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REGRESSION MODELING TO INFORM CELL INCORPORATION INTO THERAPIES FOR CRANIOSYNOSTOSIS

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Abstract

Designing an appropriate tissue engineering solution for craniosynostosis (CS) necessitates determination of whether CS derived cells differ from normal (WT) cells and what assays are appropriate to test for differences. Traditional methodologies to statistically compare cellular behavior may not accurately reflect biologically relevant differences because they poorly address variation. Here, logistic regression was used to determine which assays could identify a biological difference between WT and CS progenitor cells. Quantitative alkaline phosphatase (ALP) and MTS proliferation assays were performed on adipose, muscle, and bone marrow-derived cells from WT and CS rabbits. Data was stratified by assay, cell type, and days in culture. Coefficients of Variation were calculated and assay results coded as predictive variables. Phenotype (WT or CS) was coded as the dependent variable. Sensitivity-specificity curves, classification tables, and ROC curves were plotted for discriminating models. Two data sets were utilized for subsequent analyses; one used to develop the logistic regression models for prediction, the other independent data set was used to determine the ability to predict group membership based on the predictive equation. The resulting coefficients of variation were high for all differentiation measures. Upon model implementation, bone marrow assays were observed to result in 72–100% predictability for phenotype. We found predictive differences in our muscle- and bone marrow-derived cells suggesting biologically relevant differences. This data analysis methodology could help identify homogenous cells that do not differ between pathologic and normal individuals or cells that differ in their osteogenic potential, depending on the type of cell-based therapy being developed.

Keywords

Stem Cells; Craniosynostosis; Logistic Regression

Introduction

The prospects for the use of stem cell in medical therapy are recently coming into focus. Evidence supports the use of progenitor cells as a potential means to combat a variety of human diseases.^{1, 2} Progenitor cell transplantation is already the standard practice in the treatment of certain hematopoietic diseases.³ Clinical trials are underway to investigate other

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applications for progenitor/stem cell therapy in several fields spanning cardiology,⁴⁻⁶ hepatology,⁷⁻¹⁰ wound healing,¹¹⁻¹³ immunology,¹⁴⁻¹⁶ transplantation,¹⁷ neurology,¹⁸⁻²⁰ ophthalmology,^{21, 22} autoimmune disorders,^{23, 24} urology,^{25, 26} orthopaedics,^{27, 28} and craniofacial biology.^{29, 30} The tissue sources of the multipotent stem cells and tissue specific progenitor cells used for these therapies include adipose, muscle, and bone marrow tissues. However, further research is necessary to elucidate the mechanisms of progenitor cell effectiveness.

The variability in stem cells and stem cell response is one major obstacle to usefulness and predictive outcomes of their use in therapy. Autologous stem cells treatments have a great amount of variability in efficacy between certain progenitor cell populations, variability in host response, and the inherent biological variability of the host's autologous cells.³¹ Variability within mesenchymal stem cells has been identified in both laboratory animal and human derived populations.^{32, 33} Variability has been identified in hematopoietic bone marrow derived,³⁴ adipose,³⁵⁻³⁷ and muscle derived cells,³⁸ as well as embryonic stem cells.³⁹ Such variability is likely endemic in primary cell isolations and not an artifact of isolation technique.

Recently we reported on stem cells capable of expressing markers of osteogenic differentiation in vitro.⁴⁰ We compared bone marrow, muscle, and adipose tissues isolated from a large sample of Wild Type (WT) New Zealand White rabbits as well as a pathologic model of Craniosynostosis (CS) in a related model. We were particularly interested in the investigation of the potential connection between a tissue-specific abnormality and cells derived from seemingly un-related tissues. We stimulated these cells with bone morphogenetic protein-4 (BMP4), and performed assays for alkaline phosphatase (ALP) expression and cell proliferation. Our results suggested that in the wild-type rabbits, cells derived from muscle had more ALP activity than cells derived from adipose or bone marrow (Figure 1). We also found that cells derived from our pathological model were significantly more osteogenic than those derived from wild-type rabbits. These results suggested that cells from different tissues have different potentials for differentiation. However, we discovered our cell sources to have a great amount of variability in response between individuals and populations (WT vs. CS).

Traditionally in cell biology there are two traditional ways to “deal” with variable results: 1) compare means which adds all individual results together and divides by the number of individuals, giving a single value and a standard deviation that together are taken to be representative of the group; or pooling samples which mixes the tissues collected from several individuals and isolating a single population of cells from that pooled tissue sample, gaining a “representative” or “mean” population of cells. Neither of these methods confronts the real problem of variation in stem cell response that is the reality of clinical translation. If variation is inherent in the sample, how do we control for it while still answering the questions: 1) Which stem cells will work best for our therapeutic goals, in this case which cell will stimulate osteogenesis most readily? 2) Which groups, or groups of individuals have cells that are altered or pathologic for a specific response? 3) Can a specific stem cell population be stimulated for our targeted therapeutic purposes?

Here we suggest multivariate application of logistic regression as one potential quantitative solution to these problems. Utilization of multivariate analyses allow for the detection of segregating differences while controlling for large amounts of individual variation. The advantages of this statistical method are that it keeps individuals as individuals, can easily determine influential points, can predict (segregate) from one or multiple cell assays, and can directly compare cell assays. The purpose of the present study was to revisit the specific question of designing an appropriate tissue engineering solution for craniosynostosis (CS)

which necessitates the determination of 1) whether CS derived cells differ from wild type (WT) cells, and 2) what assays are appropriate to test for differences. Traditional methodologies to statistically compare cellular behavior may not accurately reflect biologically relevant differences because they poorly address variation. Here, as an example, logistic regression was used to determine which assays could identify a biological difference for osteogenesis between WT and CS progenitor cells.

Methods

For model development we utilized cellular data from Wild-type (WT; n=7) and Craniosynostotic (CS; n=7) rabbits. For model implementation we utilized cellular data from Wild-type (n=16) and Craniosynostotic (n=10) rabbits. All animals were housed at the University of Pittsburgh under an IACUC approved protocol. Tissue harvest, cell isolation, and quantitative proliferation and differentiation assays were performed using traditional methods as previously described.⁴⁰

Data was stratified by assay, cell type, and days in culture. Initially we calculated the coefficient of variability which is a normalized measure of dispersion of a probability distribution (standardized measure: standard deviation to the mean). Predictive variables were screened for usefulness using Rao's efficient scores which are used to test whether the logistic regression coefficient for a given explanatory variable is 0. Cut off values for group membership were determined utilizing the specificity/sensitivity curve. Maximum likelihood estimation was used for determination of predictive equations. No interaction terms or covariate analyses were conducted for the sake of simplicity. Cox and Snell R² values were used to determine a proxy for percent of variability in predicted value explained. Hosmer and Lemeshow chi-square test of goodness of fit was used to determine the overall fit of the model. ROC Curves denote the true positive rate versus the true negative rate. The reference line of the ROC curve denotes random guess. For those models of use in our analysis, sensitivity-specificity curves, classification tables, and ROC curves were plotted.^{41–45}

A separate data set described above was implemented into resulting statistical models. For purposes of example, one model that did not provide a strong derivation by phenotype (cells were the same) is also presented. For purposes of example models that did provide a strong derivation by phenotype (cell were different) is presented. For these examples, equations, ROC curves, Sensitivity and Specificity plots, and % of individuals correctly predicted for phenotype are reported. All analyses were conducted in STATA 11.2 (College Station, TX) and SPSS 18.0 (IBM Corporation, Armonk, NY).

Results

Coefficient of Variation

The coefficient of variation is a normalized measure of dispersion of a probability distribution. Coefficient of Variation values for proliferation and differentiation assays are included in Table 1. Proliferation appears to elicit little variation between samples compared to differentiation, especially after BMP4 stimulation.

Logistic Regression Variable Determination

7 CS and 7 WT derived stem cells were used to determine if an assay was useful to determine phenotype (an indication that cell type differs under certain conditions). These data were used to fit the predictive models. Assay by treatment by day in culture values were fitted to predictive equation using block method. Resulting Rao values for prediction are listed in Table 2. For proliferation assays, muscle derived cells appear to be useful to segregate between the phenotypes (WT and CS), as well as bone marrow derived cells

treated with BMP4 after seven days in culture. For differentiation assays only the seven day assays for bone marrow derived cells appear to be useful of model segregation between the phenotypes.

Model Fitting

Significant predictive independent variables were used to determine multiple single predictor logistic regression models. Table 3 contains pertinent logistic regression diagnostics including R^2 , Goodness of fit data, area under the ROC curve, and Estimation (Sensitivity/Specificity). These data are presented with 0.5 as a critical cut off value for prediction of phenotype. Estimations of phenotype were all greater than 70%, with the greatest values being for bone marrow differentiation at seven days which were over 90% predictive of phenotype.

Implementation Examples

Adipose cells, for which there was poor prediction in equation development was modeled. Results upon implementation showed no derivation between the phenotypes and thus poor prediction (Figure 2).

Three equations, bone marrow cells, are shown in which there was over 85% correct prediction of phenotype at a cut off value of 0.5 suggestive of large differences in these cells between the phenotypes. All modeled showed significant derivation by phenotype, with perfect prediction for the equation that combined the seven day untreated and BMP4 treated values for differentiation (Figure 3).

Discussion

Multivariate analyses were utilized in these analyses to determine a stem cell that was the same or predicatively different between normal and pathologic individuals, under certain conditions in culture. In our analyses as previously reported, we were not able to detect real or meaningful differences in the adipose derived cells for our biomarkers between wild type and craniosynostotic phenotypes. For muscle we find an interesting relationship given our previous report that muscle differed between our wild type and craniosynostotic phenotypes.⁴⁰ Here we find that only under certain conditions, specifically for the marker of proliferation, were the muscle cell different. These results suggest that how the stem cells are cultured or stored prior to use may modify the phenotype of these cells. In this case it is of interest that although there were differences for cell proliferation of the muscle for phenotype this did not translate into a difference in osteogenesis as measured by our ALP assay even when stimulated with BMP4. This further suggests that although our craniosynostotic derived muscle cells may be slightly more osteogenic from a grand mean of the population perspective, it was not predictive and thus muscle derived cells would be expected to act similarly for osteogenesis between WT and CS population that we studied.

There were predictive differences detected for bone marrow cells especially for differentiation at seven days. However these difference were found to be significant at the three day time point for proliferation or differentiation suggesting these cells become more different once established in an environment and especially when treated with osteogenic growth factors. Thus, for tissue engineering purposes the investigator should be aware of a potential difference in osteogenic capacity between WT derived bone marrow cells and those bone marrow cells harvested (from in this case the femur) from an animal model with an isolated calvarial pathology.

These models could also be applied to the question of which assays were predictive of an in-vivo intervention using either stem cell therapy or growth stimulation (BMP4) both of which

are predictive covariates in our statistical models. Here we can specifically speak to relationships of the independent variables with respect to phenotype while controlling for variation. Thus, logistic regression may aid our future studies to determine differences in our samples. Future research should concentrate on utilizing predictive models for quantitative cellular analyses. In addition, correlations between in vitro cellular analyses, e.g. osteogenesis, and in vivo outcomes, e.g. calvarial defect healing, could provide a powerful tool to inform bone engineering interventions.

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Figure 1. Cell comparisons for Alkaline Phosphatase Activity

From Cooper et al., 2011. Comparison in differentiation (Alkaline Phosphatase Expression) between age matched wild type (WT) and Craniosynostotic (CS) New Zealand White Rabbits. Note the great amount of variability (Mean $\pm$ Standard Deviation).

Figure 2. Example Implementation of Models that Do Not Discriminate
 Logistic Regression model to discriminate Wild Type (WT) from Craniosynostotic (CS) phenotype by adipose cell assay after 3 days in culture. Note model prediction is suggestive of all animal cells being derived from a Wild Type animal, indicative of a poor model. Note ROC Curves and Specificity and Sensitivity illustration are suggestive of prediction being no better than chance (Sensitivity and Specificity lines cross at about .50).

Figure 3. Example Implementation of Models that Discriminate

Logistic Regression model to discriminate Wild Type (WT) from Craniosynostotic (CS) phenotype by bone marrow assays including seven day in culture, seven days in culture treated with Bone Morphogenetic Protein and combined models. Note that the combined model predicts phenotype perfectly, 100% correctly assigned to Wild Type or Craniosynostotic Phenotype. This suggests predictive differences between Bone Marrow cells by phenotype, or a real biological difference. Note area under the curve (ROC curve) approaching 1.

Table 1

Coefficient of Variation for Cell Assays

Proliferation				
Adipose				
	3 DAY	3 DAY/BMP	7 DAY	7 DAY/BMP
<i>Wild</i>	21.14	24.86	35.32	36.60
<i>CS</i>	36.85	40.24	46.25	53.37
<i>Total</i>	28.59	31.94	39.42	43.61
Muscle				
	3 DAY	3 DAY/BMP	7 DAY	7 DAY/BMP
<i>wild</i>	15.87	15.35	11.65	18.27
<i>CS</i>	19.25	22.38	32.71	30.26
<i>Total</i>	19.15	21.47	27.88	33.78
Bone Marrow				
	3 DAY	3 DAY/BMP	7 DAY	7 DAY/BMP
<i>wild</i>	10.79	9.60	28.87	21.81
<i>CS</i>	14.02	13.63	26.87	21.41
<i>Total</i>	12.80	11.39	30.46	27.80

Differentiation				
Adipose				
	3 DAY	3 DAY/BMP	7 DAY	7 DAY/BMP
<i>Wild</i>	161.40	106.50	50.82	42.45
<i>CS</i>	119.30	44.33	88.97	68.96
<i>Total</i>	153.05	76.06	67.67	56.99
Muscle				
	3 DAY	3 DAY/BMP	7 DAY	7 DAY/BMP
<i>Wild</i>	132.21	134.96	52.48	53.07
<i>CS</i>	196.88	133.65	80.76	69.30
<i>Total</i>	268.91	188.29	88.03	59.12
Bone Marrow				
	3 DAY	3 DAY/BMP	7 DAY	7 DAY/BMP
<i>Wild</i>	141.74	159.60	144.92	83.22
<i>CS</i>	132.73	128.25	68.77	89.06
<i>Total</i>	153.51	162.28	129.95	150.59

Table 2Rao Screening Values with Significance, $p < 0.05$

Proliferation				
Adipose				
	<i>3 DAY</i>	<i>3 DAY/BMP</i>	<i>7 DAY</i>	<i>7 DAY/BMP</i>
<i>Rao Value</i>	0.248	0.531	0.020	0.494
<i>p-value</i>	0.619	0.466	0.889	0.482
Muscle				
	<i>3 DAY</i>	<i>3 DAY/BMP</i>	<i>7 DAY</i>	<i>7 DAY/BMP</i>
<i>Rao Value</i>	3.313	4.396	6.467	8.124
<i>p-value</i>	0.069	0.036*	0.011*	0.004*
Bone Marrow				
	<i>3 DAY</i>	<i>3 DAY/BMP</i>	<i>7 DAY</i>	<i>7 DAY/BMP</i>
<i>Rao Value</i>	1.923	0.296	2.784	5.903
<i>p-value</i>	0.166	0.586	0.095	0.015*

Differentiation				
Adipose				
	<i>3 DAY</i>	<i>3 DAY/BMP</i>	<i>7 DAY</i>	<i>7 DAY/BMP</i>
<i>Rao Value</i>	0.437	0.053	0.158	0.177
<i>p-value</i>	0.509	0.817	0.691	0.674
Muscle				
	<i>3 DAY</i>	<i>3 DAY/BMP</i>	<i>7 DAY</i>	<i>7 DAY/BMP</i>
<i>Rao Value</i>	1.642	2.958	2.782	0.577
<i>p-value</i>	0.200	0.085	0.095	0.448
Bone Marrow				
	<i>3 DAY</i>	<i>3 DAY/BMP</i>	<i>7 DAY</i>	<i>7 DAY/BMP</i>
<i>Rao Value</i>	1.664	2.341	7.348	5.645
<i>p-value</i>	0.197	0.126	0.007*	0.018*

Table 3

Regression Diagnostics for Predictive Models by Assay

Proliferation				
Cell Comparison	Assay	R² Value	ROC Curve	Estimation
Muscle	3 day/BMP	0.2709	0.8163	71.43%
Muscle	7 day	0.4095	0.8776	85.71%
Muscle	7 day/BMP	0.6584	0.9592	85.71%
Bone Marrow	7 day/BMP	0.4071	0.8776	78.57%

Differentiation				
Cell Comparison	Assay	R² Value	ROC Curve	Estimation
Bone Marrow	7 day	0.6663	0.9796	92.86%
Bone Marrow	7 day/BMP	0.7119	0.9796	92.86%