

Successful whole embryo culture with commercially available reagents

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ABSTRACT Since its development in the 1970's, whole embryo culture (WEC) has provided an important method of growing and observing murine embryos *ex utero*. During WEC, embryos are immersed in a combination of rat serum and cell culture media, and supplied with heat and appropriate mixtures of CO₂ and oxygen that mimic growth conditions *in utero*. One significant factor limiting the widespread use of WEC is the perception that commercially produced rat serum is inadequate to support normal rates of embryonic growth and development. Conversely, production of serum 'in-house' is technically demanding, time-consuming and expensive. The current study aimed to identify a WEC medium comprising commercially manufactured rat serum that would produce cultured embryos of comparable standard to those grown *in utero*. A mixed culture medium, composed of 50% commercial rat serum and 50% F12 Ham's cell culture medium with an N-2 neuronal cell growth supplement, was shown to support both a rate of growth, and the development of a range of features comparable to that which normally occur *in vivo*. Furthermore, the F12 (N-2) supplemented rat serum displayed a very low propensity to induce morphological abnormalities during the culture period. The study establishes a novel method of successful WEC using readily available commercial reagents and should enable the broader use of WEC.

KEY WORDS: *rat serum, mouse embryo culture, F12, N2*

Introduction

Embryology is dependent upon the ability to monitor the consequence of perturbed development. Perturbation can be induced by germ-line modification of an organism's genome or by physical manipulation of the embryo. The mouse has long been the vertebrate model organism of choice for genetic manipulation with techniques for transgenesis and targeted and random mutagenesis both long established and continually evolving. The combined effect of this is that alleles of every mouse gene will soon be available and the analysis of the associated phenotypes will continue to inform the study of the genetic control of mammalian development. In contrast to suitability for genetic manipulation, mammalian viviparity hinders observation following physical manipulation of post-implantation stage mouse embryos and experimental embryology approaches are most advanced in model organisms that reproduce externally such as fish and frog (Ellis-Hutchings and Carney 2010; Harris 2012; Hogan *et al.*, 1994; Piliszek *et al.*, 2011). Experimental embryology is however crucial to decipher embryonic development. Experiments

in which cells are labelled and followed overtime generate the fate maps that underpin the study of an organism's development and transplantation experiments that transfer a group of cells from a donor to a host embryo reveal the inductive interactions that direct embryonic development (Hogan *et al.*, 1994; Kaufman and Bard 1999). Combining these approaches with experimental embryology techniques provides a powerful means by which the consequence of germ-line modification can be analysed. To fully exploit mouse genetic resources, efficient and widely available protocols for the *ex utero* observation of murine embryos need to be developed.

The *ex utero* observation of murine development can be achieved

Abbreviations used in this paper: CRSD, mixed culture medium comprising 50% commercial rat serum and 50% DMEM cell culture medium; CRSDN, 50% commercial rat serum and 50% DMEM with N-2 neuronal cell growth supplement; CRSF, 50% commercial rat serum and 50% F12 cell culture medium; CRSFN, 50% commercial rat serum and 50% F12 with N-2 neuronal cell growth supplement; DMEM, Dulbecco's Modified Eagle Medium; F12, Ham's F12 Nutrient Mixture, N-2, neuronal cell growth supplement; WEC, whole embryo culture.

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by whole embryo culture (WEC) and a range of studies have been conducted with this technique to investigate normal embryonic development (Downs and Gardner 1995; Ellington 1991; Ellis-Hutchings and Carney 2010; Fleming *et al.*, 1997; Foerst-Potts and Sadler 1997; Fujinaga 2000; Lawson *et al.*, 1986, 1991; Lawson and Pedersen 1987; Moore-Scott *et al.*, 2003; Morriss and New 1979; Nakagawa *et al.*, 1997; Rivera-Perez *et al.*, 2010; Tam 1989, 1998; Trainor *et al.*, 1994; Wilson and Beddington 1996), to examine the effects of altering embryonic gene expression (Arkell and Beddington 1997; Fujinaga 2000; Pryor *et al.*, 2012; Rivera-Perez *et al.*, 2010; Takahashi *et al.*, 2008; Tam 1998), or to screen chemical compounds for teratogenicity (Chatot *et al.*, 1980; Ellis-Hutchings and Carney 2010; Harris 2012; Moore-Scott *et al.*, 2003; Morriss and Steele 1974; Rivera-Perez *et al.*, 2010; Tam 1998; Webster *et al.*, 1997). The system that enables murine WEC was developed in the 1970's (Ellis-Hutchings and Carney 2010; New 1978; New *et al.*, 1973; Tam 1998) and refined over the next two decades such that by the 1990's a standard protocol for WEC was established (Fujinaga 2000; Gray and Ross 2011; Harris 2012; Piliszek *et al.*, 2011; Rivera-Perez *et al.*, 2010; Takahashi and Osumi 2010). The method involves immersion of embryos in nutrient rich media, the supply of appropriate gas mixtures and rotation of embryos in the media at 37°C. The technique itself is relatively straight forward, requiring no more expertise than the culture of primary or transformed cell lines. As with the culture of cell lines, serum is a crucial but ill-defined component of the nutrient rich media. In contrast to the media used for cell culture however, optimal mammalian WEC requires a high proportion (typically 50% or greater) of homologous serum (i.e. derived from the organism being cultured) and that serum collection avoid haemolysis (Hunter *et al.*, 1988; Piliszek *et al.*, 2011; Priscott 1983; Sadler and New 1981; Sanyal and Wiebke 1979; Sturm and Tam 1993; Tam 1998; Tam and Snow 1980).

The use of mouse serum for mouse embryo culture is problematic because of the small blood volume of the adult mouse: one mouse typically yields sufficient sera to culture two embryos. The development of murine WEC protocols therefore focused on achieving reasonable culture success using rat serum (since one rat typically yields sufficient sera to culture twenty embryos (Hunter *et al.*, 1988)). A number of studies have shown that rat serum supports ex utero growth of post-implantation stage mouse embryos for at least 24 hours, providing that blood is withdrawn from the animal gently, immediately centrifuged, the fibrin clot carefully removed, and the sera heat-inactivated (Cockroft 1991; Harris 2012; New 1978; New *et al.*, 1976; Piliszek *et al.*, 2011; Rivera-Perez *et al.*, 2010; Steele and New 1974; Takahashi and Osumi 2010; Tam 1998; Tam and Snow 1980; Van Maele-Fabry *et al.*, 1991). The exacting requirements for the production of mouse embryo-culture-grade rat serum means commercial preparations are generally not able to support the ex utero growth of mouse embryos. Instead, current mouse WEC protocols recommend the preparation of rat serum 'in-house' as part of the embryo culture procedure (Fujinaga 2000; Harris 2012; Martin and Cockroft 2008; Piliszek *et al.*, 2011; Pryor *et al.*, 2012; Tam and Snow 1980).

The production of mouse embryo-culture-grade rat serum is not only technically demanding but is expensive and time consuming (Fujinaga 2000; Harris 2012; Martin and Cockroft 2008; Rivera-Perez *et al.*, 2010; Webster *et al.*, 1997). The lack of a readily available, consistent source of rat serum hinders comparison between

mouse WEC experiments and has constrained the use of this technique which has remained the preserve of a few specialised embryo laboratories world-wide. The current study aimed to develop a successful WEC medium that utilises only commercially produced reagents to support the ex utero growth of gastrula and neurula stage mouse embryos. Rat serum was commercially produced to custom instruction following the protocol known to be consistent with murine embryo culture. When combined with a standard cell culture medium (Ham's F12 Nutrient Mixture) and N-2 neuronal cell growth supplement the medium was found to support embryonic development that approximated in utero development and with the same frequency of success as previously published protocols that utilise in-house produced rat serum. The ability to purchase rat serum compatible with embryo culture should increase the number of researchers able to employ this technique, enable the routine coupling of murine experimental embryology with molecular genetics and likely drive further innovation in culture protocols.

Experimental Protocols

Rat serum preparation

Commercial rat serum was collected and processed by Valley Biomedical (Winchester, VA, USA catalogue number AS3061 with custom collection instructions) under sterile conditions. Rats were anaesthetised using CO₂ and exsanguinated. Collected blood was immediately centrifuged at 1200 g for 10 min, and the resulting fibrin clot squeezed and discarded. Individual bleeds were screened for haemolysis, and those selected for processing were re-centrifuged for a further 10 min at 1200 g. The resulting serum layers were collected from the individual bleeds, pooled, aliquoted and frozen at -20°C.

Culture media preparation

Under sterile conditions, Ham's F12 Nutrient Mixture with Gluta-max™ (Invitrogen catalogue number 31765-035) was supplemented with N-2 neuronal cell growth supplement (100X, Invitrogen catalogue number 17502-048) at a concentration of 500 µL per 50 mL of medium, (referred to here as F12 (N-2)). Then either Dulbecco's Modified Eagle Medium (supplemented with fresh L-glutamine (2 mM)) (Invitrogen, catalogue number 11995-073) or F12 (N-2) medium was combined with rat serum at a ratio of 1:1 to produce two distinct media referred to as Commercial Rat Serum DMEM (CRSD) or Commercial Rat Serum F12 (N-2) (CRSFN). For each stage and media condition tested a minimum of 18 embryos was scored and data combined from culture on at least five separate occasions. On some occasions, using the same basic formula of 50% rat serum and 50% medium, DMEM supplemented with N-2 neuronal cell growth supplement at a concentration of 500 µL per 50 mL of medium was used to generate Commercial Rat Serum DMEM N-2 (CRSDN) medium whilst F12 medium not supplemented with N-2 was used to generate Commercial Rat Serum F12 (CRSF) medium. For each of these media a minimum of 12 embryos was cultured and data combined from culture on at least two separate occasions. Culture medium was filter sterilised through a 0.2 µm filter into a 15 mL loosely-capped tube and allowed to equilibrate for at least 30 min at 37°C, 5% CO₂ in air in a sterile incubator.

Embryo dissection

Mice were maintained in a light cycle of 12 hours light: 12 hours

dark, with the midpoint of the dark cycle occurring at 12 a.m. and according to Australian Standards for Animal Care under protocols approved by The Australian National University Animal Ethics and Experimentation Committee. To generate staged, genetically identical embryos for culture, female BALB/c animals were paired with C57BL/6 male animals and mideday on the day of appearance of a vaginal plug was designated as 0.5 days post coitum (dpc). At the appropriate gestation point the CB6F1 embryos were dissected from the deciduum and free of maternal membranes under sterile conditions in 10% foetal bovine serum in PBS. Only embryos in which Reichert's membrane was resected without causing damage to either the embryo itself or to the trophectoderm were used for culture (Gray and Ross 2011). These embryos were staged (for pre-somite embryos according to Downs and Davies (Downs and Davies 1993), or by somite number), immersed in 500 μ L – 1 mL of equilibrated culture medium in a sterile glass embryo dish, and held in a sterile 37°C, 5% CO₂ incubator.

Embryo culture

Maintaining sterile conditions, the embryos for culture were separated into groups of up to 4 stage-matched embryos (Downs and Davies 1993) and transferred to 2 mL of equilibrated culture medium in a sterile glass vial (BTC engineering, Cambridge, UK) and placed on a roller culture apparatus (BTC engineering, Cambridge, UK) pre-warmed to 37°C. Premixed 5% CO₂ in air was supplied to the embryos at a constant rate (gas release from the bottle was at a pressure of 3 – 5 psi). After 24 hours, embryos were dissected in 10% Foetal Bovine Serum (FBS) in PBS (4°C) and examined and photographed with a stereomicroscope (Nikon SMZ1500 microscope and DS-Ri1 camera). Each embryo was staged and morphologically assessed for stage-dependent characteristics such as yolk-sac circulation, headfold development, heart rate, embryo turning (flexion), somite development, neural tube closure and forebrain elongation (see developmental and deformity scoring schemes in supplementary materials). For each characteristic scored a theoretical maximum was set and the score for each individual embryo expressed as a fraction of the maximum possible score. These individual embryo scores were averaged across all embryos for each characteristic and presented as the mean \pm S.E.M. Within a developmental stage each embryo's score for the relevant characteristics (either developmental or deformity) were summed to give a developmental and deformity score for each embryo. The total scores for each embryo were averaged and presented as the mean \pm S.E.M. These final scores were analysed via a 2 tailed *t* test to identify differences between embryos cultured in different media..

Results and Discussion

Commercial rat serum supports culture of post-implantation embryos when combined with F12 (N-2)

To determine whether the commercially produced rat serum is of sufficient grade to support the WEC of post-implantation stage mouse embryos it was used to produce two distinct media. Previous studies recommend DMEM or F12 as optimal cell culture media for combination with rat serum in a mixed embryo culture medium (Piliszek *et al.*, 2011; Rivera-Perez *et al.*, 2010), therefore the commercially produced rat serum was first used here as a direct substitute for in-house rat serum in a standard DMEM-based

embryo culture medium (50% rat serum, 50% DMEM and termed CRSD). Second, it was used to produce an F12-based media that was modified by addition of an N-2 neuronal supplement (50% rat serum, 50% F12 (N-2) and termed CRSFN). F12 is routinely used in combination with N-2 neuronal cell growth supplement for applications such as neural crest cell culture (Garcia-Castro *et al.*, 2002) and primitive streak culture (R. Arkell, personal observation). The planned culture experiments span the periods of primitive streak development and neurulation and it is therefore possible that N-2 supplement will enhance embryo culture outcomes. In order to compare the relative efficacy of these two media, embryos (dissected at 7.5 dpc or 8.5 dpc) were cultured for 24 hours under otherwise equivalent conditions and then examined for a variety of morphological criteria. As shown in Fig. 1 embryos dissected

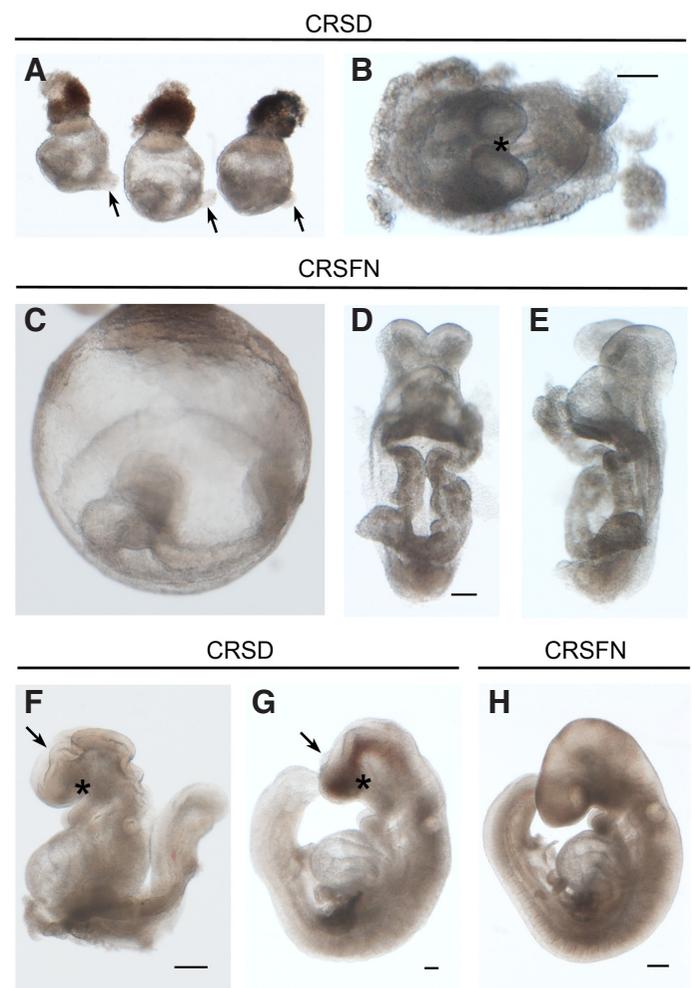


Fig. 1. Commercial rat serum can support whole embryo culture of 7.5 and 8.5 dpc murine embryos. Photographs of embryos following 24 hours of ex utero culture in each medium are shown, both with their extraembryonic membranes intact (A,C) or following membrane removal (B, D-H). Embryos are shown in lateral (A, C, E-H) dorsal (B) or ventral (D) view, with anterior to the left (A-C, E-H), or to the top (D). Culture was initiated with late primitive streak stage (7.5 dpc) embryos (A-E) or with 6-somite stage (8.5 dpc) embryos (F-H). Arrows in (A), allantois protruding from yolk sac; * in (B), poorly developed head-folds; arrows in (F,G), incomplete cranial neural tube closure; * in (F,G), poor optic cup development. Scale bar, 100 μ m.

at either 7.5 or 8.5 dpc and cultured in CRSD had overt signs of abnormal development, whereas those cultured in CRSFN appeared to develop to the equivalent in utero stage. Embryos that are grown in culture for 24 hours from the late primitive streak stage (7.5 dpc) are expected to develop ~ 6 somites and to exhibit morphological landmarks of cranial neural development (Downs and Davies 1993; Kaufman and Bard 1999; Rivera-Perez *et al.*, 2010; Takahashi and Osumi 2010; Van Maele-Fabry *et al.*, 1993). The majority of embryos cultured in CRSD did not develop somites and their allantois often protruded through the yolk sac. In contrast, embryos grown in CRSFN appeared to develop as expected for their in utero counterparts (Fig. 1 A-E). Similar results were obtained for embryos cultured for 24 hours from the 6-somite stage. During the culture period, these embryos are expected to add a further 12 somites, to have complete embryonic rotation and cranial neural tube closure, and to develop optic and otic placodes, limb buds and branchial arches (Downs and Davies 1993; Kaufman and Bard 1999; Van Maele-Fabry *et al.*, 1993). The majority of embryos cultured in CRSD did not meet all of these milestones and in fact 100% of these embryos exhibited one or more major deformities, most often including pyknosis (83% of embryos) and forebrain truncation (87% of embryos), suggesting that their development was grossly impaired. In contrast, embryos grown in CRSFN appeared to develop as expected for their in utero counterparts and only 19% of embryos exhibited one or more major deformities (Fig. 1 F-H).

To quantify and compare the success of culture with either medium each embryo was evaluated to ascertain whether their rate of growth and development was as expected for the stage at which the embryos were placed into culture. Published scoring systems did not match the precise range of developmental stages examined here and a modified scoring system was developed (Harris 2012;

Van Maele-Fabry *et al.*, 1990). For each culture period a range of morphological features was scored, consistent with an embryo of the stage equivalent to 24 hours after explantation (Tables 1 and 2). The score for these individual features were summed to give a 'developmental' score. Additionally, a range of stage appropriate features generally associated with poor embryonic development were scored and summed to generate a 'major' and a 'minor deformity' score (Tables 1 and 2) indicative of induced morphological abnormalities. Deformities severe enough to potentially confound experimental results or to necessitate exclusion of an embryo from a data set following WEC were considered major, whereas abnormalities that did not indicate impaired viability or significant growth retardation were considered minor (see supplementary material for a full description of the scoring system). Successful embryo culture is therefore associated with a high developmental score and a low deformity score. Embryos grown in CRSFN media accumulated significantly higher developmental scores than those cultured in CRSD for both age groups. Furthermore, CRSFN cultured embryos of both age groups scored significantly lower in terms of induced major morphological abnormalities. These results indicate that the F12 (N-2) based culture medium was better able to support both a normal rate of embryonic growth, and the development of expected features and was also less prone to inducing aberrant features than the DMEM based culture medium.

To measure the overall culture success of the CRSFN medium the proportion of embryos that exhibited a maximum developmental score and minimum major deformity score was calculated. For those embryos cultured from 7.5 dpc for a 24 hour period 100% of embryos achieved this score. For those embryos cultured from 8.0 or 8.5 dpc for a 24 hour period 81% of embryos received a maximum developmental score and minimum major deformity score. Pooling the data across all age groups, an overall culture success rate of 91% (n = 49) was obtained. The overall culture success rate with the CRSFN media compares favourably with that reported in studies which utilize in-house prepared rat serum. For example, previous studies report a 70 – 80% success rate for murine embryos cultured from 7.5 dpc for a 24 hour period and a success rate of 85 – 90% for embryos cultured from early somite stages for a 24 hour period (Arnell and Beddington 1997; Quinlan *et al.*, 1995; Tam 1998; Trainor *et al.*, 1994). Likewise, a recent study recommended a success rate of greater than 80% be indicative of a good WEC system for embryos cultured from 7.5 dpc (Rivera-Perez *et al.*, 2010). It is possible that a low background rate of culture failure persists even under optimal WEC conditions due to damage unconsciously incurred during the dissection/explantation experimental procedures (Klug 1991). It therefore appears that commercially available rat serum prepared specifically for embryo culture can support ex utero growth of murine gastrula and neurula stage embryos.

The difference in culture success of the two media is not determined by N-2 alone

The results presented above, in which the F12 (N-2) medium combined with commercial rat serum gave superior culture outcomes, could be explained entirely by the presence of the N-2 neuronal cell growth supplement, which to our knowledge has not previously been used in the culture of gastrulating and neurulating murine embryos. If this is correct then the addition of N-2 to the DMEM based medium would rectify the culture-induced defects

TABLE 1

GROWTH AND DEVELOPMENT OF EMBRYOS CULTURED FROM 7.5 DPC^a

Feature	Culture media		Max. possible score
	CRSD	CRSFN	
Developmental score			
Fraction of expected somite pairs added	0.00 ± 0.00	1.00 ± 0.00	1.00
Presence of heart	0.05 ± 0.05	1.00 ± 0.00	1.00
Integrity of yolk sac	0.00 ± 0.00	1.00 ± 0.00	1.00
Integrity of allantois	0.43 ± 0.11	1.00 ± 0.00	1.00
Forebrain development (presence of F/M junction/neural folds)	0.24 ± 0.07	1.00 ± 0.00	1.00
Midbrain development (presence of pre-otic sulcus)	0.00 ± 0.00	1.00 ± 0.00	1.00
Hindbrain development (presence of otic sulcus)	0.00 ± 0.00	1.00 ± 0.00	1.00
Total score	0.68 ± 0.20	7.00 ± 0.00 ^b	7.00
Major Deformity score			
Pyknosis	1.00 ± 0.00	0.00 ± 0.00	1.00
Protrusion of allantois from yolk sac	0.21 ± 0.10	0.00 ± 0.00	1.00
Head/tail fusion	0.95 ± 0.05	0.00 ± 0.00	1.00
Total score	2.16 ± 0.12	0.00 ± 0.00 ^c	3.00

^a7.5 dpc embryos were cultured for approximately 24 hours in either CRSD (n = 20) or CRSFN (n = 18) media. Each embryo was scored for rate of growth and the development of stage-appropriate morphology (developmental score) and for the presence of induced morphological abnormalities (major deformity score). A developmental score of 1.00 indicates that the embryo developed the given feature at the expected rate and with correct morphology. A major deformity score of 1.00 denotes the presence of an abnormal feature. Data were pooled for all embryos within each media group and are presented as the mean ± S.E.M.^bThe mean total developmental score of embryos cultured in CRSFN media was significantly higher than that of embryos cultured in CRSD media, P < 0.001 (2 tailed t test).^cThe mean total major deformity score of embryos cultured in CRSFN media was significantly lower than that of embryos cultured in CRSD media, P < 0.001 (2 tailed t test).

associated with this medium and the removal of N-2 from the F12 (N-2) based medium would render this medium incompatible with high rates of culture success. The effect of a 24 hour period of culture of 8.5 dpc embryos in 50% commercial rat serum, 50% DMEM (N-2) (referred to as CRSDN medium), and in 50% commercial rat serum, 50% F12 (referred to as CRSF medium) was therefore examined. Visual inspection indicated that the CRSDN cultured embryos were only marginally improved from those grown in un-supplemented DMEM, exhibiting growth retardation and failures of neural tube closure but with reduced pyknosis and headfold wrinkling (Fig. 2A). Likewise, embryos grown in the non N-2 supplemented CRSF culture medium showed growth and development that was considered approximately equivalent to that produced by culture in F12(N-2) (CRSFN). Notably, however, embryos grown in this (CRSF) medium had branchial arches that did not fuse at the ventral midline of the embryo, resulting in an open branchial pouch which was accompanied by an abnormal widening of the embryonic anterior despite anterior neural tube closure (Fig. 2B). This defect was observed in 75% of CRSF cultured embryos (n = 12). The origin of this defect is unclear, however it may involve neural crest cell migration since, though infrequently noted in CRSFN embryos, it appears to have been largely induced in the absence of the N-2 neuronal supplement from this media. Since N-2 supplementation of the DMEM based medium did not produce

culture outcomes equivalent or superior to the CRSFN medium, these (CRSDN and CRSF) media combinations were not further pursued by quantification of culture outcomes. The results imply that F12 and N-2 are together responsible for the improved culture outcomes associated with growth in 50% commercial rat serum, 50% F12 (N-2) (CRSFN).

The finding that N-2 supplementation improves WEC outcomes of gastrula and neurula embryos is perhaps not surprising given its known beneficial effects on the culture of primitive streak and developing neural tissues (Chiba *et al.*, 2005; Haubensak *et al.*, 2004; Pankratz *et al.*, 2007). It has also been found to be advantageous in the culture of mid-gestation mouse embryos using serum free media (Moore-Scott *et al.*, 2003). Failures of neural tube closure and poor forebrain elongation are often associated with WEC (Hunter *et al.*, 1988; New *et al.*, 1976; Sadler and New 1981; Tam and Snow 1980; Van Maele-Fabry *et al.*, 1995) and it may be that N-2 supplementation is a useful addition to any protocol for the WEC of post-implantation stage mouse embryos. It is however not clear why the use of F12 medium contributes to the improved culture outcome (relative to that with DMEM) when combined with the commercially produced rat serum. This is not generally the case for the culture of these stage embryos since a 1:1 combination of in-house produced rat serum with DMEM is the media combination most often recommended for murine WEC (Piliszek *et al.*, 2011; Rivera-Perez *et al.*, 2010; Tam 1998; Tam and Snow 1980; Van Maele-Fabry *et al.*, 1995). A comparison of the components of the DMEM and F12 media used in this study shows that Phenol-Red is present at a ten-fold greater concentration in the DMEM. A systematic comparison of the consequence of Phenol Red on murine WEC has not been made but Phenol Red is known to act as a weak oestrogen and to have unexpected consequences on the culture of some mammalian cell lines (Berthois *et al.*, 1986; Greiner *et al.*, 2010; Lin *et al.*, 2000). In support of the notion that commercially produced rat serum is sensitive to Phenol Red a recent description of murine WEC reports the use of commercial rat serum (Harlan Laboratories) in combination with Phenol Red free DMEM, but does not detail culture success rates (Gray and Ross 2011). Phenol Red serves as a pH indicator and

TABLE 2

GROWTH AND DEVELOPMENT OF EMBRYOS CULTURED FROM 8.0/8.5 DPC^a

Feature	Culture media		Max. possible score
	CRSD	CRSFN	
Developmental score			
Yolk-sac circulation	1.00 ± 0.00	1.00 ± 0.00	1.00
Fraction of expected somite pairs added	0.70 ± 0.05	0.98 ± 0.01	1.00
Presence of heartbeat	0.83 ± 0.08	1.00 ± 0.00	1.00
Presence of eyes	0.26 ± 0.09	1.00 ± 0.00	1.00
Fraction of expected branchial arches added	0.70 ± 0.07	0.99 ± 0.01	1.00
Extent of expected neural tube closure achieved	0.26 ± 0.09	0.94 ± 0.03	1.00
Extent of expected embryonic rotation achieved	0.45 ± 0.10	0.92 ± 0.04	1.00
Presence of expected forelimb buds	0.87 ± 0.07	0.88 ± 0.06	1.00
Total score	5.06 ± 0.42	7.71 ± 0.10 ^b	8.00
Major Deformity score			
Head truncation	0.87 ± 0.07	0.15 ± 0.07	1.00
Wrinkled headfolds	0.61 ± 0.10	0.08 ± 0.05	1.00
Pyknosis	0.83 ± 0.08	0.08 ± 0.05	1.00
Total score	2.30 ± 0.15	0.31 ± 0.13 ^c	3.00
Minor Deformity score			
Blood spotting	0.01 ± 0.00	0.05 ± 0.01	0.10
Blebbing	0.03 ± 0.01	0.02 ± 0.00	0.10
Total score	0.04 ± 0.01	0.07 ± 0.01 ^d	0.20

^a8.0 dpc and 8.5 dpc embryos were cultured for approximately 24 hours in either CRSD (n = 23) or CRSFN (n = 26) media. Each embryo was scored for rate of growth and the development of stage-appropriate morphology (developmental score) and for the presence of induced morphological abnormalities (major deformity score and minor deformity score). A developmental score of 1.00 indicates that the embryo developed the given feature at the expected rate and with correct morphology. A major deformity score of 1.00 denotes the presence of a severe abnormality, while a minor deformity score of 0.10 indicates the presence of a much less significant abnormality. Data were pooled for all embryos within each media group and are presented as the mean ± S.E.M.^bThe mean total developmental score of embryos cultured in CRSFN media was significantly higher than that of embryos cultured in CRSD media, P < 0.001 (2 tailed t test).^cThe mean total major deformity score of embryos cultured in CRSFN media was significantly lower than that of embryos cultured in CRSD media, P < 0.001 (2 tailed t test).^dThe mean total major deformity score of embryos cultured in CRSFN media was not significantly different to that of embryos cultured in CRSD media, P > 0.001 (2 tailed t test).

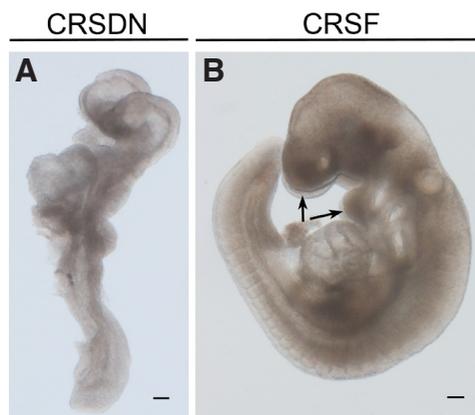


Fig. 2. N2 neuronal growth supplement does not fully account for the improved culture outcomes of the F12 (N-2) containing culture media. Lateral view of embryos cultured for 24 hours from the 6-somite stage (8.5 dpc) in the media shown. Embryos are shown with anterior to the left. Arrows in (B): Incomplete branchial arch fusion and widened forebrain. Scale bar, 100 μm.

therefore a marker of media exhaustion but the slow growth of cultured embryos and the limited culture period mean that this limit is rarely reached and that Phenol Red is dispensable for embryo culture. As with N-2 supplementation the use of Phenol Red free media may improve any murine embryo culture protocol.

The work presented here demonstrates that some companies are now willing and able to produce rat serum compatible with the WEC of murine embryos. To date we have received and tested three independent batches of rat serum from Valley Biomedical and have found all three useful for the culture of gastrula and neurula stage embryos. The ability to perform murine embryo culture with commercially available reagents should increase the number of laboratories that employ this technique and the frequency with which it is used. In addition, the ability to purchase (rather than make) mouse embryo-culture-grade rat serum should mean that researchers are more willing to dedicate it to experiments that seek only to compare the relative success of different culture media or other protocol modifications and so may drive much needed technical innovation in this area.

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