system clinical sickness score previously described (Materials and Methods, paragraph B4), we found that AFS cells improved clinical status of NEC rats (AFS cells 2.0 ± 1.6 vs. PBS 3.7 ± 2.1; $p<0.01$; ***Figure 22d***). However, both NEC rats treated with AFS cells and those treated with PBS showed higher scores than BF rats (BF 0.2 ± 0.39, $p<0.01$ vs. AFS cell rats, $p<0.001$ vs. PBS rats, ***Figure 22d***). No significant differences were observed concerning the weight of PBS vs. AFS cell rats at 96 hours of life, while there was a significant difference among BF vs. NEC rats (**data not shown**).

We assessed the bowel in situ using MRI. Images were acquired soon after sacrifice to assess the degree of ascites (T2 imaging) and after gadolinium chelate fixation to assess bowel integrity (high-resolution μMRI). ***Figure 22e***, shows MRI images; the left and right columns of images are representative slices through neonatal rats treated with AFS and PBS, respectively. Row 1, shows representative T2 maps for axial slices of an AFS (***Figure 22e-a***) and PBS rat (***Figure 22e-b***). The total number of voxels with T2>160ms (±1 SEM) for the AFS, PBS and BF rats was 224 (±135), 1682 (±453) and 278 (±27) respectively. The voxel counts were significantly different between the AFS cells and PBS groups ($p<0.05$) indicating a reduction in fluid within the abdomen following AFS cells

treatment. Rows 2 & 3, show coronal (***Figure 22e-c and d***) and axial (***Figure 22e-e and f***) μMRI slices through the bowel of the AFS cells (***Figure 22e-c and e***) and PBS (***Figure 22e-d and f***) pups following gadolinium chelate fixation. PBS treated pups displayed dilated bowel loops, with thinned gut wall; features that were not apparent in the AFS cells pup. This is clearly seen in the enlarged images of representative bowel loops (***Figure 22e-g and h***). The mean bowel wall thickness (±1 SEM) for the AFS cells, PBS and BF rats was 0.401 (±0.017), 0.284 (±0.015) and 0.63203 (±.018) mm respectively. The wall thickness measurements were found to be significantly different between groups ($p<0.05$).

Gut motility and permeability were measured to determine whether AFS cells had also a protective effect on intestinal function.

To assess motility, carmine red was administered by gavage and gastrointestinal transit tested blindly by two independent scorers after 4 hours. In comparison with BF rats, motility was decreased in NEC rats injected with PBS ($p<0.001$) but it was normal in rats injected with AFS cells (p=n.s.) (***Figure 22f***). Carmine red completed gut transit in 75% of BF rats, in 19% of rats treated with PBS, and in 47% of those treated with AFS cells. Gut weight and length were similar between PBS and AFS cell groups and decreased in comparison with BF animals indicating that differences in gut motility were not influenced by intestinal size (***data not shown***).

To study gut permeability, rats received a mannitol/lactulose solution by gavage, and the plasma lactulose/mannitol ratio was calculated. In comparison with BF rats (0.004±0.002; n=9), PBS rats had a significant increase in intestinal permeability (0.043±0.004; n=21; $p<0.001$) (***Figure 22g***). NEC rats injected with AFS cells had a decrease in lactulose/ mannitol ratio compared with those injected with PBS (0.031±0.004; n=25, $p<0.05$), indicating that injection of AFS cells decreases intestinal permeability.

In conclusion, we demonstrated that the administration of AFS cells to NEC rats significantly improved animal mortality and morbidity in rats, reduced bowel damage as assessed in situ by high resolution MRI and improved gut motility and absorption.

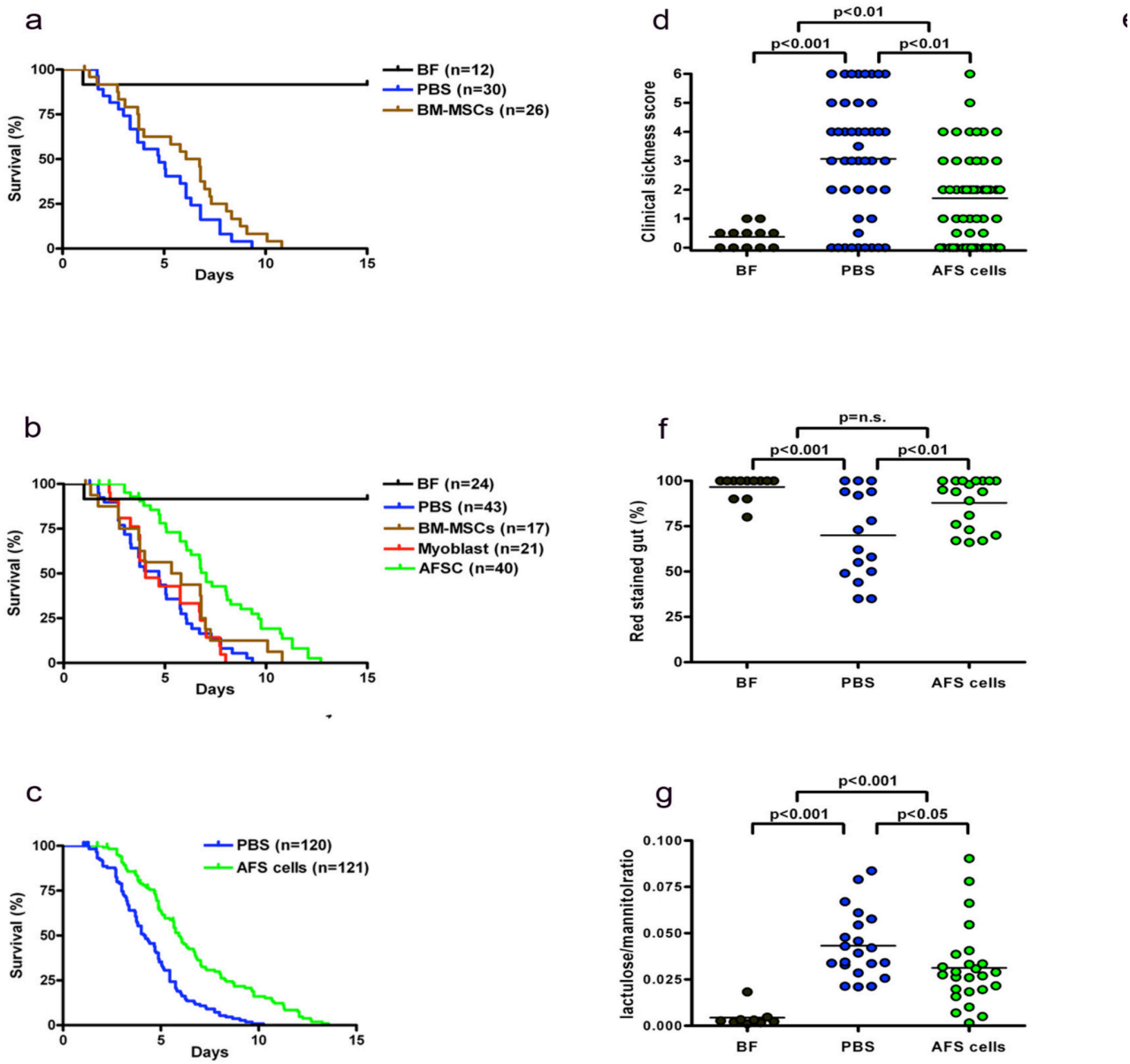
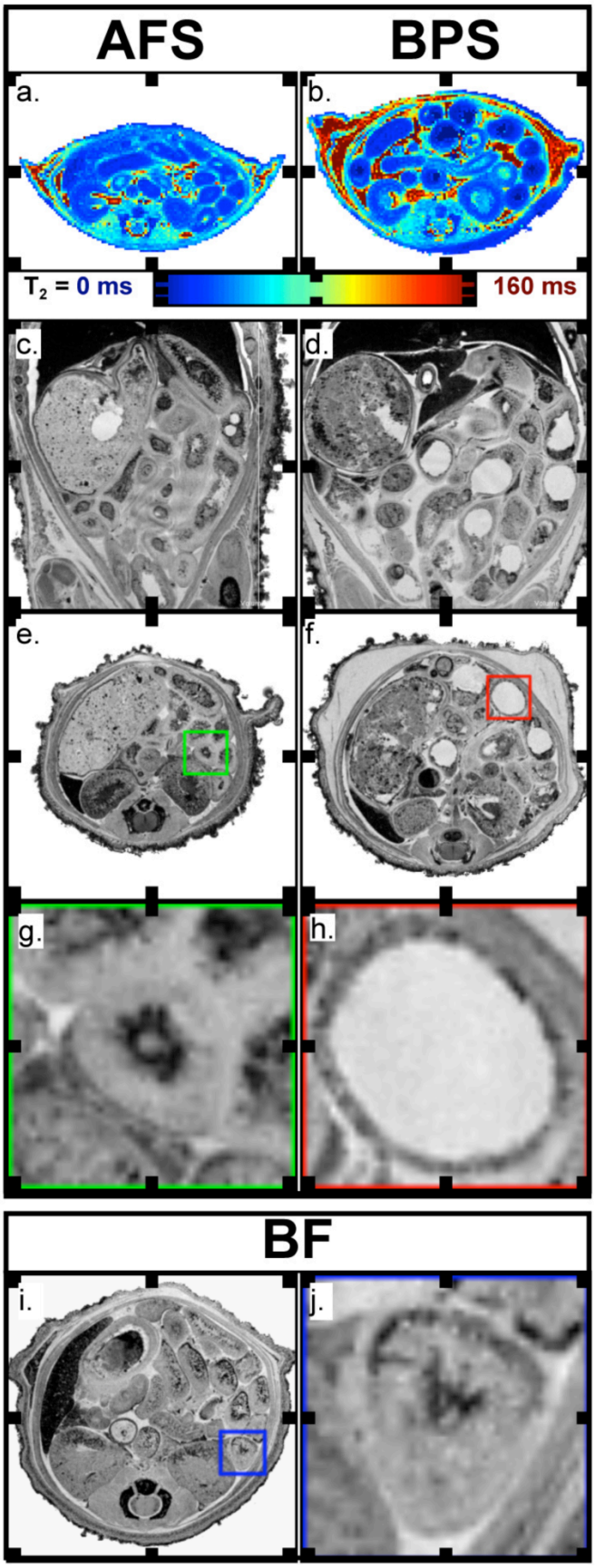


Figure 22. a. *NEC rats treated with BM-MSCs had the same survival as control NEC rats injected with PBS (p= ns), whilst BF rats survived significantly longer than both groups (p< 0.0001).* ***b.*** *When treated with AFS cells, NEC rats proved to survive significantly longer than NEC rats treated with BM-MSCs (p=0.024), PBS (p<0.0001), or myoblasts (p<0.0001).* ***c.*** *This effect of AFS cells was extremely reproducible, as cumulative results of several experiments showed a consistent survival benefit of NEC rats injected with AFS cells compared with those receiving PBS alone (p<0.0001).* ***d.*** *Morbidity, evaluated using a validated clinical sickness score, also showed a significant benefit of AFS cell treatment in comparison with PBS (p<0.001), although AFS cell treated rats were still sick compared with BF rats (p<0.01).**e.*** *please see next page* ***f.*** *Intestinal motility, assessed as carmine red transit, was severely decreased in NEC rats injected with PBS in comparison with BF rats (p<0.001), but treatment with AFS cells restored gut motility (AFS cells vs. BF: p=n.s.; AFS cells vs. PBS: p<0.01).* ***g.*** *Intestinal permeability, measured as plasma lactulose/ mannitol ratio, was dramatically increased in NEC rats injected with PBS in comparison with BF rats (p<0.001), but administration of AFS cells to NEC rats partially prevented this increase (AFS cells vs. BF p<0.01, AFS cells vs. PBS p<0.05).*

e. *MRI of rats treated with AFS (**left** column of images) and untreated rats (**right** column of images). **Row 1 (a. & b.)** Degree of ascites measured using T2 maps: dark red regions have T2 values above160ms and indicate areas of fluid accumulation. **Row 2 (c. & d.):** Bowel wall thickness using μMRI images: marked structural changes were observed in the untreated rats. **Row 3 (e. & f.)** representative axial slices demonstrate a similar pattern. **Row 4 (g. & h.):** magnified images of bowel loops from the respective axial slices highlight the loss bowel wall integrity in the untreated rats.*



E. AFS cells decrease intestinal damage, localise to the damaged gut and migrate systemically.

At 96 hours, animals were sacrificed and both macroscopic and histological appearance of the gut were assessed.

Macroscopic appearance of the gut (jejunum-caecum) was evaluated by two independent and blinded investigators using a scoring system validated by histology as reported above (Materials and Methods, paragraph B3). While NEC rats injected with AFS cells had significantly less macroscopic gut damage (0.4±0.7, n=27) than rats injected with PBS (1.4±1.1 n=24; $p<0.001$), no difference was observed between rats injected with AFS cells and BF rats (0.1±0.3; n=12, p=n.s.) (***Figure 23a***). Remarkably macroscopic signs of necrosis were frequent in rats injected with PBS, while they were not observed in rats injected with AFS cells (***Figure 23a***). No significant differences were observed among PBS and AFS cell animals concerning gut weight, length, weight/length ratio and mass (***Table 10***).

Table 10. *Gut weight, length, weight/length index, and mass index of breastfed rats and of rats injected with either PBS or AFSC. Data are expressed as (mean ± SD). p, calculated using ANOVA, refers to PBS vs. AFS cell rats.*

Parameter	BF	PBS	AFS cell	p
Gut weight (g)	0.99 ± 0.2	0.31 ± 0.1	0.30 ± 0.1	>0.05
Gut length (cm)	36.5 ± 4.4	17.0 ± 2.3	15.8 ± 3.7	>0.05
Gut w/l (g/cm)	0.03 ± 0.004	0.02 ± 0.005	0.02 ± 0.007	>0.05
Gut mass (mg/cm*100g)	238.2	368.9	412.8	>0.05

Gut was also histologically evaluated for incidence of NEC that, similarly to human, localize mainly to the ileum. Severity of bowel damage to the ileum varied, as reported in Materials and Methods (paragraph B2), based on the presence of villus core separation, epithelial sloughing and necrosis. In breastfed rats, no histological changes were detected (mean score 0.08±0.2, n= 12) (***Figure 23b***). Conversely, in NEC rats injected with PBS,

the severity of gut damage (1.78±0.7, n=50) was higher than both BF rats ($p<0.001$) and NEC rats treated with AFS cells (0.23±0.8, n= 48; $p<0.001$) (***Figure 23b***). Moreover, there were no cases of NEC in BF rats, a high incidence of NEC in rats treated with PBS (76%) and a markedly reduced incidence of NEC in rats treated with AFS cells (42%, $p=0.0009$) (***Figure 23b***).

As NEC rats injected with AFS cells had improved survival, clinical sickness score, intestinal function and histology, we hypothesised AFS cell migration and integration in the damaged intestine. Therefore the presence of AFS cells was evaluated using GFP. Freshly dissected intestines of rats sacrificed at 96 hours, examined at low magnification under UV light, displayed a GFP signal in the intestinal wall and on the mesentery which was distinguishable from green autofluorescence (***Figure 23c***). AFS cells exhibited various degrees of spreading leading, in some cases, to a characteristic ring around the longitudinal gut axis (***Figure 23c***). Microscopically, after 48 hours, bundles of cells were detected adherent to the mesenteric surface (***Figure 23d***); at 72 hours, AFS cells were present both in the serosa and in the muscle layers of the intestine (***Figure 23d***); and at 96 hours of life a few AFS cells were found in the villi (***Figure 23d***). These cells, although few in number, were in some cases positive for smooth muscle actin (***Figure 23e***) but negative for cytokeratin and anti-neuron-specific class III β-tubulin.

We have previously demonstrated that following i.p. injection to BF rats, AFS cells diffuse systemically, and can be detected in more than 60% of rat intestines (*see Results, paragraph C*). We hypothesised that this would also occur in rats with NEC and undertook PCR analysis for the *gfp* gene in genomic DNA extracted from organ specimens of 32 AFS cells injected NEC rats. Interestingly, 100% of NEC rat intestines were positive for GFP, whilst similarly to BF rats, NEC rats had GFP gene signal detected in the liver (32% of animals), kidneys (21%), spleen (20%), heart (17%), and lungs (15%) (***Figure 23f***). GFP gene was never amplified in either brain or femur specimens. 50% of NEC animals showed GFP positivity only in the intestine, 23% in the intestine plus one other organ, 17% in the intestine plus two other organs and 10% in the intestine plus three or more other organs **(*Figure 23g*)**.

In conclusion, we demonstrated that AFS cells significantly reduced the incidence of NEC and diminished bowel damage both macroscopically and microscopically. Moreover, AFS cells migrated and engrafted in the intestine, even if at a low rate, where they sometimes acquired a mesenchymal phenotype (i.e. expression of smooth muscle actin e preferential localisation into muscle layers and into the muscularis mucosae).

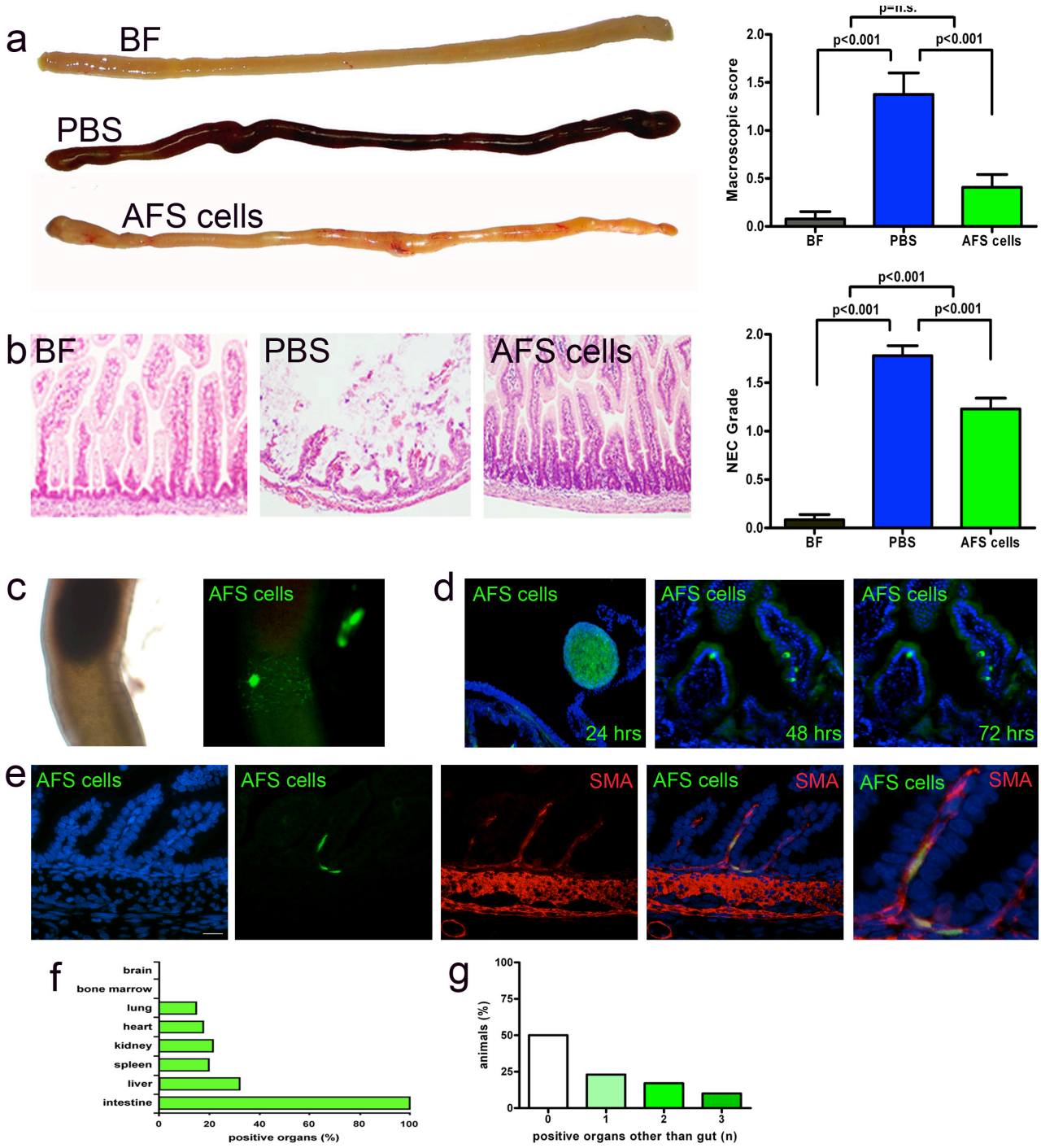
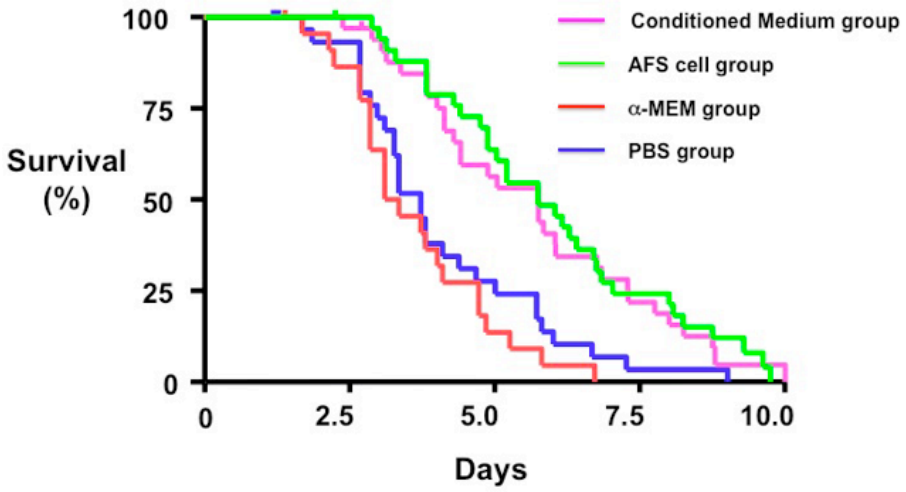


Figure 23. a. *Examples of the macroscopic gut appearance of BF rats and NEC rats injected with PBS or AFS cells is shown. Macroscopic gut damage of NEC rats injected with AFS cells was significantly less than in animals injected with PBS ($p<0.0007$), but showed no difference with BF rats (p=n.s.).* ***b.*** *Examples of the microscopic gut appearance of BF rats and NEC rats injected with PBS or AFS cells is shown. Microscopically, NEC rats treated with AFS cells had significantly less histological damage in comparison with NEC rats treated with PBS ($p<0.001$), whilst, as expected, no damage was observed in BF rats.* ***c-d.*** *Epifluorescence of GFP+AFS cells injected i.p.: 24 hours after injection cells are noted both in the mesentery and adherent to the serosa of the intestine. At 48 hours a similar aspect is observed, while by 72 hours rare AFS cells are noticed within the villus structure in close junction to the epithelial layer.* ***e.*** *Integration in the mucosa layer of AFS cells co-expressing GFP and smooth muscle actin (SMA).* ***f,g.*** *PCR analysis of GFP in 32 animals. AFS cells injected i.p. are able to migrate in all cases to the intestine and in 50% also to various other abdominal and thoracic organs, while we could not detect them in brain or bone marrow samples. In 50% of the animals AFS cells did engraft in at list 1 organ other than intestine with a maximum of 3.*

F. AFS cells decrease gut inflammation and enterocyte apoptosis and promote enterocyte proliferation/migration in rats with NEC via a paracrine mechanism of action.

Although we believe that the beneficial effect of AFS cells in NEC rats was mainly related to their presence in the gut, their low degree of tissue engraftment suggested a paracrine mechanism of action. Therefore, a survival study exploring the effect of AFS cell-conditioned medium was performed. At 24 and 48 hours of life NEC rats received an i.p. injection of either: i. $2x10^6$ AFS cells in 50μl of PBS (n=31), ii. 50μl of serum-free medium conditioned by AFS cells (n=44), iii. 50μl of serum-free medium (i.e. α-MEM) not conditioned by AFS cells (n=23) and iv. 50μl of PBS alone (n=42). The survival of rats injected with AFS cell-conditioned medium was similar to that of rats treated with AFS cells (p=n.s.), while was significantly superior to that of NEC rats injected with PBS (p=0.0011) or with α-MEM (p <0.0001) (***Figure 24***).

Figure 24. *AFS cell-conditioned medium significantly improved rat survival in comparison to PBS (p=0.0011) and* α-MEM (p<0.0001). *NEC rats treated with AFS cells and AFS cell-conditioned medium had the same survival (p=n.s.). No differences were observed among rats treated with PBS or* α-MEM *p=n.s.)*.



The above mentioned results, suggested that AFS cells exerted their beneficial effects in NEC via a paracrine mechanism. Therefore, a preliminary microarray-based expression analysis was performed to detect biological processes influenced by the i.p.

administration of AFS cells in NEC rats. cDNA arrays were used to compare the intestines (duodenum-caecum) of PBS vs. AFS cell NEC rats sacrificed at 96 hours of life. Through a hierarchical cluster analysis, 37 genes were found able to distinguish the two groups of animals (***Figure 25a***). Among these, the genes with the largest expression differences were mainly involved in inflammation and tissue repair (e.g. Aoc3, Itgb6), cell cycle regulation (e.g. Atf2, Dusp16, Gpx4, Mxd1) and enterocyte differentiation processes (e.g. Acsl5, Rab8a, Thra). Expression data prompted us to evaluate general parameters of inflammation, epithelial proliferation and apoptosis in the intestine via biochemical and immunohistochemical assays.

Inflammation: AFS cells decreased gut inflammation in NEC rats, as malondialdehyde levels, reflecting lipid peroxidation, tended to be lower in NEC rats injected with AFS cells compared with PBS-treated animals (AFS cells 3.6±2.7 nmol/mg protein; PBS 5.2±4.6; ***Figure 25b***) and significantly decreased neutrophil infiltration, measured as myeloperoxidase activity (AFS cells 5.1±0.8 U/mg protein; PBS 9.1±1.7; $p<0.05$; ***Figure 25c***).

Apoptosis: the intestine from PBS and AFS cell NEC rats was immunohistochemically stained for cleaved caspase 3. Groups were compared by two blinded investigators using a modified apoptotic index (0=no apoptosis, 5= apoptosis in crypts) (*Feng 2006; Jilling, 2004*). NEC rats injected with AFS (n=17) cells had significantly less epithelial apoptosis than rats injected with PBS (n=22; $p=0.04$), and, even more importantly, apoptosis was significantly decreased in the crypts of NEC rats treated with AFS cells (cryptal apoptosis present in 45% of PBS rats with NEC vs. 12% of NEC rats receiving AFS cells, $p<0.05$) (***Figure 25d-e***).

Enterocyte migration/proliferation: PBS and AFS cell-injected NEC rats received an intraperitoneal injection of 5-ethynyl-2'-deoxyuridine (EdU), were sacrificed 24 hours later and intestine processed for EdU detection (*Salic, 2008*). Cell migration was calculated by measuring the distance from the base of the villus crypt to the foremost labelled enterocyte (FLE) and expressing this distance as a percentage of the mucosal thickness (FLE/total mucosal thickness x 100) (*Feng, 2007*). In rats injected with AFS cells, the FLE had migrated significantly further from the villus crypt than in rats injected with PBS (75% vs. 53%, $p<0.0001$) (***Figure 25f-g***).

Within this set of experiments, we demonstrated that, via a paracrine mechanism of action, AFS cells were able to modify the intestinal gene expression profile thus reducing gut neutrophil infiltration and apoptosis, and enhancing epithelial proliferation.

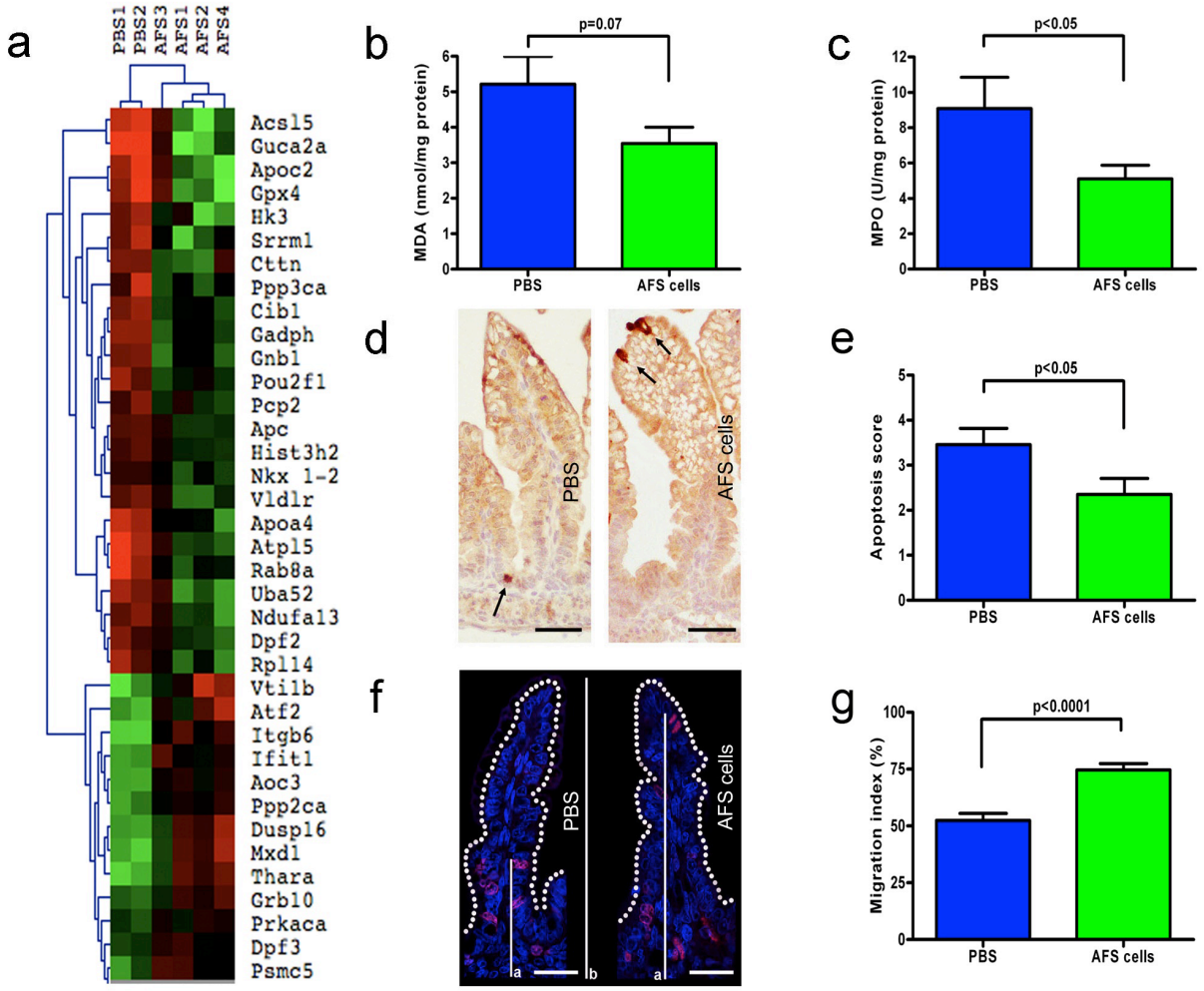


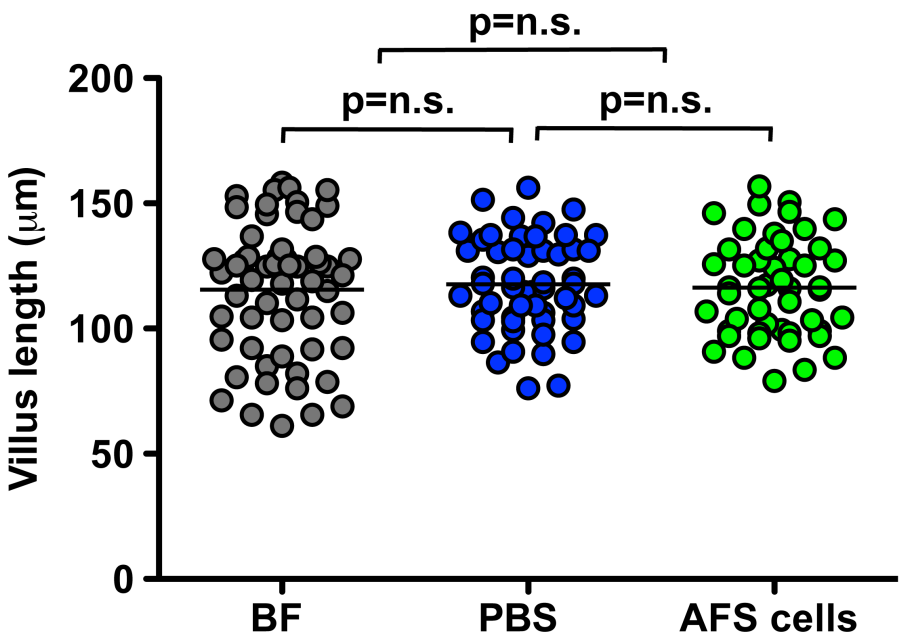
Figure 25. a. *Cluster analysis of cDNA microarray data. The intestinal gene expression profile of NEC rats receiving PBS was distinguished from that of NEC rats treated with AFS cells through a 2-D hierarchical clustering employing the Euclidean distance; 37 genes, mainly involved inflammation, tissue repair, cell differentiation and cell cycle regulation, resulted able to differentiate the two groups of animals at a molecular level. Rows correspond to genes, and columns to NEC rats treated with PBS (PBS1, PBS2) or AFS cells (AFS1, AFS2, AFS3, AFS4); red and green respectively indicate gene up- and down-regulation relative to a reference sample consisting in a pool of intestinal RNA from 5 different breast fed rats.* ***b.*** *NEC rats injected with AFS cells had a trend towards a lower degree of intestinal lipid peroxidation, measured as malondialdehyde level, than NEC rats injected with PBS.* ***c.*** *Gut neutrophil infiltration, measured as myeloperoxidase activity, was significantly lower in NEC rats treated with AFS cells than in those injected with PBS.* ***d.*** *Cleaved caspase 3 immunostaining of terminal ileum revealed apoptotic cells (arrows) in the villus crypts of NEC rats injected with PBS and at the villus tips of NEC rats injected with AFS cells.* ***e.*** *The apoptotic index, scored blindly as expression of cleaved caspase 3, was significant lower in NEC rats injected with AFS cells than in those injected with PBS.* ***f.*** *Fluorescence detection of dividing cells stained with EdU for cell migration/proliferation (EdU, purple, counterstained with DAPI, blue). Enterocyte migration/proliferation was calculated as percentage of the foremost EdU-labelled enterocyte (a) over the total mucosal thickness (b).* ***g.*** *Enterocyte migration/proliferation index assessed by EdU was significantly increased in NEC rats treated with AFS cells than in those treated with PBS (74% of mucosal thickness vs. 52%).*

G. AFS cells modulate stromal cells expressing COX-2 in the lamina propria

The inducible isoform of cyclooxygenase (COX-2) plays a role in colonic carcinogenesis such that nonsteroidal anti-inflammatory drugs (NSAIDs) retard the development of colon cancer by modulating COX-2 (*Bertagnolli, 2006; Markowitz, 2007 and 2009; Chan, 2007 and 2009*). More recently, stromal cells expressing COX-2 in the lamina propria have been shown to promote epithelial proliferation in response to injury (*Stappenbeck 2007; Walker, 2010*). We therefore evaluated whether AFS cells could modulate COX-2+ cells in the intestinal mucosa.

COX-2+ cell number and location: intestines harvested from BF rats and NEC rats receiving either PBS or AFS cells were evaluated using anti-COX-2 antibodies (**Figure 27a)**. In the lamina propria, the number of COX-2+ cells per villus unit (crypt plus adjacent villus; **Figure 27b**) and their location (i.e. underlying villus epithelial cells, **Figure 27c,** or underlying crypt cells, **Figure 27d**) were compared between the three groups of animals by two blinded investigators. NEC rats treated with PBS had fewer COX-2+ cells per villus unit (2.37±0.29; n=8) compared with BF rats (4.25±0.52; n=8, $p<0.05$) (**Figure 27b**). Interestingly, NEC rats treated with AFS cells had an increase in COX-2+ cells per villus unit (4.97±0.46; n=7) compared with those treated with PBS ($p<0.01$), so that they had a similar number of cells to BF rats (p=n.s.) (**Figure 27b**). Remarkably, while there were no differences in the number of COX-2+ cells between NEC rats treated with AFS cells or with PBS in the villus (1.95±0.28 vs. 1.32±0.16, p=n.s.) (**Figure 27c**), we observed an increase in COX-2+ cells in the cryptae of NEC rats receiving AFS cells (3.01±0.41) compared both with BF rats (1.41±0.23; $p<0.01$) and NEC rats injected with PBS (1.05±0.18; $p<0.001$). (**Figure 27d**). Moreover, both the total number of COX-2+ cells per villus unit (**Figure 27e**) and the number of cryptal COX-2+ cells (**Figure 27f**) inversely correlated with the degree of intestinal damage ($r^2= 0.5$, $p= 0.014$). Conversely, there was no correlation between COX-2+ cells in the underlying the villus epithelium and the severity of gut damage ($r^2= 0.25$, p= n.s.; **data not shown**). Remarkably, these differences could not be influenced by villus length which was similar among BF rats (115.5±3.51μm) and NEC rats treated with PBS (117.7±2.56μm) or AFS cells (116.4±2.8μm) (p=n.s.) (**Figure 26**).

Figure 26. *No significant differences in terms of villus length were observed among BF rats and NEC rats, either treated with PBS or AFS cells.*



Inhibition of COX-2 activity: to further investigate whether the beneficial effects of AFS cells were dependent on COX-2+ cells, we performed a survival study using selective and non-selective COX-1 and -2 inhibitors. BF rats (n=32) and NEC rats receiving either PBS (n=77) or AFS cells (n=78) were randomly divided in 4 sub-groups receiving by gavage: (i) vehicle (1% DMSO); (ii) a selective COX-1 inhibitor (sc-560); (iii) a non-selective COX-1+2 inhibitor (ibuprofen) and (iv) a selective COX-2 inhibitor (celecoxib). All rats were followed up from birth until death occurred and their clinical status was blindly assessed at 96 hours of life. As expected, NEC rats treated with AFS cells+vehicle survived significantly longer ($p<0.0001$) (**Figure 27g**) and had a better clinical sickness score than NEC rats treated with PBS+vehicle (0.77±0.36 vs. 3.09±1.10; $p<0.05$) (**data not shown**). The effect of AFS cells in lengthening survival was abolished by both the selective COX-2 (AFS cells +celecoxib vs. PBS+vehicle: p=n.s.) and the non-selective COX-1+2 inhibitor (AFS cells +ibuprofen vs. PBS+vehicle: p=n.s.), but maintained in rats receiving the selective COX-1 inhibitor (AFS cells+sc-560 vs. PBS+vehicle: $p=0.001$) (**Figure 27g**). Similarly, the improvement in clinical status observed in NEC rats treated with AFS cells was annulled by COX-2 inhibitor (AFS cells+celecoxib vs. AFS cells+vehicle: $p<0.001$), reduced by COX-1+2 inhibitor (AFS cells+ibuprofen vs. AFS cells+vehicle: p=n.s.) and unaffected by COX-1 inhibitor (AFS cells+sc-560 vs. AFS cells+vehicle: p=n.s.) (**Figure 27h**). None of the COX inhibitors modified the survival (**Figure 27g**) or the clinical status (**data not shown**) of PBS or BF rats.

In conclusion, based on these data, we speculate that AFS cell beneficial effects in NEC are mediated by the activation of a population of stromal cells expressing COX2 present in the lamina propria of the intestine.

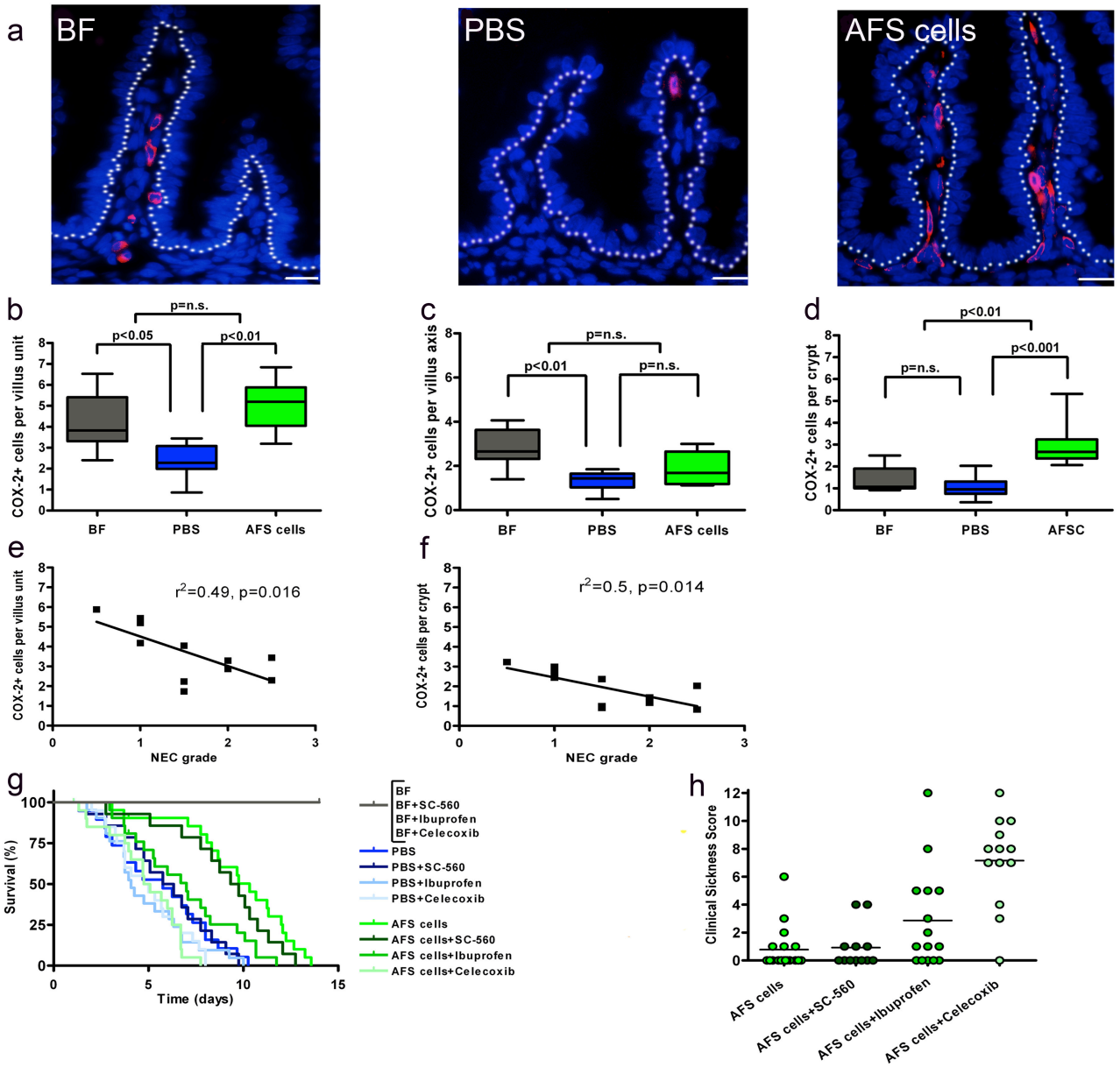


Figure 27. a. *Representative 3μm sections of the terminal ileum from BF rats and NEC rats receiving PBS and AFS cells stained with anti-COX2 Ig (red) and DAPI (blue). Scale bars: 20μm.* ***b.*** *In NEC rats treated with AFS cells, the number of COX-2+ cells per villus unit was similar to that of BF rats but higher compared to NEC rats treated with PBS. This difference was not determined by the quantity of COX-2+ cells in the villi, which was similar between the groups (**c**), but by their number underlying the cryptae which was higher in NEC rats treated with AFS cells (**d**).* ***e,f.*** *The number of COX-2+ cells per villus unit and in the cryptae inversely correlated with the histological grade of NEC by linear regression.* ***g.*** *A survival study employing selective and non-selective COX inhibitors showed that: 1) COX inhibitors did not modify the survival of BF rats (BF+vehicle vs. BF +sc-560, BF+ibuprofen, BF+celecoxib: p=n.s.) and NEC rats receiving PBS (PBS+vehicle vs. PBS +sc-560, PBS+ibuprofen, PBS+celecoxib: p=n.s.); 2) the improved survival of NEC rats receiving AFS cells was annulled by COX-2 (AFS cells+celecoxib vs. AFS cells+vehicle: p<0.0001; AFS cells+celecoxib vs. PBS+vehicle: p=n.s.) and COX-1+2 inhibitors (AFS cells+ibuprofen vs. AFS cells+vehicle: p<0.01; AFS cells+ibuprofen vs. PBS+vehicle: p=n.s.), but conserved in rats receiving COX-1 inhibitor (AFS cells+sc-560 vs. AFS cells+vehicle: p=n.s.; AFS cells+sc-560 vs. PBS+vehicle: p=0.001).* ***h.*** *The clinical sickness score improvement observed in NEC rats treated with AFS cells (0.77 ± 0.36) was abolished by COX-2 inhibitor (7.15 ± 0.89; AFS cells+celecoxib vs. AFS cells+vehicle: p<0.001), diminished by COX-1+2 inhibitor (2.86 ± 0.91; AFS cells +ibuprofen vs. AFS cells+vehicle: p=n.s.) and unaltered by COX-1 inhibitor (0.92 ± 0.39; AFS cells +sc-560 vs. AFS cells+vehicle: p=n.s.).*

DISCUSSION

NEC remains a major cause of neonatal morbidity and mortality despite changes in medical and surgical treatment. Despite more than three decades of intensive research efforts, the pathogenesis of NEC is still unproven and its treatment is difficult and often inadequate (*Henry, 2009; Lin, 2006 and 2008; Petrosyan, 2009; Schnabl, 2008; Thompson, 2008*).

There are a number of accepted animal models to study NEC. All these models aim to create a necrotic bowel in animals to simulate that of newborns affected by NEC. Many models induce intestinal damage resembling NEC through administration of pro-inflammatory cytokines but often do not contain the aspect of prematurity that is seen in human disease; in the latter, LPS, PAF and TNF-alpha are mainly used to create intestinal ischemia. LPS mimics bacterial overgrowth in the intestinal lumen, while PAF and TNF-alpha cause hypotension and shock. Other models have been described that do not physiologically resemble human NEC but aid in the study of the disease process. These include superior mesenteric artery clamping with or without PAF (*Chung, 2001*), intra-arterial injection of TNF-alpha (*Torimoto, 1990*), and placing rats into an hypoxic environment (*Hsueh, 2003*). Finally, a rat model has been described by Chan (*Chan KL, 2003*) who created intestinal ischemia by increasing intraluminal pressure and injecting E. Coli into the lumen.

Among the various experimental models of NEC described in the past 40 years, the one proposed by Barlow in 1974 remains the most reliable and the one which most closely resembles human NEC, as it reproduces the main contributory factors to the development of human disease (*Barlow, 1975; Schnabl, 2008*). This model mainly consists in the administration of bacteria (i.e. Klebsiella spp.), hyperosmolar formula and hypoxic stress to newborn rats, delivered by Caesarean section, during their first 4 days of life (*Barlow, 1974*). However, NEC reproducibility using this model varies among different studies with a prevalence ranging from 35% to 71% (*Grishin, 2006; Zamora, 2005*). In our experience, the incidence of NEC was even lower using this protocol and it appears that its success or failure in inducing NEC depends on the microbiological cleanliness of animal facilities. To overcome this source of variability and increase the severity of bowel damage in experimental NEC, we decided to administer oral bacterial lipopolysaccharide (LPS) mimicking the bacterial load that occurs in human babies affected by NEC. This approach as been used by other authors in similar NEC models (*Feng, 2006*). Thus, we established

a LPS-modified gavage model of NEC, closely resembling the one described by Barlow, based on term delivery of newborn rats by caesarean section, gavage feeding with hyperosmolar formula, hypoxic stress and LPS administration. This LPS-modified gavage model of NEC proved to be consistent and reproducible with a constant NEC incidence, estimated based on gut histology, of 70% at the end of the treatment.

To assess the safety of the intraperitoneal administration of AFS cells in vivo, preliminary experiments in which AFS cells were injected i.p. into healthy newborn rats were performed. AFS cell injection via the intraperitoneal route resulted, in the short term (1st month of life of the animals), not only a safe procedure but also an effective way to administer cells systemically. In our series, none of the animals died or presented significant adverse effects after the procedure and transplanted cells were detected in more than 90% of the treated animals: AFS cells diffused systemically within few hours from their administration and were found integrated in various organs after a 3-week period of time. Although proliferation and differentiation of stem cells is of paramount importance for their therapeutic function, the appropriate route of cell administration is an essential prerequisite for the success of cell engraftment and organ repair (*Strauer, 2003*). Despite i.v. injection of stem cells has proved to be very effective and easy to perform, this approach bears some disadvantages. First, cells can be trapped in the lung capillary vasculature in a phenomenon known as ‘‘trans-pulmonary first-pass attenuation’’. Secondly, some cell types, such as skeletal myoblasts, can harbour an emboligenic potential when delivered systemically (*Strauer, 2003*). Intraperitoneal transplantation could avoid some of these problems. The peritoneal cavity is a virtual space, which surrounds the viscera of the abdomen. Because of its large surface area (2 m^2 in adult human) and its abundant vascularisation, it provides an excellent portal of entry to general circulation. Moreover, the peritoneum can be considered as one lymph space with drainage to multiple intra-abdominal lymph node groups, in turn connected via the thoracic duct to the mediastinal lymph nodes (*Parungo, 2007*). Recently, few research studies suggested the possibility of employing the i.p. route also for systemic cell delivery. In two papers published by the same group, human umbilical cord stem cells, i.p. injected in a rodent model of toxic liver injury, were able to diffuse systemically, engraft in different organs (e.g. bone marrow, liver and spleen) and reduce liver damage (*Di Campli, 2004*). The ability of these cells to home into the liver and to differentiate towards the hepatic lineage was directly related to the severity of the organ damage (Piscaglia, 2005; Wang, 2002). In our study, we showed that the i.p. route is a safe and efficient method for cell delivery in newborn healthy rats. AFS cells might traffic spreading locally inside the peritoneal cavity

and systemically through the blood stream and the lymphatic vessels. Moreover, we demonstrated that they were able to home into different tissues of newborn animals even in the absence of organ damage. This is particularly interesting as it is well recognised that stem cell homing can be enhanced during hypoxic/ischemic conditions and in the presence of endothelial/tissue injury (*Hristov, 2009*). Stem cell homing, indeed, is a complex process primarily regulated by the interaction of many soluble/surface proteins (e.g. CXC-chemokines) with their respective receptors (e.g. CXCR4) on the cell surface *Hristov, 2007-1 and 2)*. While AFS cells do express CXCR4 (*Eddleman, 2006*), further studies are needed to elucidate AFS cells homing mechanisms and pathways of engraftment.

After i.p. injection, AFS cells mainly distributed in organs of the abdominal compartment, both intra- (i.e. gut, liver, spleen) and extra-peritoneal (i.e. kidneys). AFS cells preferentially localised in the intestine, which resulted positive for GFP in more than 60% of the animals. This could be related to the proximity of this organ to the delivery site; cell diffusion toward this tissue might occur both via lymphatic drainage and blood stream, but also directly through the serosa. In light of the speculations done above, the i.p. administration of stem cells might represent in the future a potential suitable route for cell therapy in intestinal disorders. In a minority of cases (16%), AFS cells were also able to migrate towards organs of the thoracic compartment such as lungs and heart. Remarkably, GFP signal was never detected in brain tissue, either by PCR analysis, or by immunohistochemistry. This could be due to the integrity of the blood–brain barrier. The relationship between stem cells and the endothelium of the central nervous system, which has very selective permeability due to its specific ultrastructural and functional features, has been extensively investigated in animal models of cerebral damage. Only when brain injury occurs, the inflammatory activation up-regulates homing ligands and increases the permeability of the blood–brain barrier, thus facilitating cellular adhesion and diapedesis of stem cells (*Karp, 2009; Lundberg, 2009*).

In these preliminary experiments (i.e. administration of AFS cells to healthy newborn rats), we evaluated AFS cell spreading and engraftment for only 3 weeks after their injection through the i.p. route. This was propaedeutical to AFS cell employment in the neonatal rat model of NEC previously described that is a short term model in which animal do not survive more than 2 weeks after birth. However, further studies are mandatory to evaluate AFS cell fate (engraftment, differentiation, localization) and safety in the long term. In particular, both AFS cell immune reactivity and oncogenic potential should be rigorously assessed.

Herein, we demonstrated for the first time that AFS cells significantly lengthen survival of rats with NEC. In this animal model, which is widely used and shares many features with the human disease (*Barlow, 1974*) only inhibition of nuclear factor-kB (*De Plaen, 2007*) and administration of heparin-binding epidermal growth factor-like growth factor (HB-EGF) *(Feng, 2006*) have been shown to decrease mortality at 72 and 96 hours respectively. The ability of AFS cells to lengthen survival is particularly important, as intensive care support and operative treatment given to human neonates with NEC (ventilation, cardiovascular support, intravenous fluids and nutrition, etc.) cannot be given to pup rats and this model is therefore not compatible with long term survival (*Sodhi, 2010*). In contrast with other bowel diseases, in which BM-MSCs are effective (*Bamba, 2006*; *Cassinotti, 2008; Garcia-Olmo, 2009*; *Newman, 2010 Tanaka, 2008*), the beneficial effects we obtained in experimental NEC were specific to AFS cells and were not observed with BM-MSCs. This could be due to differences in pathogenesis; in NEC, the primary causes of the enterocolitis are bowel immaturity, bacterial infection and ischemia (*Lin, 2006 and 2008*), whereas in inflammatory bowel diseases (IBDs), the pathological changes are primarily related to dysregulation of the immune system (*Cuffari, 2010; Schirbel 2010*). Rat models of IBD are usually obtained administering either dextran sodium sulfate (*Brown, 2007*) or intramural injection of peptidoglycan-polysaccharide (*Bleich, 2009*) to animals that, in the vast majority of cases, carry genetic alterations of the immune system (e.g. $IL10^{-/-}$ mice) (*Mizoguchi, 2010*). The NEC model, differently, comprises several pathogenic factors (i.e. ischemia, feeding with hyperosmolar formula, administration of LPS) that are directly implicated in the human disease (*Schnabl, 2008*). Hence, while IBD can be rescued by MSCs, beneficial effects from cell therapy in NEC appear to require a different mechanism.

In addition to the pronounced and consistent effect on survival, several clinical indicators also demonstrated the beneficial effects of AFS cell treatment. Firstly, NEC rats injected with AFS cells had an improvement in clinical status using a validated scoring system, which is a marker of less severe gut damage in this animal model (*Zani, 2008-2*). While human NEC can be suspected at radiology, confirmed at surgery and graded at histology (*Lin, 2006; Rees, 2008*), only the latter have been used in experimental models. Herein, for the first time, we were able to define bowel appearance by using MRI imaging. Similarly to human infants with NEC, we demonstrated that peritoneal fluid collection and dilated bowel loops are features of rats with NEC, whilst MRI images of animals treated with AFS cells were indistinguishable from BF rats.

Treatment with AFS cells both improved intestinal function and reduced intestinal damage. AFS cells rescued gut motility and partially restored intestinal permeability in NEC rats. Animals injected with AFS cells had an improvement in gut macroscopic appearance as evaluated employing a novel scoring system reflecting the histological gravity of the intestinal damage (*Zani, 2008-2*). Villus sloughing, venous congestion and villus core separation are classic histological features of NEC, and we showed that AFS cell-treated animals had normal intestinal architecture with decreased incidence of all of these hallmarks. These findings corroborate previous studies in which stem cell therapy reversed colonic damage in an established model of IBD (*Khalil, 2007*).

The beneficial role of AFS cells on clinical outcome and survival was closely related to their presence in the gut. While AFS cells administered intravenously home primarily in the lung, and subsequently colonize spleen and liver, with no distribution to the gut (*Perin, 2010*), we have shown that AFS cells injected intraperitoneally colonize the gut in 80% of BF pup rats. Remarkably, in this rat model of NEC, when AFS cells were injected i.p., they localized in 100% of intestines, homing to the mesentery or the gut where they were mainly found on the serosal surface. In most of the animals, 48 or 72 hours after injection, AFS cells were also present, albeit in small numbers, in the smooth muscle and submucosal layers and/or in the villi. As the improvements in morbidity and mortality occurred within hours after injection, at which time relatively small numbers of AFS cells were found in the bowel, their direct contribution to tissue regeneration is unlikely to be the major mechanism for AFS cell beneficial effects. This is also in accordance with the low engraftment rate of AFS cells in the intestinal wall observed at 96 hours and with the restricted differentiation of engrafted cells towards the epithelial, mesenchymal and neuronal lineage. Thus, we hypothesised that in this environment, rather than differentiating into target tissue, AFS cells released specific growth factors that acted on resident progenitor cells. This hypothesis was confirmed from experiments demonstrating no differences in terms of survival among NEC rats receiving AFS cells vs. AFS cell-conditioned medium. Microarray analyses of NEC guts receiving AFS cells vs. PBS showed modification of the transcriptional profile of genes involved in inflammation and tissue repair (e.g. Aoc3, Itgb6), cell cycle regulation (e.g. Atf2, Dusp16, Gpx4, Mxd1) and enterocyte differentiation processes (e.g. Acsl5, Rab8a, Thra) suggesting, in accordance to other authors (*Ford, 2006*), that damage resolution in NEC is achieved via activation of multiple pathways acting on tissue inflammation, cell apoptosis and proliferation.

Severe intestinal inflammation leading to intestinal damage is the main pathological event that characterises NEC (*Halac, 1990; Ford, 2006*). In the attempt to reduce

incidence and severity of NEC, studies have tried to directly impact on the inflammatory cascade in both human (*Halac, 1990*) and experimental NEC (*Caplan, 1997*; *De Plaen, 2007*; *Zuckerbraun, 2005*). Similarly, we observed that AFS cell injection reduced gut lipid peroxidation and neutrophil sequestration in NEC rats. Stem cells are well known to have anti-inflammatory effects, which are exerted in different ways on different organs (*Guo, 2007; Gupta, 2007; Wang, 2006; Xu, 2007*). When injected in a model of endotoxin-induced lung inflammation, BM-MSCs down regulate the pro-inflammatory response while increasing production of anti-inflammatory IL10 (*Gupta, 2007*). Interestingly, in a similar model of lung injury, BM-MSCs decrease both the systemic and local inflammatory responses induced by endotoxin. These effects do not require either lung engraftment or differentiation of the stem cells and are due at least in part to the production of stem cell chemoattractants by the lungs and to humoral and physical interactions between stem cells and lung cells (*Xu, 2007*). Adult progenitor cells can also improve post-ischemic myocardial function both when used as a preventive measure, by inducing a 50% reduction in pro-inflammatory cytokine production (*Wang, 2006*), or when used as a therapy after myocardial infarction (*Guo, 2007*). In the latter scenario, MSCs decreased pro-inflammatory cytokines, inhibited collagen deposition, decreased expression of MMP-1 and TIMP-1 and attenuated left ventricle cavitary dilation and transmural infarct thinning, thus preventing myocardial remodeling (*Guo, 2007*).

In addition to inflammation, intestinal apoptosis has also been shown to be a key factor in gut barrier failure, in both human and experimental NEC (*Nadler, 2000*). Abundant epithelial apoptosis of the villi is observed in histological specimens collected at the time of bowel resection in patients with NEC (*Ford, 1997*). It usually precedes widespread tissue damage (*Jilling, 2006*) and its reduction in experimental NEC has been achieved using various agents such as epidermal growth factor (*Clark, 2005*) anti-TNF-alpha (*Halpern, 2006*), HB-EGF (*Feng, 2006*), IGF-1 (*Baregamian, 2006*), Lactobacillus GG (*Lin, 2008*), Lactobacillus bulgaricus (*Hunter, 2009*). Herein, we demonstrated for the first time that stem cells also reduce apoptosis in NEC rats. Strikingly, AFS cells abolished apoptosis in the crypts where intestinal endogenous stem cells reside. These results also parallel findings in animal models of IBD, in which administration of BM-MSCs decreases apoptosis.

Finally, we have shown that AFS cells have been able to influence villus cell proliferation. Impairment of cell proliferation and migration, which extends beyond the crypts, is continuous, irregular or spreads into the covering villi (*Le Mandat, 2007*), and is commonly observed in both human (*Vieten, 2005*) and experimental NEC (*Cetin, 2004*).

Administration of HB-EGF protects the intestine from NEC via preservation of enterocyte migration and proliferation (*Feng, 2007*). Similarly, we found that proliferation and migration of EdU positive enterocytes along the whole villus length were markedly increased in AFS cell treated vs. untreated NEC rats. This is in keeping with results obtained in a model of radiation-induced intestinal injury, where human MSCs transplanted into immuno-tolerant NOD/SCID mice increase small intestinal villus height and increase gut self-renewal (*Dempke, 2001; Semont, 2006*).

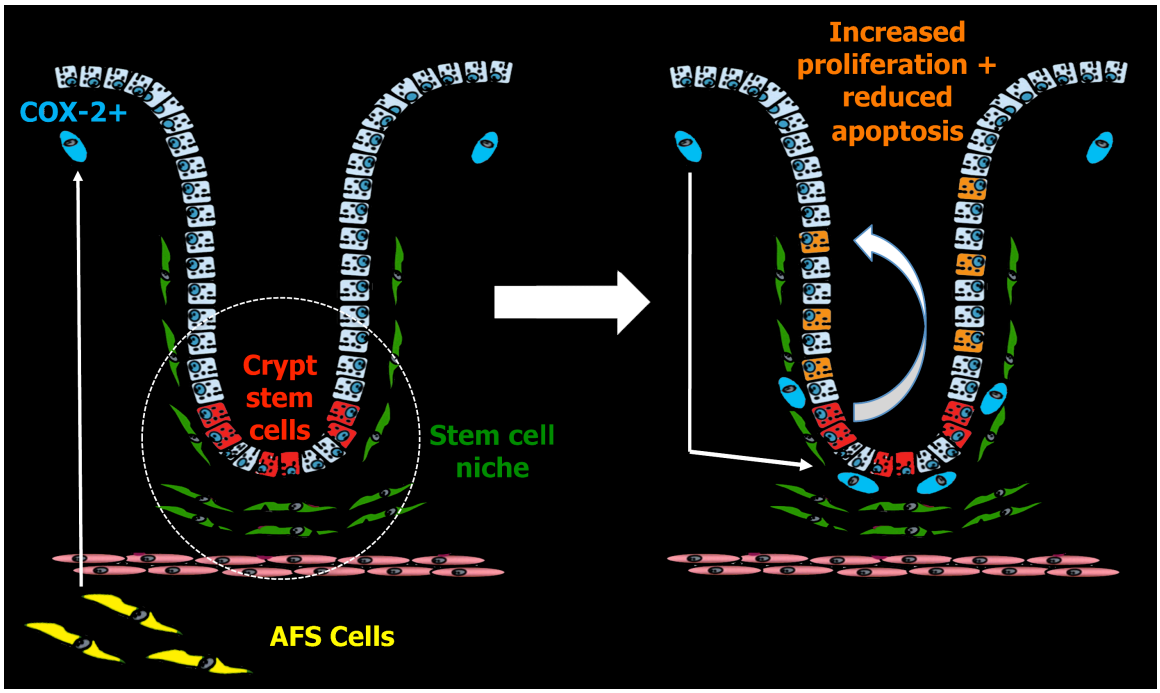
Hence, AFS cells diminish apoptosis and inflammation, and promote enterocyte proliferation. Intriguingly, COX-2, normally expressed at very low levels in intestine (*Dempke, 2001*) has been shown to have similar effects: it decreases enterocyte apoptosis (*Joseph, 2005*) (*Chen, 2002; Joseph, 2005*; *Tessner, 2004*), diminishes granulocyte infiltration (*Ajuebor, 2000; Gilroy, 1999*) and promotes epithelial proliferation (Arber, Eagle et al. 2006) (*Bertagnolli, 2006; Ko, 2001; Shao, 2006; Sheng, 2002)*. We therefore questioned whether AFS cells could act through a COX-2 related mechanism. To this end, we evaluated the number and the localisation of COX-2+ cells in the lamina propria of the terminal ileum of BF rats and NEC rats treated with PBS or AFS cells. COX-2+ cells, constitutively present in the lamina propria of BF rats, were markedly diminished in NEC rats injected with PBS while they were maintained in number in NEC rats treated with AFS cells. Remarkably, the higher number of COX-2+ cells in AFS cell treated rats was due to their increased presence in the crypts. Moreover, in both PBS and AFS cell treated rats, the number of COX-2+ cells inversely correlated with the severity of microscopic intestinal damage. In support of the role of COX-2+ cells in AFS cell-induced effects, administration of a specific COX-2 inhibitor or a non-selective COX-1+2 inhibitor, but not a COX-1 inhibitor, abolished the beneficial effects of AFS cells on animal morbidity and mortality. COX-2 inhibition did not affect BF rats or NEC rats injected with PBS. Together, these data support the hypothesis that the beneficial effects of AFS cells in this model of NEC may be mediated by COX-2 activity. It has been suggested that COX-2 may have a dual role in NEC, with high activities being pro-inflammatory, and lower levels protective (*Lugo, 2007*). Grishin et al. have shown that systemic administration of selective COX-2 inhibitors worsened intestinal inflammation and increased animal mortality in a rat model of NEC (*Grishin, 2006*) and it is well-known that peri- and post-natal exposure to glucocorticoids and NSAIDs, which inhibit COX-2 expression and activity respectively, are risk factors for the development of NEC (*Guthrie, 2003; Lugo, 2007*). On the other hand, it has been suggested that the high levels of COX-2 expression observed in both human and experimental NEC may contribute to the pathogenesis of the disease via pro-inflammatory

effects (*Lugo, 2007*). In contrast to our study, in these reports COX-2 expression was evaluated throughout the whole intestinal wall or the entire mucosa and no correlation was found between COX-2 expression and the severity of intestinal injury (*Grishin, 2006; Zang, 2003*). We postulate that these findings can be reconciled when the localisation of COX-2+ cells is taken into account, with the presence of COX-2+ cells in the crypts promoting epithelial proliferation and migration whilst preventing apoptosis. This is in agreement with the recent demonstration that the repositioning of COX-2+ cells in the cryptae is necessary to maintain the proliferation of colonic epithelial progenitors after damage (*Brown, 2007*). Although the identity of the COX-2+ cells remains to be established; only one previous report states that they consist of a population of stromal CD44 positive/hematopoietic lineage-negative/myofibroblast lineage-negative cells (*Brown, 2007*). Concerning the mechanisms involved in COX2 activation on the one hand it is well known that COX2 expression is generally enhanced by different pathways (eg TLR-Myd88, cytokines, growth factor receptors) (*Tsatsanis, 2006*) and on the other hand it has been recently demonstrated that COX2+ cells in the lamina propria are specifically activated by FGF9 (*Walker, 2010*). Based on our actual results we can only speculate that the activation of COX2+ cells in the lamina propria is mediated by a factor specifically released by AFS cells and not produced by BM-MSCs and myoblasts. Further studies are needed to identify the molecular pathways/signals determining COX2+ cell activation in the intestinal mucosa.

CONCLUSIONS

In conclusion, we have demonstrated that when injected in a widely-used model of experimental NEC, AFS cells ameliorated survival of the animals, clinical status and gut function. In addition, MRI and gut macroscopic and microscopic appearance were also significantly improved in NEC rats injected with AFS cells. These beneficial effects did not appear to be due to repopulation of damaged intestine by AFS cells, but instead were probably related to their paracrine effects. These latter included decreased inflammation and apoptosis and a concomitant increase in enterocyte proliferation and migration, thus aiding epithelial restitution. We believe these effects may be mediated by COX-2+ cells, as their presence in the cryptae was significantly enhanced by AFS cell injection, while the beneficial effects of AFS cells were abolished by COX-2 inhibitors (***Figure 28***). Future work should focus on both the potential clinical use of AFS cells and further elucidation of their mechanism of action in order to develop pharmacological agents suitable for neonates affected by NEC.

Figure 28. *Proposed AFS cell mechanism of action in rat experimental NEC. Firstly, AFS cells injected i.p. activate COX2+ cells in the lamina propria of the intestine through a paracrine mechanism. Once activated COX2+ cells increase in number and migrate from the villus axis to the bottom of intestinal crypts, where they respectively stimulate the proliferation and reduce the apoptosis of intestinal epithelial cells.*



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RESEARCH ACTIVITY

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