F. Animal Use. Justify the use of animals vs. non-animal alternatives, the choice of species (why this is the most appropriate species/strain to use in these studies), and the numbers of animals to be used (provide a breakdown of the animals into experimental group, identifying each experimental group and the numbers of animals in each group).

In discussing alternatives, consider the "3 Rs":

a. Replacement: Those methodologies (computer programs, tissue culture techniques, epidemiological data, etc.) which replace the use of animals.
b. Reduction: Those methodologies which reduce the numbers of animals used in the protocol.
c. Refinement: Those methodologies which refine the procedure to minimize the amount of discomfort that the animal may experience.

Include the computer database searched (e.g., Medline, Index Medicus, etc.) or other sources, such as journals or meetings that you used to determine that: a) there are no appropriate alternatives for this research and/or b) this protocol does not unnecessarily duplicate previous experiments by yourself or others. A computer search of at least two databases is required. A search for alternatives may include words such as "cell culture", "in vitro", or "computer models" or it may include a less sentient animal species. The search should be for reduction and refinement, not just replacement of animals.

Provide the databases searched, the key words used, the years searched, and the date of your search. Summarize the outcome of your search. If there are any hits, explain: a) why these would not be acceptable as a replacement for your in vivo work and/or b) how your study differs from previous work.

Two databases designed specifically to search for alternatives that you might want to use are:

http://www.vetmed.ucdavis.edu/Animal_Alternatives/altsearch.htm
http://altweb.jhsph.edu/

F. Animal use

Replacement

The embryonic development of vertebrate organisms is extremely complex and requires a multitude of intercellular signals and positional cues that cannot possibly be replicated in cultured cells. Thus, in order to study the role of Wnt signals in the specification of somite and neural crest lineages, it is absolutely necessary to use an animal model system. Furthermore, the examination of Wt signaling gradients requires a 3 dimensional tissue-signaling environment, which is not possible in cultured cells. We have chosen to use the chick embryo for a variety of reasons. First, they are the lowest vertebrate model organism available that largely recapitulates human development (with respect to somites and neural crest). Thus, our research will be applicable to human biomedical research. Second, unlike mammalian systems, the mother need not be sacrificed in order to gain access to the embryos. Third, the embryos are not sentient and feel no pain. Lastly, the embryos can be manipulated in a variety of ways that make them useful models for our experiments.

We have searched for model non-animal model systems that would allow us to conduct our experiments without the use of animals. I have searched http://www.avar.org/ for "chick AND somite" and found no alternatives. I also broadened the search to "somite" and still found no matches. Similarly, there were no matches for "neural crest". I also searched the http://altweb.jhsph.edu/about-us/alt_search.htm website for alternatives. When I searched for "somite", three hits came back as follows:

1. In Vitro Cell. Dev. Biol - Animal 37:440-444, July/August 2001
EFFECTS OF FIBROBLAST GROWTH FACTOR 2 AND INSULIN-LIKE GROWTH FACTOR II ON THE DEVELOPMENT OF PARTHENOGENETIC MOUSE EMBRYOS IN VITRO
LEONID I. PENKOV, EVGENY S. PLATONOV, AND DENIS A. T. NEW

2. In Vitro Cell. Dev. Biol - Animal 36:593-599, October 2000
ROLES OF β-CATENIN IN SOMITOGENESIS IN RAT EMBRYOS
MOTOK0 MATSUDA

3. Screening Chemicals for Reproductive Toxicity: The Current Alternatives
The Report and Recommendations of ECVAM Workshop 121,2
Reprinted with minor amendments from ATLA 23, 868-882.

Nigel A. Brown3, Horst Spielmann4, Rudolf Bechter5, Oliver P. Flint6, Stuart J. Freeman7, Richard J. Jelínek8, Elisabeth Koch5, Heinz Nau9, Derek R. Newall10, Anthony K. Palmer11, Jean-Yves Renault12, Marina F. Repetto13, Richard Vogel14 and Richard Wiger15

Strangely, the first two involve the use of mice and rats. As mammals, both of these are considered to be in a higher category of animal than chicks. The last reference on alternatives to screening toxic chemicals is not at all relevant to my studies. When I searched the same database for "neural crest", 67 hits were identified. After perusing these hits, it seems that at a minimum, cell culture models for neural crest required primary cultures of explants (which is something we also propose). As these primary cultures are directly established from embryos, this does not represent an effective replacement.

Reduction

For all studies, it is necessary to take into account the fact that not all eggs delivered to us have viable embryos. In some months, as few as 25% of the eggs have viable embryos. This causes a dramatic increase in our estimated usage of eggs. Also storage of eggs at 4^{o}C reduces viability. As we are only able to get shipments once/week, we observe decreased viability of embryos used at the end of the week.

The number of embryos required to analyze the expression of a single gene is dependent on a number of factors. First, in order to fully examine the temporal and spatial expression of a gene and/or protein throughout somite and/or neural crest cell development, it is necessary to examine multiple embryos at a number of different developmental stages. A minimum of 20 embryos per stage must be successfully stained in order to thoroughly analyze the expression pattern. If one analyzes 5 different stages of development, that would require 100 embryos/gene analyzed. Both the dissection and the whole mount in situ analysis are technically advanced procedures with a high failure rate for students who are just learning the technique. In addition, chick embryos do not always develop at exactly the same rate (Hamburger and Hamilton, 1951). Thus, one often has to dissect several embryos to get a single embryo of the desired developmental stage. To maximize the amount of information derived from each embryo, we cut embryos into thin sections, which allow us to generate as many as 10-50 images from a single embryo. Due to these experimental factors, we anticipate needing ~500 embryos/gene or protein analyzed. Each year, it is reasonable to estimate that we will be analyzing the expression of at least five genes and/or proteins, thus requiring a total of 2500 embryos/yr for this part of the project.

We anticipate that the explant/grafting studies will not be the major focus of this project. However, it is reasonable to expect that we would use ~500 embryos/yr for this type of study.

Electroporation studies represent the largest use of embryos. All members of my lab (~10) use this technique. In addition to requiring viable embryos, we also require that they be of a particular

developmental stage when we use them. The survival rate of embryos that have been electroporated ranges from 40-80%. Unfortunately, the success rate for the electroporations is much lower, typically around 10-20%. Thus, in a worst case scenario, where 40% of the embryos survive and 10% of those are electroporated, only 4% of the total embryos used will be suitable for analysis. Given that we are over and underexpressing approximately 10 different genes either alone or in different combinations, we predict that we will use 7000 embryos/year. In order to maximize information gained from successfully electroporated embryos, they are generally analyzed in thin sections, thus yielding 10-50 data points per embryo. We have retained a statistician who helps us determine the number of data points needed in order for our results to be statistically significant. Thus, the total number of embryos will need is approximately 10,000/year.

Refinement (Those methodologies which refine the procedure to minimize the amount of discomfort that the animal may experience.). Because our embryos do not experience pain, no refinement is necessary.